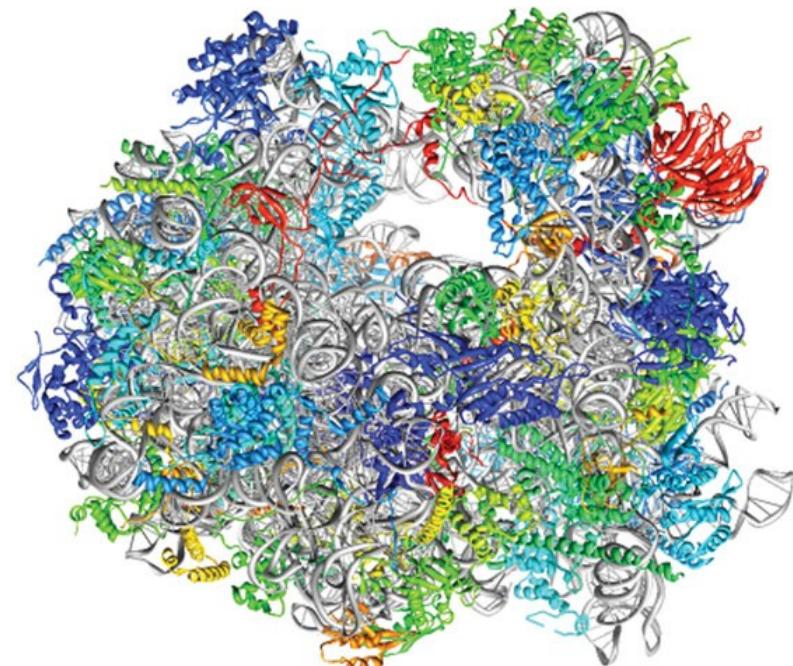


Purification of ribosomes for X-ray



KAZAN
2016



1974 Nobel Prize in Physiology or Medicine

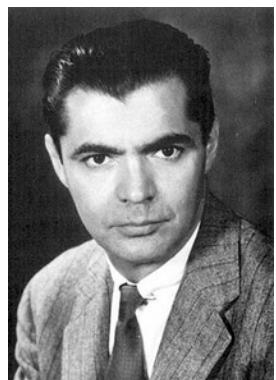
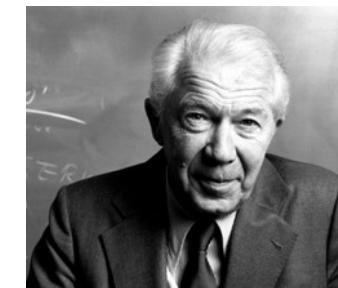
"for their discoveries concerning the structural and functional organization of the cell"



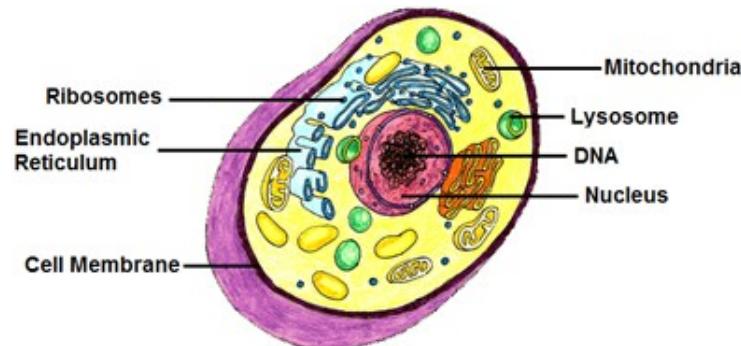
Albert Claude (24.08.1899 – 22.05.1983)

Belgian medical doctor and cell biologist.
Work with cell fractionation
in the 1930s and 1940s

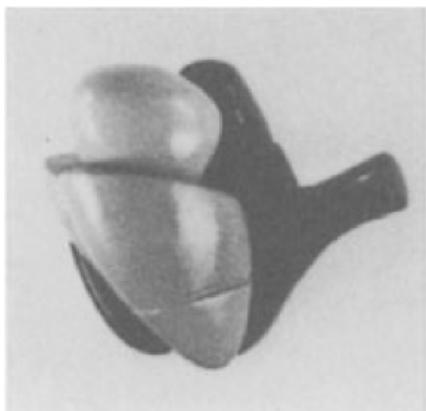
Christian René, viscount de Duve (02.10.1917 – 04.05.2013).
Belgian cytologist and biochemist.
Discovered of two eukaryotic
organelles, peroxisome and lysosome



