Desalting, Rebuffering, Renaturation – Dialysis for Optimized Sample Preparation

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Introduction

Sample preparation of macromolecules is one of the most often tasks in the daily lab work. For example, desalting, rebuffering, renaturation, precipitation, resolving of DNA, RNA, carbohydrates and proteins are necessary to purify and to characterize the molecules of interest into samples. Fortunately for the sample preparation are well established methods available. A well-known example is the removal of salts in a purified protein after FLPC separation. A further example is the purification of DNA sequencing reactions where labelled dNTP's or primers have to be removed. Were proteins, for example, denaturated by urea to improve their solubility, in further the urea has to be removed. The question for the user is in every case which method and tools are the most suitable for their specific process. Size exclusion chromatography, ultrafiltration and dialysis are methods of choice in lab. This paper should help the user to receive a generally overview about the advantages and disadvantages of common used methods based on size of molecules within the sample. Main focus will be at sample preparation of proteins with new developed tools for the diaylsis of variable sample numbers in microplate format with applications.

Separation methods

There are three separation methods entrenched in laboratories (Figure 1):

- Gelfiltration
- Ultrafiltration
- Dialysis

Each method has its specific advantages and disadvantages which have to considered individually depending on the personally requirements.

Gelfiltration

Gelfiltration is a non-absorptive chromatography method with isocratic elution.The sample is separated by molecule size. The solved sample is transported by the mobile phase (buffer) through the stationary phase (matrix). Smaller molecules enter into the matrix whereas larger molecules cannot enter the matrix. The result is that the larger molecules were transported faster through matrix and elutes earlier.

The gelfiltration material is packed in columns with different lengths and diameters. Usual gelfiltration material is dextran (Sephadex™), agarose (Sepharose™, Bio-GelA) or polyacrylamide (Bio-Gel®P, BioRad). A wide range of gelfiltration columns are available on the market today.

The gelfiltration is a widely used method in particular for desalting or separation of not completely purified protein solutions. Users can obtain high sample recovery rates with gelfiltration and is typically characterized by simple handling.

But there are some cons which have to treat before application. The sample volume defines the columns size (length) because the separation between different molecule sizes and resolution increases with the columns length. Another con is that sample will be diluted and have to be concentrated in certain circumstances after

gelfiltration. Also limitations in high throughput screening applications and with the use of liquid handling devices occur.

[1]Sephadex™ and Sepharose™ are trademarks of GE Healthcare, Little Chalfont, USA. Bio-Gel® is a registered trademark of Bio-Rad, Hercules, USA.

Ultracentrifugation

Common applications of the ultracentrifugation are desalting, exchange of buffer and concentration of the sample. The molecule separation takes place through a semipermeable membrane. Molecules with larger size than the pores of the membrane cannot pass and will be retained. Molecules with smaller sizes will pass the membrane. The process is accelerated by the use of pressure due to centripetal acceleration in a centrifuge. Since the solvent of the sample passes the membrane the volume of the retained sample (over the membrane) decreases and therefore the concentration of the sample increases. This is an advantage and disadvantage: Sample concentration can be increased in an easy and fast way. The significant disadvantage is that the sample could precipitate, membrane fouling or clogging and this is normally associated with sample losses.

There is a large selection of ultrafiltration cartridges in different sizes, membrane types and cut offs on the market available. Polyethersulfone, nylon, hydrophilicpolypropylene, and cellulose acetate are common membrane materials. Cut offs between 3:10:30:100 and 300 kDa ensure a wide application field.No special skills or equipment were required, only a standard laboratory centrifuge is necessary.

