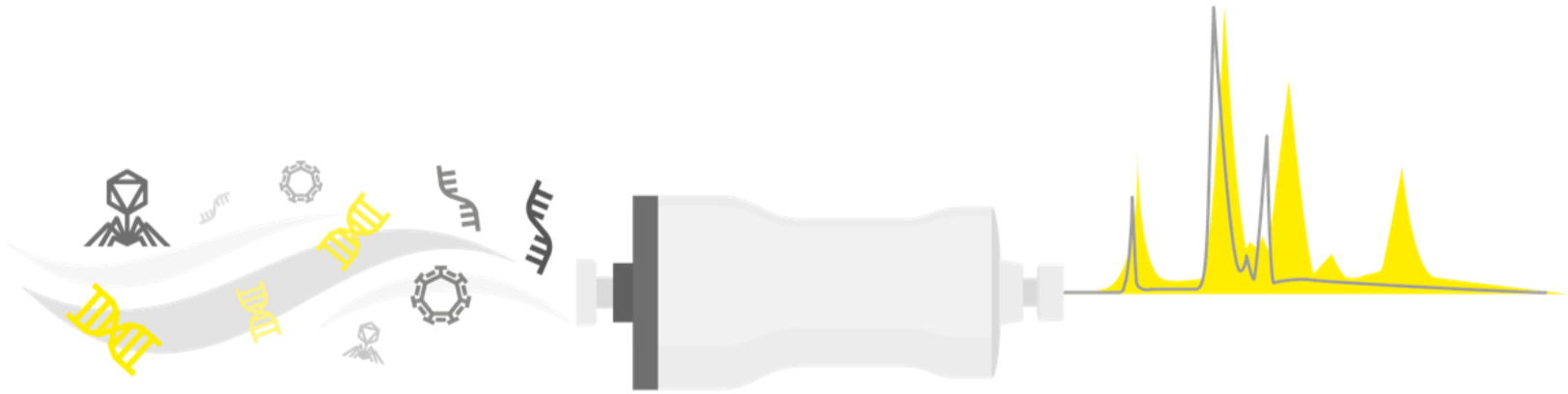
Simplifying Progress



Chromatographic Tools for high-yielding mRNA production process

17 June 2022 Monolith Summer Symposium

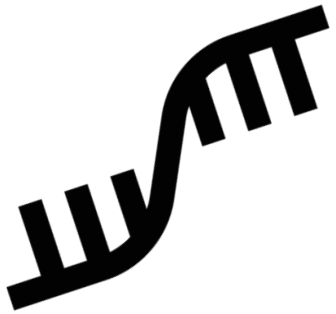
Rok Sekirnik, Head mRNA/pDNA process development

BIA separations

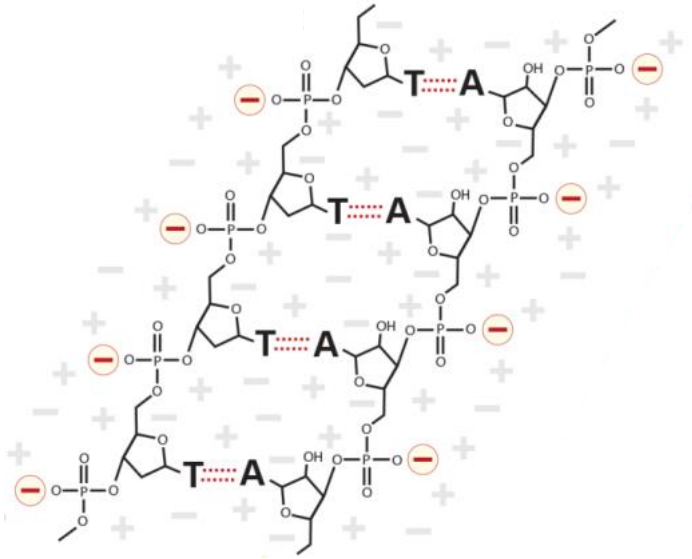
SARTORIUS

mRNA is large, charged, (somewhat) hydrophobic and has useful sequence attributes

IgG – 150 kDa



4000 nt mRNA – 1.3 MDa



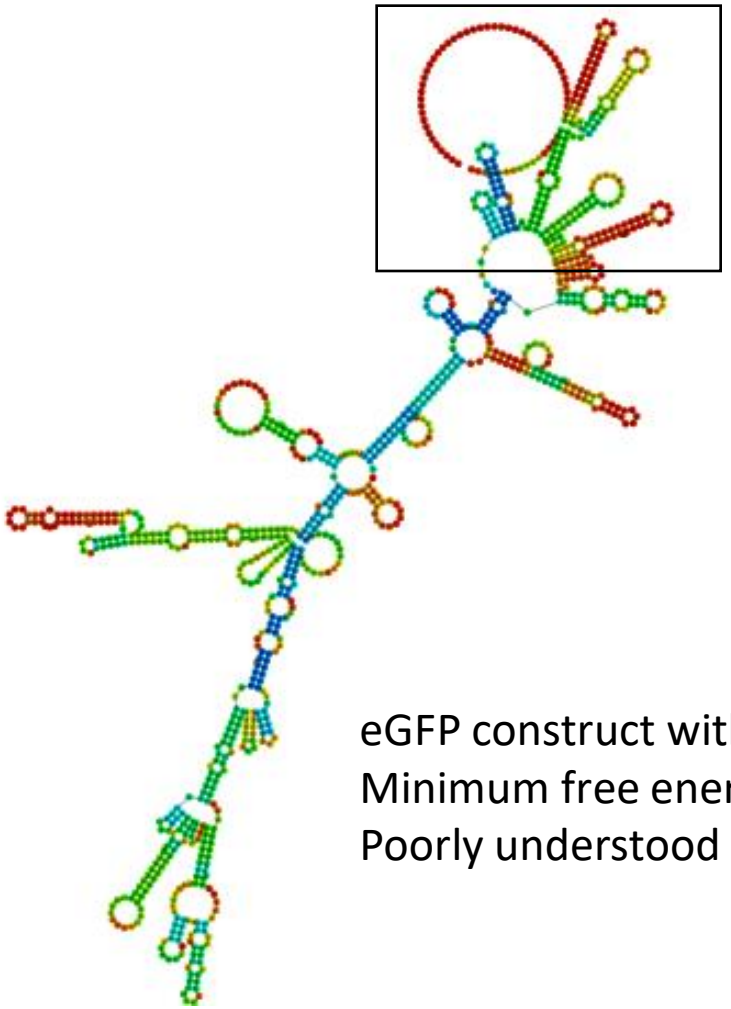
mRNA is much larger than a traditional biologic

mRNA is negatively charged and binds strongly to anion exchangers

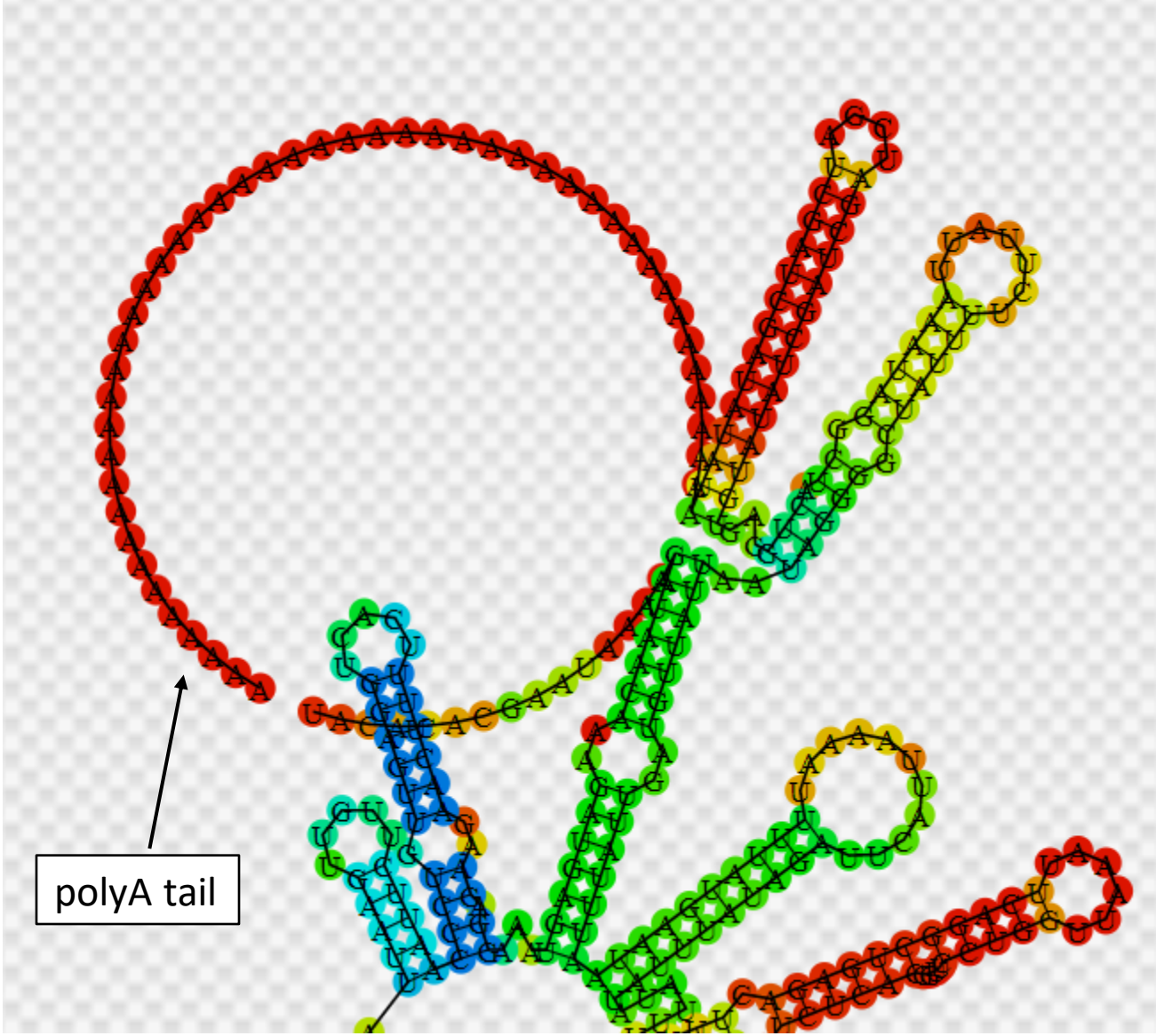
mRNA is hydrophobic if exposed to high concentration of salts

