Characterization of Residual DNA in rAAV Made in the Baculovirus/Sf9 Platform

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Residual DNA in rAAV

Health authorities have recommended very restrictive residual DNA level limits for recombinant AAVs.[1,2]

As a result, characterization of residual DNA in rAAV is very important for making well-informed risk assessments.

Methods used in this study

- Digital-droplet PCR (ddPCR), Quantitative PCR (qPCR), and Southern blot

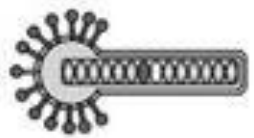
References: [1]FDA. 2021. Toxicity Risks of Adeno-associated Virus (AAV) Vectors for Gene Therapy (GT)

[2]Wright JF. 2014. Product-Related Impurities in Clinical-Grade Recombinant AAV Vectors: Characterization and Risk Assessment.

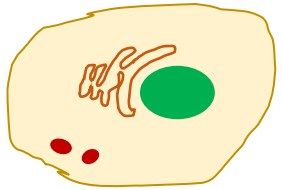
Penaud-Budloo et al. 2018. Pharmacology of recombinant adeno-associated virus production.

Baculovirus / Sf9 production system

rBV (recombinant baculovirus)

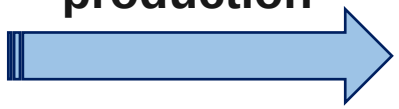


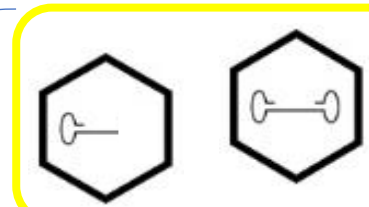
- ITR-flanked GOI
- Rep cassette
- Cap cassette



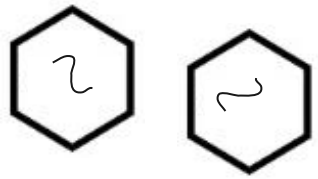
Sf9 insect cells

rAAV production

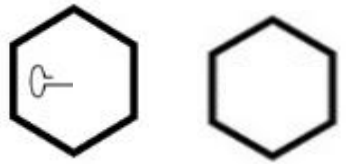




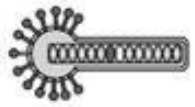
rAAV capsids containing **Vector Genomes** (VGs)



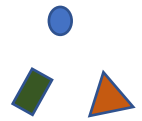
rAAV capsids containing **non-Vector Genome** DNA



Empty rAAV capsids or containing short VGs or non-VG DNA

baculoviruses 

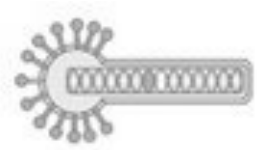
Free DNA 

Proteins & other cell debris 

Baculovirus / Sf9 production system

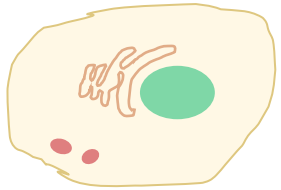
Encapsidated residual DNA
difficult to remove during purification

rBV (recombinant baculovirus)



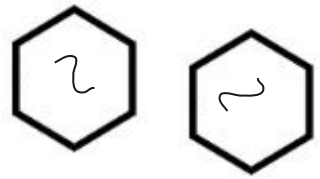
- ITR-flanked GOI
- Rep cassette
- Cap cassette

rAAV production

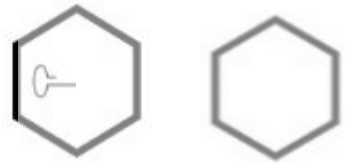


Sf9 insect cells

rAAV capsids containing **Vector Genomes** (VGs)

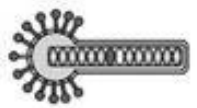


rAAV capsids containing **non-Vector Genome** DNA



Empty rAAV capsids or containing short VGs or non-VG DNA

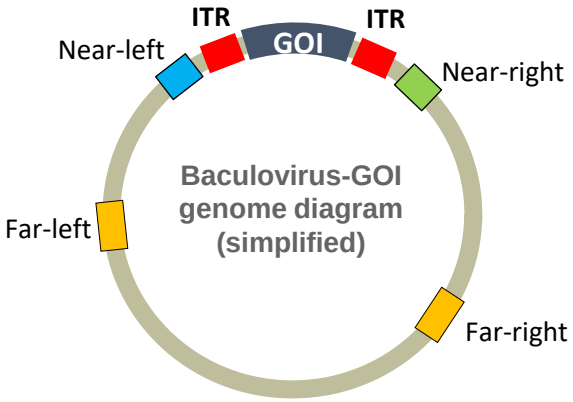
Non-encapsidated residual DNA
easy to remove during purification

