Bi7430c MOLECULAR BIOTECHNOLOGY

practice

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01 course information

COURSE MOTIVATION

The practice provides an interesting combination of practical problems solved under the guidance of experts from academia (Masaryk University and Veterinary Research Institute) and business environment (a biotechnology company Enantis). At the end of the course, students will be familiar with a variety of techniques of molecular biotechnology, such as design of primers, DNA cleavage by restriction enzymes, transformation of bacterial cells, methods of fermentation culturing E. coli, application recombinant microorganisms and isolated enzymes in biosensing and remediation of hazardous substances or synthesis of pharmaceutically pure chemicals. The course will enable students to deepen their theoretical knowledge acquired in lectures Molecular Biotechnology. Students will become familiar with the currently used methods and the principle and operation of modern laboratory equipment. The course also aims to strengthen communication skills of students in a discussion with experts of the field and exercise stylistic skills of students in a writing an essays and protocols. The learning materials in English support the ability to work with scientific texts, including strengthening the knowledge of the English terminology used in this field.

CAPACITY two groups of 10 students

LANGUAGE materials – EN
spoken language – CZ
protocols – student chooses between EN and CZ

ABSENCE official excuse in the Information System (IS) + written essay (2 pages A4)

INTERACTIVE SYNOPSIS available in Bi7430c in the Information System

PREREQUISITES

1. parallel attendance of Molecular Biotechnology lecture (Bi7430)
2. the course of Molecular Biology (Bi4020) and Basics of Molecular Biology (Bi4010) completed

PRACTICE ORGANISATION

1. theoretical introduction (presentation given by lecturer)
2. introduction to practice and assignment control (homework)
3. experimental work in the laboratory

TEACHING AND ASSESSMENT METHODS

Students are expected to study the manual and prepare the homework before each practice. In the laboratory, each student works independently or in pairs under the guidance of the teacher. Each student works with own sample. The results of experiments are evaluated by each student in separate essays and protocols based on the individual practice. Students will obtain positive assessment of the practice for active participation in exercises and preparation of protocols or essays from each exercise. Protocols should be uploaded to IS within one week from each practice. Delay would result in extra written essay (1 page A4).

syllabus

IN SILICO CLONING OF A HALOALKANE DEHALOGENASE

Lecturer: Ing. Andrea Schenkmayerová, Ph.D.
Aims: software tool Clone Manager, design of primers, restriction cleavage, cloning, transformation

RECOMBINANT PROTEINS: FROM SMALL- TO LARGE-SCALE EXPRESSION TECHNOLOGIES

Lecturer: Ing. RNDr. Martin Marek, Ph.D.,
Ing. Andrea Schenkmayerová, Ph.D.
Aims: from DNA to recombinant protein production, small-scale expression screening, large-scale (fermentor) overproduction, protein purification strategies, and quality control of the purified proteins

PREPARATION AND TESTING OF MICROFLUIDIC CHIP

Lecturer: Mgr. David Kovář, Ph.D.
Mgr. Michal Vašina
Aims: capillary microfluidic platform, preparation and microscopy of microdroplets, microscopy of microdroplets with cells

BIOCATALYTIC PREPARATION OF PHARMACEUTICAL PRECURSOR

Lecturer: Assoc. Prof. Radka Chaloupková, Ph.D.
Aims: biocatalytic synthesis of pharmaceutical precursor by using recombinant enzyme

PREPARATION AND TRANSFORMATION OF LIPOSOMES

Lecturer: RNDr. Jaroslav Turánek, CSc.
Aims: preparation of liposomes, transformation, high-pressure extrusion, preparation of SUV (small unilamellar vesicels), DLS (dynamic light scattering) analysis, ZetaSizer

ANALYSIS OF LIPOSOMES

Lecturer: RNDr. Jaroslav Turánek, CSc.
Aims: single particle tracking analysis by using Nanosight 500, transmission electron microscopy

PREPARATION OF ENZYMATIC BIOSENSOR

Lecturer: Mgr. Šárka Nevolová, Ph.D.
Aims: co-immobilization of recombinant enzyme and fluorescence probe to optical transducer, detection of selected analyte

workplaces

LOSCHMIDT LABORATORIES

http://loschmidt.chemi.muni.cz/peg/

The Josef Loschmidt Chair was established at the Faculty of Science of Masaryk University by a donation of Alfred and Isabel Bader to honour the name of great Czech scientist of the 19th century Jan Josef Loschmidt. Jiri Damborsky was appointed to the Chair in 2003 and the Loschmidt Laboratories were founded two years later. The Laboratories hold prestigious award from the European Molecular Biology Organization and the American foundation Howard Hughes Medical Institute. The research is focused to the areas of protein and metabolic engineering. Especially understanding the structure-function relationships of haloalkane dehalogenase enzymes and improve their functionalities for bioremediation, biocatalysis and biosensing.

ENANTIS, S.R.O.

http://www.enantis.com/

Enantis is a Czech R&D company founded in 2006 as the first biotechnology spin-off from Masaryk University, Brno, Czech Republic. Since 2008, the company has its own fully-equipped laboratories in the Biotechnology Incubator operated by the South Moravian Innovation Centre, where research, service and production operations are being carried out. Enantis provides consulting and development services in the field of enzyme technologies and protein engineering for biomedical, environmental, agrochemical and military-defence applications. Employing the latest technologies, the company offers its own range of products based on proteins and microorganisms.

VETERINARY RESEARCH INSTITUTE

http://www.vri.cz/

The Veterinary Research Institute in Brno (VRI) was founded in 1955. Collection of Animal Pathogenic Microorganisms which joined European Culture Collections Organisation in 1985 is a part of the Institute. The institution specializes in the veterinary medicine research. Its activity was focused on performing exact experiments with the aim to solve health problems in farm animals, protect people from zoonoses and to guarantee safety of foodstuffs and raw materials of animal origin.

02 lecturers

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Prof. RNDr. Zbyněk Prokop, Ph.D.

EDUCATION

Zbyněk Prokop is Professor in the Loschmidt Laboratories at Masaryk University, where he leads research team engaged in the study of fundamental principles of protein chemistry, enzyme mechanism and kinetics. He received Ph.D. degree in Environmental Chemistry from Masaryk University and extended his expertise during his research stays at University of Cambridge (UK), ETH Zurich (CH), TU Wien (A) and University of Groningen (NL).

PROFESSIONAL ASSIGNMENTS

His current research interests are the development of advanced biophysical methods for the structural and functional study of proteins, enzyme technology development and applications of microfluidics in life and biomedical sciences. He has co-authored 80 papers in refereed journals (h-index 21), five book chapters, five international patents and has given more than 20 invited lectures at conferences and universities worldwide. He received Alfred Bader Prize for Bioorganic Chemistry in 2005 and Werner von Siemens Award for Excellence in Innovation in 2015. He is a co-founder of Enantis Ltd, a Masaryk University biotechnology spin-out company, and a member of the Advisory Board of Centre for Technology Transfer at Masaryk University. He teaches several courses, such as Molecular Biotechnology, Methods in Biophysical Chemistry and Summer School of Protein Engineering.

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Mgr. Šárka Nevolová, Ph.D.

EDUCATION

Šárka Nevolová received her master's degree in Microbiology and Secondary School Teacher Training in Biology in 2007 and Ph.D. degree in Environmental Chemistry in 2012 from the Masaryk University in Brno. She extended her expertise during her research stay at the Institute of Chemical Process Fundamentals at the Academy of Sciences of Czech Republic and at Institute of Analytical Chemistry/Chemo- and Biosensors at the University of Regensburg in Germany.

PROFESSIONAL ASSIGNMENTS

Šárka Nevolová worked as a researcher in Loschmidt Laboratories at Masaryk University from 2009 to 2016, she is currently on maternity leave. She focused on the development, optimization and characterization of optical enzyme-based bioanalytical devices for environmental applications and military-defense. Her research interests also included immobilization and characterization of enzymes and pH indicators. She is co-author of twelve publications and two book chapters. She obtained the Award of the Dean of the Faculty of Science of Masaryk University in 2013 and the Award of the Czechoslovak Microbiological Society in 2014.

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Assoc. Prof. Radka Chaloupková, Ph.D.

EDUCATION

Radka Chaloupková received her master's degree in Theoretical and Physical Chemistry in 2001, a Ph.D. degree in Biochemistry in 2008 and habilitation from Environmental Chemistry in 2017 at the Masaryk University. She has gained an international experience, theoretical knowledge and practical skills during the three months' research visit to the University of Warwick (Coventry, UK) supported by Marie Curie Fellowship, five months' research visit at the Diamond Light Source Ltd. (Didcot, UK) and several international and national courses organized by EMBO, EPSRC, COST, Academy of Sciences and Masaryk University.

PROFESSIONAL ASSIGNMENTS

Radka Chaloupková worked as a leader of a research team focused on structure and thermodynamics of biomolecules within the Loschmidt Laboratories from 2006 till 2018. Currently she is working as Chief Science Officer in Enantis company. She has more than 17 years' experience with various biophysical techniques exploited in protein research. She is an expert in application of optical spectroscopic techniques, microcalorimetry and X-ray crystallography for analysis of structure-function relationships of enzymes. She has published more than 60 scientific papers in international journals, 5 book chapters and 2 international patents (h-index 19). She received the Award of the Dean of the Faculty of Science, Masaryk University in 2005 and the Award of the Rector of the Masaryk University in 2007.

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Ing. Andrea Schenkmayerová, Ph.D.

EDUCATION

Andrea studied Biotechnology at the Slovak University of Technology in Bratislava, Slovakia and obtained her PhD in Biotechnology while doing research at the Slovak Academy of Sciences in Bratislava. Her studies were focused on fermentation and biotransformation processes, whole-cell immobilization techniques and construction of biosensors.

PROFESSIONAL ASSIGNMENTS

After finishing her PhD she was awarded a Claude Leon Fellowship for her two year PostDoc in Biochemistry at Stellenbosch University in South Africa. She was doing research on new antistaphylococcal drug targets. She is currently doing her second PostDoc in Protein Engineering at the Masaryk University in Brno, Czech Republic, being employed by the International Clinical Research Center of St. Anne's University Hospital.

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Ing. RNDr. Martin Marek, Ph.D.

EDUCATION

Martin Marek received his master's degrees in Agricultural Engineering (2002) from the Czech University of Life Sciences (CULS) and in Molecular Biology (2007) from the Charles University, and a PhD degree in Phytopathology and Plant Protection from the CULS, all in Prague (CZ). He extended his scientific expertise in recombinant multi-expression technologies, biochemistry, biophysics and structural biology (X-ray crystallography) during post-doctoral stays at the Wageningen University (Wageningen, NL), Généthon (Paris, FR) and IGBMC (Strasbourg, FR).

PROFESSIONAL ASSIGNMENTS

Martin Marek works as a senior researcher in the Loschmidt Laboratories at the Masaryk University since 2017, where he leads a team working in molecular and structural biology. In 2018, he was awarded by Marie Skłodowska-Curie Actions individual fellowship. He is co-author >35 scientific publications, three book chapters and one international patent in the field of expression technologies, virology, molecular structural biology and drug discovery.

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Mgr. David Kovář, Ph.D.

EDUCATION

David Kovář received his master's degree in Analytical Biochemistry in 2009 and Ph.D. degree in Biomolecular Chemistry in 2015 at the Masaryk University. His Ph.D. studies were focused on immunospecific sensors exploiting nanoparticles and nanostructures with the main application of magnetic nanoparticles. He then joined the group of Prof. Maria Hepel at the State University of New York (SUNY; Potsdam, NY) as a postdoctoral researcher in physical electrochemistry.

PROFESSIONAL ASSIGNMENTS

In 2016, he joined Loschmidt Laboratories and has gained new experience and knowledge in the field of protein engineering. He extended his expertise in microfluidic during his research stays at the ETH Zurich (Switzerland) in the group of prof. deMello. Currently, his research activities relate to microfluidic platforms for screening and selection methods in directed enzyme evolution. He is also focused on development and testing of new pH sensitive fluorescent assays.

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Mgr. Michal Vašina

EDUCATION

Michal Vašina obtained his Bachelor and Master's degree at the Masaryk University in Biophysical Chemistry. During these studies, he was working in Loschmidt Laboratories, where he has developed a microfluidic platform for characterization of enzymes' activity, substrate specificity, and temperature profiles.

PROFESSIONAL ASSIGNMENTS

Michal continues in Loschmidt Laboratories during his Ph.D. studies in Environmental Health Sciences. He is focused on the development of novel microfluidic platforms, to be used in metabolic and protein engineering.

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RNDr. Jaroslav Turánek, CSc.

