Techniques in Molecular Genetics

2010 edition

H.E. Schellhorn

Day 2

- Fire Safety/Chemical Spills
- Discussion sigma factors (brief) and protein expression
- SDS Page (1:30pm-BSB130E)
- Set up and pour gels, inoculate cultures (lab)

Day 1 Lecture 2- Protein Expression

- Protein over-expression and purification are key techniques in Molecular Biology and Biotechnology.
- In this course we will over express two proteins, a transcription factor and Taq polymerase, to demonstrate important considerations in protein purification.

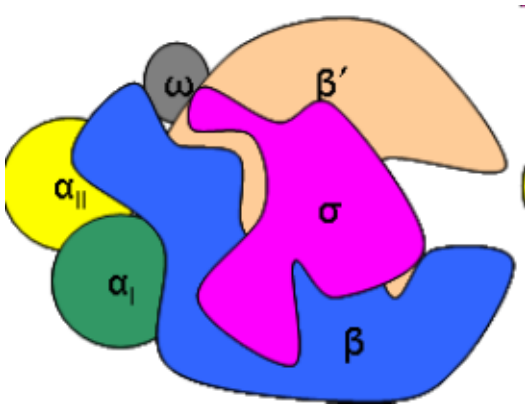
Sigma Factors of *Escherichia coli*

E. coli produces several sigma factors

- RpoD - main sigma factor, transcribes most genes
- RpoN - nitrogen-limitation sigma factor
- RpoS – alternative starvation/stationary phase sigma factor
- RpoH - heat shock sigma factor
- RpoF - flagellar sigma factor
- PpoE - extracytoplasmic/extreme heat stress sigma factor
- FecI - the ferric citrate sigma factor regulating iron transport

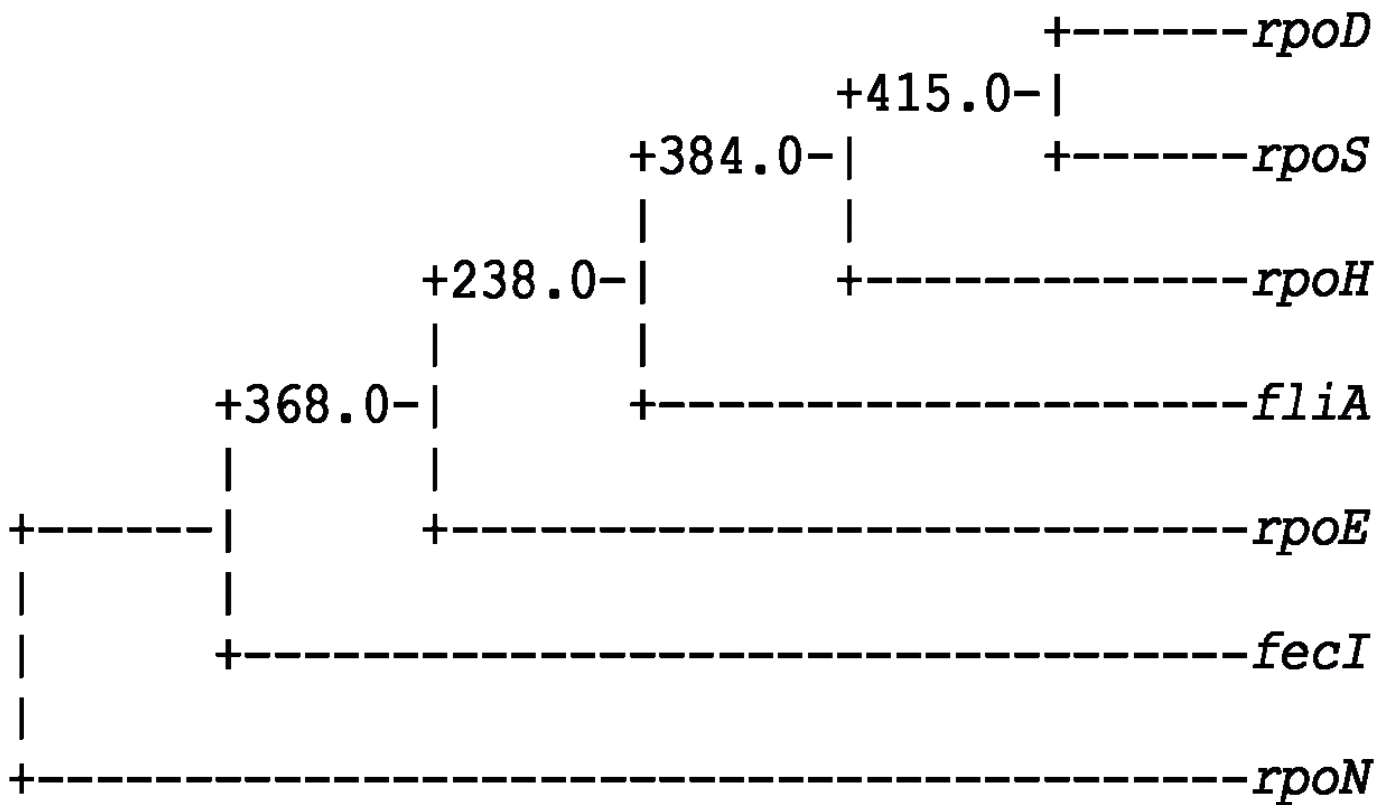
Escherichia coli RNA Polymerase

Parts of the Procaryotic
RNA polymerase



www.steve.gb.com/science/transcription.html

E. coli sigma factors: Phylogenetic relationship



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Sigma Factors of *Escherichia coli*

Expression of sigma factors is not independent

e.g. RpoD controls RpoS, RpoS negatively controls RpoF, RpoN may regulate RpoS etc

In addition, presence of a given sigma factor may affect mount of core polymerase available for other sigma factors→sigma factor competition.

Protein Expression- General Considerations

Will discuss factors to consider in expressing proteins..

Techniques in Molecular Genetics

Polyacrylamide Gel Electrophoresis (PAGE) of Proteins

H.E. Schellhorn

PAGE Electrophoresis-Principle

PAGE can be used to separate proteins and nucleic acids.

Polyacrylamide is a crosslinked polymer of acrylamide and bis-acrylamide

Properties of the gel, especially limiting pore size, are determined by the total concentration of acrylamide-bisacrylamide (%T) and conc. of bis-acrylamide to total acrylamide (%C)

PAGE Electrophoresis-Polymerization

Two additional chemicals play important roles in the polymerization process.