mRNA is frequently polyadenylated in IVT

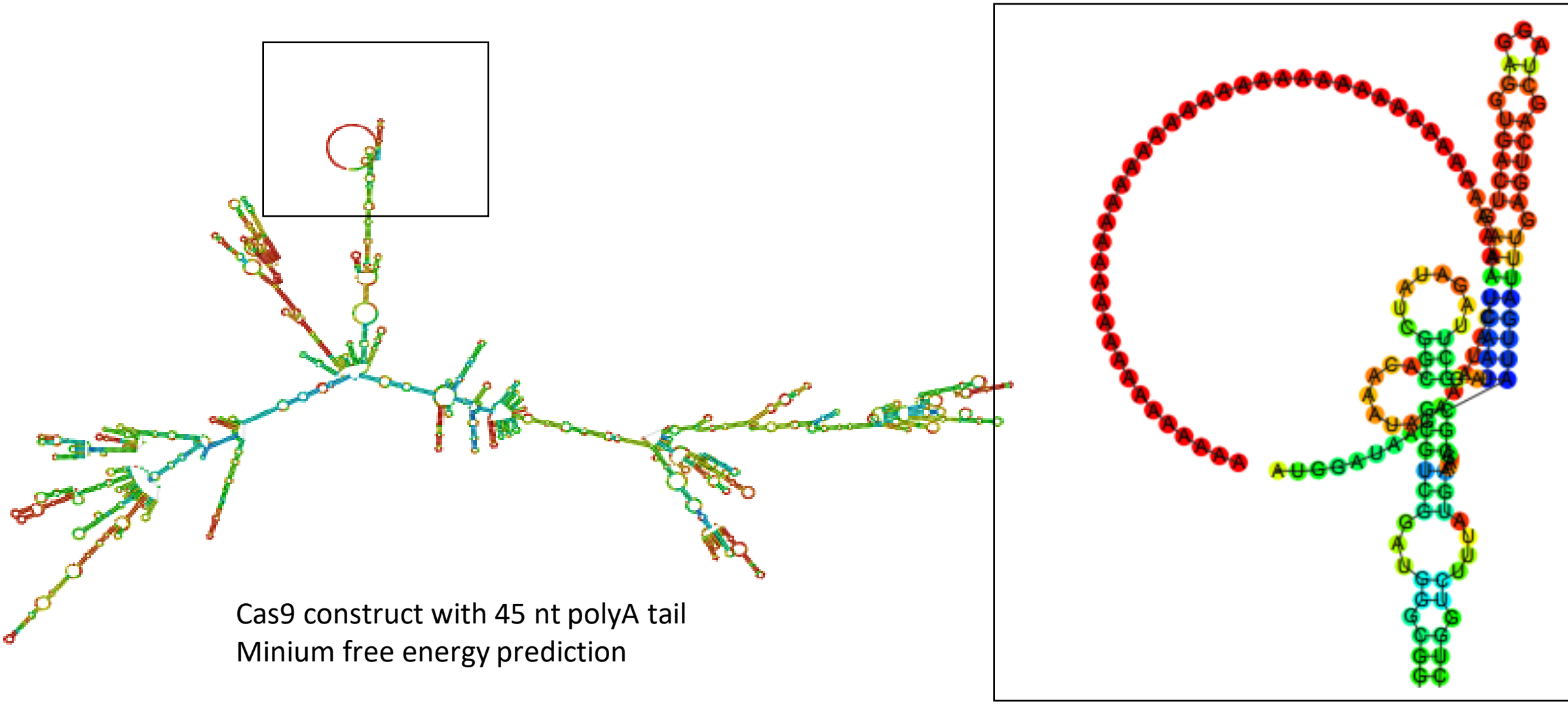
mRNA has secondary structure



eGFP construct with 45 nt polyA tail
Minimum free energy prediction
Poorly understood

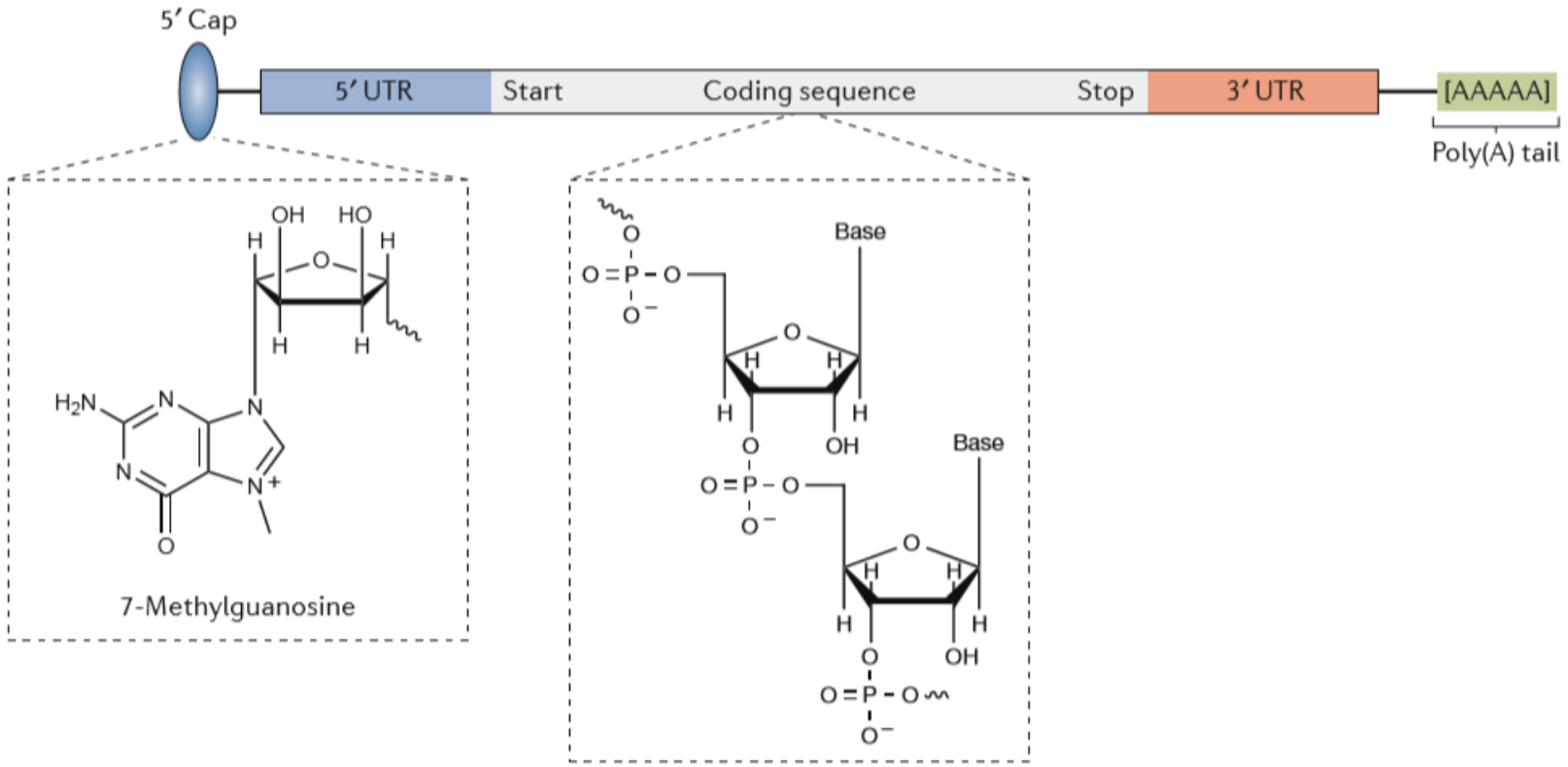


http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi; Jure Ličen, Anže Martinčič Celjar



Cas9 construct with 45 nt polyA tail
Minium free energy prediction

Mature mRNA Structure

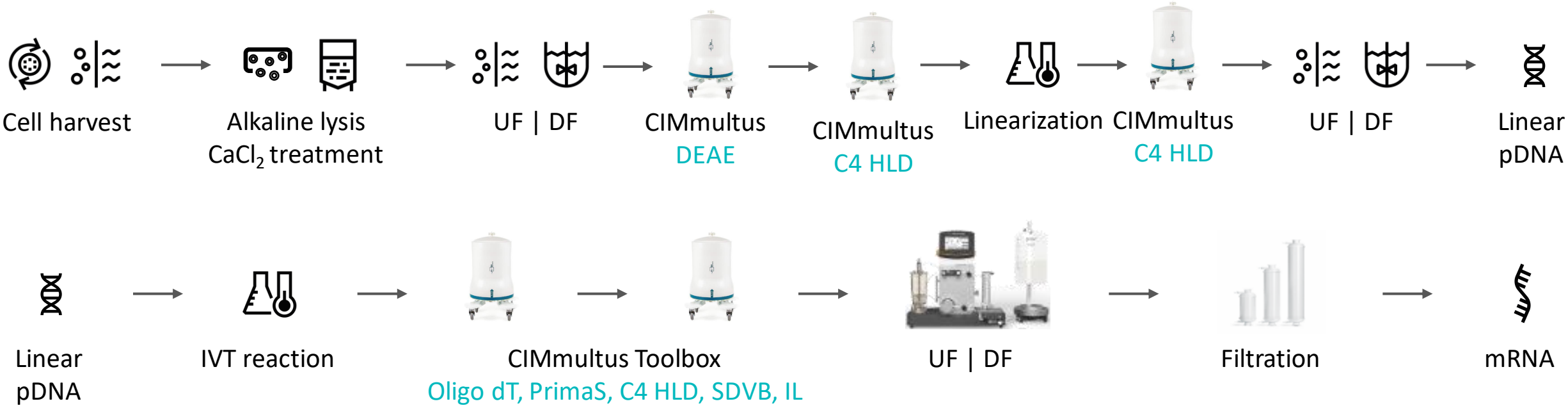


Hajj, Khalid A., and Kathryn A. Whitehead. "Tools for translation: non-viral materials for therapeutic mRNA delivery." *Nature Reviews Materials* 2.10 (2017): 17056.

- 5’ Cap and poly-A tail are required for protein expression in cells.
- *In Vitro* transcription produces RNA from a DNA template, often plasmid DNA
- 5’ Cap can be added co-transcriptionally (during IVT), or post-transcriptionally
- Poly-A tail can be encoded in the DNA template, or added enzymatically after IVT

mRNA Drug Substance Production workflow

Analytical workflow (PATfix pDNA and CIMac pDNA)



Analytical workflow (CIMac PrimaS, CIMac Oligo dT, CIMac SDVB)

World Health Organization

POST-ECBS version
ENGLISH ONLY

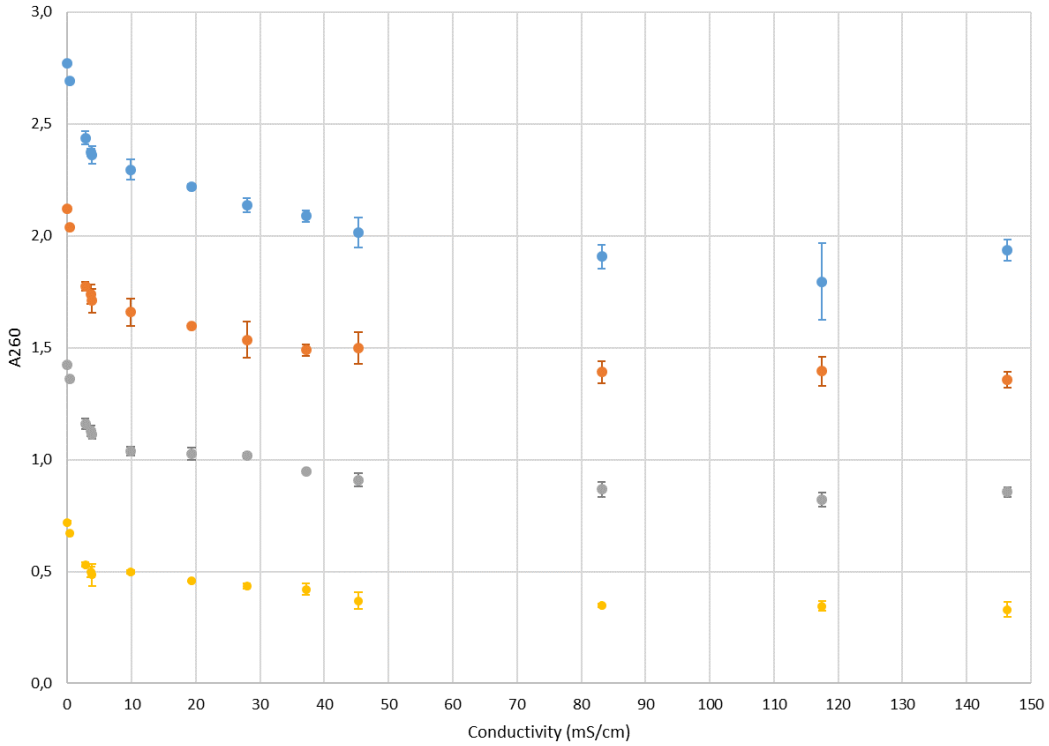
Evaluation of the quality, safety and efficacy of messenger RNA vaccines for the prevention of infectious diseases: regulatory considerations

6.7.1.3 Quantification and physical state

The integrity of the structure of the mRNA is considered to be a critical quality attribute for release of the mRNA. Thus, control is needed of mRNA integrity, 5′ capping efficiency, 3′ poly(A) tail presence or length, percentage intact mRNA, percentage mRNA fragments, percentage of dsRNA and so on. The need to measure 3′ poly(A) tail presence or length depends upon the way in which this sequence is added to the mRNA. If encoded in the DNA template, then all full-length mRNA should include the poly(A) tail but if it is added enzymatically after IVT, then it would be appropriate to address this attribute through testing or process validation. Likewise, the presence of dsRNA depends on whether the processes used are capable of producing it. Tests such as gel electrophoresis, PCR or chromatographic detection methods might be considered for these purposes. It should be borne in mind that quantification of the mRNA is the basis for vaccine dosing and the presence of intact mRNA is key to the mechanism-of-action of the vaccine. Thus, the methods used for quantifying the mRNA (for example, ultraviolet spectrophotometry) and for quantifying the intact mRNA (for example, gel electrophoresis) should be described.