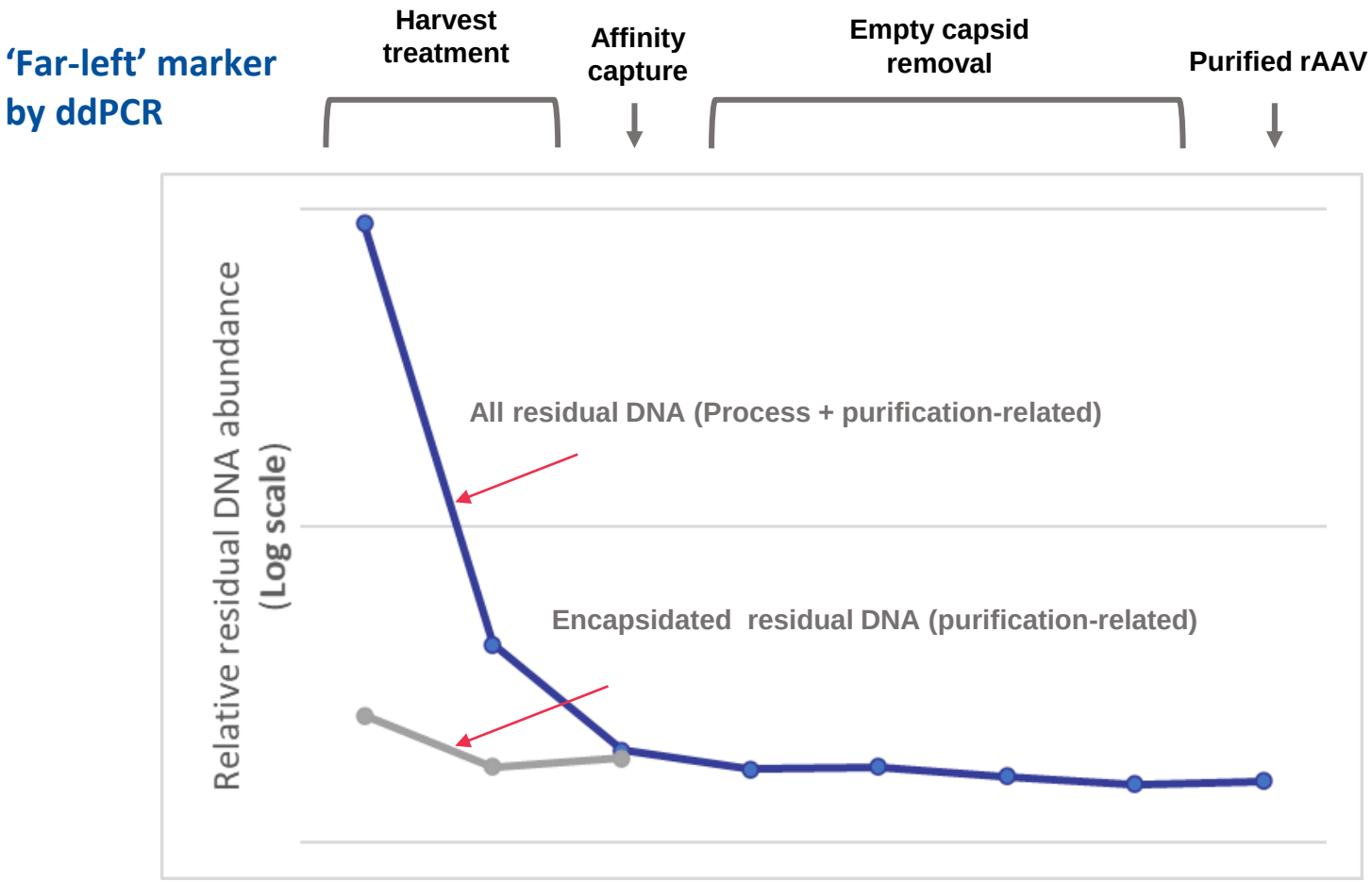
baculoviruses 

Free DNA 

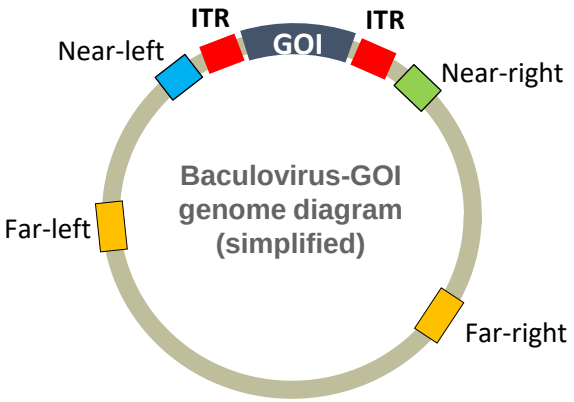
Proteins & other cell debris 

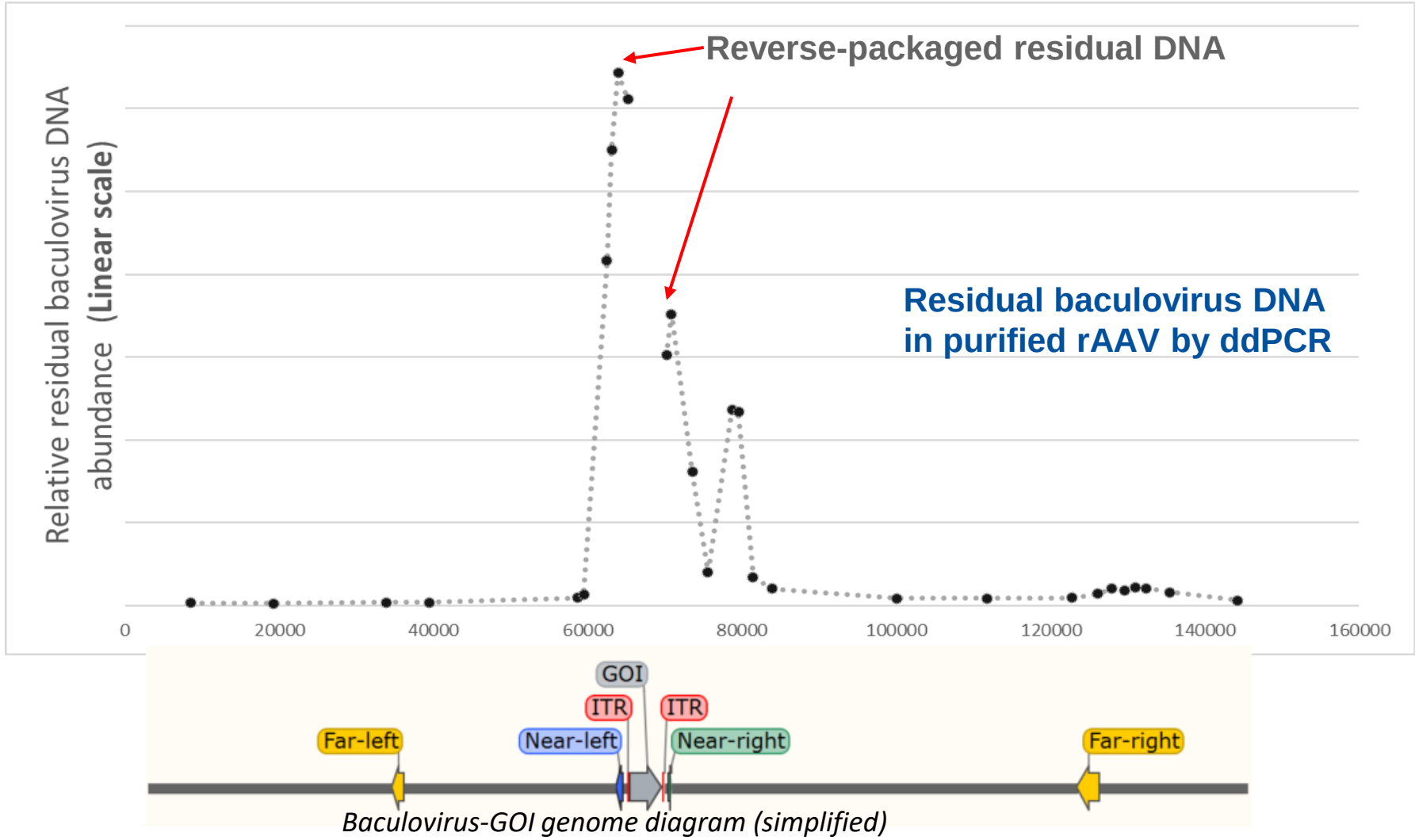
- Non-encapsidated residual DNA is removed efficiently
- Encapsidated residual DNA copurifies with rAAV





Expect all DNA (baculovirus and Sf9) to be present in rAAV, but not at the same ratios as in the cell substrate

