BIA separations
Leaders in Monolith Chromatography

Simplifying Progress

High yield mRNA production process from E. coli to highly pure mRNA – platform 2

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February 2021

BIA separations

SARTORIUS

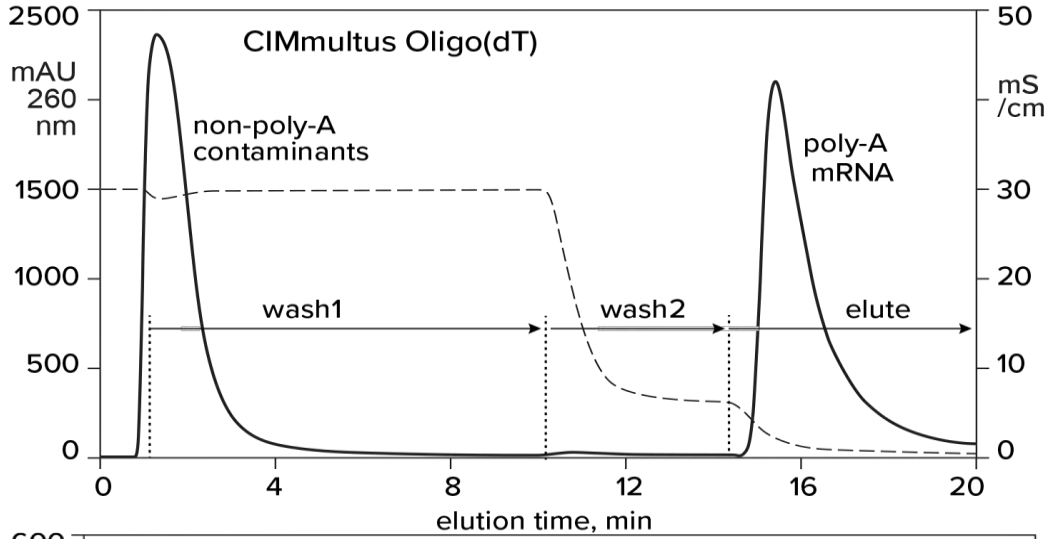
Expert DSP bioprocess knowledge:

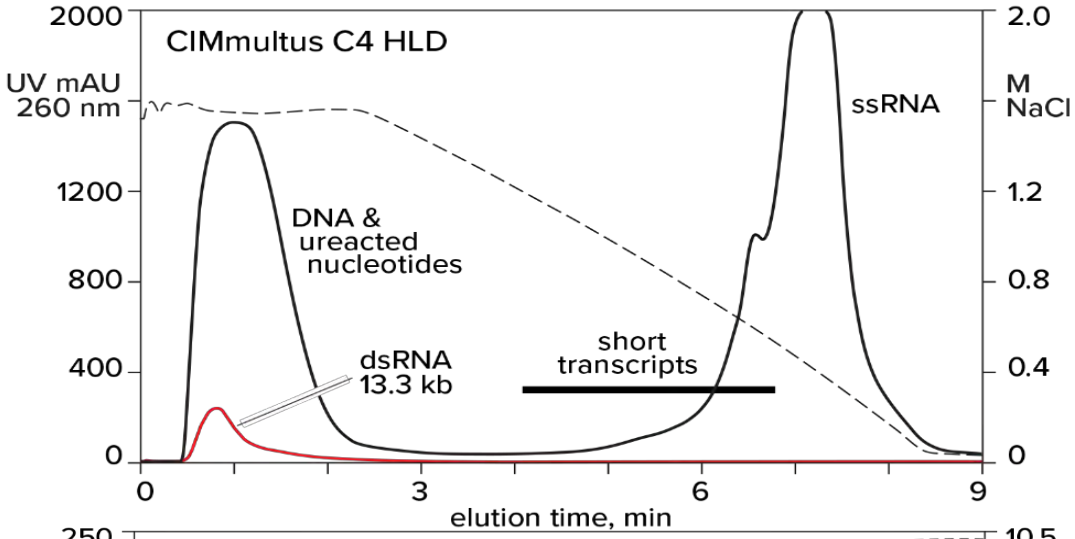
- **30 DNA, RNA, virus DSP processes tech transferred to CMOs, sponsors**

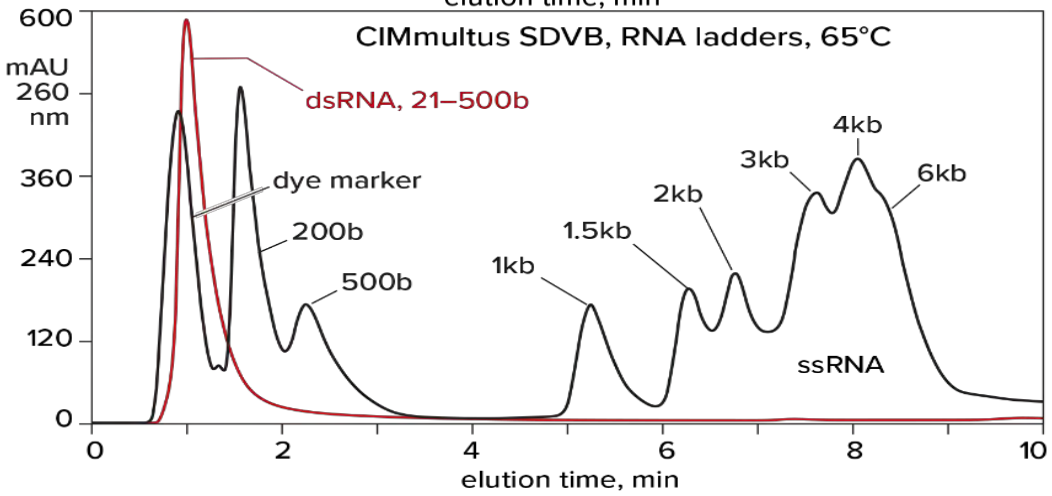
- **10 AAV DSP processes tech transferred to CMOs, sponsors**

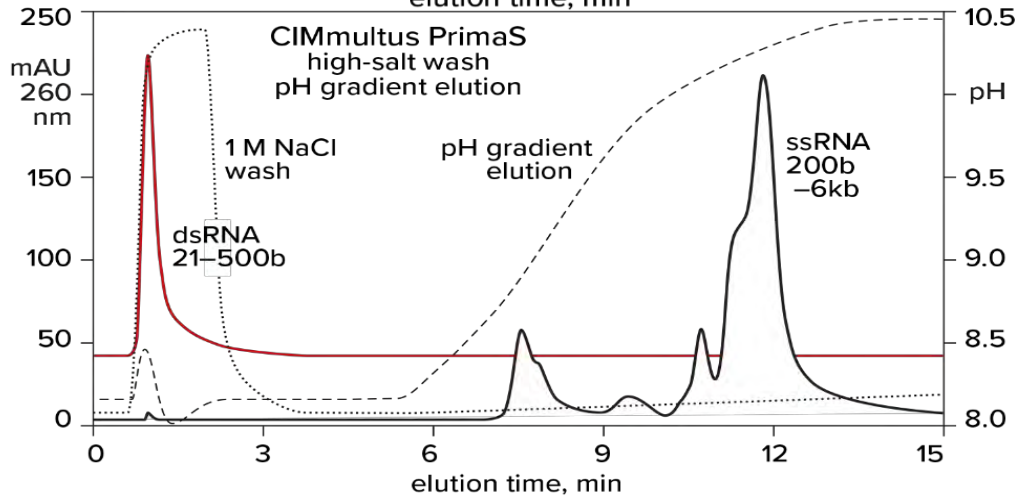
- pDNA (incl. plasmids larger than 30 kbp) - **pure pDNA, THE key for better transfection and for pure mRNA**
- mcDNA (shorten the pDNA)
- ssRNA and dsRNA, **platform process from E.coli to mRNA**
- Adeno virus
- AAV (**all serotypes, > 20 tested**)
- Influenza virus (all serotypes)
- Vaccinia/MVA
- **Exosome**
- Bacteriophage
- IVIG
- IgM and many more

Chromatographic tools for mRNA analytics and purification









BIA separations
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Simplifying Progress