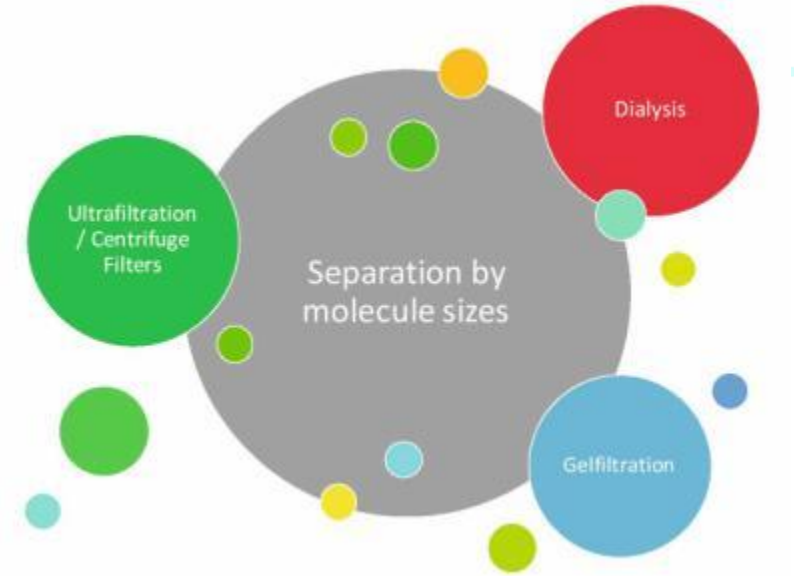

Figure 1| The three common ways for scientist to separate sample compounds by molecular weight

Dialysis

The third separation method is the diffusion driven transport through a semipermeable membrane. In the following the paper will focus on the method dialysis.

Introduction to Dialysis

Dialysis is the diffusion based size selective transportation of molecules or particles through a semipermeable membrane from higher concentration to lower concentration through Brownian Motion. Selectivity of dialysis is determined by pore size of semipermeable membranes. The end point of dialysis is the concentration equilibrium. Dialysis is an often-used method to separate bigger molecules like proteins or DNA from accompanying substances such as salt or detergents. It is a very gentle method for sensitive substances.

Example: A protein is dissolved in a highly concentrated salt solution, eg. Sodium Chloride NaCl solution (Figure *2*). NaCl disturbs following step such as ion exchange chromatography. The sample volume is pipetted into a dialysis device with a semipermeable membrane. The sample volume is in contact with higher volume of dialysis buffer through the semipermeable membrane. Only NaCl ions can pass through membrane pores. Protein molecules cannot pass through pores and will be retained. The NaCl concentration in the sample will be clearly reduced during dialysis process. The salt concentration can be adjusted to any desired level through multiple exchanges of dialysis buffer volume.

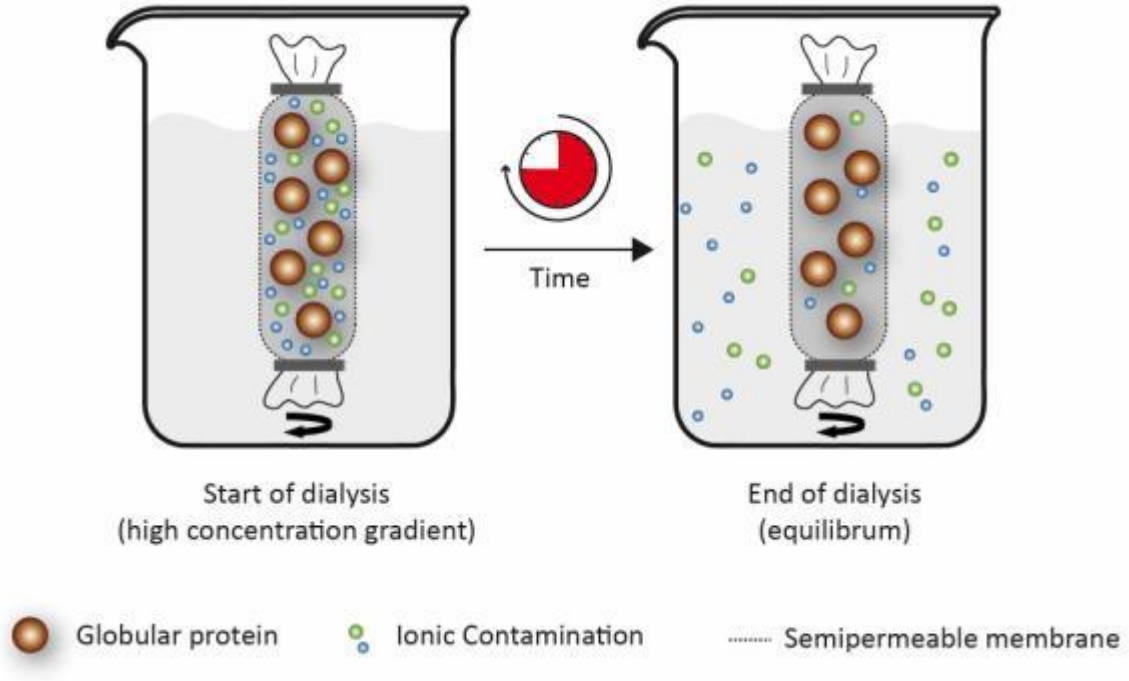

Figure 2 | Principle of protein sample dialysis.