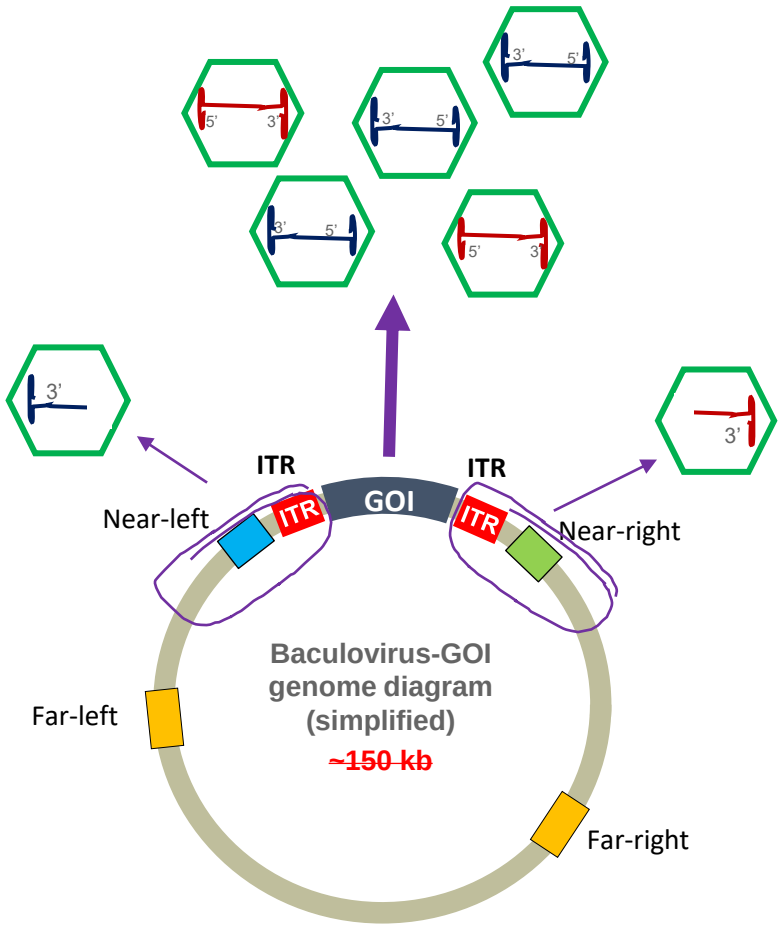




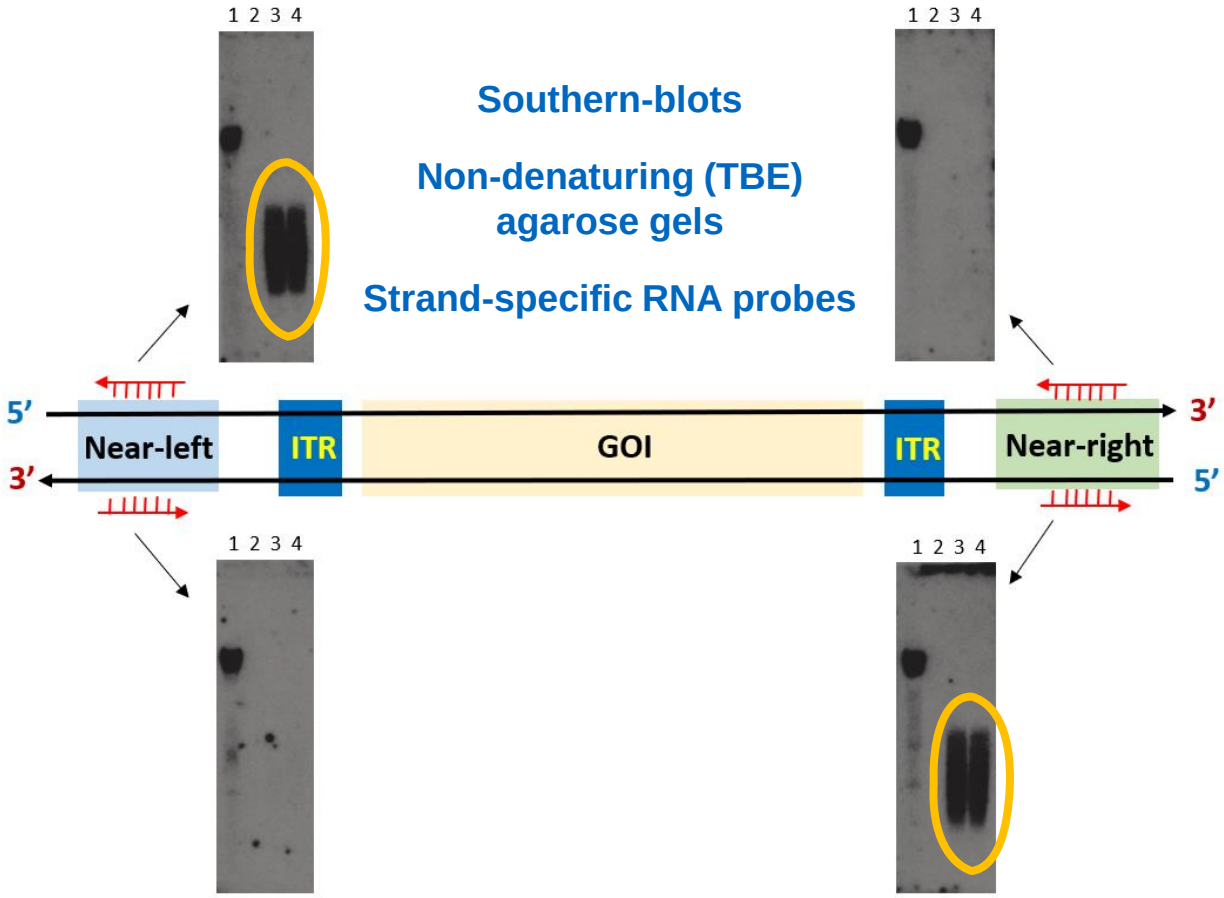
Residual DNA dependence on ITRs

Residual DNA from regions adjacent to ITRs

- **ITR-dependent** encapsidation (reverse-packaging)



Reverse-packaged residual DNA corresponds to only one of the baculovirus DNA strands