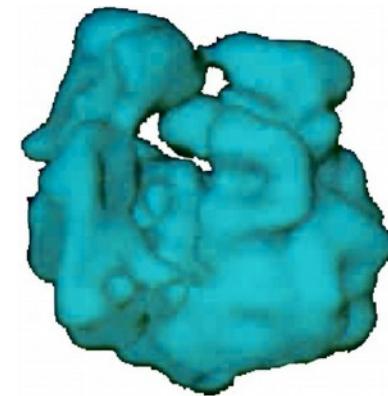
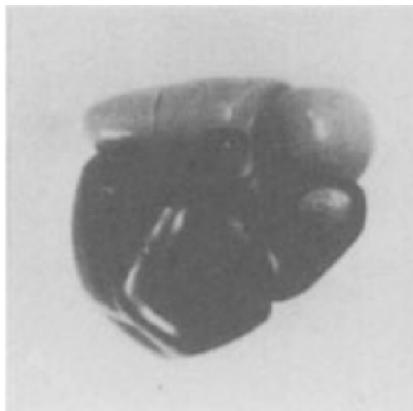
George Emil Palade
(19.11.1912 – 08.10.2008). Romanian-American cell biologist.
Discovered of the ribosomes of the endoplasmatic reticulum in 1955.



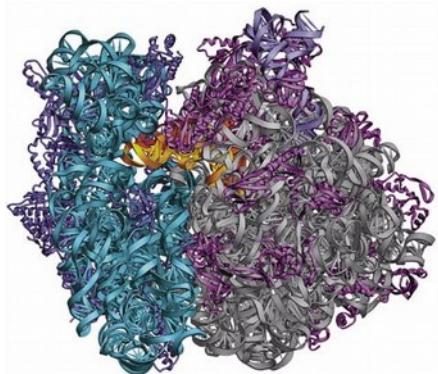
Structural investigations of the ribosome



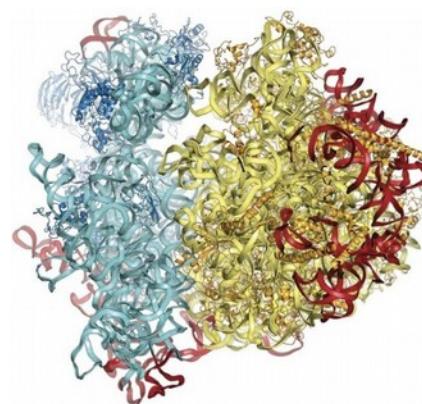
E. coli. Electron microscopy [Vasiliev V, 1983]



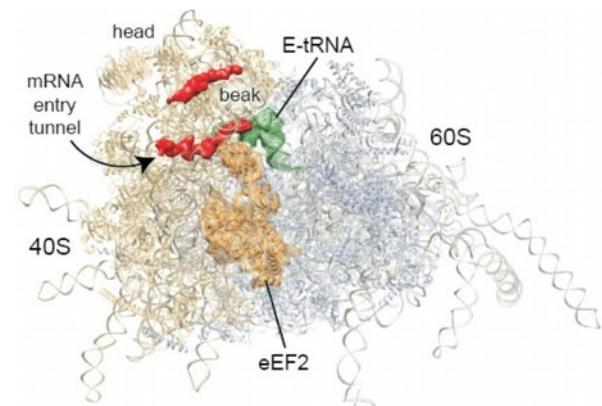
E. Coli. Electron microscopy
[Frank J et al., Science, 1996]



