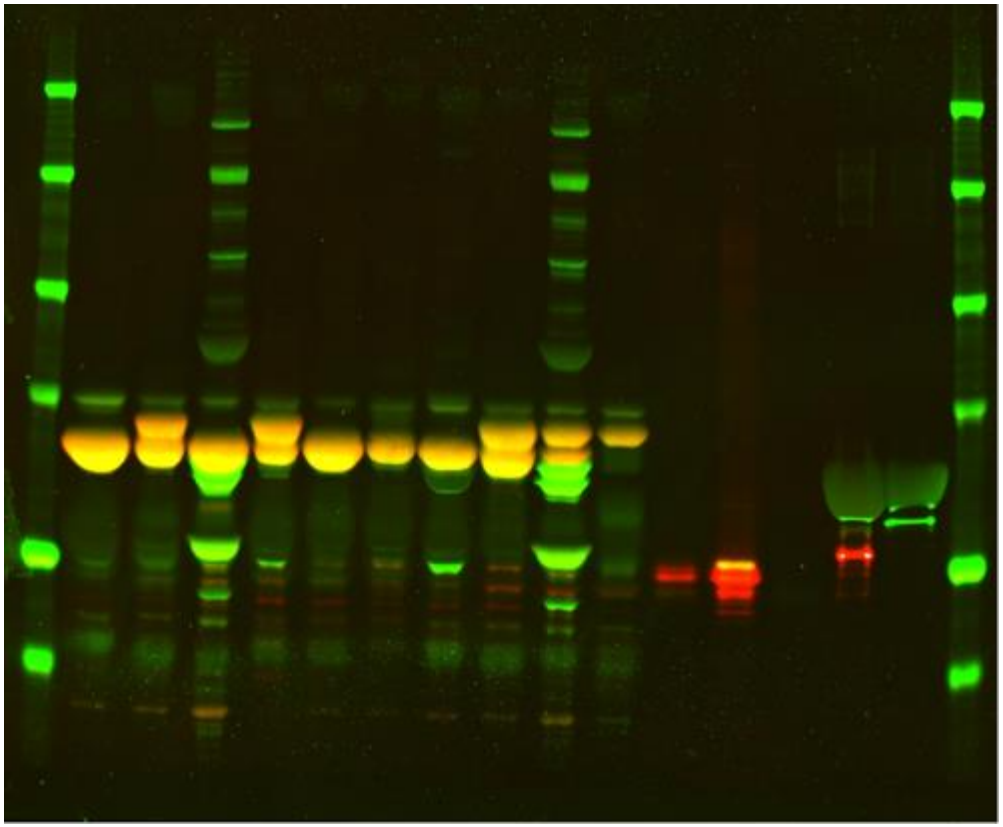


Oliver Pötz
CEO, SIGNATOPE GmbH

November 13th 2017

Laboratory Techniques and Medical Device Approvals: Western Blot

- Electrophoresis
- Blotting
- Detection
- Antibody databases

Western-Blot concept

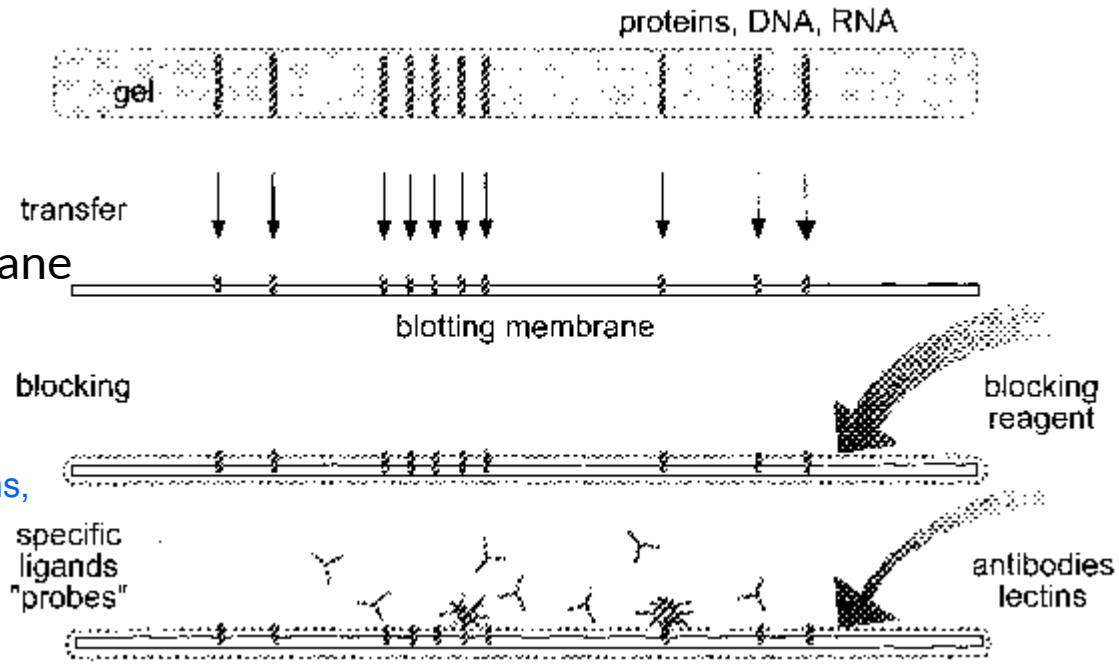
separation to detect the protein you are interested in

first sample with different protein e.g. blood plasma, tissue lisate
approx. 20.000 genes and even more proteins

1. Separation (Electrophoresis)
2. Transfer onto membrane ("Blotting")
3. Blocking

block space which is not occupied by proteins, so no unspecific binding is done

4. Detection



R. Westermeier, Electrophoresis in Practice, Wiley-VCH, Weinheim, 2005
F. Lottspeich, J.W. Engels, Bioanalytik, 2. Auflage, Elsevier, 2006

- Electrophoresis
- Blotting
- Detection
- Antibody databases

Electrophoresis

What does electrophoresis mean?

- “Electro-“ applying voltage between two electrodes
- “-phor“, (Greek) means “carry”
- Movement of charged molecules, particles, cells … in a constant electric field

solution with electrolytes

- Differentiate carrier-free electrophoresis (capillary electrophoresis) and gel electrophoresis
- Separation based on differently charged masses and shapes (molecular forms)

only for certain Electrophoresis procedures!

History

1937 Electrophoresis

Arne Tiselius: Noble prize in chemistry 1948

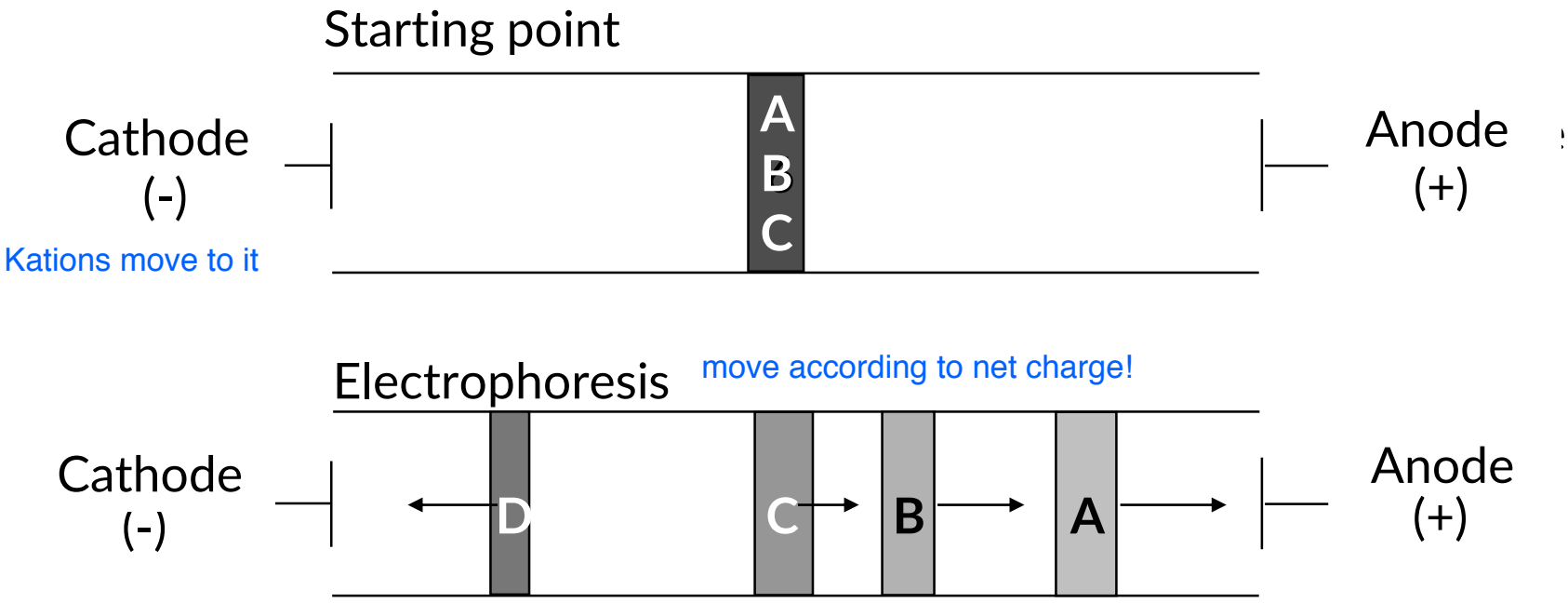


For his work on electrophoresis and adsorption, especially for the discovery of complex plasma protein heterogenity



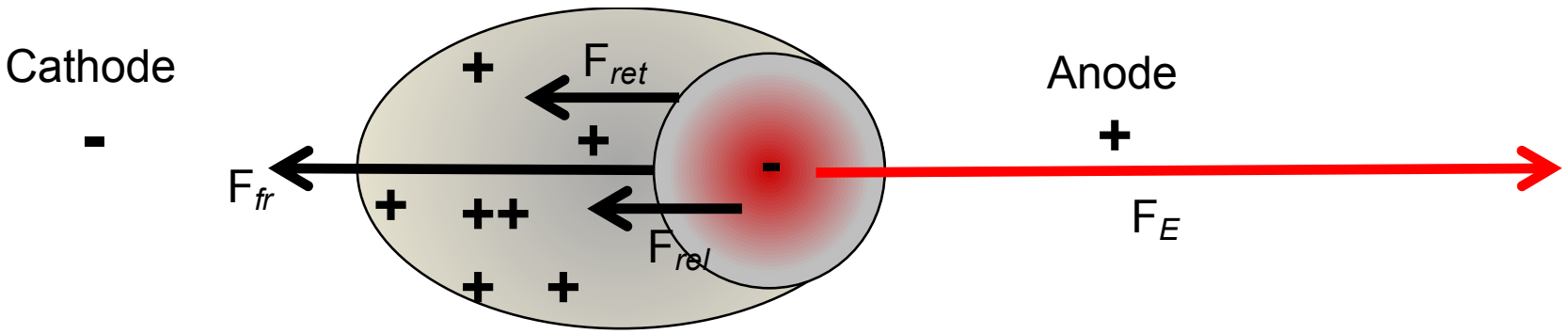
Aus Lottspeich/Engels, *Bioanalytik*, 2. Aufl., © 2006 Elsevier GmbH

Electrophoresis principle



Particles different in charge and size move in the electric field with different velocities. Substances move in discrete zones.

Electrophoresis – Theoretical Basics



modified according to Lottspeich/Engels Bioanalytik, 2. Aufl., © 2006 Elsevier GmbH

Forces which influence electrophoretic mobility

F_e= electrostatic force = q· E

nicht klausurrelvant, alle Kräfte zu wissen!

F_{fr}= friction force retards ion

~ velocity

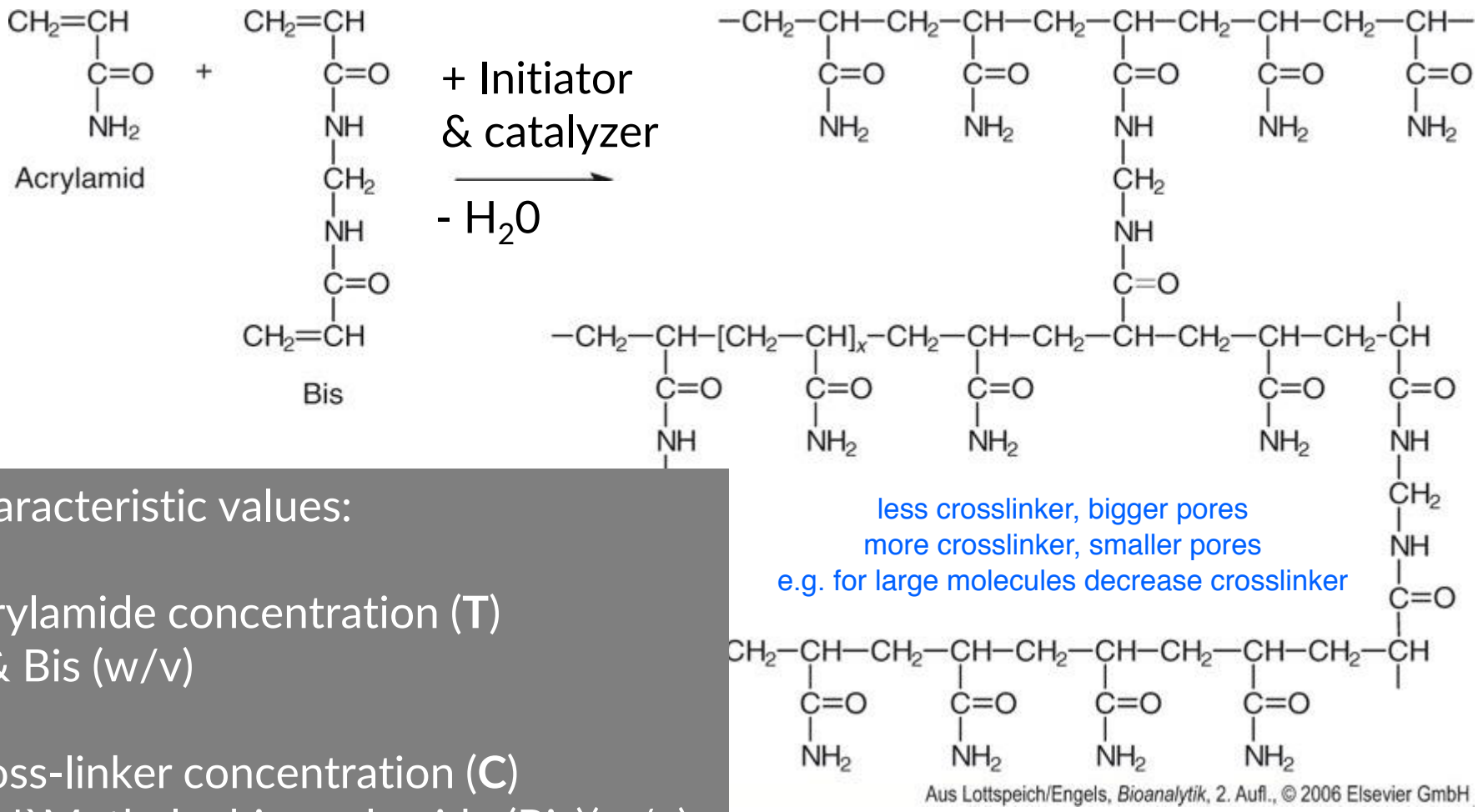
~ viscosity & pore size of media

~ molecule size

F_{ret}= retardation force = ion cloud-induced solvent polarization retards ion movement

F_{rel}= relaxation force = ion cloud retards central ion

Polyacrylamide gel electrophoresis (PAGE) most common in western regions!



Characteristic values:

Acrylamide concentration (**T**)
A & Bis (w/v)

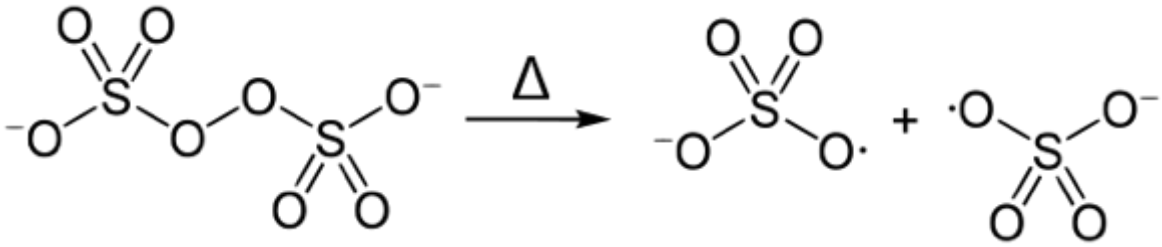
Cross-linker concentration (**C**)
N`N`Methylenbisacrylamide (Bis)(w/v)

crosslinker, links molecules that are composed of acrylamid molecules

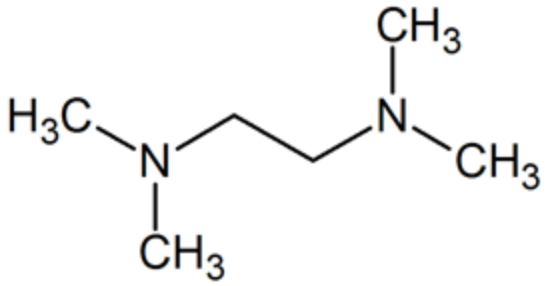
T & **C** determine pore size!
Typical mixture 30:1

Polyacrylamide gel electrophoresis (PAGE)

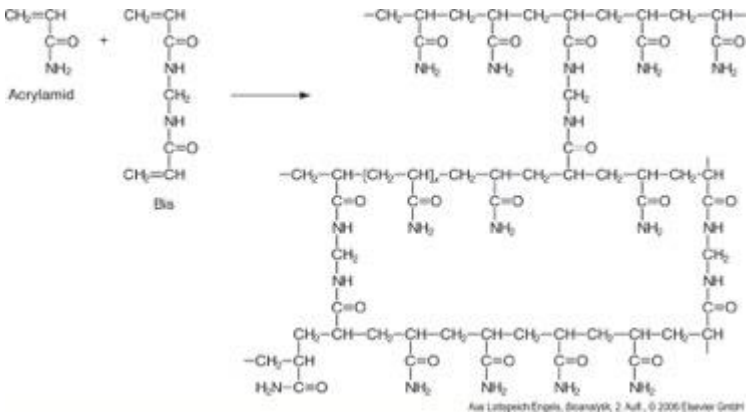
Initiator/ radical starter
Ammonium persulfate (APS)
Conc. 1-10 mM



Catalyzer
N‘,N‘,N‘,N‘-tetramethylethylendiamine (TEMED)
Conc. 1-10 mM



Polyacrylamide gel electrophoresis (PAGE)