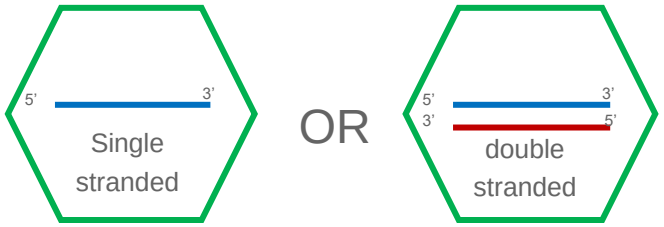


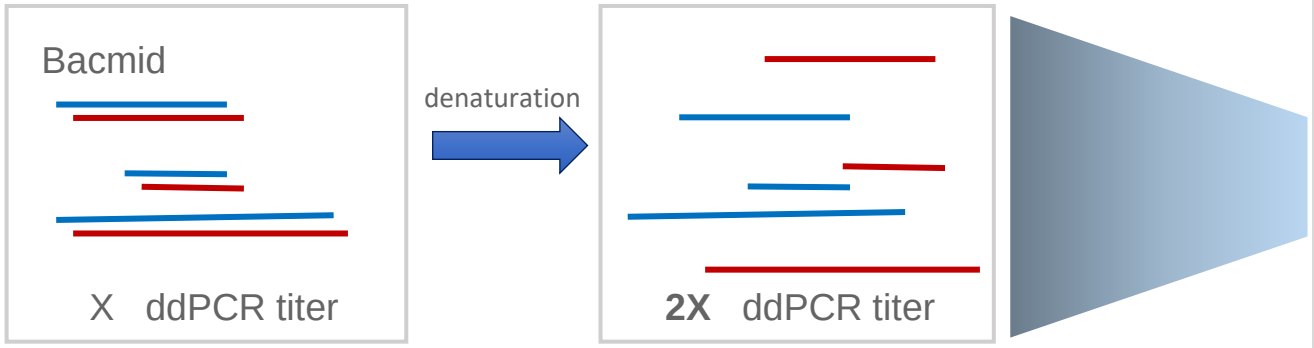
Lane #1: DNA from Sf9 cells infected with Baculovirus
Lane #2: No DNA
Lane #3: Final (purified) rAAV
Lane #4: Final (purified) rAAV, treated with nuclease before DNA extraction

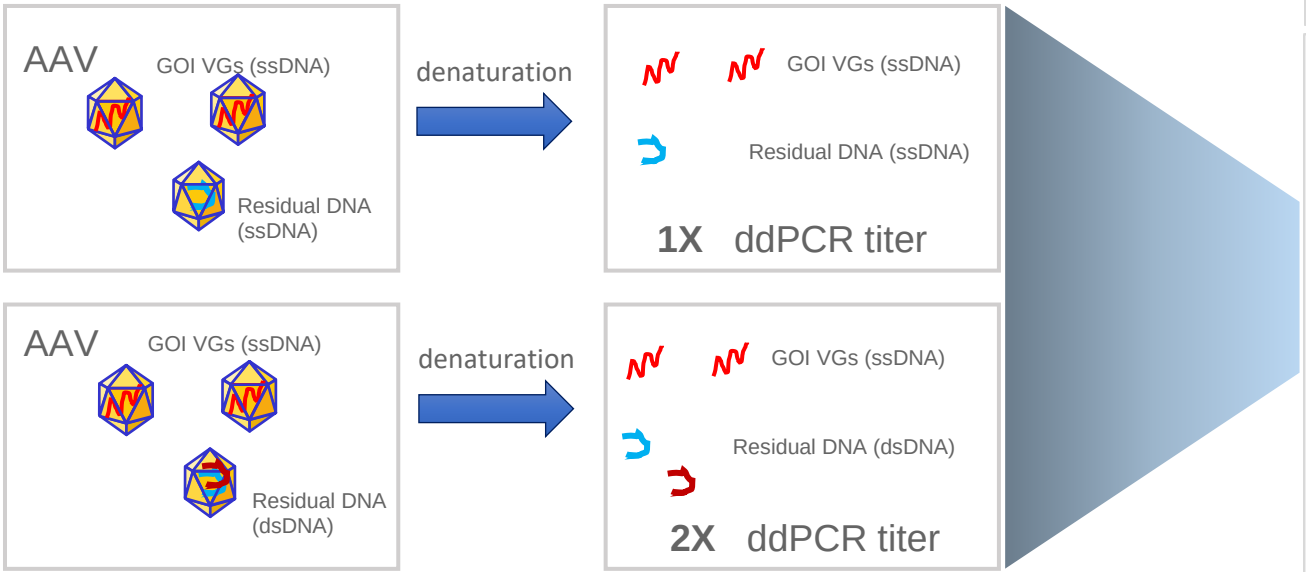
Single-stranded or double-stranded?

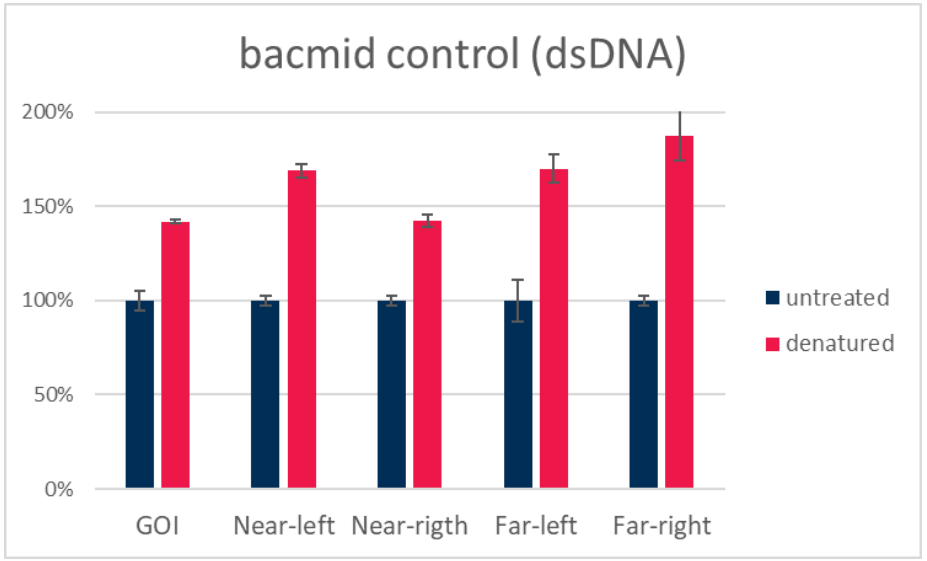
dsDNA denaturation experiments:

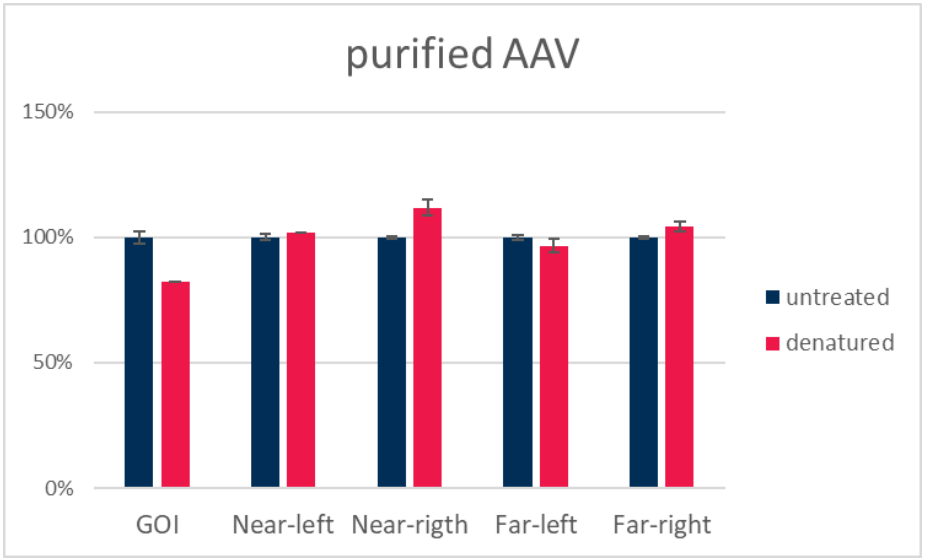
Residual DNA in AAV capsids is present as single-stranded DNA











Residual DNA size

Lane #1: DNA from Sf9 cells infected with Baculovirus
Lane #2: No DNA
Lane #3: Final (purified) rAAV
Lane #4: Final (purified) rAAV, treated with nuclease before DNA extraction

DNA purified from AAV

↓

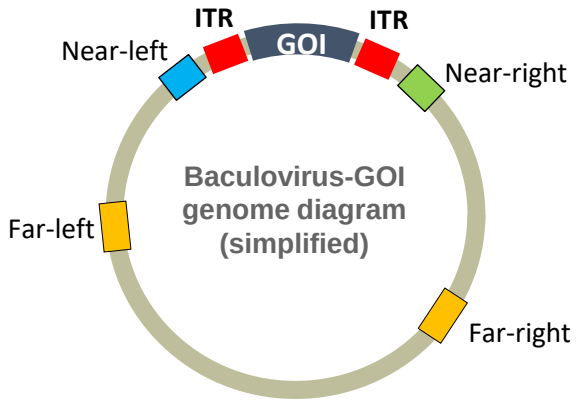
Denaturing (Alkaline agarose gel) electrophoresis
To run DNA as single-stranded