BIA Separations SARTORIUS

UV not a reliable method for quantification of mRNA



- Matrix conductivity affects absorbance at 260 nm, potentially leading to errors in content determination
- Higher matrix conductivity results in lower absorbance values. Variations ≤30% can be observed in matrices containing 0-1 M NaCl.
- Also affects mass balance calculations for process intermediates in different matrices!

Chromatographic tools required

Raw material control
- pDNA content/purity
- NTP
- Capping reagent
- Enzyme purity

IVT reaction monitoring
mRNA content
NTP content
Capping reagent content
dsRNA content

mRNA purification
- mRNA capture, polish

DS Stability
- Stability indicating methods
- Content

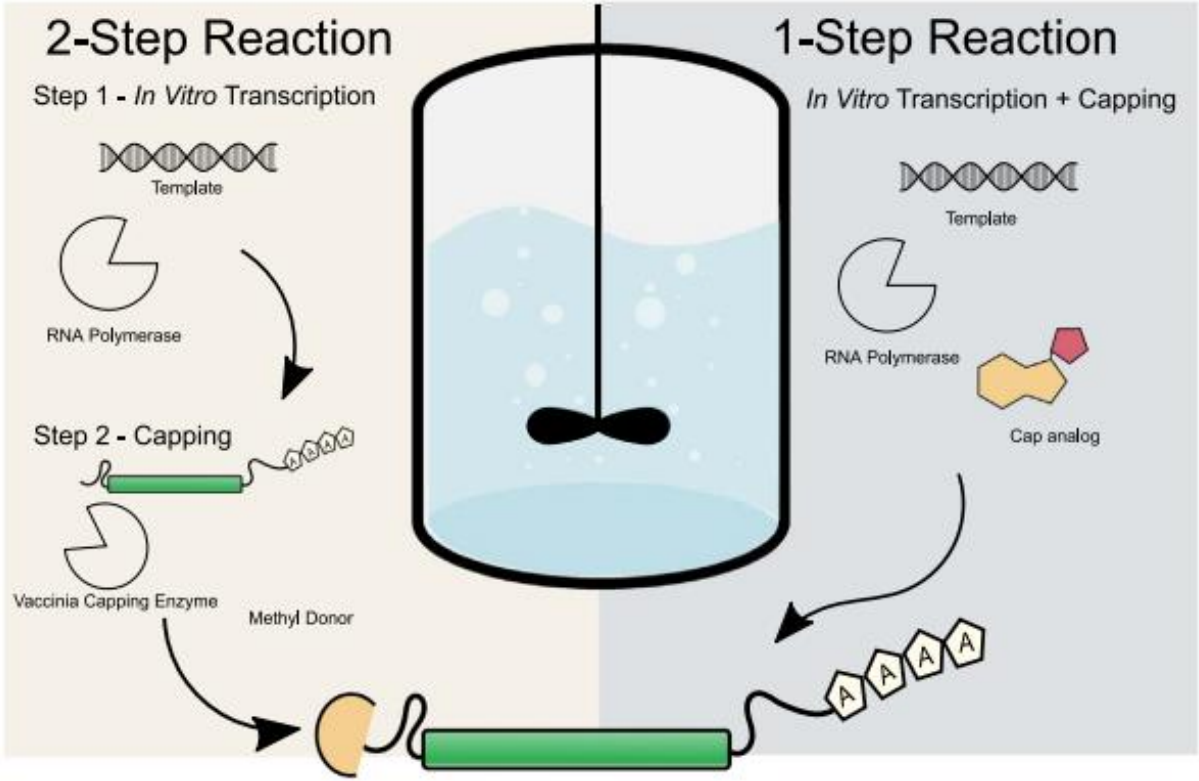
DSP IPC analytics
- Purity
- Content of mRNA

DS analytics
- Content
- Purity

Sekirnik and Kostelec
'Chromatography in mRNA Production Workflow'
BPI December 2021

IVT reaction → converting pDNA to RNA

- Multi component reaction:
  - Plasmid (dsDNA)
  - RNA polymerase (e.g. T7)
  - NTPs (optional modified NTPs)
  - Capping reagent (optional)
  - $MgCl_2$
  - Pyrophosphatase (optional)
  - RNAse inhibitor
  - Spermidine
  - DTT
  - Buffer

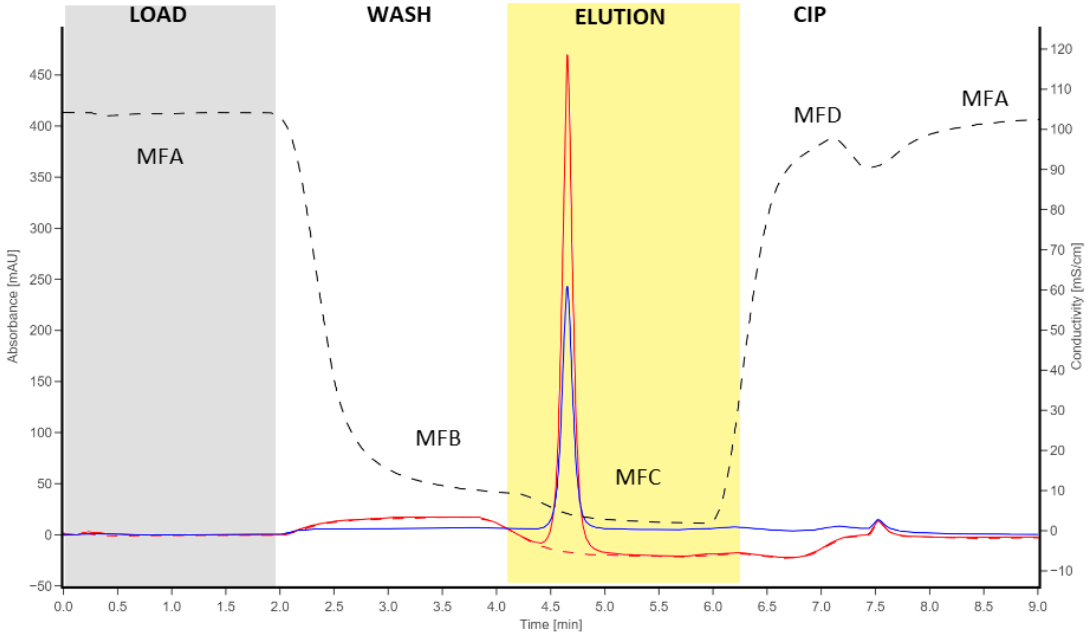


from Rosa, S. S., et al., Vaccine. 2021 Apr 15; 39(16): 2190–2200.

Monitoring of IVT: two paradigms for rapid at-line analytics

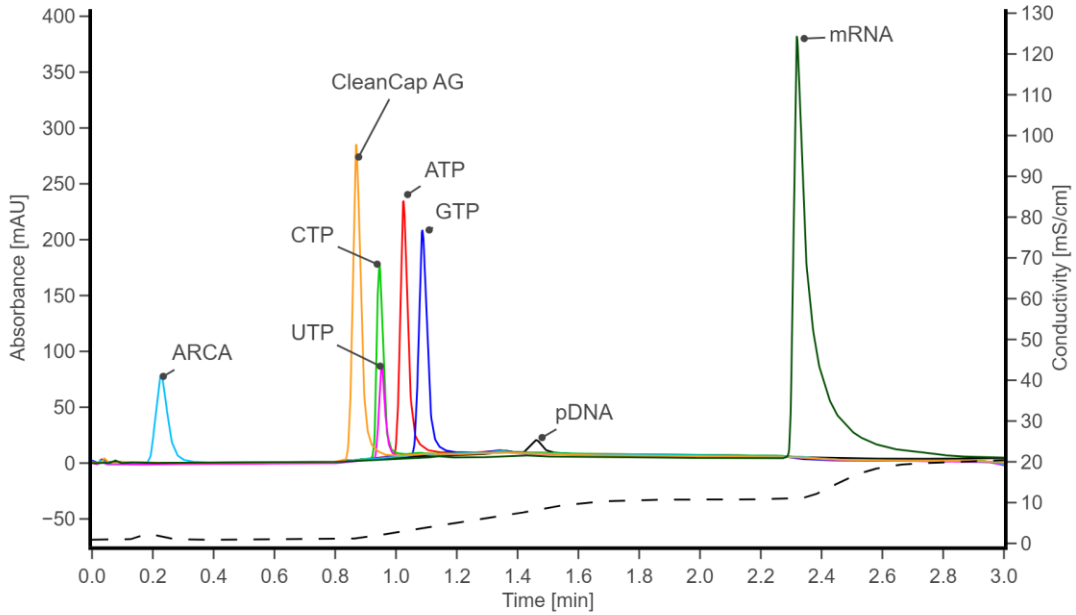
CIMac Oligo dT - affinity

- One-parameter-at-a-time, faster than Ribogreen
- ‘Protein A – mAb’ paradigm for mRNA
- Titre of polyadenylated mRNA throughout process