Ammonium Persulfate---produces initiating free radicals when dissolved in water

TEMED- also produces free radicals

PAGE Electrophoresis-Other Chemicals

Sodium Dodecyl Sulfate

ß-mercaptoethanol

PAGE Electrophoresis-Discontinuous

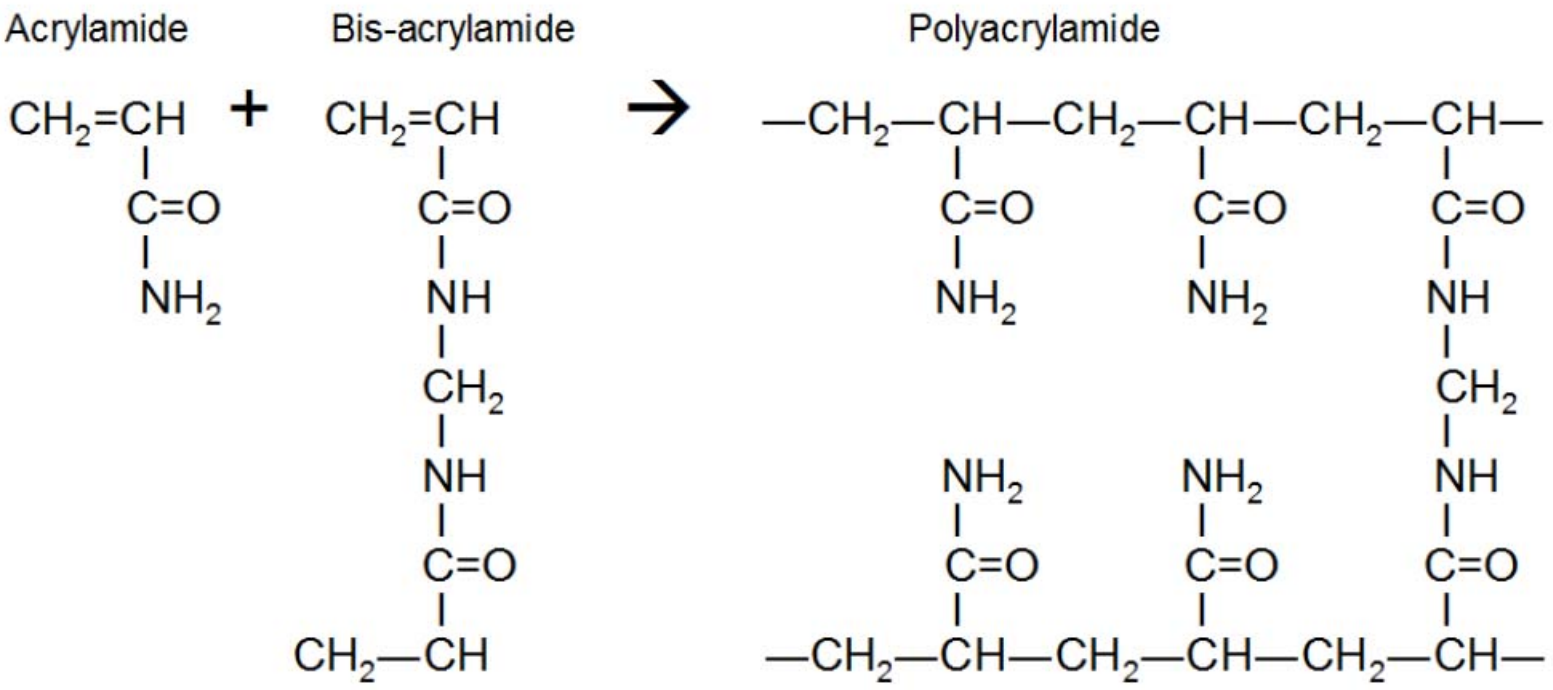
Most denaturing gels are composed of a *stacking* gel and a *separating* gel

Stacking gel- low porosity, 2 pH units below running buffer

-allows proteins to form a compressed band after a few min.

Separating gel- sieves protein according to size

PAGE Electrophoresis-Monomers



PAGE Electrophoresis-Types

- Native (non-denaturing)
- Two dimensional
- Gradient
- Isoelectric focussing

Common Problems in Using PAGE

Problem	Common Cause	Solution
Unequal lane width	Varying salt conc	Wash samples before
Vertical streaking	overload	Reduce sample
No bands	Not enough protein	Increase protein
Skewed bands	various	Use equal volumes, equal salt conc. in samples
Skewed bands at sides	Standard volume different from sample volume	Make standards up in sample buffer and use same volume as test samples

PAGE Electrophoresis Equipment

A-Electrophoresis Cell
B-Glass Plates
C-Combs
D-Casting Stand

Electrophoresis-Principle

Electrophoresis i

Fig. 2. Assembling the Mini-PROTEAN 3 cell.