T. thermophilus. X-ray
[Yusupov M., et al., Science, 2001]



S. cerevisiae. X-ray
[Ben-Shem A., et al., Science, 2011]



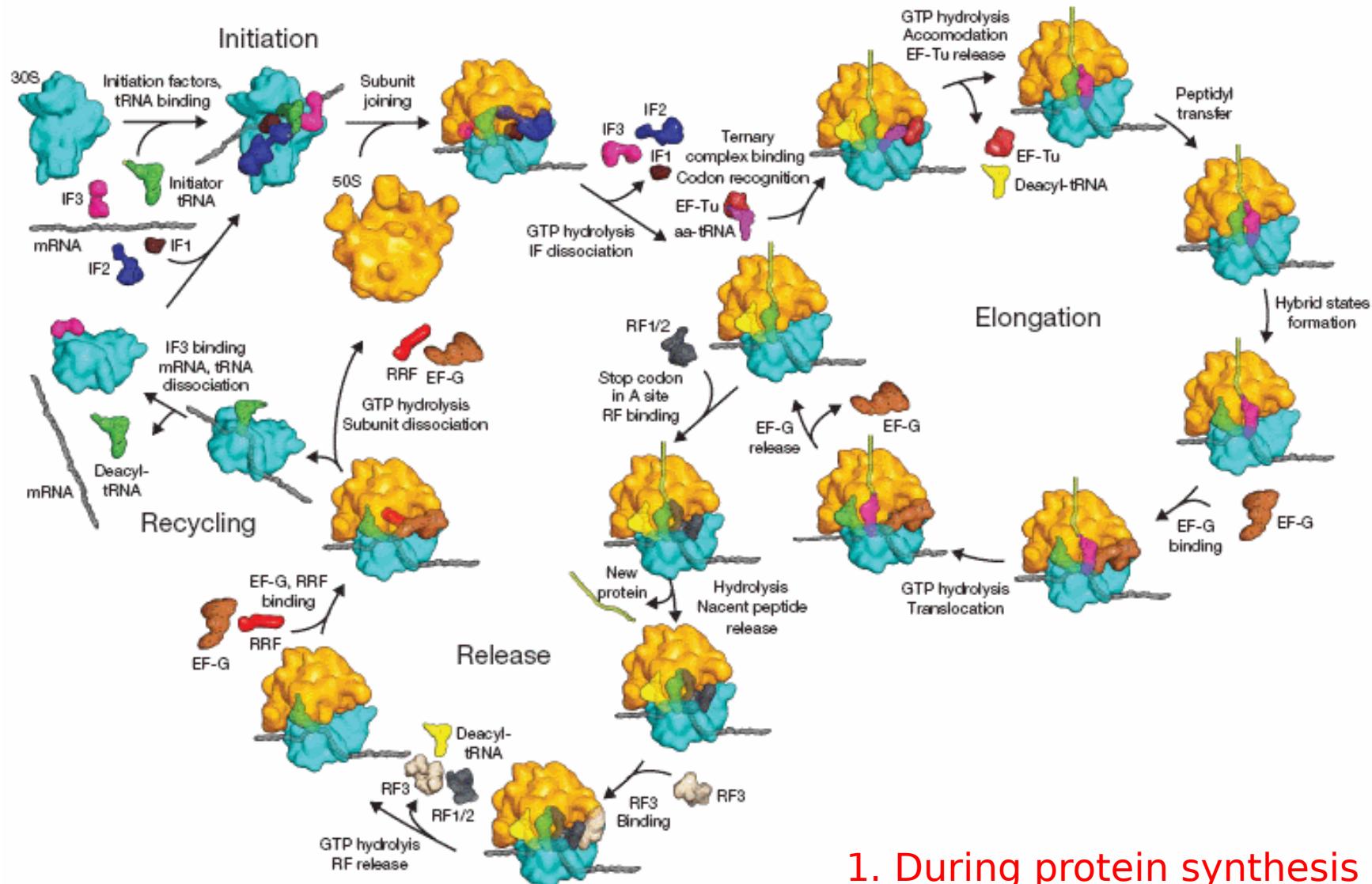
H. sapiens. Electron microscopy
[Anger A., et al., Nature, 2013]

Complexity of the ribosomes within different domains of life

Bacteria (<i>T. thermophilus</i> or <i>E. coli</i>)	The common core	Lower eukaryotes (<i>S. cerevisiae</i>)	Higher eukaryotes (<i>H. sapiens</i>)
 L1-stalk 	 P-stalk 	 	
2.3 MDa	2.0 MDa	3.3 MDa	4.3 MDa
54 proteins 3 rRNA	34 proteins 3 rRNA	79 proteins 4 rRNA	80 proteins: 4 rRNA
Large subunit (50S): 33 proteins 23S rRNA—2,904 bases 5S rRNA—121 bases	Large subunit: 19 proteins 23S rRNA—2,843 bases 5S rRNA—121 bases	Large subunit (60S): 46 proteins 5.8S rRNA—158 bases 25S rRNA—3,396 bases 5S rRNA—121 bases	Large subunit (60S): 47 proteins 5.8S rRNA—156 bases 28S rRNA—5,034 bases 5S rRNA—121 bases
Small subunit (30S): 21 proteins 16S rRNA—1,542 bases	Small subunit: 15 proteins 16S rRNA—1,458 bases	Small subunit (40S): 33 proteins 18S rRNA—1,800 bases	Small subunit (40S): 33 proteins 18S rRNA—1,870 bases

What should we think about when purifying the ribosomes for structural studies?