From *E.coli* to pure mRNA

BIA separations

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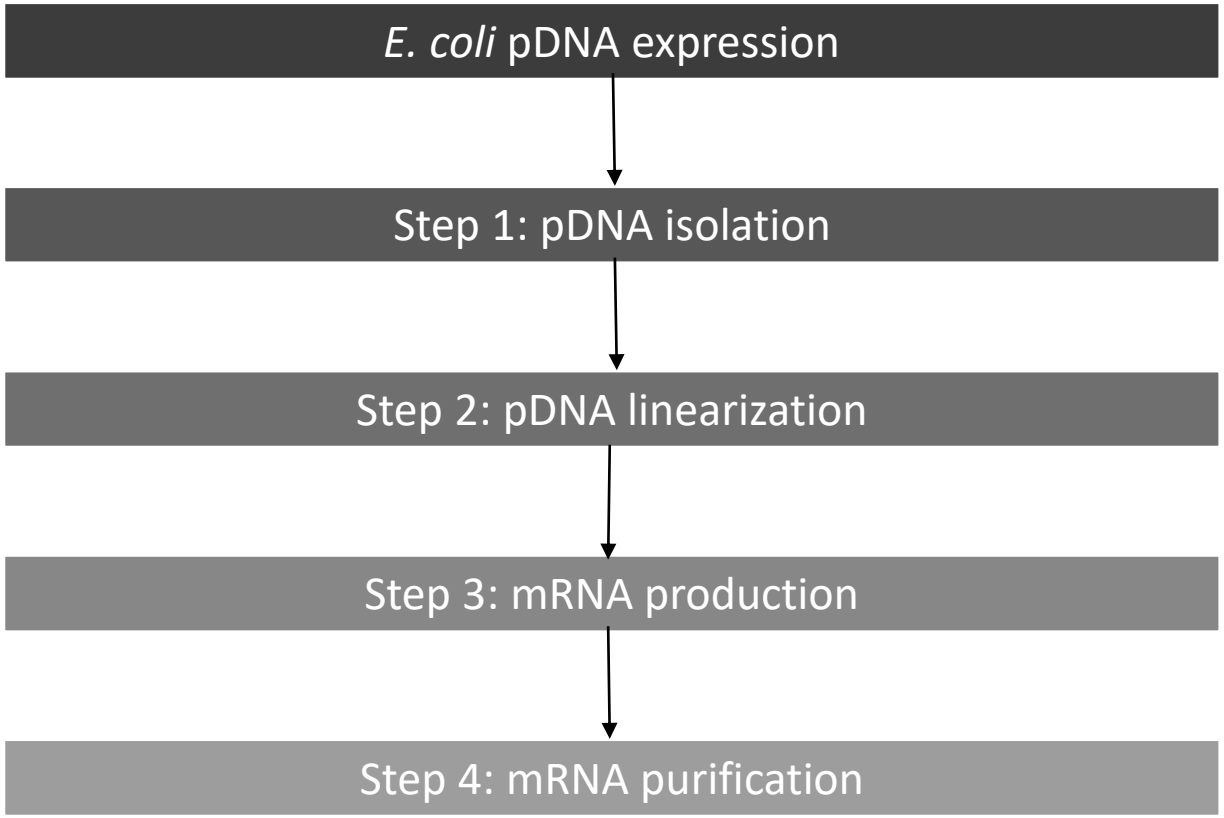
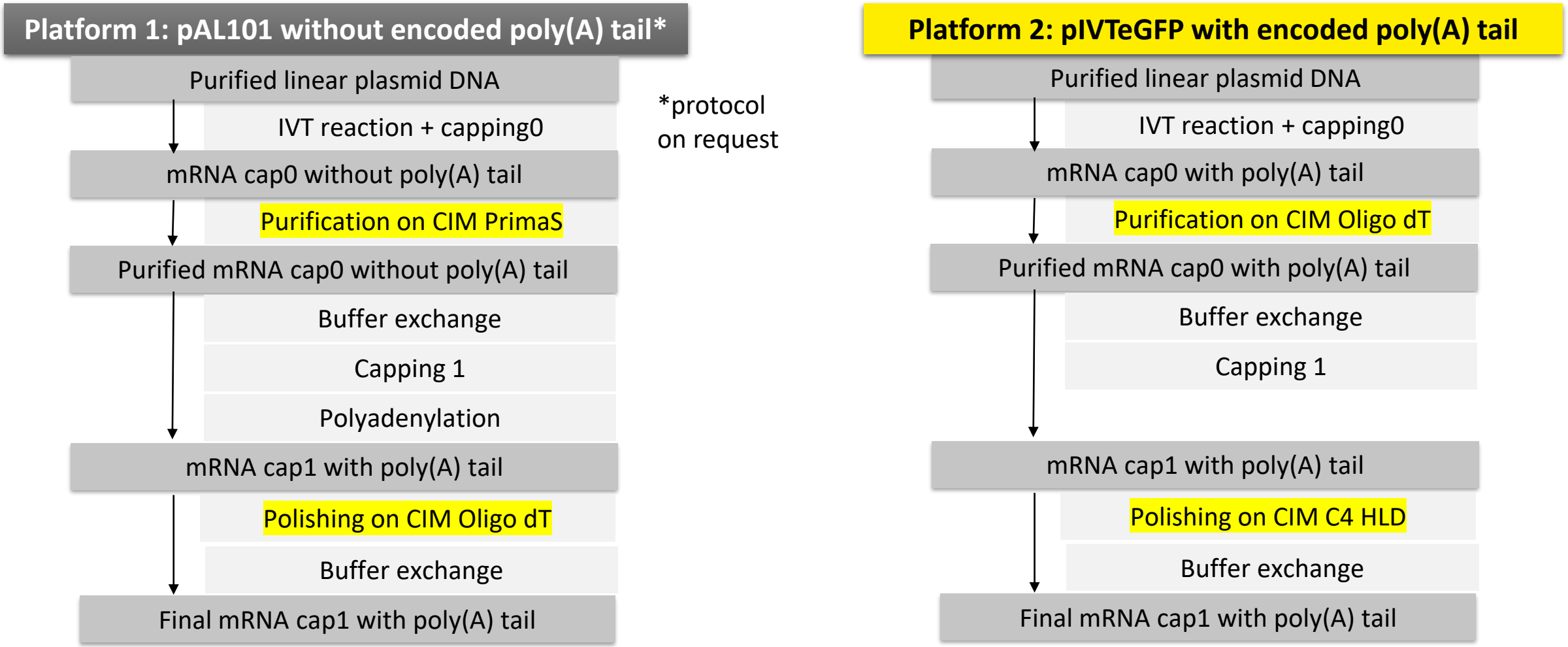
From *E. coli* to mRNA



mRNA production and purification workflow (4 major platforms)



Preparative LC methods conditions

pDNA purification:

Column**: CIMmultus DEAE 1 mL**

- Method: step 3 min MPA, 10 min step 50% MPB, 15 min linear gradient from 50% to 100% MPB, 3 min strip 100% MPB, flow 1 mL/min
  - Mobile phase A (MPA): 50mM Tris 10mM EDTA pH 7.2
  - Mobile phase B (MPB): 50mM Tris 10mM EDTA 1M NaCl pH 7.2

Linear pDNA purification:

Column: **CIMmultus™ C4 HLD 1 mL**

- Method: 15 min MPA, 10 min step 36% MPB, strip 100% MPB, flow 1 mL/min
  - Mobile phase A (MPA): 50mM Tris 10mM EDTA 2.5M AS pH 7.2
  - Mobile phase B (MPB): 50mM Tris 10mM EDTA pH 7.2

Preparative LC methods conditions

mRNA purification:

Column: **CIMmic™ Oligo dT 18 C12 0.1mL**

- Method: 8 min 100% MPA, 8 min step 100% MPB, 5 min 100% MPC
  - MPA: 50mM Na-phosphate, 500mM NaCl, pH 7.0
  - MPB: 50mM Na-phosphate, pH 7.0
  - MPC: 10mM Tris, pH 7.2

mRNA polishing:

Column: **CIMmic C4HLD 0.2 mL**

- Method: 6min step 80% MPA, 5 min linear gradient from 80 % MPA to 0% MPA, 5 min step 0% MPB
  - MPA: 50 mM TRIS-HCl, 3M NaCl, pH 7.2
  - MPB: 50 mM TRIS-HCl, pH 7.2

HPLC analytical methods conditions

pDNA analytics:

Column: **CIMac™ pDNA 0.3 mL**

- Method: 1.5 min step 26% MPB, 1.5 min linear gradient from 26% to 77% MPB, 8 min linear gradient from 77% to 92% MPB, 1 min step 100% MPB, flow 1 mL/min
  - MPA: 50 mM HEPES, 1% Tween, pH 7.5
  - MPB: 50 mM HEPES, 1M guanidine HCl, 1% Tween, pH 7.5

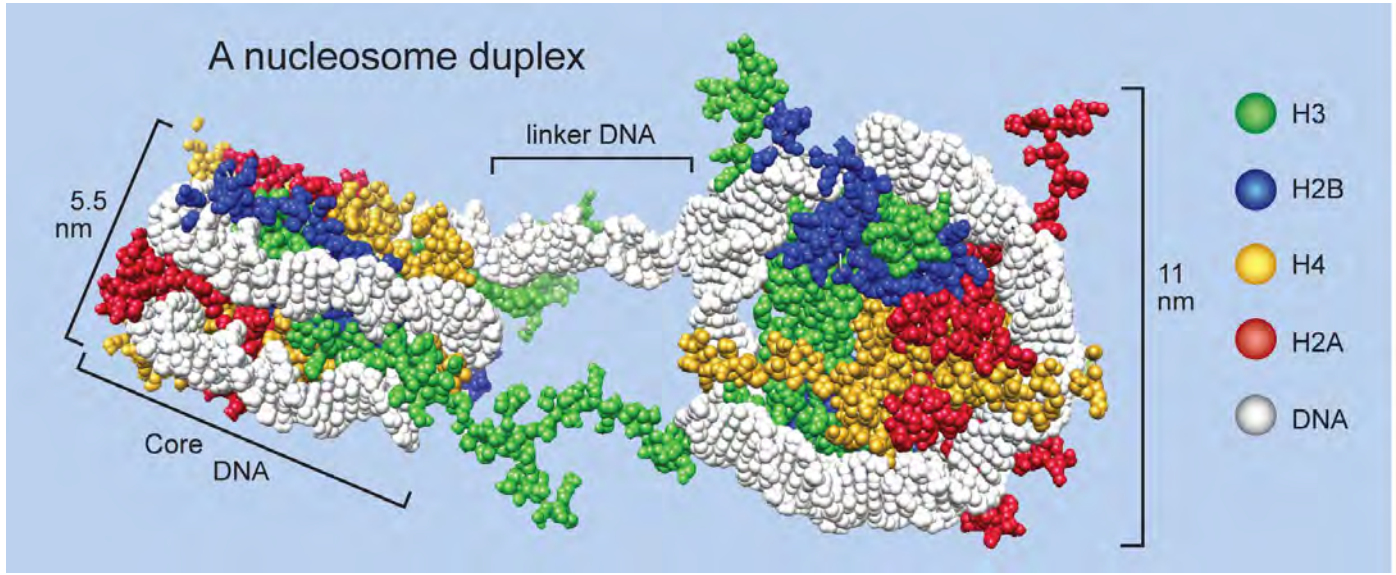
mRNA analytics:

Column: **CIMac™ PrimaS 0.1 mL**

- Method: 1 min linear gradient from 100% MPA to 80% MPA and 20% MPB, 0.8 min step 20% MPB and 80% MPA, 0.7 min step 50% MPC and 50% MPB, 0.6 min step 100% MPB, 0.9min step 100% MPC, 0.5min step 100% MPD, 1min step 100% MPA.
  - MPA: 50mM HEPES pH7
  - MPB: 50mM HEPES and 200mM sodium pyrophosphate pH 8.5
  - MPC: 0.1M NaOH 2M NaCl
  - MPD: 2M HEPES

Raw material purity – avoid using material with residual Chromatin/Nucleosome

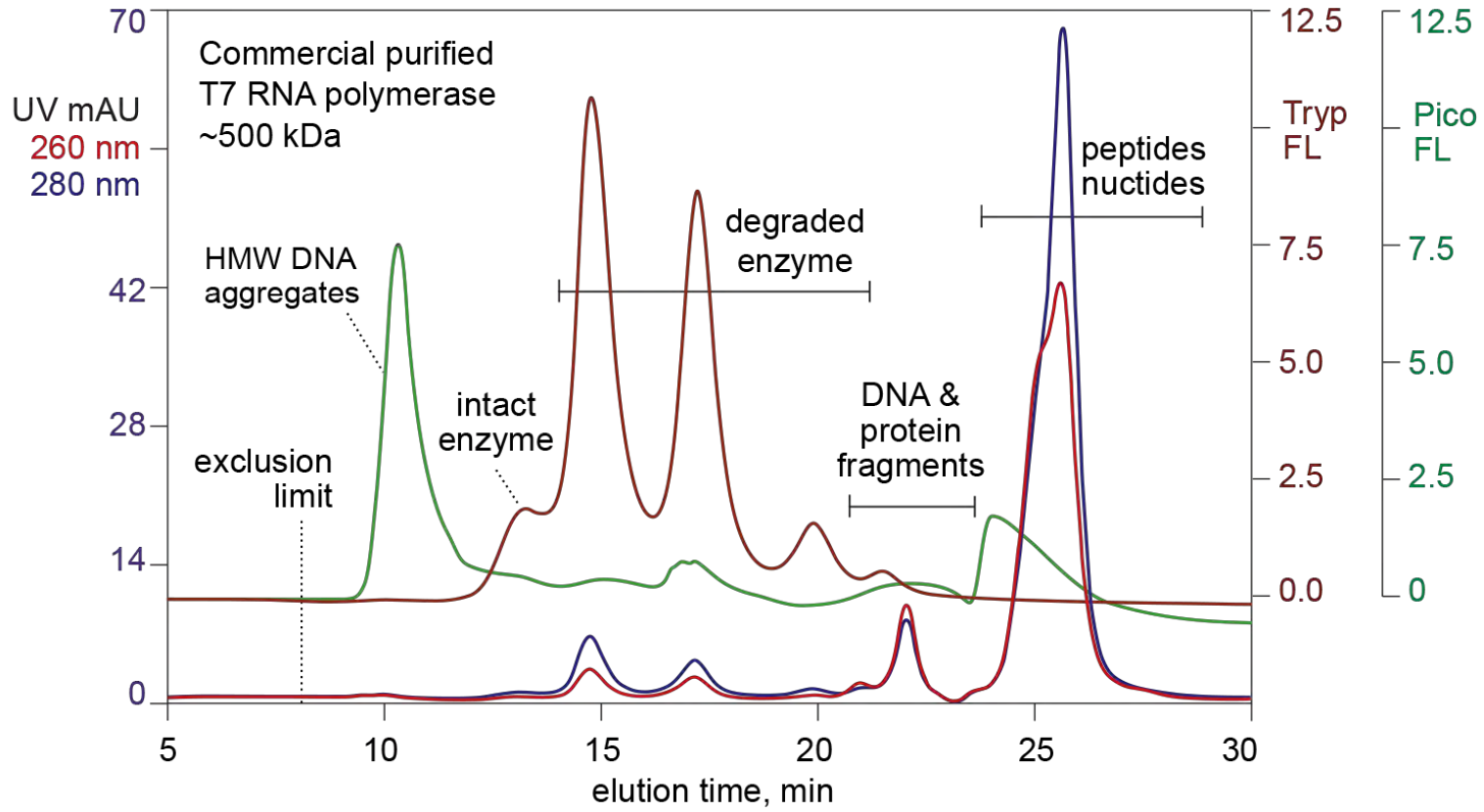
The basic structural subunit of chromatin is a nucleosome. It consists of a histone octamer wrapped with 1.6 turns of DNA; about 150 bp. RNA acts similar as the pDNA



Histones are extremely hydrophobic and highly positively charged, with isoelectric points ranging from 9 to 11. DNA has a pK of about 2.6.