EDUCATION

He graduated from the Faculty of Science at Masaryk University, specialization biochemistry, and obtained the degree RNDr. in 1982. In 1987, he terminated post-graduated studies in the field of biochemistry and physical chemistry at the Faculty of Science, MU and South Bohemian Biological Centre, and obtained the degree CSc. Jaroslav Turánek extended his expertise during a research stay at GTC, Imperial College in London and by taking a number of specialist courses.

PROFESSIONAL ASSIGNMENTS

Jaroslav Turánek is Head of the Department of Pharmacology and Immunotherapy, Veterinary Research Institute, Brno, Czech Republic. His current research interests are development of recombinant vaccines and molecular adjuvants, immunotherapy of infectious diseases and cancer, nanodelivery systems for drugs (e.g., liposomes, micelles and dendrimers), and *in vivo* and *in vitro* pharmacological and toxicological models. Dr. Turánek is a lecturer of the following courses: Advanced Immunology (Masaryk University), Immunochemistry (Technical University), and Cellular Biotechnology (Technical University). He is a member of the Committee for state examination (Masaryk University) and supervisor of postgradual and pregradual students.

03 manuals

IN SILICO CLONING OF A HALOALKANE DEHALOGENASE

location: Loschmidt Laboratories, Kamenice 5/A5 room 114 or A17/332
lecturer: Ing. Andrea Schenkmayerová, Ph.D. (andrea.schenkmayerova@fnusa.cz)

I. WORKFLOW

During this practical course/computer exercise we will perform all steps of the cloning of the haloalkane dehalogenase gene (*dhaA*) from *Rhodococcus rhodochrous*. The gene will be cloned in the pET21b vector for recombinant (over)expression in *Escherichia coli*.

- design of the primers for cloning the dhaA gene in pET21b
- *in silico* PCR reaction is performed using the gene sequence and the created primers to create the RE site containing DNA fragment
- the restriction digest is performed on both the vector and insert using the chosen restriction enzymes
- the two fragments are ligated to obtain the final plasmid
- finally the sequence obtained from sequencing is compared with the plasmid that was made in CloneManager [1]

II. MOTIVATION

An important step in biochemical research and biocatalytic processes is the production of the protein of interest. Therefore, the corresponding gene will be transferred into a vector (e.g. plasmid) which can be transformed into expression hosts. This exploitation of nature allows scientists to express their protein of interest at larger scale but also to alter it by mutagenesis or attach specific tags to facilitate purification.

III. THEORETICAL BACKGROUND

What is Molecular Biotechnology? In its broadest sense, molecular biotechnology is the use of laboratory techniques to study and modify nucleic acids and proteins for applications in areas such as human and animal health, agriculture, and the environment. Thus, molecular biotechnology is the branch of biology that deals with the formation, structure, and function of macromolecules essential to life, such as nucleic acids and proteins, and especially with their role in cell replication and the transmission of genetic information. Molecular biotechnology results from the convergence of many areas of research, such as molecular biology, microbiology, biochemistry, immunology, genetics, and cell biology (Figure 1).

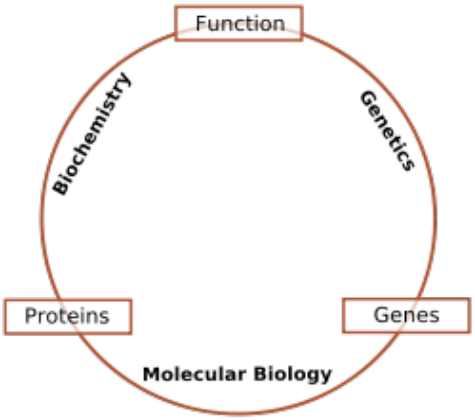


Figure 1. Schematic relationships the different research areas in Molecular biotechnology (biochemistry, genetics, and molecular biology).

More recently, much work has been done at the interface of molecular biology and computer science, namely in bioinformatics and computational biology. Molecular biotechnology is an exciting field fuelled by the ability to transfer genetic information between organisms with the goal of understanding important biological processes or creating a useful product. The central dogma (Figure 2) of molecular biology confirms the importance of genetic information or DNA (and RNA) in nature and the biological sciences.

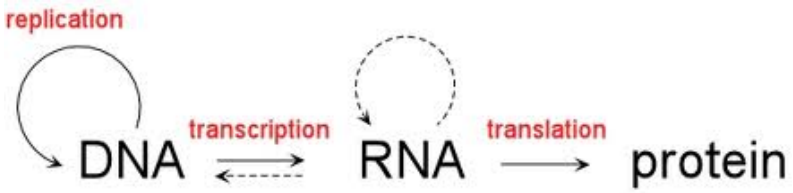


Figure 2. The Central dogma of molecular biology is an explanation of the flow of genetic information within a biological system

The transfer, use or changing of genetic information (manipulation of DNA) is a very important step – and in many cases the first experimental step – in biotechnological research since this enables to introduce, delete or alter the DNA and hereby change and study the characteristics of cells, proteins, enzymes, … Molecular biology is the branch of biology that deals with the manipulation of DNA so that it can be sequenced or mutated or used to study the biological effects of the mutation(s), gene product, …

In a standard molecular cloning experiment, the cloning of the desired DNA fragment can be seen as a seven step process (Figure 3). Several types of DNA manipulations are used here, including amplification, digestion and ligation by polymerase chain reaction (PCR), restriction enzymes and a ligase, respectively. Standard protocols for molecular cloning and DNA handling are well described in several (practical) handbooks [2].

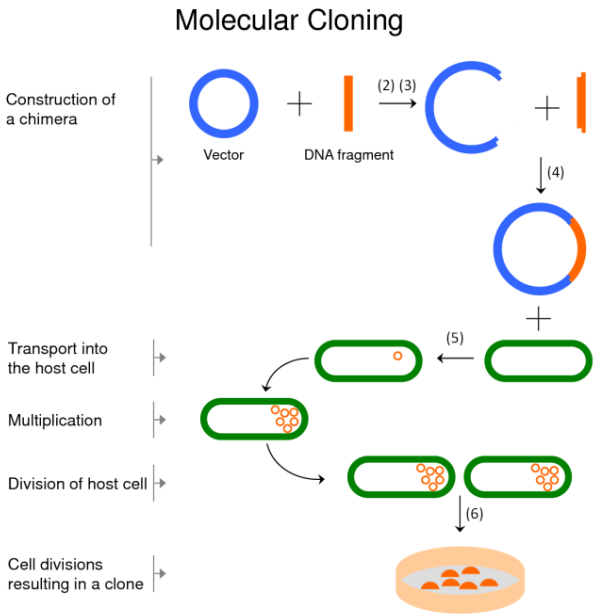


Figure 3. The different steps of a typical molecular cloning process. Numbers between brackets correspond to the seven step mentioned in the text.

The seven steps of a standard molecular cloning experiment

1. Choice of a host organism and a cloning vector

Several host organisms and molecular cloning vectors are available, however, for the vast majority of molecular cloning experiments a lab strain of the bacterium *E. coli* is used together with an appropriate plasmid cloning vector. *E. coli* and its vectors are widely used due to their relative simplicity, yet technically sophisticated nature, versatility, and wide availability. In addition, they offer rapid growth of recombinant organisms with minimal equipment.

2. Preparation of vector DNA

To prepare the cloning vector to accept the gene of interest it is treated with restriction endonucleases in order to create (specific) sites at which the foreign DNA can be inserted.

3. Preparation of DNA to be cloned

The gene of interest can be picked up from genomic DNA extracted from the organism of interest. Any tissues source can be used as long as the DNA is intact. In case of starting from RNA, a reverse transcriptase step is needed to create complementary DNA (cDNA). Nowadays, it is also possible to order synthetic DNA sequences, for example from hard to amplify fragments, for variants containing multiple mutations, eukaryotic genes (cfr. introns and RNA splicing) or unnatural sequences. Artificial gene synthesis has the advantage of to build in or removal of specific restriction sites as well as codon optimization for your specified host organism.

PCR methods (Figure 4) are the techniques used for amplification of specific DNA or RNA (RT-PCR) sequences prior to molecular cloning. While picking up the gene of interest one can simultaneously 'attach' the desired restriction sites to the PCR product by using primers containing these restriction sites.

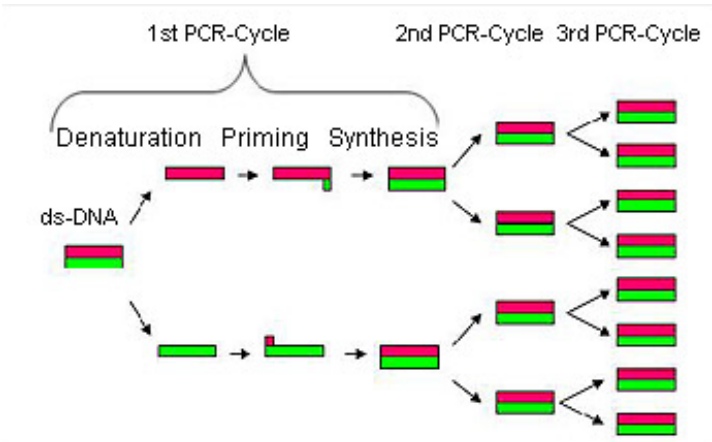


Figure 4. Amplification of a DNA fragment is achieved by PCR

4. Creation of recombinant DNA

In this step, the vector and foreign DNA are mixed at appropriate concentrations and an enzyme called DNA ligase will covalently link the ends together, creating a single DNA molecule which can be introduced into the host organism. Nowadays, new techniques have been developed like restriction/ligation free cloning, such as Gibson assembly [3] which allows for the joining of multiple DNA fragments in a single, isothermal reaction.

5. Introduction of recombinant DNA into a host organism

The *in vitro* prepared DNA can now be inserted back into a living cell, namely the host organism. Several options are available depending upon the experimental method and host cells (e.g. transformation, transduction, transfection, electroporation).

6. Selection of organisms containing recombinant DNA

The introduction of recombinant DNA into host organisms is usually a low efficiency process. Only a small number of cells will actually take up DNA. Therefore, efficient selection of the good clones is needed. When using bacterial cells, this can easily be achieved by a selectable marker on the vector. Most common are antibiotic resistance markers which allow the good clones to survive in the presence of antibiotic while the other cells die.

7. Screening for clones with desired DNA inserts and biological properties.

To make sure that the surviving colonies contain your gene of interest a very wide range of

experimental methods is available, including the use of nucleic acid hybridizations, antibody probes, polymerase chain reaction, restriction fragment analysis and/or DNA sequencing. DNA sequencing is also used to confirm the absence of unwanted mutations and correct integration of the DNA fragment.

PCR Primer Design Guidelines

(adapted from http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html)

1. Primer Length: The generally accepted optimal primer length for PCR primers is 18-22 bp. This length is long enough to ensure adequate specificity and short enough for primers to bind easily to the template

2. Primer Melting Temperature (T_m): T_m is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. T_m in the range of 52-58°C generally produce the best results. The formula T_m = 2°C x (A+T) + 4°C x (G+C) is popular for its simplicity and roughly accurate prediction of oligonucleotide T_m.

3. Primer Annealing Temperature: Too high T_a will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low T_a may possibly lead to non-specific products caused by a high number of base pair mismatches.

4. GC Content: The GC content (the number of G's and C's in the primer as a percentage of the total bases) of primer should be 40-60%.

5. GC Clamp: The presence of G or C bases within the last five bases from the 3' end of primers (GC clamp) helps promote specific binding at the 3' end due to the stronger bonding of G and C bases. More than 3 G's or C's should be avoided in the last 5 bases at the 3' end of the primer.

6. Primer Secondary Structures: Presence of the primer secondary structures produced by intermolecular or intramolecular interactions can lead to poor or no yield of the product. Interactions can be intramolecular resulting to hairpin formation or self-dimerization or intermolecular when different primers in the PCR mix form a dimer.

7. Repeats (repetitive presence of di-nucleotides) and Runs (long runs of a single base) should be avoided because they can misprime.

8. 3' End Stability: It is the maximum ΔG value of the five bases from the 3' end. An unstable 3' end (less negative ΔG) will result in less false priming.

9. Avoid Template Secondary Structure, Avoid Cross Homology, …

Luckily, these rules have been integrated in Molecular Biology software like CloneManager and free online primer design tools.

In the case of this cloning experiment, specific sites have to be introduced where the restriction enzymes (RE) can cleave the DNA for easy and correct fusing of the insert (gene of interest) and plasmid vector. Therefore, it is important to choose the right restriction enzymes for cloning and add the according RE sites to the primers. Some simple rules: the chosen restriction enzymes cannot cleave anywhere else in the insert or vector fragment, sticky ends are better than blunt ends, non-compatible ends are desired as they allow unidirectional cloning.

