Laboratory 2:
RNA isolation / Reverse-Transcription

Objectives

- To isolate total RNA from mouse tissue
- To determine the quantity and quality of the isolated RNA
- To synthesise cDNA using the reverse transcription reaction

Introduction

Amy2 is specifically expressed in the mouse pancreas. This tissue contains large amounts of RNases and isolation of intact total RNA is delicate. Therefore, we have prepared total RNA from pancreas and you will instead isolate total RNA from mouse liver tissue (containing lower amounts of RNases). You will use pancreatic RNA provided by us to prepare cDNA by reverse transcription in order to amplify the *Amy2* coding sequence in the next lab.

A mammalian cell contains about 10^{-5} µg of **total RNA** comprised of:

- 80-85% ribosomal RNA (28S, 18S, 5.8S, and 5S)
- 15-20% transfer RNA, miRNA, small nuclear RNA etc
- 1-5% mRNA

The Size of ribosomal RNA (rRNA) is species-specific:

Species	rRNA	Size (bp)	Ratio 28S/18S
Human	28S 18S	5034 1870	2.69
Mouse	28S 18S	4729 1869	2.53

Table 1: Size of ribosomal RNA in human and mouse

Since 18S and 28S rRNA species are derived from the same transcript, cells have equal molar amounts of both molecules. The intensity of the rRNA bands on (denaturing) agarose gels is proportional to the transcript length and the 28S/18S ratio serves as a proxy of RNA integrity. A 28S/18S ratio of 2 is considered to be good quality RNA, although the theoretical ratios are higher (Table 1). In case RNA is degraded, shorter products will appear (as discrete bands or a smear). Note that 28S rRNA is more susceptible to degradation than 18S due to its larger size. The following assumption is made: if ribosomal RNA (80-85% of total RNA) is intact then the mRNA (1-5% of total RNA) is usable. Other techniques for quantitative measurements of nucleic acids exist, for example fluorescent dyes that bind specifically to RNA (or DNA) or microfluidic systems that are essentially miniature gels requiring very little sample.

Working with RNA

RNA which has hydroxyl groups in both the 2' and 3' positions of ribonucleotides is chemically more reactive than DNA and susceptible to degradation by RNases. These enzymes are released from cells upon lysis and are present on the skin (bacteria and fungi).

Guidelines when working with RNA:

- Practice good sterile technique to prevent microbial contamination: clean your bench well with 70% ethanol or a special RNase decontamination solution.
- Always wear gloves and a lab coat.
- Change gloves if you touch surfaces that might be contaminated with RNase.
- Only use filter tips, tubes and solutions that are RNase-free.
- Keep RNA on ice while assembling a reaction and keep the tubes closed as long as possible.
- Addition of RNase inhibitor protects the RNA from degradation.

1. RNA isolation

Isolation of total RNA from mouse tissues will be performed using Trizol (Chomczynski and Sacchi, 1987). This method is simple and widely used for purification of RNA, DNA or proteins, which can be recovered from the same biological sample. Trizol is a mono-phasic solution of phenol and guanidine isothiocyanate that preserves the integrity of the RNA during sample homogenization and lyses the cells. Guanidinium thiocyanate denatures proteins, including RNases, and separates rRNA from ribosomes. Phenol-chloroform extraction followed by centrifugation separates the solution into an aqueous (upper clear) phase and an organic (lower red) phase. RNA remains exclusively in the aqueous phase, while DNA and proteins are at the interphase and organic phase. RNA is recovered from the aqueous phase by precipitation with isopropyl alcohol and further purified on silica spin-columns to remove small RNA species <200 bp (5S rRNA, tRNA, miRNA etc).

During this laboratory session we will use a combination of the Trizol RNA isolation method with the RNeasy procedure (Qiagen). We have performed the RNA extraction with Trizol. You will start with the aqueous phase that is mixed with ethanol to provide appropriate binding conditions. The sample is then loaded onto an RNeasy spin column, where RNA >200 bp binds to the silica membrane and smaller contaminants are washed away. The purified RNA is finally eluted in RNase-free water. Note for quantitative real-time PCR studies, RNA samples can be treated with DNase during column purification.

Materials

- Trizol/ liver extract from a C57 Black 6 mouse (20 µl; from about 5 mg liver)
- 100% Ethanol
- RNeasy Mini Kit reagents (Qiagen): RW1 buffer, RPE buffer, RNase-free water, RNeasy mini spin column, 2 ml collection tubes, 1.5 ml collection tube
- Microcentrifuge

Procedure

Trizol and chloroform are carcinogens- work with **gloves and goggles**. For safety reasons, we performed homogenization of mouse tissue in Trizol and phenol-chloroform extractions. You will continue the RNA isolation using the aqueous phase of a liver Trizol extract. All steps are performed at room temperature (RT). Remember to balance the tubes in the rotor during centrifugation.

1. To the **Trizol extract (20 µl)** add **80 µl RNase-free wate**r and **120 µl of 100% EtOH**, mix well by vortex and spin down.
2. Load the sample onto a RNeasy spin column, placed in a 2 ml collection tube. **Spin 15 sec at 13'000 rpm**. Discard the flow-through. Reuse the collection tube in the next step.
3. Add **700 µl RW1** wash buffer to the RNeasy spin column, **spin 15 sec at 13'000 rpm** to wash the spin column membrane. **Carefully** remove the RNeasy spin column from the collection tube (the column should not contact the flow-through at this and subsequent steps to avoid contamination). Discard the flow-through. Reuse the collection tube in the next step.
4. Add **500 µl RPE** buffer to the RNeasy spin column. Close the lid gently, and spin **15 sec at 13'000 rpm** to wash the spin column membrane. Discard the flow-through and reuse the collection tube.
5. Wash a second time with **500 µl RPE buffer**, and **spin 2 min at 13'000 rpm**. Discard the flow-through. The long centrifugation dries the spin column membrane, which is to remove all ethanol. Residual ethanol in the elution may interfere with downstream reactions.
6. Place the spin column into an RNase-free 1.5 ml microfuge tube, add **30 µl RNase-free water directly to the center of the spin column membrane**. **Spin 1 min at 13'000 rpm** to elute the RNA. Remove the RNeasy spin column from the tube and put the purified RNA on ice.

2. RNA quality control

The **chemical purity** of an RNA sample can affect the efficiency of downstream enzymatic reactions, like reverse transcription. **Integrity** of RNA samples is essential for quantitative RT-PCR or next-generation sequencing analysis. RNA integrity is less critical for PCR amplification and cloning of a target sequence from a cDNA pool, since a few copies of intact mRNA are sufficient.

2.1 Determination of RNA concentration and purity

Using the NanoDrop spectrophotometer, you will determine the concentration and chemical purity of the purified RNA. 1 ul of sample is directly pipetted onto the optical fiber. Upon contact of the liquid with the second fiber a liquid column forms between the fibers by surface tension. The pedestal automatically adjusts for an optical path length of 1 mm during the measurement. Absorbance values are then normalized to a 10 mm (1 cm) path. The concentration of nucleic acids can be determined from the absorbance measurement using the

Beer-Lambert Equation **$A = \varepsilon c l$**

A = absorbance
ε = extinction coefficient
c = concentration (in units corresponding to ε)
l = light path length.

Both DNA and RNA have absorption maxima at 260 nm. Extinction coefficients have been calculated for double stranded DNA, single stranded RNA, and single stranded DNA (see table below). The extinction coefficients can be converted into standard coefficients for a 1 cm path length which are commonly used for nucleic acids.

Standard coefficient = path length 1 cm/ extinction coefficient.

Nucleic acid	**Extinction coefficient** (µg/ml) cm^{-1}	**Standard Coefficient** 1 cm path length (µg/ml)
Double stranded DNA	0.020	50
Single stranded RNA	0.025	40
Single stranded DNA	0.027	33

Table 2: Extinction and standard coefficients for nucleic acids measured in a 1 cm cuvette

For converting absorbance (at 260 nm) of nucleic acids the following relationship holds (1 cm path):

- 1.0 A260 unit of dsDNA = 50 µg/ml
- 1.0 A260 unit of ssDNA = 33 µg/ml
- 1.0 A260 unit of RNA = 40 µg/ml

Thus, the equation for calculating concentration of nucleic acids (path length 1 cm) with standard coefficients reads:

nucleic acid concentration = OD260 X standard coefficient X dilution factor
Example of a typical NanoDrop output profile (Figure 2):
undiluted RNA measured OD260= 49.148
c = 49.148 X 40 µg/ml= 1965.9 µg/ml

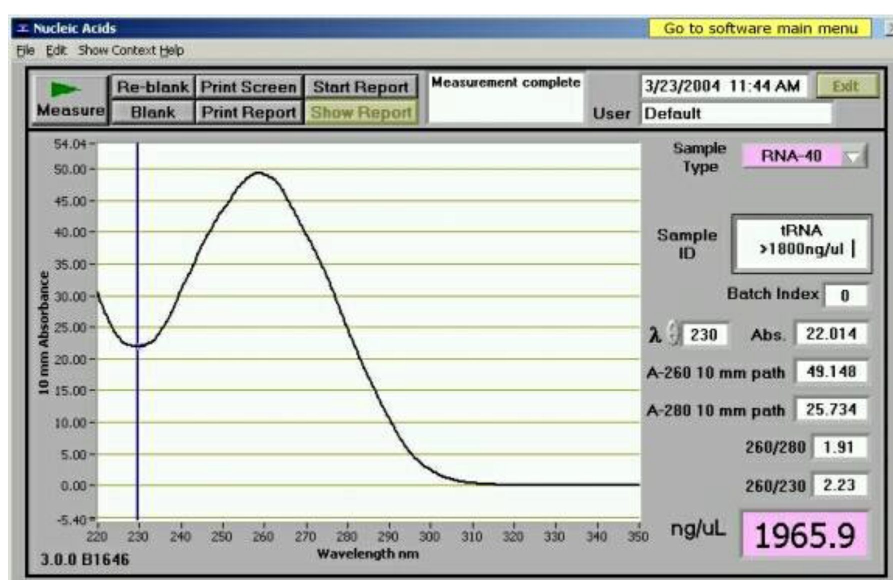

Figure 1. NanoDrop profile of RNA with corresponding concentration as well as 260/280 and 260/230 ratios.

A260/A280 ratio

The ratio of the absorbance at 260 nm and 280 nm is used to assess the **purity** of nucleic acids. Expected values for pure samples:

	RNA	DNA
A260/A280	2.0	1.8

Ratios below 1.6 may indicate contaminants that absorb at or near 280 nm (aromatic residues in proteins and phenol etc).

RNA contains uracil (ratio 4.00) instead of thymine (ratio 1.47) and therefore its A260/280 value is higher compared to that of DNA. The A260/A280 ratio is influenced by pH (Wilfinger et al. 1997). Since water is not buffered, the pH and the resulting A260/A280 ratio can vary greatly. Remember to blank the instrument with the same buffer as for the sample, since acidic pH results in a decreased ratio (by 0.2 – 0.3).

A260/A230 ratio

The A260/A230 ratio is a second purity assessment and indicates the presence of contaminants that absorb near 230 nm (e.g. guanidinium isothiocyanate, Trizol, phenol, proteins or EDTA). Contamination of your RNA sample with trace amounts (0.5%) of guanidinium isothiocyanate (present in the RNA isolation buffer) will result in a drastic drop of the A260/A230 ratio without affecting the A260/A280 ratio.

Expected A260/A230 values for nucleic acids are in the range of 2.0 – 2.2.

NanoDrop specifications

- Minimal sample volume: 0.5 µl
- Path length: 1 mm
- Wave length: 180-840 nm
- Accuracy of Absorbance: 2%
- Range of Absorbance: 0.02-300
- Detection limit: 2 ng/µl ds DNA
- Maximal detection limit: 15’000 ng/µl ds DNA

Materials

- RNA you purified from liver and control liver RNA
- RNA elution buffer (here: RNase free water, Qiagen)
- NanoDrop 2000 spectrophotometer (Thermo scientific)

Procedure

Always blank the spectrophotometer with the same solution as your sample!

1. Double click on the Nanodrop software and select application of interest (e.g. *Nucleic Acid,* then *RNA*).
2. Establish a blank with 1.5 µl buffer (same as sample); lower the arm carefully and click the Blank button. To ensure the blank was successful you may then click *Measure.*
3. Once the measurement is done wipe away the blank from both upper and bottom pedestals with Kimwipe
4. To measure each 1.5 µl sample or control, enter the sample ID (with group number), lower the arm and click *Measure*.
5. After each measurement, print screen the spectrum and save images with group number.
7. Wipe sample from both upper and bottom pedestals with Kimwipe. **Cleaning**: only use dH_2O and wipe with *Kimwipe.* Never use detergents or alcohol!! They un-condition the patented pedestal surfaces.

2.2 Determination of RNA integrity by gel electrophoresis

RNA quality is critical for the success of subsequent downstream molecular applications such as RT-qPCR analysis and library preparation for next-generation sequencing. Size determination of RNA and DNA is a useful quality metric, since degradation leads to predominantly small fragments.

We will analyse the integrity of RNA samples by **agarose gel electrophoresis** and specific staining of nucleic acids with a dye (GelRed). Binding of the dye is proportional to the length of the RNA, and thus 28S rRNA will bind two times more dye than 18S and its intensity on the gel will increase accordingly. Since RNA is negatively charged, it migrates toward the anode in an electric field. Smaller molecules will migrate faster through the gel matrix than larger ones. For size determination of RNA a different method, denaturing agarose gel electrophoresis containing a chemical for denaturation (such as urea, formaldehyde) can be used; for safety reasons we are not using this technique in the course. Many modern automated systems provide quantitative nucleic acid quality metrics based on electrophoresis of small volumes (for example in a capillary) combined with a fluorescent dye.

Materials

- Purified liver RNA
- Commercial liver RNA (control)
- RNase-free water
- RNase-free 1% agarose gels (non-denaturing in TAE buffer) and trays
- 6X loading buffer

Procedure

1. Prepare **1 µg** of purified liver and control liver RNA in a final volume of 18 µl 1X loading buffer. Keep samples on ice. Remember that an RNase free environment is essential when working with RNA samples.

   Concentration of purified liver RNA: ________ ng/µl

   Concentration of control liver RNA: ________ ng/µl

Volume	µl	µl
Purified liver RNA		–
Commercial liver RNA	–	
Water		
6X loading buffer		
Final volume	18	18

2. Load RNA samples onto an agarose gel (2 groups per gel) and run the gel at **100 Volts for 30 minutes**.
3. Take an image at the UV transilluminator. Save image with group number.

Before you insert the gel picture into your notebook, **crop** parts that contain no information (parts without gel, empty lanes) and **label** the image outside of the image (do not cover data, no handwriting) by indicating the samples as well as the bands corresponding to 18S and 28S RNA. You may use any suitable program (Powerpoint, Inkscape etc). Describe the pattern of the bands that you observe. Do you see the expected bands? What do you see in the control? Compare the intensity of 28S and 18S RBA. Is the purified RNA intact? Was there degradation? Based on your results, is the quality of the purified liver RNA sample good enough to be reverse-transcribed in order to get full-length cDNA copies? Discuss the results in your lab notebook.

3. Reverse Transcription (RT)

The aim of this experiment is to produce cDNA copies (first-strand cDNA) from total pancreatic RNA. The target sequence corresponding to themRNA of interest (*Amy2*) will then be amplified by PCR from this tissue-specific cDNA pool during the next lab session.

Many viruses contain RNAas genetic material and encode a specific enzyme called reverse transcriptase, which can catalyze the synthesis of DNA from an RNA template. **Reverse transcription** has become an important tool in molecular biology. We will use Moloney murine leukemia virus-derived reverse transcriptase (M-MLV RT). It allows the synthesis of DNA complementary to an mRNA template and DNA prepared in this manner, called complementary DNA (cDNA), which can be PCR amplified and cloned. Random oligonucleotides or oligo dT primers are used to prime reverse transcription reactions. Oligo dT primers only prime at the polyA tail of mRNAs (1%-5% of total RNA) and all cDNAs made this way will contain the 3' end of the gene. This primer is commonly used to produce full-length cDNAs. Random hexamers bind to mRNA, rRNA or tRNA at a variety of complementary sites and lead to partial length (short) cDNAs.

Materials

- RNA template: total RNA from mouse pancreas; 250 ng/ µl
- Oligo dT_{12-18} (50 µM; Invitrogen)
- dNTPs (10 mM each; Invitrogen)
- DTT (0.1 M, Invitrogen)
- 5X RT buffer (250 mM Tris-HCl pH 8.3; 375 mM KCl, 15 mM $MgCl_2$, Invitrogen)
- Reverse transcriptase (Superscript III, 200 U/µl; Invitrogen)
- Ribonuclease inhibitor (RNasin, 40 U/µl; Promega)
- Nuclease-free water
- Thermal cycler (Biometra)

Procedure

To detect potential DNA contamination, you perform two reactions: one sample with reverse transcriptase (+) RT and a control reaction without (−) RT. In the negative control (−) RT no synthesis of first-strand cDNA should occur and thus allows detection of potential DNA contamination of the RNA sample or reagents.

1. Primer annealing

1. In sterile RNase-free 0.2 ml PCR tubes add:

	(+) RT sample in µl	**(−) RT control** in µl
Pancreas RNA (1 µg)		
oligo dT primers	1	1
Nuclease-free water		
Total volume	12	12

2. Heat the tubes to **65°C for 5 min** to melt the secondary structure within the template. Cool the tubes immediately on ice to prevent secondary structure from reforming.
3. Spin briefly to collect the liquid at the bottom of the tube.

2. Reverse Transcription

4. Add the following components to the annealed primer/template in the order shown.

	(+) RT sample in µl	**(−) RT control** in µl
Annealed primer/RNA from step 1	12	12
5X Reaction Buffer	4	4
DTT, 0.1M	1	1
dNTP, 10 mM	1	1
Ribonuclease inhibitor	1	1
RT enzyme	1	-
Nuclease-Free Water	-	1
Total volume	20	20

5. Vortex briefly to mix followed by a quick spin and put your tube in the Thermal cycler.
6. Start the RT program:
    - 50°C, 60 min
    - 72°C, 15 min (to inactivate the enzyme)
    - 4°C on hold

7. Store the cDNA at -20°C until use.

Task

Draw a diagram that shows both the (+)RT and (-)RT control reactions, including the key molecules involved. You can either draw it by hand or use BioRender (navigate to Icons > Nucleic acids to find the relevant symbols). Ensure your diagram clearly distinguishes the presence and absence of reverse transcriptase in the reactions. Paste the diagram in your electronic lab notebook with a figure legend. Remember to cite the source of any tools or images used.

References

Chomczynski P, Sacchi N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162(1), 156-9.

Wilfinger WW, Mackey K, Chomczynski P. (1997). Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques. 22(3), 474-6, 478-81.