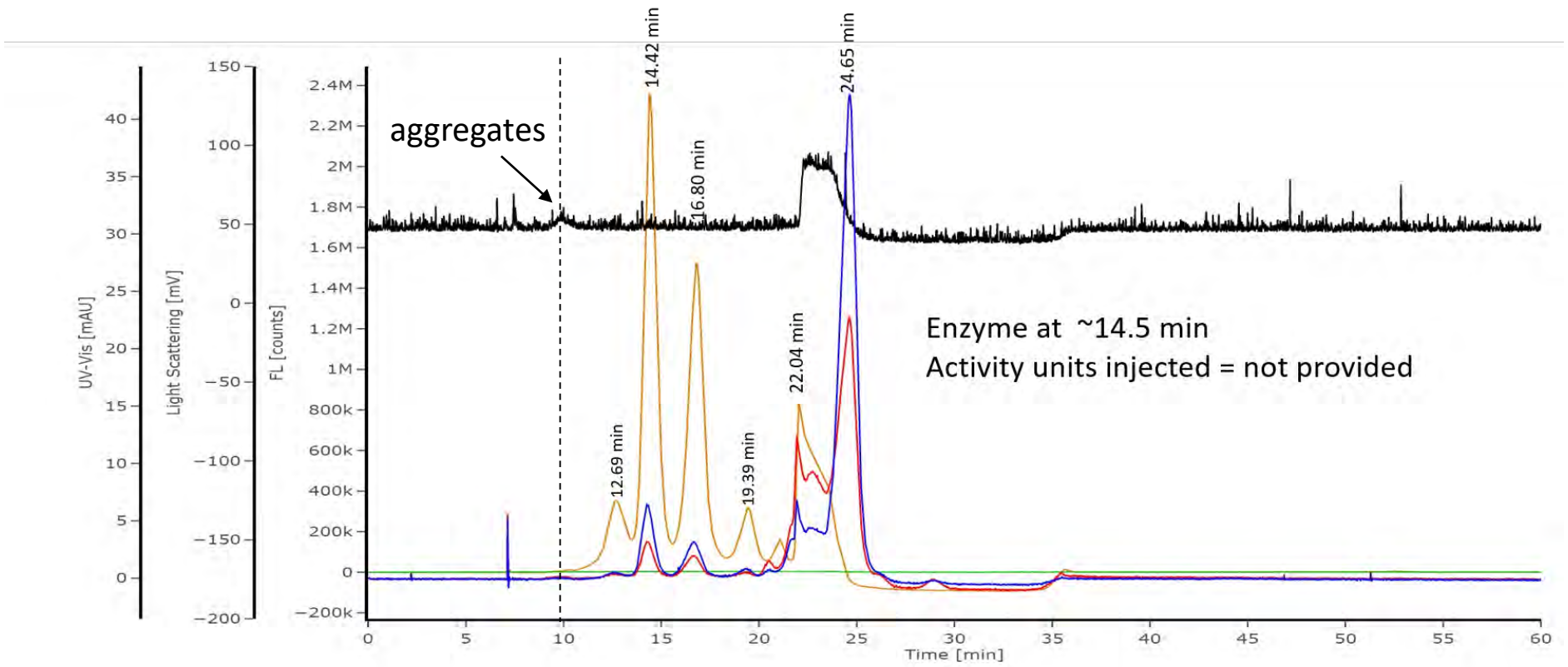
The net charge of **chromatin** is roughly neutral but its exposed components still retain their extreme charge characteristics. Both also participate in metal affinity, hydrogen bonding, and van der Waals interactions.

SEC using multidetector approach - very powerful tool to check Raw material purity



Size exclusion chromatography (SEC). TSKgel™ G3000SWxl. 0.5 mL/min. Sample pre-stained with **Picogreen fluorescence** increases sensitivity of DNA/RNA related impurities. **Tryptophan fluorescence** increases sensitivity of protein detection over UV.

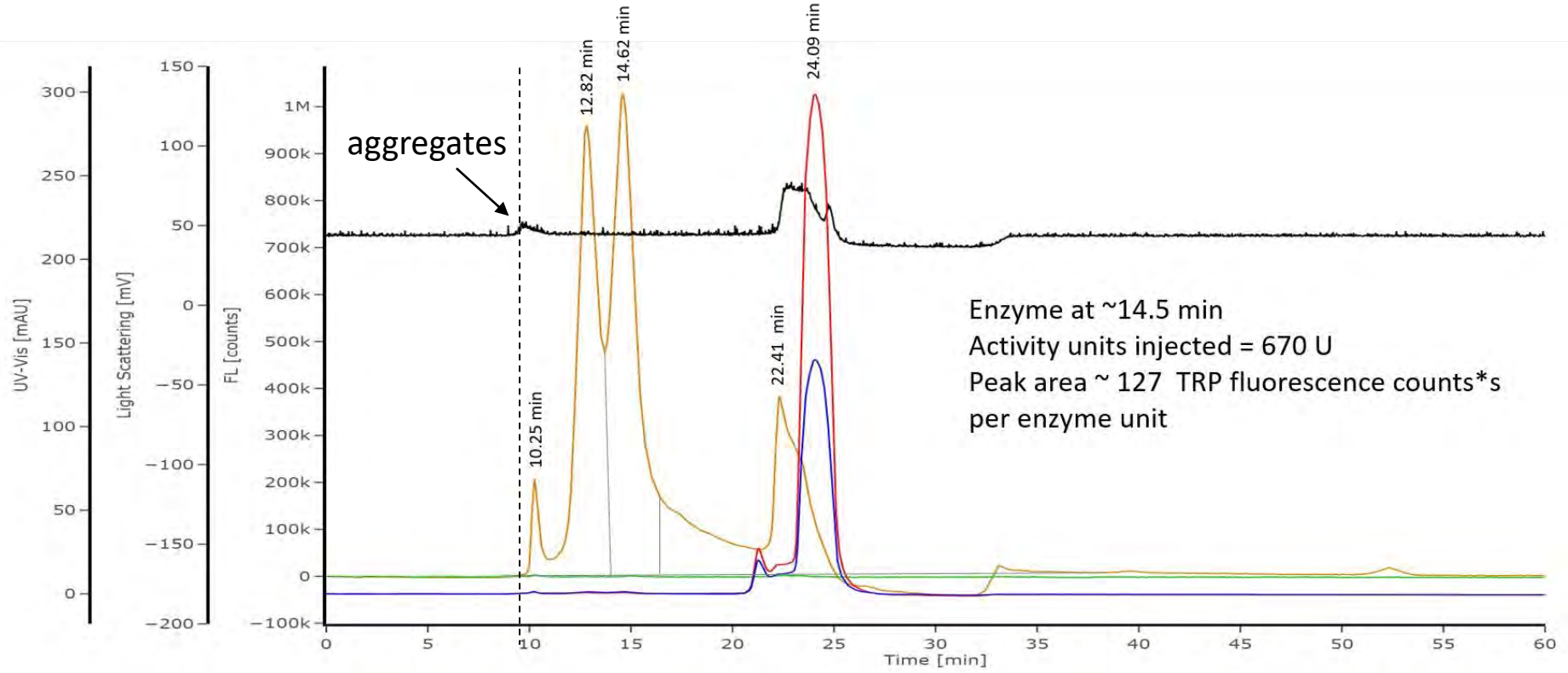
Raw material purity – Jena T7 RNA polymerase



Absorbance 260 nm (mAU)
Absorbance 280 nm (mAU)
Fluorescence PG (mV)
Fluorescence TRP (mV)
Light scattering @ 90° (mV)

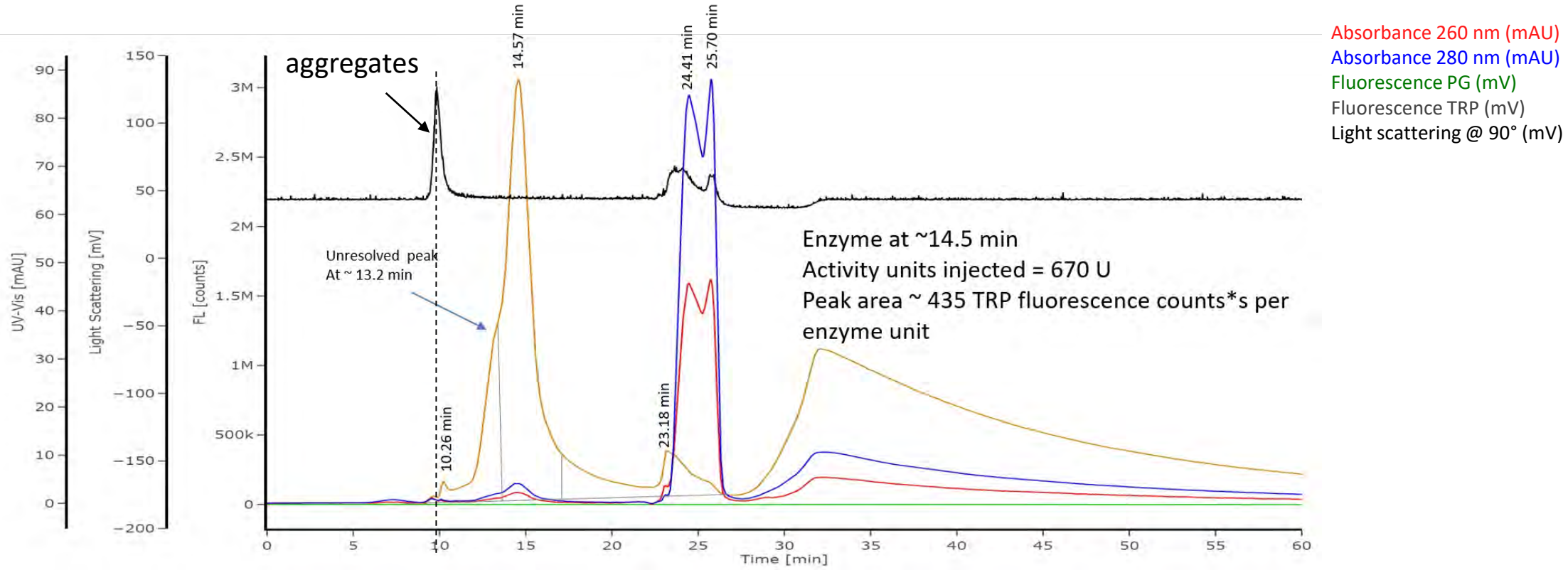
Size exclusion chromatography (SEC). TSKgel™ G3000SWxl. 0.5 mL/min. Sample pre-stained with Picogreen fluorescence increases sensitivity of DNA/RNA related impurities. Tryptophan fluorescence increases sensitivity of protein detection over UV.