IV. DESIGN OF EXPERIMENT

Equipment:

– PC with CloneManager software
– sequence of DhaA and pET21b plasmid

Workflow:

1. design of the primers for cloning the *dhaA* gene in pET21b
2. *in sillico* PCR reaction is performed using the gene sequence and the created primers to create the RE site containing DNA fragment
3. the restriction digest is performed on both the vector and insert (created PCR fragment) using the chosen restriction enzymes
4. the two fragments are ligated to obtain the final plasmid
5. finally the sequence obtained from sequencing is compared with the plasmid that was made in CloneManager [1].

V. EXERCISES

1. Design primers for the cloning of the haloalkane dehalogenase gene (*dhaA*) from *Rhodococcus rhodochrous* in the pET21b vector for recombinant expression in *Escherichia coli*. Write down the primer and underline the restriction enzyme sites.

Fwd:

Rev:

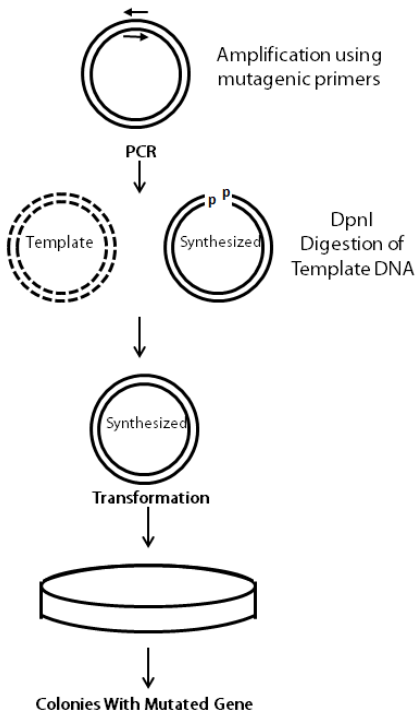
2. Calculate the melting temperatures of both primers using the A/T=2°C, G/C= 4°C rule and compare it with the melting temperatures found using CloneManager.

Check the New England Biolabs website (www.NEB.com) for more information about the restriction enzymes for question 3-5.

3. What is the advantage of using two different restriction enzymes for the cloning of a gene into a vector, for example *Nde*I and *Eag*I instead of *Nde*I at both ends? Which are the two problems that are avoided?

4. What is the advantage of using for example *Nde*I and *Eag*I for cloning rather than *Nae*I and *Bsr*BI?

5. Below you find a protocol for mutagenesis. You created a mutant library and accidently added *Dpn*II to the PCR mix instead of *Dpn*I. Do we have a problem now? Important to know: *E. coli* has a system for DNA methylation, which is not present in a PCR reaction.



6. You are provided with two sets of sequencing data (Fwd + Rev). Which one confirms the correct sequencing and which sample contains the error? What is the result of the error at DNA level?

VI. LITERATURE

1. CloneManager (SciEd software, USA), http://www.scied.com/pr_cmpro.htm
2. Green MR & Sambrook J (2012) Molecular cloning: A laboratory manual, 4th ed. (Cold Spring Harbor Laboratory Press, New York)
3. Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, III, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature Methods 6:343-345

RECOMBINANT PROTEINS: FROM SMALL- TO LARGE-SCALE EXPRESSION TECHNOLOGIES

location: Loschmidt Laboratories, Kamenice 5/A13, 2nd floor, room 332
lecturer: Ing. RNDr. Martin Marek, Ph.D. (martin.marek@recetox.muni.cz)
Ing. Andrea Schenkmayerová, Ph.D. (andrea.schenkmayerova@fnusa.cz)

I. WORKFLOW

Selection of an expression host – purpose of protein production – combinatorial screening in small-scale formats – bench-scale and large-scale (fermentor) overproductions – downstream processing and purification strategies – quality/quantity check and final formulation

II. MOTIVATION

Proteins are produced in heterologous systems because of the impossibility to obtain satisfactory yields from natural sources. The production of soluble and functional **recombinant proteins** is among the **main goals in the biotechnological and pharmaceutical industries**. The selection of an optimal expression organism (host) and the most appropriate growth conditions to minimize the formation of insoluble proteins have to be done according to the protein characteristics and downstream requirements. Escherichia coli is the most popular recombinant protein expression system despite the great development achieved so far by eukaryotic expression systems. However, it is important to mention that E. coli expression system possesses, in many cases, severe limitations for a successful recombinant protein production. Therefore, eukaryotic systems, including mammalian cells, insect cells, yeasts, filamentous fungus, and microalgae, are an important alternative for the production of those difficult-to-express proteins. During this course we will combine theoretical designs with practical demonstrations concerning all aspects of recombinant protein (multi)-expressions, ranging from small- to large-scale (fermentor) overproduction technologies. In the second part, we will focus on the downstream processing and purification strategies. The students will get knowledge on:

- How to select a heterologous system (expression host)
- Pilot small-scale (transient) expression screening to determine optimal expression conditions, and buffer screening strategies
- Scaling-up of production process, bench-scale and large-scale (fermentor) productions
- Harvesting process and timing
- Downstream processing and purification strategies to yield highly pure recombinant proteins in sufficient amounts
- Control (quality/quantity) of the purified protein, final formulation and long-term storage

III. THEORETICAL BACKGROUND

Selection of **expression organism (host)**: each heterologous expression system has benefits and drawbacks with respect to their capacity for recombinant protein production (**Figure 1**). The gram-negative bacterium ***Escherichia coli*** is frequently the first expression host chosen for the production of a recombinant protein, owing to the rapid, affordable and technically straightforward culturing associated with its use. The *E. coli* system offers a mean for rapid, high yield, and economical production of recombinant proteins (**Figure 2**). However, high-level production of functional eukaryotic proteins in *E. coli* may not be a routine matter, sometimes it is quite challenging. Techniques to optimize heterologous protein overproduction in *E. coli* have been explored for host strain selection, plasmid copy numbers, promoter selection, mRNA stability, and codon usage, significantly enhancing the yields of the foreign eukaryotic proteins.

Yeasts is a single-celled eukaryotic organism capable of producing very large quantities of recombinant protein. ***Pichia pastoris*** is the most used strain of first choice for yeast expression. The **baculovirus/insect cell expression vector system (BEVS)** is a popular choice for the production of recombinant proteins, particularly those requiring complex post-translational modifications or integral membrane proteins. **Mammalian cell-based expression** (e.g. HEK293 cell line) is the dominant system for the production of therapeutic recombinant proteins. Their capacity to handle complex post-translational modifications, folding and assembly of recombinant proteins and protein complexes is superior to other systems. The Leishmania tarentolae extract (LTE) in vitro translation cell-free expression system is a rapid, convenient, flexible and cost effective tool to produce recombinant proteins for biochemical, biophysical and structural analysis.

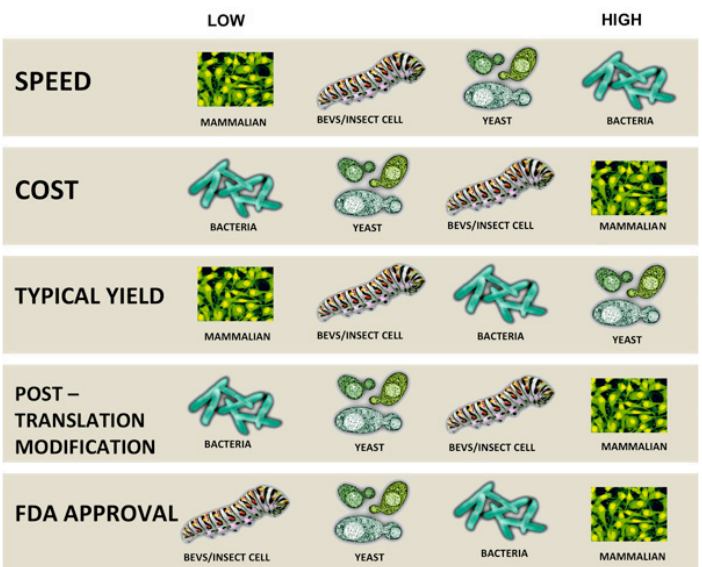


Figure 1. Comparison of the most industrially used expression systems.

At the beginning of a protein expression project, **small-scale expression screenings** in minimal volumes (2-6 mL) are recommended prior to running large-scale overproductions. The pilot small-scale expression tests are designed to test different expression parameters (host strain selection, timing of induction and harvesting, expression temperature, pH, ionic strength etc.) to find an optimal condition and buffer screening. **Large-scale expression** in bacteria, yeast, insect and mammalian cell systems in culture volumes from 400 mL to 10 L are available in shake flask and from 2 L to 20 L in a stirred-tank bioreactor or up to 25 L in a Wave bioreactor. Large-scale production in **bioreactor (fermentor)** is very complex procedure where several factors play a crucial role on the performance of the culture. Composition of cultivation broth, sterilization efficiency, proper agitation and aeration, temperature or feeding strategy can all together influence final yield of biomass and recombinant protein from the fermentation. Since culture in fermentor is half opened system, it is very susceptible to contamination. It is very important for the operator of the fermentor to maintain sterility throughout the fermentation. In this practice, the students will learn how to assemble the fermentor vessel and prepare it for sterilization, how to calibrate pH and DO (dissolved oxygen) sensors and set up the fermentation together with creation of recipes for fed-batch cultivations.

Recombinant protein expression in *E. coli*: basic principles and advantages versus limitations

ADVANTAGES
- Inexpensive setup and running costs
- High recombinant protein production levels
- Short timeline from cloning to protein recovery (1 week)
- Limited technical knowledge required for culturing
- Scalability from small (2 mL) to very large industrial culture (>10,000 L) volumes

DISADVANTAGES
- Inability to perform post-translation modifications (PTMs)
- Limited formation of disulphide bond

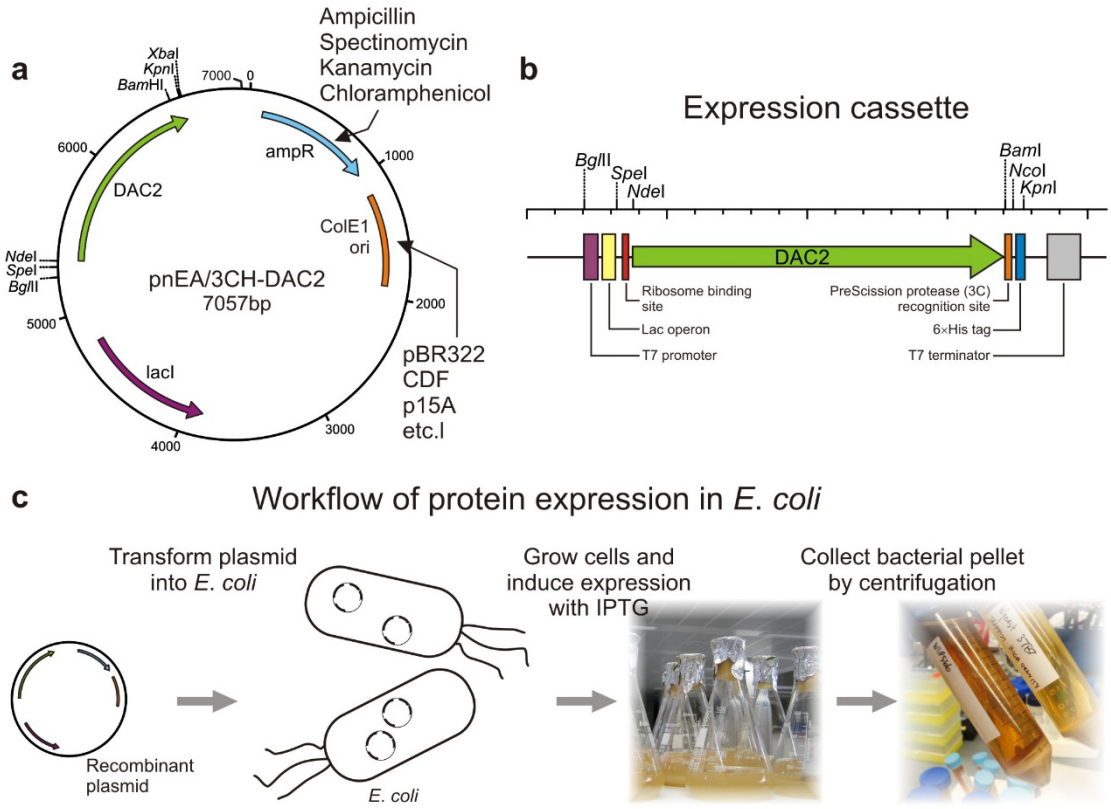


Figure 2. Schematic representation of the plasmid used for the protein expression in *E. coli*. (A) Map of the typical expression plasmid. The gene of interest (DAC2) is inserted between NdeI and BamHI restriction sites. Selection in E. coli is performed by the beta-lactamase ampicillin resistance gene (ampR). Origin of replication sequence (ColE1) is available for maintenance in *E. coli* cells, and the lacI gene is present for expression of the Lac repressor protein. (B) Details of the expression cassette. The gene of interest (DAC2) is controlled by the T7 promoter and T7 terminator. The DAC2 gene is in cloned in frame with a sequence coding for a C-terminal thrombin cleavage site followed by a poly-histidine tag. (C) Overview of recombinant protein expression workflow in *E. coli*.

