SUBJECT BOOK
ERN 4210

AUTUMN 2011

NUTRIENTS and GENE REGULATION

Study program for Nutrition
Department of Nutrition
Medical Faculty

University of Oslo

PROGRAM AND TIME SCHEDULE

WEEK	44	45	46	47	48	49	50
	SUBJECT BLOCK I			SUBJECT BLOCK II			
TOPIC	TOPIC 1	TOPIC 2	TOPIC 3	INTRODUCTION TO METHODS AND LAB WORK	LAB WORK	LAB WORK	STUDIES & EXAM
Monday	31.10.2011 Presentation of course and topics (LMG-W) Lecture TOPIC 1 (FH) Room: 2180 Time: 13-15	07.11.2011 Lecture TOPIC 2 (LMG-W) Room: 2180 Time: 12-14	14.11.2011 Lecture TOPIC 3 (KTD) Room: 2183 Time: 09-11	21.11.2011 Introduction to promoter studies (KTD) Room: 2183 Time: 09-11	28.11.2011 Day 1 Room: 1130 Time: 09-15	05.12.2011 Day 5 Hand in laboratory report from lab week 1. Room: 1130 Time: 09-12	12.12.2011 STUDY DAY
Tuesday	01.11.2011 TOPIC 1 Lecture (FH) Room: 2180 Time: 12-14	08.11.2011 TOPIC 2 Lecture (HN) Room: 2180 Time: 12-14	15.11.2011 TOPIC 3 Lecture (KTD) Room: 2183 Time: 09-11	22.11.2011 Introduction to RNA work (KTD) Room: 2183 Time: 09-11	29.11.2011 Day 2 Room 1130 Time: 09-12	06.12.2011 Day 6 Room: 1130 Time: 09-16	13.12.2011 STUDY DAY
Wednesday	02.11.2011 STUDY DAY	09.11.2011 STUDY DAY	16.11.2011 STUDY DAY	23.11.2011 STUDY DAY	30.11.2011 STUDY DAY	07.12.2011 Day 7 Room: 1130 Time: 09-16	14.12.2011 STUDY DAY
Thursday	03.11.2011 TOPIC 1 Lecture (FH) Room: 2183 Time: 09 - 11	10.11.2011 TOPIC 2 Lecture (LMG-W) Room: 2180 Time: 12-14	17.11.2011 TOPIC 3 Lecture (KTD) Room: 2180 Time: 09-11	24.11.2011 Introduction to Protein work (LMG-W) Room: 2183 Time: 09-11	01.12.2011 Day 3 Room: 1130 Time: 09-15	08.12.2011 Day 8 Hand in answers to questions from lab week 2.	15.12.2011 STUDY DAY
Friday	04.11.2011 TOPIC 1 Lecture Summary Room: 2183 Time: 09 - 11	11.11.2011 TOPIC 2 Lecture Summry Room: 2183 Time: 09 - 11	18.11.2011 TOPIC 3 Lecture Summary Room: 2180 Time: 09 - 11	25.11.2011 Summary How to write a Laboratory report (LMG-W) Room: 2183 Time: 12-14	02.12.2011 Day 4 Summary PCR (laboratory report) Room: 1130 Time: 09-15	09.12.2011 Day 9 STUDY DAY	16.12.2011 Exam Room: 2180/ and 2183 Time: 9-14

ERN 4210 module nutritional biology

This subject will give a broad presentation of the mechanisms for the effects of central nutrients, hereunder general mechanisms for regulation of transcription and gene expression. There will be an emphasis on nutrients that regulate expression of target genes via their specific transcriptional factors and protein modifications. This includes fatty acids (saturated and unsaturated fat), cholesterol, retinol and glucose. Our focus is on lifestyle-related diseases, such as cardiovascular disease (CVD), type 2 diabetes and obesity (these metabolic disorders are collectively called metabolic syndrome) which has become a major public health problem with large social health care costs. Metabolic syndrome is a complex physiological process shaped by nutrition, metabolism and hormones, such as insulin responsible for glucose absorption from the blood. Defining the regulatory signalling pathways involved as well as the genetic and dietary factors is a prerequisite for efficient disease risk reduction and novel therapeutic strategies in metabolic disorders. Our ultimate goal is to give an integrated molecular-to-organism view of how nutrient regulated transcription factors and key proteins affect the metabolic syndrome. This will be achieved by giving you state-of-the-art articles as the literature prescribed for ERN 4210. In addition you will have three weeks of laboratory work that will highlight in a practical way the study of how nutrients regulate genes in glucose and lipid metabolism.

The module consists of two subject blocks and endures 7 weeks:
Subject block I lasts 3 weeks, and consists of lectures and self study under tight supervision from scientists at the department. Three topics will be studied during this period, together covering central parts of the subject nutrients and gene regulation.

Subject block II lasts 3 weeks; week 1 includes lectures describing methods and techniques in more detail that have been introduced in subject block I and methods that will be used in the practical laboratory part in week 2 and 3 of subject block II. How to write a laboratory report will also be presented as you must write a laboratory report from the practical laboratory work in week 2+3. The aim of subject block II is to learn variable techniques in

molecular biology and to illustrate how nutrients regulate gene transcription. The gene expression will be studied at the level of RNA and protein.

The module will be completed by a written exam at the end of the last week.

We wish to give you all a warm welcome to ERN 4210. We will be happy to be contacted at any time for discussions and supervision.

Best regards,

Associate Professor Line M. Grønning-Wang
Phone 22851342

Professor Hilde Nebb
Phone 22 85 15 10/ 41 55 29 25

ERN 4210 AUTUMN 2011

44 45 46	47 48 49	50
SUBJECT BLOCK I	**SUBJECT BLOCK II**	
Lectures Start 31st of October 2011 Time: 12:00-14:00 room 2180 Lectures and self studies Course based on research 3 topics that will cover: Key proteins and key transcription factors regulating energy balance and insulin signalling pathways. The themes will give insight into how metabolism of fat and sugar are closely interrelated and what factors interplay to regulate this metabolism. Every topic lasts 1 week. 4-5 central scientific papers will be gone through. Four teachers will give the lessons.	**Laboratory course** **Introduction to methods:** Start: 21st of November 2011 Time: 09:00 room 2183 **Laboratory practical course:** Start 28th of November 2011 Time: 09:00 room 1130 Week 1 (47): Introduction to methods from subject block I and methods that will be used in the practical course. You will also learn how to write a laboratory report. Week 2+3 (48+49): Practical course that teaches you basic techniques used in molecular biology. The techniques are used to study gene regulation by nutrients. The topics in the lab course are based on the topics of SUBJECT BLOCK I.	**Written exam** 16th of December Time: 09-14 Rooms 2180 and 2183 5 hours The exam contains questions from the lectures (week 44-47) and papers.

TOPICS

SUBJECT BLOCK I LECTURES

TOPIC 1:
SREBP transcription factors - regulators of cholesterol and fatty acid metabolism.
Lecturer: Post.Doc. Fred Haugen

TOPIC 2:
Nuclear receptors in control of glucose and lipid homeostasis
Lecturers: Associate Professor Line M. Grønning-Wang and Professor Hilde Nebb

TOPIC 3:
Lipid droplet-associated proteins
Lecturer: Post.Doc. Knut Tomas Dalen

SUBJECT BLOCK II LABORATORY COURSE

Responsible: Line M. Grønning-Wang/Knut Tomas Dalen

Teachers: Knut Tomas Dalen and PhD-student Ole Berg
Line M. Grønning-Wang and PhD-student Christian Bindesbøll

SUBJECT BLOCK I

TOPIC 1

SREBP TRANSCRIPTION FACTORS - REGULATORS OF CHOLESTEROL AND FATTY ACID METABOLISM

Fred Haugen, Dr.philos

The sterol regulatory element-binding protein (SREBP) transcription factors are critical regulators of cholesterol/lipid homeostasis, which act by controlling the expression of many cholesterogenic and lipogenic genes. We will look into scientific work that indicates a role for polyunsaturated fatty acids in regulation of gene expression.
A summary of SREBP regulation will be given and several articles will be presented in depth.