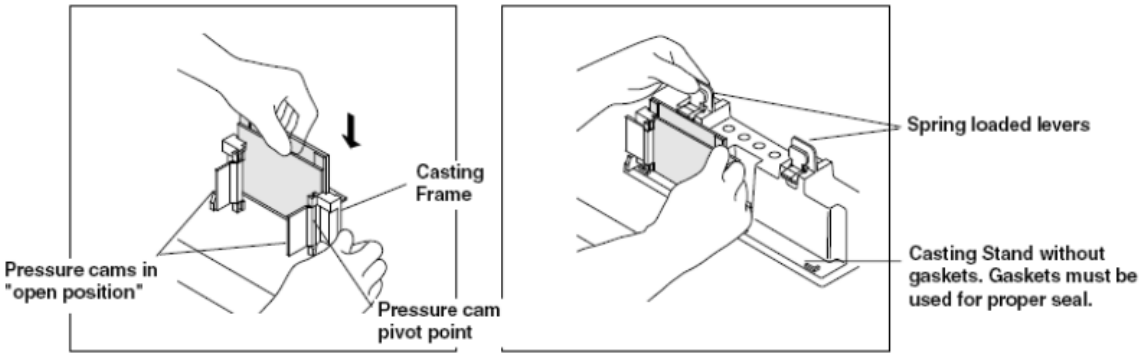
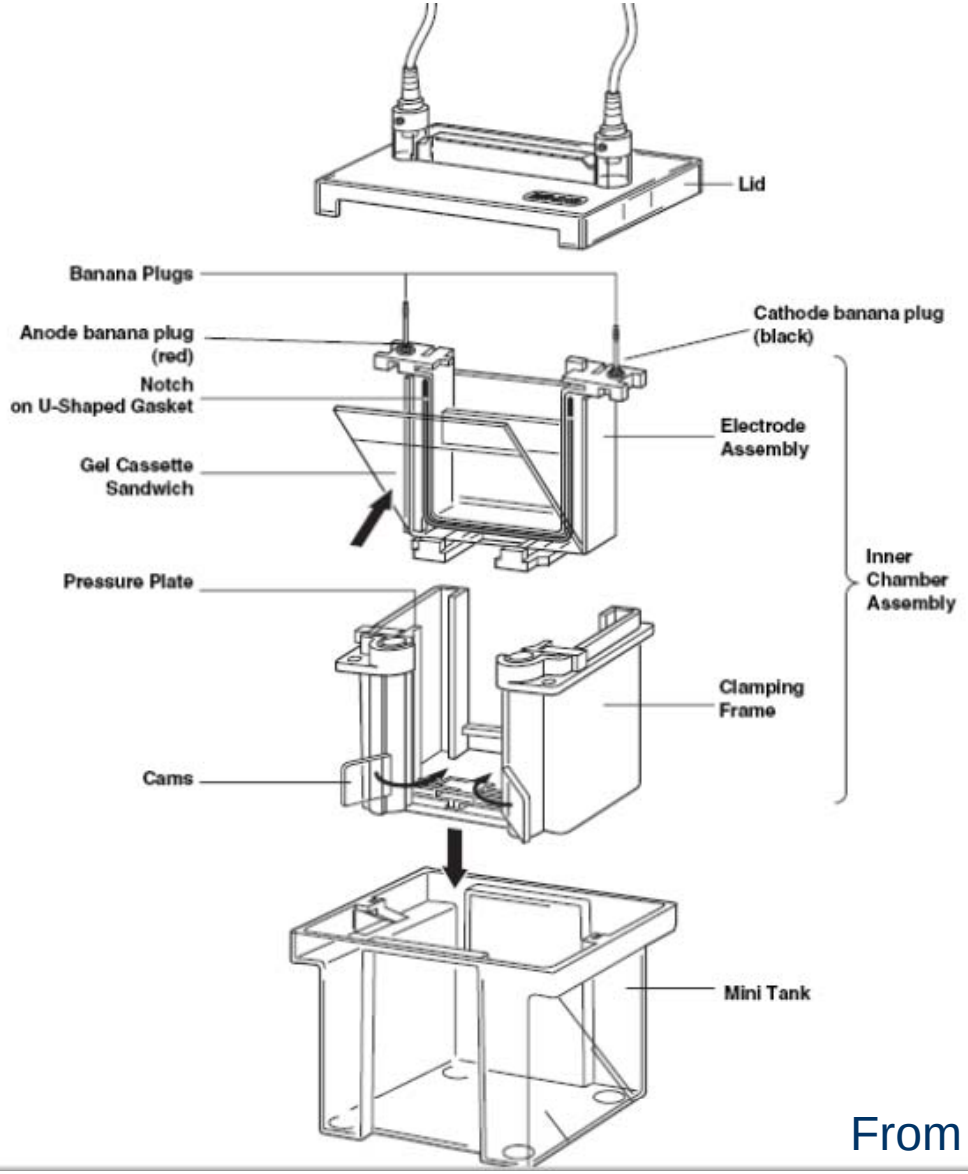


Fig. 3. Assembling the Mini-PROTEAN 3 Casting Frame and Casting Stand.

From Biorad Manual

Using the Biorad PAGE System…



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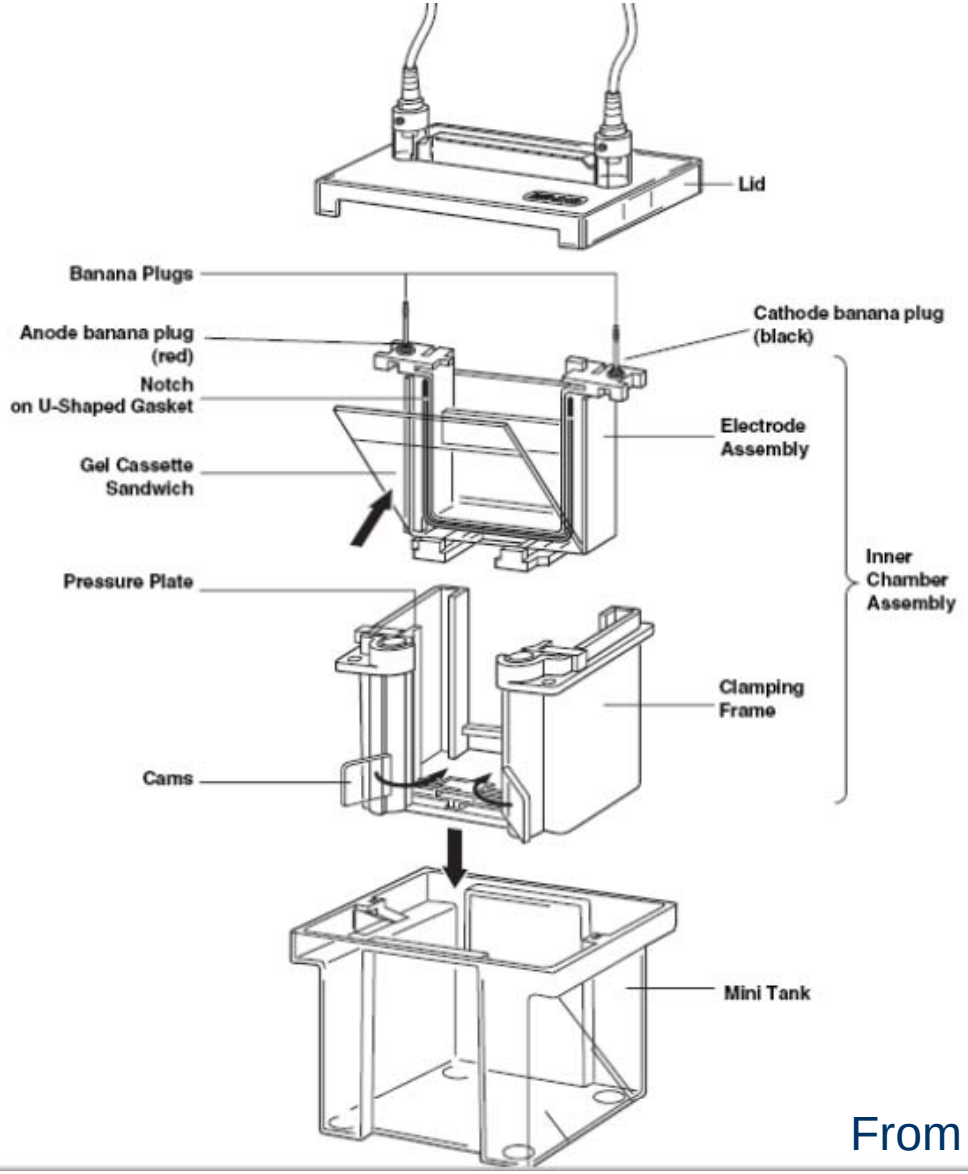