Recombinant protein purification: the aim of a purification procedure is to obtain a highly pure and stable protein at an appropriate concentration in a buffer compatible with the intended downstream application (**Figure 3**). Chromatographic techniques are the most powerful and commonly used means of purifying recombinant proteins. Each technique separates proteins based on different properties, so it is often advantageous to combine several types to maximise separation of the recombinant protein from host cell proteins. The use of **fusion tags** (polyhistidines, Strep-tag, GST, MBP, SUMO, thioredoxin etc.) not only facilitates affinity chromatography steps, but also can dramatically improve protein expression, stability, resistance to proteolytic degradation and solubility. Variety of **proteases** (thrombin, protease 3C, TEV etc.) for fusion tag removal during downstream processing is available.

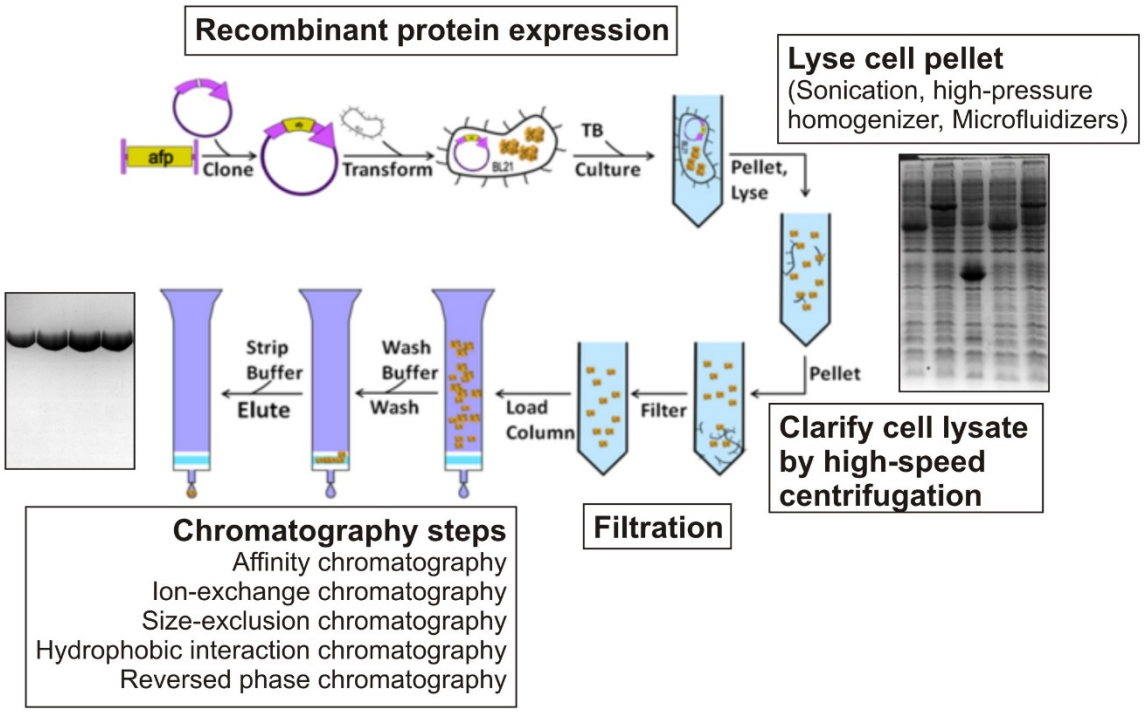


Figure 3. Workflow of recombinant protein purification includes (i) the lysis of a cell pellet by sonication or high-pressure homogenizer, (i) the clarification of cell lysate by high-speed centrifugation, (iii) filtration, and (iv) one or more chromatographic purification steps.

Recombinant protein characterization: an aggregation problem. A key challenge in recombinant protein production is to maintain and store the target protein in a soluble and stable form. Protein aggregation can compromise protein function and thus it is necessary to overcome this challenge to generate functionally active protein (**Figure 4**). Protein aggregates can be detected by (i) analytical size-exclusion chromatography (SEC), (ii) dynamic light scattering (DLS), and (iii) analytical ultracentrifugation (AUC).

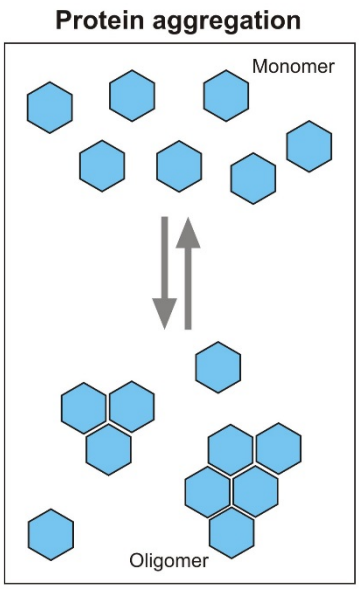


Figure 4. Schematic representation of unwanted protein aggregation problem that can compromise protein (enzyme) function.

How to avoid (minimize) the protein aggregation problem:

- Culture conditions (e.g. reducing temperature)
- Buffer composition (ionic strenght, pH, reducing agents)
- Presence co-factors (Acetyl-CoA, metal ions)
- Fusion tags (Trx, MBP, SUMO)
- Minimising sample handling
- Avoiding time delays between purification steps
- Performing purification steps at 4°C
- Store purified proteins in -80°C

!!! Concluding remark: The recombinant protein production project is ultimately determined by the end-use of the recombinant protein. The overall success of a project lies in an effective project design.

IV. THEORETICAL DESINGS AND PRACTICAL DEMONSTRATIONS

Small-scale expression tests: finding optimal condition and buffer screening

1. Day 1, the E. coli BL21 (DE3) cells are transformed with the expression plasmid. Transformed bacteria are seeded on a 6-well plates containing 2xLB agar medium with ampicillin (100 µg/ml); the plates are then incubated at 37°C overnight.
2. Day 2, ampicillin-resistant colonies are inoculated in minicultures (2 ml 2xLB, 0.5% glucose and 100 µg/ml ampicillin).
3. After incubation (275 rpm, 37°C) for 6h, induction is done at 22°C overnight by adding 2 ml of 2xLB medium with 0.6 % lactose, 0.5 mM IPTG, 20 mM HEPES pH 7.4 and ampicillin 100 µg/ml.
4. Day 3, cells are centrifuged (3,500 rpm, 10 min), re-suspended in four different lysis buffers (10 mM Tris-HCl pH 8.0 with 50 mM, 100 mM, 200 mM or 400 mM KCl) and lysed by sonication (1 min, 40 % amplitude).
5. The lysates are clarified by centrifugation (4,000 rpm, 20 min), and then the supernatants are loaded on Talon Superflow Metal Affinity Resin (Clontech) pre-equilibrated in the lysis buffer.
6. The samples are incubated on roller shaker (4°C, 2 h), and the resins are then washed with the corresponding lysis buffers twice by centrifugation (1,000 rpm, 2 min).
7. Finally, the resins are re-suspended in Laemmli Buffer (40 µl), and the proteins bound on the resin are analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and visualised by Coomassie Brilliant Blue staining.

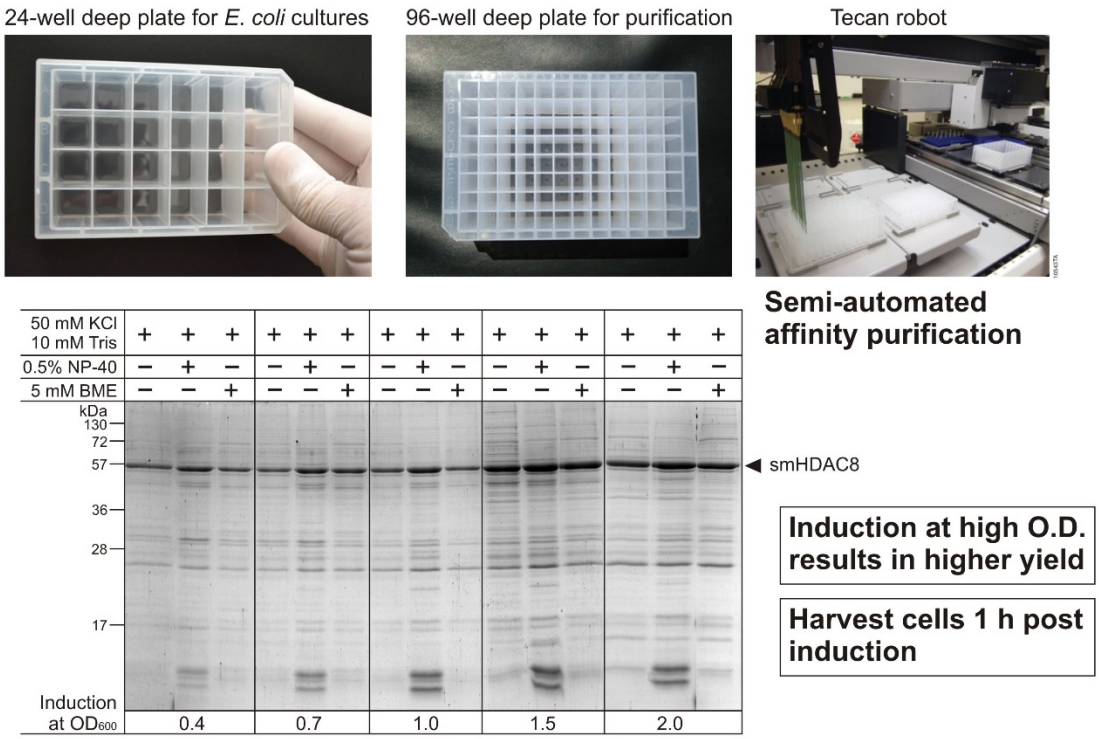


Figure 5. The example of small-scale expression screening in 24-well plates that revealed induction and harvesting times as the key parameters in successful production of soluble smHDAC8 protein.

Protocol for large-scale recombinant protein production in bioreactor (fermentor)

Protocol for assembly of the fermentation vessel:

1. Prepare and dissolve all components of the fermentation broth. The volume of the medium should not be higher than working volume of the fermentor (usually 2/3 of total volume).
2. Calibrate pH probes (See Protocol for calibration of pH probes).
3. Pour dissolved medium to the vessel, add antifoaming reagent Struktol SB2020 to the final concentration 100 µL/L.
4. Insert baffles to the vessel. Make sure the O-ring is present on the top of the vessel.
5. Attach the lid to the glass part of the vessel by tightening large screws on the top of the lid.
6. Attach small plastic tubes to reagent inlet ports and sampling port, and big tubes to the sparger inlet and gas exhaust. Fix by tightening of screw clamps if necessary.
7. Attach the pH and DO probes to the vessel lid.
8. Place rubber septa to remaining ports and fix them with metal rings.
9. Close all plastic tubes with clamps, close sparger inlet tube with Hofman screw clamp.
10. Insert air filter to sparger inlet and gas exhaust tubing, cover ends with aluminium foil. Cover all plastic tube ends with aluminium foil.
11. Make sure all ports and tubes are closed, except for exhaust tube.
12. Sterilize the fermentor in autoclave.
13. After sterilization, let the fermentor cool down to room temperature.

Protocol for calibration of pH probe:

1. Connect the pH probe to the connector on fermentor control unit.
2. Select calibration mode on the control panel.
3. Put the probe in the container with calibration solution with pH 4.1.
4. Wait until the signal is stable, confirm the value.
5. Rinse the probe with distilled water.
6. Repeat measurement with calibration solution with pH 7 or 9.
7. Confirm new calibration slope.

Protocol for preparation of seed cultured:

1. Prepare night culture by picking one colony of freshly transformed E. coli BL21(DE3) cells to 10 mL LB medium with respective antibiotics.
2. Cultivate the cells overnight at 37 °C and 200 rpm.
3. Approximately 8-10 hrs prior to fermentation, prepare seed culture by transferring 2 mL of night culture to 200 mL of LB medium with respective antibiotics.
4. Incubate at 37 °C and 200 rpm.