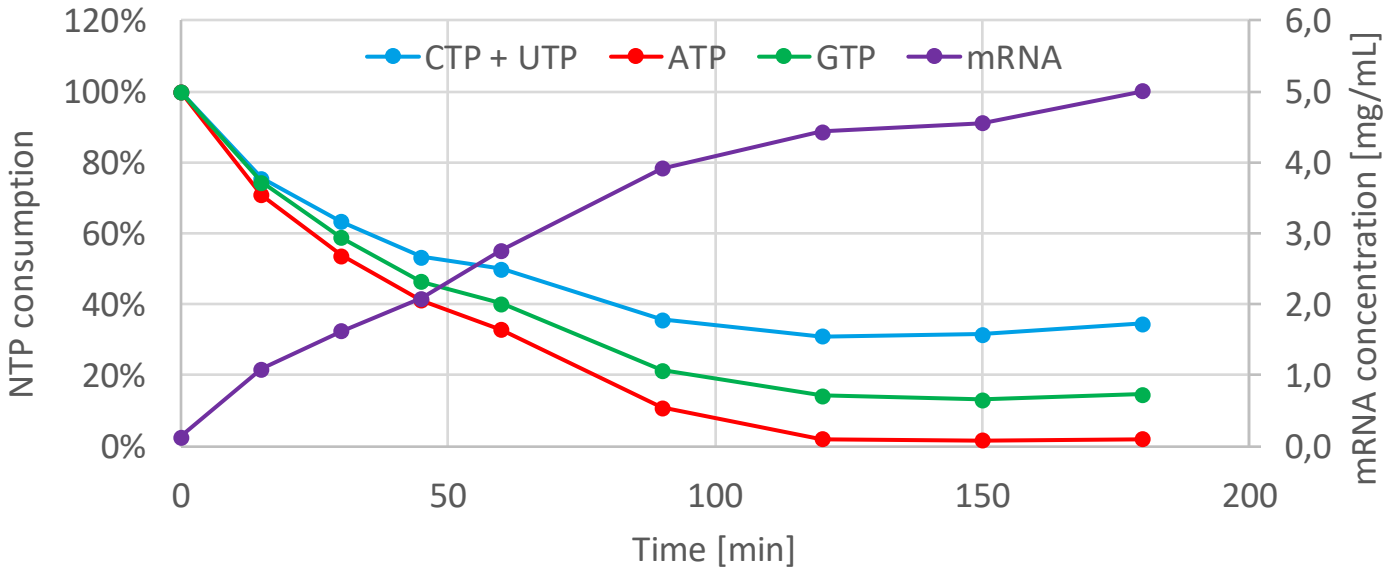
CIMac PrimaS - multimodal

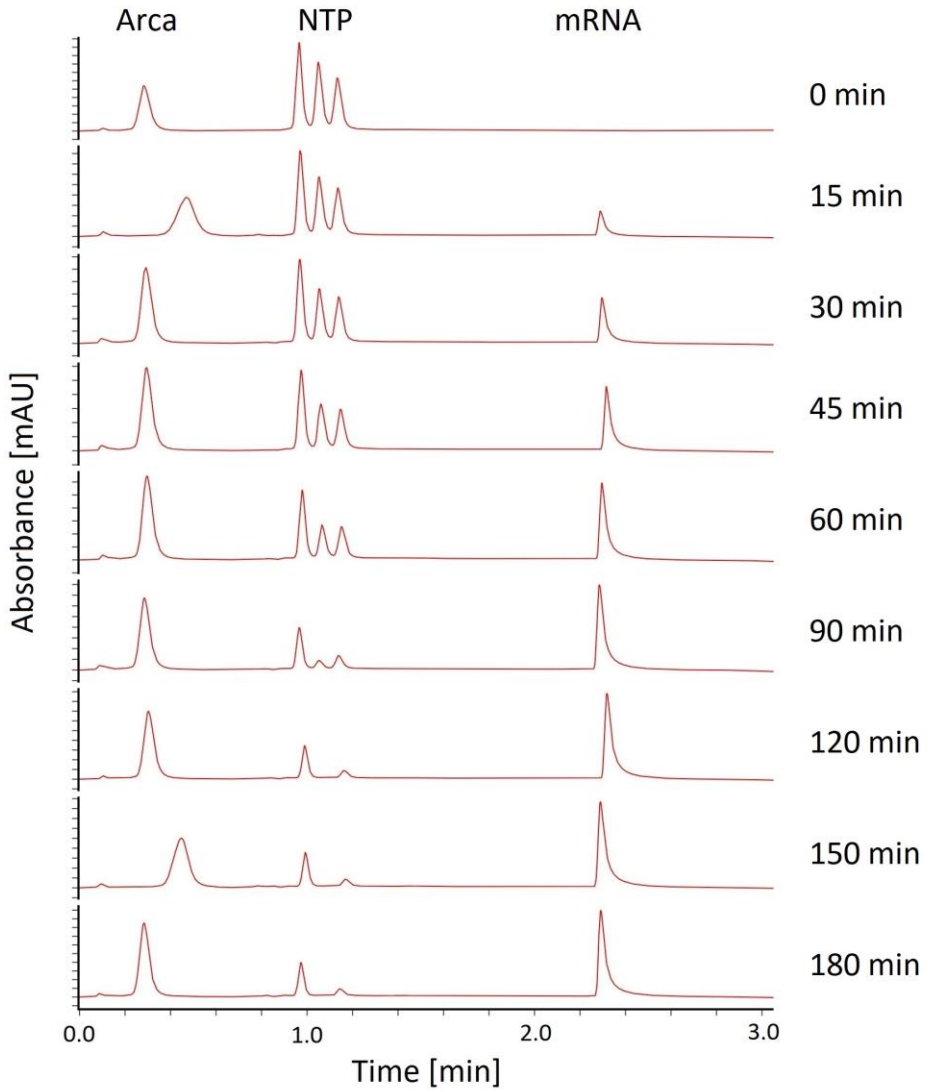
- Multi-parameter method
- New paradigm for mRNA
- NTP, capping, RNA content. Applies to all RNA modalities



Building Up IVT Understanding With Analytics

- The IVT reaction can be monitored at-line **by CIMac PrimaS**
- mRNA production kinetics is monitored. Productivity maximum can be identified, to prevent degradation.
- Consumption of nucleotides and concentration of capping reagent can simultaneously be studied
- Effects of feed addition can be studied





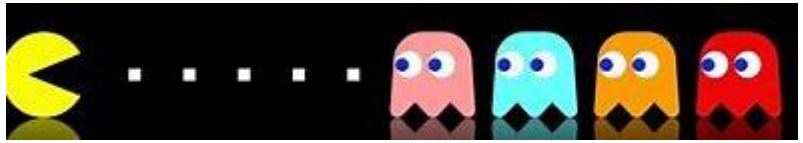
CIMac PrimaS: Capping Efficiency (with Enzymatic Digestion)

IVT reaction

IVT with capping reagent, e.g. ARCA or CleanCap

Systematic study of variables, e.g. GTP:ARCA ratio, addition of NTPs

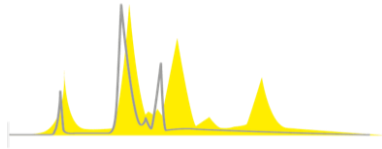
5’ polyphosphatase + terminator exonuclease



Enzymatic treatment selectively degrades uncapped mRNA

(concept based on Chiron and Jais, 2016)

CIMac PrimaS

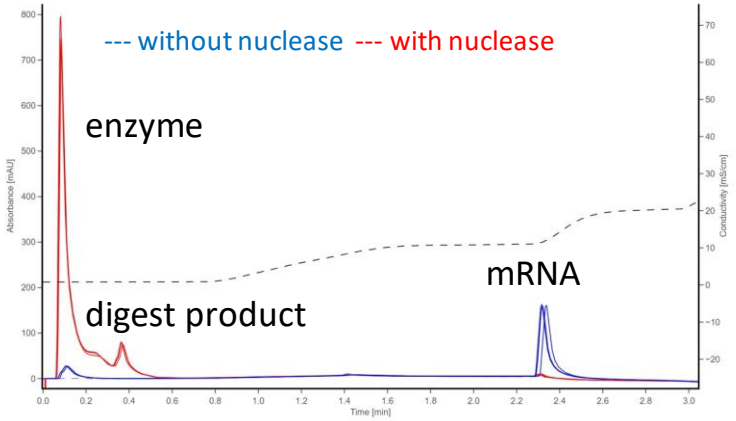


Quantification of undigested mRNA by HPLC

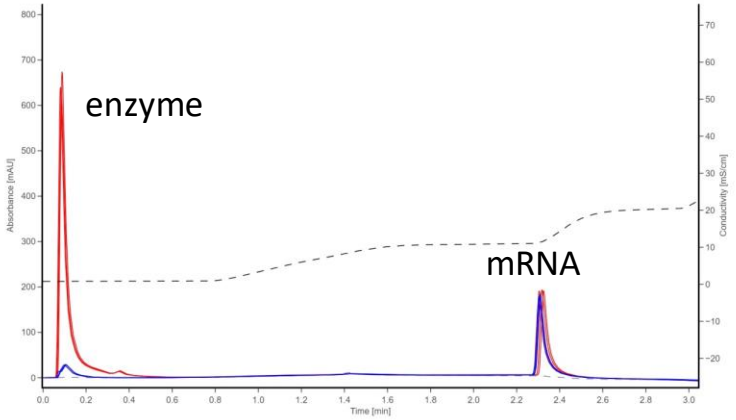
Calculation of %undigested mRNA by comparison to no-enzyme control