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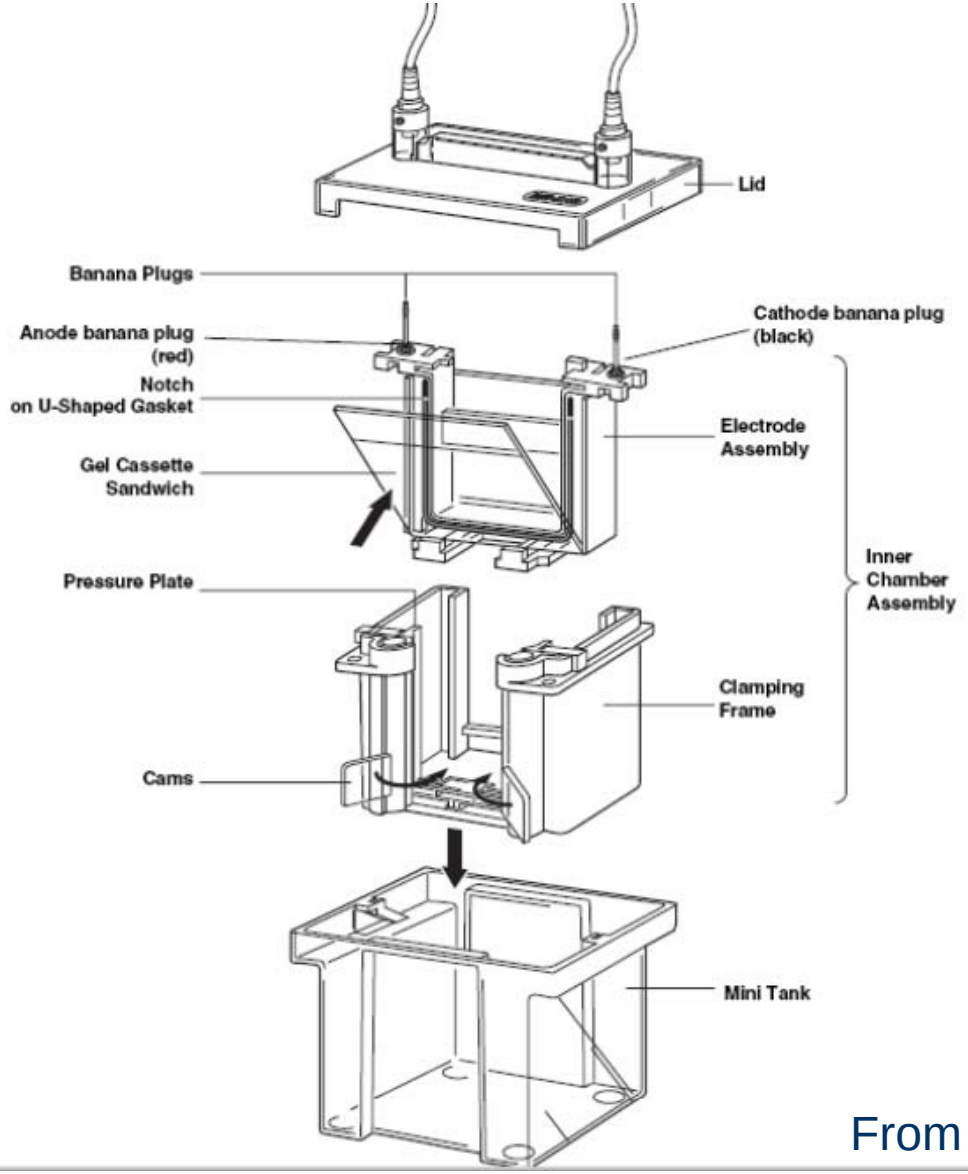

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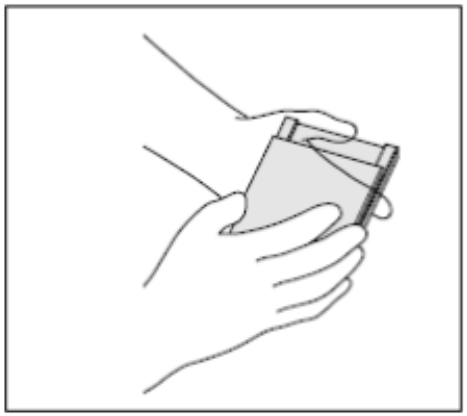
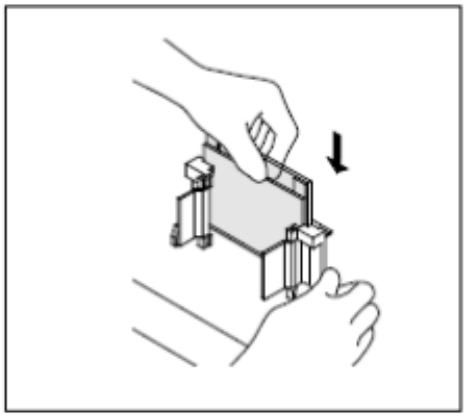
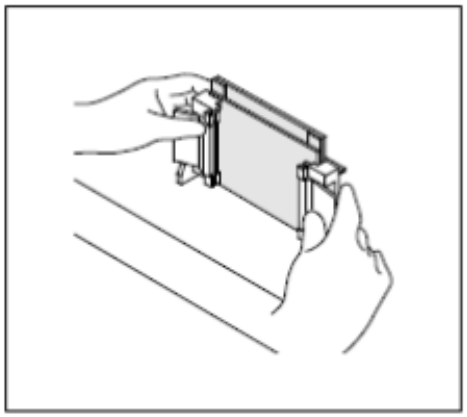


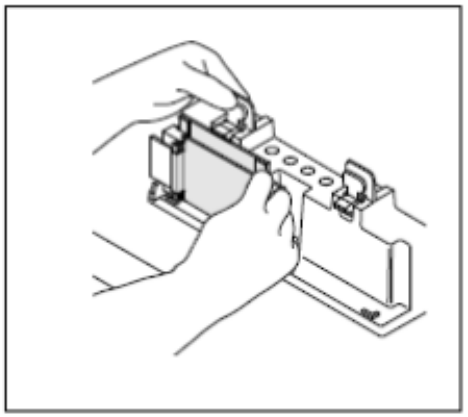
4a. Place a Short Plate on top of the Spacer Plate.