Preparation of the vessel for fermentation protocol:

1. Fill up the jacket with tap water if necessary.
2. Connect the hose connectors to the thermostat.
3. Connect all probes to appropriate connectors on the control unit.
4. Turn on heating by setting the fermentation temperature (30 °C in our case).
5. Attach the motor on the top of the lid.
6. Wait until the DO probe is polarized.
7. Calibrate DO probe (See Protocol for calibration of DO probe).
8. Connect tubes to appropriate pumps and solution bottles (citric acid and NaOH).
9. Connect the sparger inlet tube to mass flow control.
10. Release all clamps on tubing with exception of clamp on sampling tube.
11. Set the cultivation parameters on control panel or in computer.
12. Inoculate fermentor with seed culture. For this purpose, seed culture volume corresponding to 1/100 of volume of medium in fermentor is used.
13. After 1 hr, add 2 g/L lactose to induce expression of heterologous protein. 14. Let the fermentation run overnight, harvest the cells by centrifugation and store the culture at -80 °C.

Protocol for calibration of DO probe:

1. Wait until the temperature of the medium reaches demanded temperature. Be sure the stirring is ON.
2. Connect bottle with pure nitrogen to sparger inlet tube via the sterile filter.
3. On control panel, select DO probe calibration module.
4. Wait until medium is fully saturated by nitrogen. Collect signal for 0 % O2.
5. Connect air tube to the sparger inlet.
6. Wait until the medium is saturated with air. Collect signal for 100 % O2.
7. Confirm new calibration slope.
8. This calibration has to be done prior to every fermentation! Oxygen solubility differs with medium composition and viscosity and its temperature.

Settings for batch fermentation:

1. Temperature 30 °C
2. Stirring cascade parameters: 30 % saturation, setpoint 500, minimum 500, maximum 1000 rpm
3. Aeration 0.5 vvm
4. 4. pH 7

Protocol for sampling from the fermentor:

1. Connect the sterile syringe to sampling tube. Release the clamp. Suck ca. 5 mL to rinse the tube with fresh culture. Fix the clamp again. Unplug the syringe and discard whole volume. Repeate this to take your sample. Transfer the culture from syringe to sterile glass tube.
2. Transfer 1 mL of the culture to a plastic cuvette and measure optical density at 600 nm.
3. Prepare 6 tubes with 900 µL of PBS buffer. Transfer 100 µL of the culture to first tube, mix properly. Transfer 100 µL of suspension from first to second tube, repeat until the sixth tube. Take 100 µL of the final suspension and spread it with glass spreader on two plates with Plate Count Agar. Incubate the plates overnight at 37 °C.
4. Pre-weigh 15 mL plastic falcon, transfer 10 mL of the culture to the falcon and centrifuge for 5 min at 5,000 g. Discard supernatant and weigh the cells.
5. Re-suspend the cells in 1 mL of water, transfer the suspension to pre-weighted aluminium foil. Put the foil with suspension for 2 hrs to dryer (110 °C). Weigh the foil again, calculate the dry cell weight.

Equipment: Biostat B Plus bench-top fermentor (Sartorius Stedim) – Labfors 3 bench-top fermentor (Infors HT) – table centrifuge – ultracentrifuge Avanti J30I (Beckman-Coulter) – LabStak M10 membrane filtration system (AlfaLaval) – spectrophotometer and plastic cuvettes – bench-top dryer STZ 5,4 (FALC)

Purification of His-tagged recombinant protein

1. The following protocol is provided considering the use of cell pellets from 3 L of cultures. First, if required, thaw the re-suspended cell pellets. Adjust the volume of the cell re-suspension to 40 mL per liter of culture (i.e. final volume of 120 mL for 3 L of culture) using the lysis buffer (identical to the resuspension buffer).
2. Lyse the cell suspension using a high-pressure homogenizer at high pressure (18,000 psi) using a single round of lysis. After the lysis, centrifuge the disrupted cell suspension at 210,000 × g for 1 h and collect the supernatant in an ice-cold bottle.
3. Apply the supernatant to a column with 2 ml of Talon Metal affinity resin pre-equilibrated in lysis buffer. Briefly, connect the column with pre-equilibrated Talon resin to a peristaltic pump and pump the supernatant from Step 1 through the column with Talon resin at a flow rate of 4.0-5.0 ml/min. Every 30 min, disconnect the column from the peristaltic pump and mix the resin to release the excess pressure. After the loading, wash the column extensively with approximately 100 ml of the lysis buffer to remove non-specifically bound proteins.
4. Release the protein from the Talon resin by thrombin treatment. Briefly, re-suspend the Talon resin with bound smHDAC8-His fusion protein with the lysis buffer and transfer it to a new sterile 15-mL Falcon tube. The volume of the resin suspension should be approximately 5 ml. Add 60 µl of thrombin (1U/ µl) and place the tube on a rolling mixer overnight at 4°C.
5. Next morning, separate the released protein from the Talon resin particles by applying the resin suspension onto an Econo-Pac column and collect the unbound flow-through fraction into a fresh sterile 15-mL Falcon tube. Wash the resin with additional 3 ml of the lysis buffer to harvest all thrombin-released protein. Check the presence and concentration of protein in the flow-through fraction by the Bio-Rad Protein Assay.
6. Load the flow-through containing smHDAC8 enzyme from the Step 4 onto a 1-mL HiTrap Q FF column pre-equilibrated with low-salt ion-exchange chromatography buffer (10 mM Tris-HCl pH=8.0; 50 mM KCl). Elute the bound protein with a gradient of KCl (50 mM to 1 M KCl): see **Figure 5A** for a typical ion-exchange purification of smHDAC8. Identify fractions containing the protein by SDS-PAGE.
7. Pool the peak fractions from the ion-exchange chromatography from Step 5 and load this sample onto a gel filtration column (16/60 Superdex 200) equilibrated with gel filtration buffer. Identify fractions containing the target protein by SDS-PAGE. See **Figure 5B** for a typical gel filtration purification of smHDAC8.

8. Pool the peak fractions from gel filtration from Step 6, and concentrate the smHDAC8 protein with an Amicon Ultra centrifugal filter unit to reach a final concentration of 2.5 mg/ml. Check purity of the purified smHDAC8 enzyme by SDS-PAGE and determine protein concentration by the Bio-Rad Protein Assay reagent. Flash-freeze the final product with liquid nitrogen and store at -80°C.

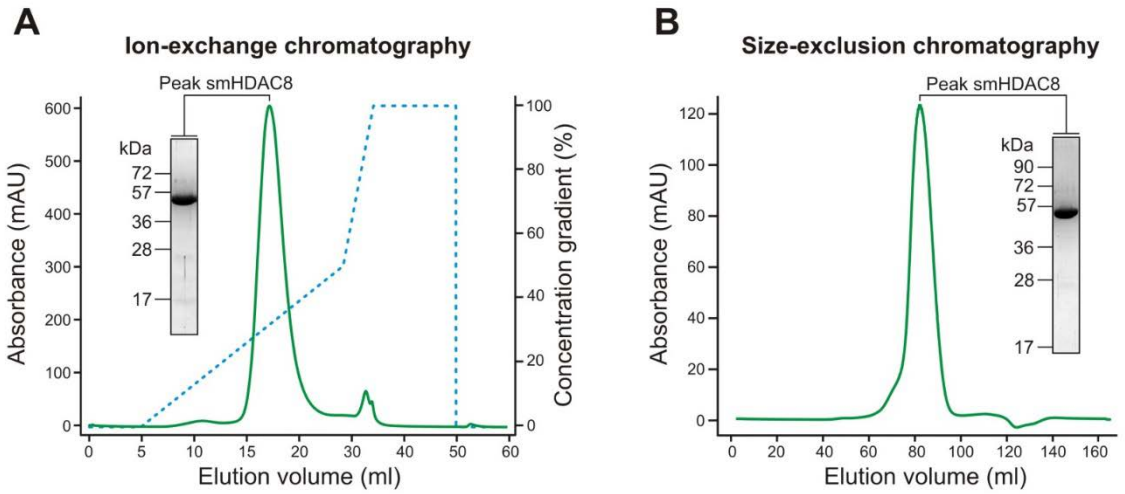


Figure 5. (A) Chromatogram of ion-exchange purification of smHDAC8 protein. The gradient used for this purification step is displayed (blue broken line). (B) Chromatogram of gel filtration purification of smHDAC8 protein.

Quality/quantity control of the purified protein

1. **Protein concentration** is measured on DeNovixR DS-11 Spectrophotometer (DeNovix Inc., USA) using the absorbance A280 mode, where molar extinction coefficient (ε) and molar mass (Mr) are provided.
2. Proper protein folding is detected by **circular dichroism (CD)**. CD spectra are recorded at protein concentration 0.2 mg.mL-1 and at 20 °C using a spectropolarimeter Chirascan (Applied Photophysics). Data are typically collected from 185 to 260 nm, at 100 nm/min, 1 s response time and 1 nm bandwidth using a 0.1 cm quartz cuvette. Each spectrum shown is the average of five individual scans and is corrected for absorbance caused by the buffer. Collected CD data are expressed in terms of the mean residue ellipticity (ΘMRE) using the equation

$$\Theta_{\mathrm{MRE}} = \frac{\Theta_{\mathrm{obs}} \cdot M_{\mathrm{w}} \cdot 100}{n \cdot c \cdot l}$$

where Θ_{obs} is the observed ellipticity in degrees, *Mw* is the protein molecular weight, *n* is number of residues, *l* is the cell path length, *c* is the protein concentration (in mg/ml) and the factor 100 originates from the conversion of the molecular weight to mg/mol.

3. **Dynamic light scattering (DLS):** The dynamic light scattering (DLS) experiments are conducted typically with protein solutions (1-2mg/ml) in a corresponding buffer containing using instrument DynaPro NanoStar (Wyatt). Protein solutions are centrifuged (13,000 rpm/10 min) prior to DLS measurement in order to remove impurities. Before measurement temperature is equilibrated to 20°C.
4. **Differential scanning fluorimetry (nanoDSF) measurements:** Thermal stability of recombinant proteins is analysed by a label-free differential scanning fluorimetry (DSF) approach using a Prometheus NT.48 instrument (NanoTemper Technologies). Briefly, the shift of intrinsic tryptophan fluorescence of proteins upon gradual temperature-triggered unfolding (temperature gradient 20–95°C) is monitored by detecting the emission fluorescence at 330 and 350 nm. The measurements is carried out in nanoDSF-grade high sensitivity glass capillaries (NanoTemper Technologies) at a heating rate of 1°C/min. Protein melting points (Tm) are inferred from the first derivative of the ratio of tryptophan emission intensities at 330 and 350 nm.

V. LITERATURE

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VI. HOMEWORK

Your goal is to recombinantly produce of 2 g of highly pure haloalkane dehalogenase DhaA enzyme from Desulfobacterium autotrophicum. See amino acid and corresponding nucleotide sequences below:

>ACN15444.1 DhaA [Desulfobacterium autotrophicum HRM2]
MVTRDPAEQSRNIKSPGIRRKINGTMVGTKDFYEIYPFVPHFMTLDRHKLHYLDLGKGSPVVMVH
GNPTWSFYFRRLARDLSVNHRVIVPDHMGCGLSDKPSTRDYDYTLASRVRDLDRLIQSLDLGKKITL
VVHDWGGMIGCAWALRHLDRIDRIIITNTSGFHLPGAKRFPLRLWLIKYLPWFAIPGIQGLNLFAR
AALYMAPKQSLSTTVRQGLTAPYNSWKNRIATLKFVQDIPLSPRDKSYELVNWVDTHLEGLKTVP
MMILWGRHDFVFDLSFLDEWNKRFPHAQTHIFEDAGHYLFEDKPDETSNLIKKFIEEY

>CP001087.1:2679075-2680040 Desulfobacterium autotrophicum HRM2, complete genome
ATGGTAACCAGGGATCCAGCGGAGCAAAGCAGAAACATCAAAAGTCCGGGCATCAGAAGAA
AGATCAACGGCACCATGGTCGGCACCAAGGATTTTTATGAAATATATCCCTTTGTTCCCCATTT
CATGACCCTGGACCGGCACAAACTCCACTACCTTGACCTGGGTAAGGGAAGTCCAGTTGTCAT
GGTCCACGGTAATCCCACCTGGTCGTTTTATTTTCGCAGGCTTGCCCGGGATCTTTCGGTGAAC
CACCGGGTCATTGTTCCCGACCACATGGGGTGCGGCCTGTCTGACAAGCCGTCCACCAGGGAT
TACGACTATACCCTTGCATCAAGGGTCCGGGACCTGGACCGTCTGATCCAGAGCCTTGACCTTG
GAAAAAAGATCACCCTGGTCGTCCACGACTGGGGCGGTATGATCGGCTGCGCCTGGGCCCTTC
GTCACCTGGACAGGATAGACAGGATCATCATCACCAACACCTCGGGGTTTCATCTTCCCGGGG
CAAAACGATTTCCCCTGCGGCTTTGGCTGATCAAATACCTTCCCTGGTTTGCCATTCCAGGGAT
TCAGGGCCTGAATCTCTTTGCCAGGGCAGCCCTTTACATGGCTCCGAAACAATCACTTTCAACA
ACGGTCAGGCAGGGGCTCACGGCACCCTACAACTCGTGGAAAAACAGGATCGCCACCCTCAA
ATTTGTCCAGGACATTCCCCTTTCACCCAGGGACAAAAGCTACGAACTTGTCAACTGGGTGGA
CACCCACCTTGAAGGTCTTAAAACCGTTCCCATGATGATCCTATGGGGCAGACACGATTTTGTG
TTTGATCTGTCGTTCCTTGACGAGTGGAACAAACGGTTTCCCCATGCCCAAACACATATTTTCG
AGGATGCAGGCCATTATCTGTTTGAGGACAAACCCGATGAAACATCAAATCTTATCAAAAAAT
TCATAGAGGAGTACTAA

1. Select a suitable expression host (heterologous system) for the DhaA enzyme overproduction and explain why the selected host is the best choice:

2. Propose and design a strategy for the DNA template synthesis, including primer design:

3. Propose a cloning strategy – ligation-dependent versus ligation-independent cloning, selection of expression vector, affinity/solubility tags etc.? How will you check the error-free clones?