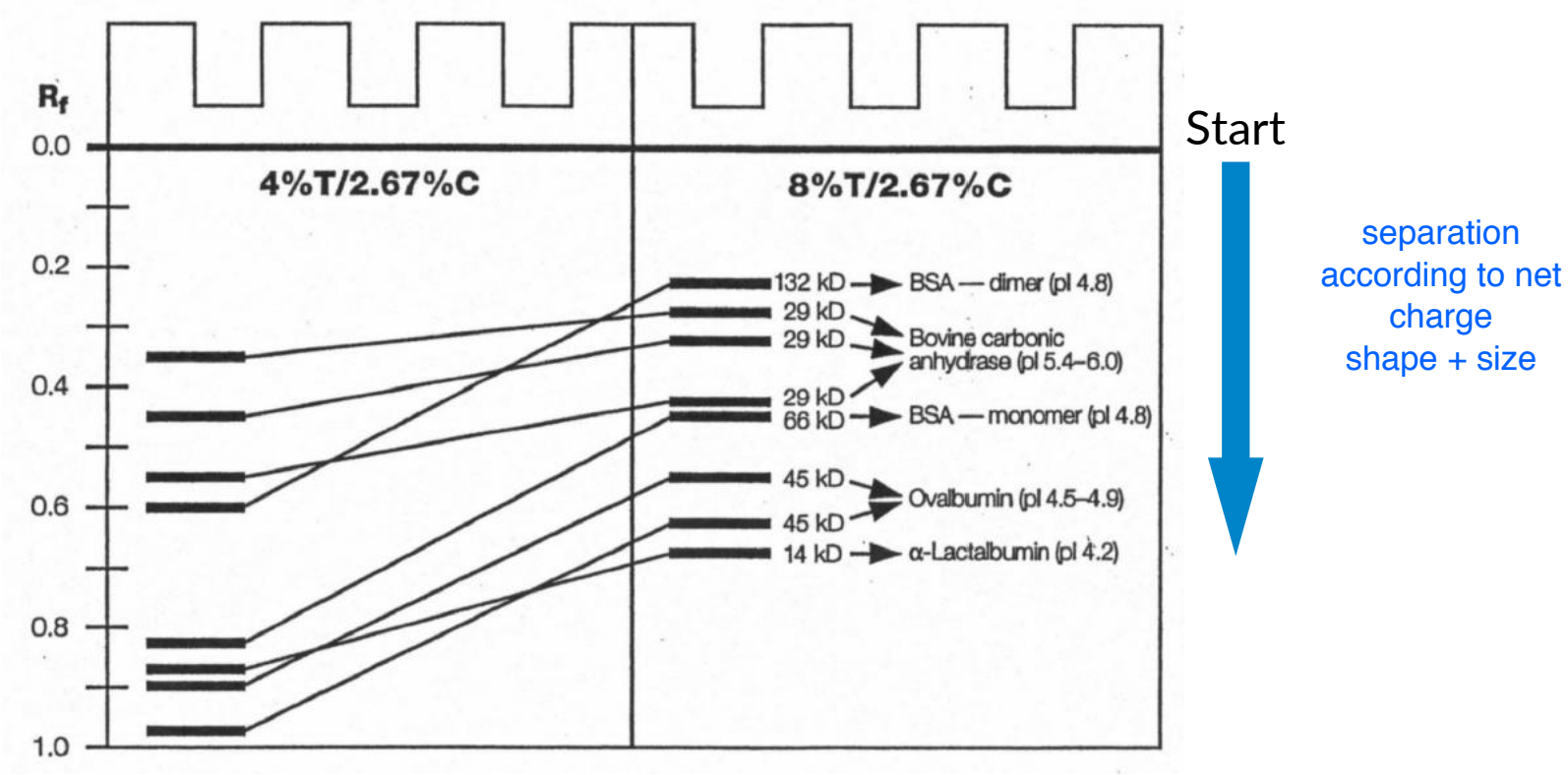
Copolymere of acrylamide and N,N‘-Methylenbisacrylamide (Bis)

- Stable, transparent, nearly no electro-endoosmosis
- Excellent separation
- Storable
- Suitable for many staining methods different dies to make protein visible, e.g. Ag+ Ions

Note: Monomeres are toxic, pore size limited

Polyacrylamide gel electrophoresis (PAGE)

Impact of T and C on the separation of a native protein mixture

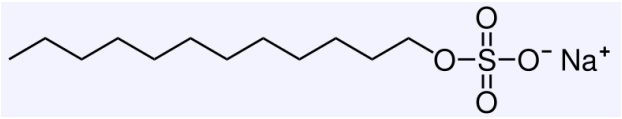


- 4 % T no sieving
  → separation based on free mobility (charge/ shear coefficient)

- 8 % T sieving effect
  → **Charge, shape and size – based separation**

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE Protein separation based on size



SDS: Sodium Dodecyl Sulfate

- anionic detergent
- masks protein charges
- all proteins negatively charged
- supports protein denaturation and unfolding
- relatively constant mass : charge

➔ **proteins separate according to their molecular mass (not charge!)**

➔ **negative surface charge prevents protein aggregation**

➔ **allows fast separations**

the bigger the protein, the more ist interacts with
masks alls proteins negative,
masks to charge ratio!
no protein accurigation with SDS

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE Protein separation based on size

Sample Buffer

2 x „Laemmli Buffer“

- 125 mM Tris-HCl, pH 6.8,
- 4 % SDS
- 20% (w/v) glycerol
- 0.004% bromophenol blue
- 10% 2-mercaptoethanol

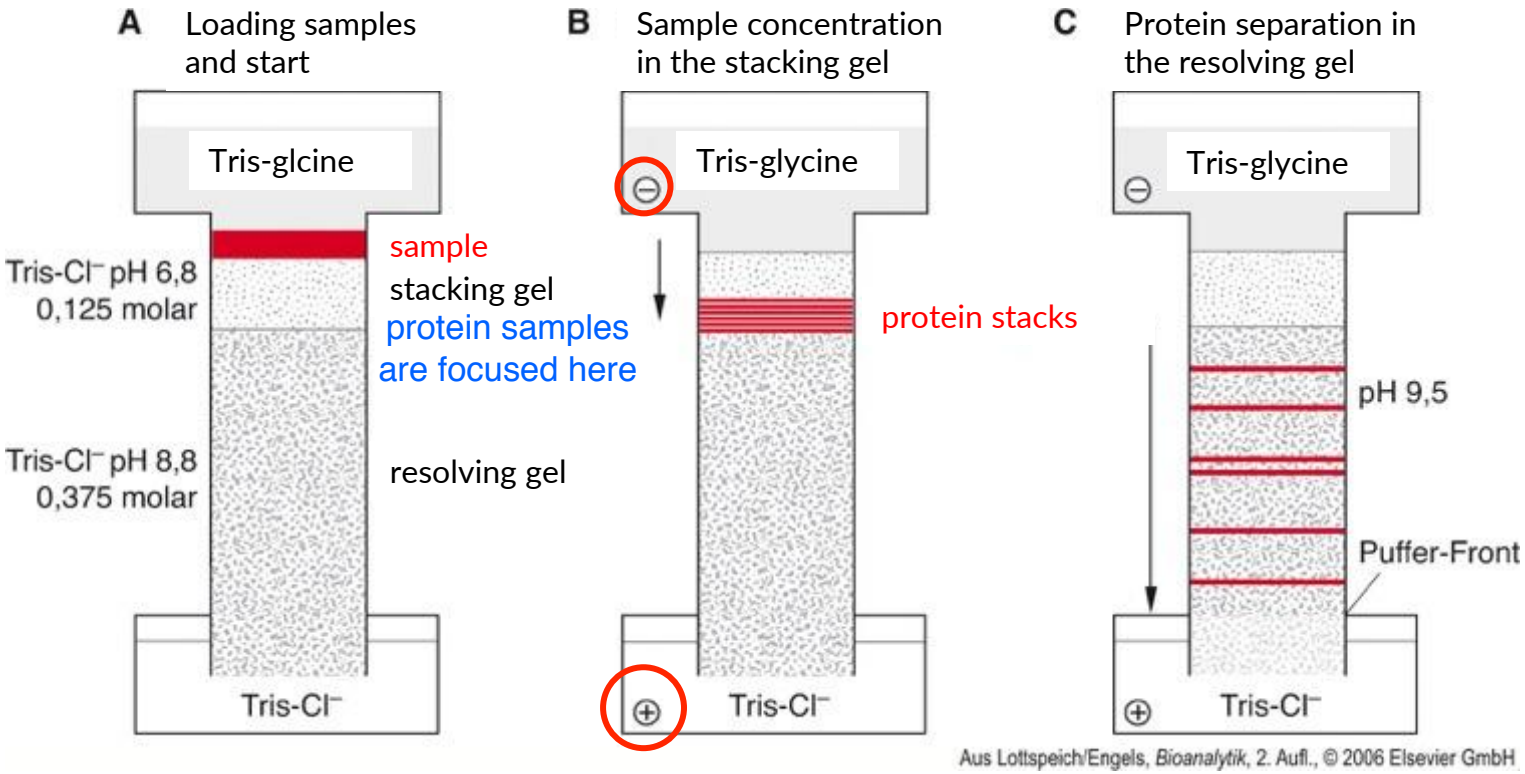
  to get rid of the sulfate bonds!

Denaturate sample 5 min at 95°C

Polyacrylamide gel electrophoresis (PAGE)

Discontinuous electrophoresis (Disc-electrophoresis)

ratio,
smaller ones at bottom, bigger ones at the top (are slower)

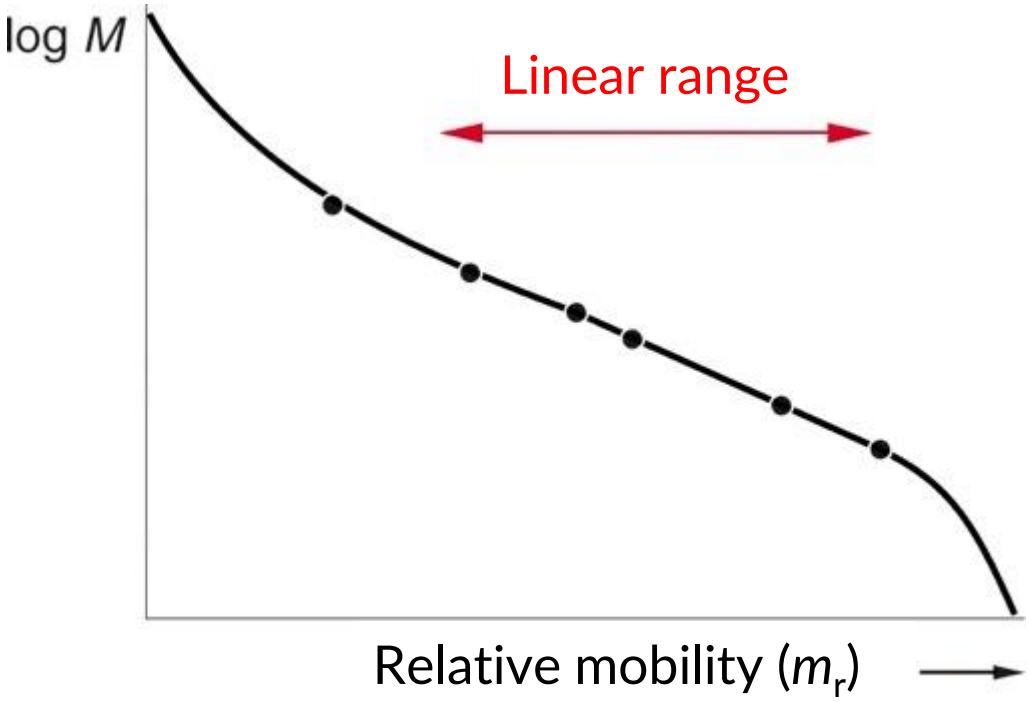


Aus Lottspeich/Engels, *Bioanalytik*, 2. Aufl., © 2006 Elsevier GmbH

- prevents aggregation; higher resolution
- proteins are concentrated at the interface between stacking and resolving gel
- different ion mobilities in stacking gel

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE Protein separation based on size



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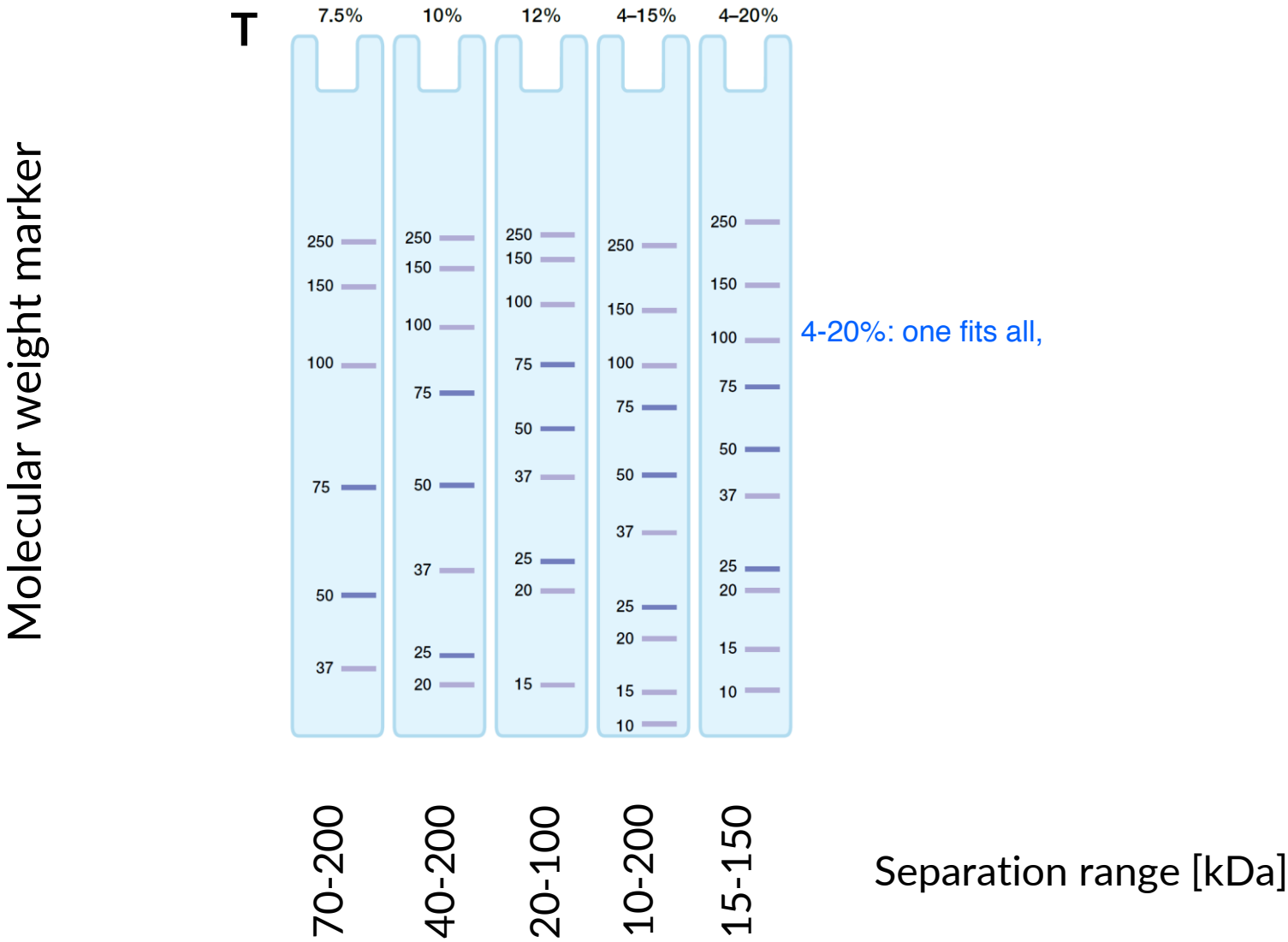
M = Molecular weight

Rel. Mobility = Relative distance of the protein to the starting point

- Linear range depends on molecular weight : pore size
- Highest resolution in combination with DISC-PAGE

Polyacrylamide gel electrophoresis (PAGE)

Selection of T



SDS-PAGE

Staining methods

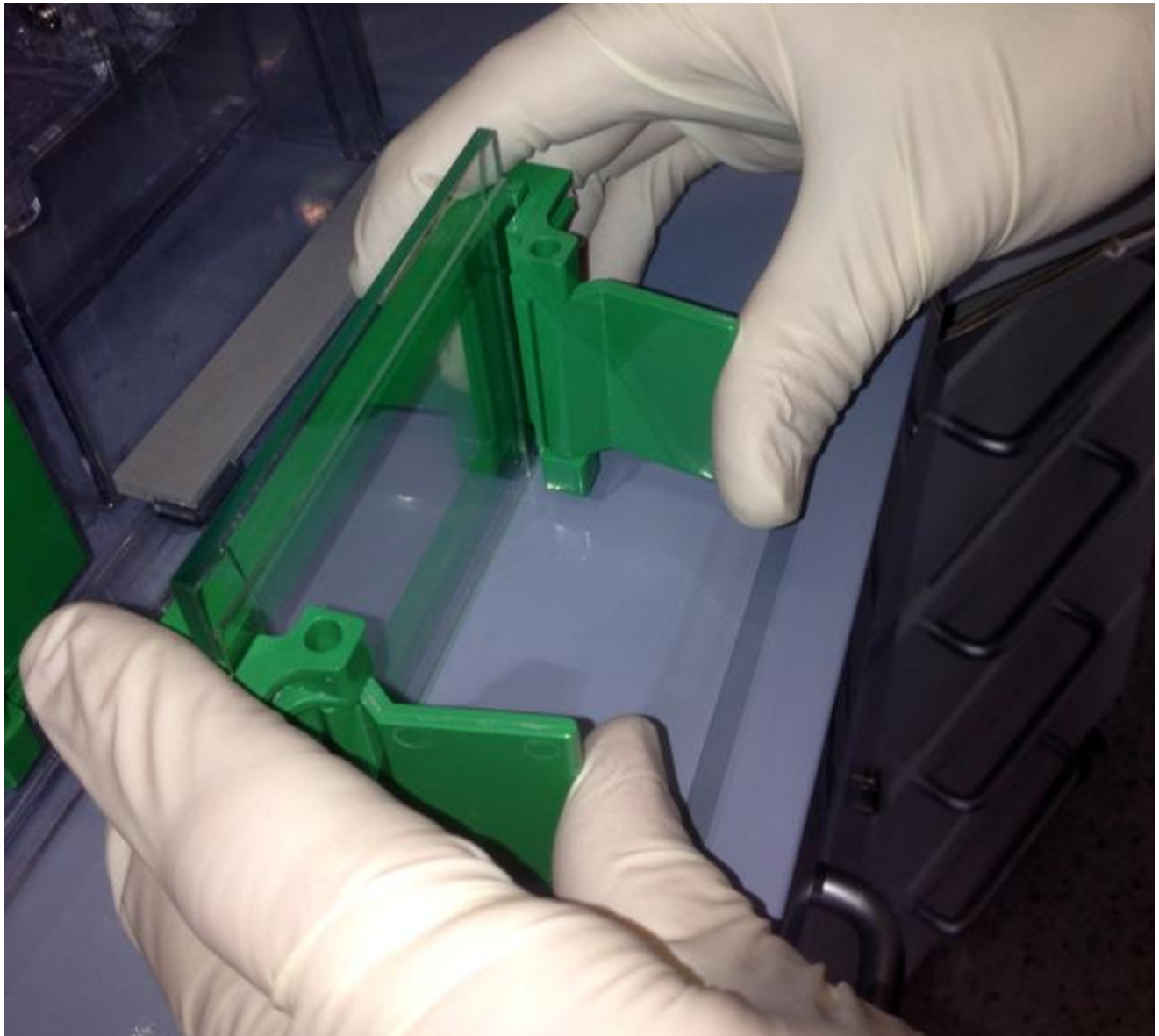
- **Coomassie-blue** (anionic triphenyl dye):
  Simple
  Sensivity: 100 ng to 1µg per band

- **Silver staining**
  Proteins bind silver cations
  Cations are reduced to elementary silver
  Silver forms seed crystals for further chemical redox reactions
  Sensitivity: < 1 ng per band
  Note: Very sensitive! Requires clean and precise work!