Raw material purity – NEB T7 RNA polymerase



Size exclusion chromatography (SEC). TSKgel™ G3000SWxl. 0.5 mL/min. Sample pre-stained with Picogreen fluorescence increases sensitivity of DNA/RNA related impurities. Tryptophan fluorescence increases sensitivity of protein detection over UV.

Raw material purity – Thermo Scientific T7 RNA polymerase



Size exclusion chromatography (SEC). TSKgel™ G3000SWxl. 0.5 mL/min. Sample pre-stained with Picogreen fluorescence increases sensitivity of DNA/RNA related impurities. Tryptophan fluorescence increases sensitivity of protein detection over UV.

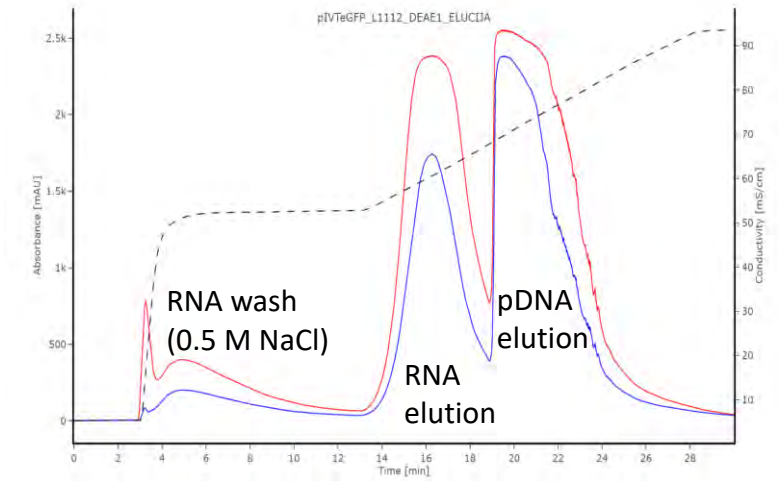
Step 1: pDNA isolation workflow

Plasmid pIVTeGF, provided by Biomay GmbH, Vienna, Austia, size 3.3 kbp

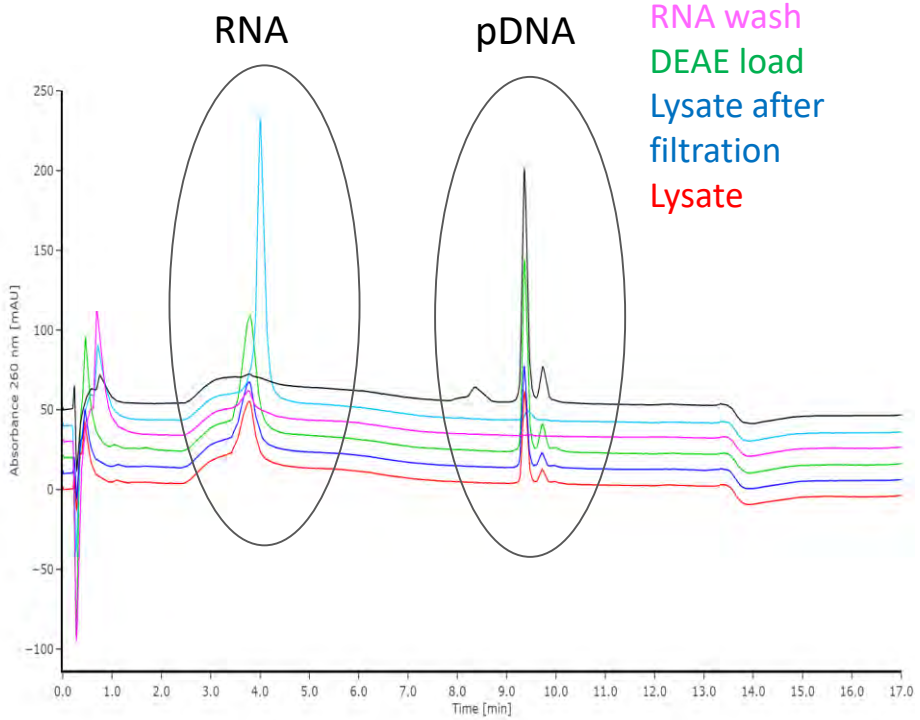
- Alkaline lysis steps:
  - E. coli biomass lysed with 0.1M NaOH and 0.5 % SDS
  - RNA precipitation with 1.0M $CaCl_2$ for impurities
- Two step filtration ($CaCl_2$ improves the filterability)
- DEAE isolation (lysate diluted with water to 35 mS/cm)

Purification of pDNA (3.3 kbp) using CIMmultus DEAE column

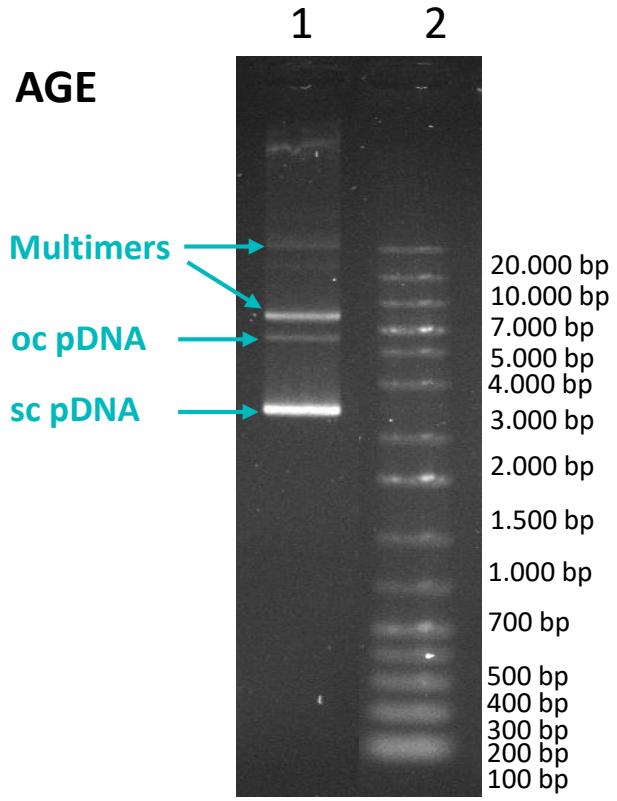
pDNA purification using CIMmultus DEAE column	
pDNA recovery in elution (%)	87
RNA removal (%)	> 99 %
oc pDNA (%)	3
Multimers (%)	25



HPLC CIMac™ pDNA column

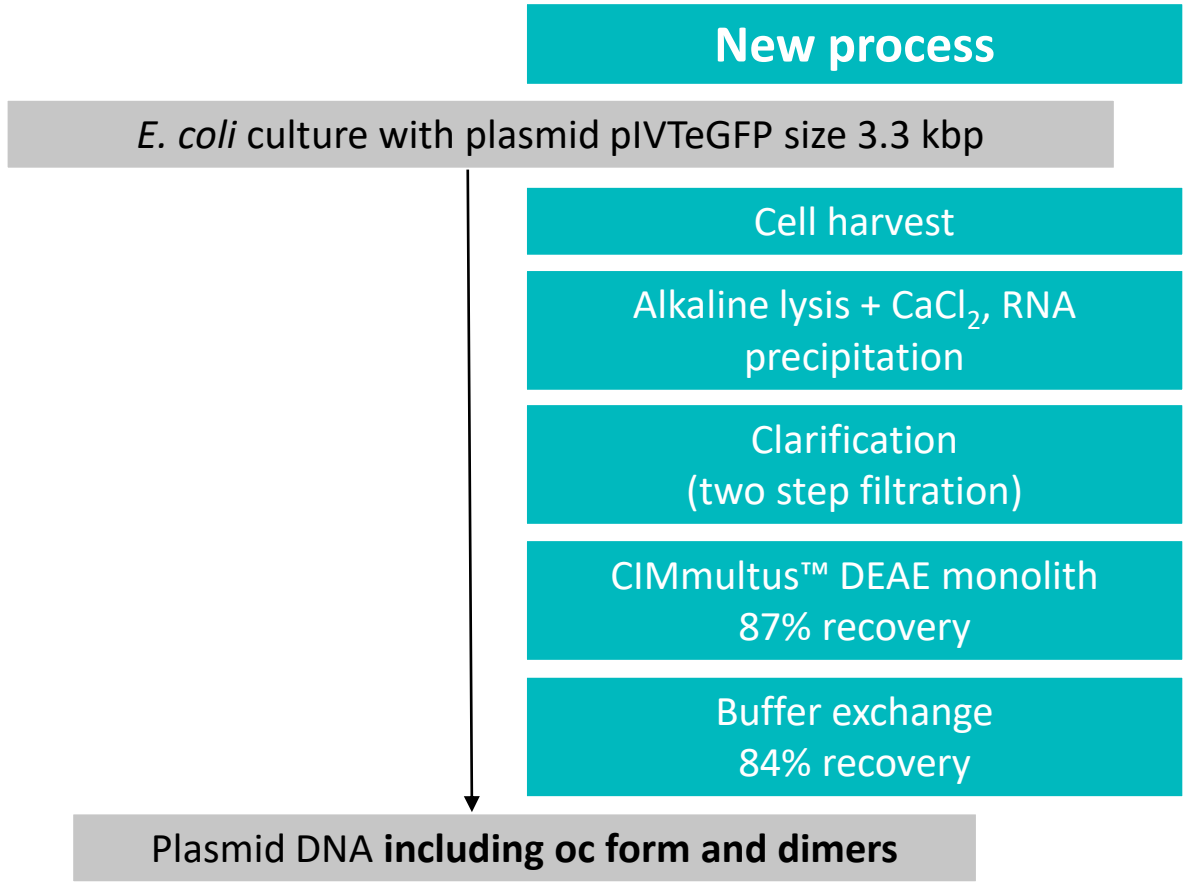


AGE



1: GeneRuler 1 kb Plus
2: purified plasmid

Step 1: pDNA isolation summary



Step 2: pDNA linearization workflow

- Linearization of pDNA (3.3 kbp) with restriction enzyme NotI-HF (NEB) and buffer **without BSA**, 37°C, 21h