4b. Slide the two plates into the Casting Frame keeping the Short Plate facing front.



4c. Lock the pressure cams to secure the glass plates.

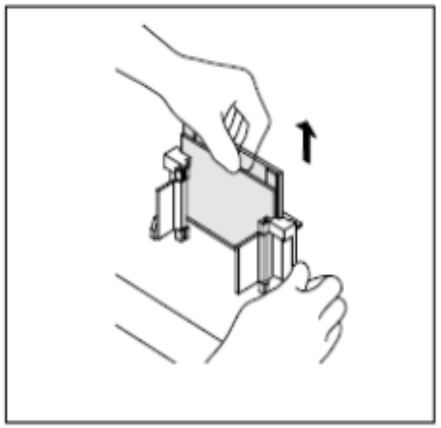


4d. Secure the Casting Frame in the Casting Stand by engaging the spring loaded lever.

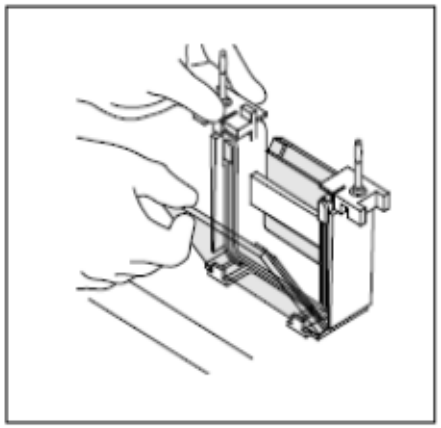
Fig. 4. Assembling the Mini-PROTEAN 3 casting stand and frame.

From Biorad Manual

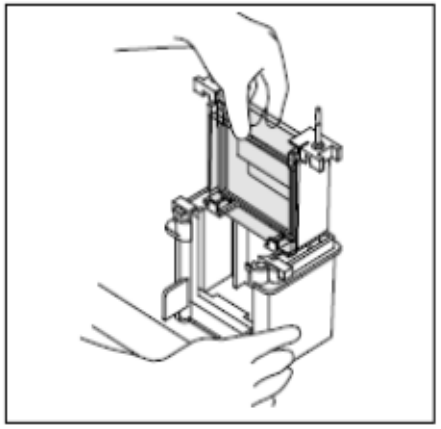
Using the Biorad PAGE System…



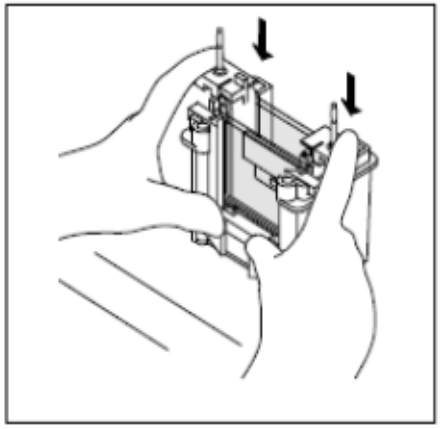
5a. Remove the Gel Cassette Sandwich from the Casting Frame.



5b. Place Gel Cassette Sandwich into the Electrode Assembly with the Short Plate facing inward.



5c. Slide Gel Cassette Sandwiches and Electrode Assembly into the clamping frame.



5d. Press down on the Electrode Assembly while closing the two cam levers of the Clamping Frame.

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Using the Biorad PAGE System…

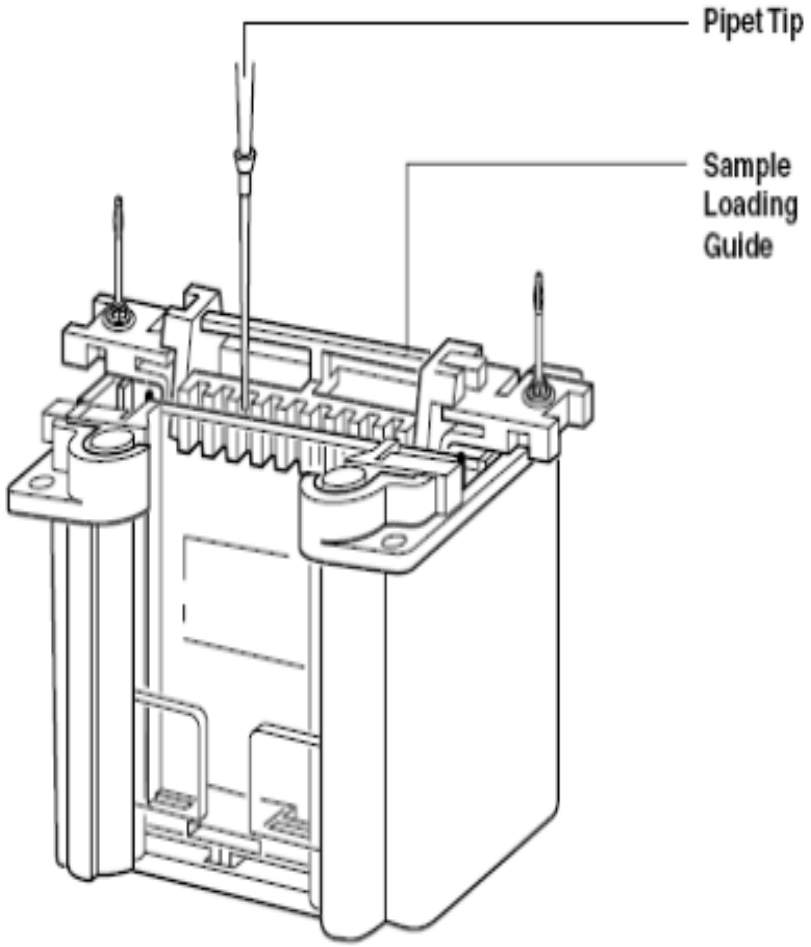
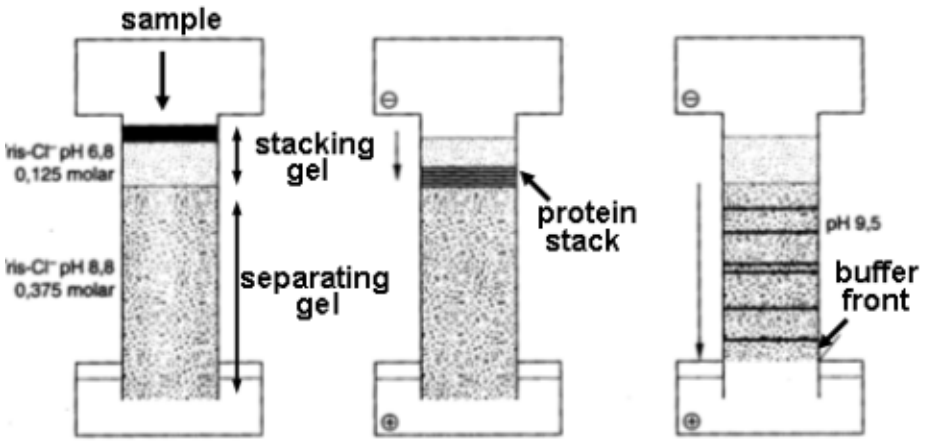