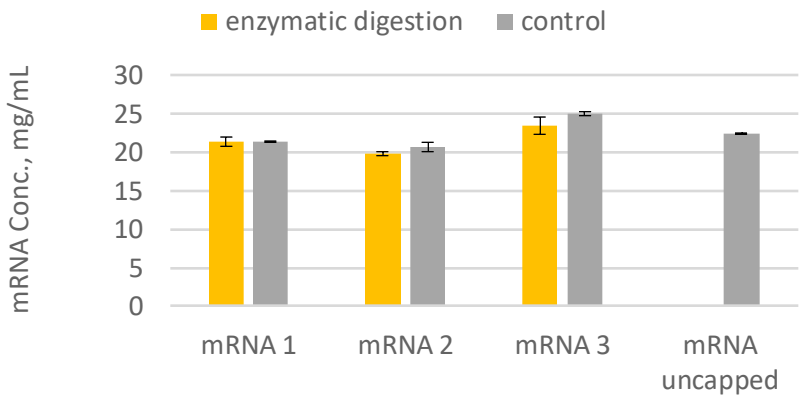
Capping analysis of mRNA

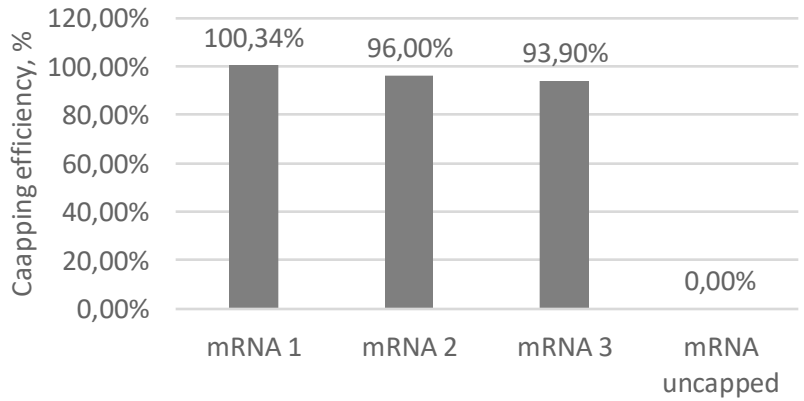
Uncapped mRNA



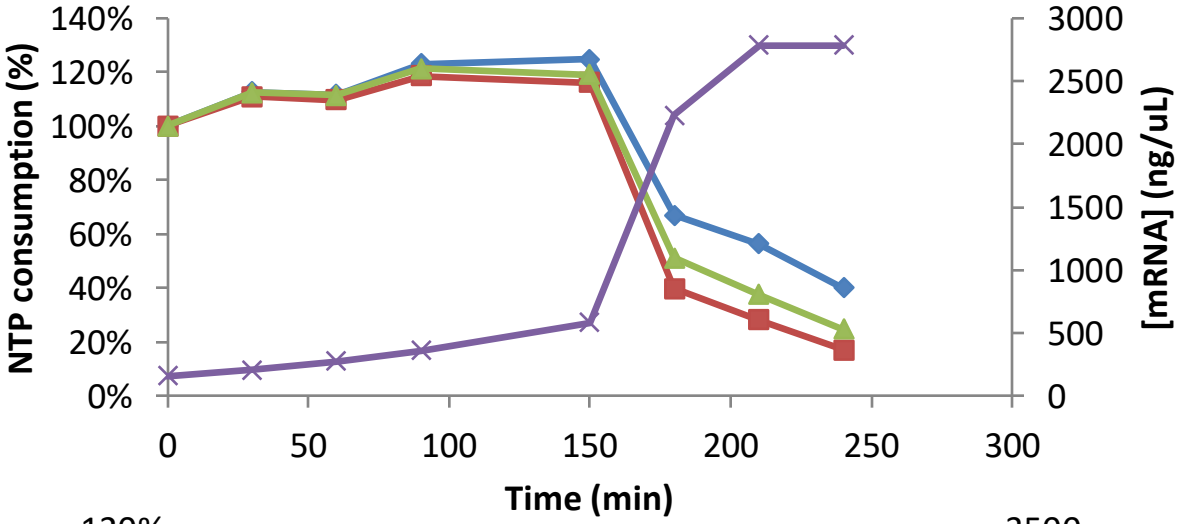
Capped mRNA
CleanCap AG

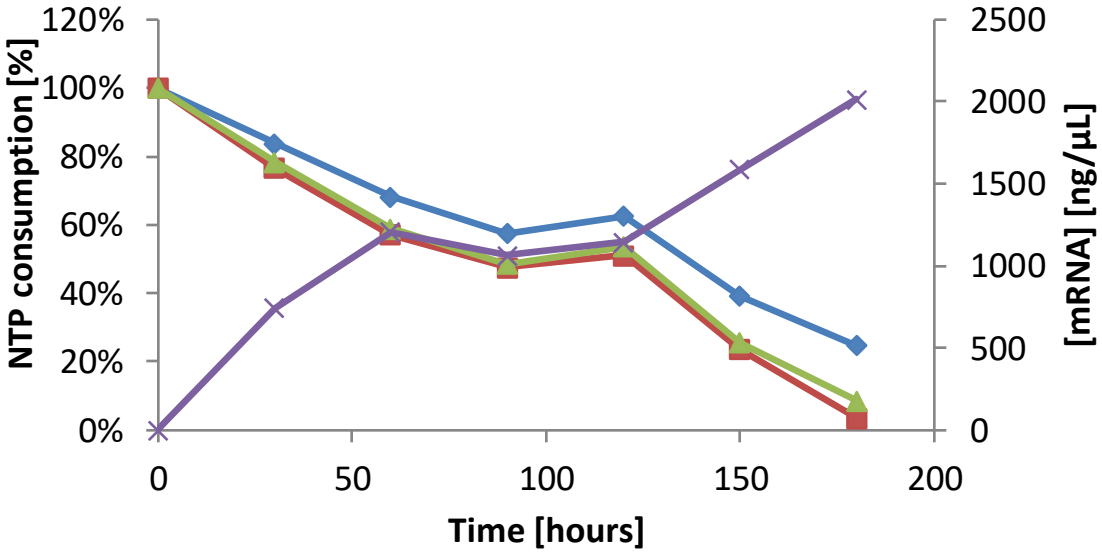


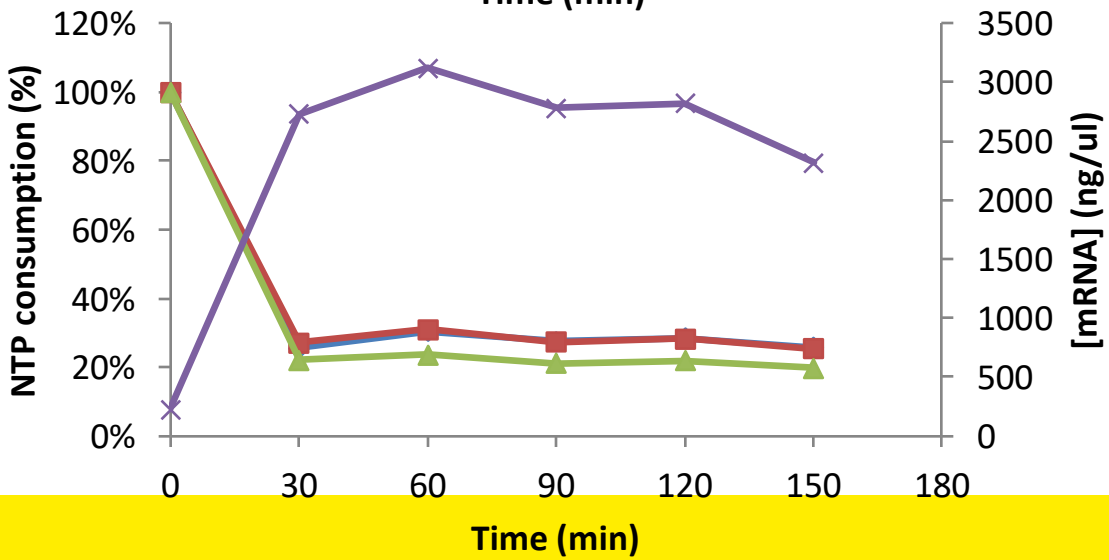


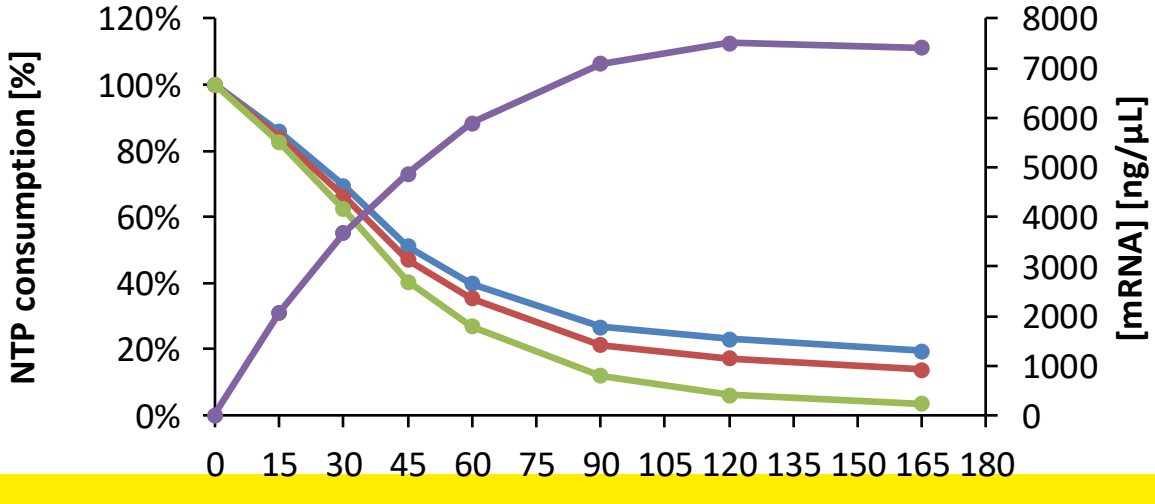


Playing the IVT game with CIMac PrimaS gaming console

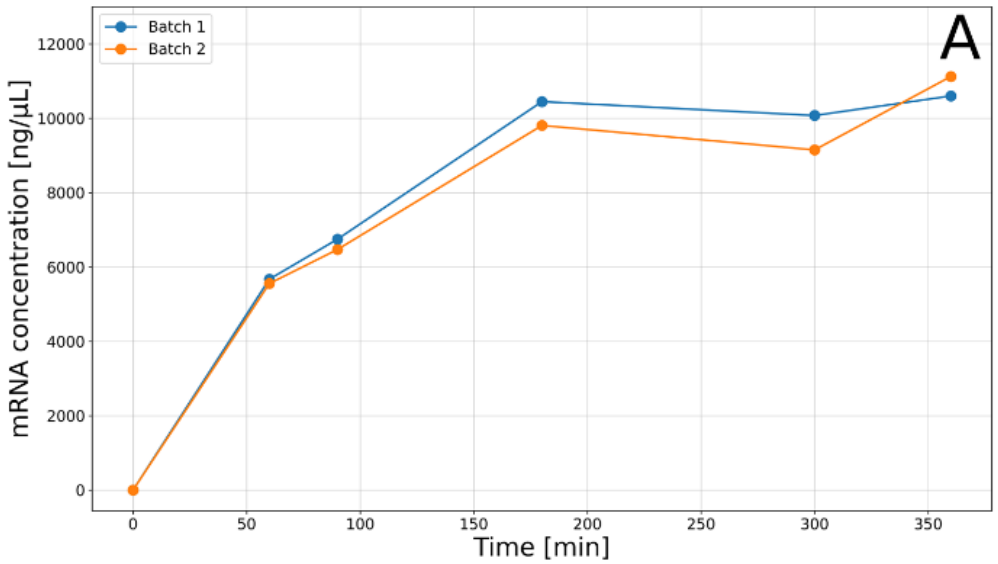


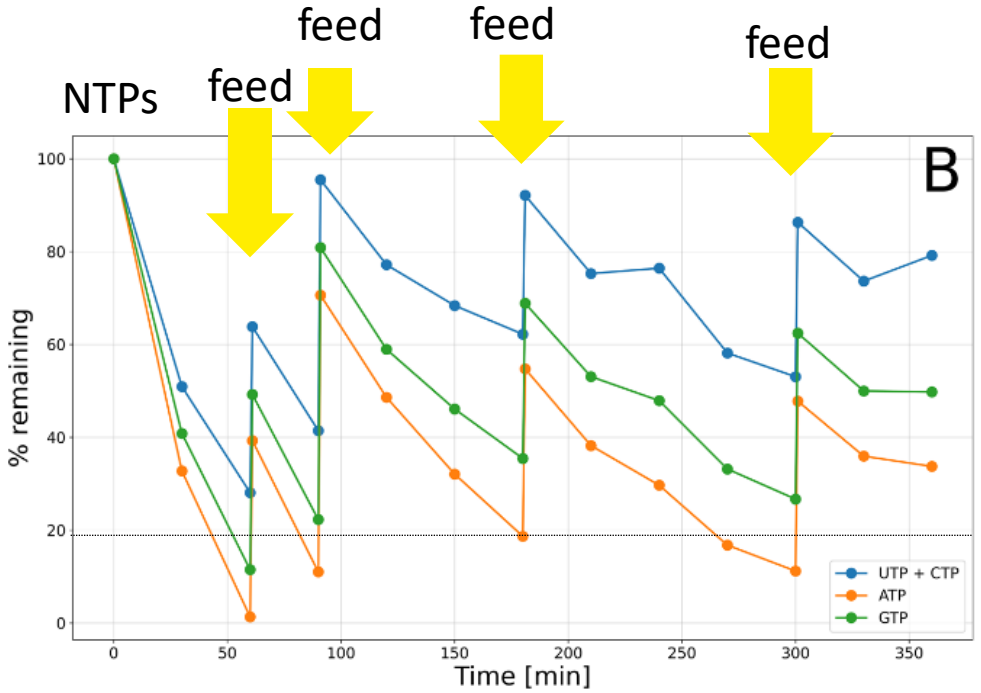






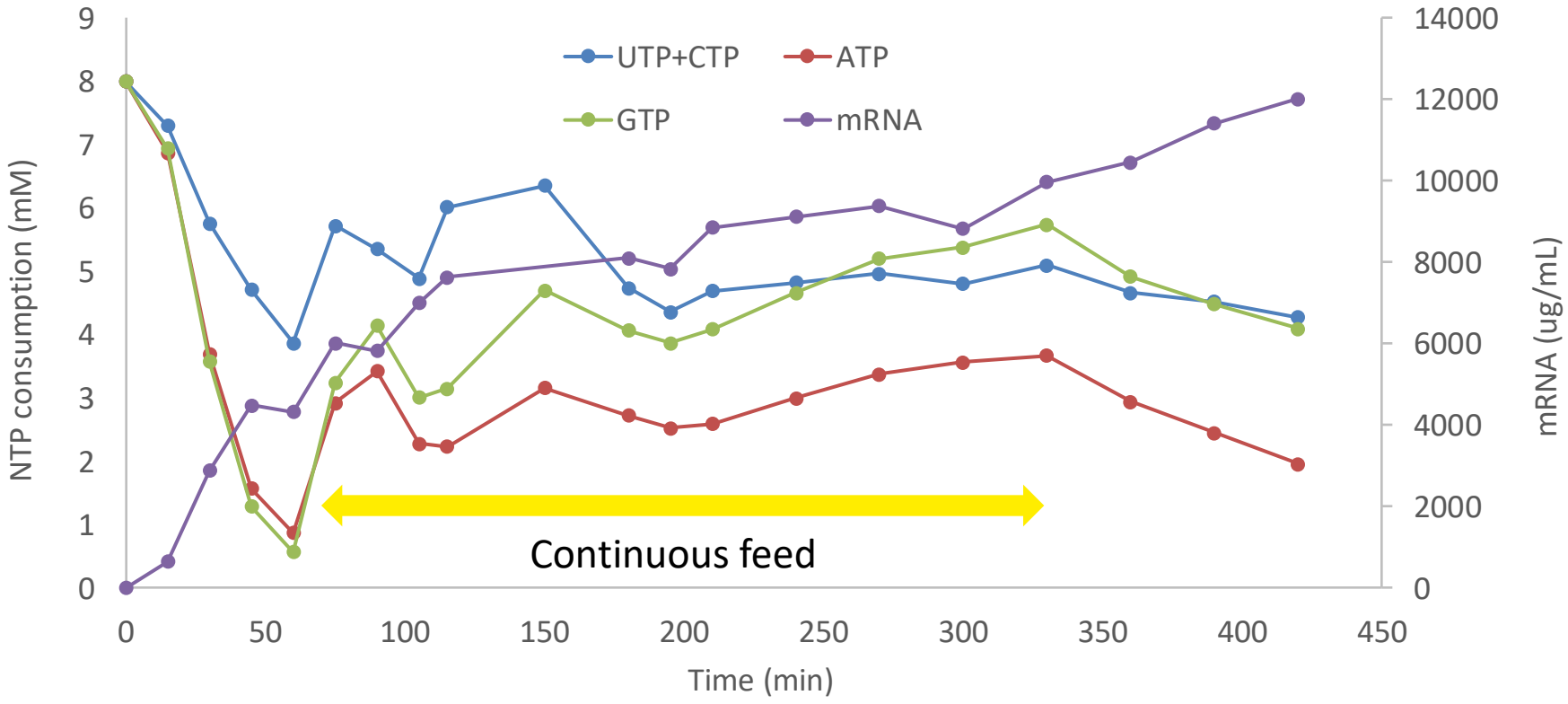
Batch to fed-batch – monitoring NTPs and mRNA