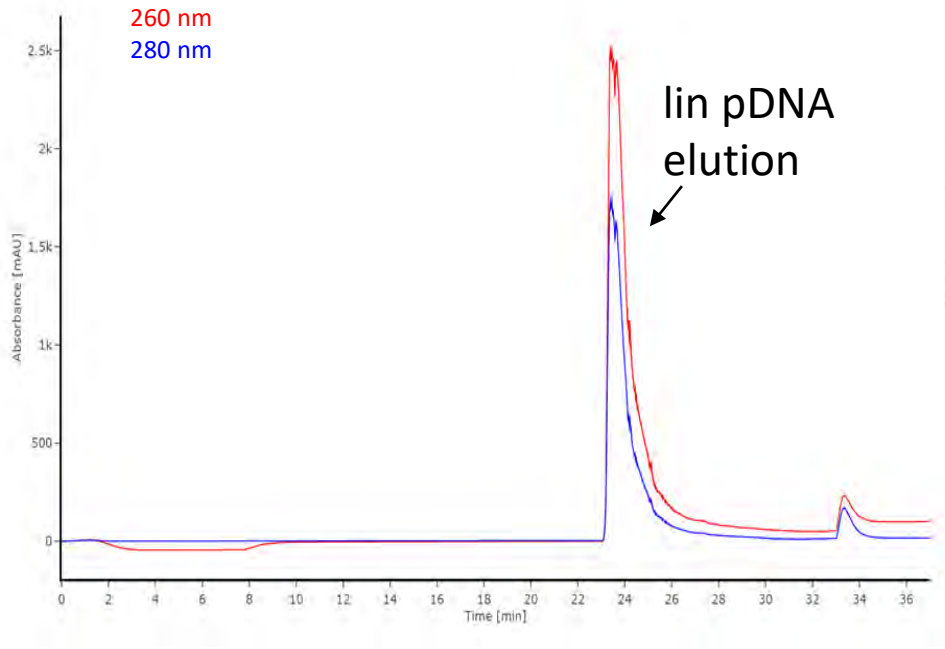
- Purification on CIMmultus™ C4 HLD using step elution from 2.5M to 0.9M ammonium sulphate **(proteins elute with 1M NaOH only - powerful protein removal – to prevent Chromatin/Nucleosome formation)**

- Buffer exchange: PD Midi Trap

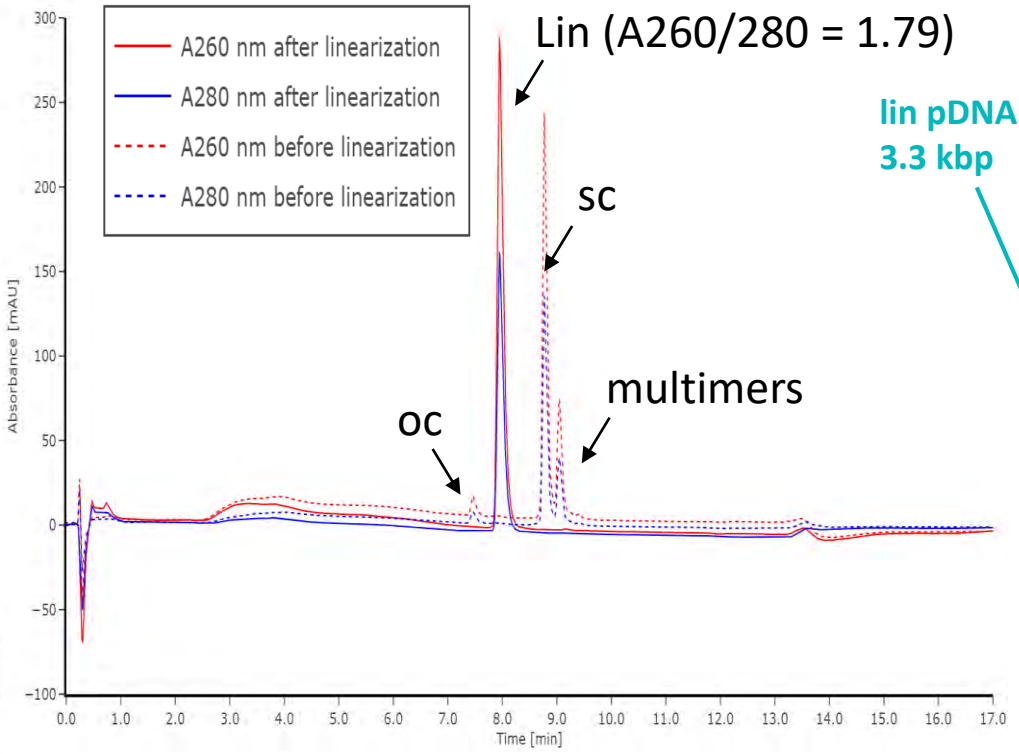
Purification of linear pDNA using CIMmultus™ C4 HLD column

Preparative CIMmultus™ C4 HLD

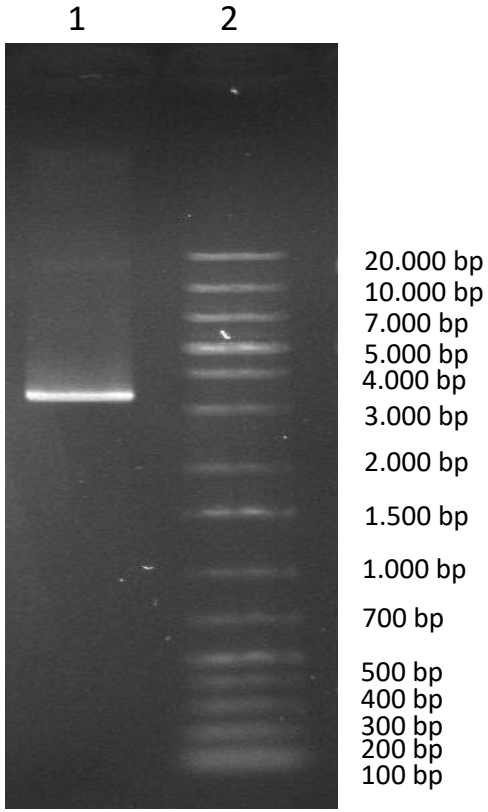
Efficient protein removal (proteins stick and elute in 1M NaOH only)



HPLC CIMac™ pDNA analytics

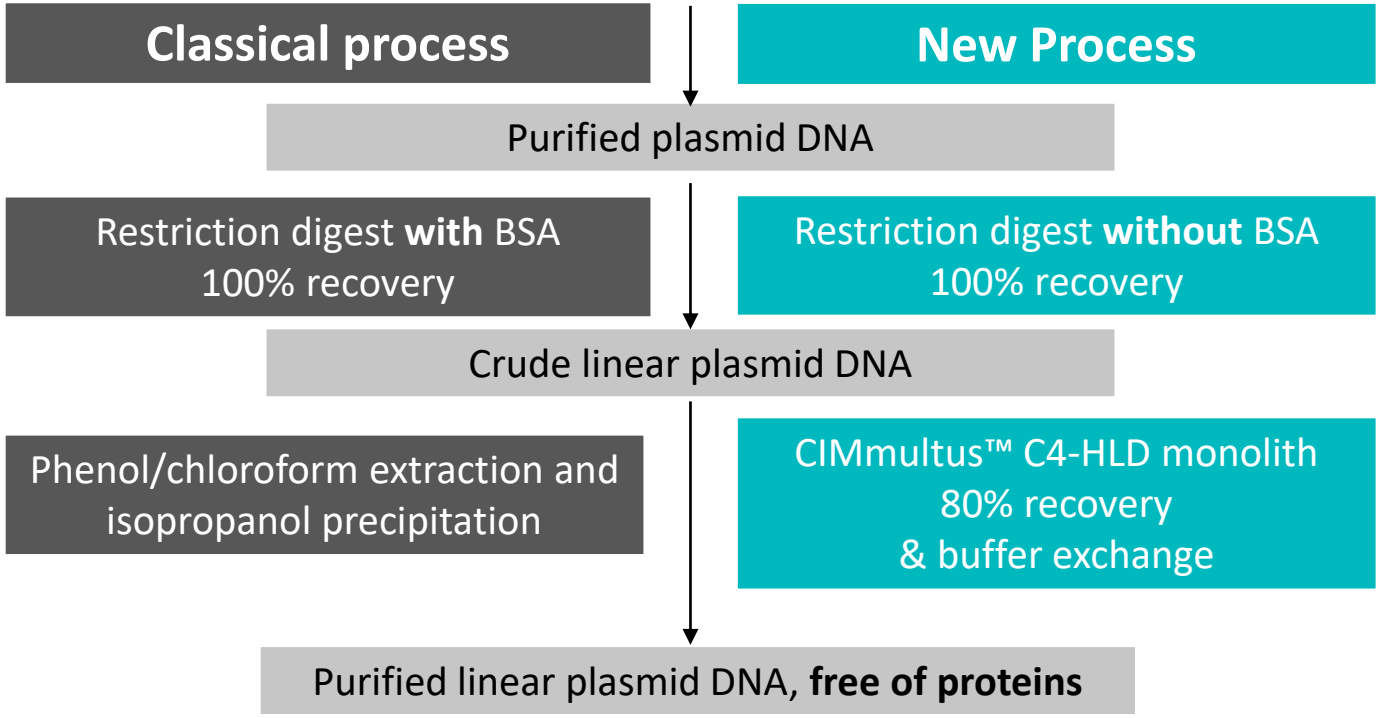


AGE



1: linear plasmid
2: GeneRuler 1 kb plus

Step 2: pDNA linearization summary



Step 3: mRNA production – IVT process workflow

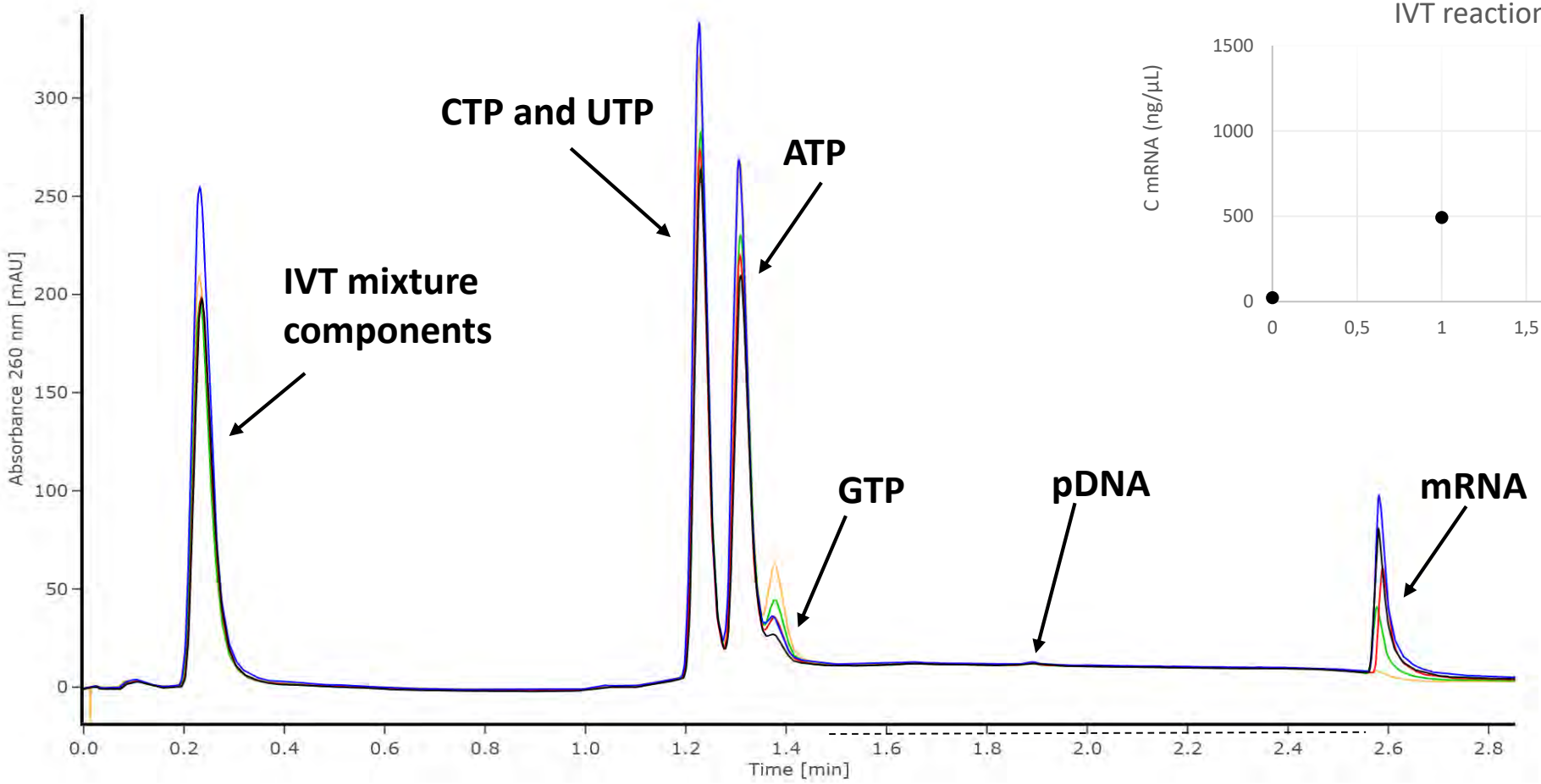
IVT reaction (THE key step to lower manufacturing costs):