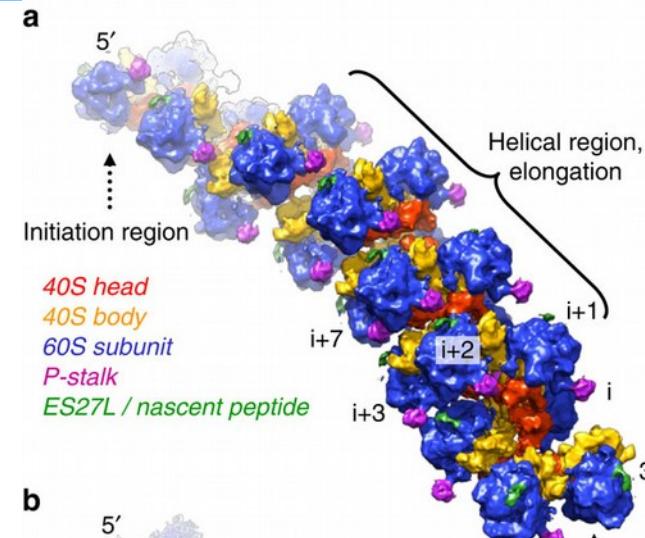
What should we think about when purifying the ribosomes?



1. During protein synthesis
the ribosome is present in
different states

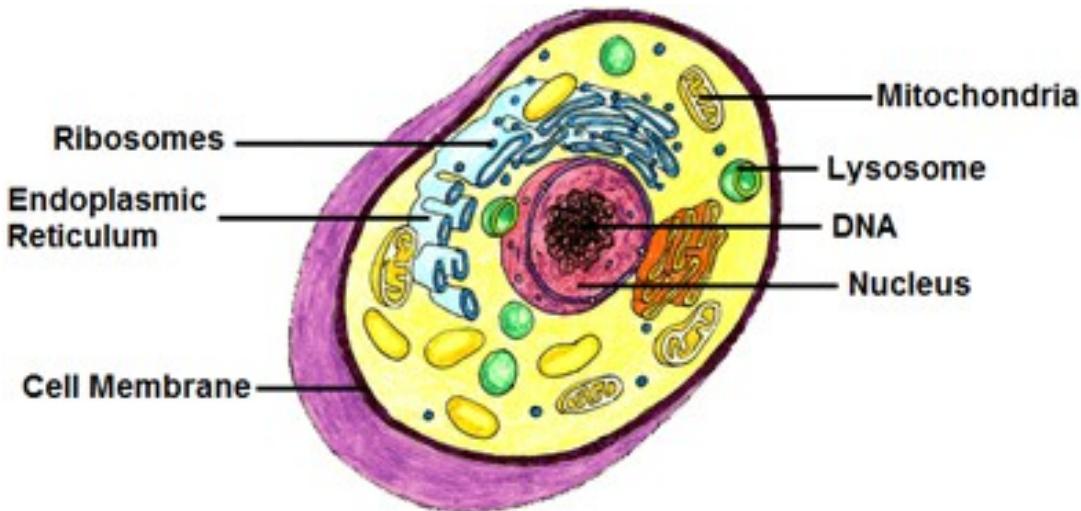
What should we think about when purifying the ribosomes?