4. Briefly describe production process – how will you introduce a foreign gene into the host, from pre-culture to large-scale overproduction, inducible versus stable expression, cytotoxicity issue, timing, harvesting strategy etc.

5. How will you determine the quality and yield of the purified enzyme?

6. How will you determine oligomeric state of the DhaA enzyme?

MICROFLUIDICS & LAB ON A CHIP

location: INBIT, Kamenice 34, ground floor, room 023

lecturer: Mgr. David Kovář, Ph.D. (kovard@mail.muni.cz)
Mgr. Michal Vašina (437248@mail.muni.cz)

I. WORKFLOW

– on-demand droplet generation and fusion
– droplet microfluidics and microscopy
– capillary microfluidic platform

II. MOTIVATION

Microfluidics can be defined as the science and technology manipulating and analysing fluid flow in sub-millimeter dimensions. It is becoming important technology for many emerging applications and disciplines, especially in the fields of chemistry, biology and medicine. Concrete application examples are biosensor devices for molecular diagnostics, polymerase chain reaction chips, high-throughput screening, controlled drug delivery systems, drug discovery methods, forensic analysis instruments, and so on (1).

III. THEORETICAL BACKGROUND

III-A1. On-demand droplet generation

A nice technique for droplet generation, when multiple distinct samples are necessary, is the on-demand generation using the commercial microfluidic device, Mitos Dropix from Dolomite Microfluidics, UK. The scheme of the droplet generation principle is shown in Figure 1.

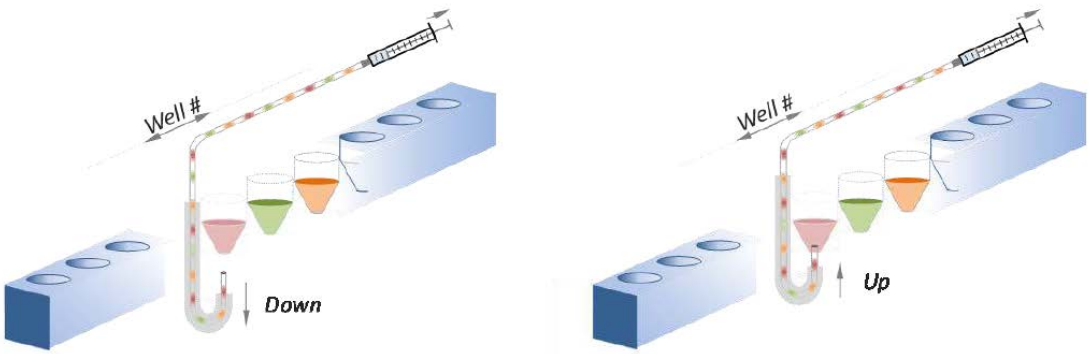

Figure 1 *Droplet on demand – a constant suction driven flow results in the creation of a segmented flow. The timing of the 'up' or 'down' position of the sampling hook dictates the droplet volume and spacing volume respectively. The transverse position of the hook dictates the selection of the sampling well. (Source: https://www.dolomite-microfluidics.com/wp-content/uploads/mitos-dropix-droplet-splitting-application-note-1.pdf)*

III-A2. Droplet merging

The setup of a capillary microfluidic platform with the application of negative pressure enables the creation of concentration gradients using droplet merging. To achieve this, the initial droplets should be generated in a low flow rate and there should be droplet pairs, consisting of one smaller droplet in the front and one larger droplet right behind it (in between both of them, there is a short oil plug). After the droplet generation, the flow of droplets is stopped and subsequently accelerated to around 20x higher flow rate than the initial one. This results in paired droplets getting closer to each other, due to an imbalance of oil leaking through the corner gutters of both droplets, which behave as leaky pistons [2]. This process is visualized in Figure 2.

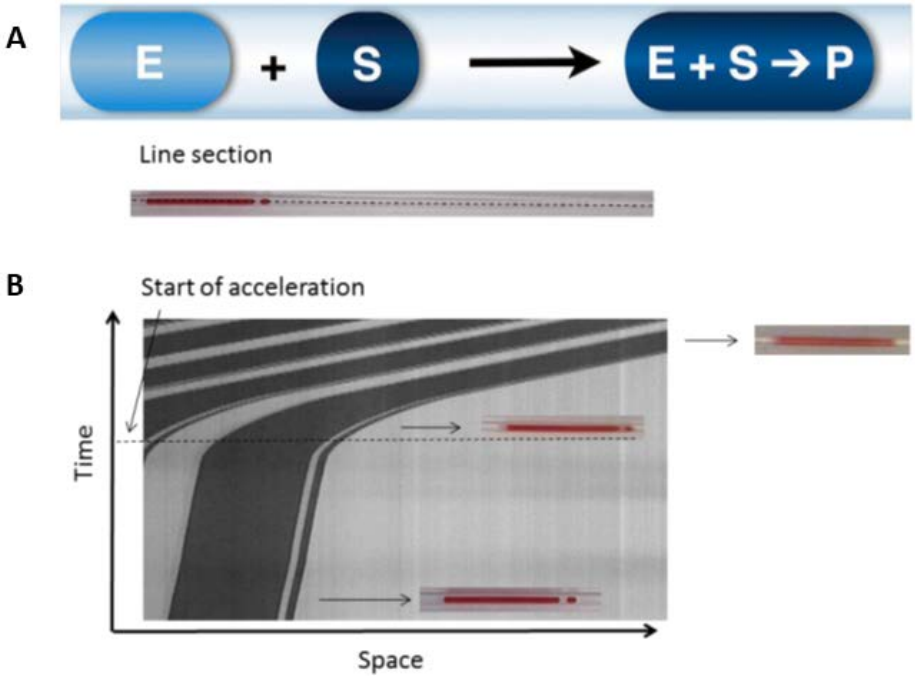

Figure 2 *Droplet merging. A: Merging scheme for a pair of droplets inside a tubing. A large compartment loaded with an enzyme (E) will catch up with a smaller compartment loaded with substrate (S) placed immediately in front of it. Merging triggers the enzymatic reaction leading to the formation of product P. B: Visual representation of droplet merging (catch-up). The x-axis represents space while the y-axis represents time. Adapted from Gielen et al [2].*

III-B. On-chip droplet formation

Formation of water in oil droplets in microfluidic chip has several benefits when compared to standard technology. Amongst such benefits belong low volume of reagents consumed, chip modularity, low cost and simple fabrication. When all pros combined properly one may encounter drop costs of screening million fold [3,4].

In this practice students will put hands on microfluidic chip technology. Prepared chips are to be used for water in oil droplet generation [5]. An example will follow with encapsulation of single E.coli BL21 DE3 cells to droplets. Finally, there will be observation of single cells in emulsions generated.

III-C. Capillary microfluidic platform

Technical setup (Figure 2) can be described separately as an optical, mechanical and microfluidic part. A precise calibration is one of the primary processes. First, the concentration of HCl must be determined by acid-base titration with potentiometric detection. Once we know the concentration of the acid, the titration of the working buffer must be performed to know the dependency between the proton concentration (pH respectively) and the fluorescence intensity of the probe. Working buffers with three different pH values are used for the "on-platform" calibration of the proton-sensitive assay – the buffer with initial pH ≈8.00 and several mixtures adjusted by titration with HCl to desired pH, respectively. The fluorescence intensity of the pH-sensitive probe must be measured at all check-points (loops) and for all ratios of reaction mixture and acid. Please note, that the fluorescent dye is temperature sensitive and therefore the calibration must be measured for all the combinations.

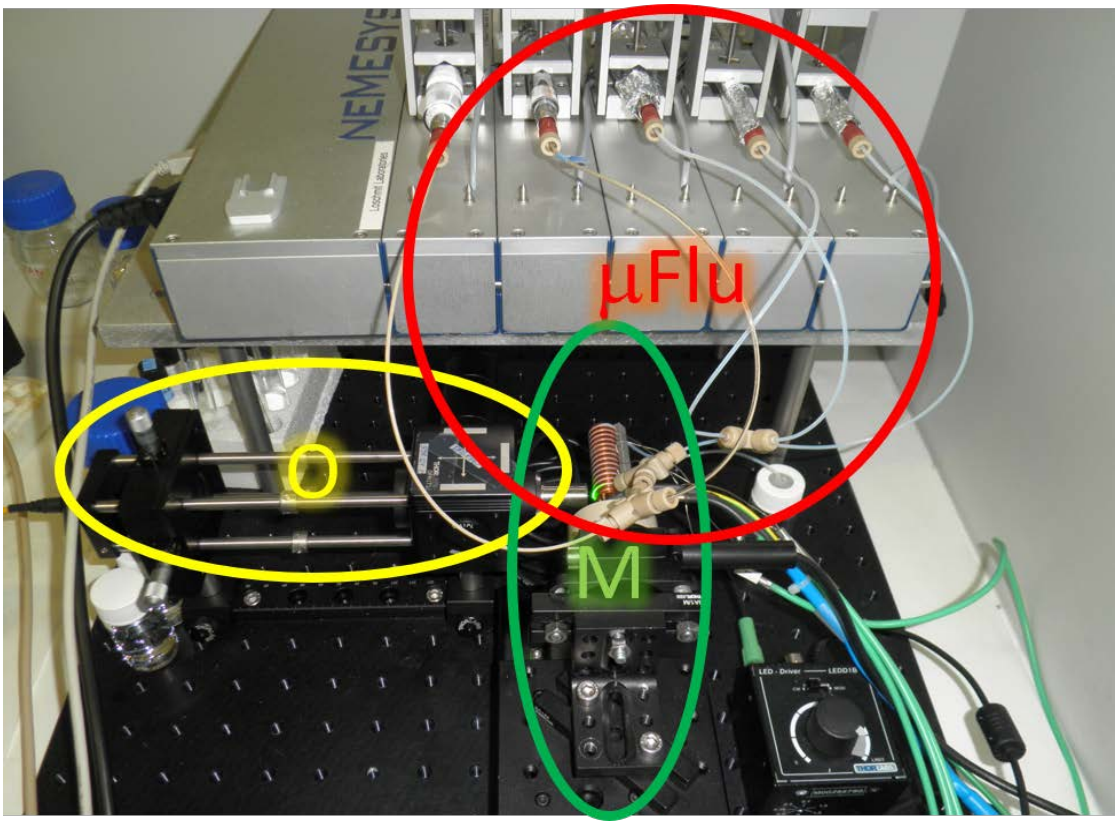

Figure 2 *A photography of the capillary-based microfluidic platform with highlighted optical (O), mechanical (M) and microfluidic (μFlu) parts.*

IV-A. PROTOCOL (On-demand droplet generation and fusion)

1. Prepare 1mL of 50 µM HPTS solution out of 1 mM HPTS stock solution. Afterward, prepare 500 µL of 2 mM HCl solution out of 100 mM stock solution, diluting it with the just prepared 50 µM HPTS solution.
2. Put 20 µL of both 50 µM HPTS solution and 2 mM HCl solution into separate wells of the rack. Markdown the numbers of the wells.
3. Start the microfluidic pump at 10 µL/min in a withdraw mode.
4. Write the sequence of droplets for droplet merging into the Dropix software according to your homework.
5. Start the droplet generation on Dropix.
6. When finished wait a while, until you visually see that the generated droplets in the tubing. Then stop the pump.
7. Start the detection on the LabView detection window.
8. Change the flow rate of the microfluidic pump to 200 µL/min and start it.
9. Observe how the droplets merge – both with the naked eye and on the detector.