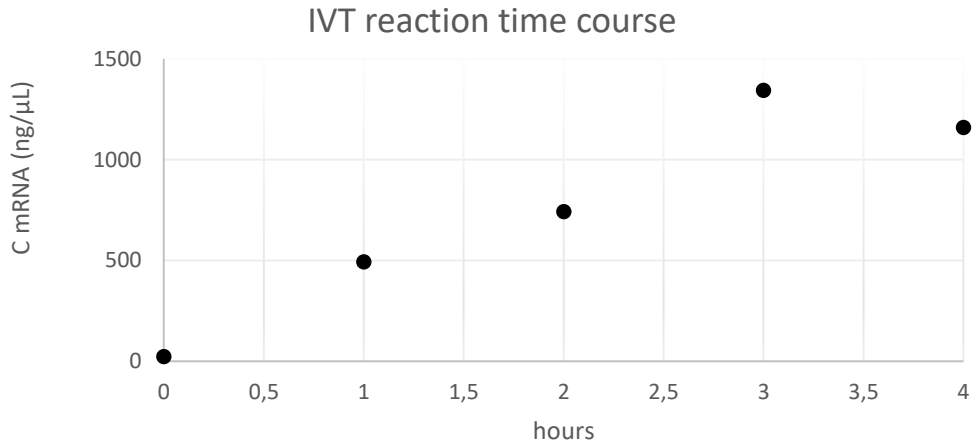
- Plasmid pIVTeGFP with encoded poly(A) tail (tail length 45 nt), T7 RNA polymerase (NEB), pyrophosphatase (NEB), RNAse inhibitor (NEB), and capping reagent ARCA (NEB); incubation 40°C, 3h; inactivation with EDTA; subsequent *O*-methylation (cap1 formation), expected mRNA size: 950 nt

LC purification:

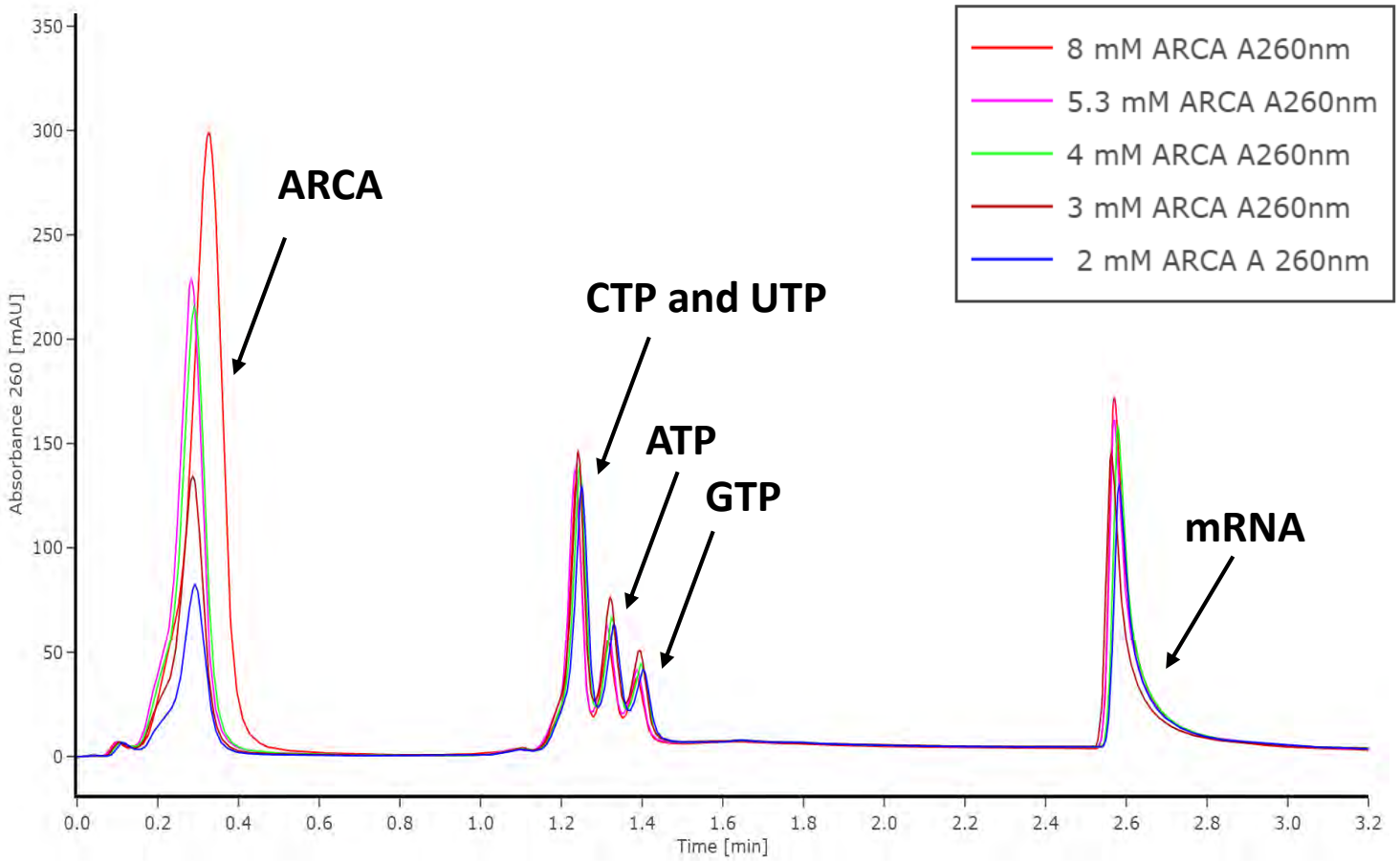
- **CIMmultus™ Oligo dT**: removal of components without poly(A) tail before capping 1 reaction and removal of product related mRNA impurities (dsRNA)

HPLC in-process control using CIMac™ PrimaS column





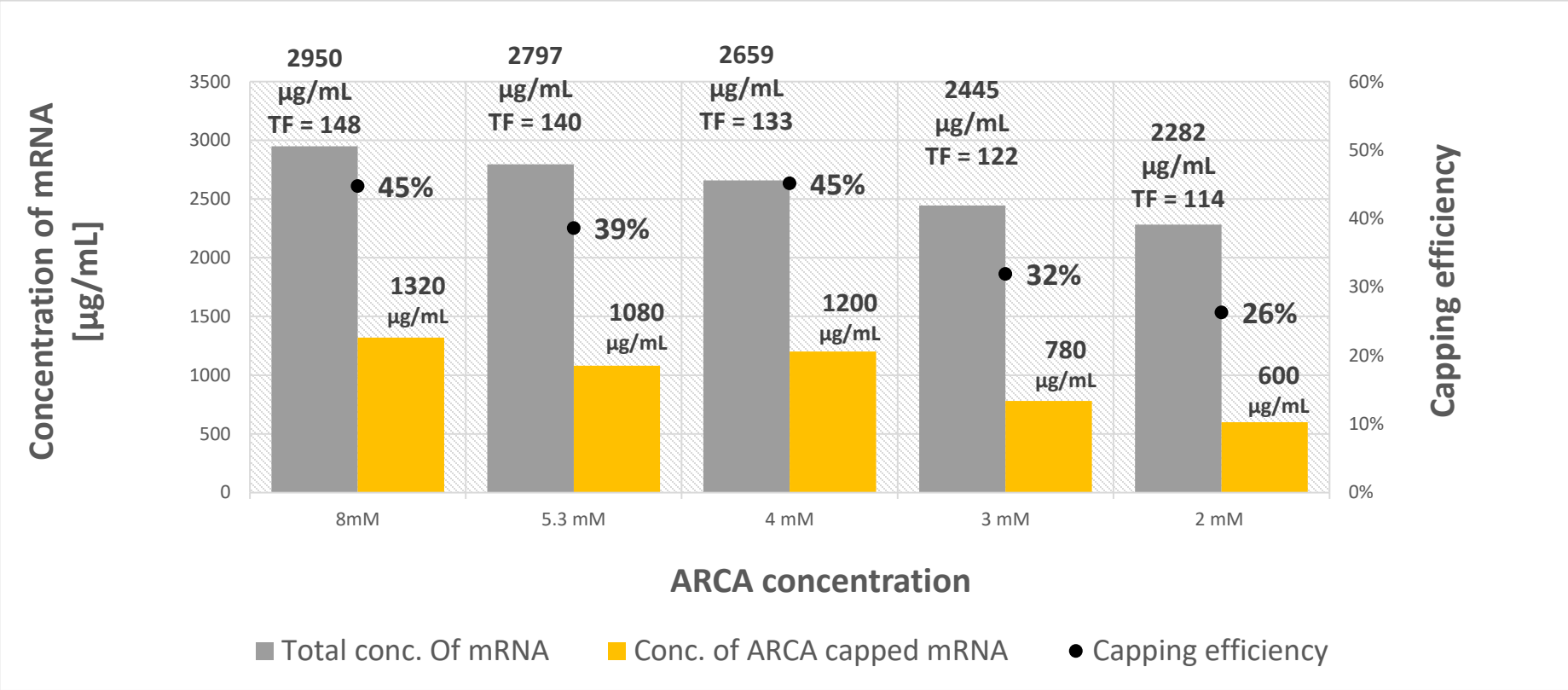
Determination of optimal ARCA concentration using HPLC CIMac™ PrimaS



IVT reaction:
Plasmid pIVTCas9 (4430 nt) with encoded poly(A) tail (tail length 45 nt), T7 RNA polymerase (NEB), pyrophosphatase (NEB), RNAse inhibitor (NEB), NTPs (each 4 mM) and different concentrations of capping reagent ARCA (NEB); incubation 37°C, 3h; inactivation with EDTA