↓

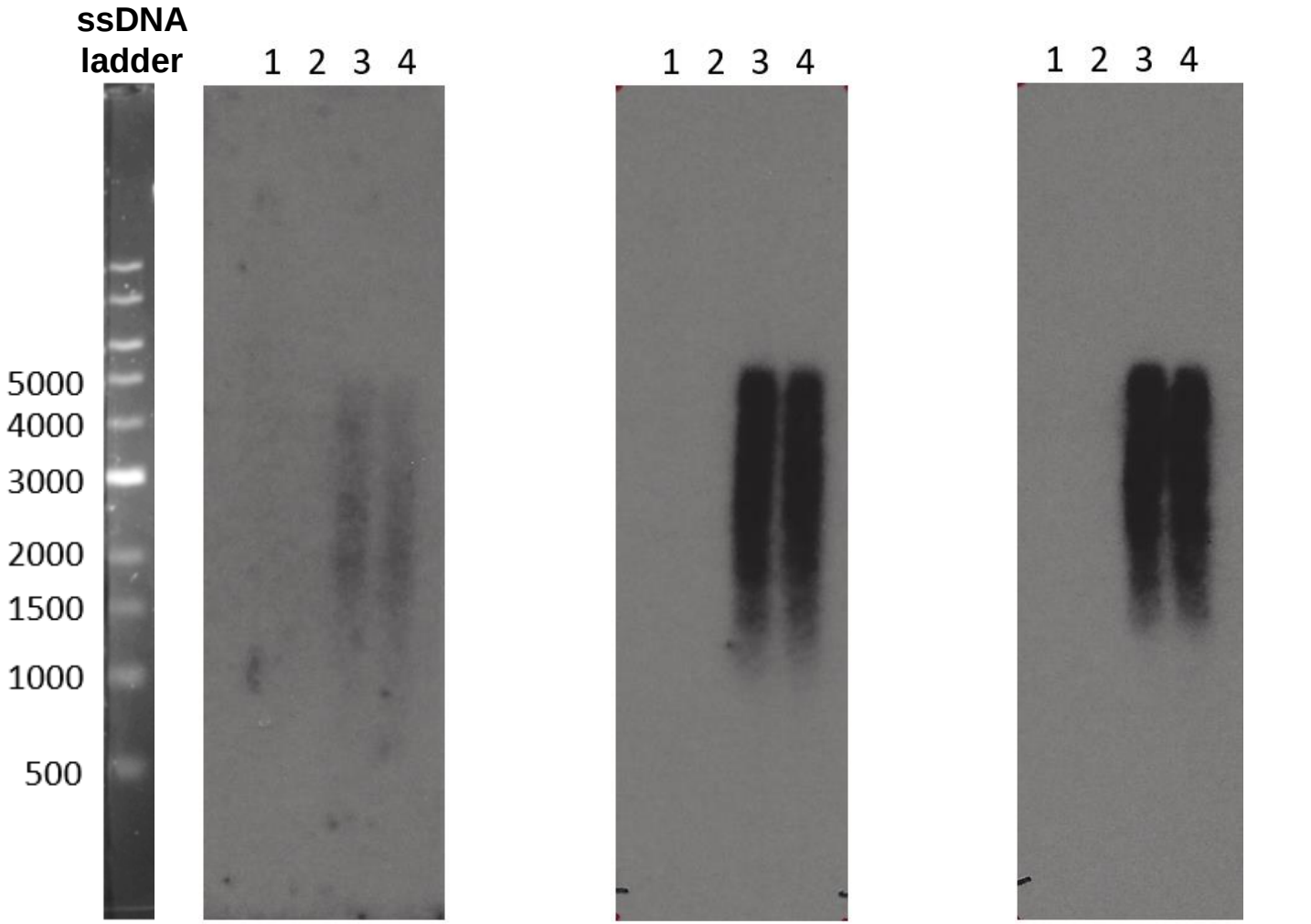
Transfer to nylon membrane

↓

Hybridization with 32P-labelled probe



Residual DNA size from packaging limit (~4.7 kb) to less than 1.5 kb



Baculovirus residual DNA (**Far-right** region)

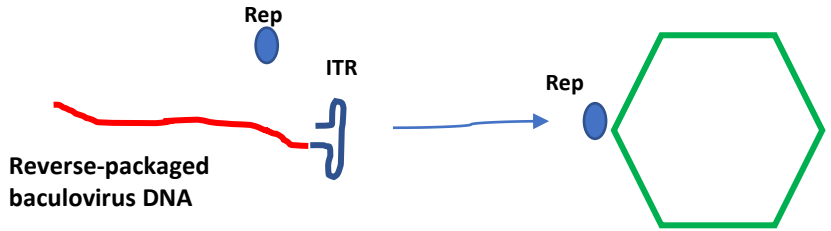
Baculovirus residual DNA (**Near-right ITR** region)

Baculovirus residual DNA (**Near-left ITR** region)

Hypothetical mechanisms of residual DNA encapsidation:

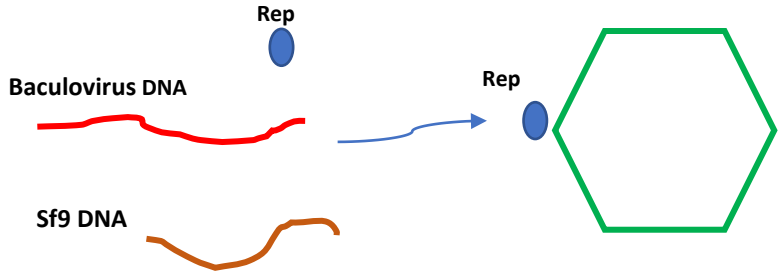
1) Rep and ITR-dependent:

- Baculovirus regions flanking the GOI (near ITRs) (Reverse-packaging)



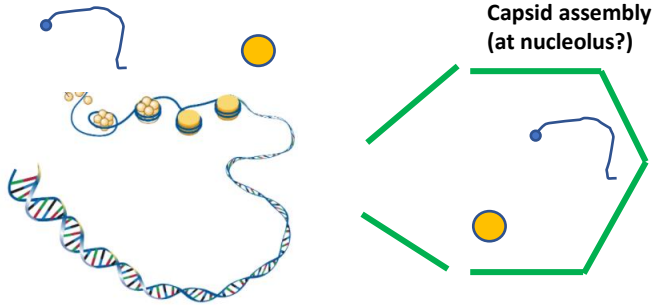
2) Rep-dependent, (not ITR-dependent):

- Sequences similar to Rep binding elements (RBE) or ITRs?
- Rep less than 100% for RBE and ITRs?