Fig. 6. Using the Sample Loading Guide (patent #5,656,145).

From Biorad Manual

SDS-PAGE:
Separation process



SDS (Na-dodecylsulfate) -> charge

stacking of proteins between Cl^- and Gly

Solutions for 2 Laemmli gels

		7 %	10 %	12.5 %
Separating gel	4x Lower Tris	3.7 ml	3.7 ml	3.7 ml
	40% Acrylamide Solution	2.6 ml	3.8 ml	4.7 ml
	H_2O	8.7 ml	7.5 ml	6.6 ml
	APS	75 µl	75 µl	75 µl
	TEMED	7.5 µl	7.5 µl	7.5 µl
Stacking gel	4x Upper Tris	1.25 ml	1.25 ml	1.25 ml
	40% Acrylamide Solution	0.5 ml	0.5 ml	0.5 ml
	H_2O	3.25 ml	3.25 ml	3.25 ml
	APS	75 µl	75 µl	75 µl
	TEMED	7.5 µl	7.5 µl	7.5 µl

Solutions for SDS-PAGE:

Ammoniumperoxodisulfate (= APS): 10%

4x Lower Tris (buffer for separating gel): 1.5 M Tris/Cl pH 8.8 + 0.4% SDS

4x Upper Tris (buffer for upper gel): 0.5 M Tris/Cl pH 6.8 + 0.4% SDS

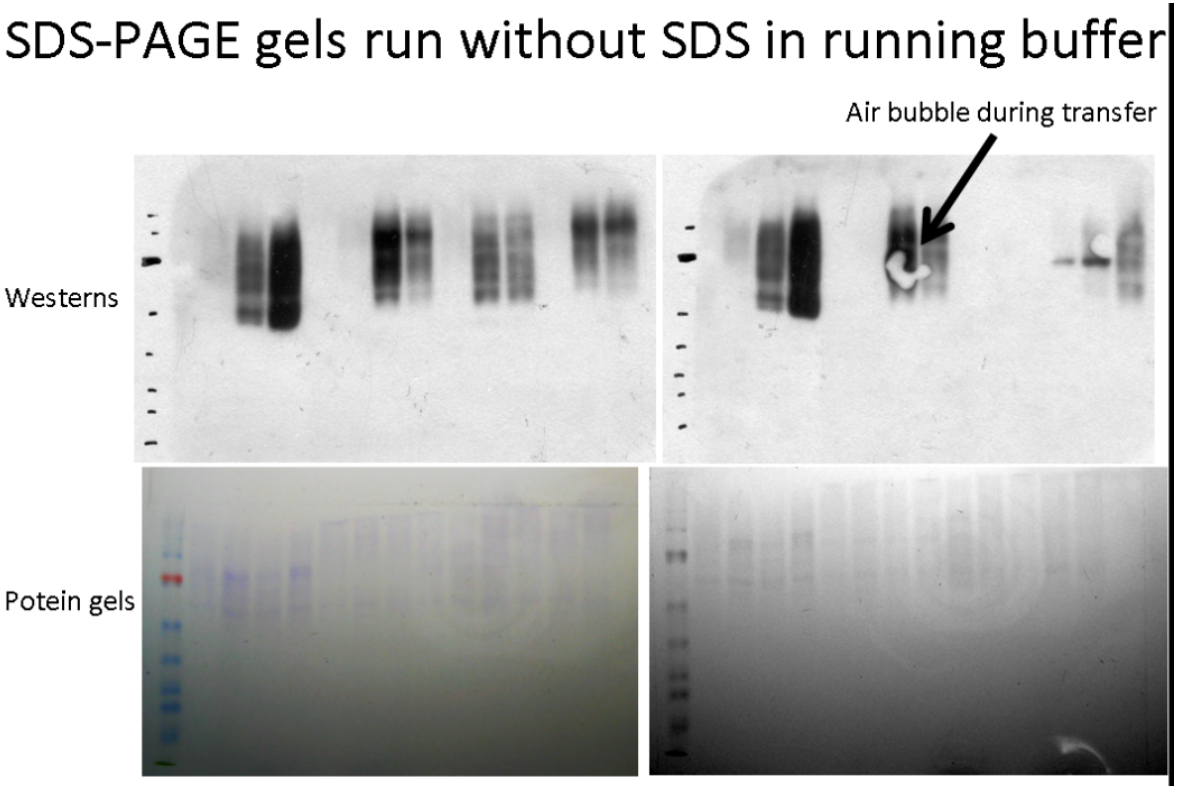
10x Laemmli running buffer (1 l):

Tris	30.25 g
Glycine	144 g
SDS	10 g

2x concentrated sample buffer (=SB) (40 ml):

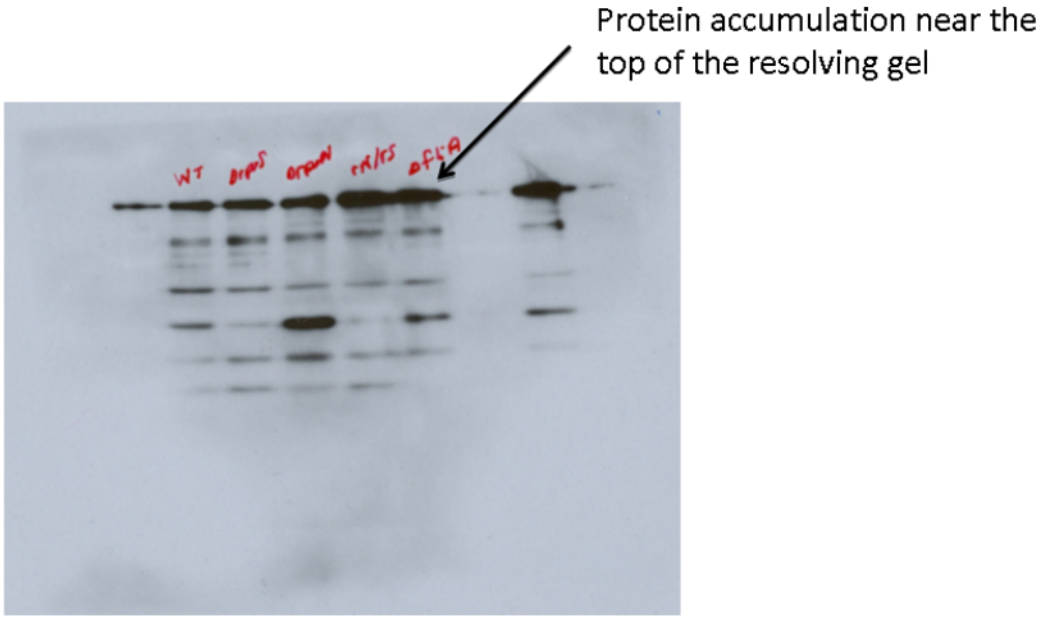
1 M Tris/Cl pH 6.8	5 ml
β-Mercaptoethanol	4 ml
SDS	1.84 g
Glycerol	8 ml (heat in Microwave)
Bromphenol blue	(a few grains until colored)
H_2O	23.2 ml

Bad SDS-PAGE gel examples



Bad SDS-PAGE gel examples

SDS-PAGE GEL: WESTERN

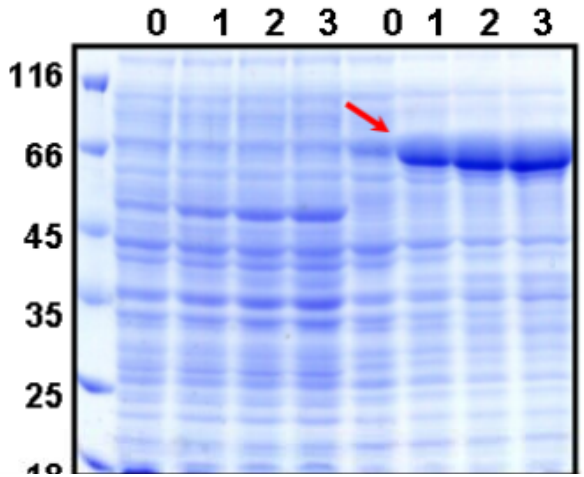


SDS-PAGE: Staining and de-staining

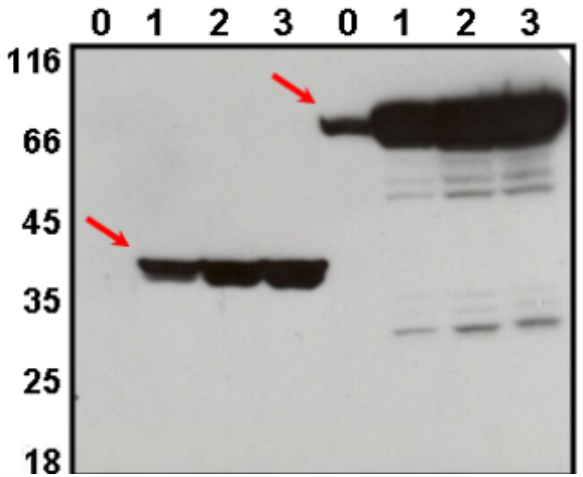
Staining solution: 30% MeOH, 10% Acetic Acid, 0.1% Coomassie Blue R250

Destaining solution: 20% MeOH, 8% Acetic Acid

Coomassie stain



Western blot with StrepII-specific antiserum

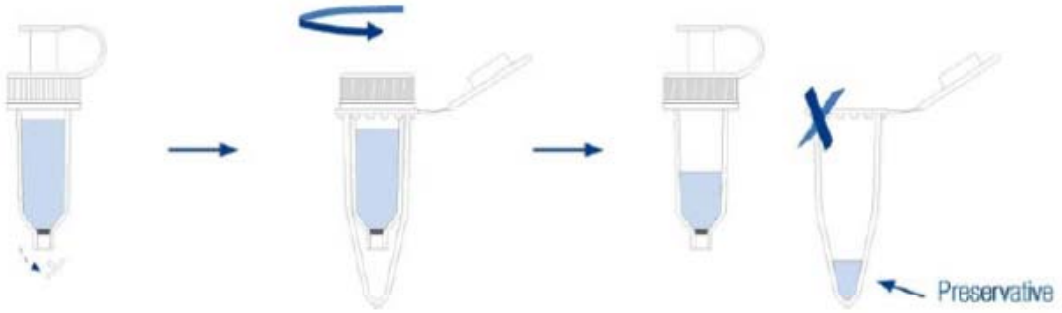


His-Tag purification-Steps

1. Elimination of the Preservative

Remove the lower cap of the Spin-Column, place in a microtube and centrifuge, then discard the preservative residue collected in the tube.

Note: In all centrifugation processes carried out in the procedure, normally a mild centrifugation (1,000 – 1,500 rpm) is sufficient.