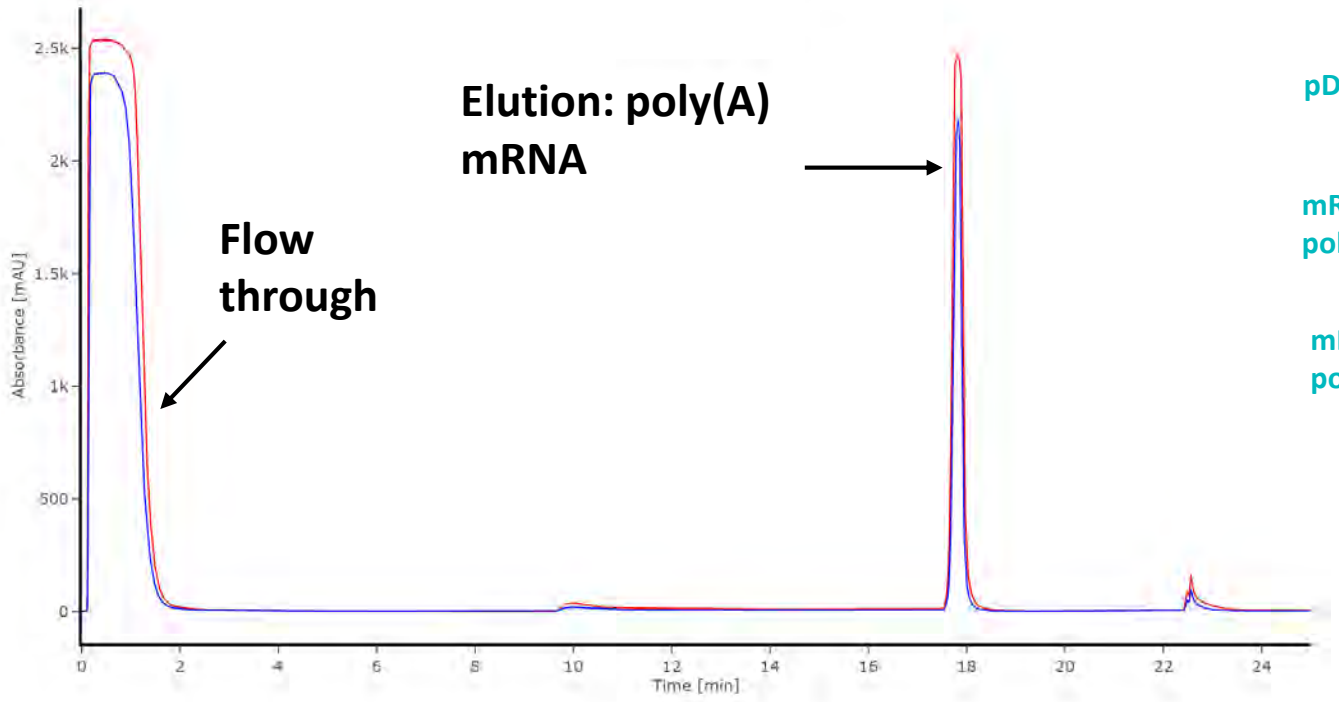
Tested ARCA concentrations:
8 mM
5.3 mM
4 mM
3 mM
2 mM

Determination of optimal ARCA concentration using HPLC CIMac™ PrimaS

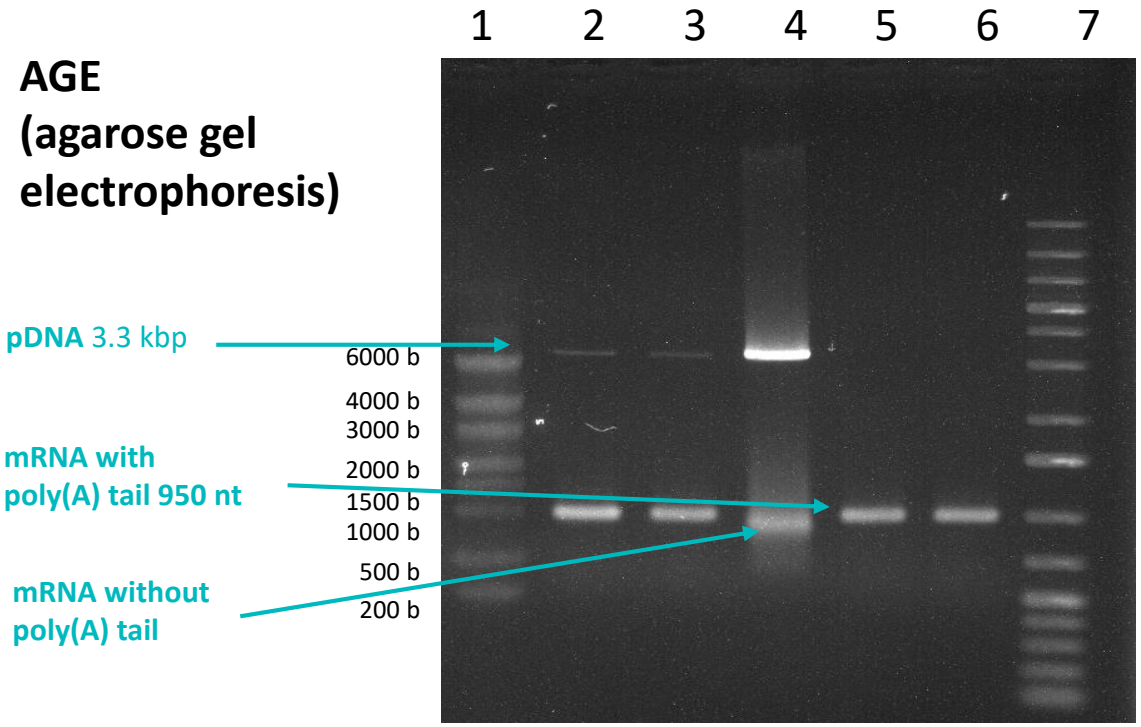


Concentration of ARCA can be successfully lowered from 8mM (manufacturer's protocol) to 4mM and thus cutting the main cost of the IVT reaction (88 % of the total cost) by half.

Purification of mRNA cap0 using CIMmultus™ Oligo dT



AGE (agarose gel electrophoresis)



1 RiboRuler HR

2 IVTmix

3 IVT mix - OligodT load

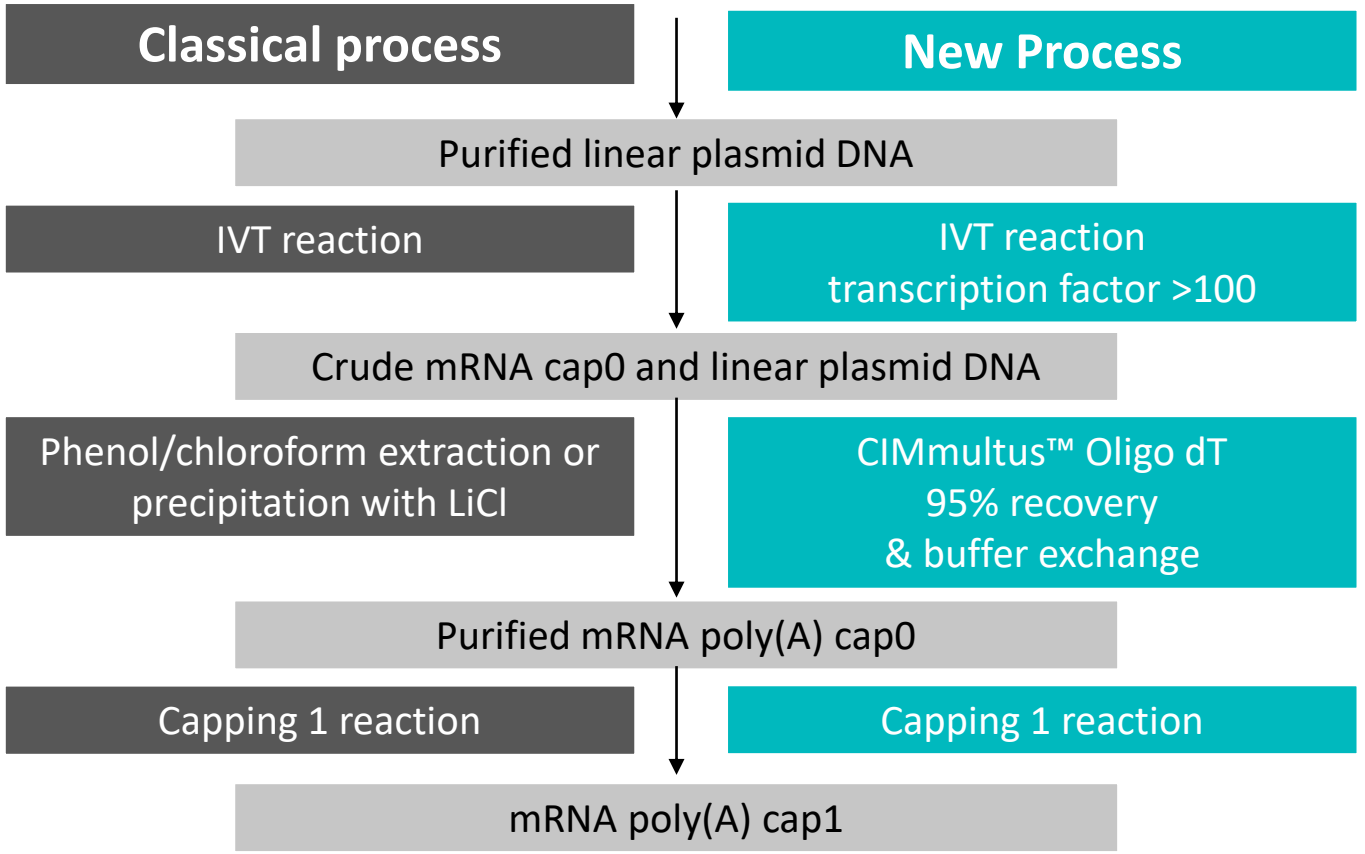
4 IVT mix- FT (50x concentrated)