2. Actively translating ribosomes assemble in the huge ansambles called polysomes

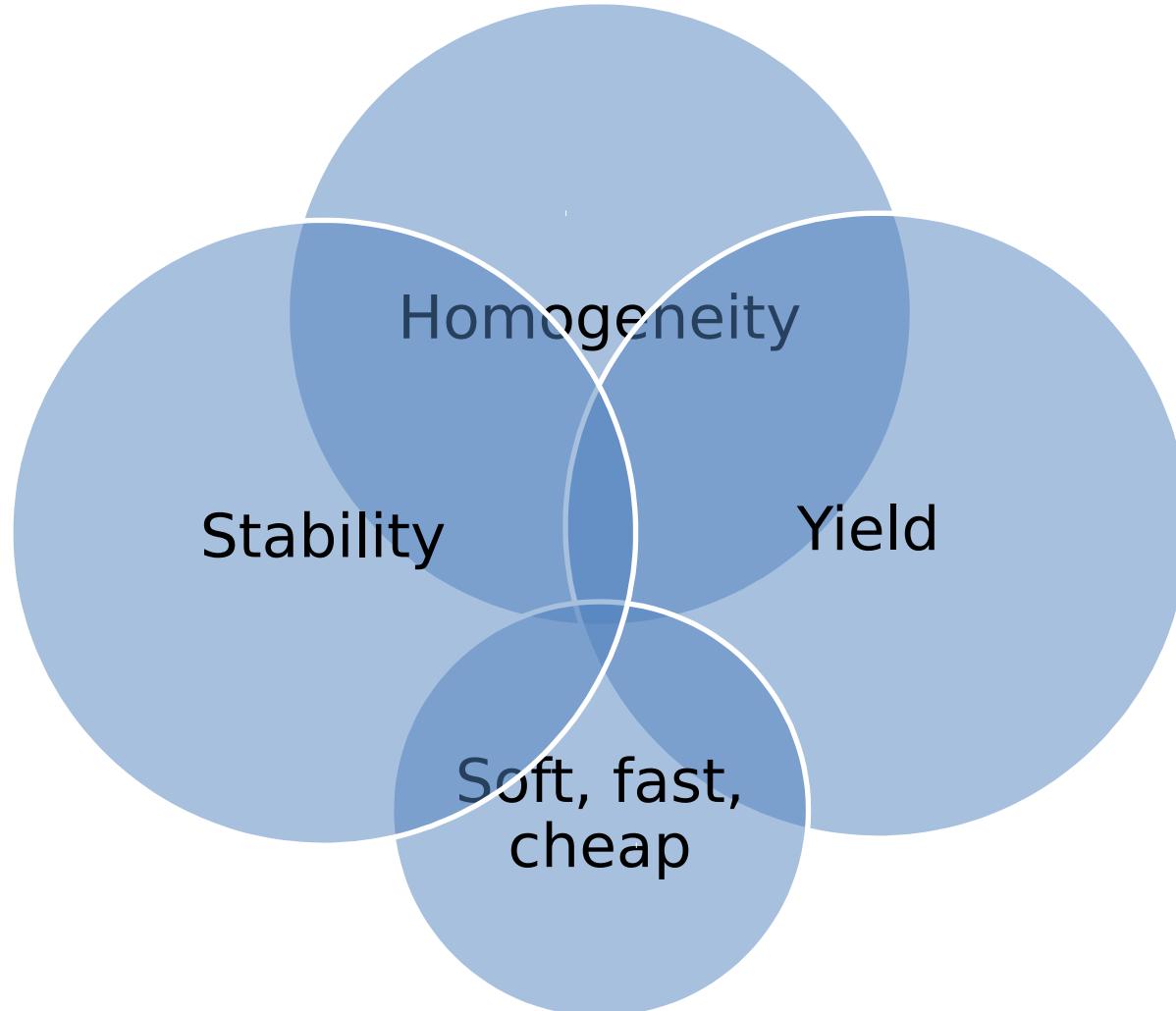


Myasnikov A., et al., Nat.Commun., 2014

3. Ribosomes are intermediate compounds of the cell, bigger than proteins but smaller than organelles (mitochondria, endoplasmic reticulum, etc..)



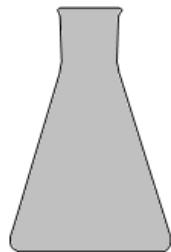
Basic criteria for the protocols of ribosome purification



Purification of bacterial ribosome

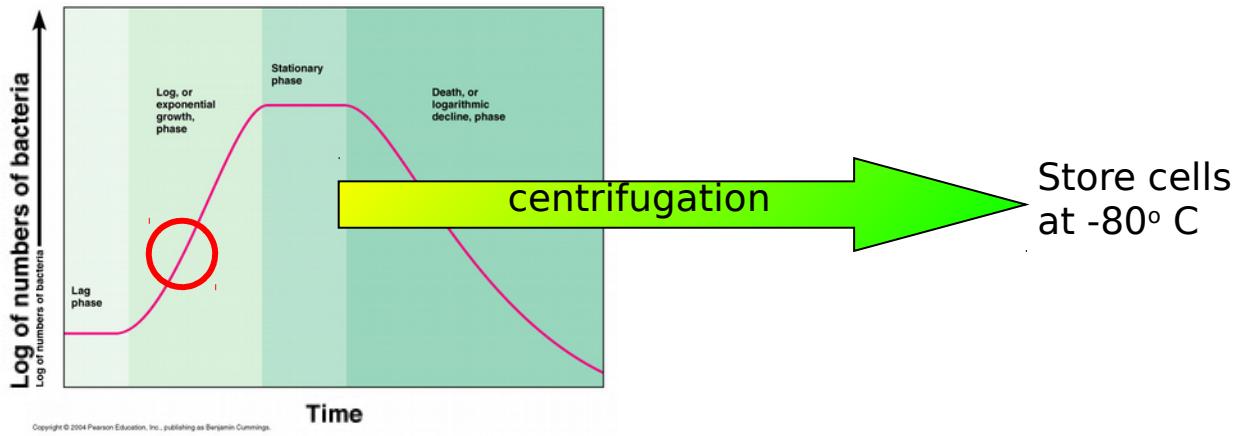
Selmer, et al., Science, 2006.
T. thermophylus.
MRC, Cambridge, UK
Venkatraman Ramakrishnan lab

Cell growth



75° C

5 g Polypeptone
2 g Yeast extract
2 g NaCl
0.4 ml KOH 10M



Purification of 70S ribosomes

All buffers contained:

6 mM 2-mercaptoethanol Reducing S-S bonds
(kept in all buffers)

0.1 mM PMSF

(Phenyl-methyl-sulfonyl fluoride).
Deactivates proteases by binding to the active sites of enzymes

0.5 mM benzamidine

Inhibitor of proteases

Add just before use!