- Monitor depletion of NTPs; react with feed addition
- Control scale-up of IVT reaction
- Control tech transfers
- Calculate kinetics of NTP consumption → transform to continuous feeding (e.g. AMBR250)

Scaling up mRNA production with HPLC support



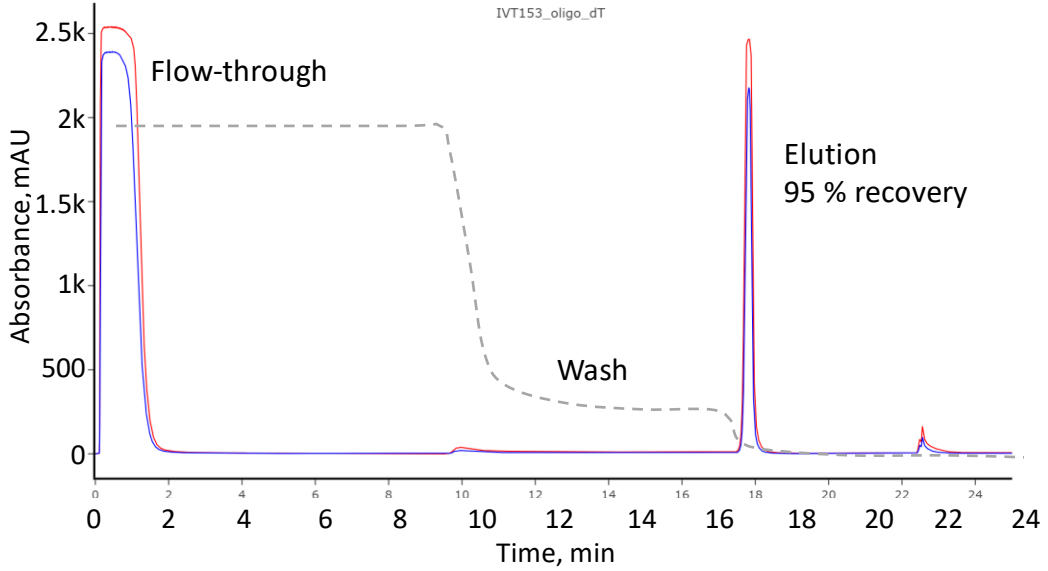
AMBR250

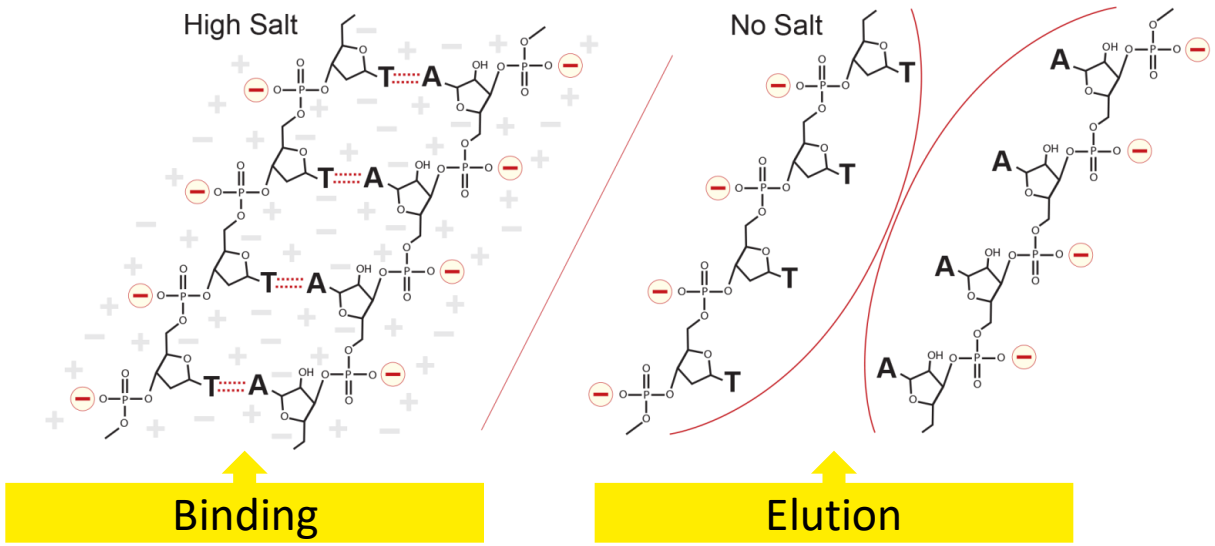
+

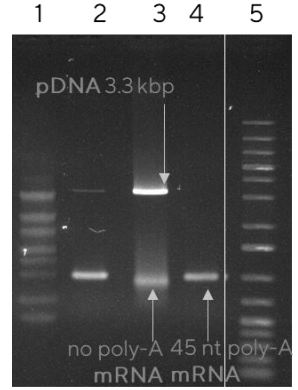
PATFix

- 2 g of mRNA produced in a single batch (11 mg/mL yield) by coupling automated bioreactor system (AMBR250) with PATFix HPLC analytics

Affinity Capture of polyadenylated mRNA from IVT by Oligo dT18





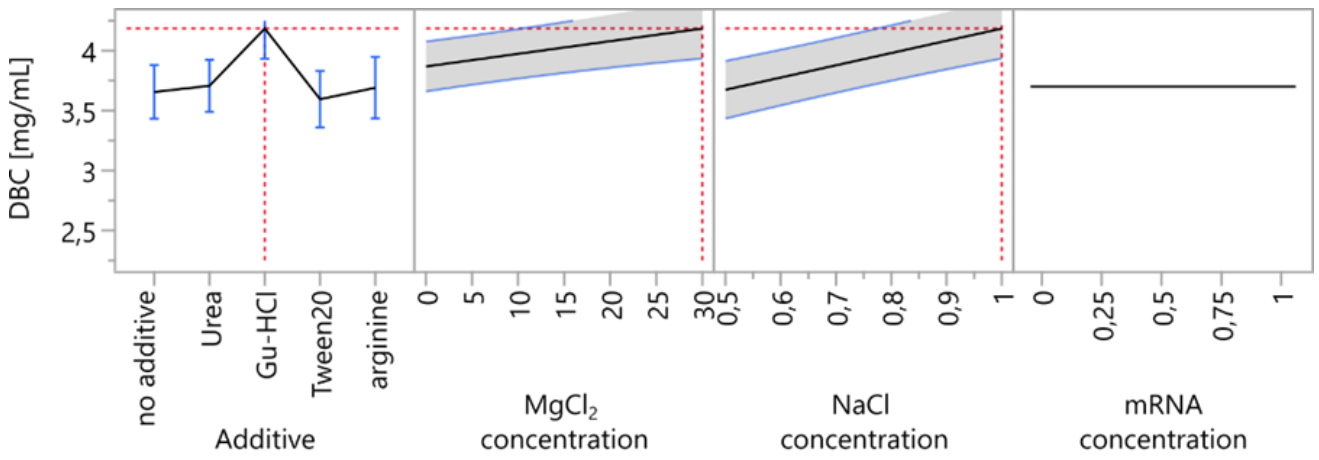


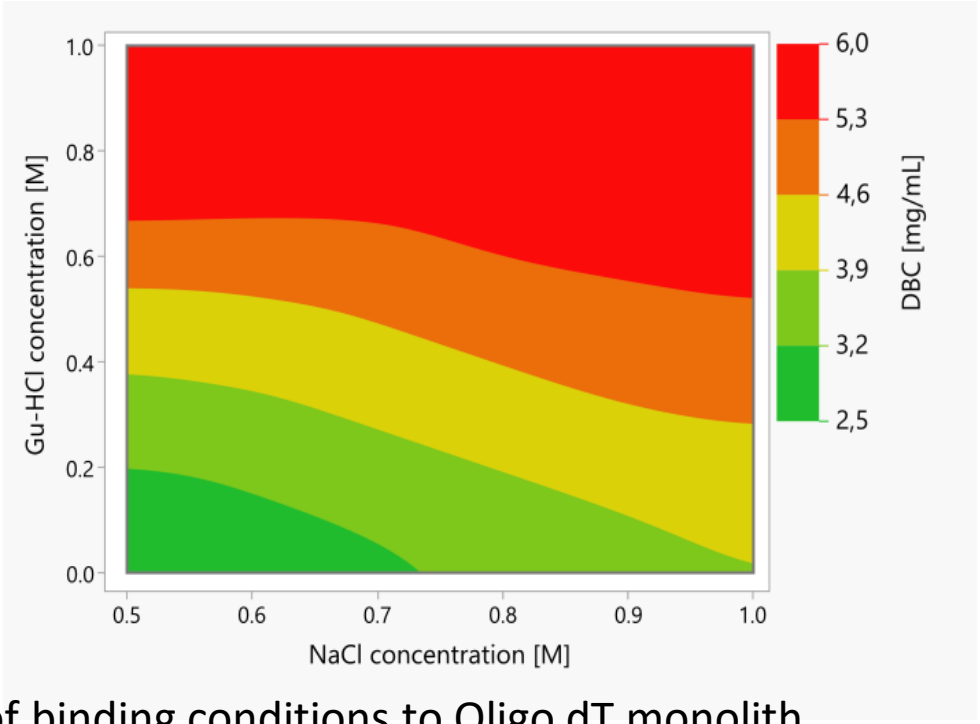
AGE

1 RiboRuler HR
2 IVTmix
3 Flow-through (concentrated)
4 Elution
5 GeneRuler 1kb Plus

- Binding in moderate NaCl concentrations (250 mM – 1.5 M)
- Dynamic Binding Capacity with NaCl 3-4 mg/mL
- Elution in low concentration buffer or in water

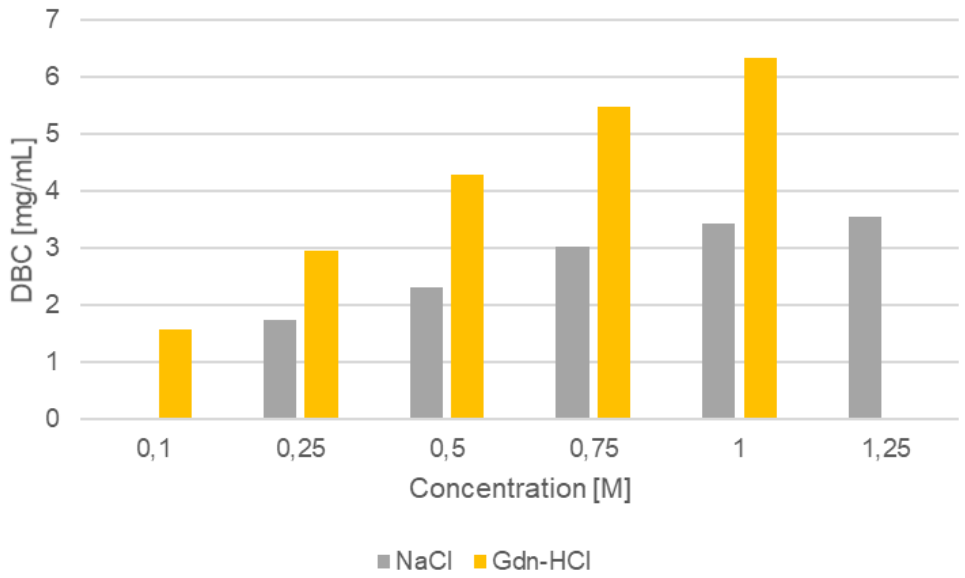
Increasing binding capacity of Oligo dT18 for mRNA

