



FRACTIONATION AND QUANTITATIVE DETERMINATION OF SERUM PROTEINS

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2016

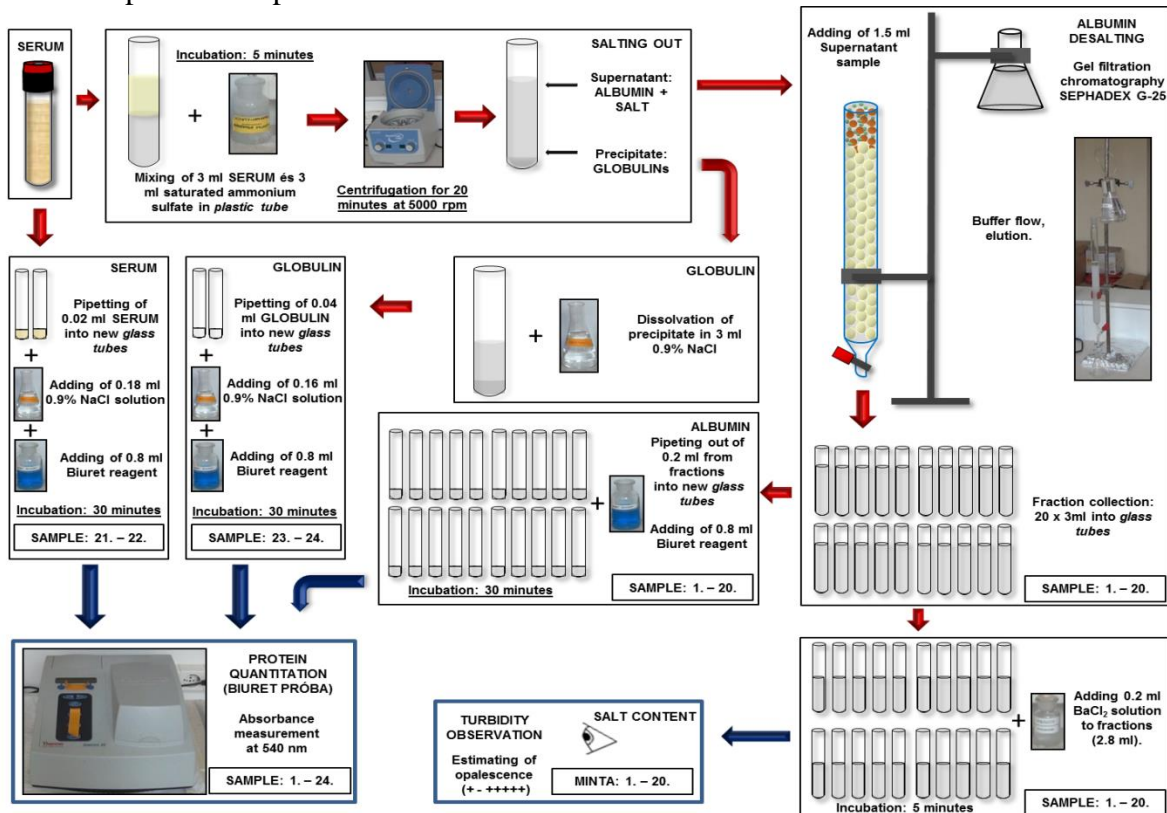
1. Aim of the practice

During the practice the composition of the serum is examined by various methods. Serum globulins are salted out and isolated by centrifugation. Albumin and salt from the precipitation are separated by gel filtration chromatography. For determination of the salt content of the collected fractions a turbidity assay is used. Biuret reaction is used for determining the protein concentration of blood serum sample; collected fractions from gel filtration) and the dissolved globulin fraction. A specific reagent Bromcresol purple is used for determination of albumin level in serum sample.

In the second part of the practice the protein composition of serum, purified albumin and gamma globulin samples is investigated by cellulose acetate membrane electrophoresis (demonstration).