Cell paste

Step 1

Resuspend in **buffer A:**

100 mM	NH_4Cl
10.5 mM	Mg-acetate
0.5 mM	EDTA
20 mM	K-Hepes
pH 7.5	

1.5 ml of buffer per gram of cell paste

- Ionic strength
- Prevents subunits dissociation
- Chelating agent.
(Desactivates proteases)
- Buffer. Effective for maintaining enzymes structure and function at low temperatures.

Step 2

Treatment with **DNAze I**

(8 µg per gram of cell paste)

→ Cleave the DNA

Step 3

High pressure homogenizer

→ Disruption of cells



CF 1h/+4°C/30,000rpm.
Removes cells debris.
Result: S30 fraction.

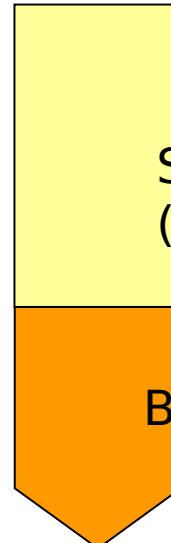
Step 4



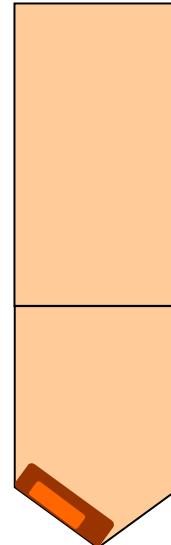
“Sucrose cushion”

Layer the
supernatant over

1.1 M buffer B
Sucrose
0.5 M KCl
10.5 mM Mg-acetate
0.5 mM EDTA
20 mM Hepes-KOH pH 7.5



Buffer B (contains sucrose 1.1M)



CF 17.5h/+4°C/45,000rpm
Beckman 45 Ti
rotor

Step 5

Wash and resuspend the pellet lightly in **buffer C**:



Salting-out agent for HIC

1.5 M	$(\text{NH}_4)_2\text{SO}_4$
10.5 mM	Mg-acetate
0.4 M	KCl
20 mM	Tris-HCl pH 7.5

Step 6

Hydrophobic Interaction Chromatography (**HIC**)

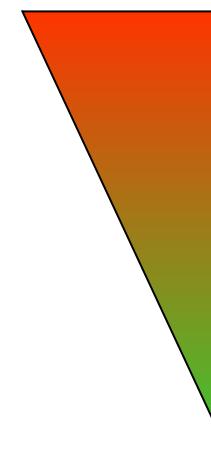


Toyopearl butyl 650 S column:
• Equilibrated in **buffer C**;

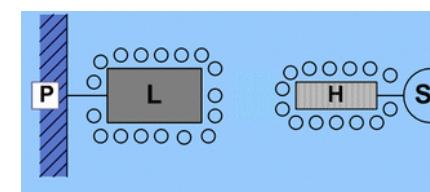
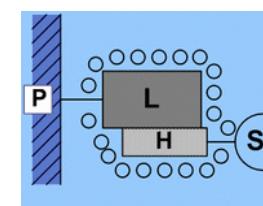
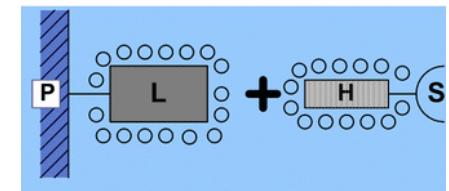
- Eluted by a reverse gradient of $(\text{NH}_4)_2\text{SO}_4$



1.5 M $(\text{NH}_4)_2\text{SO}_4$



~0.4 M $(\text{NH}_4)_2\text{SO}_4$



Step 7

70S ribosomal peak dilute 2-fold
in **buffer E**:

50 mM KCl
10 mM NH₄Cl
10.25 mM Mg-acetate
0.25 mM EDTA
10 mM Hepes-K pH 7.5

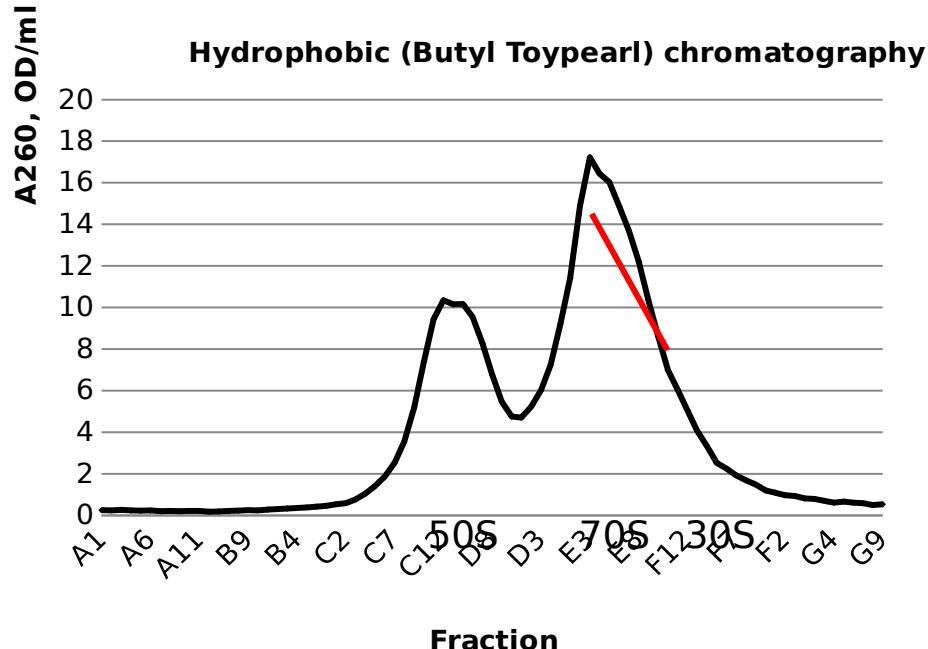


CF 12h/+4°C/43,000rpm and
then resuspend in buffer E. (**45Ti**
rotor)

Step 8

Sucrose gradients

SW28 rotor with a
gradient of
sucrose 5 - 20% in
buffer E



Separation of 70S from
excess of 50S and 30S
subunits



Step 9

70S ribosomes dilute in **buffer E**
without sucrose.



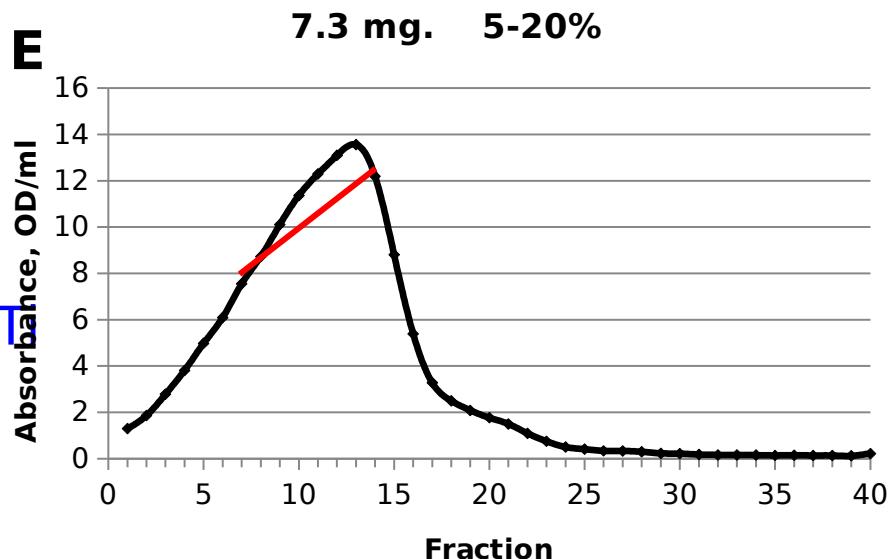
CF 12h/+4°C/43,000rpm and
then resuspend in buffer E. (**45T**
rotor)



Step 10

Resuspend in **buffer G**:

50 mM	KCl
10 mM	NH ₄ Cl
10 mM	Mg-acetate
5 mM	Hepes
	pH 7.5



ield: 35 mg of RS per 25 g of cells



Freeze in liquid nitrogen
Store at -80° C

Purification of eukaryotic ribosome

Ben-Shem et al., Science 2011.
S. cerevisiae
IGBMC, Strasbourg, France
Marat Yusupov lab

Grow in YPD media

**Glucose starvation
in YP media**

**Wash and disrupt in
buffer M (+
heparin)**

**4,5 % PEG20K
precipitation**
10K rpm /
10min

**8,5% PEG20K
precipitation**
10K rpm /
10min

**Sucrose
gradients 15-
30%**
18K rpm/15h

**7% PEG20K
precipitation**
10K rpm / 10min

**Dissolve in
buffer G**

Disrupt with glass beads to save mitochondria

30 mM	Hepes-K 7.5
50 mM	KCl
10 mM	MgCl ₂
8%	Mannitol
2 mM	DTT
0,5 mM	EDTA

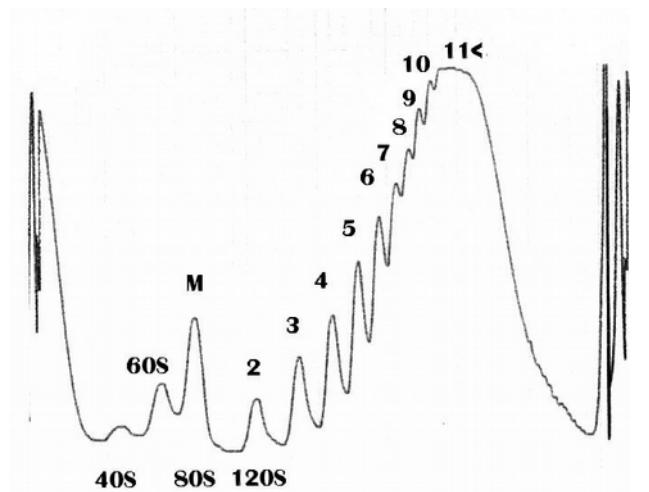
All remaining cell debris is precipitated.
Ribosomes are in solution.

Ribosomes and HMW components are
precipitated.

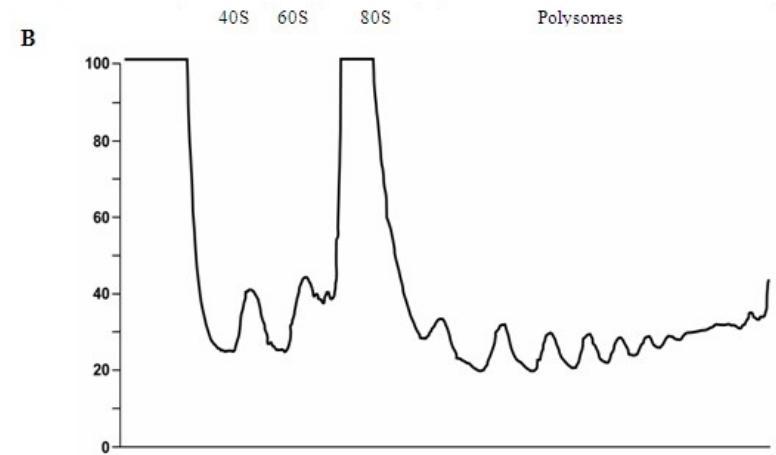
20 mM	Hepes-K 7.5
120 mM	KCl
8,3 mM	MgCl ₂
2 mM	DTT
0,3 mM	EDTA

10 mM	Hepes-K 7.5
50 mM	KAc
10 mM	NH ₄ Cl
5 mM	MgCl ₂
2 mM	DTT

1. Glucose starvation



Growing in YPD media (+Glucose)



Growing in YP media (-Glucose)

During starvation ribosomes transform mostly from polysomal form to monosomal 80S form.

2. Cell disruption



**Wash and disrupt in
buffer M (+
heparin)**

Disrupt with glass beads to save mitochondria

30 mM	Hepes-K 7.5
50 mM	KCl
10 mM	MgCl ₂
8%	Mannitol
2 mM	DTT
0,5 mM	EDTA

- Buffer contains inhibitors of proteases and RNases;
- Heparin - anticoagulant;
- Glass beads allow not to break mitochondria that helps to avoid mitochondrial ribosomes in the sample.

3. PEG precipitation



4,5 % PEG20K precipitation

10K rpm /
10min

8,5% PEG20K precipitation

10K rpm /
10min

All remaining cell debris is precipitated.
Ribosomes are in solution.

Ribosomes and HMW components are precipitated.

- PEG provides soft precipitation
- Fast procedure
- Variation of PEG concentration allows to purify ribosomes from HMW and LMW compounds

4. Sucrose gradients and ribosome pelleting



**Sucrose
gradients 15-
30%**
18K rpm/15h

20 mM	Hepes-K 7.5
120 mM	KCl
8,3 mM	MgCl ₂
2 mM	DTT
0,3 mM	EDTA

**7% PEG20K
precipitation**
10K rpm / 10min

**Dissolve in
buffer G**

10 mM	Hepes-K 7.5
50 mM	KAc
10 mM	NH ₄ Cl
5 mM	MgCl ₂
2 mM	DTT

Yield: 12.5 mg of RS per 10 mg of cells

- Separation of 60S and 80S ribosomes during gradient . For better separation concentration of Mg decreased to 8 mM while concentration of KCl increased to 120 mM.
- Last PEG precipitation provides fast and soft pelleting of pooled 80S peak.



The Nobel Prize in Chemistry 2009

"for studies of the structure and function of the ribosome"



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Ramakrishnan

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Steitz

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New Haven, CT,
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Ada E. Yonath

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Institute of
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Israel