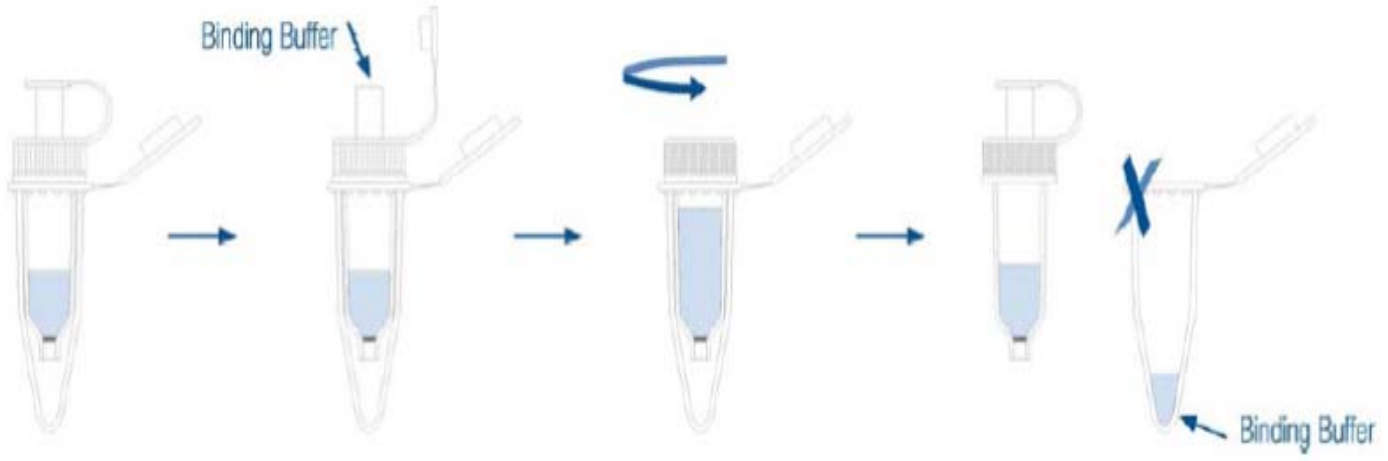


His-Tag purification-Steps

2. Equilibration of the Spin-Column

Introduce the Spin-Column in a microcentrifuge tube and add binding buffer through the top. Centrifuge and discard the residue obtained.

Note: As binding buffer, generally 20 mM disodium phosphate, 500 mM NaCl, 10 mM imidazole pH 7.5 is used.



ABT Manual

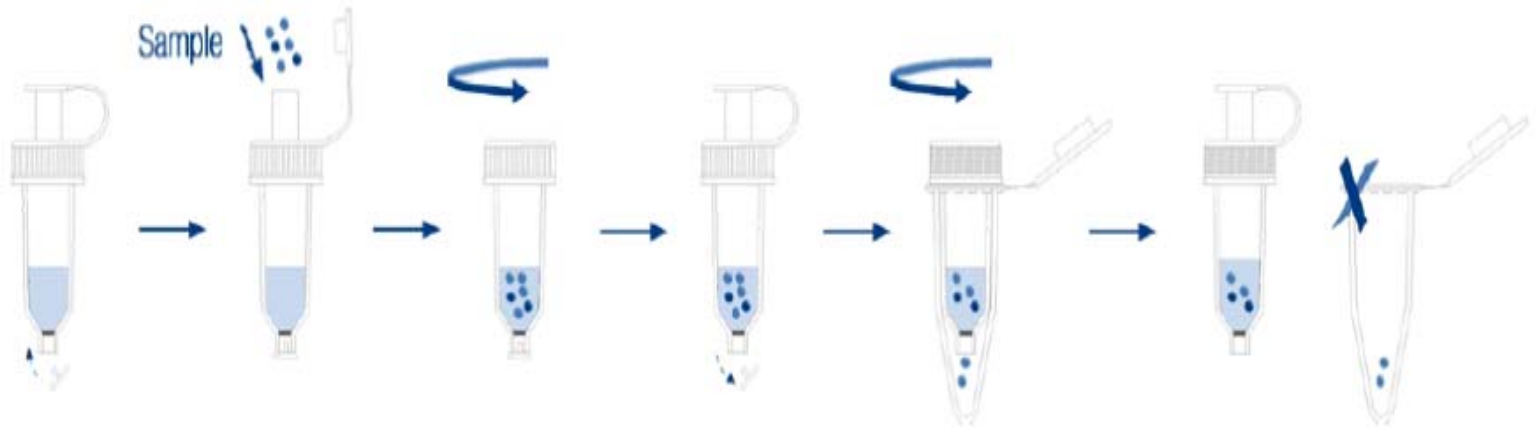
His-Tag purification-Steps

3. Application of the Sample

Add the sample containing the histidine-tagged protein keeping the lower cap in its place.

Manually shake the Spin-Column to maximize contact between the resin and the target-protein.

Remove lower cap, introduce the Spin-Column in a microcentrifuge tube and centrifuge (thus eliminating the proteins not retained in the column).

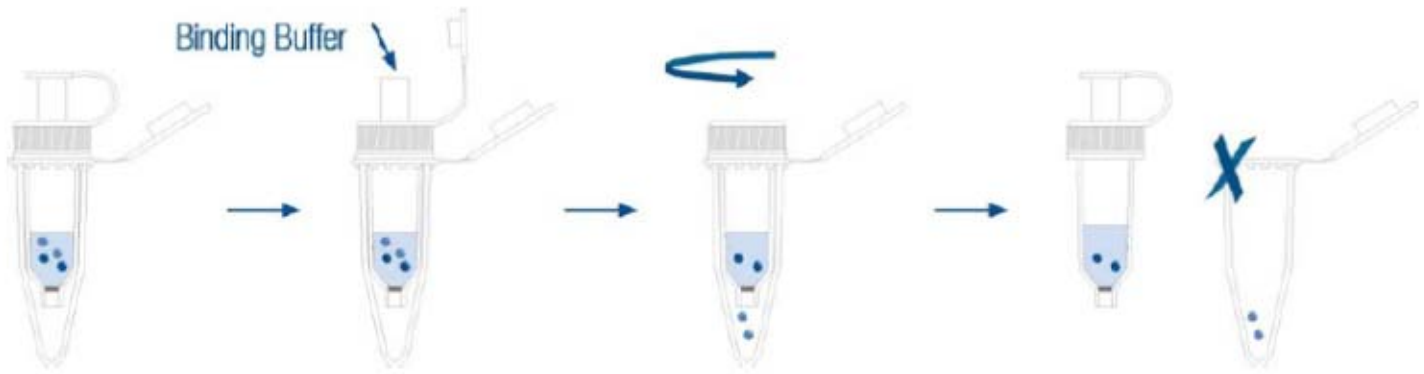


ABT Manual

His-Tag purification-Steps

4. Washing of the Spin-Column

Introduce the Spin-Column in a microcentrifuge tube and add the binding buffer through the top. Centrifuge and discard the residue gathered in the tube.



ABT Manual

His-Tag purification-Steps

5. Elution of the pure protein

Add the elution buffer with the lower cap of the Spin-Column in place. Manually shake to drive the elution of the target-protein. Remove the lower cap, introduce the Spin-Column in a microcentrifuge tube and centrifuge, finally collecting the pure protein in the tube.

Note: As elution buffer, 20 mM disodium phosphate, 500 mM NaCl, 500 mM imidazole pH 7.5 is generally used. This concentration of imidazole is usually enough to provoke the elution of the target-protein. However if the desired result is not achieved then the concentration may be increased up to 2.0 M.

Note: Other reagents that may be used to elute the protein are histidines and ammonium chloride.

ABT Manual