Dialysis duration

Several aspects influence the dialysis duration: Substance, concentration, membrane, or length of diffusion pathway, as well as temperature. Some manufacturers, like scienova™, depict protocols for frequently used substances in their technical data sheets. These can be used as a basis for optimization of dialysis conditions for similar substances. When operating under special conditions or with less common substances, it is often

necessary to perform preliminary tests to find out the optimal parameters, especially required dialysis time, necessary to obtain the desired result.

Diffusion, concentration and size of the dialysed compound

Flux of molecules and particles are described by Fick's first law:

$$J = -D\frac{\partial c}{\partial x}$$

J:	*Diffusion flux*
D:	*Diffusion coefficient*
c:	*Concentration*
x:	*Diffusion length*
D:	*depends on characteristics especially of the size*

Concentration difference between sample and dialysis buffer: diffusion speed depends on the concentration difference between inside the dialysis sample and the corresponding dialysis buffer. Duration dialysis the concentration of membrane passing compounds is balanced and dialysis speed goes down. At the equilibrium the resulting flux is zero.

Rule dialysed compound size and concentration: The diffusion speed depends on molecular size (in solution hydrodynamic diameter). A smaller molecule diffuses faster. Therefore, the required dialysis time for most salts is shorter as for dyes with typical molecular weights of 200-500 Da. Use a pretest to determine optimal dialysis time if there is not an application example for your compound.

Length of diffusion pathway

Time consumption of the dialysis grows exponentially with the length of the diffusion pathway to the dialysis membrane. Therefore, it is very important to select a dialysis device with a short diffusion length (Example Figure *3*). Stirring or shaking the dialysis buffer and sample is recommended if possible. It will increase the transportation of substance through diffusion.

Rule diffusion length: as short as possible

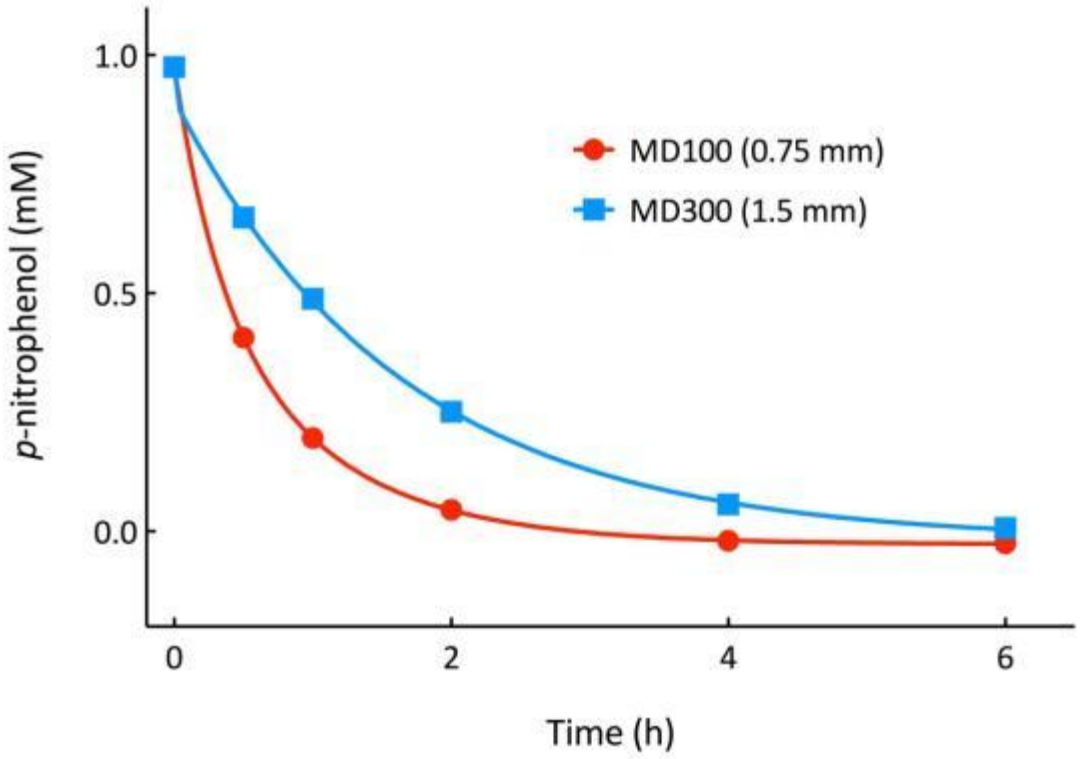

Figure 3| Comparison of dialysis speed at different diffusion lengths: scienova *Xpress* Micro Dialyzer MD100 (0.75 mm diffusion length) and MD300 (1.5 mm diffusion length) in Deep Well Plate Riplate (Co. Ritter Medical), sample 1 mM *p*-nitrophenole, dialysis buffer: 1-fold PBS pH 7.4. Dialysis duration 30 min, absorption at 420 nm (Tecan Sunrise)