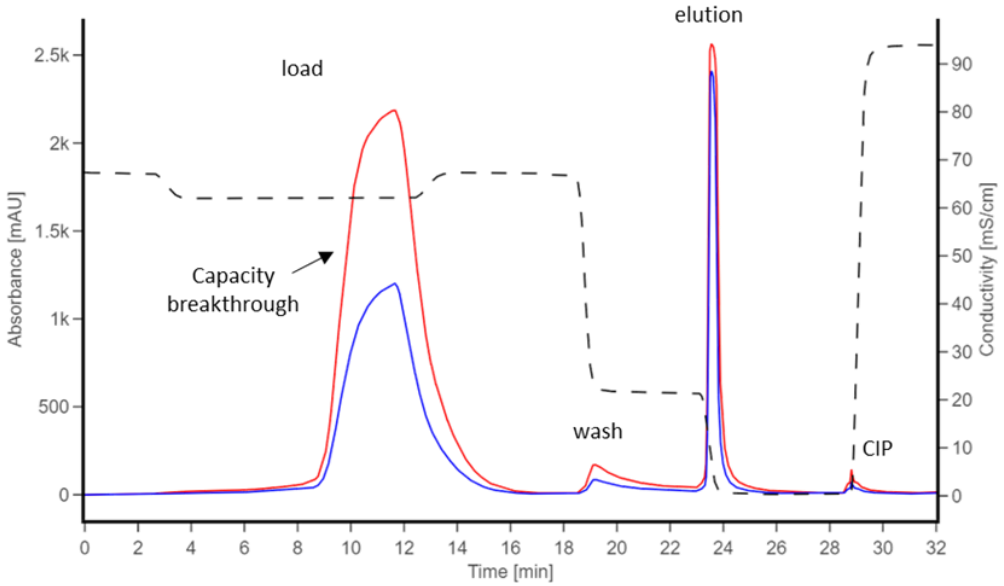




- CIM® Oligo dT18 0.05 mL 96-well lates used for multi-parallel screening of binding conditions to Oligo dT monolith.
- Three main contributing factors to DBC identified: NaCl, Gu-HCl, $MgCl_2$; effect of Gu-HCl not described before
- DoE suggested capacity of 6 mg/mL can be achieved on Oligo dT
- Contour plots of Gu-HCl/NaCl indicated a significantly more pronouced effect on DBC of Gu-HCl than NaCl

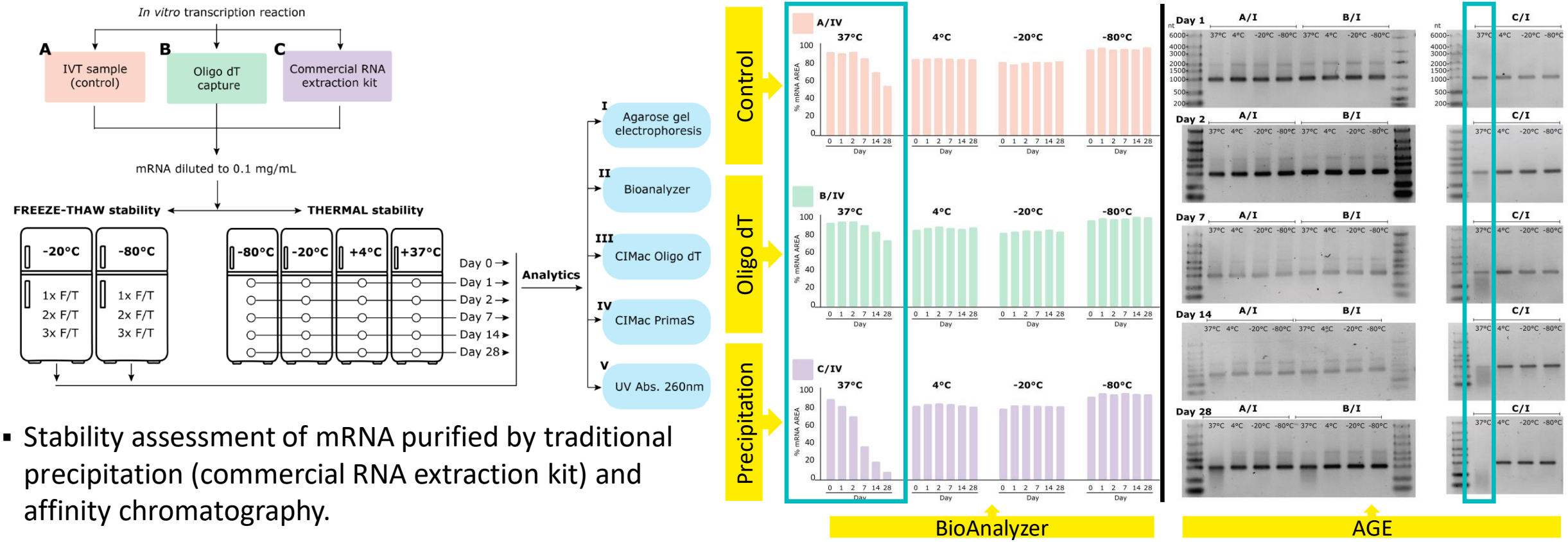
Up to 6 mg/mL binding capacity reached with CIMmultus Oligo dT





- Titration of guanidinium vs NaCl confirmed a stronger effect of Gdn (higher chaotropicity)
- DBC >6 mg/mL demonstrated in 96-well format for loading mRNA in guanidinium hydrochloride
- Transfer to axial monolith chromatography (CIMmic 0.1 mL) resulted in DBC 5.5 mg/mL
- Scale-up to CIMmultus Oligo dT: mRNA diluted in 0.75 M Gu-HCl, 5.5 mg/mL determined by UV in elution

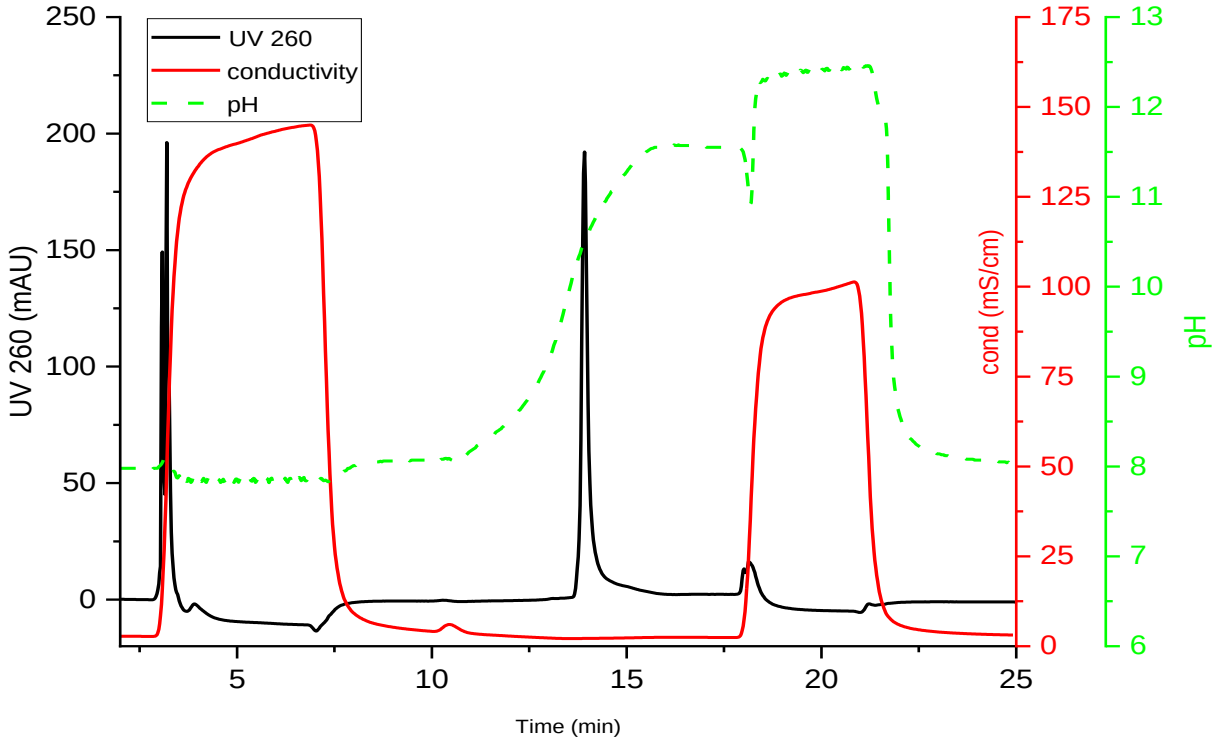
Oligo dT purification increases mRNA Stability Post-Capture

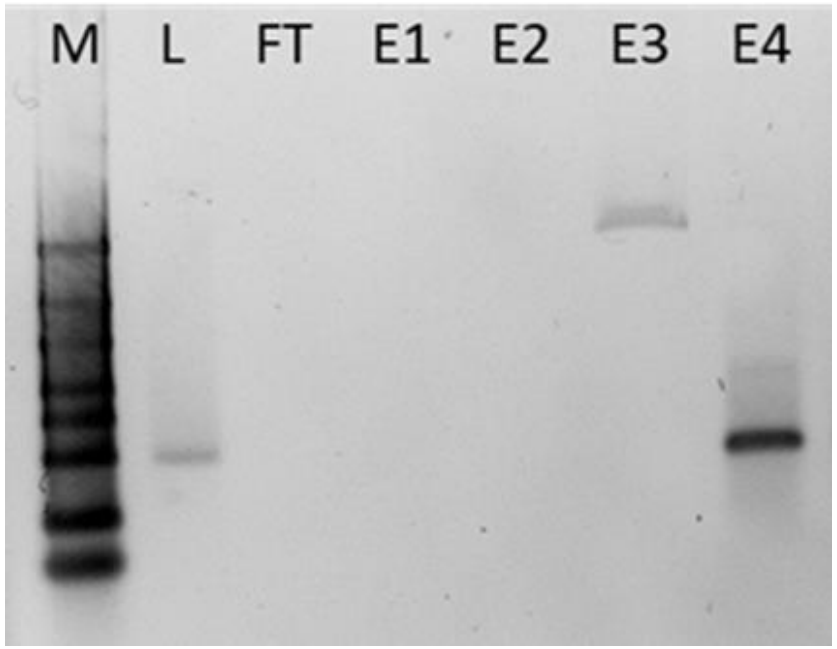


- Stability assessment of mRNA purified by traditional precipitation (commercial RNA extraction kit) and affinity chromatography.