2. Experiments

1. Salting out of globulins from human serum – Ammonium Sulfate Precipitation
2. Desalting of albumin by gel filtration chromatography.
3. Protein content determination with different methods – (reference curves, graphs, calculations).
4. Electrophoretic separation of serum proteins on cellulose acetate membrane (demonstration).
5. Preparation of practical notebook.





3. Theoretical questions

1. Compare blood plasma and serum:

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2. What are the major protein components of the human plasma?

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3. What is happening during "salting out"?

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4. What is the principle of the gel filtration chromatography?

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5. Compare the Biuret and BCP method!

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6. How the salt content of fractions will be determined?

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7. What is the principle of the cellulose acetate membrane electrophoresis?

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1. Experiment: Salting out of globulins from human serum – Precipitation with Ammonium Sulfate

During the experiment globulins are salted out with saturated Ammonium Sulfate and the precipitate is separated by centrifugation. The supernatant contains the albumin, while the precipitate contains globulins. After separation the precipitate can be dissolved in 0.9% NaCl solution.

Materials:

- human serum
- saturated Ammonium Sulfate
- 0.9 % NaCl solution

Equipment:

- pipets with tips
- centrifugal tubes (plastic)
- glass test tubes

Procedure:

1. Pipette **3 ml** serum and **3 ml** saturated ammonium sulfate solution into a centrifuge tube.
2. Shake the sample and incubate it for 5 minutes at room temperature.
3. Centrifuge it for **20 minutes** at 5000 rpm.
4. Transfer the supernatant (ALBUMIN) into a clean test tube.
5. Dissolve the precipitate remained in the centrifugal tube in 3 ml of 0.9 % NaCl solution (GLOBULINS).

Observation:

- Estimate the volume of the supernatant: ml

2. Experiment: Desalting of albumin by gel filtration chromatography

After ammonium sulfate precipitation serum albumin and globulin fractions are separated. The albumin containing supernatant is desalted by **Sephadex G25 gel filtration column**. During gel filtration, NaCl solution is flowed through the column continuously and fractions are collected.

Materials:

- ALBUMIN sample from **Experiment 1**.
- 20 cm Sephadex G-25 column
- 0.9 % NaCl solution

Equipment:

- pipets with tips
- glass tubes
- racks for tubes
- holder for column



Before separation, please try:

- ✓ How to connect the 0.9 % NaCl solution containing flask to the column.
- ✓ How much you have to open the outlet on the bottom of the column to be able to get the eluate uniformly.
- ✓ **ATTENTION! Do not let the gel column dry** and no air bubbles get into the gel, because it affects your separation and damages the gel structure of the column too.
- ✓ Equilibrate the column by the flow of 0.9 % NaCl solution.
- ✓ Prepare in a rack 20 clean, numbered test tubes for fractions you collect (glass tubes – **SAMPLE 1 - 20.**)
- ✓ Pipet 3 ml distilled water into a test tube to use as comparison how much volume of the fractions you have to collect.

Separation:

1. Let the buffer layer lowered up to 5mm on the top of the gel column.
2. Close the outlet valve on the bottom.
3. Pipette 1.5 ml ALBUMIN fraction from the **Experiment 1.** onto the top of the column.
4. Open the outlet and allow the sample to percolate into the gel.
5. Wash the inner wall of the column with 5 ml 0.9 % NaCl.
6. Connect the flask containing 0.9 % NaCl to the column and wash continuously the gel with the eluent.

➤ Collect 20 (3 ml each) fractions into numbered test tubes: **SAMPLE 1 - 20.**

3. Experiment: Protein content determination with different methods – reference curves, graphs, calculations

1. Determination of protein concentration by biuret reaction

During practical, this method is used to determine the protein concentration of blood serum; collected fractions during gel filtration and dissolved globulin fraction.