Temperature

At higher temperature, the Brownian Motion as force of diffusion has a higher intensity. Therefore, the dialysis efficiency is higher at higher temperature (Figure *4*).

Rule temperature for dialysis: higher temperature = faster dialysis. The limitation is the temperature stability of your substance. Check the decomposition temperature of compounds of your sample especially for proteins and select a lower temperature as the decomposition temperature.

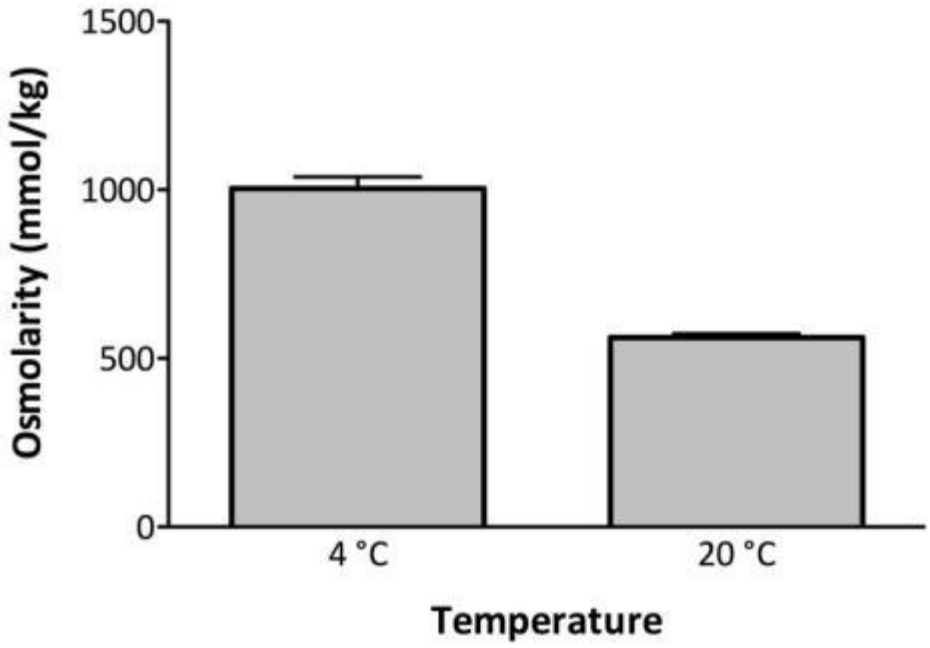

Figure 4 | Dialysis of PBS at different temperatures: scienova *Xpress* Micro Dialyzer MD100 in Deep Well Plate Riplate (Co. Ritter Medical), sample 100 µl of 1-fold PBS *p*-nitrophenole, dialysis buffer: destilled water, dialysis duration 30 min, measuring of osmolarity (Vapro Osmometer 5520. Kreienbaum)

Membrane selection

The flow through the membrane depends on the active membrane area, the ratio of active membrane area to sample volume, pore size, and porosity. A larger area to sample volume ratio, higher porosity, and broader pores enhances dialysis speed. The unit for pore size is most often a Dalton (Da). The selected pore size of the membrane should be as wide as possible while still retaining the desired sample substance. The pore size or cutoff (MWCO) in Da should be about double the molecular weight in Da of the desired sample substance. The characteristic of the membrane is indicated in the technical data of the manufacturer. Cut-off means that at least 90 % of globular substance with the same molecular weight of cutoff should be retained. For example, non-globular molecules or chain like molecules of similar molecular cut-off could pass pores easier. Therefore, preliminary tests should be performed for molecules with different cut-offs. To remove a low molecular substance, the cutoff should be selected at lower than half the molecular weight of the retained substance. Semipermeable membranes have a selective passage for molecules. Semipermeable membranes for dialysis have pores and can retain molecules according to their sizes. Selectivity depends on a tight size distribution of pore widths. Pure water tends to pass through semipermeable membrane toward higher concentration of molecules like salts or glucose. Osmotic pressure is the pressure needed to compensate the inward flow of pure solvent across a semipermeable membrane. Osmotic pressure can cause significant solvent flux during dialysis.