Korenč, M. et al, Chromatographic purification with CIMmultus™ Oligo dT increases mRNA stability, Cell & Gene Therapy Insights 2021; 7(9), 1207–1216

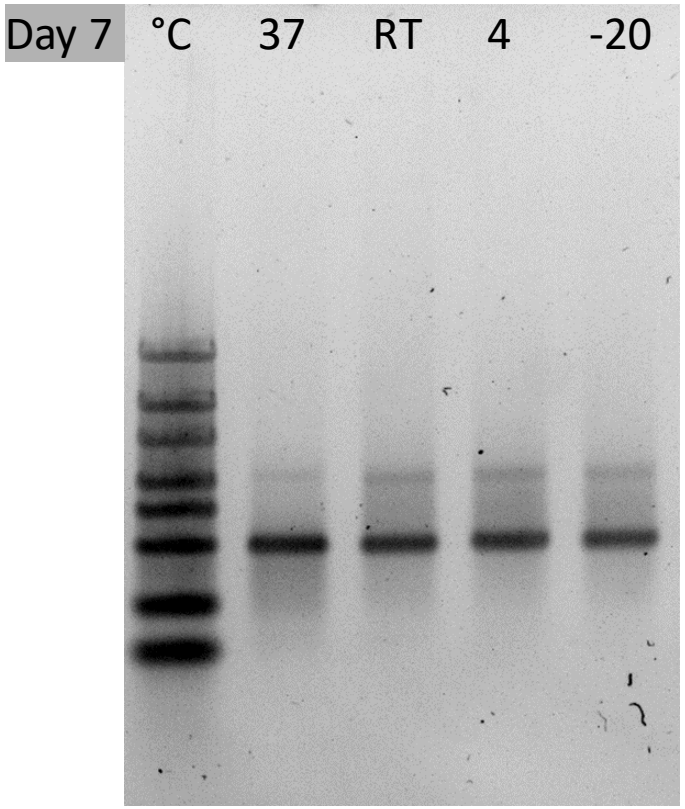
Non-affinity capture of RNA from IVT with PrimaS

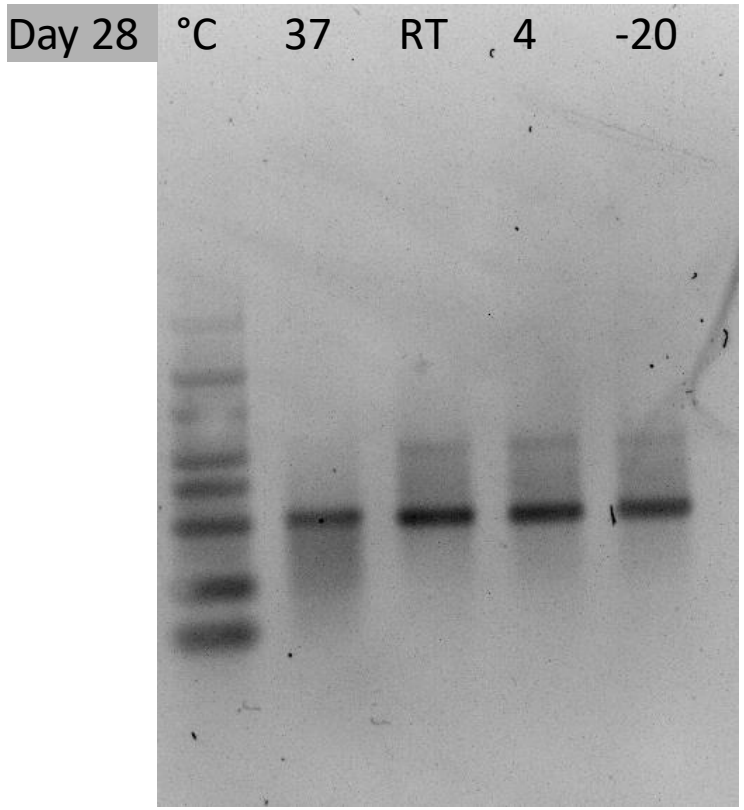


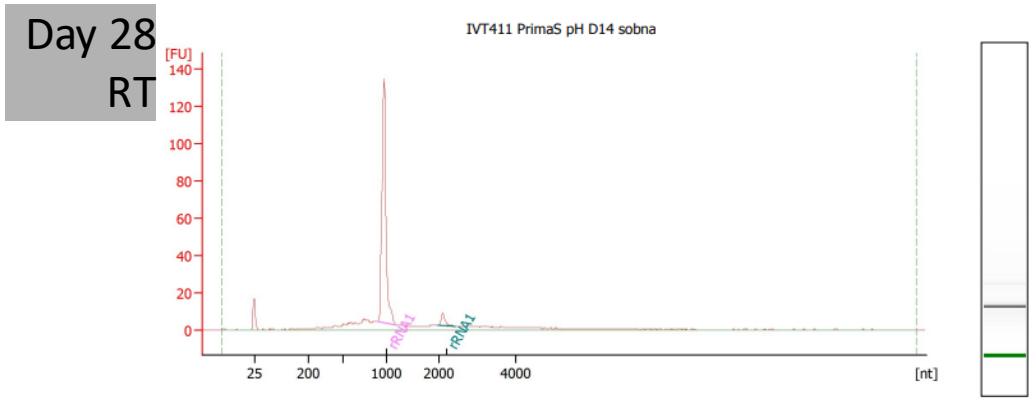


- Elution at pH gradient (or step) can separate mRNA from IVT components.
- Capture of mRNA without poly A tail, saRNA, circRNA, protein & plasmid clearance
- Robust, IVT is applied to the column after initial dilution with loading buffer.

mRNA Stability After PrimaS pH Gradient Elution

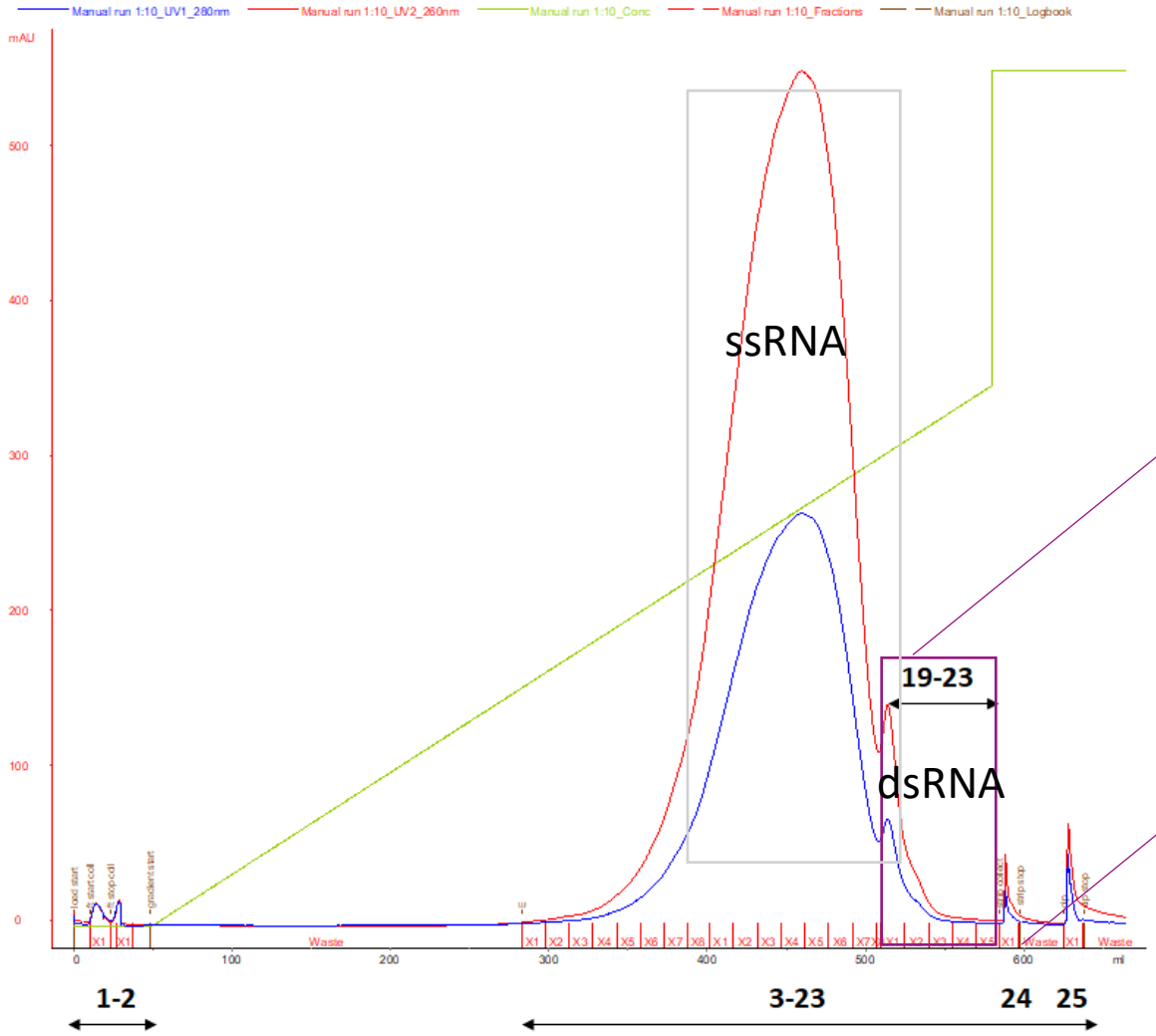


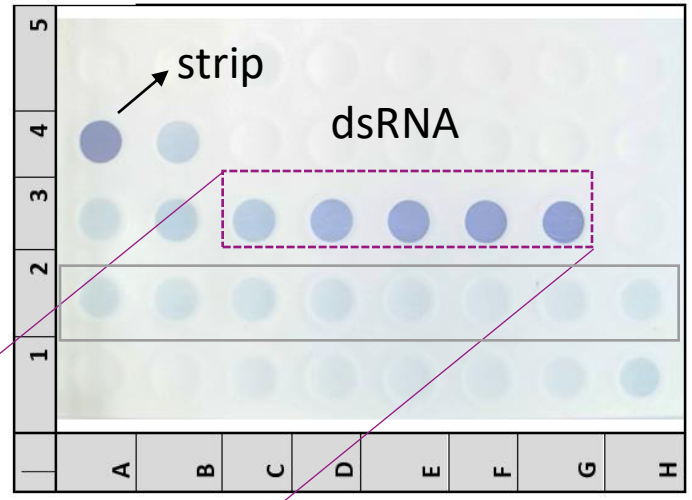




- mRNA is stable for at least 28 days at RT after purification with CIMmultus PrimaS performed with pH gradient followed by neutralization in KOAc

Removal of dsRNA by reverse-phase chromatography (CIMmultus SDVB)



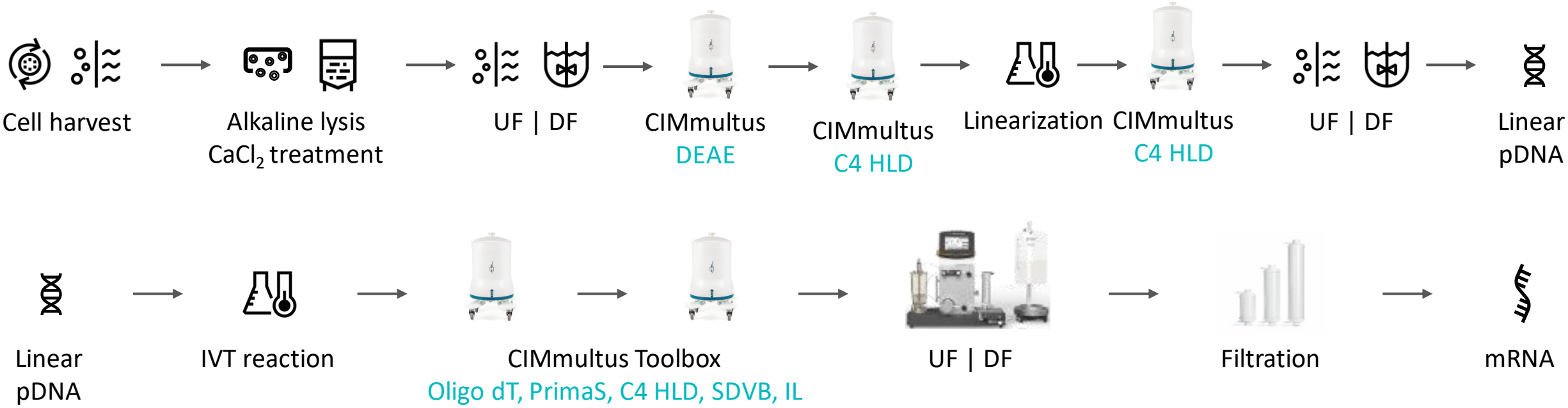


ssRNA (main elution)

- CIMmultus Oligo dT-purified Cas9 mRNA (4000 nt)
- Loaded onto CIMmultus SDVB 8 mL
- Room temperature separation 7.5→18% ACN in 50 mM TEAA, pH=7.0
- Removal of dsRNA demonstrated by J2-dot-blot

mRNA Drug Substance Production workflow

Analytical workflow (PATfix pDNA and CIMac pDNA)



Analytical workflow (CIMac PrimaS, CIMac Oligo dT, CIMac SDVB)

Big thank-you to the wonderful PC2 team

Thank you!

Rok Sekirnik
Head Process Development mRNA/pDNA
Rok.sekirnik@biaseparations.com