IV-B. PROTOCOL (On-chip droplet formation)

Solutions and reagents:

- HFE-7500 or FC-40 oil
- PicoSurf-2 surfactant
- Trichloro(1H,1H,2H,2H-perfluorooctyl)silane
- 1.5M NaCl
- deionized H_2O
- *E.coli* BL21 DE3 (calculate the concentration!)
- Percoll ®
- Isopropanol

Equipment:

- Chemyx Fusion 200 syringe pumps or precise neMesys syringe pumps
- gas-tight syringes (various volumes)
- PTFE tubing
- Microfluidic chips – various designs
- glass slide
- Inverted microscope

1. load syringes with HFE-7500 oil and 150 mM NaCl, 25 % (v/v) Percoll and properly diluted cells
2. attach PTFE tubing to the syringe and remove any bubbles
3. put syringes into the syringe pumps, lock tight and set proper syringe diameter
4. connect syringe via tubing into the chip
5. set liquid flow – 300 $\mu L.h^{-1}$ for oil phase and 30 $\mu L.h^{-1}$ for the aqueous phase
6. observe droplet formation under microscope at various magnification
7. verify cell occupation in emulsions on inverted microsope

IV-C. PROTOCOL (Enzyme kinetic on the microfluidic platform - calibration)

Solutions and reagents:

- FC-40 oil
- 1.0M NaCl
- 50 mM Tricine
- 50 mM BisTrisPropane
- deionized H_2O

Equipment:

- neMesys precise pump
- gas-tight syringes (various volumes)
- PTFE tubing
- Microfluidic platform
- DAD spectrophotometer

1. Prepare 5 mL of 50 µM HPTS solution (use the 1 mM HPTS stock and UB1 buffer) and 0.5 mL of 2 mM HCl solution (use 100 mM stock solution and DW)
2. Fill the gastight syringes (HPTS, HCl, FC-40) and mount them into pump
3. Start the pumps at flowrates (Oil 25 $\mu L.min^{-1}$ / HPTS 10 $\mu L.min^{-1}$) – generating plugs
4. Increase the flowrate of HCl for increment of 0.05 $\mu L.min^{-1}$ till 0.5 $\mu L.min^{-1}$
4. Collect data and plot the calibration curves

V. HOMEWORK

1. Calculate the volumes to be pipetted to prepare both reaction solutions according to the protocol IV-A.

2. You have an oil reservoir and two starting solutions: 0mM and 2mM HCl solution. Your task is to think up a sequence of droplets and the exact volumes of aqueous and oil phase to generate droplets by the above-stated mechanism of droplet fusion (Figure 2, first droplet is smaller, the second droplet is larger). The merged droplets should have the final concentration of HCl in the range of 0.2mM – 1.8mM HCl. You should take into account the following requirements:

- The volumes of droplets should be in nanoliters (only whole numbers).
- The volume of the smaller droplet should be constant – calculate it as a minimum sphere-droplet to fit the tubing with inner diameter d = 0.4 mm.
- The oil space between the smaller and larger droplet is of the same volume as the smaller droplet.
- The oil space between the larger droplet and a next smaller droplet should be at least 400 nL.
- You should think up at least 5 distinct final concentration of HCl, including the mandatory 0.2mM and 1.8 mM.

An example of such a sequence (random volumes) is in the following

Order	1	2	3	4	5	6	7	8
Sample	0 mM	Oil	2mM	Oil	0 mM	Oil	2 mM	Oil
Volume (nL)	100	10	500	45	324	900	20	70

3. Estimate volume in pico-/femto- litres for monodisperse droplet formed at channel having dimensions 5, 10 and 20 µm, respectively (assume square cross-section forms spherical droplets of the same diameter).

4. For calculated droplet volumes estimate approximate cell density in $x.10^{y}$ per mL, to put single cell per droplet. There is approximately 1.10^{8} cells in medium with OD_{600} 0.5. Cultivated cells have OD_{600} 4.8. In case that grown culture has insufficient density, calculate the factor for thickening cell media to sufficient level. Account for 10 per cent pipetting error.

VI. LITERATURE

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BIOCATALYTIC PREPARATION OF PHARMACEUTICAL PRECURSOR (*S*)-2-BROMOPENTANE

location: Loschmidt Laboratories, Kamenice 5/A13, 2nd floor, room 227
lecturer: Assoc. Prof. Radka Chaloupková, Ph.D. (chaloupkova@enantis.com)

I. WORKFLOW

- preparation of reaction mixtures
- enantioselectivity measurements
- gas chromatography analysis
- calculation of enantiomeric excess and yield

II. THEORETICAL BACKGROUND AND MOTIVATION

Enzymatic enantioselectivity may be defined as the ability of an enzyme to distinguish between two enantiomeric substrates. The discriminating capacity of the enzyme is quantitatively measured by the ratio *E* of the corresponding specificity constants V_m/K_m (or k_{cat}/K_m). The enantioselectivity of enzymes has been exploited in organic synthesis, for example in kinetic resolution of racemic mixtures as well as in the synthesis of chiral building blocks from achiral precursors. Enzymatic kinetic resolution of racemic mixtures is an effective tool for the preparation of enantiomerically enriched compounds which is a continuous social demand due to the clinical advantages that enantiopure drugs offer over the racemic forms. Haloalkane dehalogenase DbjA from *Bradyrhizobium japonicum* USDA110 showed high enantioselectivity with β-substituted bromoalkanes. This enzyme is selectively acting on (*R*)-enantiomer, allowing the preparation of enantiopure (*S*)-enantiomer using kinetic resolution.

III. DESIGN OF EXPERIMENT

Solutions and reagents:

- glycine buffer (100 mM, pH 8.6)
- (*rac*)-2-bromopentane
- haloalkane dehalogenase DbjA (c = 1.5 mg/ml)
- diethyl ether

Equipments:

- 25-ml Reacti Flask with Mininert Valves
- automatic pipette 5 ml
- Hamilton syringe 1 ml
- test tubes
- glass vials with crimp caps
- Pasteur pipettes
- water shaking bath
- vortex mixer
- gas chromatograph equipped with a chiral column

Procedure:

1. Inject 10 µl of (*rac*)-2-bromopentane into 15 ml of glycine buffer in 25-ml Reacti Flask closed by Mininert Valves.
2. Prepare the required number of test tubes with aliquots of diethyl ether (1 ml).
3. Incubate the reaction mixture in a water shaking bath at the room temperature (approx. 20 °C) for 15 min.
4. Withdraw 1 ml from the reaction mixture and mix it with 1 ml of diethyl ether in the test tube to stop the reaction (sample at 0 min).
5. Vortex the sample for 30 s for the extraction.
6. Start the reaction by adding 0.5 ml of the enzyme solution (c = 1.5 mg/ml).
7. Withdraw 1 ml of the reaction mixture at 5, 10, 15 and 20 min and mixed samples with 1 ml of diethyl ether in test tubes. Do not forget to vortex each sample for 30 s for the extraction!
8. Transfer the ether (upper) part of the sample into a clean vial.
9. Analyse samples by gas chromatograph equipped with a chiral column.

IV. HOMEWORK

1. Calculate:
   a. concentration of enzyme (mM) in the reaction mixture
   b. theoretical concentration of substrate (mM) at the beginning of measurement
   c. enantiomeric excess (%) and yield (%) of (*S*)-2-bromopentane.

2. Prepare protocol.
3. Read article related to the topic of practice. The article can be found in study materials.

Protocol:

BIOCATALYTIC PREPARATION OF PHARMACEUTICAL PRECURSOR (*S*)-2-BROMOPENTANE

Name:

Date:

MOTIVATION:

EXPERIMENTAL PART:

RESULTS AND CALCULATIONS:

DISCUSSION:

V. LITERATURE

1. Faber, K. (2000). Biotransformation in Organic Chemistry, Springer-Verlag, Berlin.
2. Straathof, J. J., Jongejan, J. A. (1997). The enantiomeric ratio: origin, determination and prediction. Enzyme and Microbial Technology 21: 559-571.

PREPARATION AND TRANSFORMATION OF LIPOSOMES

location: Veterinary Research Institute, Hudcova 297/70, 2nd floor, Department of Pharmacology and Immunotherapy

lecturer: RNDr. Jaroslav Turánek, CSc. (turanek@vri.cz)

I. WORKFLOW

- preparation of liposomes
- transformation: high-pressure extrusion, preparation of SUV (small unilamellar vesicles)
- analysis: DLS (dynamic light scattering) analysis, ZetaSizer

II. MOTIVATION

Liposomes are very common delivery system in medicine because of their biocompatibility, non-toxicity, encapsulation of hydrophilic and hydrophobic compounds (e.g. drugs) and specific transport to the target region/area. Composition and surface of liposomes could be modified for better targeting, long circulation in the body or better stability. These modifications are a great tool for designing the liposomes according to current needs. After preparation a verification of the result is needed.

III. THEORETICAL BACKGROUND

Liposomes (closed bilayer phospholipid system) are known for more than 50 years and as delivery system they have many advantages. Their diameter is in the range of few nanometers to few micrometers. They can be unilamelar or multilamelar or even multivesicular and the bilayer is hydrophobic inside and hydrophilic outside. The process of assembling is not spontaneous like in micelles, it must be controlled and certain steps must be done. There are many possibilities of liposome preparation and many form to prepare, also there are some following steps to attached ligands to them or just make them more stabilized. In the method of Lipid film hydration the first step is to dissolve lipids in chloroform and this mixture evaporates by vacuum rotary evaporator. The resulting lipid film is hydrated by water PBS or other solution/buffer. Ultrasound or other techniques can help during hydration. At this point the liposomes are polydisperse and other process is needed to make them monodisperse.

Sonication and filtration through polycarbonate filters with defined pore size adjusts liposome diameter and lamilarity (extrusion). Also a method of freezing and unfreezing (FTMVL) can be used. Detergent removal method is used for preparation of unilamelar liposomes. When the detergent reaches its critical micelle concentration, it solubilizes lipids.

Dynamic light scattering is used for determining the size of small particles (proteins, polymers, micelles, carbohydrates, and nanoparticles) in suspension. It is also possible to measure the concentration and the size of surface. The principle is based on measuring the fluctuation of particles, when the smaller particle the bigger fluctuations.

TEM (transmission electron microscope) uses a beam for electrons transmitted through an ultra-thin specimen/sample. The electrons are interacting with the specimen and the resulting image is detected by a sensor/camera. TEM is able to detect and imagine at higher resolution than light microscope.