Membranes can bind substances in an unspecific manner, potentially causing loss of the sample. Especially proteins have very different binding affinities. Cellulose and cellulose ester are well-proven low binding materials for a lot of substances, especially proteins. The test of membrane binding for samples is recommended if loss of sample substance was detected.

Rule membrane selection: molecular weight of your molecule should be at least the double of the cut-off of the membrane to be retained, molecular weight of compounds which should be removed, should be no higher as half of the membrane cut off. If available use dialysis devices with vertical membranes to avoid membrane clogging.

Dialysis buffer

The dialysis buffer is in contact with the dialysis sample. In the simplest case the dialysis buffer can be water. Small compounds of dialysis buffer can pass through the semipermeable membrane. The chosen dialysis buffers have to be compatible with the required steps of sample preparation. It can be used for stabilization of pH or redox potential to protect sensitive substances. If the sample has a high concentration of a low molecular weight substance like saccharose, then the concentration gradient will cause an osmotic pressure resulting in significant transportation of water into the sample. In this case it is recommended to first use a step with a dialysis buffer that contains the same substance in a lower concentration to reduce the concentration gradient and the osmotic pressure.

Frequently used buffers for protein samples are phosphate buffer, TRIS, MOPS, and HEPES. Additional substances for protein dialysis are amino acids, EDTA, inhibitors, activators, cofactors, or DTT.

Buffer volume: essential for the dialysis result is the ratio buffer volume to sample volume. Dialysis is like a dilution of dialyzable compounds in the buffer volume plus sample volume. For example the concentration of NaCl into a sample is 100 mM. It should be reduced to 1 mM by dialysis. Factor for the reduction of NaCl concentration is 100 mM to 1 mM = 100. Sample volume is 1 ml. Therefore a buffer volume of 99 ml is necessary to get the reduction to 1 mM at dialysis equilibrium. For a reduction to 0.1 mM NaCl the buffer volume should be 999 ml, for a reduction to 0.01 mM NaCl it should be 9999 ml. In order to save dialysis buffer it is possible to use a stepwise approach with the exchange of the dialysis buffer after a time of dialysis. With the same example 1 ml sample NaCl concentration 100 mM and the wished end concentration 0.01 mM, for the first dialysis step the dialysis buffer volume is 99 ml, end concentration of NaCl of the first step is 1 mM, after first step dialysis buffer is removed and and replaced by 99 ml fresh dialysis buffer. End concentration of step two is a concentration of 0.01 mM. For the first example with one step dialysis the needed buffer volume is 9999 ml, the two step dialysis needs for the same result 198 ml dialysis buffer.

Rule dialysis buffer: buffer should be compatible with sample molecules and the following analysis or preparation steps, if necessary check the osmotic pressure in a pretest. Selection of buffer volume depends on the required end concentration of the substance which should be removed. Mostly an exchange of dialysis buffer is recommended in order to save dialysis buffer and time.

Selection of the best dialysis device

Main point for optimal dialysis process is the selection of a suitable dialysis device. There are different products of several suppliers on the market (table 1).

Company	scienova Gmbh	EMD Bioscience	Geno Technology	GN Biosystems	Spectrum Labs	Spectrum Labs	Thermo Fisher	Thermo Fisher
Name	Xpress Dialyzer Family	GeBAflex-Tubes	Tube-O-DIALYZER™	Rapid-Dialyzer™	Tubes Dialyseschlauch	Float-A-Lyzer® G2	Slide-A-Lyzer Cassettes	Slide-A-Lyzer MINI Dialysis
Sample Volume	10-1000 µl	10-20000 µl	20 µl - 2,5 ml	1- 20 µl	10-100 ml	0,1 - 10 ml	0,1 - 30 ml	10 µl - 2 ml
MWCO	3,5 / 6-8 / 12-14/ 140 kDa	3,5 - 12/14 kDa	1-50 kDa	3,5-25 kDa	3,5 - 40 kDa	0,1 - 1000 kDa	2 - 20 kDa	10 kDa
Handling	+++	+++	++	++	+	++	++	++
Scalability Sample	+++	++	+	+	+	+	+	+
Dialysis time/sample*	+++	++	++	++	+	++	++	++
Automatable	+++	+	0	0	0	0	0	+
Costs/sample * in EUR	4,41	5,77	7,18	5,70	8,00	8,50	10,00	0,15

* for 96 samples a' 100µl, costs per device and handling effort (40€/man hour), floating dialysis

Table 1| Overview dialysis devices

Every user has to ask the following simple questions to find the optimal device for his task.

Which sample volume is needed?	Selection according to manufacturer's data
Suitable Cut off?	Half of the molecular weight of the retained molecule, double of molecular weight of the removable compounds.
Costs per sample?	Price for the dialysis device + costs for operational hours + costs for the dialysis buffer
Requirement of additional equipment?	Selection according to manufacturer's instructions and application protocol for additional equipment.
For higher sample numbers: Ready for automated handling?	Selection according to manufacturer's instructions and application protocol for compatibility with microplate format and automation.

Diffusion is the force for the transportation process of the dialysis. According to Fick's first law the flux depends in inverse proportion of the diffusion length. Therefore is the geometry of the dialysis device is essential for dialysis speed. The diffusion length is the distance between the membrane at one side to the membrane at the other side. It should be as short as possible. The dialysis buffer should be stirred or shaken. The dialysis buffer movement avoids a concentration gradient of the buffer and raise the dialysis speed. A new development is a Deep Well Plate in combination with dialysis cartridges (Fig 5).

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Figure 5| 96 deep well plate Riplate (Ritter Medical) with 12 cartridges ED300 (scienova GmbH) dialysis cartridges for 96-fold dialysis

Deep Well Plates are common for storage of samples with higher volumes then 300 µl in microplate format but also as reaction vessel and bioreactor for cell culture. In case of the new dialysis application, the Deep Well Plate is used as dialysis buffer container and the cartridge is placed in the wells of the plate. The system has following advantages:

1. Geometry: short diffusion ways and vertical membranes
2. High volume recovery
3. Easy handling with standard lab pipettes
4. Ready for automation
5. Easy and downscaling of sample number

An example for an application with this new system is given below.

Rule dialysis device: Select according to manufactur's data the sample volume and cut-off. The diffusion length should be as short as possible. Easy handling with the available lab equipment.

Dialysis application

Dialysis is suitable and gentle method for the removal of denaturing agents. But it can be time-consuming and difficult and was limited in handling high sample quantities.

In the following application example of enzyme reactivation by urea removal, we demonstrate the possibility to handle 48 separate dialysis samples with an automated liquid handling device in one single process.

Trypsin is reversibly inhibited in the presence of high urea concentrations. To regain tryptic activity, by para-nitroaniline (PNA) release, urea removal is needed, which is achieved through dialysis (see equitation below).

$$\text{DL-Benzoyl-Arg}p\text{-nitroaniline} \xrightarrow{\text{Trypsin}} p\text{-nitroaniline} + \text{benzoyl-Arg}$$

To show that there is no sample loss in consequence of protein binding with the membrane, different bovine serum albumin (BSA) solutions were dialyzed for two hours and the recollected samples were analyzed.

Methods & Material

Dialysis samples: 100 µl trypsin samples (0.5 mg/ml trypsin in 8 M urea, 20 mM $CaCl_2$), reference sample (0.5 mg/ml trypsin in 20 mM $CaCl_2$), **dialysis buffer:** 4.4 ml of dialysis buffer (35 mM Tris·HCl pH 7.8, 20 mM $CaCl_2$).

Measurement solution: 200 µl (4.7 mM DL-Benzoyl-Arg-*p*-nitroaniline(DL BAPNA) in 10 % DMSO + trypsin 0.05 mg/ml in35 mMTris·HCl pH 7.820 mM $CaCl_2$).

Determination method: Photometer BioTek ELx800 405 nm (measurement wavelength) and 620 nm (reference wavelength), PNA-release rate (rr) indicating tryptic activity is evaluated (see below):

$$rr = \frac{\text{absorbance}_{405\ nm} - \text{absorbance}_{620\ nm}}{\Delta t \text{ in min}}$$

Urea determination: Wescor VAPRO 5520 Osmometer.

Dialysis device: scienova *Xpress* Micro Dialyzer MD100 GridKit48 (48 dialysis samples in 6 cartridges with 8 sample segments each, delivered in a Riplate 48-deep well plate, Ritter Medical). **Liquid handling device:**CyBi®-FeliX, multi-channel/single-channel pipettor for automated liquid handling,head R96/250 µl, AnalytikJena.

Micro Dialyzers were placed in 4.4 ml dialysis buffer into the grid and filled with 100 µl trypsin sample by CyBi®-FeliX. Forty samples (n=40) with urea and eight samples (n=8) without urea as reference were dialyzed 60 min at room temperature (22°C). After incubation time the samples were transferred to a 96-well microplate for measurement.

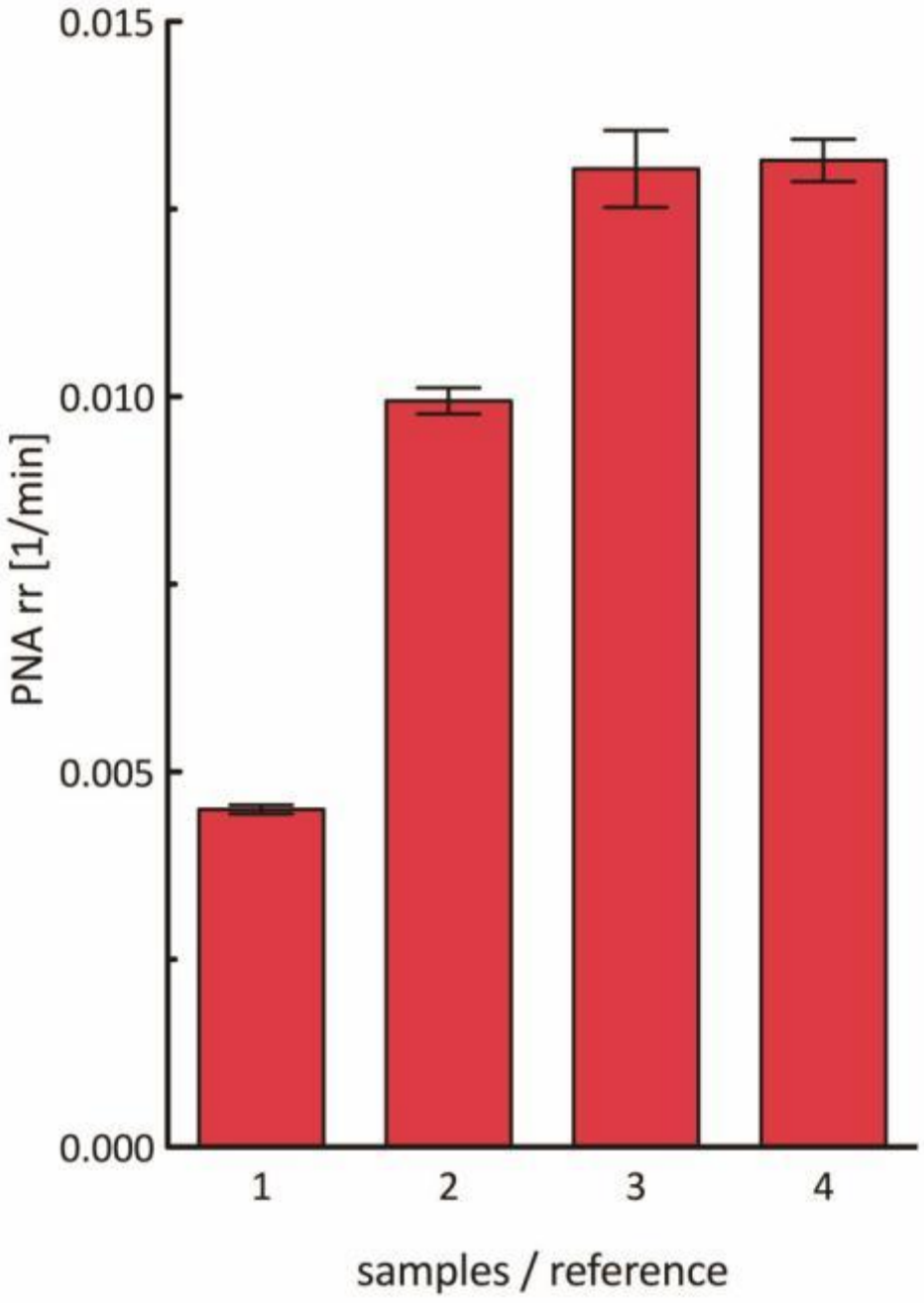

Figure 6| Regain of tryptic activity after dialysis (urea removal):

1: sample non-dialyzed, 2: samples 60 min dialyzed, 3: reference 60 min dialyzed, 4: reference non-dialyzed. Trypsin activity is illustrated in PNArr.

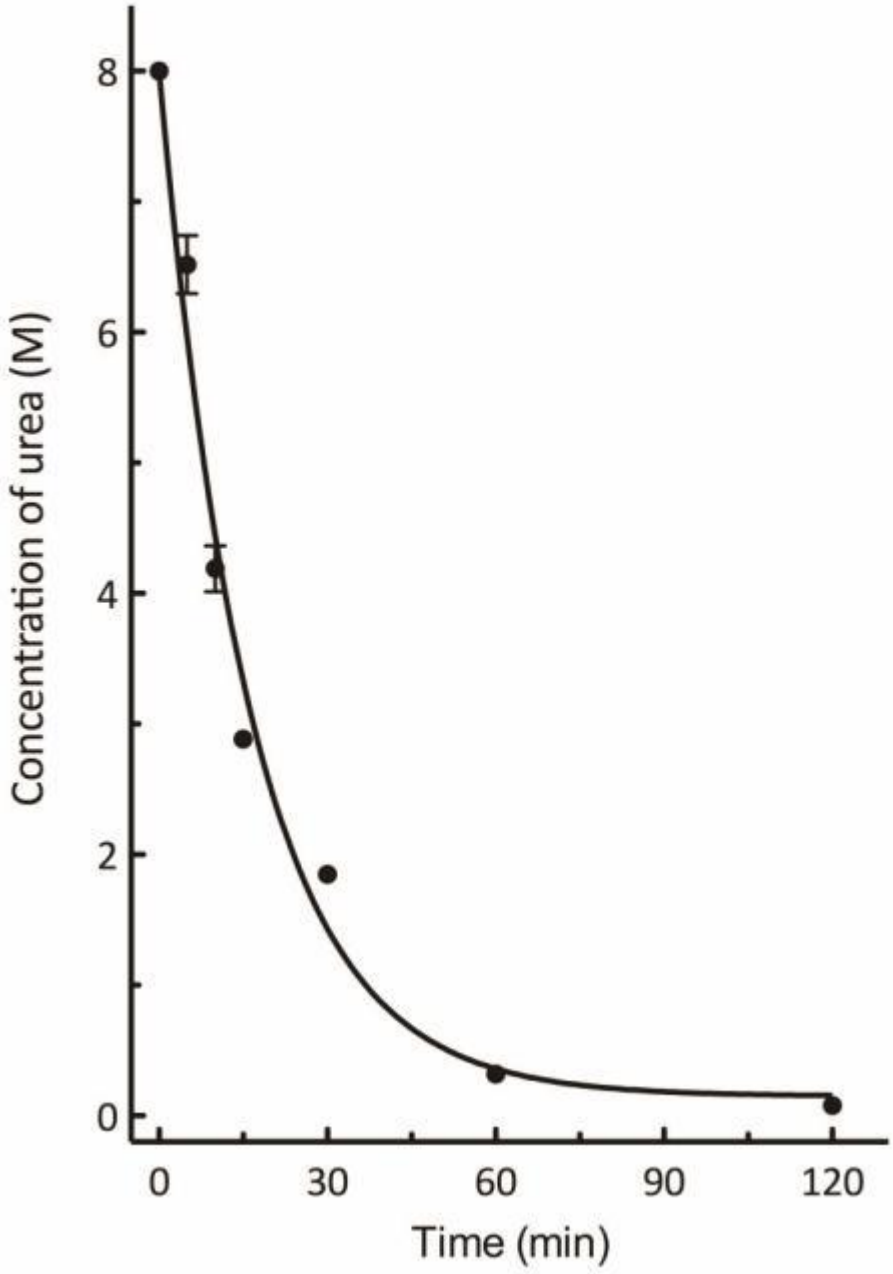

Figure 7| Urea removal during dialysis:

The concentration of urea is shown in dialyzed samples. A scienova Xpress Micro Dialyzer MD100 GridKit48 in 48 deep well Riplate (Ritter Medical) and AnalytikJena CyBi®-FeliX (head R96/25 µl) were used. Performed at room temperature.

Results

After 1 hour of dialysis the urea concentration dropped from 8 M to 0.30 M. The results show that a regain of about 75 % tryptic activity could be achieved after one hour. In total, all 48 samples have a low standard deviation which indicates a good constancy and reproducibility.

The combination of modern dialysis device and liquid handling automate enables a high sample throughput without losing quality, useable for all dialysis applications.