Materials:

- Protein standard solution (10mg/ml)
- 0.9 % NaCl solution
- Biuret reagent (6 g *K-Na-tartrate*, 1.5 g $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 30 g *NaOH*, 1 g *KI* in 1000 ml distilled water)
- Fractions from **Experiment 2.**
- Human SERUM sample
- GLOBULIN sample from **Experiment 1.**
- Distilled water

Equipment:

- pipets with tips
- glass tubes
- racks for tubes
- spectrophotometer, plastic cuvettes



a) Prepare reference series from protein standard solution (10 mg/ml):

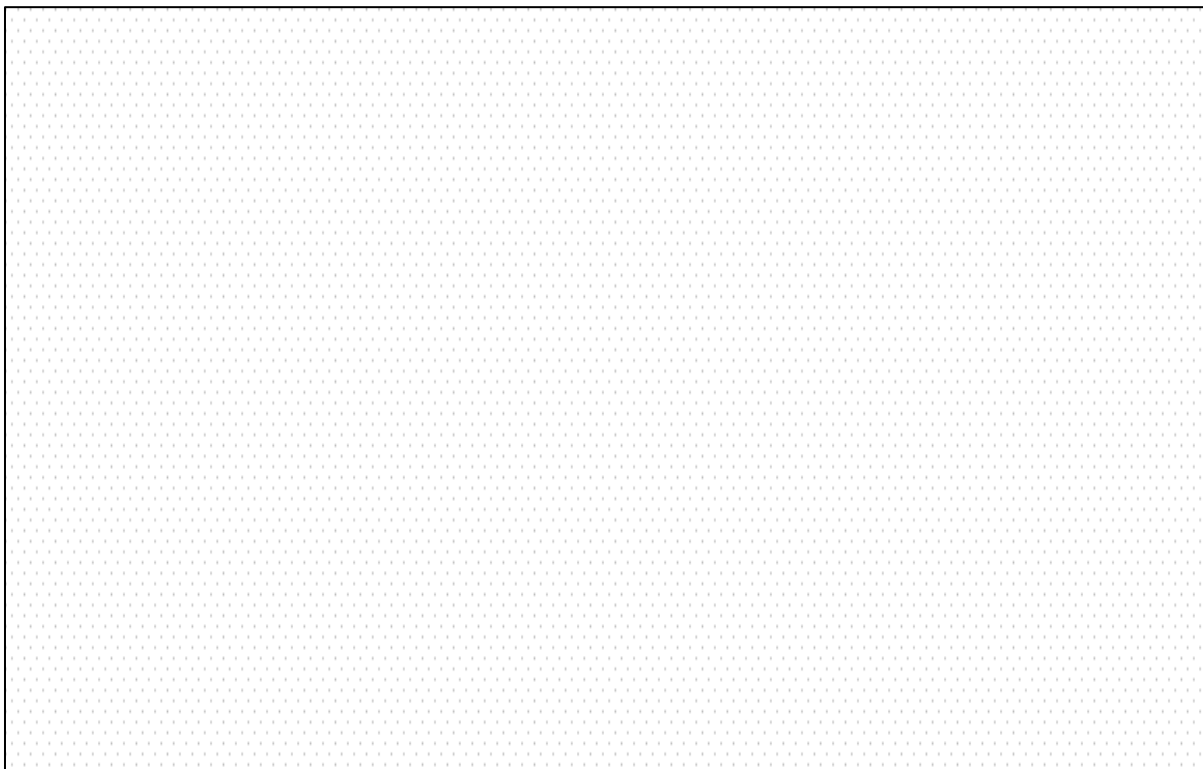
1. Prepare 5 glass tubes.
2. Mix the following solutions into the tubes:

Reference samples	1 st tube	2 nd tube	3 rd tube	4 th tube	5 th tube Blank
10 mg/ml protein standard solution (ml)	0.050	0.100	0.150	0.200	-
0.9 % NaCl solution (ml)	0.150	0.100	0.050	-	0.200
Biuret reagent (ml)	0.800	0.800	0.800	0.800	0.800

3. After addition of Biuret reagent shake the tubes.
4. Incubate the samples at room temperature for **30 minutes**.
5. Set the wavelength on the photometer to 540 nm, and set it to zero with distilled water.
6. Measure the absorbance values of the samples, and record them:

Reference sample results	1 st tube	2 nd tube	3 rd tube	4 th tube	5 th tube Blank
10 mg/ml protein standard solution (ml)	0.050	0.100	0.150	0.200	-
Protein content in the tube (mg)		1			0
A_{540}					
$A_{540} - A_{540 \text{ blank}}$					-

7. Prepare the reference curve. Plot the absorbance ($A_{540} - A_{540 \text{ blank}}$ (y)) against the protein content in the tubes (**Protein amount in tube (mg)** (x)):





8. Fit a line on the points, and calculate the slope of the reference curve:

➤ Slope:

b) Protein content of the ALBUMIN fractions:

1. Prepare 20 new glass tubes.
2. Take the **ALBUMIN fractions** from Experiment 2.
3. Mix the following solutions into the new tubes:

SAMPLE:	1	2	3	4	5	6	7	8	9	10
From ALBUMIN fractions (ml) (1-10)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Biuret reagent (ml)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8

SAMPLE:	11	12	13	14	15	16	17	18	19	20
From ALBUMIN fractions (ml) (11-20)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Biuret reagent (ml)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8

4. After addition of Biuret reagent shake the tubes.
5. Incubate the samples at room temperature for **30 minutes**.
6. Measure the absorbance values of the samples, and record them into the table below:

➤ Calculate the albumin content of the samples (**SAMPLE 1 – 20**):

[#]Albumin content in 0.2 ml: $(A_{540} - A_{540\text{blank}}) / \text{slope from 3.1.a}$

^{##}Albumin content in 3 ml: e.g. if 0.2 ml contains X mg albumin, then 3ml contains $(3/0.2=15)$; $15 \times X$ mg albumin

SAMPLE results:	1	2	3	4	5	6	7	8	9	10
A_{540}										
$A_{540} - A_{540\text{ blank}}$										
[#] Albumin content in 0.2ml (X mg)										
^{##} Albumin content in 3ml fraction (15 x X mg)										

SAMPLE results:	11	12	13	14	15	16	17	18	19	20
A_{540}										
$A_{540} - A_{540\text{ blank}}$										
[#] Albumin content in 0.2ml (X mg)										
^{##} Albumin content in 3ml fraction (15 x X mg)										

➤ Calculate the total amount of recovered albumin (**SAMPLE 1 – 20**):

..... mg **total ALBUMIN** in fractions



c) Protein content of the SERUM sample:

1. Prepare 2 new glass tubes.
2. Take the **SERUM** sample.
3. Mix the following solutions into the new tubes:

SAMPLE:	21	22
Serum (ml)	0.02	0.02
0.9 % NaCl solution (ml)	0.18	0.18
Biuret reagent (ml)	0.8	0.8

4. After addition of Biuret reagent shake the tubes.
5. Incubate the samples at room temperature for **30 minutes**.
6. Measure the absorbance values of the samples, and record them into the table below:

- Calculate the protein content of the SERUM samples (**SAMPLE 21 – 22**):
 - # Albumin content in 0.02 ml: $(A_{540\text{Average}} - A_{540\text{blank}}) / \text{slope from 3.1.a}$
 - ## Total protein content in 1 ml serum: if 0.02 ml serum contains Y mg protein, then 1ml contains $(1/0.02=50)$; 50x Y mg protein.

SAMPLE:	21	22
A_{540}		
A_{540} Average		
A_{540} Average - A_{540} blank		
#Protein content in 0.02ml serum (Y mg)		
##Protein content in 1ml serum (50 x Y mg)		
Protein content in 3 ml SERUM (mg)		

d) Protein content of GLOBULIN sample:

1. Prepare 2 new glass tubes.
2. Take the **GLOBULIN** sample from the **Experiment 1**.
3. Mix the following solutions into the new tubes:

SAMPLE:	23	24
Globulin (ml)	0.04	0.04
0.9 % NaCl solution (ml)	0.16	0.16
Biuret reagent (ml)	0.8	0.8

4. After addition of Biuret reagent shake the tubes.
5. Incubate the samples at room temperature for **30 minutes**.
6. Measure the absorbance values of the samples, and record them into the table below:

- Calculate the protein content of the GLOBULIN samples (**SAMPLE 21 – 22**):
 - # Protein content in 0.04 ml: $(A_{540\text{Average}} - A_{540\text{blank}}) / \text{slope from 3.1.a}$
 - ## Total protein content in 1 ml serum: if 0.04 ml serum contains Z mg protein, then 1ml contains $(1/0.04=25)$; 25 x Z mg protein.



SAMPLE:	23	24
A ₅₄₀		
A ₅₄₀ Average		
A ₅₄₀ Average - A ₅₄₀ blank		
#Protein content in 0.04ml globulin (Y mg)		
##Protein content in 1ml globulin (25 x Y mg)		
Protein content in 3 ml GLOBULIN (mg)		

2. Determination of albumin content using bromcresol purple (BCP)

In our experiment, the bromcresol purple (BCP) reagent will be used for specific determination of albumin levels in human serum sample.

Materials:

- Protein standard solution (10mg/ml)
- 0.9 % NaCl solution
- Bromcresol purple (BCP) reagent (0.005 % BCP dissolved in 50 ml ethanol)
- Human SERUM sample
- Distilled water

Equipment:

- pipets with tips
- glass tubes
- racks for tubes
- spectrophotometer, plastic cuvettes

a) Prepare reference series from protein standard solution (10 mg/ml):

1. Prepare 5 glass tubes.
2. Mix the following solutions into the tubes:

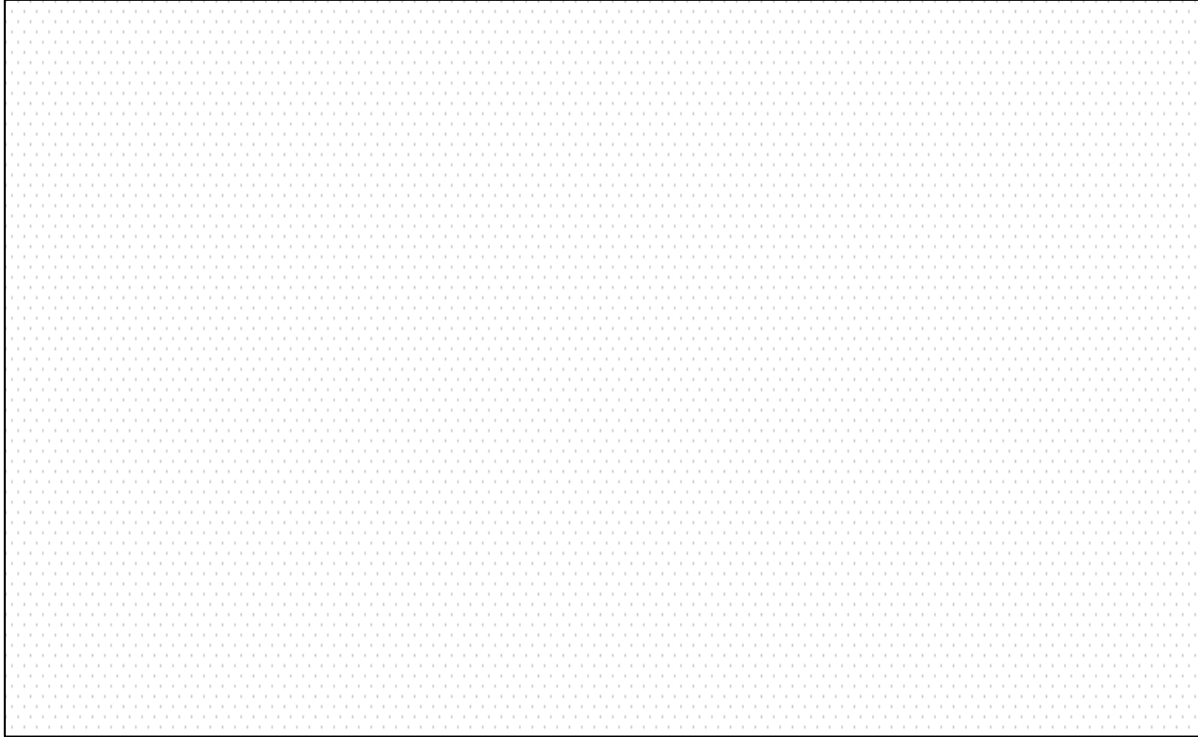
Reference samples	1 st tube	2 nd tube	3 rd tube	4 th tube	5 th tube Blank
10 mg/ml protein standard solution (ml)	0.050	0.075	0.100	0.150	0
0.9 % NaCl solution (ml)	0.150	0.125	0.100	0.050	0.200
BCP - reagent (ml)	0.800	0.800	0.800	0.800	0.800

3. After addition of BCP reagent shake the tubes.
4. Incubate the samples at room temperature for **5 minutes**.
5. Set the wavelength on the photometer to 620 nm, and set it to zero with distilled water.
6. Measure the absorbance values of the samples, and record them:

Reference sample results	1 st tube	2 nd tube	3 rd tube	4 th tube	5 th tube Blank
10 mg/ml protein standard solution (ml)	0.050	0.075	0.100	0.150	-
Protein content in the tube (mg)			1		0
A ₆₂₀					
A ₆₂₀ - A ₆₂₀ blank					-



7. Prepare the reference curve. Plot the absorbance ($A_{620} - A_{620\text{blank}}$ (y)) against the protein content in the tubes (**Protein amount in tube (mg) (x)**):



8. Fit a line on the points, and calculate the slope of the reference curve:

➤ Slope:

b) Albumin content of SERUM sample:

1. Prepare 2 new glass tubes.
2. Take the SERUM sample.
3. Mix the following solutions into the new tubes:

SAMPLE:	25	26
Serum (ml)	0.1	0.1
0.9 % NaCl solution (ml)	0.1	0.1
BCP - reagent (ml)	0.8	0.8

4. After addition of BCP reagent shake the tubes.
5. Incubate the samples at room temperature for **5 minutes**.
6. Measure the absorbance values of the samples, and record them:
 - Calculate the albumin content of the SERUM samples (**SAMPLE 25 – 26**):
 - # Albumin content in 0.1 ml serum: $(A_{620\text{Average}} - A_{620\text{blank}}) / \text{slope from 3.2.a}$
 - ## Total albumin content in 1 ml serum: if 0.1 ml serum contains W mg protein, then 1ml contains $(1/0.1=10)$; 10 x W mg protein.



SAMPLE:	25	26
A ₆₂₀		
A ₆₂₀ average		
A ₆₂₀ average - A ₆₂₀ blank		
*Albumin content in 0.1ml-ben (W mg)		
**Albumin content in 1ml serum (10 x W mg)		
Albumin content in 3 ml SERUM (mg)		

3. Determination of the sulfate salt of the chromatography fractions with turbidimetry

For determination the approximate concentration of the sulfate ion (salt) of the collected fractions a barium-chloride (BaCl₂) based **turbidity assay** is used.

Materials:

- 10 % BaCl₂ solution (**Poison!**)
- FRACTIONS from **Experiment 2**.

Equipment:

- pipets with tips
- racks for tubes

Procedure:

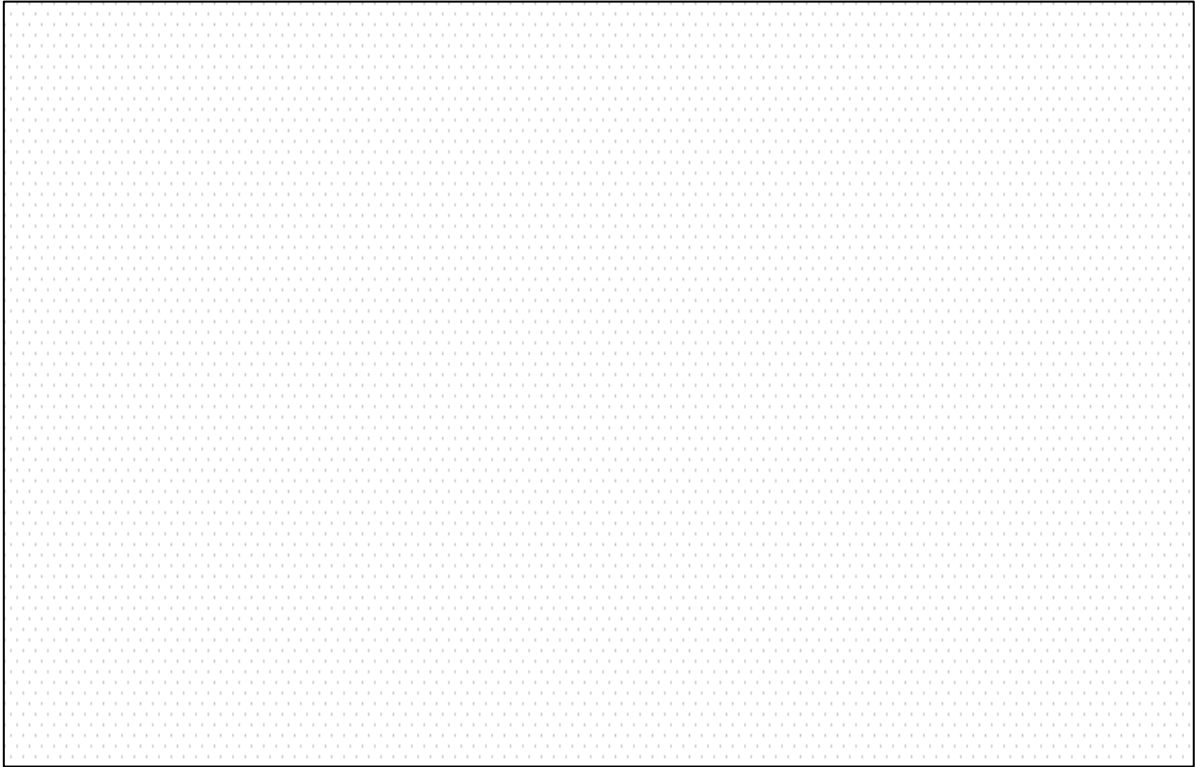
1. Prepare the **ALBUMIN Fractions** from **Experiment 2**. with the remaining 2.8 ml samples (Do not use new tubes!).
2. Add 0.2 ml BaCl₂ solution to each fraction.
3. After addition of BaCl₂ solution shake the tubes.
 - Estimate the turbidity of fractions by eye inspection on a five grade scale using one to five crosses (where “+” is the least turbid and “+++++” the most turbid fraction) and record the result:

Results:	1	2	3	4	5	6	7	8	9	10
Turbidity (+ - +++++)										

Results:	11	12	13	14	15	16	17	18	19	20
Turbidity (+ - +++++)										

4. Comparison of protein and salt content of different fractions from gel filtration chromatography and components of serum sample

- Plot the protein concentration (in 3ml) (y₁-axis-left) (**3.1.b**) and the turbidity (y₂-axis-right) (**3.3**) against the number of gel filtration chromatography fractions (x-axis) (**SAMPLE 1-20**) in one graph.



- Conclude the results! How successful was the separation procedure?
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- What is the yield of the gel filtration chromatography in your experiment?
 - Albumin content of 3 ml serum used for desalting procedure determined by BCP reaction (3.2.b):
.....**mg/3ml**

 - Volume and albumin content of the supernatant after desalting and centrifugation (Experiment 1.):
.....**ml****mg/total volume of supernatant (ml)**

 - Albumin content of 1.5 ml supernatant loaded on the column:
.....**mg/1.5 ml 100%**

 - Total recovered albumin (3.1.b)
.....**mg**

 - Yield of the gel filtration (%):
(Total recovered albumin / loaded albumin) x 100



.....%

- Protein content of the serum determined by Biuret reaction (3.1.c):

.....mg/3 ml 100%

- Albumin content of the serum determined BCP reaction (3.2.c):

.....mg/3 ml

- Globulin content of the serum determined by Biuret reaction (3.1.d):

.....mg/3 ml

- Globulin content of the serum according to the BCP reaction?

(Protein content of the serum (Biuret) – albumin content of the serum (BCB)=
globulin content)

.....mg/3 ml

- Conclude the results! Compare the globulin content of serum determined by Biuret method and those obtained after BCP reaction and calculation! Why is it different?

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4. Experiment: Electrophoretic separation of serum proteins on cellulose acetate membrane (demonstration)

The protein composition of serum, purified albumin and gamma globulin fractions are investigated by cellulose acetate membrane electrophoresis.

Material:

- cellulose acetate membrane
- running buffer (50 mM Na-veronal, 10 mM veronal, 60 mM TRIS, pH 8.6)
- SERUM sample
- ALBUMIN sample
- GLOBULIN sample
- Bromphenol Blue stained marker (*albumin*)
- AmidoBlack 10B staining solution (*in methanol:water:acetic acid=5:4:1*)
- Destaining solution (*methanol:water:acetic acid=5:4:1*)

Equipment:

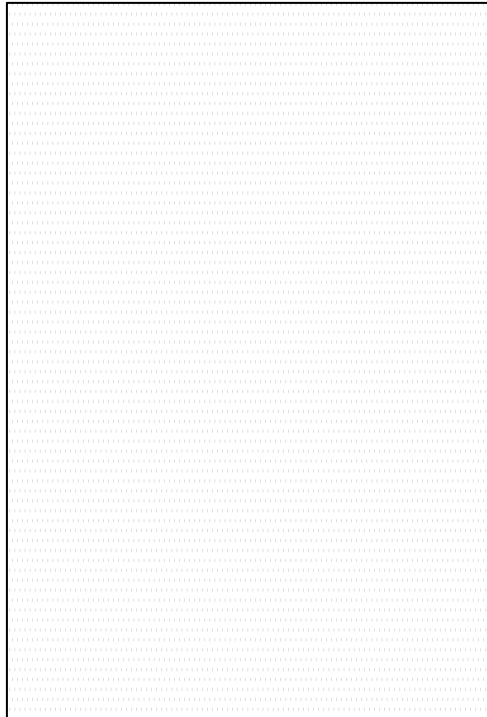
- Pipets with tips
- Power supply
- Electrophoresis device
- Dishes, forceps, filter papers

Separation process:

- ✓ Wet the cellulose acetate membrane by placing it with forceps on the surface of the buffer in a dish.
- ✓ Lightly blot the excess of the buffer with filter paper.
- ✓ Place the membrane into the device as a bridge between the support frames connected by filter papers to the buffer chamber.
- ✓ Apply the samples with pipets as 0.5 cm long lines at the cathode (-) side of the strip, about 1 cm far from the contact point of the strip and the filter paper.
- ✓ Close down the electrophoretic device, and turn on the power.
- ✓ Set the amperage to 0.5 mA/cm membrane width (e.g. 2 mA for 4 cm membrane)
- ✓ After the separation turn of the power supply, disconnect the device and put the membrane into the staining solution with forceps.
- ✓ After 5 minutes, flush the membrane with water and put it into the destaining solution while the base color of the membrane becomes white.
- ✓ Flush it again with water and dry it between filter papers.



- Evaluate the results and draw the pattern of the separation (indicate the samples, poles, start line and identify protein bands):



- Conclude the results!

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