Preparation:

1. Lipid film hydratation
2. Detergent removal method
3. Sonication, extrusion, FTMVL

Analysis: DLS (size and polydispersity, zetapotential)

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

- Lipids: 1-octadecanoyl-2-hexadecanoyl-sn-glycero-3-phosphocholine (SPPC), L-α-phosphatidylcholine (EPC)
- Sodium cholate
- NaCl
- Chloroform
- PBS
- Liquid N2

Equipments:

- vacuum rotary evaporator
- Liposofast
- Sonicator
- ZetaSizer

A) Lipid film hydration:

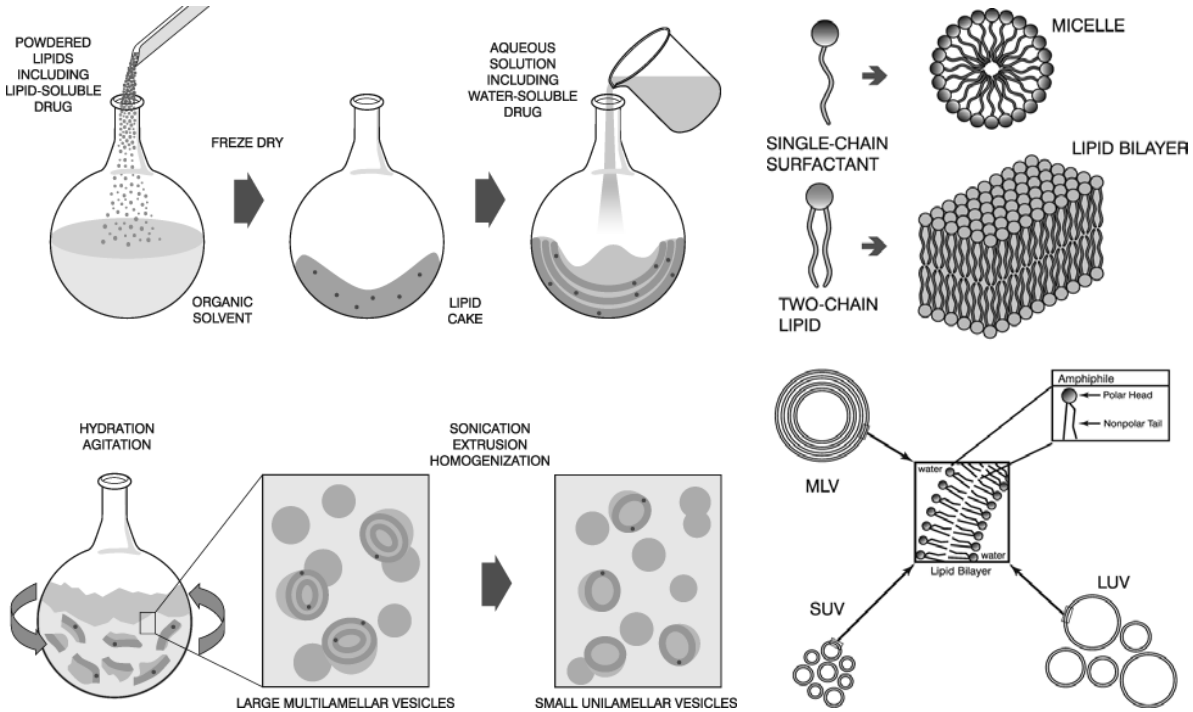
1. weight out: 100mg PPC-SPC
2. hydratation: 10ml 0,9% NaCl for infusio, final concentration of lipid: 10mg/ml

B) Detergent removal method

1. sodium cholate – dilution with amount up to critical micelle concentration (CMC)
2. dialysis

C) Sonication, extrusion, FTMVL

1. extrusion: filters 200nm (app. 15x)
2. sonication : total time 1 min, pulse mode: 10s pulse/10s pause, amplitude: 60%
3. FTMLV: frozen-thawed 2 times



D) DLS (Malvern) – size, size distribution, PDI

MLV, after extrusion, after sonication

V. LITERATURE

1. Avanti Lipids
   http://www.avantilipids.com/index.php?option=com_content&view=article&id=1384&Itemid=372

ANALYSIS OF LIPOSOMES

location: Veterinary Research Institute, Hudcova 297/70, 2nd floor, Department of Pharmacology and Immunotherapy

lecturer: RNDr. Jaroslav Turánek, CSc. (turanek@vri.cz)
RNDr. Jana Plocková (plockova@vri.cz)
MVDr. Pavel Kulich (kulich@vri.cz)

I. WORKFLOW

– single particle tracking analysis by using Nanosight 500– size, size distribution
– transmission electron microscopy (TEM) – size, morphology

II. MOTIVATION

Single particle tracking is a method for measuring the movement of every particle in a solution in time and for its quantifying. With this it is possible to study intracellular transport of these particles and their kinetics in real time. NanoSight instruments accurately and rapidly sort liposomes in water according to their size requiring only small volumes and very little sample preparation. The system enables individual liposomes in suspension to be visualized and their Brownian motion tracked. It anables specific and general nanoparticle tracking (presence, size distribution, concentration and fluorescence of all types of nanoparticles from 10nm to 2000nm depending on the instrument configuration and sample type). The particle size will determine the distribution of the particles within the body and the concentration and size of the particles will govern the amount of drug delivered.

III. THEORETICAL BACKGROUND

Single particle tracking is used to quantify specific behaviour of particles, e.g. liposomes, in colloid solution. The trajectory of every particle is measured and then the volume, hydrodynamic diameter and frequency of the particles are evaluated. This method is used to determine different kinds of particles in the mixture of variable materials.

The rate of movement is related only to the viscosity of the liquid, the temperature and size of the particle and is not influenced by particle density or refractive index. Absolute numbers of particles can be measured and the relative number of monomer versus aggregated particles is calculated in real time.

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

– liposomes prepared in the previous exercise

Equipments:

– NanoSight 500

– TEM

A) NanoSight 500

Using the solution of lipids from previous exercise, dilution as needed

B) TEM

1. Preparation of specimen for TEM
2. Imaging

V. LITERATURE

1. NANOSIGHT: http://www.nanosight.com/technology/nanoparticle-tracking-analysis-nta

PREPARATION OF ENZYMATIC BIOSENSOR

location: Loschmidt Laboratories, Kamenice 5/A13, 2nd floor
lecturer: Mgr. Šárka Nevolová, Ph.D. (77580@mail.muni.cz)
Ing. Markéta Zámečníková

I. WORKFLOW

- preparation of biosensor discs
- preparation of reaction mixtures
- biosensor measurements
- evaluation of biosensor response
- calculation of detection limit

II. MOTIVATION

The need for simple, rapid, cost-effective, and portable screening methods has boosted the development of practical biosensors with applications in medical diagnostics, food safety, process control and environmental monitoring. Compared to traditional analytical methods, enzymatic bioanalytical devices have several distinct advantages such as high sensitivity and specificity, portability, cost-effectiveness, and the possibilities for miniaturization and mass production. Additionally, they can be developed for real-time and high-frequency testing without extensive sample preparation.

III. THEORETICAL BACKGROUND

Enzymatic biosensors employ the affinity and selectivity of catalytically active proteins, towards their target molecules. The transducer converts the effect created by the interaction of enzyme with the analyte, usually into an electrical signal. Depending on the assay type, two fundamental classes of enzymatic sensors can be distinguished. First, the enzyme detects the presence of a substrate, or co-substrate/co-factor. This is then, by way of a transducer, used to monitor the increase of enzymatic activity. The second group is based on the detection of inhibitors in the presence of a substrate. With this system the decrease of signal (caused by enzyme inhibition) is monitored. In this practice, optical biosensor based on enzyme haloalkane dehalogenase and fluorescent pH indicator will be introduced. Haloalkane dehalogenase catalyses conversion of halogenated hydrocarbons to a halide ion, an alcohol and a proton, the last being responsible for the signal change of fluorescence pH indicator. Described biosensor is useful for assessment of contamination at a particular environmental site or for monitoring the concentration of known halogenated contaminant.

IV. DESIGN OF EXPERIMENT

Solutions and reagents:

- enzyme haloalkane dehalogenase (EC 3.8.1.5)
- bovine serum albumin
- fluorescence pH indicator 5(6)-carboxynaphthofluorescein
- substrate 1,2-dibromoethane
- 1 mM HEPES buffer, pH 9.0: 1 mM HEPES, 20 mM Na_2SO_4, 1 mM EDTA
- 50 mM phosphate buffer, pH 9.0: 400 mM K_2HPO_4, 90 mM KH_2PO_4
- 25% (v/v) glycerol
- 70% (v/v) glutaraldehyde
- mixture of methanol with 1,2-dichloroethane as an internal standard

Safety precautions:

Glutaraldehyde, 1,2-dichloroethane and 1,2-dibromoethane are toxic chemicals. 1,2-dichloroethane and 1,2-dibromoethane are possibly and probably carcinogenic to humans, respectively. Therefore, these chemicals must be handled carefully under appropriate safety conditions. A fume hood with good ventilation is dedicated to preparation of the samples and biosensor measurements. The material is handled using appropriate personal safety equipment, including laboratory coats, glasses and gloves. All halogenated waste needs to be disposed separately.

Equipments:

- EnviroPen device with glass stick
- laboratory stand with a burette holder
- analytical balances
- pH meter with calibration solutions
- stirrer with stirring bar
- 10 ml vials, GC vials, vial crimper
- set of automatic pipettes
- glass discs
- Eppendorf tubes
- minishaker
- 10 microlitre syringe
- beaker
- graduated cylinder
- tweezer
- exsiccator
- computer with software FluorPen and Origin

Protocol for preparation of biosensor and measurement:

1. Weigh 4 mg of lyophilised enzyme and 8 mg of pH indicator in Eppendorf tube for preparation of enzymatic layer.
2. Weigh 4 mg of bovine serum albumin and 8 mg of pH indicator in Eppendorf tube for preparation of reference layer.
3. Dissolve each mixture in 65 µl of 25% glycerol by careful mixing.
4. Wash the glass discs with ethanol and distilled water.
5. Dry the glass discs with paper wipe.
6. Prepare enzymatic and reference discs - apply 5 µl of the corresponding mixture on each glass disc.
7. Expose the glass discs to vapour of 70% glutaraldehyde for 30 min.
8. Store the prepared glass discs in a Petri dish with 50 mM phosphate buffer, pH 9.0.
9. Put the glass disc with enzyme layer on the glass stick and attached into EnviroPen.
10. Switch on the device by pressing the SET key for 1 s.
11. Press SET key to measure Ft.
12. Press MENU key to scroll down into the Main menu.
13. Find Setting and adjust the F-fulse to 0.90 using SET key.
14. Press SET key to confirm the selection and MENU key to return to measurement.
15. Switch on the computer and run the program FluorPen.
16. Check that the program FluorPen and the EnviroPen device are properly paired.
17. Adjust the pH of 50 mM phosphate buffer to 9.0 using 1 mM NaOH if necessary.
18. Pipette 5 ml of this buffer into vial and add stirring bar.
19. Fix the EnviroPen device into the laboratory stand and immerse the tip into the vial with phosphate buffer.
20. Record the baseline while stirring for 10 min.
21. Dissolve chemicals for preparation of 1 mM HEPES buffer in 1 l of distilled water.
22. Adjust its pH to 9.0 using 1 M NaOH or 1 M HCl.
23. Pipette 5 ml of this buffer into vial and add stirring bar.
24. Inject 1 µl of substrate 1,2-dibromoethane into HEPES buffer and shake the reaction mixture on vortex (20 s).
25. Transfer the EnviroPen tip from phosphate buffer to HEPES buffer with substrate.
26. Record the enzymatic reaction with substrate for 2 min.
27. Save the measurement record using menu option File and Export in .txt format.
28. Remove the used glass disc.
29. Put a new glass disc with a reference layer on the glass stick.
30. Repeat the whole measurement procedure as described for disc with enzymatic layer.
31. Prepare sample for GC analysis – pipette 0.5 ml of methanol with 1,2-dichloroethane into GC vial.

32. Pipette 0.5 ml of 1 mM HEPES buffer with substrate to the methanol.
33. Cap the GC vial using vial crimper and shake the mixture on vortex (20 s).

Protocol for data analysis:

1. Copy the raw data into Excel file.
2. Plot the signal from enzymatic and reference layer in dependence on time of measurement in minutes.
3. Calculate a slope from the 1 min linear signal of enzymatic and reference layer.
4. Calculate biosensor response by substraction the reference slope from the enzymatic slope.
5. Plot the biosensor responses in dependence on substrate concentrations using software Origin and provided biosensor data.
6. Determine detection limit of biosensor for 1,2-dibromoethane.

V. HOMEWORK

1. Calculate amount of chemicals used for preparation of 1 l of 1 mM HEPES buffer: 1 mM HEPES (Mr 238.30 $g.mol^{-1}$), 20 mM Na_2SO_4 (Mr 142.04 $g.mol^{-1}$), 1 mM EDTA (Mr 292.24 $g.mol^{-1}$)

2. What is the concentration of 1,2-dibromoethane (Mr 187.86 $g.mol^{-1}$; ρ=2.18 $g.cm^{-3}$) if 1 µl of this chemical is dissolved in 5 ml of HEPES buffer?

3. Determination of halogenated pollutants using biosensor utilising haloalkane dehalogenase is based on measurement of:

A: enzyme inhibition
B: increase of product concentration

4. Which immobilization methods are commonly used in development of biosensors? What are their advantages and disadvantages?

5. What is the difference between selectivity and sensitivity of the biosensor?

VI. LITERATURE

1. Wencel D., Abel T., McDonagh C. (2014): Optical chemical pH sensors. Anal. Chem. 86: 15-29.
2. Long F., Zhu A., Shi H. (2013): Recent advances in optical biosensors for environmental monitoring and early warning. Sens. 13: 13928-13948.
3. 6Sassolas A., Blum L.J., Leca-Bouvier B.D. (2012): Immobilization strategies to develop enzymatic biosensors. Biotechnol. Adv. 30: 489-511.

Protocol template

Practice XY

Name:

Date:

Motivation:

Experimental part:

Results:

Conclusions: