Molecular virology
- Laboratory exercises -

VIRAL NUCLEIC ACIDS (NA) ISOLATION

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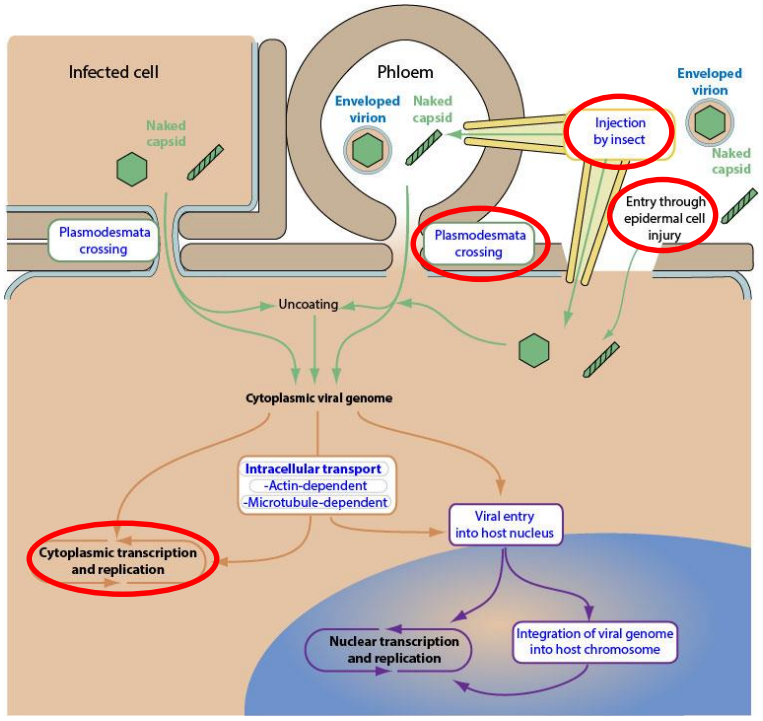
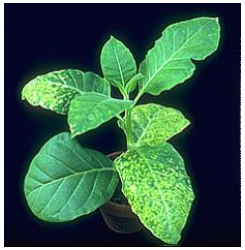


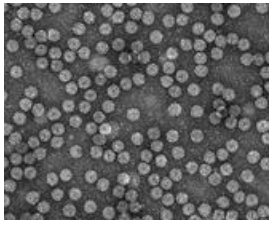
VIRUSES – intracellular pathogens



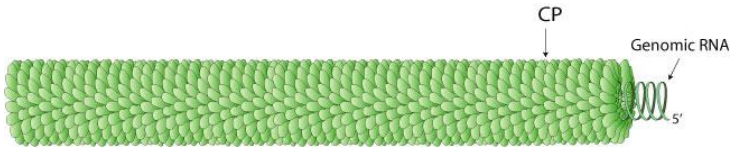
Isolation of viral RNAs from plant tissue:

- Total RNAs (plant RNA + viral RNA; fraction of viral RNAs is small relative to plant RNAs)
- viral dsRNAs (replicative form)

→ Isolation of genomic viral RNAs from previously partially purified viral particles (increasing the fraction of viral RNAs in sample, i.e. separating it from plant RNAs)

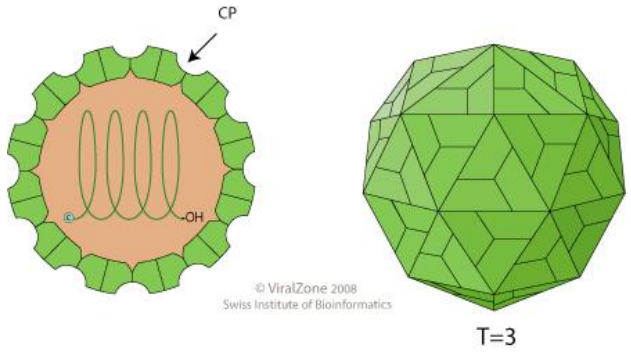


VIRAL NUCLEIC ACIDS ISOLATION (RNA):



Tobacco mosaic virus **(TMV)**

ssRNA(+) genome, 6.3 - 6.5 kb
anisometric virus
5 % NA in the virus particle



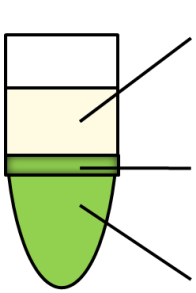
Turnip yellow mosaic virus **(TYMV)**

ssRNA(+) genome, 6.3 kb
isometric virus
28 % NA in the virus particle

PURIFICATION OF VIRUS PARTICLES

HOMOGENISATION OF INFECTED TISSUE
(+ ribonuclease inhibition)

ORGANIC SOLVENT ADDITION



water phase (virus particles, leftover plant proteins, leftover ribosomes, low-molecular components)

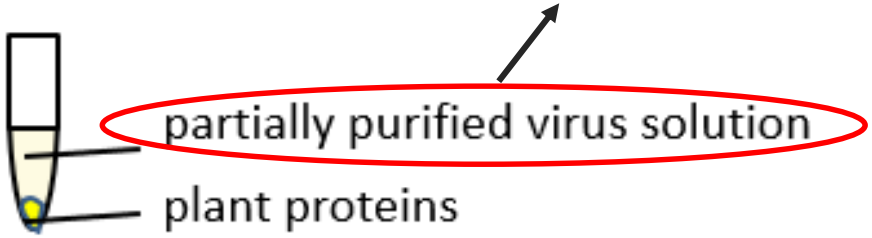
clot/interphase (rough cellular debris, denatured proteins and nucleoproteins on surface)

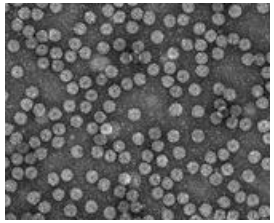
organic solvent phase (pigments, membranous structures)

CLARIFICATION

ULTRACENTRIFUGATION or alternative PHYSICO CHEMICAL procedure (addition of ammonium sulphate + low speed centrifugation)

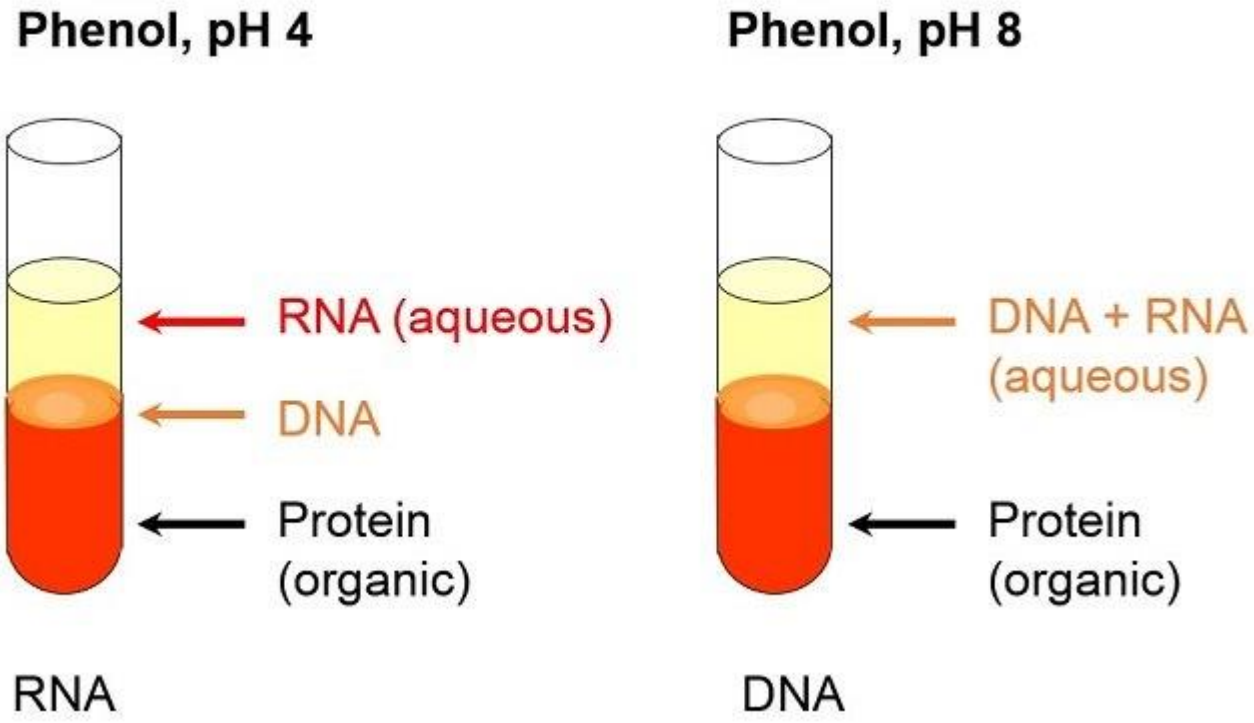
From here, we isolate viral NA.





TMV	**E=3**	**A_{260}/A_{280}= 1.19**
TYMV	**E=9,6**	**A_{260}/A_{280} =1.51**

Traditional Phenol Extraction



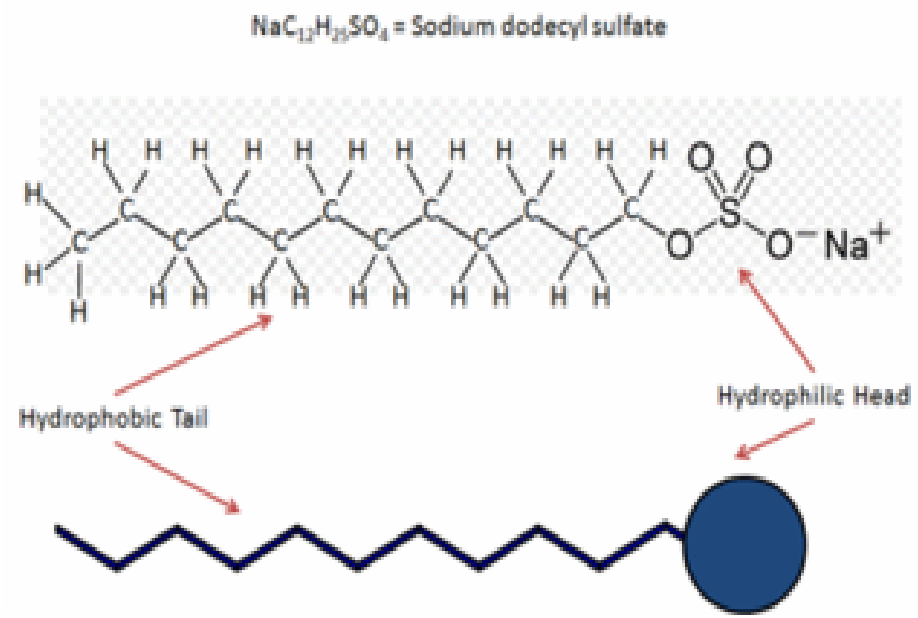
Disadvantages:
Phenol – toxic, carcinogenic