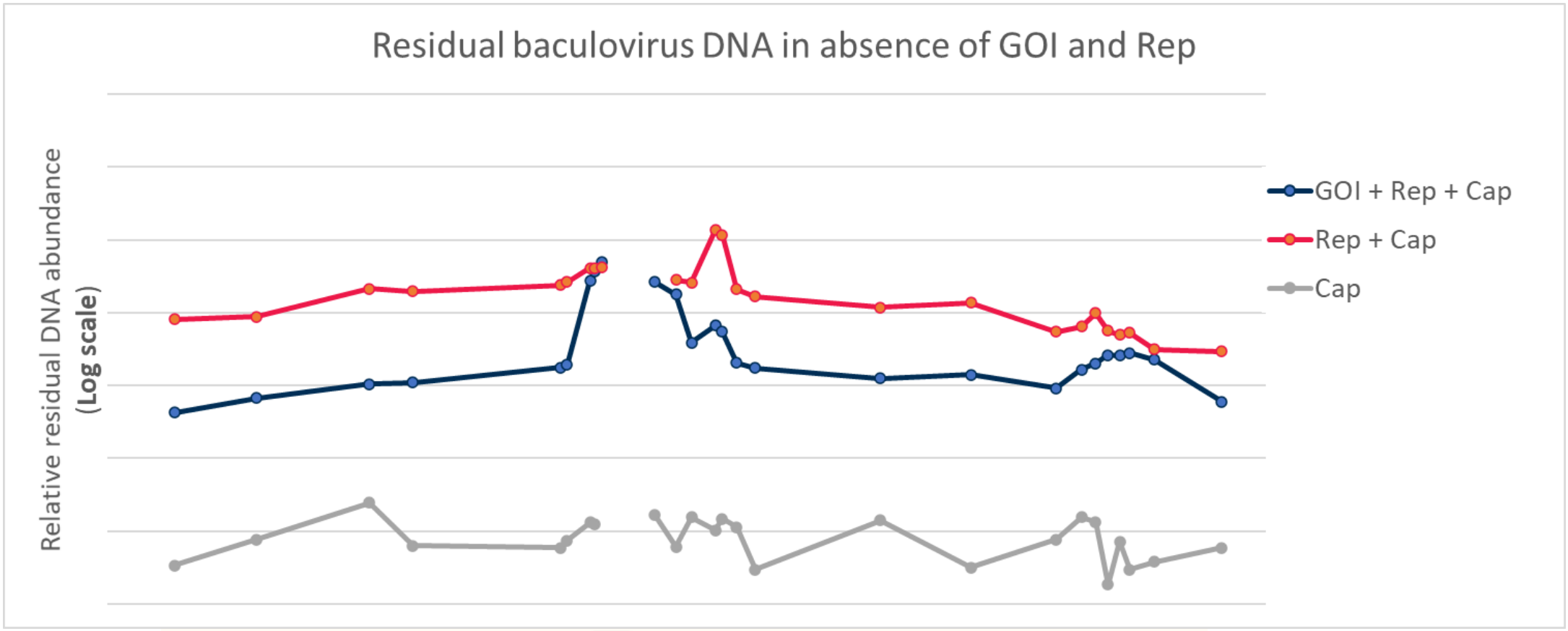
3) Not dependent on Rep or ITRs

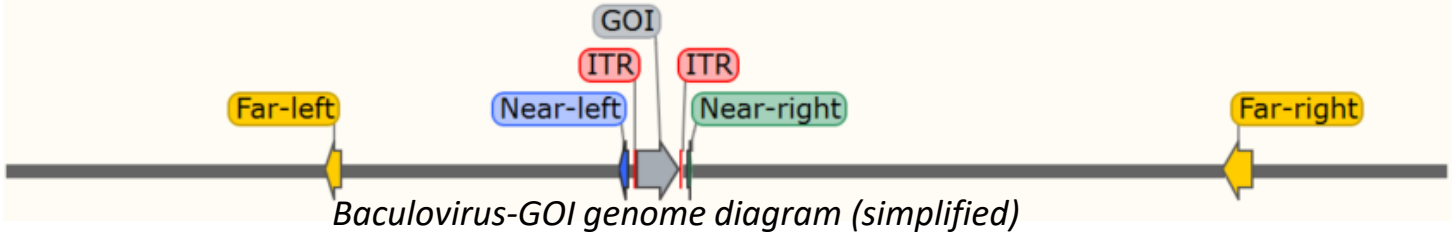
- Capsids trapping nucleolar material during assembly?



Residual DNA dependence on Rep proteins

- Residual DNA is Rep-dependent (~100x lower in the absence of Rep)





Baculovirus-GOI genome diagram (simplified)

Residual DNA has physical characteristics resembling that of Vector Genomes:

- AAV-encapsidated
- Size : ~4.7 kb and shorter
- Encapsidated as single-stranded DNA
- Encapsidated in a Rep-dependent manner (most of it):
- baculovirus regions adjacent to ITRs encapsidated in an ITR-dependent manner (only DNA strand from 3’ end)

Acknowledgements

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Thank you for attending!

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