- **Fluorescent dyes**:
  Several dyes available (e.g. Sypro ruby)
  Sensitivity: < 1 ng per band

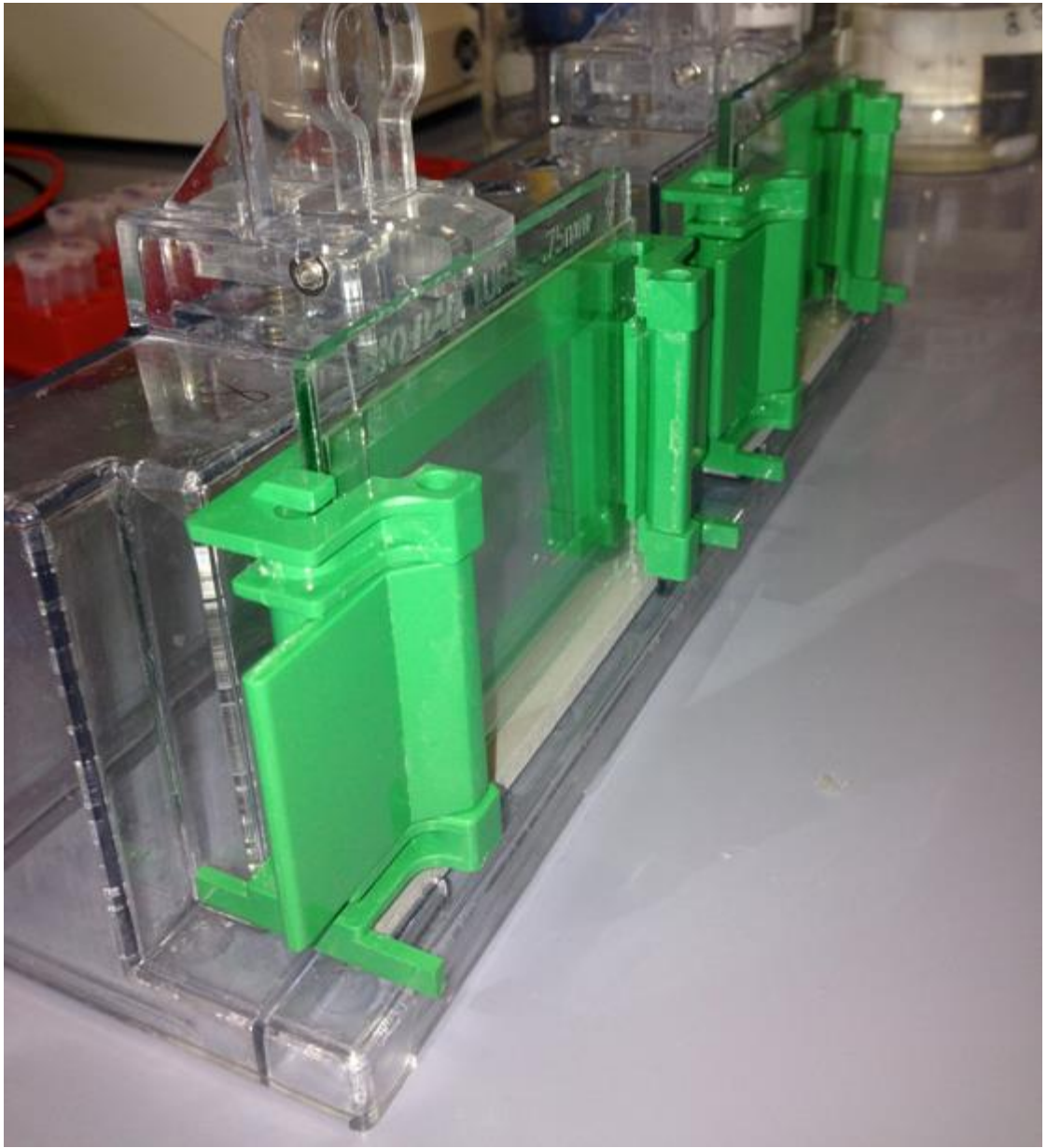
How to prepare a gel?

Assemble the casette



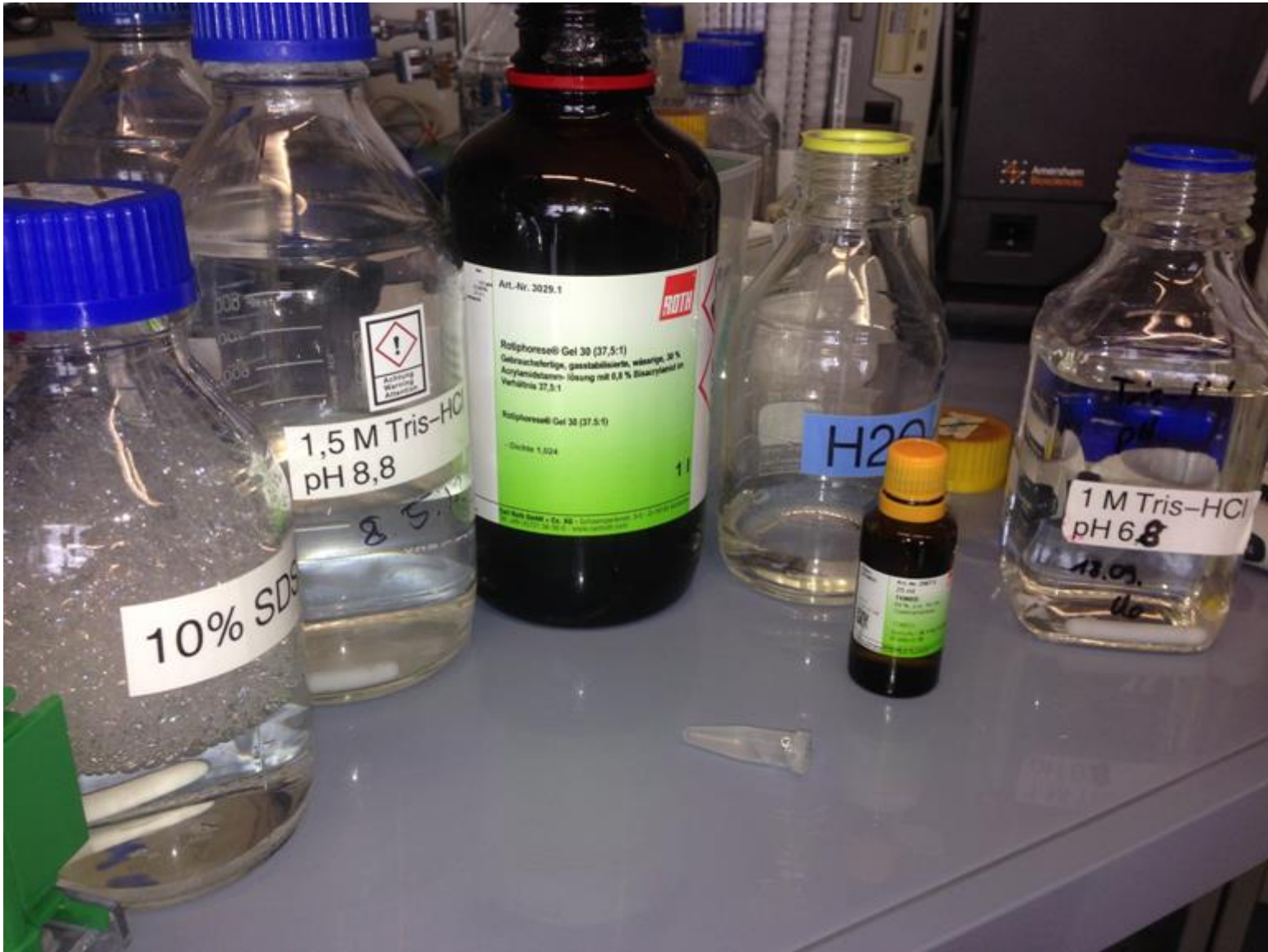
How to prepare a gel?

Put the gel into a clamp



How to prepare a gel?

Prepare resolving gel



How to prepare a gel?

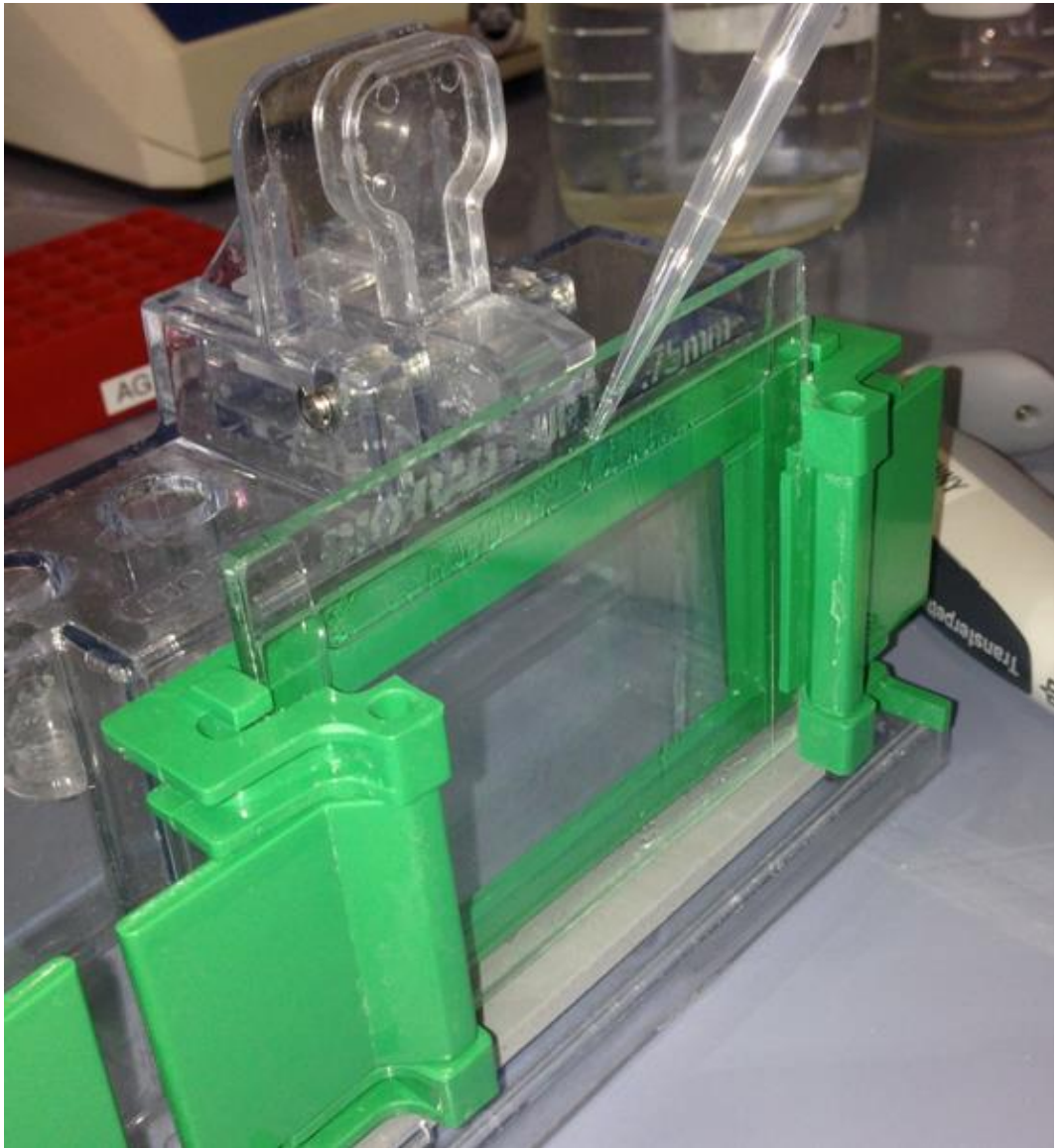
Pour resolving gel



How to prepare a gel?

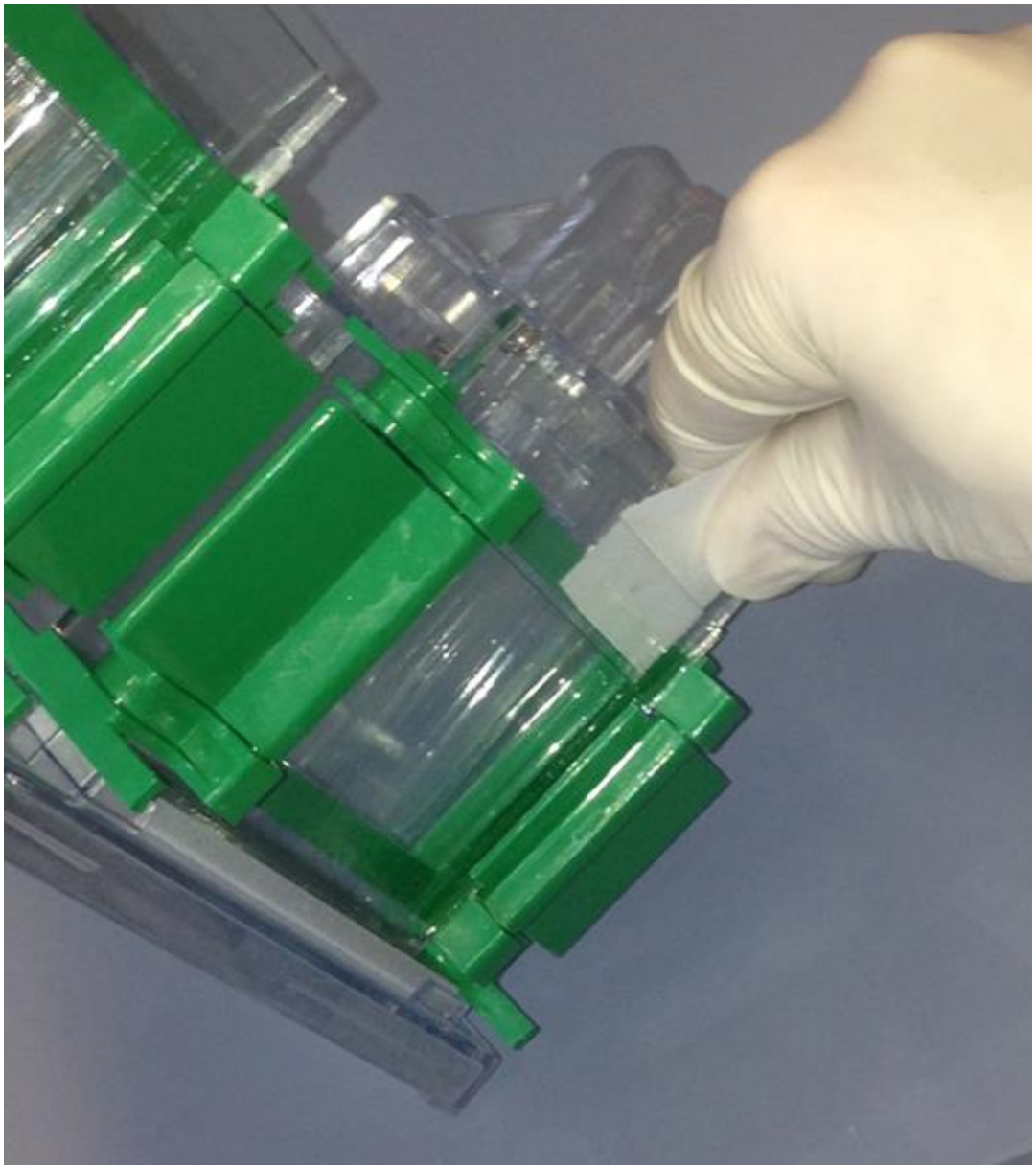
Overlay monomer solution with n-butanol

Let polymerize for 45-60 min



How to prepare a gel?

Remove n-butanol



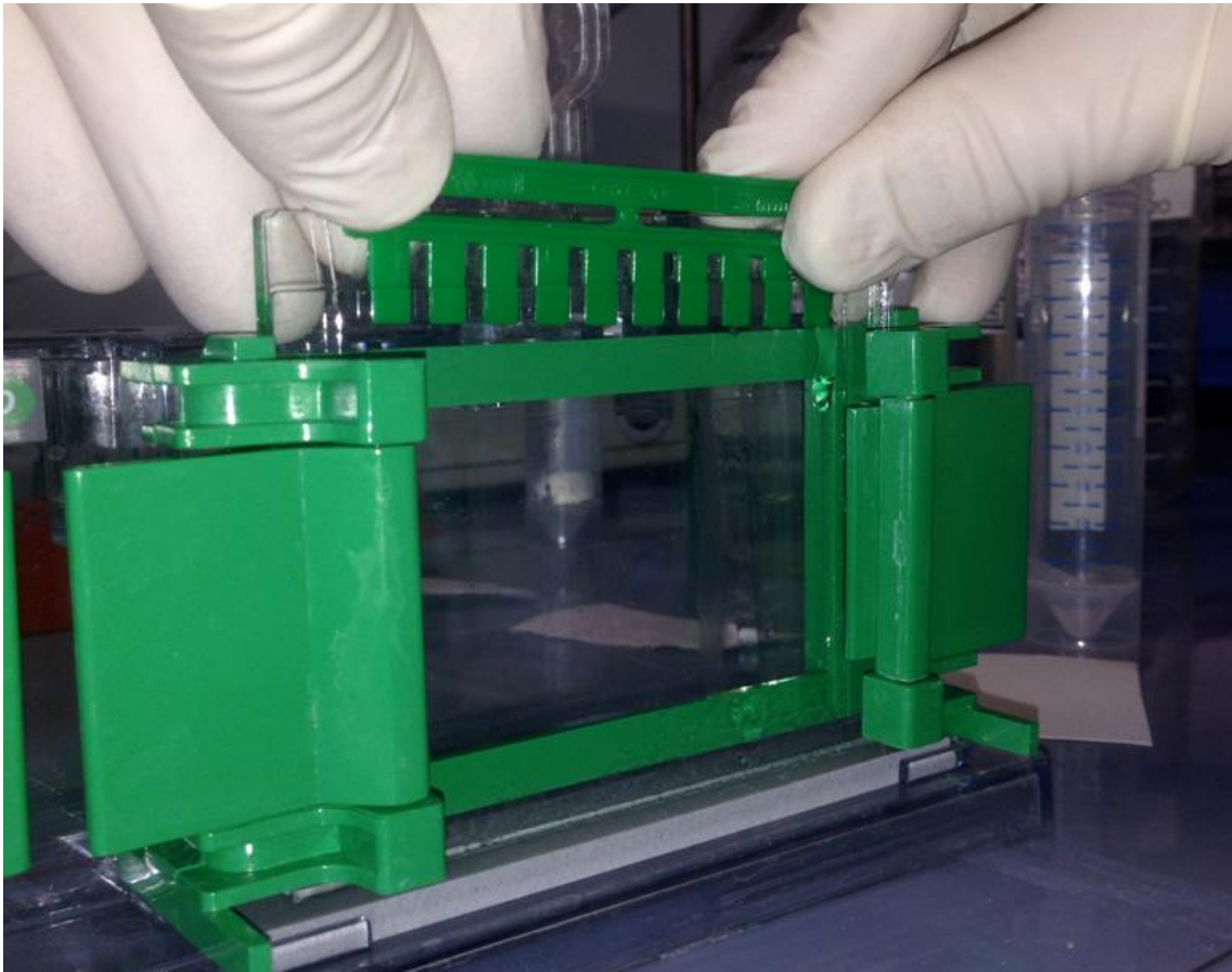
How to prepare a gel?

Pour stacking gel



How to prepare a gel?

Place comb in the stacking gel



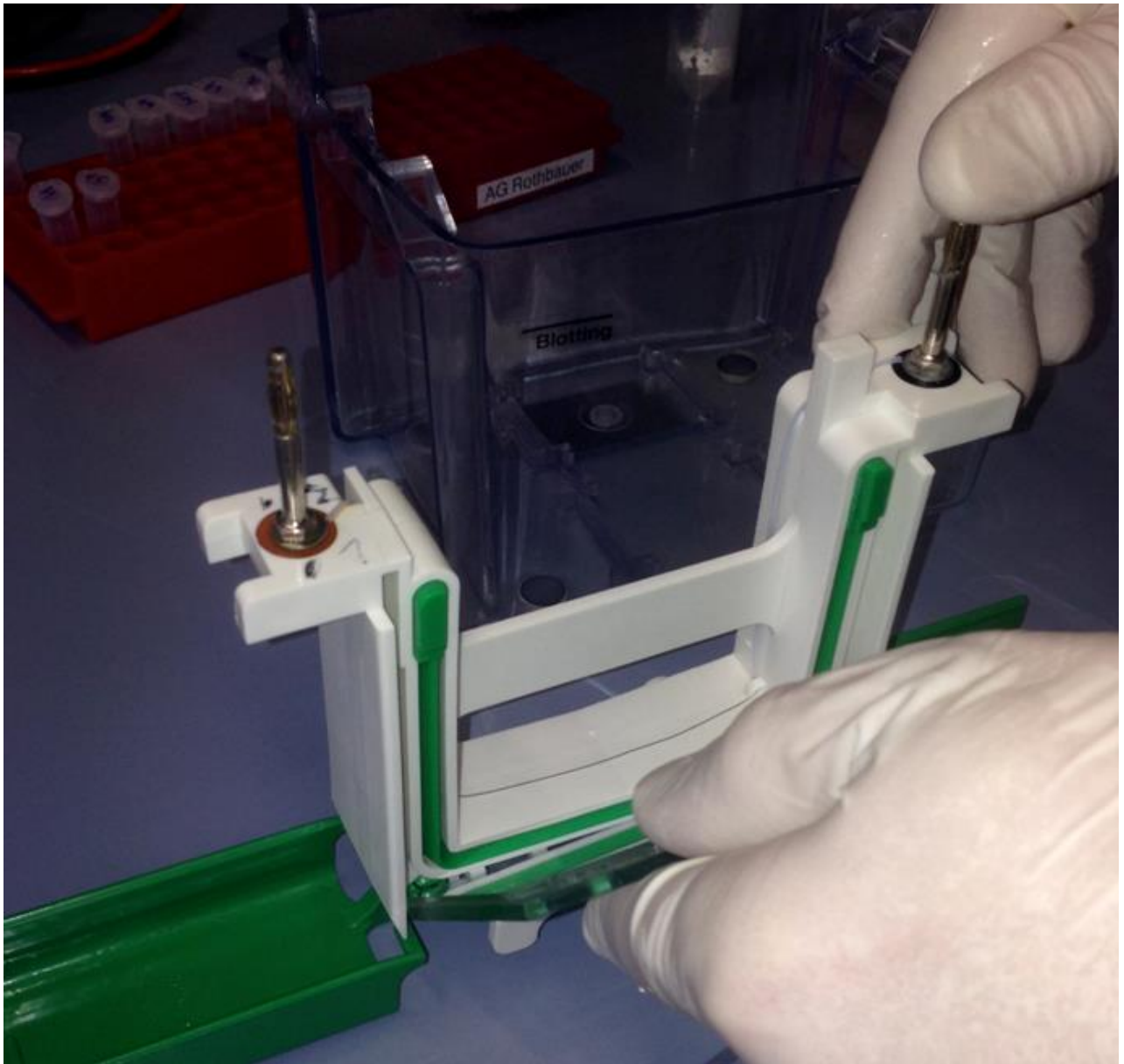
How to prepare a gel?

Remove comb



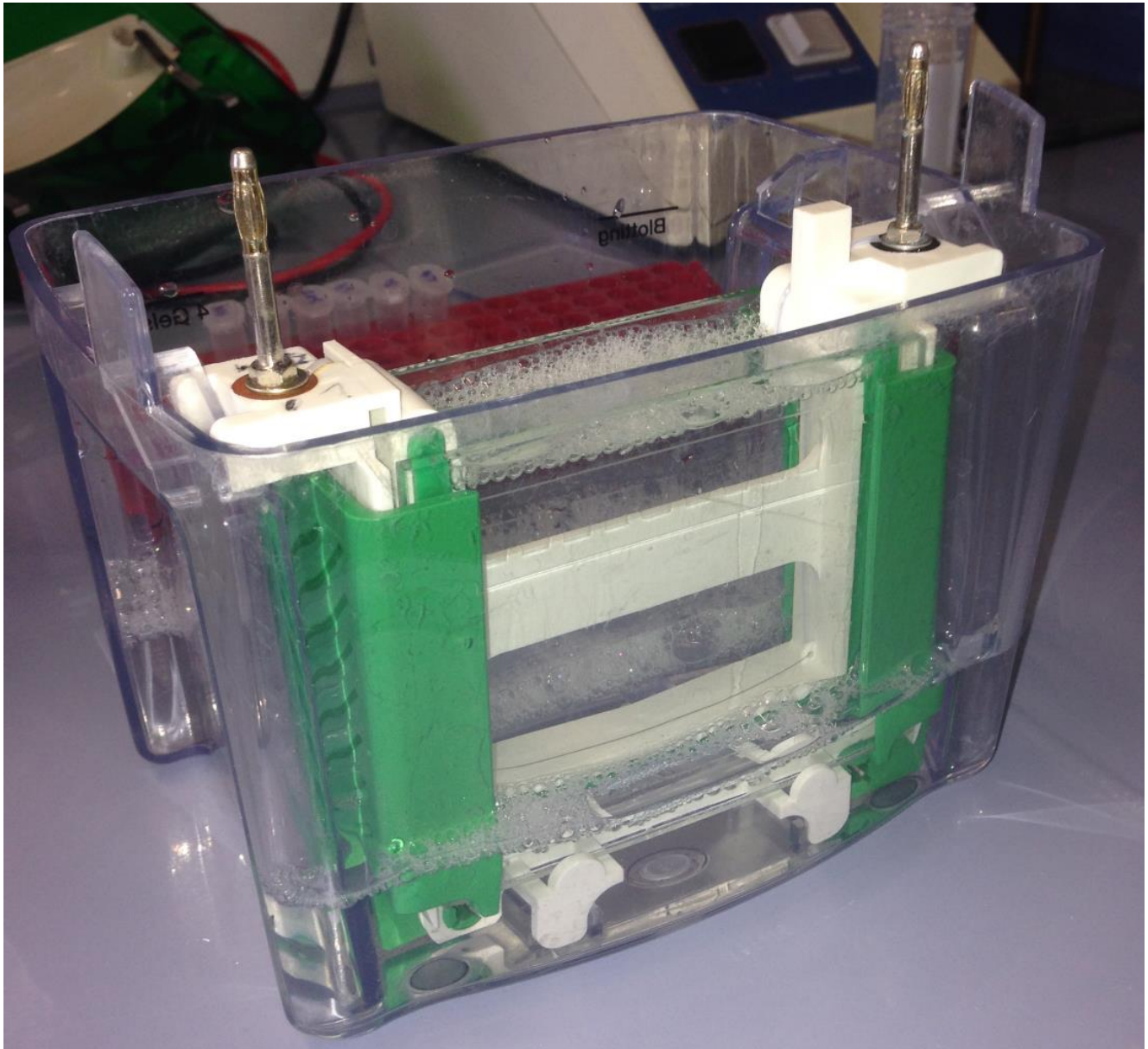
How to prepare a gel?

Place gel in the tank



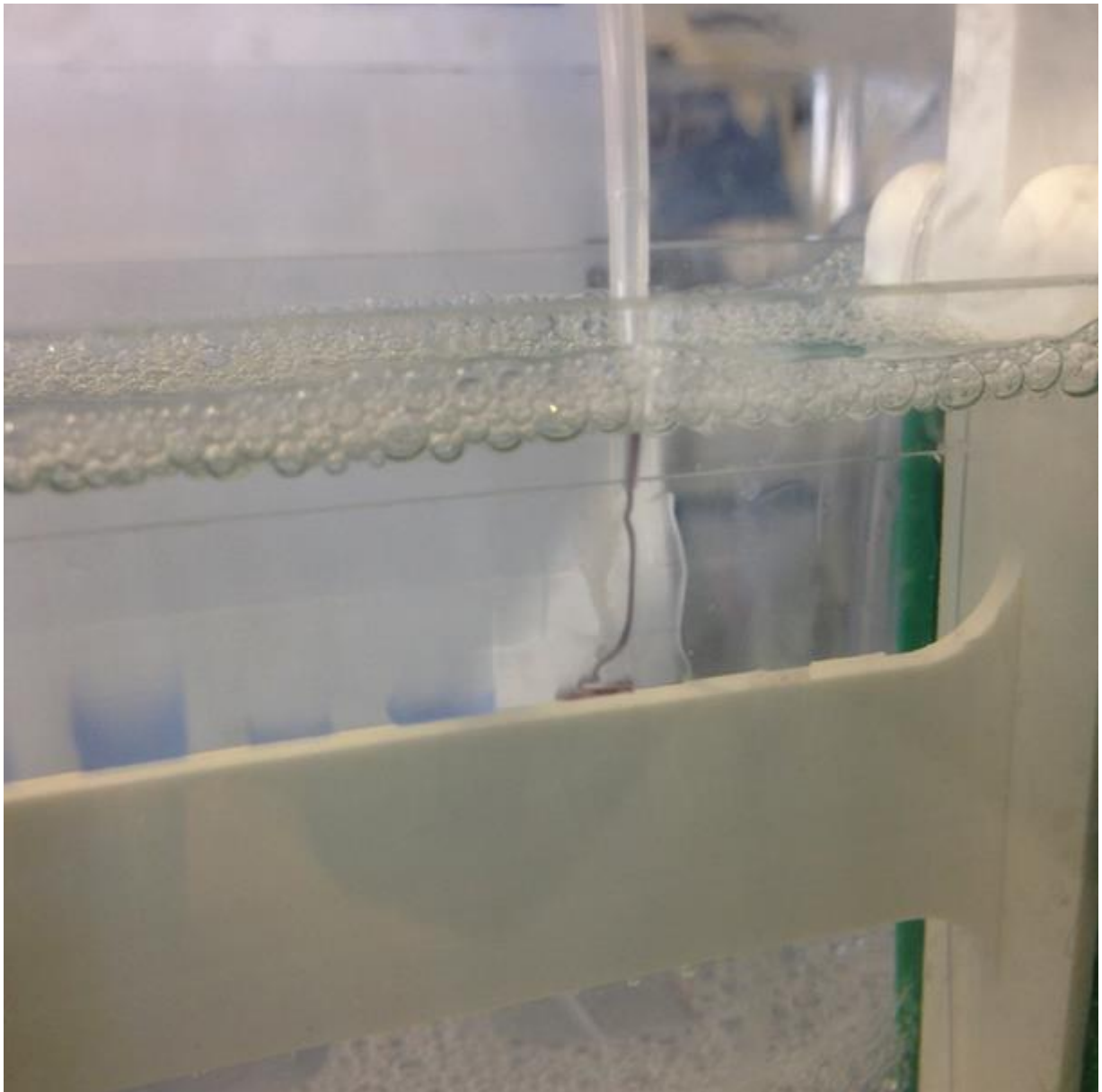
How to prepare a gel?

Place gel in the tank



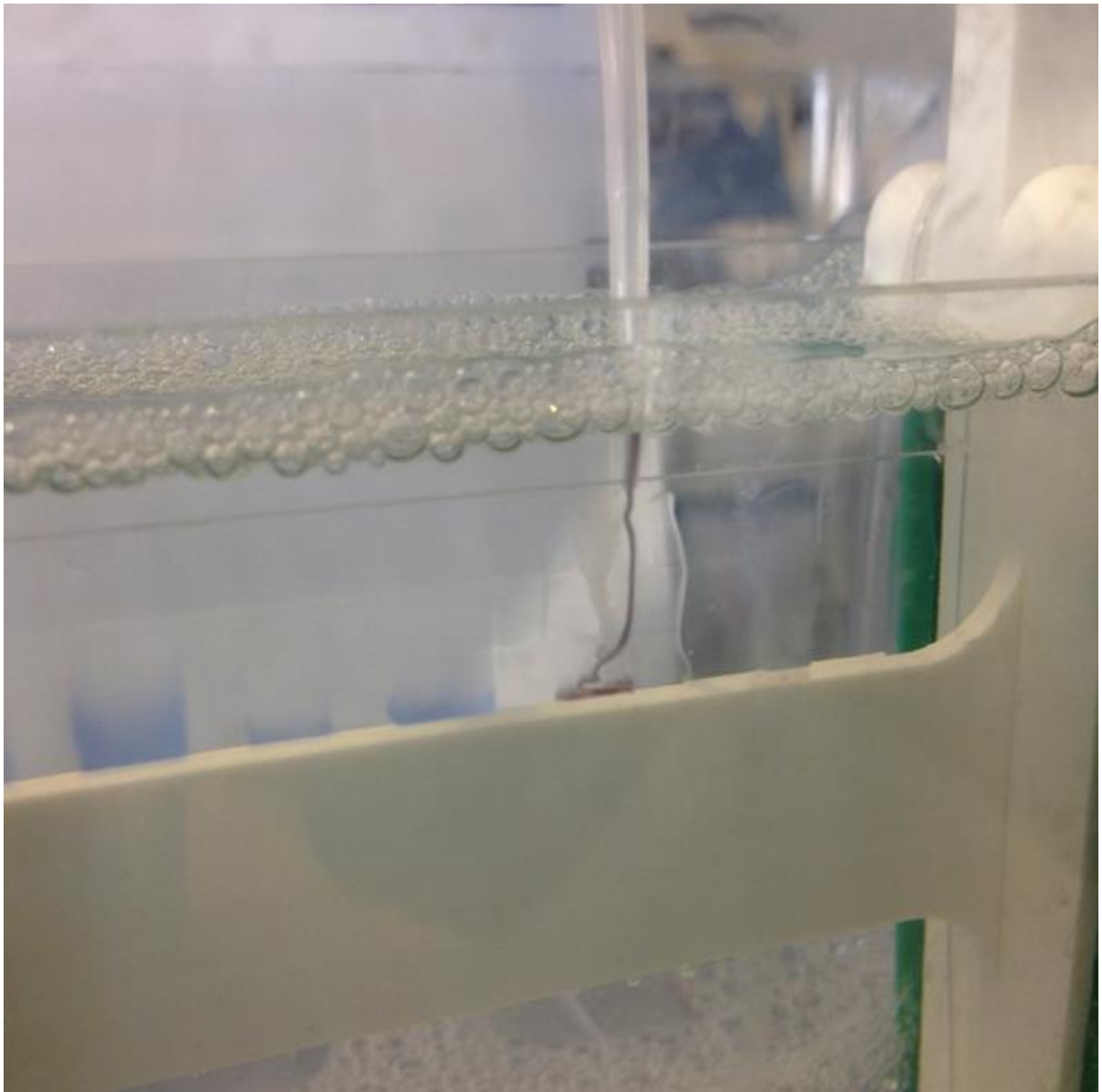
How to prepare a gel?

Load samples



How to prepare a gel?

Run Gel



- Electrophoresis
- Blotting
- Detection
- Antibody databases

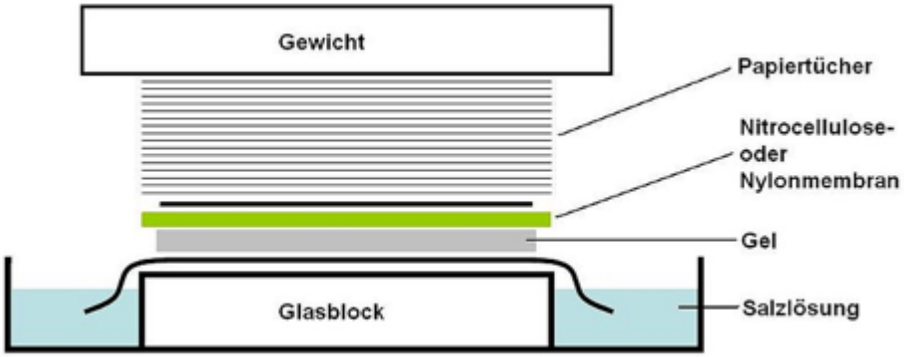
History: „Blotting“

Southern blot – detection of specific DNA



Inventor: Sir Edwin M. Southern

1. Partial DNA cleavage by restriction enzymes
2. Agarose gel electrophoresis
3. Blotting onto membrane
4. Hybridisation of complementary labelled probe
5. Removal of unbound probe
6. Detection of bound probe



Bildquelle: http://de.wikipedia.org/w/index.php?title=Datei:Southernblot_aufbau.JPG&filetimestamp=20090911223518
http://de.wikipedia.org/w/index.php?title=Datei:Southern-Blot-Agarosegel.jpg&filetimestamp=20050929203856

Blotting-“atlas“



RNA → Membran (1977)

James C. Alwine, David Kemp, George Stark (Stanford CA, USA)



Protein → Membran (1979)

Harry Towbin, T. Staehelin, J. Gordon (ETH Zürich, CH)
W.N. Burnette (Term: Western Blot 1981)
Jaime Renart, J. Reiser, G.R. Stark (Stanford CA, USA)



?? → Membran



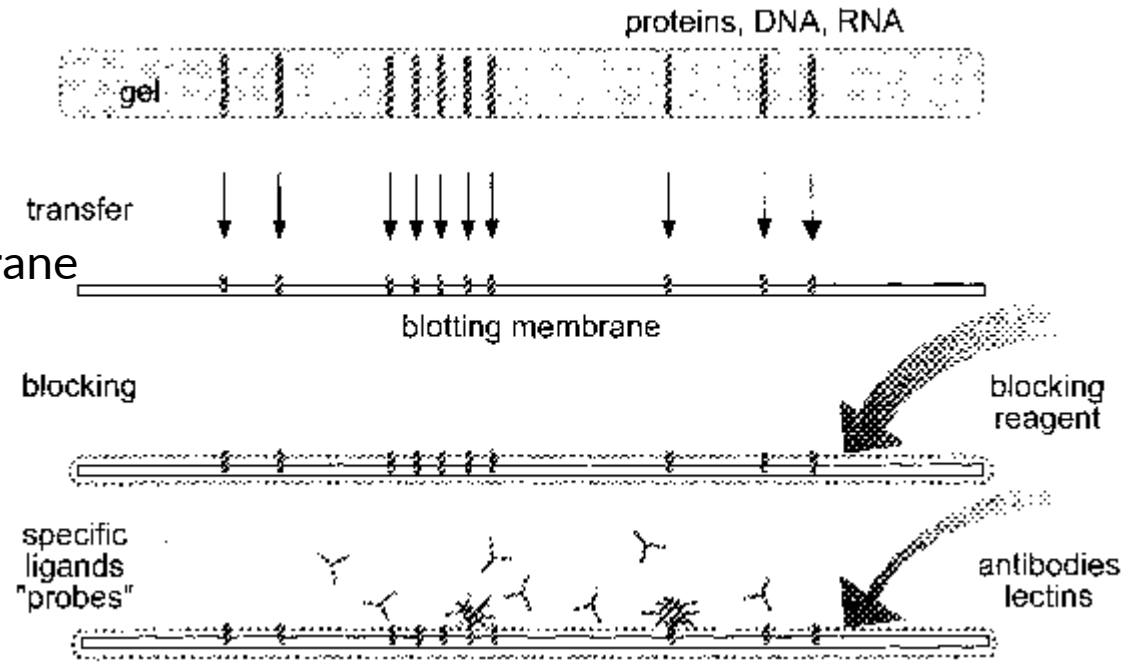
DNA → Membran (1975)

Sir Edwin M. Southern (University of Edinborough, Great Britain)

http://www.easydealz.de/wp-content/uploads/Weltkarte.jpg
http://de.wikipedia.org/w/index.php?title=Datei:Southern-Blot-Agarosegel.jpg&filetimestamp=20050929203856
http://nihrecord.od.nih.gov/newsletters/2005/11_04_2005/images/Khourylect250.jpg

Western-Blot

1. Separation (Electrophoresis)
2. Transfer onto membrane ("Blotting")
3. Blocking
4. Detection



R. Westermeier, Electrophoresis in Practice, Wiley-VCH, Weinheim, 2005
F. Lottspeich, J.W. Engels, Bioanalytik, 2. Auflage, Elsevier, 2006 .

Blotting

Method	Physico-chemical transfer principle
Diffusion blotting	Diffusion
Thermal blotting	Diffusion & temperature
Capillary blotting	Capillary forces
Pressure blotting	Capillary forces & pressure
Vacuum blotting	Vacuum
Electro-blotting	Electric field
Tank blotting	
Semi-dry blotting	

not efficient

long-term, but more efficient than only diffusion

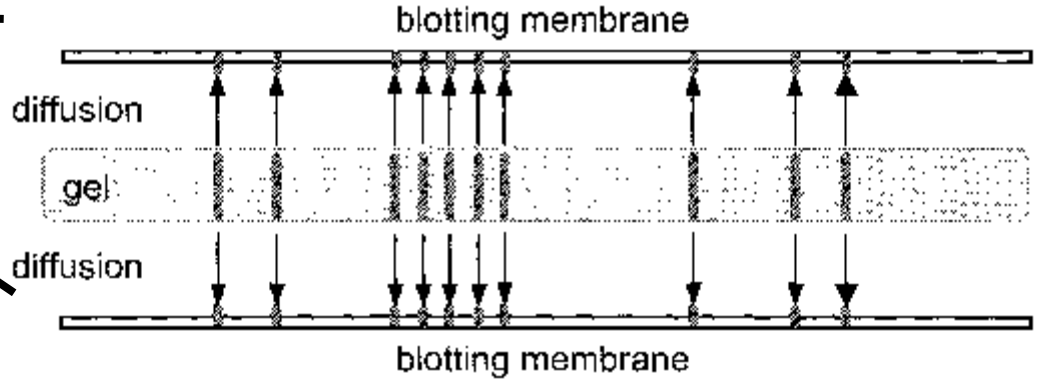
same concepts but different machines

HIGHLY efficient

R. Westermeier, Electrophoresis in Practice, Wiley-VCH, Weinheim, 2005.

Blotting

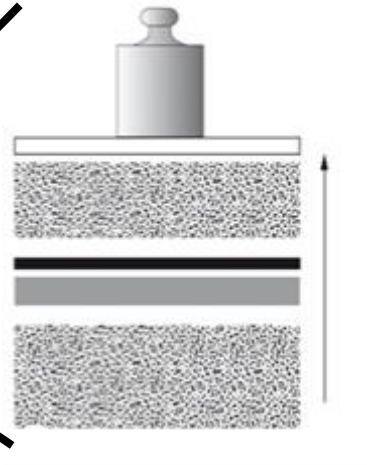
Method	Physico-chemical transfer principle
Diffusion blotting	
Thermal blotting	
Capillary blotting	
Pressure blotting	
Vacuum-blotting	
Electro blotting	
Tank blotting	
Semi-dry blotting	



R. Westermeier, Electrophoresis in Practice, Wiley-VCH, Weinheim, 2005.

Blotting

Method	Physico-chemical transfer principle
Diffusion blotting	
Thermal blotting	
Capillary blotting	
Pressure blotting	
Vacuum blotting	
Electro-blotting	
Tank blotting	
Semi-dry blotting	

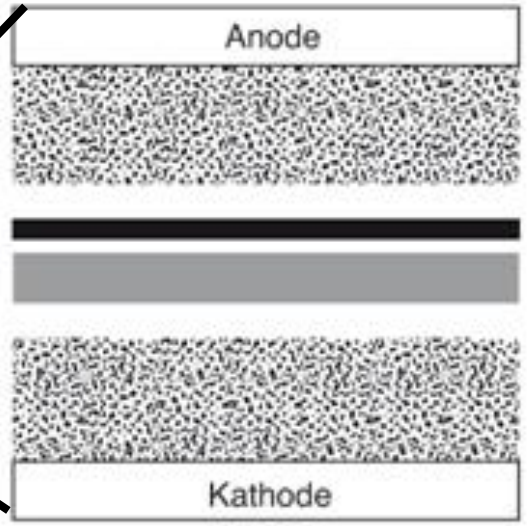


4: dry Whatman paper
3: Blotting membrane (Nitrocellulose/PVDF)
2: Gel
1: Whatman paper + Puffer

R. Westermeier, Electrophoresis in Practice, Wiley-VCH, Weinheim, 2005.

Blotting

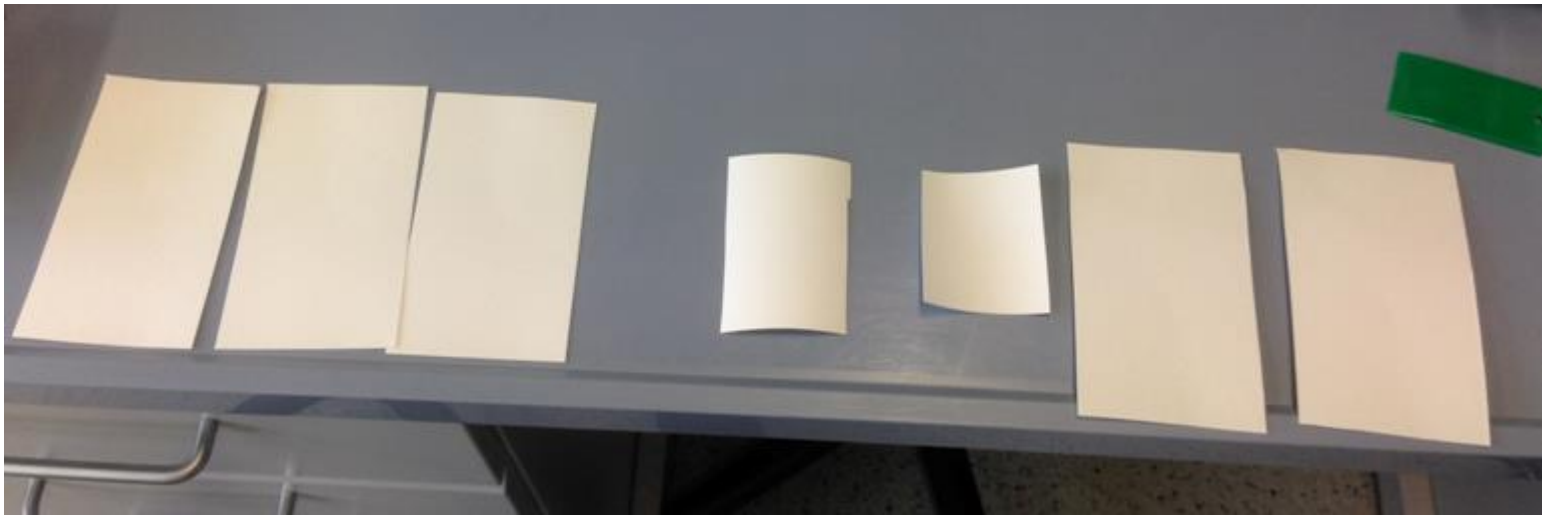
Method	Physico-chemical transfer principle
Diffusion blotting	
Thermal blotting	
Capillary blotting	
Pressure blotting	
Vacuum blotting	
Electro-blotting Tank blotting Semi-dry blotting	



4: Whatman paper + Puffer
3: Blotting membrane (Nitrocellulose/PVDF)
2: Gel
1: Whatman paper + Puffer

R. Westermeier, Electrophoresis in Practice, Wiley-VCH, Weinheim, 2005.

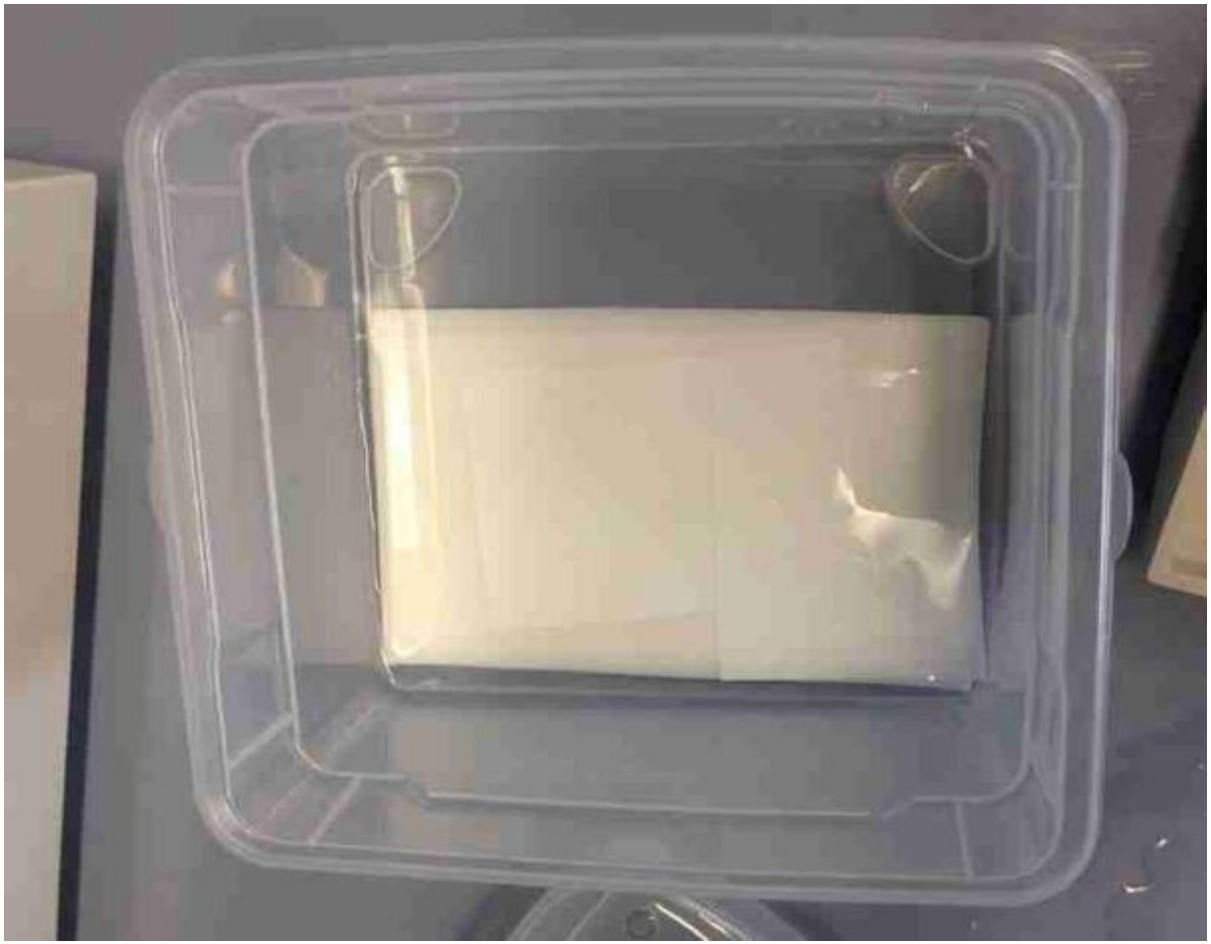
How to blot a gel?



3 Paper Membranes 2 Paper

How to blot a gel?

Pre-wet membranes and paper



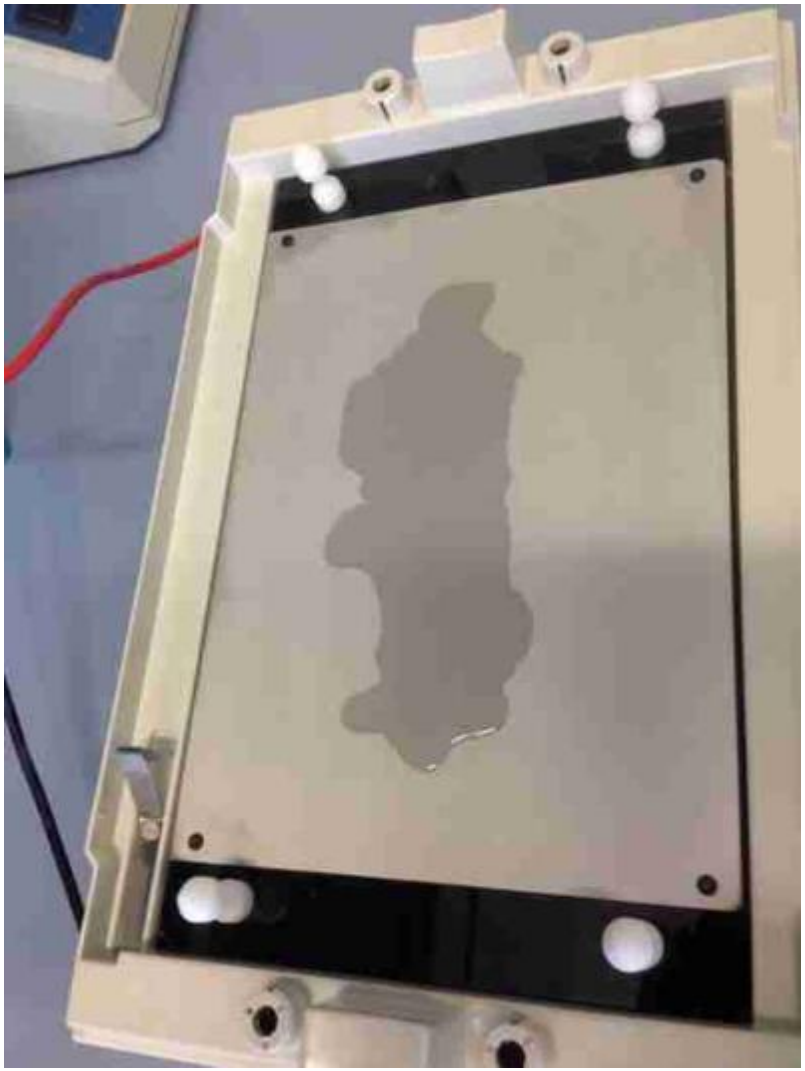
How to blot a gel?

Disassemble gel



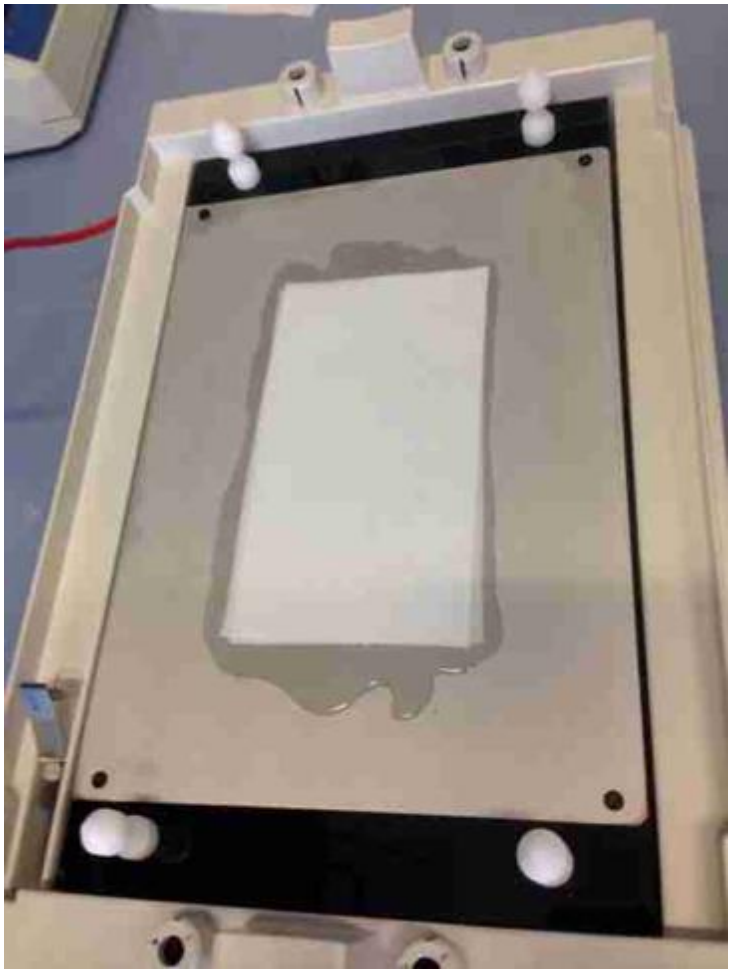
How to blot a gel?

Pre-wet anode



How to blot a gel?

Place blot paper



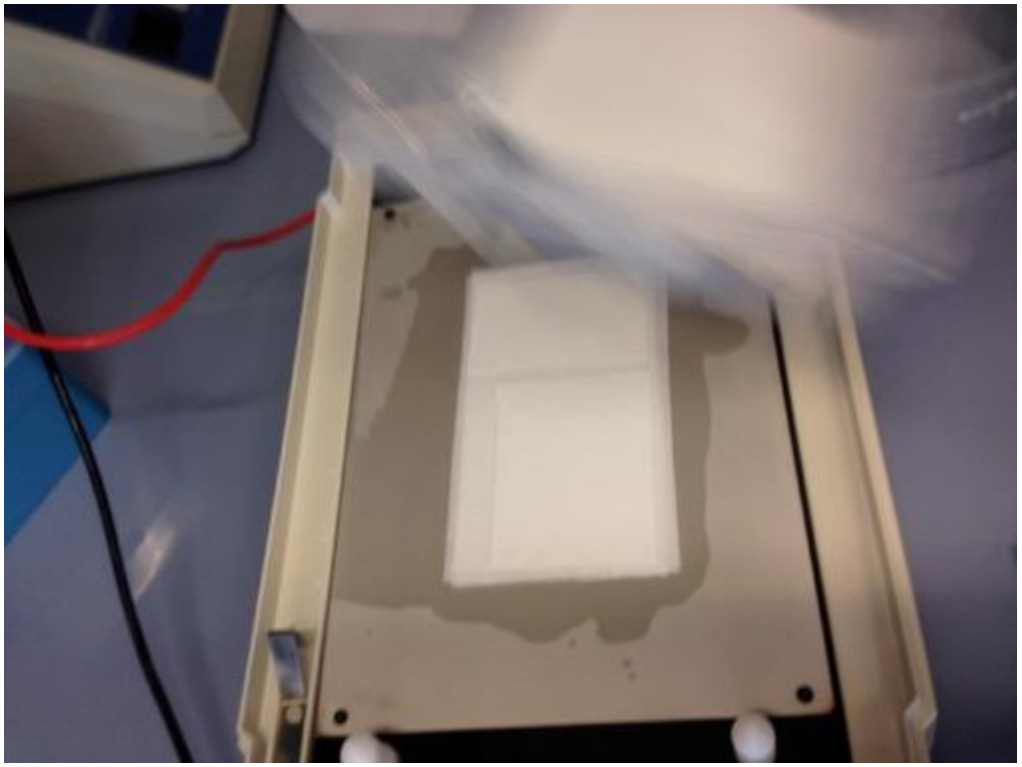
How to blot a gel?

Remove air bubbles



How to blot a gel?

Place membranes



How to blot a gel?

Place gels



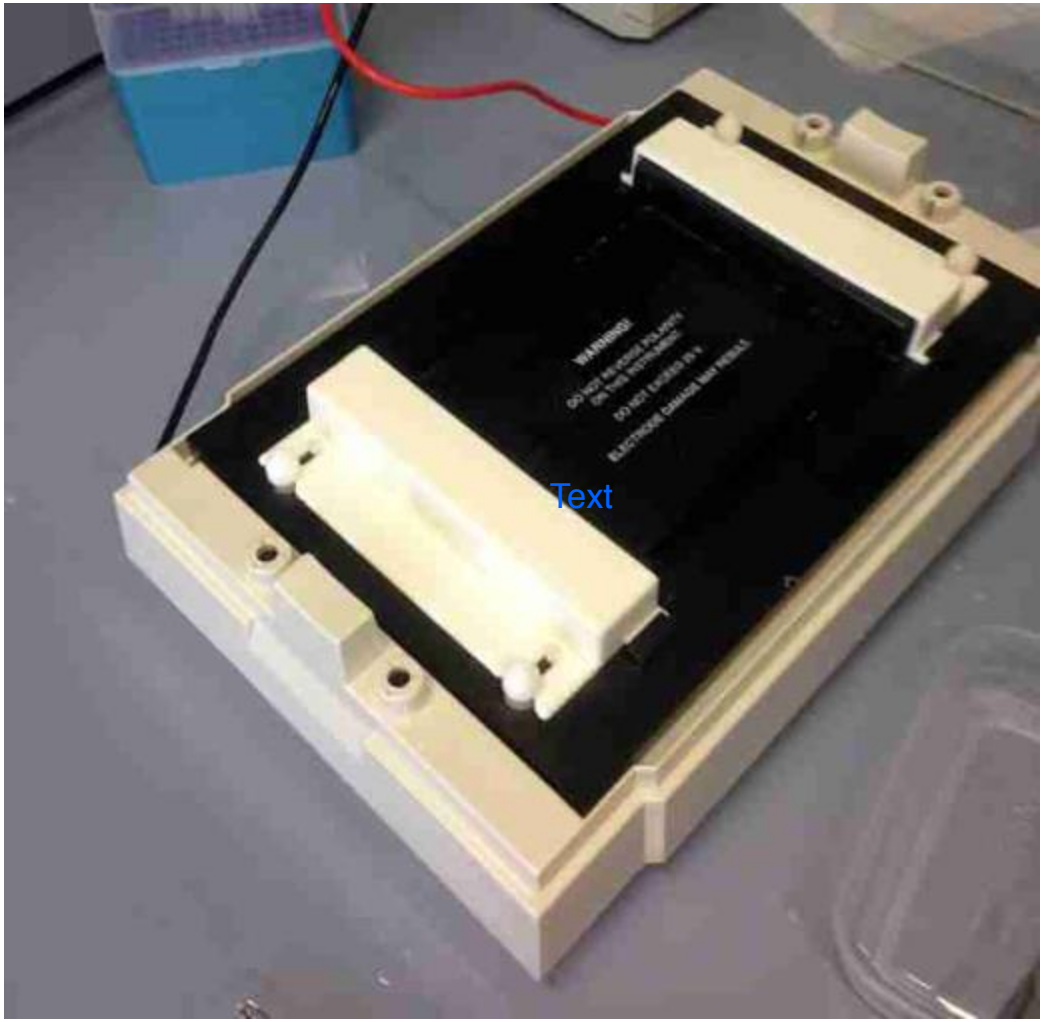
How to blot a gel?

Remove air bubbles



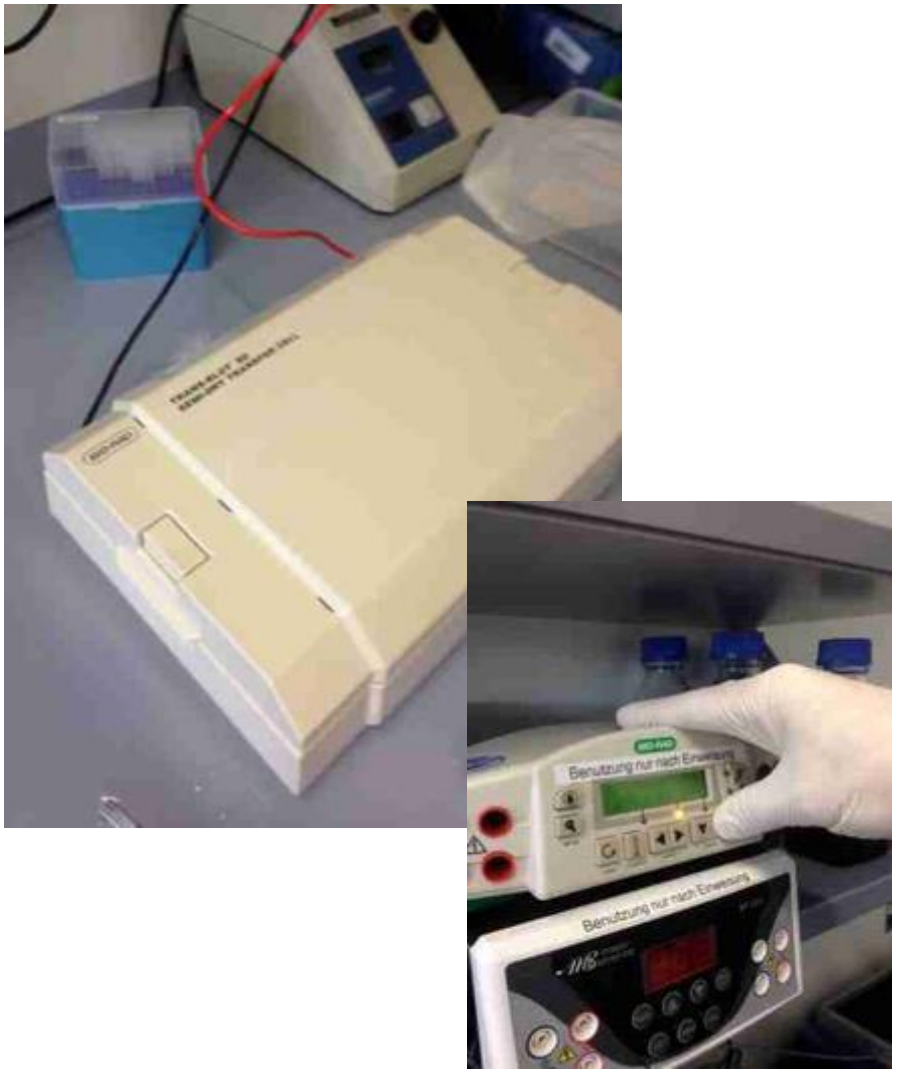
How to blot a gel?

Place cathode

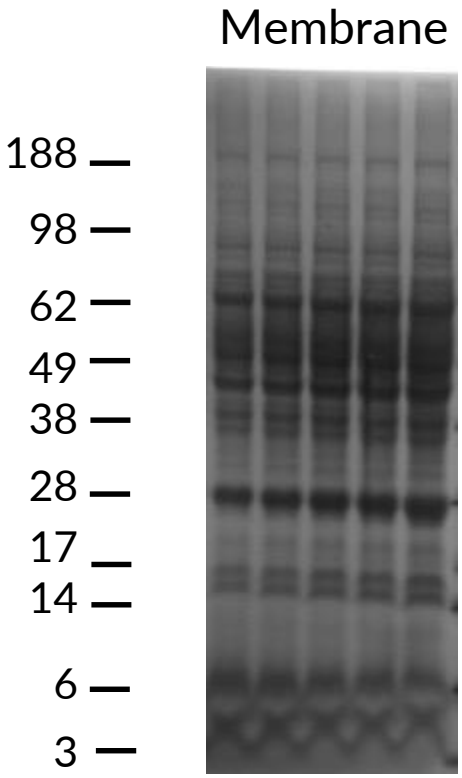


How to blot a gel?

Start transfer

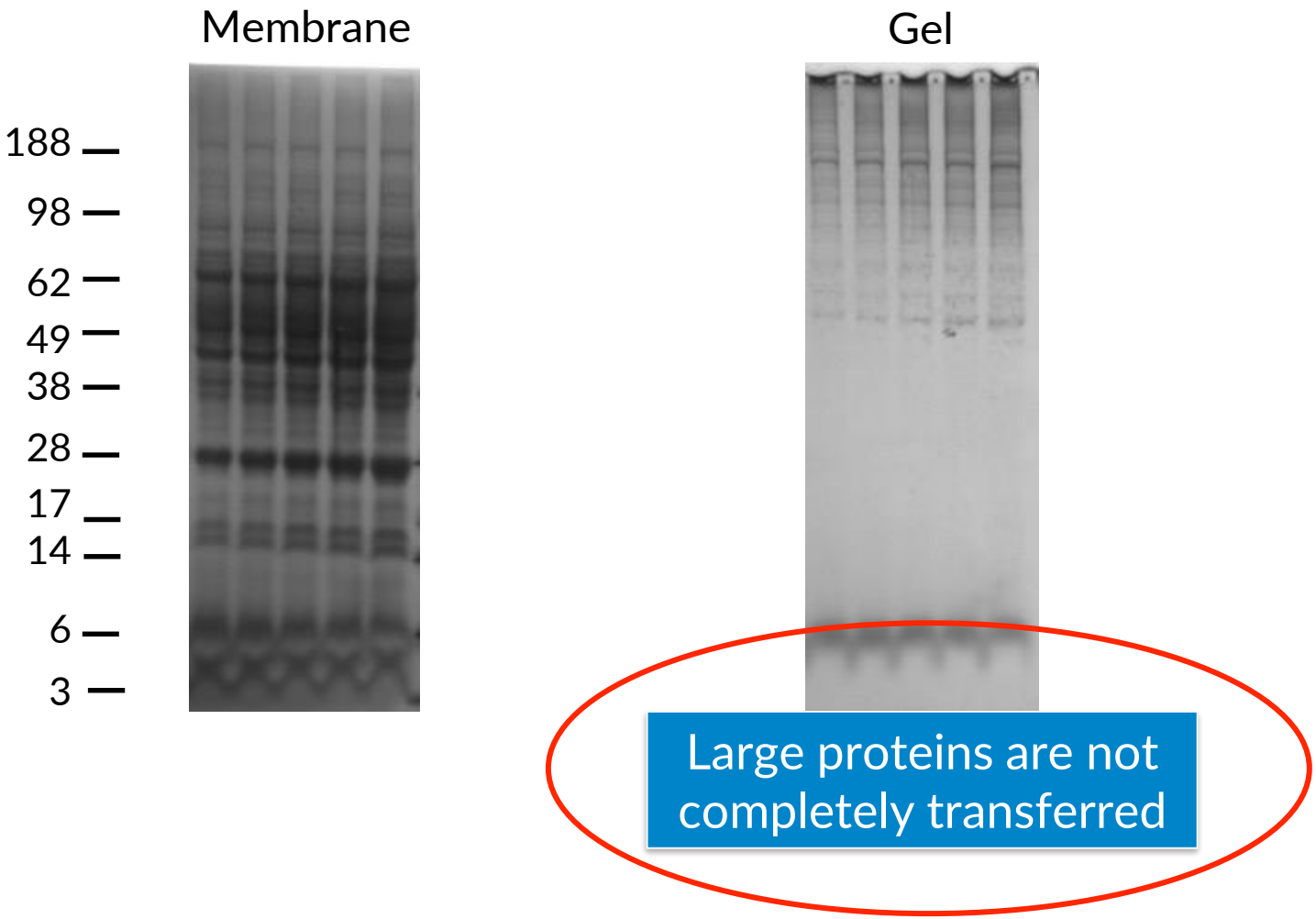


Blotting
Size does matter!

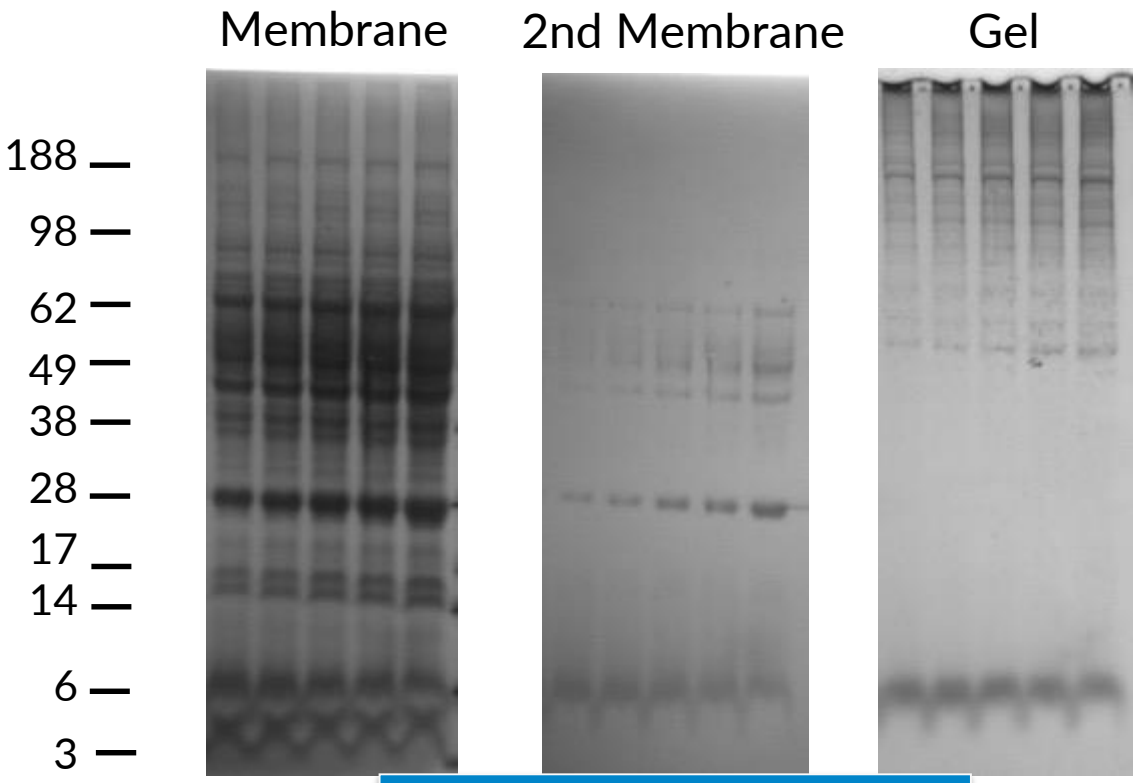


dye proteins on gel or membrane

Blotting
Size does matter!



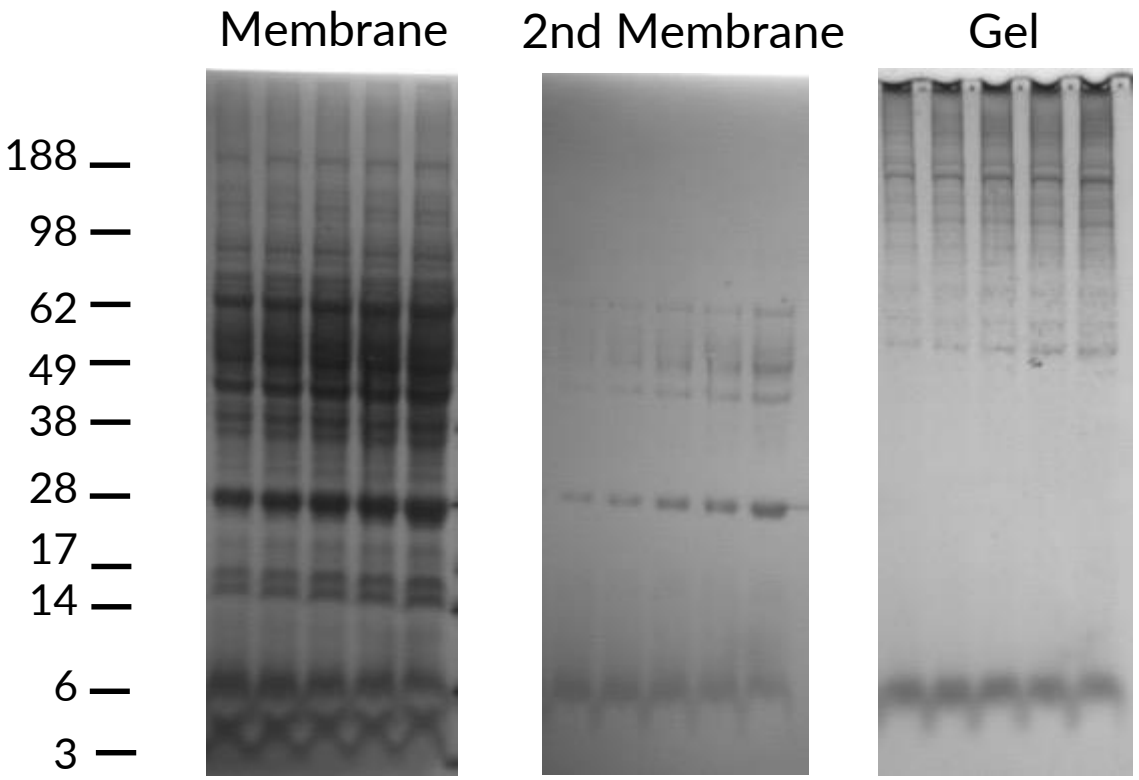
Blotting
Size does matter!



Small proteins partially passed membrane

some proteins might have gone through the membrane

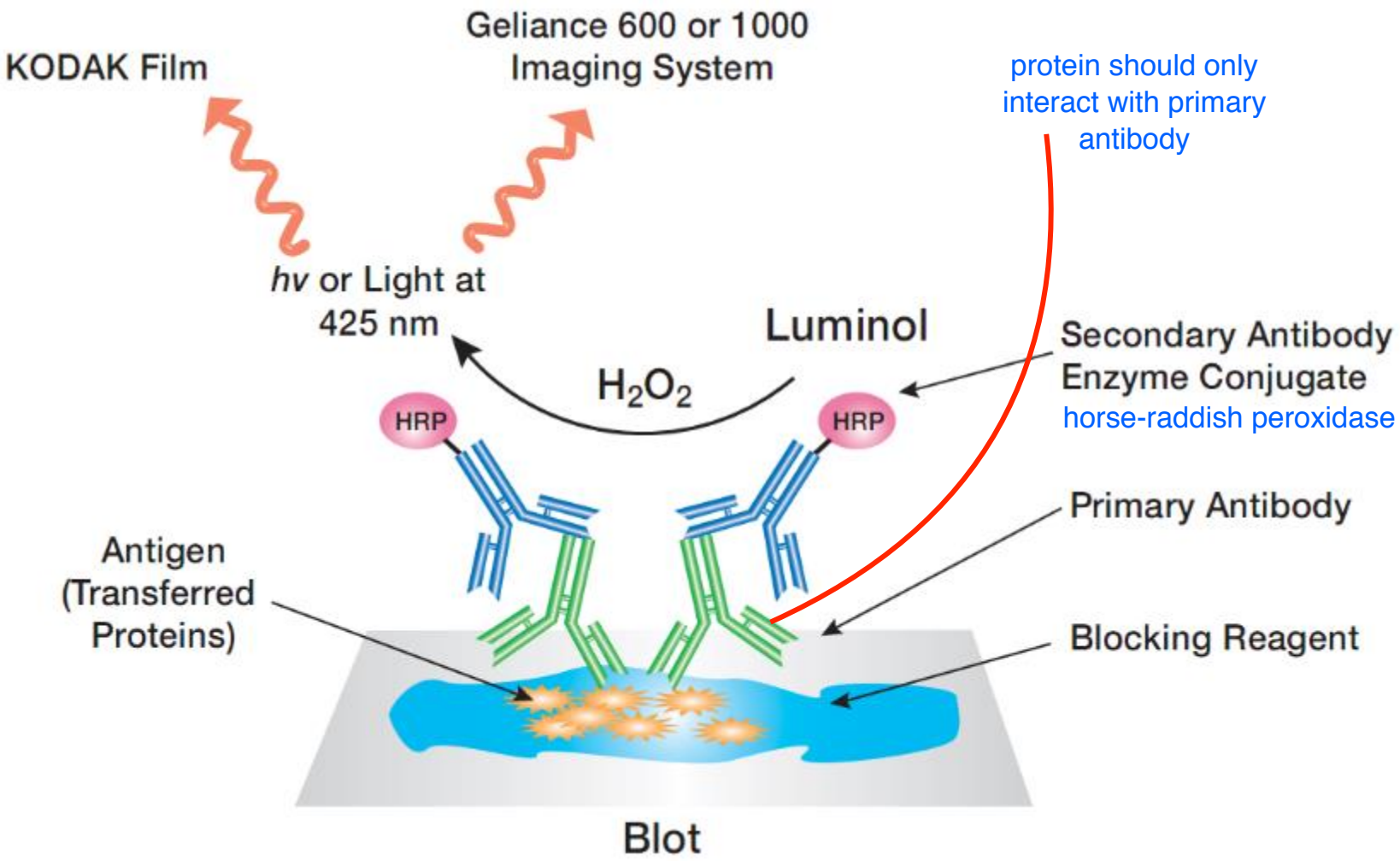
Blotting
Size does matter!



For best sensitivity adjust transfer time according to size of analyte!

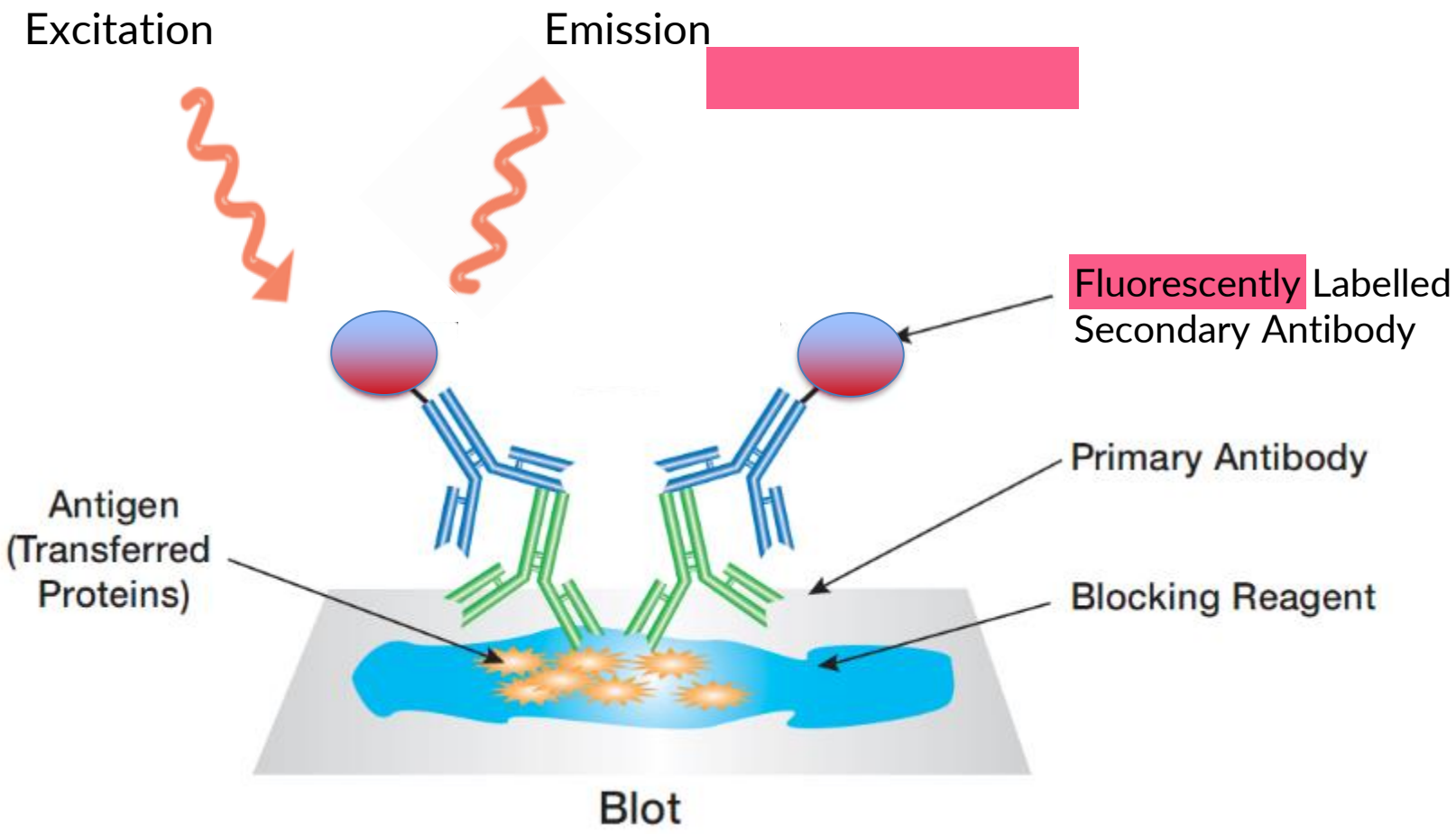
- Electrophoresis
- Blotting
- Detection
- Antibody databases

Western Blot



Western Blot Application Guide, Perkin Elmer, 2008 (Nr. 0072005C_01)

Western Blot



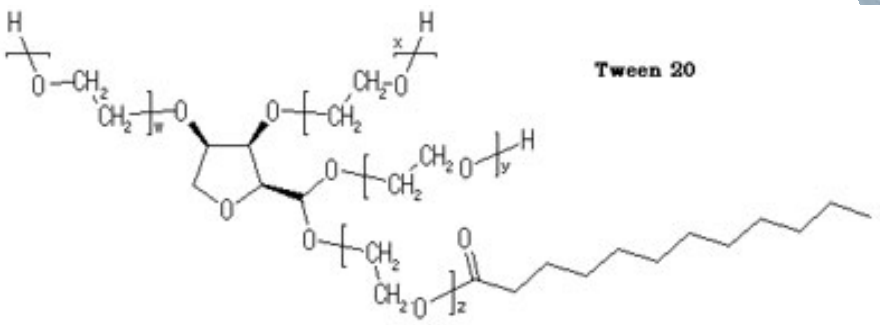
Western Blot Application Guide, Perkin Elmer, 2008 (Nr. 0072005C_01)

Western Blot – Blocking Step

Blotting membranes provide high binding capacity
→ uncoated space has to be blocked between the proteins

Examples for blocking reagents:
Tris-buffered saline + 0.05% Tween 20 (TBST)
+ 5% skimmed milk powder
or 2% - 10% bovine serum albumin (BSA)
or 3% fish gelatine

without black Western Blot, because primary antibody would bind everywhere





Western Blot - Detection

1st step: Specific primary antibody

- Concentration range: 0.01-0.5 µg/ml
- Incubation time: 1h - O/N
- Incubation temperature: RT – 4°C RT: 1h, 4°C: overnight
- Incubation buffer: 1% skimmed milk powder or
  1% BSA in TBST



Western Blot - Detection

2nd step: Visualization of specific primary antibody

Non-enzymatic:

- biotinylated secondary antibody
- fluorescently labelled secondary antibody
- labelled protein A

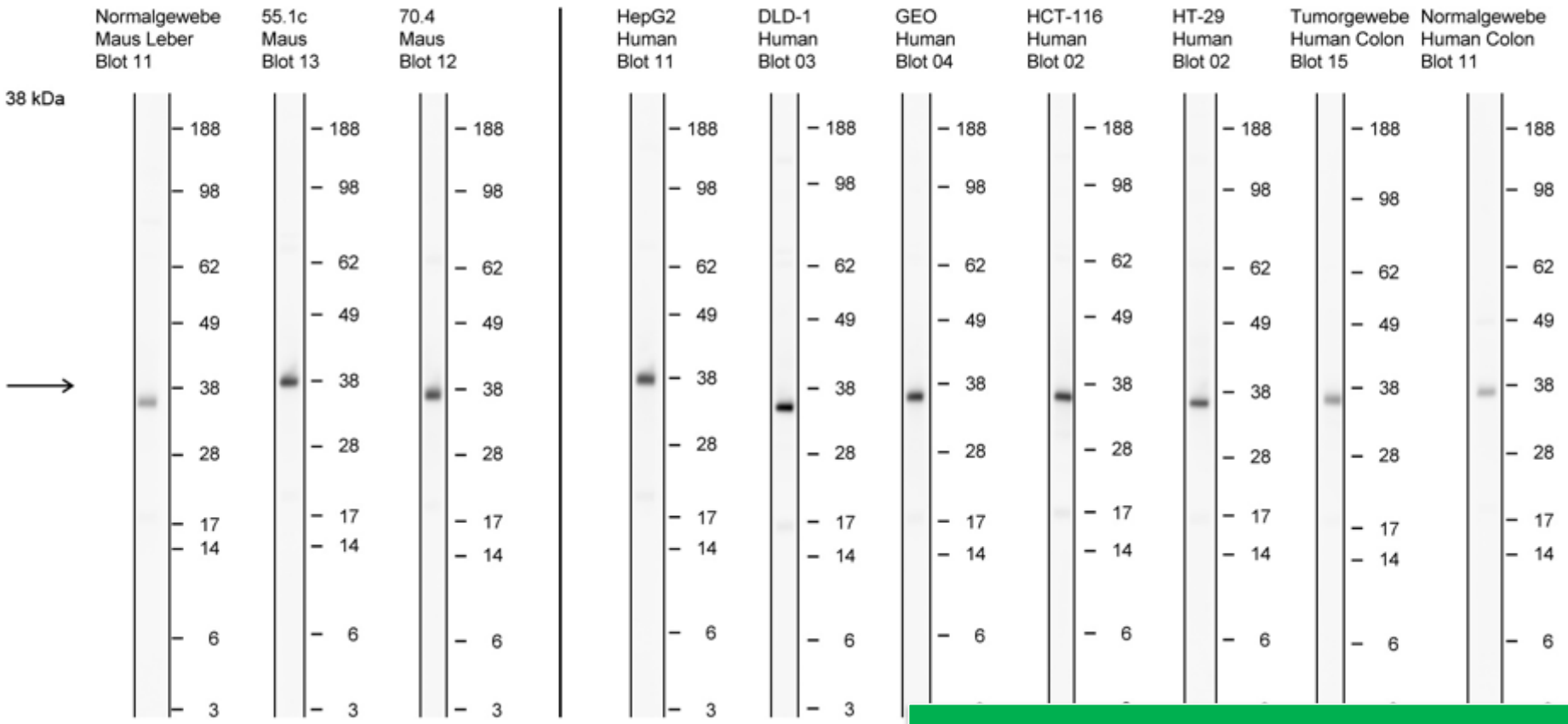
Enzymatic: SIGNAL AMPLIFICATION

- enzyme-conjugated secondary antibody
  → chemoluminescence, fluorescence, dye
- biotinylated secondary antibody
  → enzyme-avidin/streptavidin conjugate
  → dye detection or chemoluminescence

Enhanced detection by enzymatic signal amplification

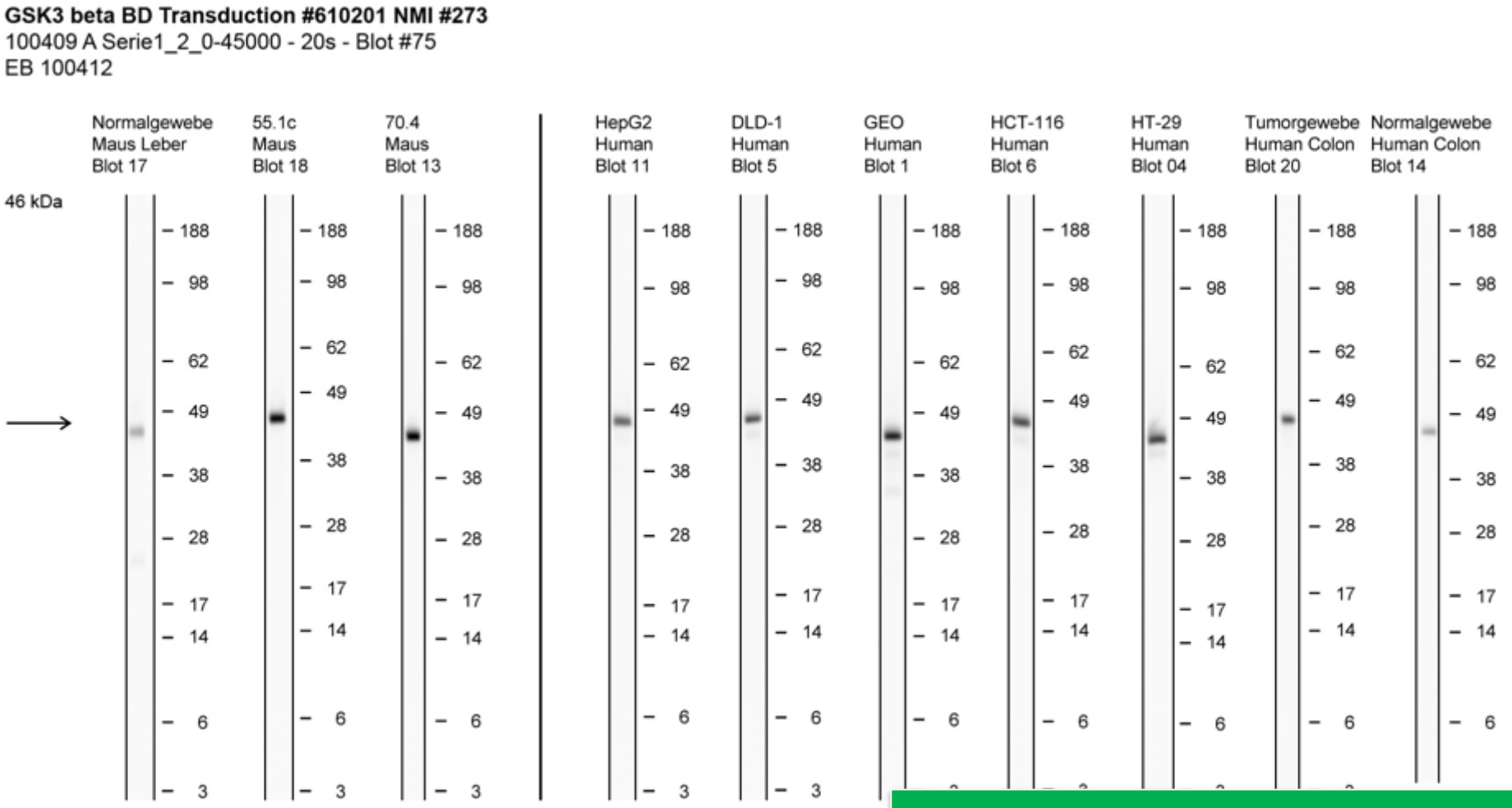
Western Blot – “good and bad“ antibodies

one (strong) signal, with molecular weight, antibody is highly specific



good

Western Blot – “good and bad“ antibodies

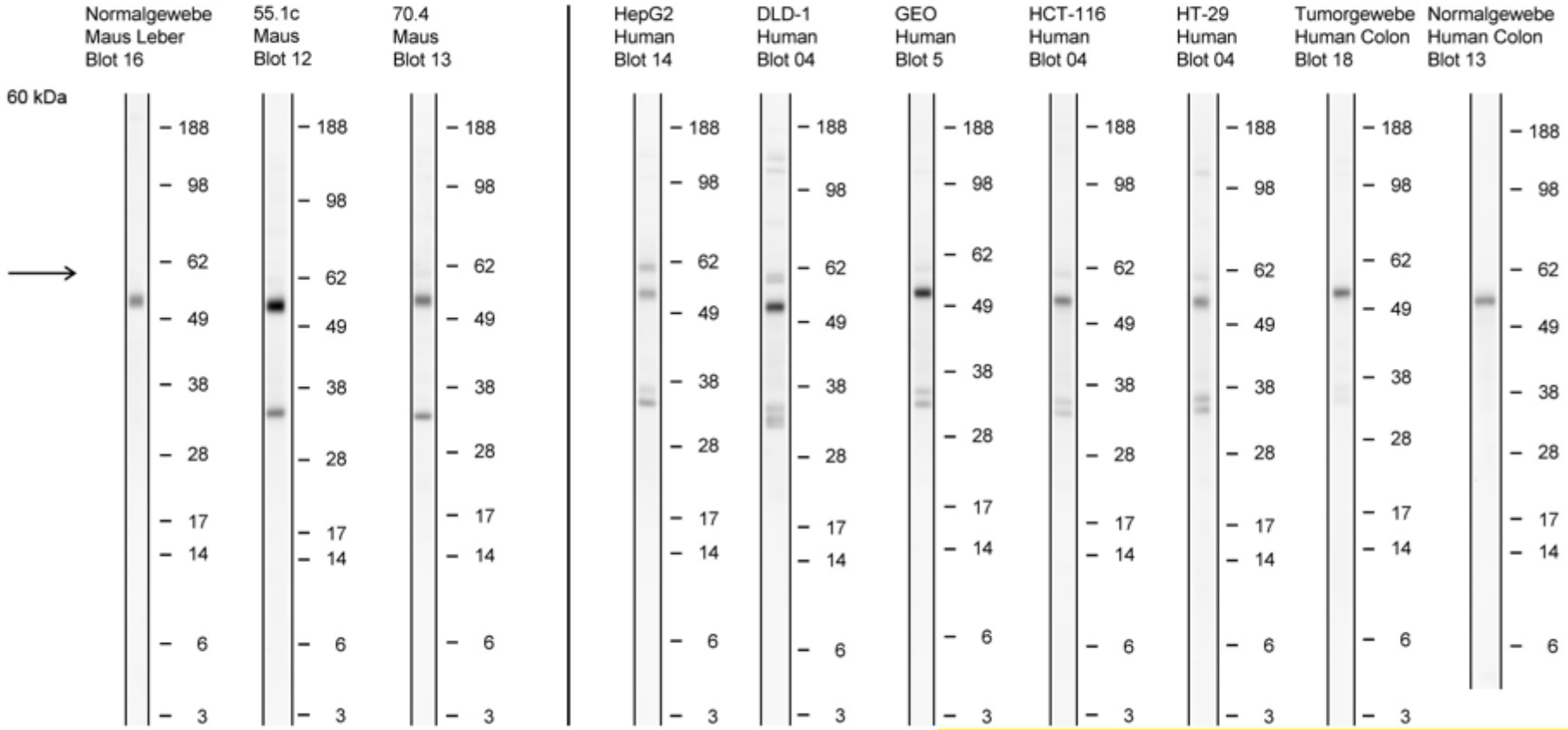


good

Western Blot – “good and bad“ antibodies

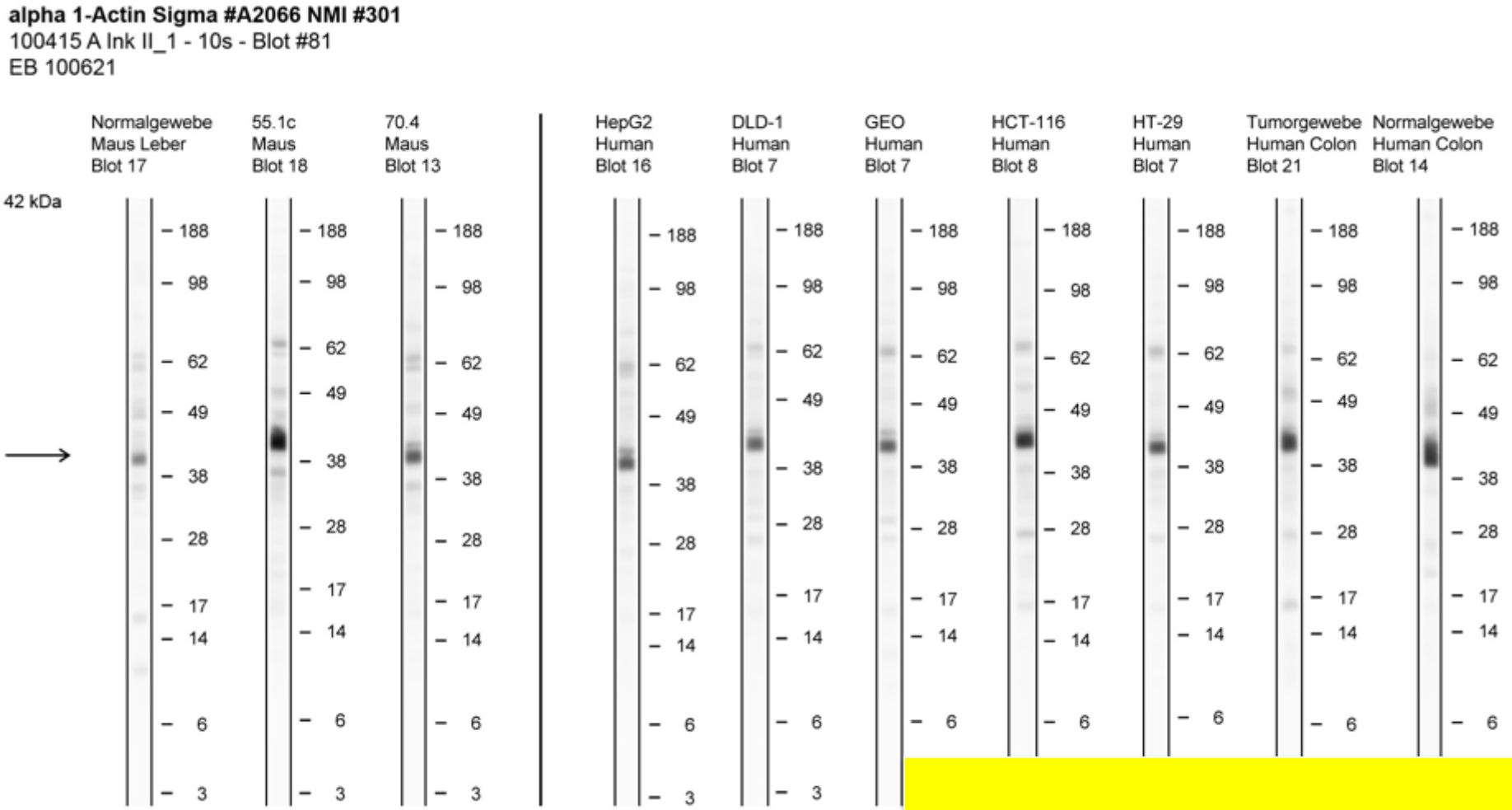
Akt - phospho Ser473 CST #4060 NMI #503
100226 A Serie 2_5 - 225s - Blot #70
EB 100308

around 60 kD still strongest signal but also other signals



medium

Western Blot – “good and bad“ antibodies

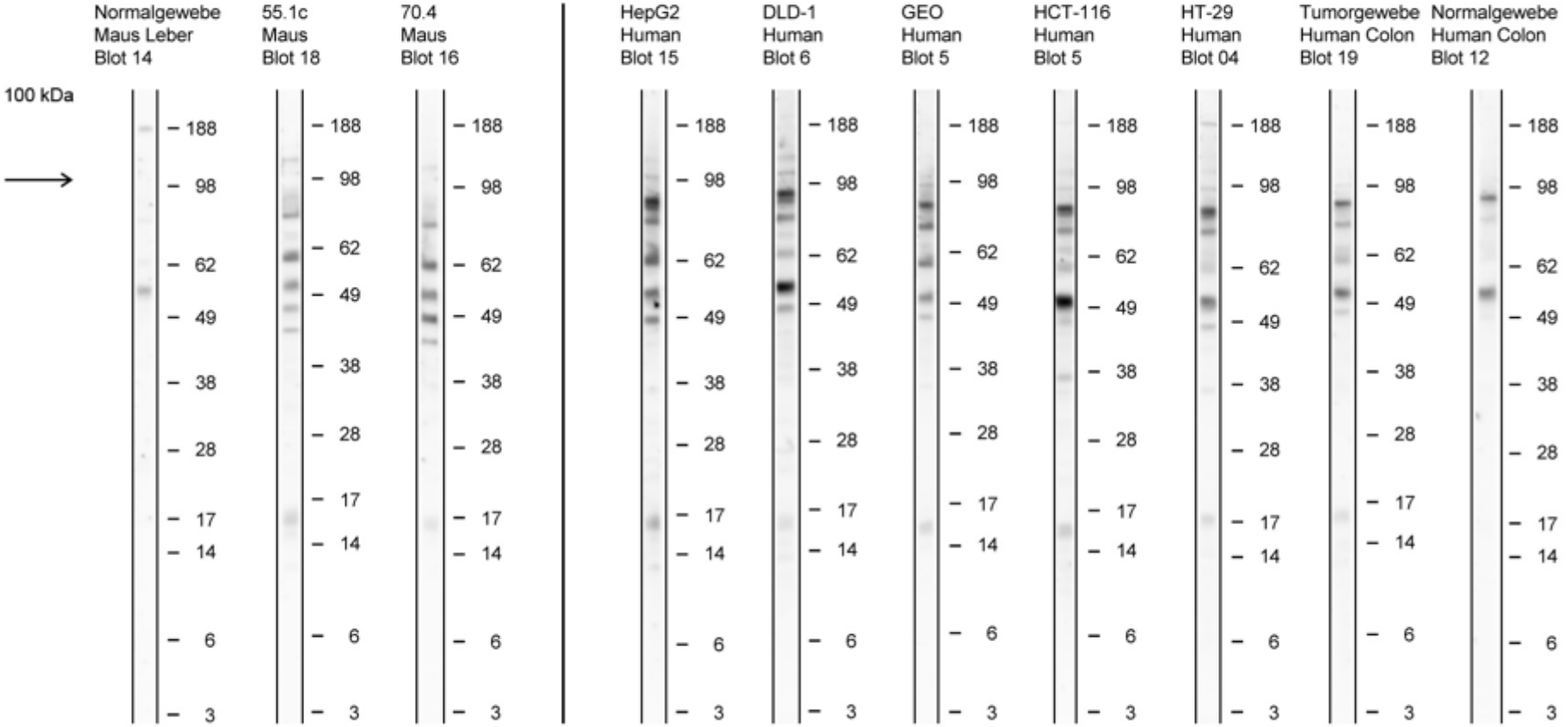


medium

Western Blot – “good and bad“ antibodies

Nucleolin - phospho Thr76/84
Acris #AM06020PU-N NMI #483
100216 A Serie 1_3 - 30s - Blot #68 - EB 100225

second antibody generated with different antigen, validate one antibody with another

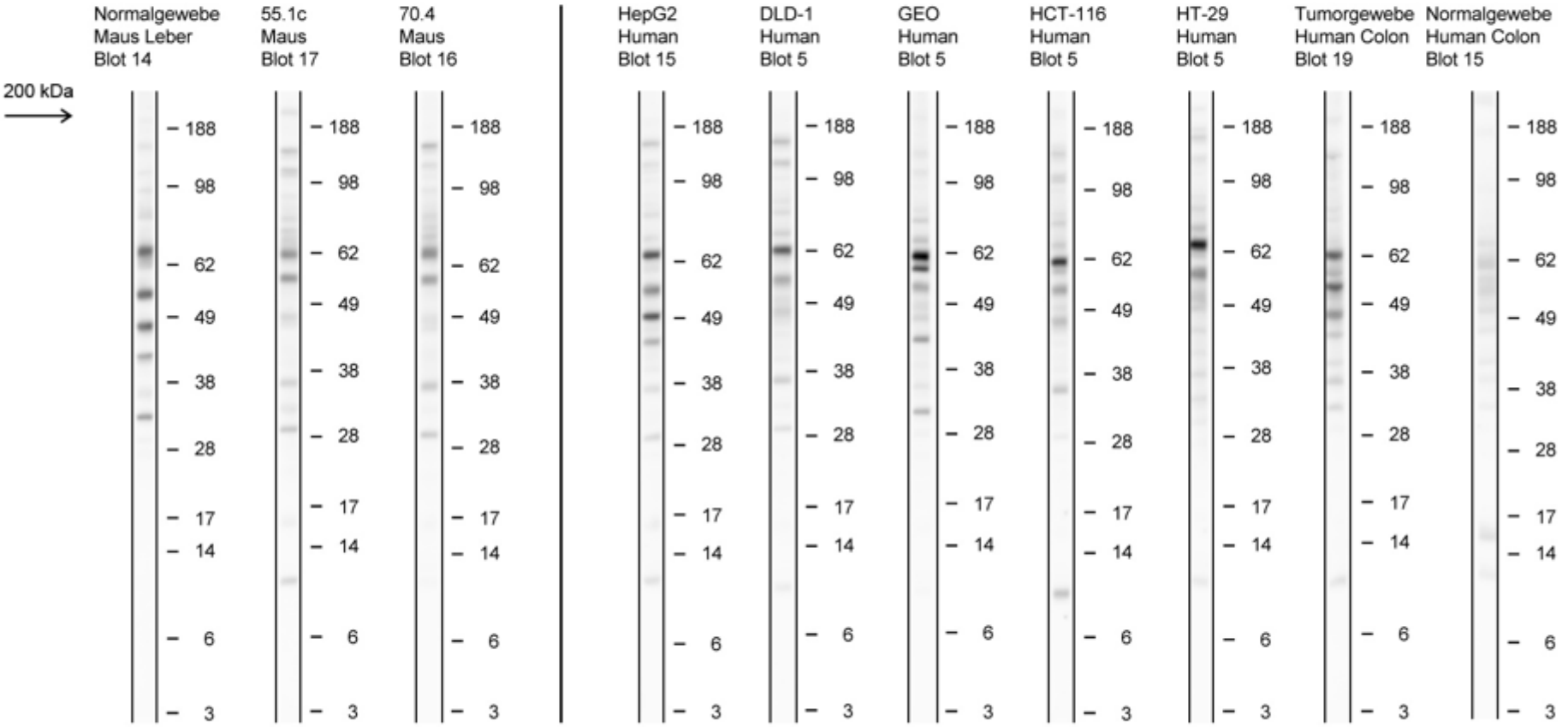


bad

Western Blot – “good and bad“ antibodies

MEKK4 Epitomics #1693-1 NMI #440
100212 C Serie I_2_0-58000 - 20s - Blot #67
EB 100224

maybe transfer time not 100% or play with voltage



bad

- Electrophoresis
- Blotting
- Detection
- Antibody databases

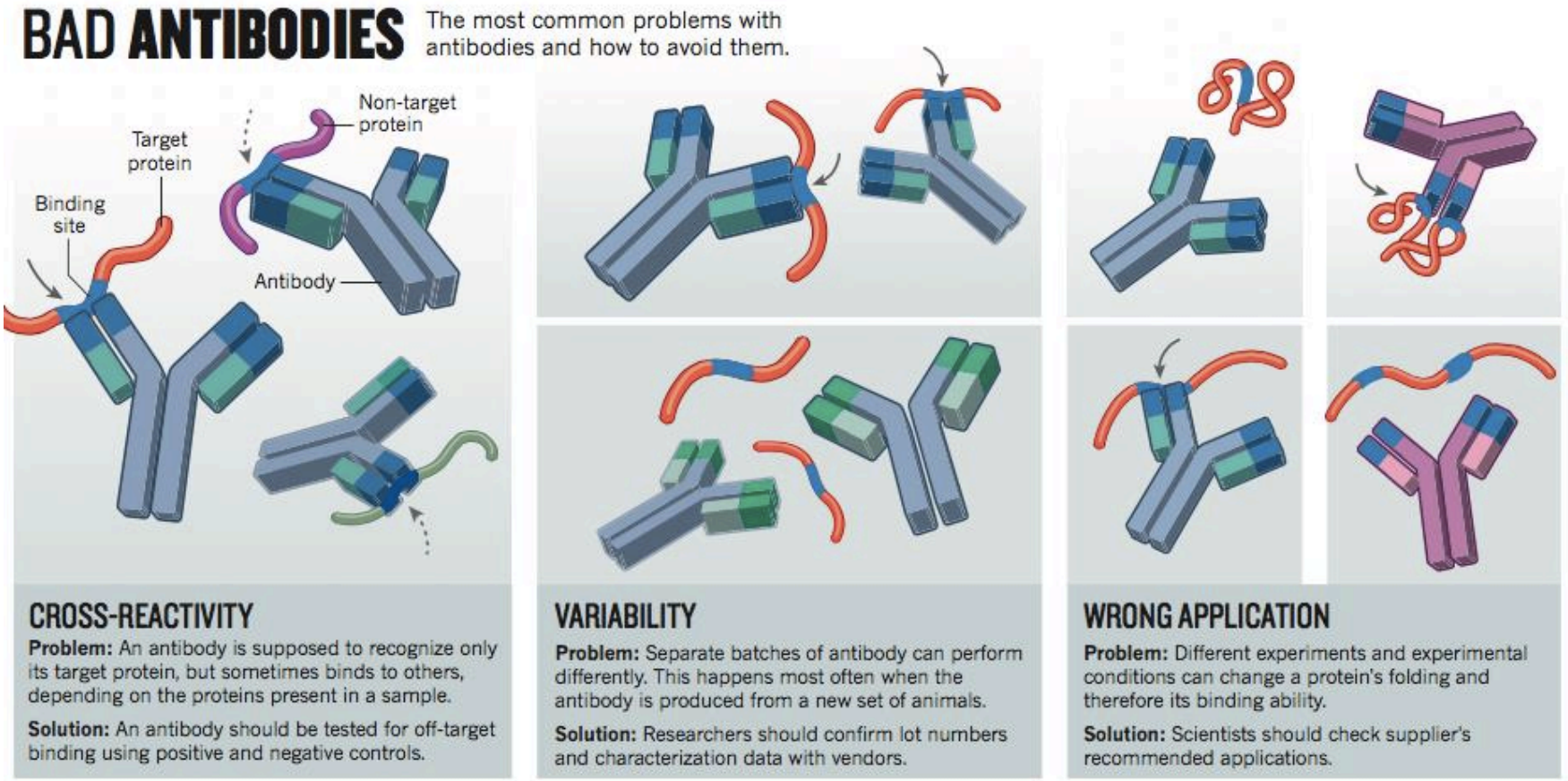
Finding the right antibody for the job

Vivien Marx

As new research applications for antibody-based assays emerge, the quest for quality intensifies in a crowded marketplace.

NATURE METHODS | VOL.10 NO.8 | AUGUST 2013

The good, the bad, and the ugly



Baker (2015) Nature

Where can I find information on suitable antibodies?

Antibodypedia (academic)

http://www.antibodypedia.com

Human Protein Atlas

http://www.proteinatlas.org

Linscott´s directory (commercial)

http://www.linscottsdirectory.com

Biocompare (commercial)

http://www.biocompare.com

distributors and companies...

FAQ aka what can be asked in the exam

1. Describe the basic concept of gel electrophoresis.
2. Describe the basic concept of SDS-PAGE.
3. How does a stacking gel work?
4. How can you stain proteins in an SDS-gel?
5. What do you need for Western blot?

MULTIPLE CHOICE!