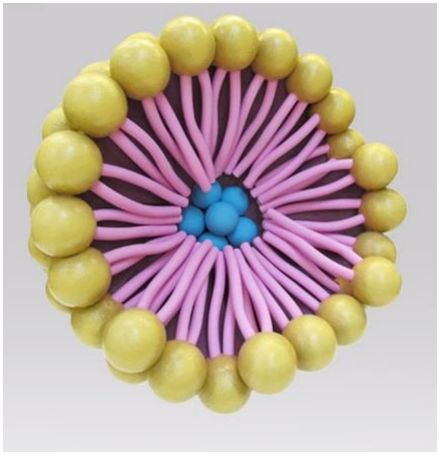
→ Alternative methods

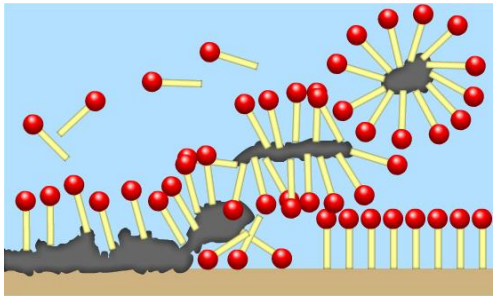
PERCHLORATE METHOD OF NA ISOLATION – specific steps

1. SDS (Sodium dodecyl sulphate) – anionic detergent

- amphiphilic molecule – it disrupts interactions between non-polar amino acids (AA) in proteins – protein denaturation (enhanced by heating)







Globular proteins + detergent molecules = micellae (good solubility, clear solution)

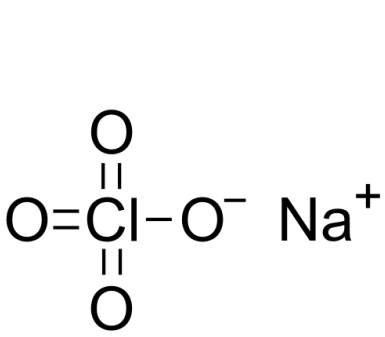
PERCHLORATE METHOD OF NA ISOLATION – specific steps

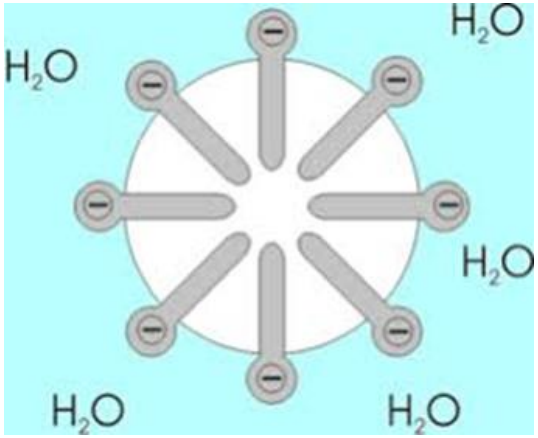
2. Sodium perchlorate ($NaClO_4$) – hygroscopic

- **It extracts water from hydration shell of micellae – turbidity („flocks") →**

→ Proteins separate from aqueous phase containing NA

- **„Flocks" are lighter than the aqueous phase – they float**





3. Separation of aqueous phase with NA – centrifuge

4. NA precipitation with EtOH (3 volumes for RNA, 2 volumes for DNA)

PERCHLORATE METHOD OF VIRAL NUCLEIC ACIDS (RNA) ISOLATION – protocol

0.8 mL partially purified virus
\+ 0.2 mL **SDS** (25%)

heat for 3 min at 60°C — **denaturation of capside proteins**

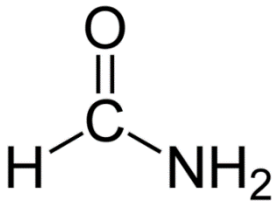
\+ 3 mL **$NaClO_4$** (8 M)

vortex for 1 min — **separation of proteins and NA (turbidity)**

- centrifuge for 10 min at 6000 rpm (4 °C)
- separate aqueous phase containing RNA (<u>at the bottom</u> of the tube), add 3V of cold **EtOH** and incubate for 20 min at -20 °C — **nucleic acids precipitation**
- centrifuge for 20 min at 11000 rpm (4 °C)
- dry the precipitate and dissolve it in 25 µL **TE-buffer** (pH 7.5)

TE buffer
- 10 mM Tris + 1mM EDTA
- pH 7.5 for RNA, pH 8 for DNA
- nuclease inactivation – protects NA from degradation

PARTIAL DENATURATION OF NA (preparation for electrophoresis)