Literature:

Hannah et al.
Unsaturated Fatty Acids Down-regulate SREBP Isoforms 1a and 1c by Two Mechanisms in HEK-293 Cells.
J. Biol. Chem., Vol. 276, Issue 6, 4365-4372, February 9, 2001

Takeuchi et al.
Polyunsaturated fatty acids selectively suppress sterol regulatory element-binding protein-1 through proteolytic processing and autoloop regulatory circuit.
J Biol Chem. 2010 Apr 9;285(15):11681-91.

Gong et al.
Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake.
Cell Metab. 2006 Jan;3(1):15-24.

Radhakrishnan et al. Sterol-regulated transport of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig.
Proc Natl Acad Sci U S A. 2007 Apr 17;104(16):6511-8. Epub 2007 Apr 11.

Najafi-Shoushtari et al.
MicroRNA-33 and the SREBP host genes cooperate to control cholesterol homeostasis.
Science. 2010 Jun 18;328(5985):1566-9.

TOPIC 2

NUCLEAR RECEPTORS IN CONTROL OF GLUCOSE AND LIPID HOMEOSTASIS

Associate Professor Line M. Grønning-Wang and Professor Hilde Nebb

It is known that fat plays an important role for development of obesity, diabetes type II, coronary heart disease and cancer. Both heritage and environmental factors are crucial for the predisposition of these diseases, and the diet is a pivotal factor. The food contains nutrients used for construction and maintenance. Transcription factors are messengers that convey signals between intracellular signals and the genes. Nuclear receptors such as Liver X Receptor (LXR), Peroxisomal Proliferator-Activated Receptor (PPAR) and Retinoid X Receptor (RXR) are such cellular messengers, conveying signals from the nutrients cholesterol compounds, fatty acids and vitamin A, respectively.

The nuclear receptor superfamily consists of about 48 receptors (in man), including steroid hormone receptors and receptors for vitamin A, thyroid hormone and vitamin D. This group also includes receptors for both saturated and unsaturated fatty acids, cholesterol metabolites like oxysterols and bile acids, in addition to orphan receptors where the physiological ligand is still not known.

This family of transcription factors has a crucial role in regulation of gene expression, and mediates a communication between extracellular signals and transcriptional responses. They recognize short DNA sequences on target genes that are specific for the receptors.

These DNA motifs are called DNA response elements (RE). Other names are hormone response elements (HRE) or cis-elements. The elements are localized upstream (in front of the coding region) of the target genes. A transcriptional activation of the gene occurs when the ligand and the receptor make a complex that bind to the specific HRE.

Several nuclear receptors participate in central metabolic pathways in glucose and lipid homeostasis. Glucose and lipid homeostasis is a delicate balance between food intake, synthesis and degradation. The liver, muscle and adipose tissue are main organs in this balance.

Topic 2 discusses some of these regulatory pathways where nuclear receptors play a key role, the influence of these receptors on glucose and lipid homeostasis – alone or in an interaction.

Literature:

1. Evans RM The nuclear receptor superfamily: A rosetta stone for physiology Mol Endo. 2005. 19(6):1429-1438 http://mend.endojournals.org/cgi/reprint/19/6/1429

2. Lonard DM, Lanz RB nad O'Malley BW. Nuclear receptor coregulators and human disease. Endocrine Reviews. 2007. 28(5):575-587 http://edrv.endojournals.org/cgi/reprint/28/5/575

3. Chawla, A. et al. Nuclear receptors and lipid physiology: opening the x-files. Science. 2001, 294:1866-1870 http://www.sciencemag.org/cgi/reprint/294/5548/1866.pdf

4. Steffensen, KR and Gustafsson, JA. Putative metabolic effects of the liver X receptor. Diabetes. 2004,Feb;53, suppl 1:S36-42 http://diabetes.diabetesjournals.org/cgi/reprint/53/suppl_1/S36

5. Anthonisen, EH. Et al, Nuclear Receptor Liver X receptor is O-GlcNAc-modified in response to Glucose. 2010, Januray 15, vol 285, no 3 http://www.jbc.org/content/285/3/1607.full.pdf+html

TOPIC 3

LIPID DROPLET-ASSOCIATED PROTEINS

Knut Tomas Dalen, Dr. philos

Lipid droplets (LDs) are found ubiquitously; in bacteria, yeast, plants and most eukaryotic cells studied. They exist in multiple sizes, ranging from droplets invisible to the light microscope to LDs that occupy most of the cellular cytoplasm (<1 – 100 µm). Depending on the scientific field, they have been described as (among others): liposomes, lipid bodies, oil bodies and fat droplets. Today, the terminology "Lipid droplets" is usually used by scientists working with mammalian organisms.

The LD surface consists of a phospholipid monolayer that surrounds a core of neutral lipids; such as cholesterol esters and triacylglycerols. LDs were for a long time perceived as inert fat particles, and given little scientific attention by cell biologist. However, the discovery of proteins that attach to the surface of LDs (such vimentin and perilipin) in the late 80-ies, ignited attention to this "new organelle". Today, it is clear that the high number of proteins associated with the LDs reflects their complex nature, and they are recognized as important components of cells. They are important in divergent biological processes such as energy storage, a reservoir of lipids for synthesis of membranes, binding of histones during cell division, virus replication, detoxification of intracellular lipids and lipophilic toxins, proteasome function, autophagy, and intracellular transport.

Proteins found associated with LDs are either loosely attached to the LD surface (or other bound proteins) or bound with high affinity when embedded into the phospholipid monolayer or covalently linked to phospholipid head groups. The LD surface is highly dynamic, where the type of associated proteins varies among different cell types and nutritional supply. Such proteins include motor proteins for vesicle transport and fusion, effector/activator proteins, metabolic enzymes and lipid binding transporter proteins.

This week's *topic-session* will focus on the role of lipid droplets in general, with detailed focus on the LD-binding perilipin proteins. The human and mice genomes contain 5 different perilipin genes (*Plin*1-5), which encode for the proteins perilipin1-5. When located at the LD surface, these are believed to either facilitate or prevent recruitment of lipases to the LD, thereby controlling the release of lipids stored inside the LD core. Similar to a large number of genes involved in lipid metabolism, the Plin genes are transcriptionally regulated by the nuclear receptors Peroxisome proliferator-activated receptors (PPARs).

Literature:

Handouts:

Krahmer N, Guo Y, Farese RV Jr, Walther TC.
SnapShot: Lipid Droplets. Cell. 2009 Nov 25;139(5):1024-1024.e1.

Kimmel AR, Brasaemle DL, McAndrews-Hill M, Sztalryd C, Londos C.
Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. J Lipid Res. 2010 Mar;51(3):468-71.
(Explains Plin nomenclature).

Reviews:

Lipid droplets: Walther TC, Farese RV Jr.
The life of lipid droplets. Biochim Biophys Acta. 2009 Jun;1791(6):459-66.

Perilipins: **Bickel PE, Tansey JT, Welte MA.**
PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. Biochim Biophys Acta. 2009. Jun;1791(6):419-40. (**sections 1-5**).

Scientific articles:

Perilipin-1: Tansey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, Gavrilova O, Reitman ML, Deng CX, Li C, Kimmel AR, Londos C. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity.
Proc Natl Acad Sci U S A. 2001 May 22;98(11):6494-9.

Perilipin-2 and 3: Bell M, Wang H, Chen H, McLenithan JC, Gong DW, Yang RZ, Yu D, Fried SK, Quon MJ, Londos C, Sztalryd C. Consequences of lipid droplet coat protein downregulation in liver cells: abnormal lipid droplet metabolism and induction of insulin resistance. Diabetes. 2008 Aug;57(8):2037-45.

Perilipin-5: Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, Sztalryd C, Nebb HI. LSDP5 is a PAT protein specifically expressed in fatty acid oxidizing tissues.
Biochim Biophys Acta. 2007 Feb;1771(2):210-27.

SUBJECT BLOCK II

Introduction to methods

In week 1 (47) of subject block II we will go through relevant methods dicussed in topic I and methods that will be used in the practical laboratory course in week 2+3 (48+49). We will also go through how to write a laboratory report.

Literature:

Dalen KT et al, Expression of the insulin-responsive glucose transporter GLUT4 in adipocytes is dependent on liver X receptor alpha. J Biol Chem. 2003.
Vol 278, NO 48

Anthonisen EH et al, Nuclear receptor Liver X Receptor is O-GlcNAc-modified in Response to Glucose. J Biol Chem.2010. Vol 285, NO 3.

Compendium of laboratory course

Purpose of the study

RNA and protein expression of FAS (fatty acid synthase) will be studied in liver lysates isolated from fasted and refed mice.

Lab course overview

Day 1 (Monday)	Isolate total RNA from liver lysates RNA quantification
Day 2 (Tuesday)	cDNA synthesis
Day 3 (Thursday)	Real time PCR 1.Prepare tubes for real-Time PCR 2.Run Real-Time PCR
Day 4 (Friday)	Summary Real-Time PCR
Day 5 (Monday)	Protein quantification from liver lysates Prepare protein for Western blotting
Day 6 (Tuesday)	Western blotting, part 1

Day 7 (Wednesday) Western blotting, part 2
Summary Western blotting

NB! Some days of the lab course will be longer, while others will be shorter.
At least day 5 will be long, and day 2 will be short.

Day 1 Purification of Total RNA

Purpose of today's lab work (day 1):

Task 1: Isolate total RNA from liver lysates prepared from fasted and refed mice.

Task 2: Measure absorbance and calculate RNA concentration

Task 1: Isolation of total RNA from mouse liver lysates

Precautions:

RNA is easily degraded by the enzyme RNase. This enzyme is present almost everywhere – on our hands, clothes, bottles, etc. Therefore, working with RNA is very demanding. Please take these precautions:

i) Use lab coat and gloves, and change gloves as required.
ii) Wash the bench before use, and use a new bench cover.
iii) Use sterile disposable equipment only.
iv) RNases are not destroyed by autoclavation. Water should be treated with DEPC to deactivate RNases.
v) RNA must be stored at -70°C and thawed on ice only shortly before use

With the RNeasy procedure, all RNA molecules longer than 200 nucleotides are purified. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded.
RNA stabilization is an absolute prerequisite for reliable gene expression analysis. Immediate stabilization of RNA in biological samples is necessary because, directly after harvesting the samples, changes in the gene expression pattern occur due to specific and nonspecific RNA degradation as well as to transcriptional induction. Such changes need to be avoided for all reliable quantitative gene expression analyses, such as microarray analyses, quantitative RT-PCR, such as TaqMan® and LightCycler® technology, and other nucleic acid-based technologies.

Purification of Total RNA from Animal tissue Using Spin Technology
Mouse liver homogenized in Buffer RLT is distributed to each group (sample 1: fasted mice, sample 2: fed mice). Ethanol is then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane. The sample is then applied to the RNeasy Mini spin column. Total RNA binds to the membrane, contaminants are efficiently washed away, and high-quality RNA is eluted in RNase-free water. All bind, wash, and elution steps are performed by centrifugation in a microcentrifuge. (From Qiagen handbook)

Bench Protocol: Purification of Total RNA;
RNeasy Mini Kit cat no 74104, Qiagen

***Note**: Before using this bench protocol, you should be completely familiar with the safety information and detailed protocols in the RNeasy Mini Handbook.*

Important points before starting
Perform the procedure at room temperature (15–25°C). Work quickly.
Perform centrifugation at 20–25°C.

If necessary, redissolve any precipitate in Buffer RLT by warming. Before using Buffer RPE for the first time, ensure ethanol is added. If performing on-column DNA digestion, prepare DNase I stock solution.

Procedure

1. Thaw tubes containing mouse liver homogenized in RLT buffer. Repipette 10 times to disrupt and homogenize. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting.

2. Transfer sample to RNeasy column in 2 ml collection tube. ▲ Close lid, centrifuge for 15 s at 8000 x g, and discard flow-through.

(Optional DNase digest: Follow steps 1–4 of "Bench Protocol: Optional On-Column DNase Digestion" after this step.)

3. Add 700 µl Buffer RW1 to RNeasy column. ▲ Close lid, centrifuge for 15 s at 8000 x g, and discard flow-through.

Skip this step if performing optional DNase digestion or if performing RNA cleanup.

4. Add 500 µl Buffer RPE to RNeasy column. ▲ Close lid, centrifuge for 15 s at 8000 x g, and discard flow-through.

5. Add 500 µl Buffer RPE to RNeasy column. ▲ Close lid and centrifuge for 2 min at 8000 x g.

6. Place RNeasy column in new 2 ml tube, close lid, and centrifuge at full speed for 1 min.

7. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at _8000 x g (_10,000 rpm) to elute the RNA.

Optional: Repeat elution with another volume of water or with RNA eluate.

Mark the 1,5 ml collection tube "**RNA, date, your initials**" and put the tube on ice. From this point, always keep the RNA sample on ice, for storing in a freezer at -86°C.

Adapted from Rneasy Handbook (Qiagen).

Task 2: Measure absorbance and calculate RNA concentration

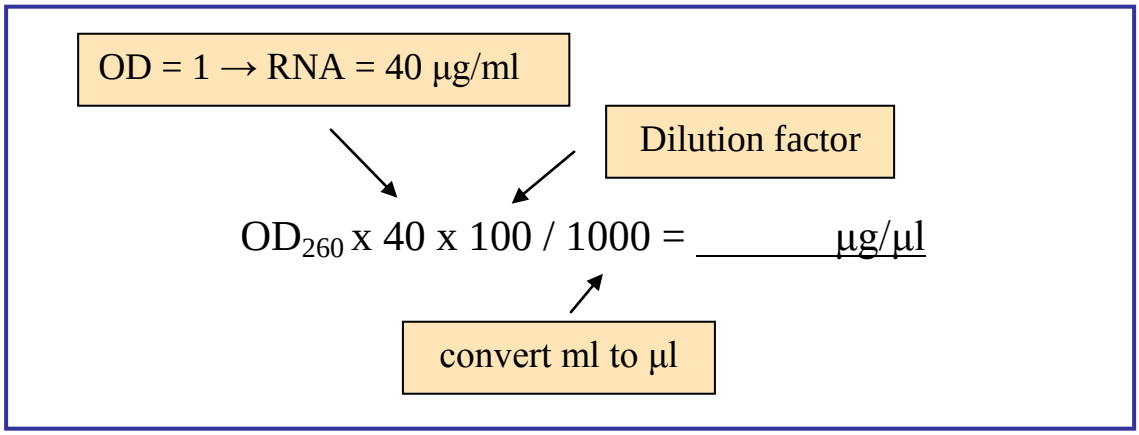
Precautions:

RNA is easily degraded by the enzyme RNase. This enzyme is present almost everywhere – on our hands, clothes, bottles, etc. Therefore, working with RNA is very demanding. Please take these precautions:

vi) Use lab coat and gloves, and change gloves as required.
vii) Wash the bench before use, and use a new bench cover.
viii) Use sterile disposable equipment only.
ix) RNases are not destroyed by autoclavation. Water should be treated with DEPC to deactivate RNases.
x) RNA must be stored at -70°C and thawed on ice only shortly before use

RNA absorbs light with a maximal peak at wavelength 260 nm, just like DNA. RNA (and single-stranded DNA) has an absorbance of 1 at 40 µg/ml (50 µg/ml for DNA).

Make a 100 X (times) dilution, totally 200µl with MQ H_2O. Mix the RNA tubes by whirlmixing briefly before you take out the volume RNA needed to make the dilutions. Measure the absorbance at OD_{260} and OD_{280} in a spectrophotometer. Calculate the concentration of RNA using the formula below and the OD_{260}/OD_{280} ratio.



Fill in:

Sample	**OD_{260}**	**OD_{280}**	**OD_{260}/OD_{280}**	**Concentration**

OD_{260}/OD_{280} ratio indicates the purity of the RNA. A ratio between 1,9- 2,1 indicates a RNA sample with satisfactory purity. Lower ratios indicate high protein contamination.

Day 2 Quantitative RT-PCR – day 1
Production of cDNA from total RNA

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) is the foremost sensitive and reliable technique for detection and quantification of mRNA transcripts present in a sample. Before introduction of the qRT-PCR technique in the late 90'ies, other techniques such as Northern blot analysis and RNase protection assay were used. Quantitative real-time RT-PCR, in contrast to end-point RT-PCR, is today the preferred method for quantification of changes in gene expression in experimental samples, and for validation of results obtained from micro array analyses [micro arrays is often used to compare and identify differences in expression levels of *all* mRNAs (the whole transcriptome) or a large selection on mRNAs expressed in a group of samples simultaneously in **one** experiment). In qRT-PCR, up to 364 samples can be run simultaneously, and the expression levels of pre-selected mRNAs present these samples can be determined in a single experimental run within hours.

The qRT-PCR reaction involves two steps, using two different enzymes. **The first step** copies each mRNA transcripts in an experimental sample into cDNAs catalyzed by the enxyme *reverse transcriptase*. Depending on the primers used in the reaction, each mRNA gives rise to one large cDNA (if using oligo dT primer, TTTTTTTTTT) or multiple cDNAs (if using a random hexamer primer, NNNNNNNNNN). The use of random primers is the preferred choice of primer to use, since this technique is less susceptible to experimental errors caused by RNA degradation. **The second step** amplifies one selected cDNA (among all the cDNAs present in the sample) in a Polymerase Chain Reaction catalysed by a *DNA polymerase*. More detailed information of the second step is found in the next lab section.

The qRT-PCR technique is sensitive enough to detect the presence of *a single copy* of an mRNA transcript in an experimental sample. Hence, the technique is highly susceptible to experimental errors, if not performed accurately. To reduce the risk of experimental errors, it is common to use only solutions and reagents that are tested to be free of contaminants that could interfere with the assay.

Purpose of today's lab work (day 2):

Task 1: Generate cDNA from RNA isolated on day 1.

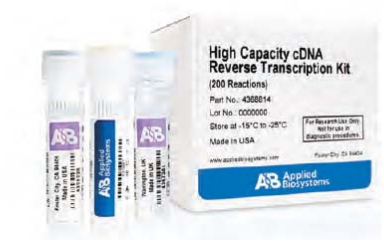
Task 1: Make cDNA from RNA samples isolated day 1

Each group should make cDNA from all RNA samples isolated on day 8 (sample 1-6) and an additional negative control (no mRNA template).
The high capacity cDNA Reverse Transcriptase Kit from Applied Biosystems will be used for the synthesis of cDNA. This kit is designed to amplify total RNA into cDNA.

High Capacity cDNA Reverse Transcription Kit
(formerly the High Capacity cDNA Archive Kit)
First Strand cDNA Synthesis Reverse Transcription Kit

The High Capacity cDNA Reverse Transcription Kit provides:

- Highest Level of Performance for Accurate and Precise RNA Quantification
- High Capacity for Maximum Dynamic Range
- High Reverse Transcription Efficiency
- Unsurpassed Value



Before you start, make sure you have enough RNA (at sufficient concentration) isolated from day 8. The total RNA that should be used in the cDNA synthesis step is **500 ng/µl**.

Protocol – pipeting of cDNA master mix

Prepare the 2x RT Master Mix (10µl / reaction):

1. Allow the kit components to thaw on ice.
2. Refer to the table below to calculate the total volume of components needed for all reactions (add a little extra, +0.5). **Note!** Prepare and store the RT master mix on ice.

Mastermix

Component	Volume µl/sample	Volume µl X (# samples +0,5)
10X RT Buffer	2,0	
25X dNTP Mix (100mM)	0,8	
10X RT Random primers	2,0	
MultiScribe™ Reverse Transcriptase	1,0	
(RNAse Inhibitor)	0	
Nuclease-free H_2O	4,2	
Total per Reaction	**10,0**	

Protocol – cDNA pipetting and RT reaction

1. Calculate the amount of RNA sample and RNAse free water needed for each sample based on the concentration of RNA in each sample.

Sample number	RNA (µg/µl)	To cDNA (µg)	RNA (µl)	H_2O (µl)
1		500		
2		500		
3		500		
4		500		
5		500		
6		500		
Neg. Contr.		0		10

2. Prepare the cDNA Reverse Transcription reaction:
    a. Mark each individual PCR tube from 1-7. For convenience, we might use a PCR tube strip containing 8 tubes.
    b. Pipette 10µl of 2X master mix into each tube (one will not be used).
    c. Pipette RNAse free water according to the table above.
    d. **Samples:** Pipette the calculated volume of each RNA sample into the corresponding tube. Pipett up and down two times to get all the RNA into the solution.mix.
    Negative control: Pipette10 µl nuclease free water.
    e. Seal the tubes.
    f. Briefly centrifuge the tubes to spin down the contents, mix by tapping on the tube strips, and spin down again to get the samples to the bottom of the tube and eliminate any air bubbles.
    g. Place the reaction tubes on ice until you are ready to put it the thermal cycler. Put the RNA samples back at -80 ºC.

3. Program the thermal cycler with the temperature settings shown below.

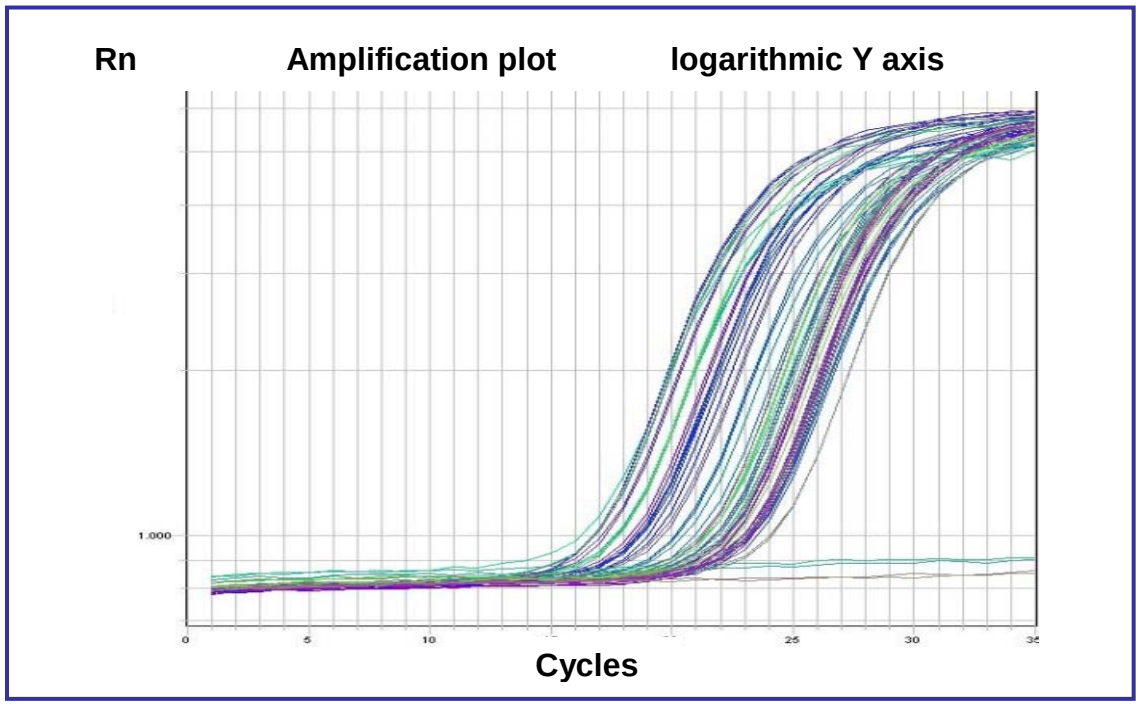
	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25°C	37°C	85°C	4°C
Time	10 min	120 min	5 min	∞

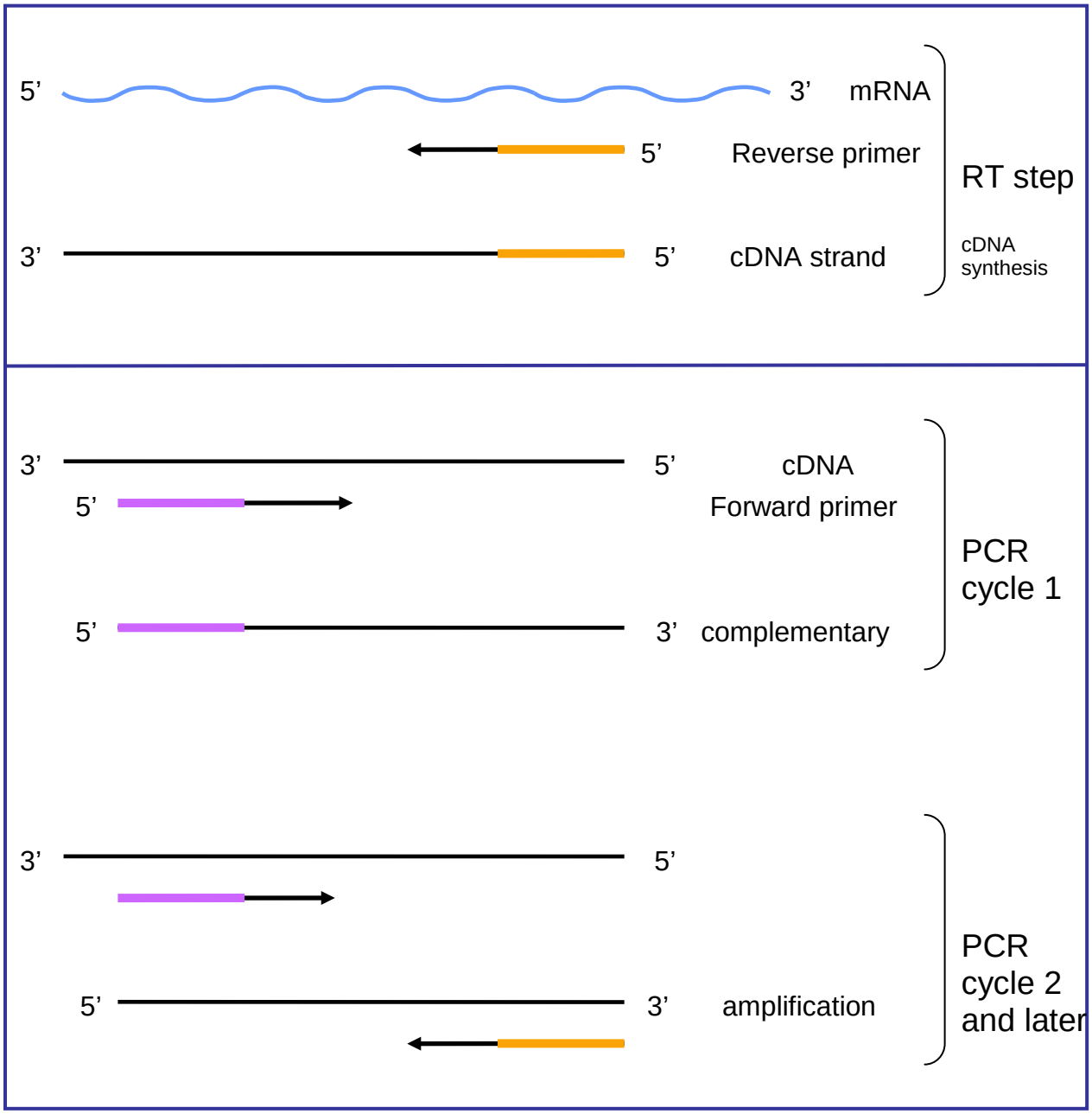
4. Start the thermal cycler. Put the samples at -20ºC after the run.

Day 3 Quantitative RT-PCR – day 2
qRT-PCR amplification of cDNAs

The first step in qRT-PCR is the synthesis of cDNA from mRNA using the reverse transcriptase (RT) enzyme (performed at day 10). The next step is amplification of selected transcript among all the cDNAs present in the cDNA reaction. In one tube/well a small segment (70-200 nucleotides) of a single cDNA is amplified using a DNA polymerase enzyme and template specific primers. In the first PCR reaction, the cDNA strands give rise to a new complementary DNA strand and generate a double stranded DNA. After the first step, amplification of the target sequence proceeds at an exponential rate (doubles for each cycle). Depending on the amount of target cDNA present in the sample, a variable number of cycles will be needed to amplify the target and produce enough copies to produce a fluorescent signal that can be detected by the qRT-PCR instrument. If the conditions are optimal, the reaction will occur with almost exponential efficiency (for each cycle, each DNA fragment gives rise to one new DNA fragment).
After some additional cycles (>10 cycles), primers and reagents will no longer be in excess. Consequently, at this point the amplification rate leaves the exponential phase and enters a variable ("linear") phase. At additional cycles, the amplification rate will approach zero (plateau), where only a negligible amount of product is made.

The amplification is very most reproducible at the start of the exponential phase. The number of cycles needed for a target to give rise to a signal above a threshold value (set at the exponential phase for all samples) is used to calculate the amount of the target in the sample (C_t value). Genes that are expressed at a high levels reach the threshold levels early, whereas genes that are low expressed need additional cycles.





Overview of qRT-PCR reaction

The qRT-PCR reaction consists of two steps, catalysed by two different enzymes. The first step copies all RNAs in a sample into cDNA sequences using the enzyme *reverse transcriptase*. The next step amplifies one selected target sequence among all the cDNAs present in a polymerase chain reaction using the enzyme *DNA polymerase*. The number of cycles needed to reach a certain number of copies of the target sequence is used to calculate the amount of this target in the sample.

Fluorescence for detection of DNA

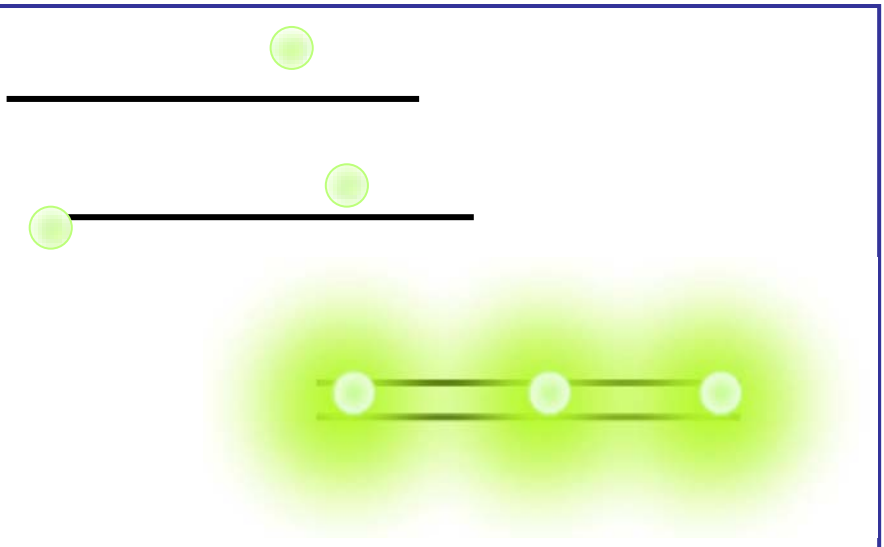
Two different technologies have been developed to detect the amplified DNA fragments. TaqMan probes and SYBR Green dyes have different mechanisms for detection of DNA products.

The TaqMan probe system requires three primers that bind to the target sequence**.** Two primers amplify the target sequence, whereas a third primer binds in the region in-between the two primers. The internal binding primer is called a probe and contains a fluorescent reporter dye attached to the 5' end and a quencher attached to the 3'end. The quencher absorbs any emission from the reporter dye when they are in proximity (the probe is intact). The DNA polymerase used in the PCR reaction has 5' exonuclease activity, and when the probe is bound to a template sequence, it will and hydrolyzes the probe into single nucleotides and release quencher from the the fluorescent reporter, which then emits fluorescence. Because TaqMan probes bind to the template in a sequence-specific manner, this type of assay needs little optimization. However, synthesis of a unique TaqMan probe for each target is expensive.

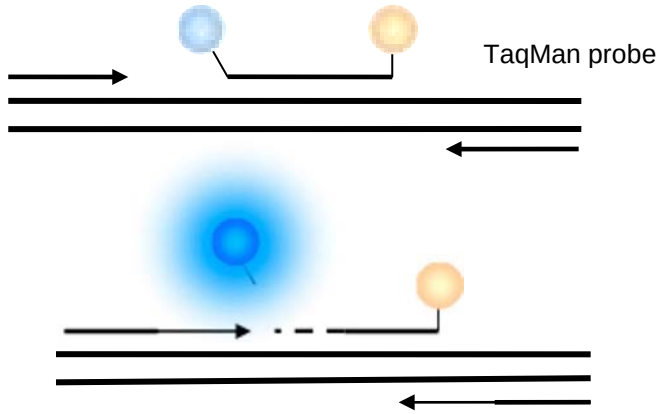
SYBR Green dyes binds to any double-stranded DNA and emits strong fluorescence upon excitation. Several molecules of dye can bind to each double-stranded DNA product, and the emission is a measure of the total mass of double stranded DNA, and not only the number of target copies produced. This system is therefore more susceptible to unspecific fluorescence signals caused by primer-dimers and amplification of non-specific products, which could lead to overestimation of target concentration, especially in very late cycles. Different methods must be used to confirm product specificity and validate primers for each target before SYBR green technology can be used.

Quantification - determination of the amount of targets in a sample

Two different strategies are used to determine the amount of a target in a sample.
In the **standard curve method**, an mRNA of known concentration is first used to make a standard curve. This curve can then be used as a reference for extrapolation of mRNA targets. The **comparative C_t method** (comparative threshold method) involves comparison of the sample against a control or calibrator (e.g. RNA from untreated sample or from normal tissue). The samples and the control are normalized against an appropriate endogenous housekeeping gene [= Reference gene, unregulated by treatment or metabolic status].



SYBR Green exhibits weak fluorescence in solution, but strong fluorescence upon binding to double-stranded DNA



When they are both attached to the TaqMan probe, the quencher (orange) inhibits fluorescence from the reporter dye (blue).
When reporter dye is cleaved off the probe and separated from the quencher, it emits fluorescence.

Purpose of today's lab work (day 3):

Task 1: Amplify FAS mRNA from cDNA prepared from mouse liver lysates.

Protocol - TaqMan qRT-PCR

Pre-considerations

Set up the following negative controls:

1) Negative controls without cDNA (a water sample with regular M-mix) to control the master-Mix (set up one for each assay).

2) Negative controls without RT enzyme to control for DNA contaminants in the RNA samples (cDNA samples generated at the cDNA synthesis step).

3. Pipette samples in the following order in a 96 well PCR plate: A1 sample 1, A2 sample 2. If samples are pipetted in singlicate, multi channel pipette can be used. *Alternatively, 8-strips PCR tubes might be used.*

4. Pipette negative controls as the last samples on Assay plates. This will simplify data analysis.

Set up Assay plate

5. Thaw 2x TaqMan®Universal PCR Master Mix, TaqMan®Gene Expression Assay, PCR-water and cDNA on ice, vortex, and spin down before use.

6. Dilute synthesized cDNA 5 times in RNase free H_2O (final cons. 2.5 ng/ul).

7. Set up qRT-PCR reaction. Final volume should be 20 µl reaction/well. Pipett assay according to the table below (multiply with n samples + 10%):

2x TaqMan® Universal PCR Master Mix	10 µl
Taqman® Gene Expression Assay	1 µl
PCR-water	4 µl
Total volume	15 µl
Add later	
cDNA (diluted 5 times)	5 µl

8. Mix the assay, and pipette into selected wells on an ABI Prism® 96-Well Optical Reaction Plate.

9. Add 5 µl diluted cDNA/well (gives 12.5 ng cDNA/reaction).

10. After all pipeting is done, cover the top of the plate by firmly putting on an ABI Prism® Optical Adhesive sealing. Make sure the film adhere and close properly around all wells.

11. Vortex the plate, and spin at 1000 rpm for 1 min. Placed the plate in the ABI 7900HT machine.

Running the 7900 instrument

Set up the run using the SDS 2.3 Software from ABI. Run using standard thermal cycling conditions. After the run, analyze results in RQ Manager (ABI).

12. Start the SDS 2.3 program.
13. Choice "File" – "New" – select on new window:
    1. Assay: ΔΔCt
    2. Container: 96 wells,
    3. Template: blank template
    4. Press OK.
14. Select all wells to be analysed for one assay/control. On "Setup"-page, choose "Sample Name", write a convenient assay name (gene-assay). Press "Enter". Repeat for different assays and/or negative controls.
15. Select all samples to be analysed. Press "Add Detector". Select detector (or make new). Press Copy to plate Document. Press "Done".
16. Select Instrument Window. Adjust "mode" to **standard**. Adjust reaction volume to **20 µ**l on thermal Cycler protocol. Use **40 cycles**.
17. Save the method/analysis file – choose proper location (D:\username).
18. Select [Real-time]-window. Press connect to instrument.
19. Press Open.
20. Place a gummy-plate on top of the assay plate. Put in samples (A1 upper left corner).
21. Press Close.
22. Start analysis by pressing "Run".

Day 4 Summary RT-PCR results

Day 5 Protein Quantification

Purpose of today's lab work (day 5):

Task 1: Measure protein concentration in liver lysates prepared from fasted and refed mice .
Task 2: Dilute samples for Western blotting.

Task 1 : Determine protein concentration in mouse liver lysates

Mouse liver lysates prepared from fasted and refed mice will be used to compare FAS protein expression in liver using a method called Western blotting. Prior to Western analysis, we need to measure the protein content in each sample, to ensure that we assay the same amount of protein from each sample. Protein concentrations can be determined using several methods, but the most widely used are based on addition of substances that absorbs light when bound to proteins.

The protein concentration is determined against a standard curve generated from a range of samples with known concentrations (standards). Absorption of light in the samples and standards are measured simultaneously in a spectrophotometer. The absorption of each standard is plotted against the known concentration and used to generate a standard curve. The standard curve is then used to estimate the concentration of each standard.

Protocol – Prepare protein samples

1. Defreeze the tubes with cell lysate harvested from dish A on ice.
2. Sonicate the cells for 5 seconds to disrupt cell membranes.

Always keep the samples on ice after thawing!

Protocol – Prepare standard curve and measure samples

Protein concentrations are measured with the BC Assay method (Interchim France, #FT-40840)

This method is based on a two-step reaction, in which Cu^{2+} is first reduced to Cu^{1+} forming a complex with protein amide bonds (Biuret reaction). Secondly, bicinchoninic acid (BCA) forms a purple complex with Cu^{1+} which is detectable at 562nm.

The protein concentration in the samples will be analyzed in a 96 well format.

1. Pipett standards into the 96 well plate. The standards are generated by using increasing volume of a bovine serum albumin (BSA) solution with known protein concentration (2 µg/ml).

standard	BSA standard (2µg/µl)	H2O	Sample	BC reagent
Std 0µg	0 µl	10 µl	-	200µl
Std 0µg	0 µl	10 µl	-	200µl
Std 6µg	3µl	7 µl	-	200µl
Std 6µg	3µl	7 µl	-	200µl
Std 10µg	5 µl	5 µl	-	200µl
Std 10µg	5 µl	5 µl	-	200µl
Std16µg	8 µl	2 µl	-	200µl
Std16µg	8 µl	2 µl	-	200µl
Prøve	-	-	5µl	200µl

2. Pipette 5µl from your sample in triplicates to the 96 well plate. Remember to whirl mix each sample before pipetting.
3. Calculate the amount BC reagent you need for both standards and samples, and add to the wells containing standards / samples. (200 µl reagent/well * X wells + 10 % extra).
4. Mix gently by shaking the plate carefully, seal the plate with plastic cover, and incubate at 37°C for 30 minutes.
5. Read the absorbance at 562nm.

Remember that the concentration measured is the total concentration in the well. Calculate the mean concentration pr µl in your samples.

Task 2 : Dilute samples and prepare proteins for western blotting/immuno blotting

1. Dilute the samples to 2 µg/µl using lysis buffer (containing proteinase inhibitor).
2. Prepare tubes with 40 µg protein (20 µl) in each tube for the SDS protein gel at day 6.

Tube 1a	Fasted
Tube 1b	Fasted
Tube 2a	Fed
Tube 2b	fed

3. Make duplicates from each treatment. Store the tubes at -70°C until running the gel at day 6.

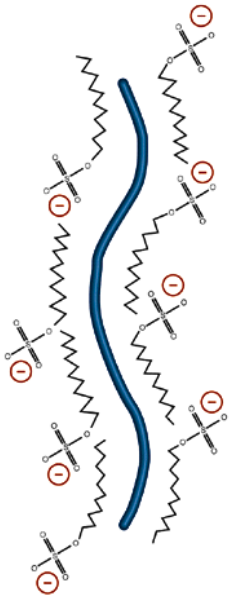
You should apply 20 µg proteins of each sample on the gel. In addition, you need to add loading buffer prior to gel running (see day 6).

Remember to always keep the samples and lysis buffer on ice after thawing!

Day 6 Western blotting

Western blotting (one of several different immunoblotting techniques) is used to detect and compare the expression levels of proteins in biological samples. Proteins are first separated by size in a matrix (polyacrylamide) prior to transfer of the proteins to a supporting membrane, usually a nitrocellulose or a PVDF (polyvinylidene difluoride) filter membrane, and finally detection and comparison of the expression levels of a selected protein among the samples using antibodies.

Several of the amino acids that are used as building blocks in proteins are negative or positive charged. Hence, similar to DNA, proteins can be separated by electrophoresis in an electric field. The electrophoresis can be performed in either *native* (non-reducing) or *reducing* conditions (often called SDS-PAGE). In native conditions, proteins are separated with their tertiary structures intact (the proteins folded), where migration speed depends on size and number of charged residues at the surface.



In denatured conditions, the proteins are unfolded with detergents (e.g. SDS, Tween, NP-40) and reducing agents (e.g. β-mercaptoethanol, dithiothreitol /DTT). Proteins solubilised in SDS bind the detergent uniformly along their length to a level of 1.4g SDS/g protein. This creates a charge/mass ratio which is consistent between proteins. For this reason, separation on a polyacrylamide gel in the presence of SDS occurs by mass alone, SDS PAGE offers a rapid and relatively accurate way to determine protein molecular weights within 5 - 10% accuracy. Occasionally proteins may retain enough secondary structure or contain sufficient charged groups to migrate anomalously. The migration of histones, which carry a strong intrinsic charge, is an example of this phenomenon. Since denatured conditions give a better estimate of the proteins size, protein separation is generally run under reducing conditions.

After polyacrylamide gel electrophoresis, the gel content is transferred to a membrane.
Selveral techniques might be used to detect a specific protein on the membrane. The most commonly used technique is the use of a two step antibody (Ab) procedure. The membrane is first exposed to an unlabeled Ab specific for the target protein (e.g Plin2). The bound Ab is then bound by a secondary Ab that specifically recognises the first antibody (but not the target protein). The second Ab is coupled to an enzyme, e.g. horseradish peroxidase (HRP), which catalyse conversion of a substrate that emits light.
The presence and amount can in the end be detected by incubating the membrane in the substrate for the enzyme, followed by detection of the emitted light using film or special equipment that can detect emitted light. Enhanced chemilumeniscence (ECL) Plus technology is based on enzymatic generation of acridinium ester intermediates which reacts with peroxide and produces a high intensity chemiluminescence with maximum emission at a wavelength of 450 nm.

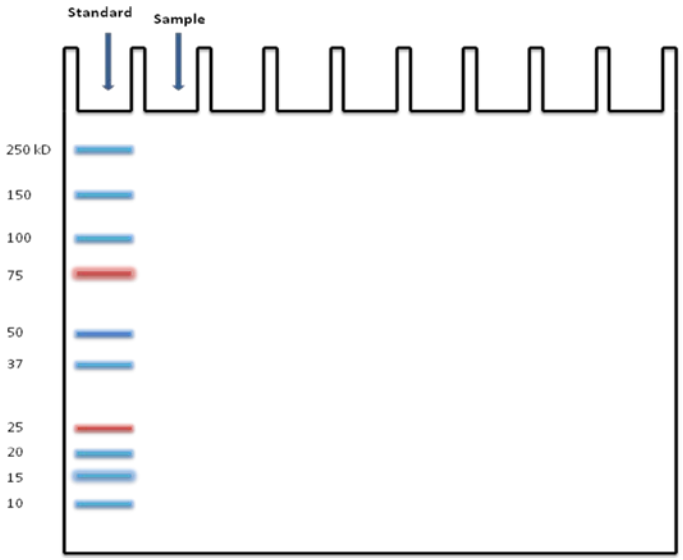
Purpose of today's lab work (day 6):

Task 1: Run SDS-PAGE gelelectrophoresis.
Task2: Electrotransfer to membrane (blotting)
Task3: Block membrane, and incubate with primary antibody (Ab)

Task 1 : Run SDS- polyacrylamide gel

SDS- polyacrylamide gel separation

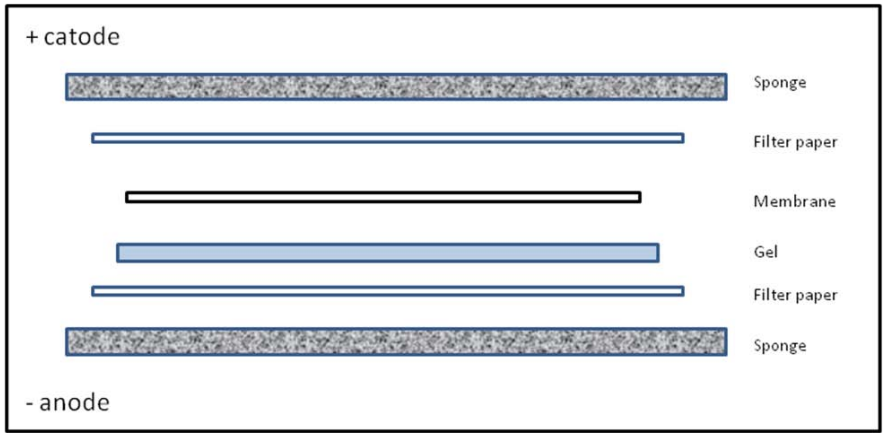
1. Thaw the samples with proteins from liver (20 µl=40 µg) made at day 5 (you will need one tube from fasted mice, one from fed).
2. Prepare 500 ml running buffer. Dilute 10 X running buffer to 1 X with MilliQ filtered H_2O (ultrafiltered/ deionised).
3. Rinse the wells with running buffer before adding sample.
4. When the samples are thawed, add 5 µl 5x loading buffer to each tube, mix and spin briefly (*see next page*).
5. Denature the proteins by heating for 5 minutes at 95ºC on a heat block. After heating spin the tubes for a short time, keep the tubes at room temperature and apply the samples as soon as possible onto a 7.5 % Tris-HCl SDS gel. Remember to apply a molecular weight marker.
6. Run the gel at 150 V for about 1,5 hour (until the blue colour from the loadingbuffer has run out of the gel).



Task 2 : SDS gel blotting

Methanol is volatile and toxic. Always keep an open bottle in a ventilated hood.
Prepare the transfer buffer while the gel is running.

1. Make 1600 ml buffer pr blotting tray. Dilute 10 X transfer
2. Buffer to 1 X. Add metanol to 20% of the total volume and
3. Dilute with H_2O (deionised). Keep at 4°C until use.
4. Cut two peaces of MM-Watman filter paper, a bit bigger than the size of the gel. Cut a the PDF membrane at the same size of the filter paper. Do not touch the surface of the membrane. Use a ink pen (or pencil) to mark the membrane with experimental details. Soak the membrane in methanol for 1 min, rinse in water and place in a tray with transfer buffer.
5. Place a stacking of filter-paper, gel, and membrane in the order shown below. Make sure you have the correct orientation (the proteins in the gel are transferred against the catode).
6. Transfer the gel to the PVDF membrane by electro transfer for about 1h at 30 V.



Reagents

10 X Running Buffer, pH 7,4
96 mM (30 g) TrisBase
1,9 M (144 g) Glycin
10 % (10 g) SDS
H_2O (MQ) ad 1l

10 X Transfer Buffer
25mM TrisHCl
190mM Glycin
H_2O (MQ) ad 1l

Task 3 : Blocking membrane

While the gel is transfered to the membrane, prepare washing buffer and blocking buffer.

1. Dismount the filter paper, membrane, gel-stack, and carefully transfer the membrane to a chamber containing washing buffer. Wash well, and make sure all gel is removed from the membrane (the stacking (the top containing the wells) tend to stick to the membrane.
2. Rinse the membrane twice for 5 minutes in water.
3. Wash quickly with washing buffer and block the membrane in 20 ml blocking buffer for 1 hour on an orbital shaker at room temperature. The blocking buffer is added to the membrane to prevent background binding.

Task 4 : Ab binding to FAS

All incubating and washing steps should be performed on an orbital shaker.

1. Drain off the blocking solution.
2. Wash 4 x 5 minutes with washing buffer on an orbital shaker (all incubations and washing is performed on an orbital shaker).
3. Add primary Ab: anti FAS diluted ~1: 1000 (will depend on Ab used) in 10 ml blocking agent. Incubate over night at 4°C on an orbital shaker.

Recipes

Washing buffer

Make 2 l of washing buffer by dissolving PBS- tablets in 2 litre of H_2O. Add 2 ml Tween- 20 (0,1 %) by pipetting, add a magnet and stir until everything is dissolved.

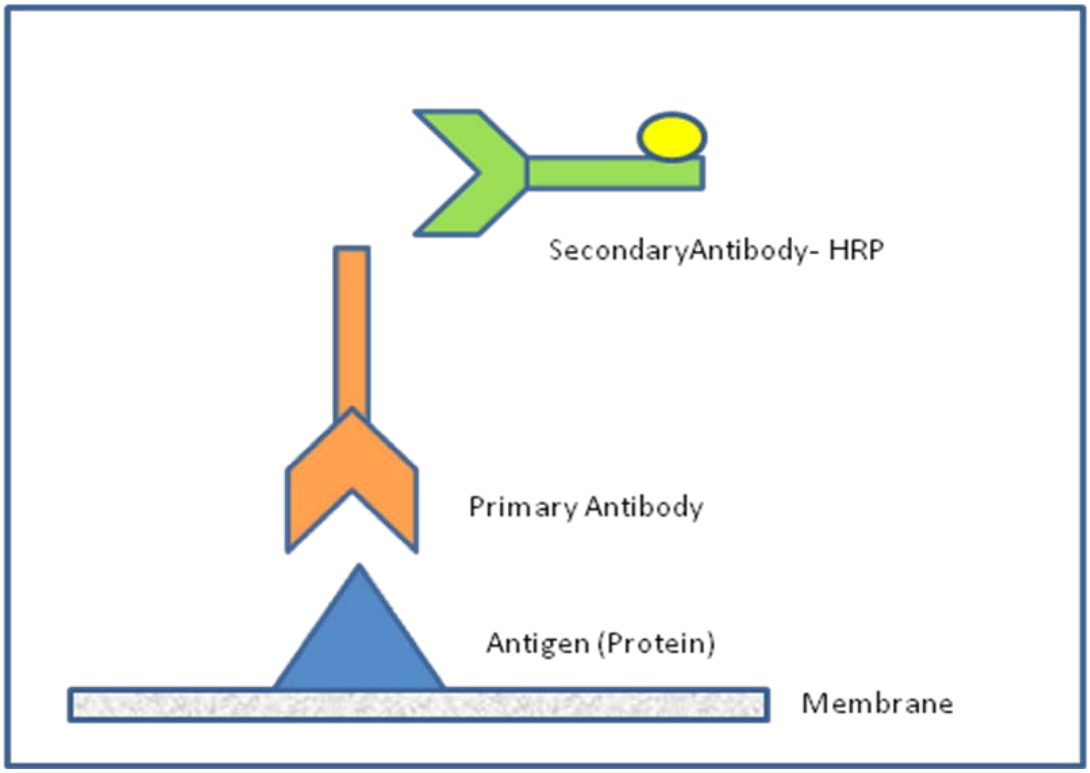
Blocking buffer

Make 150 ml of blocking buffer by adding 7,5 g skim milk powder (0,5 %) to washing buffer. Add a magnet and stir until dissolved.

Day 7 Continue Antibody binding

Task 1: Washing and 2.Ab binding

4. Wash 4 x 5 minutes with washing buffer.
5. Add secondary HRP-conjugated Ab specific to the primary Ab: diluted 1: 10 000 in 10 ml blocking agent. Incubate for 1 hour at room temperature.
6. Wash 4 x 5 minutes with washing buffer.
7. Rinse the membrane 2 x 5 minutes in H_2O.
8. Finally the membrane is ready for enhanced chemiluminescence (ECL) detection of the bound secondary HRP-conjugated Ab.



Task 2 : Chemiluminescence and visualization

The non- radioactive method of ECL Plus - enhanced chemiluminescence (Amersham Biosciences (RPN2132) is performed to visualize the proteins. A chemiluminescent reaction with horseradish peroxidase produces light with exitation of 430 nm and emision of 503 nm.

Detection:

The ECl Plus detection kit contains two solutions; A and B, which should be mixed at the ratio 40:1. Use 2 ml solution A + 50µl solution B. (0,1ml/cm^2 is required for the membrane).

1. Remove all fluid from the membrane (membrane must not dry completely!).
2. Carefully cover the whole membrane with the enhanced chemiluminescence (ECL) solution.
3. Incubate at room-temperature for 5 minutes without disturbing the membrane.
4. Remove the chemiluminescence solution and wrap the membrane in a thin plastic bag. The plastic is required to prevent the membrane form drying.

There are numerous different ways of detecting the fluorescent signal from the bound HRP-conjugated Ab. We will visualize the signal by exposing the membrane to Hyperfilm MP (Amersham Biosciences) and develop the film on a Kodak processor machine.

Important: Films are sensitive to light, and all handling of the film prior to development ***must*** *be carried out in a dark room under special lightening*

Summary Western blotting results