- **Formamide**
  - **Disrupts hydrogen bonds between base pairs → stabilisation of ssRNAs in electrophoretic gel**
  - **Affects Tm (higher order structures dissociate at lower temp.)**
  - **NA in semi-denatured state: better electrophoretic properties, stability, retains infectivity (possibility of downstream biological experiments)**

PARTIAL DENATURATION OF NA – protocol

10 µL nucleic acid isolate
\+ 40 µL **formamide**

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heat for 2 min at 85 °C

add 10 µL **coloured electrophoresis sample buffer (SB**)

ELECTROPHORESIS OF NUCLEIC ACIDS – protocol

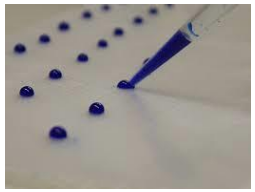
Agarose gel preparation:

0.5% agarose	1% agarose	1.2% agarose
150 mg agarose	300 mg agarose	360 mg agarose
30 mL 1xTBE	30 mL 1x TBE	30 mL 1x TBE

Agarose = agar purified from agaropectin (charged groups disrupt electrophoresis – electro-osmosis)

Electrophoresis:

90 V, 40 min



Electrophoresis of tracking dye in 6xGel Loading Dye, SDS +

Composition and role of SB (sample buffer, gel loading buffer, loading „dye")

- dyes (xylene cyanol, bromphenol blue) → sample visibility (loading, run tracking)
- sample density increase (sucrose, glycerol, ficoll)
- water

ADVANTAGES AND DISADVANTAGES OF THE PERCHLORATE METHOD

+	-
• Fast • Non-toxic • Excess of perchlorate in EtOH inhibits the precipitation of proteins leftover in the aqueous phase	• Samples are never as pure as after phenol purification

ALTERNATE METHODS FOR NA ISOLATION

- Phenol method – „classical method";
  - phenol – toxic, mutagen, harmful for environment;
  - method: slower, „finer" results

- "Flash heating"
  - opening of virus particle by heating (physical method)
  - several cycles of heating for 1 min at 95°C
  - risk of NA denaturation

SILVER STAINING

- **Very sensitive method, developed for PAGE (Ag^+ does not bind strongly to polyacrylamide gels, but it does bind to agarose)**

1. Fixation:

- **Formaldehyde (reducing agent) – increase of contrast due to blocking of charged groups of agarose**
- **Simultaneous NA fixation (to unable diffusion) and complete denaturation**

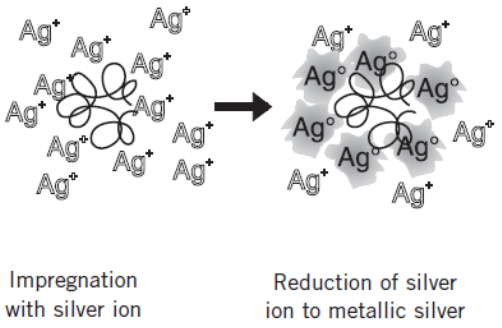
2. Washing:

- **Rinsing of buffer, ions, denaturing agent**
- **Formaldehyde should be COMPLETELY rinsed off! (to avoid unspecific Ag^+ reduction in staining)**

3. Silver impregnation:

- **$AgNO_3$ – binds to gel and NA**

(rinsing removes $AgNO_3$ unspecifically bound to gel)



4. Developing:

- **Developing solution – Ag^+ reduction to Ag forms a precipitate (formaldehyde = reducing agent, sodium carbonate increases pH)**

SILVER STAINING – protocol

Solution **A** **(FIXATION + REDUCTION)**

110 mL water
\+ 14 mL formaldehyde
---
incubate gel for 20 min; 3 x 10 min rinsing with water

Solution **B** **(SILVER IMPREGNATION)**

250 mg $AgNO_3$
\+ 125 mL water
---
incubate gel for 10 min in dark, 3 x short rinse with water (15")

Solution **C** **(DEVELOPING SOLUTION)**

6,867 g Na_2CO_3 x 10 H_20
70 µL formaldehyde
\+ 125 mL water
---
incubate gel until bands become visible

Store the gels in water in dark!

Solution **D** **(FIXATIVE)**

0,5 mL HNO_3
\+ 125 mL water
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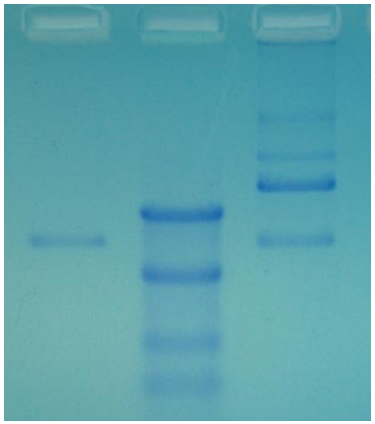
incubate gel for several min

NA STAINING WITH NON-FLUORESCENT DYES

- Methylene blue
- Toluidine blue

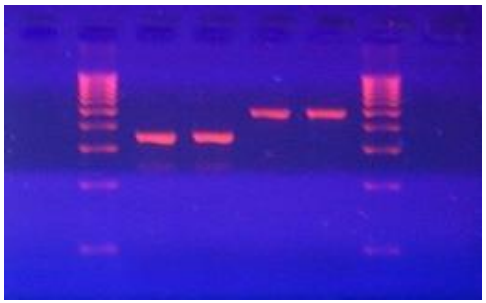


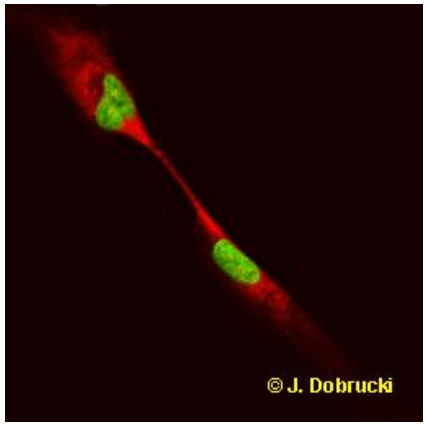
NA STAINING WITH FLUORESCENT DYES

- Ethidium bromide (EtBr)

  max A (EtBr) = 360 nm

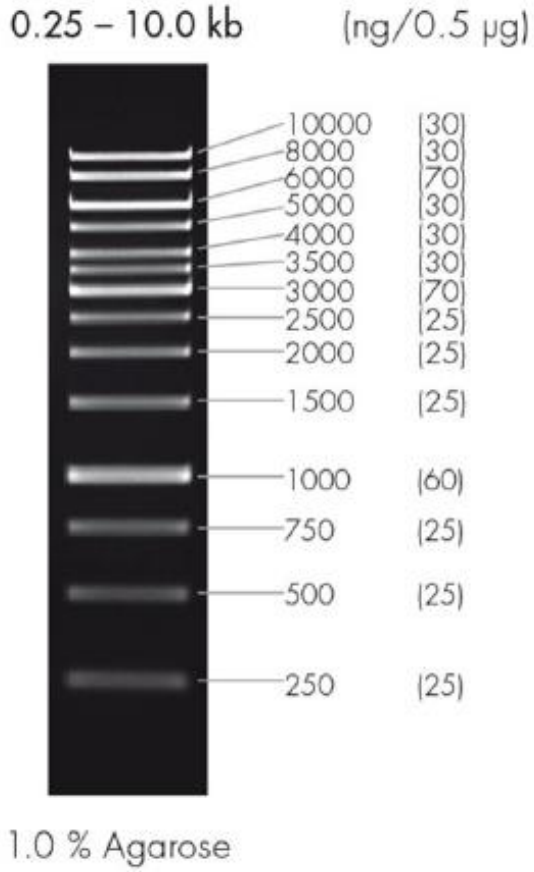
  max A (NA) = 260 nm – nucleotides excited by UV-light
  transfer energy to EtBr
- Acridine orange – differentiates DNA (green) and RNA (red)
- Commercially available fluorescent dyes (Sybr Green, Sybr Safe, GelRed, Gel Green, Stain G…)



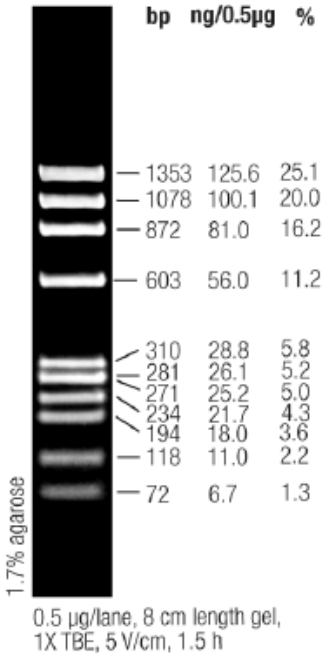


MARKERS

1 kb DNA Ladder, 250 - 10,000 bp



ΦX174 DNA/BsuRI (HaeIII) Marker, 9, ready-to-use



RESULTS – GROUP 1

Electrophoresis:
1.2% gel, 1xTBE
40 min, 100 V
a) Silver staining
b) Ethidium bromide staining

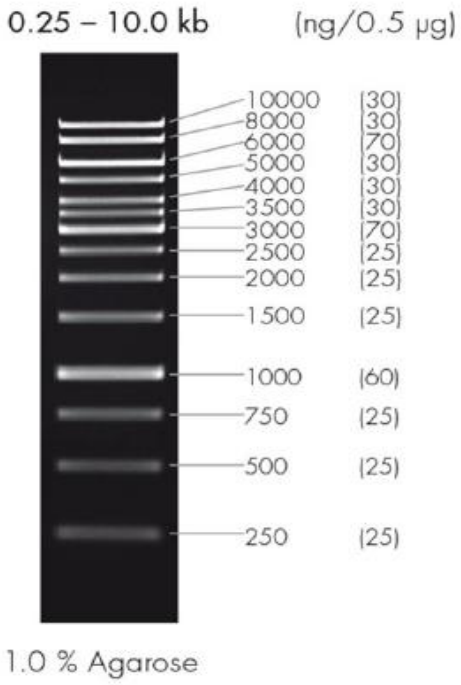
Total RNA concentration after NA isolation:

	Group 1
TMV	390,1 µg/mL
TYMV	1273,6 µg/mL

Load : TMV ~ 1.5 - 2 µg per well
TYMV ~ 5.2 - 6.5 µg per well

a)

M TMV TYMV



b)

M TMV TYMV

viral genomic RNA

decomposed RNA

RESULTS – GROUP 2

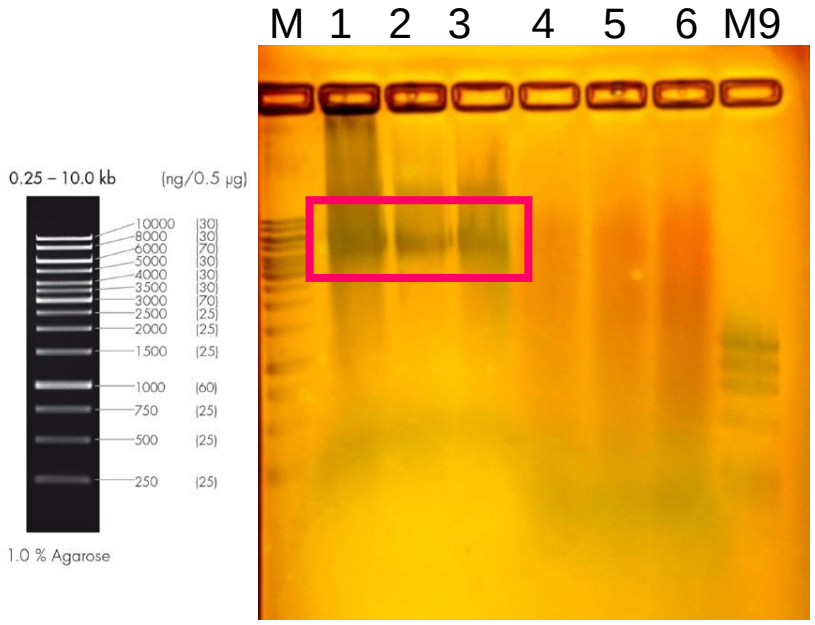
Total RNA concentration after NA isolation:

	Group 2
TMV	241,4 µg/mL
TYMV	217,8 µg/mL

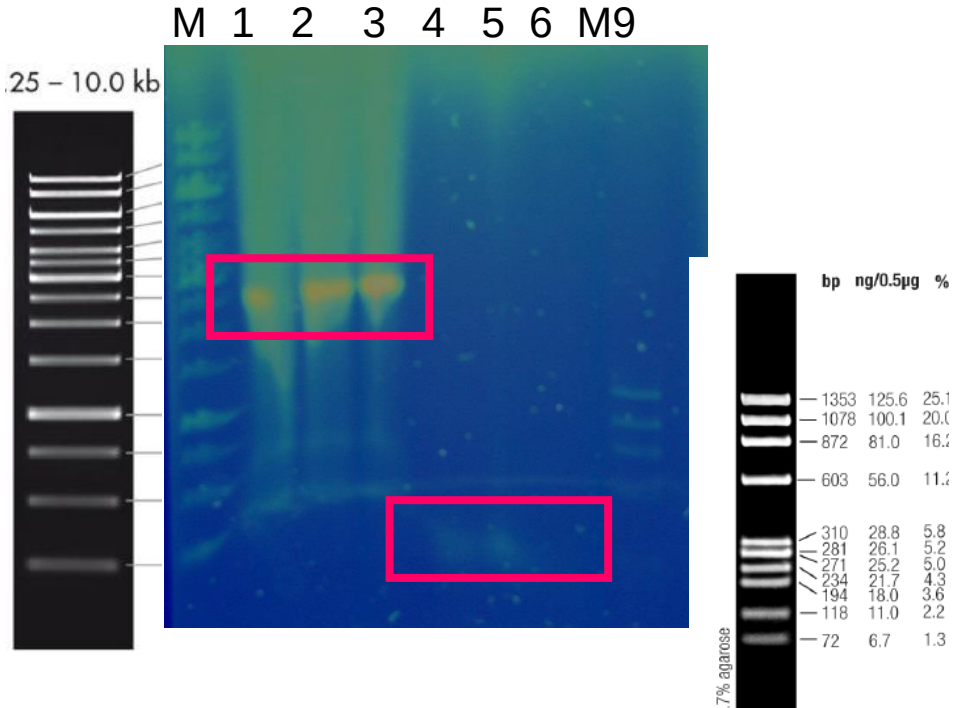
Load:

1 – TMV - 0.5 µg
2 – TMV - 0.7 5µg
3 – TMV - 1 µg
4 – TYMV - 0.5 µg
5 – TYMV - 0.75 µg
6 – TYMV - 1 µg

a)
1% gel, 1xTBE, 40 min, 100 V
Silver staining



b)
0.5% gel, 1xTBE + 1 µL Serva DNA Stain G
~1 h, 100 V



RESULTS – GROUP 3

Total RNA concentration after NA isolation:

	Group 3
TMV	426,6 µg/mL
TYMV	83,1 µg/mL

Load:

1 – TMV - 1 µg
2 – TYMV - ?
3 – ?

a)
1% gel, 1xTBE, 40 min, 110 V
Silver staining

M 1 2 3 M9

b)
0.5% gel, 1xTBE + 1µL Serva DNA Stain G
~50 min, 110 V

M 1 2 3 M9