5 IVTmix – OligodT Elution

6 IVTmix – OligodT Elution (buffer exchanged and concentrated into water)

7 Gene Ruler 1 kb Plus

Step 3: mRNA production – IVT process summary



Step 4: mRNA cap 1 synthesis and purification workflow

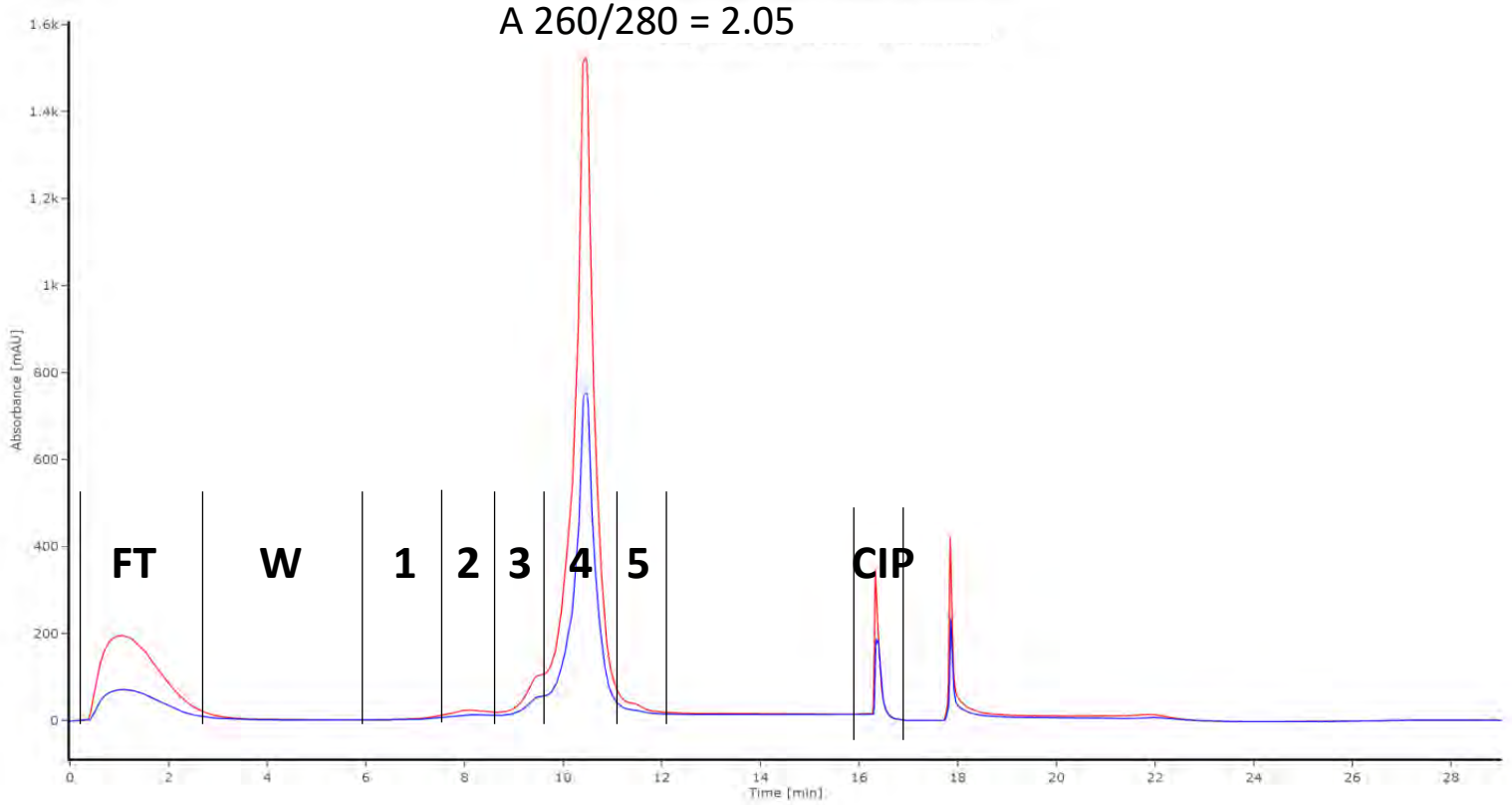
LC purification:

- **CIMmic™ C4 HLD**: removal of process related impurities from capping1 reaction (capping reagent, buffer components, proteins) and removal of product related mRNA impurities (dsRNA)

Purification of cap1 polyadenylated mRNA using CIMmic C4 HLD 0.2mL

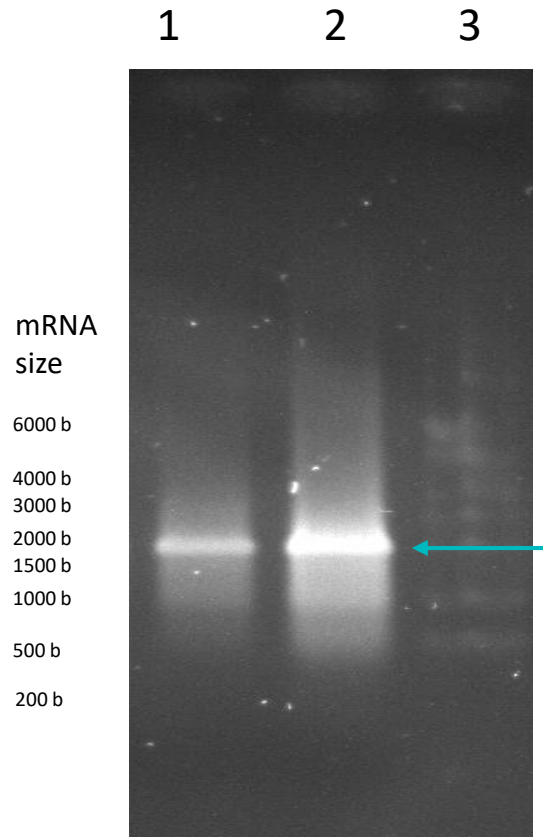
Elution mRNA

A 260/280 = 2.05



Poly(A) mRNA cap 1 purity assessment

AGE (agarose gel electrophoresis)

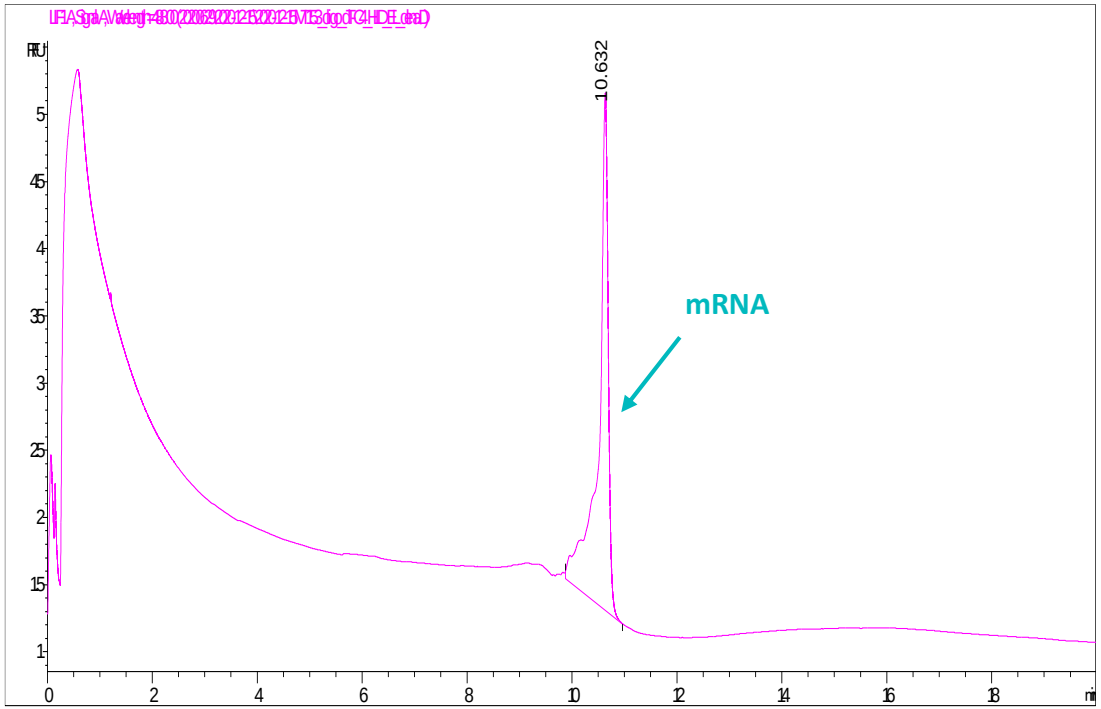


1 Final mRNA eGFP

2 Final mRNA eGFP denaturated at 80 °C, 5 min

3 RiboRuler HR

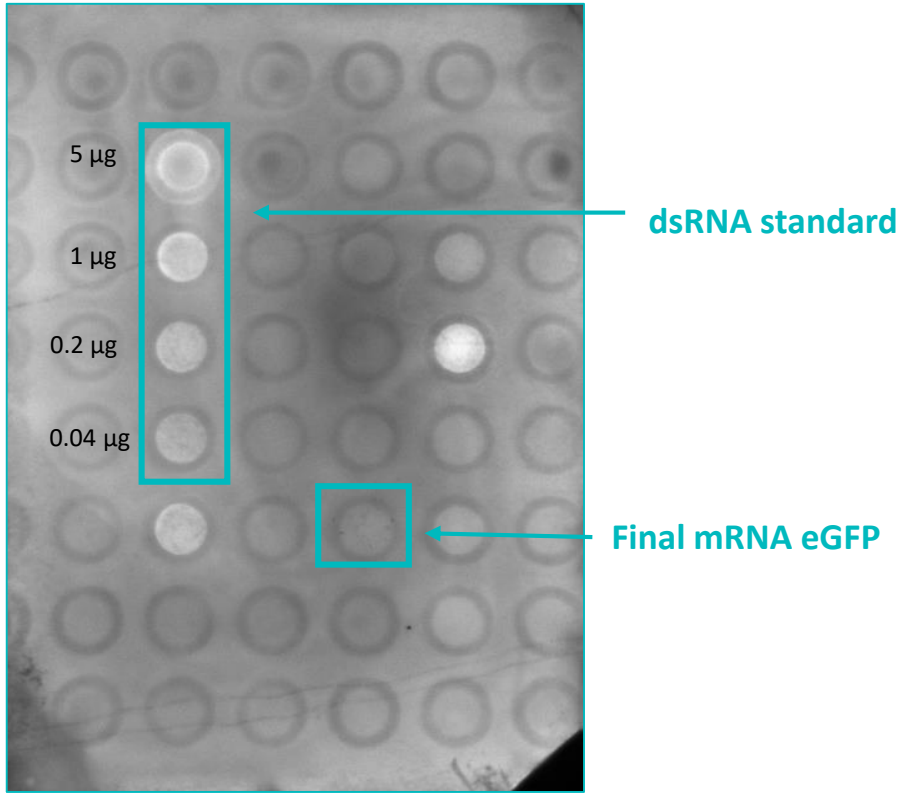
Capillary electrophoresis



Sample denaturated at 80°C, 5 min

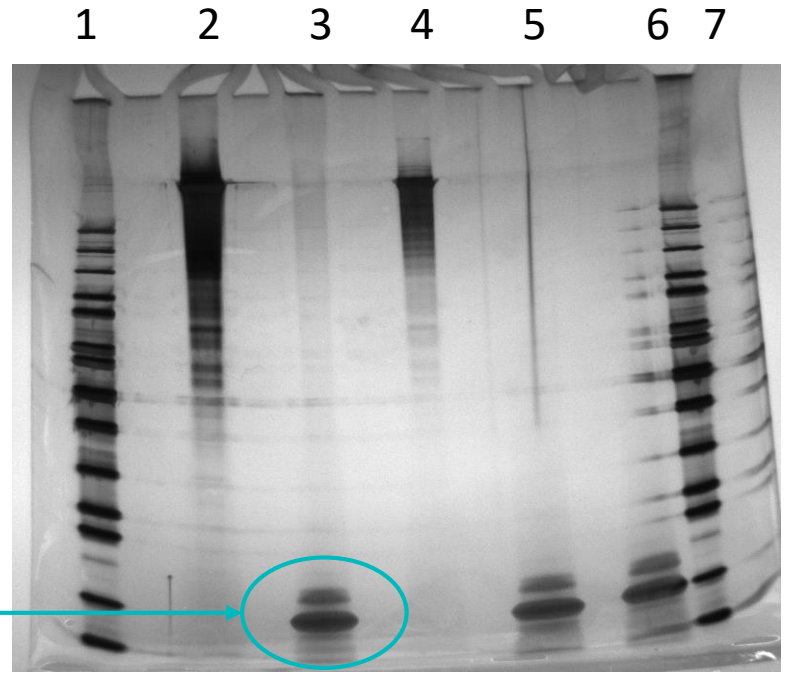
Poly(A) mRNA cap 1 purity assessment

Dot blot
dsRNA detection limit 40 ng per well



no dsRNA detected (< 1% of dsRNA in sample)

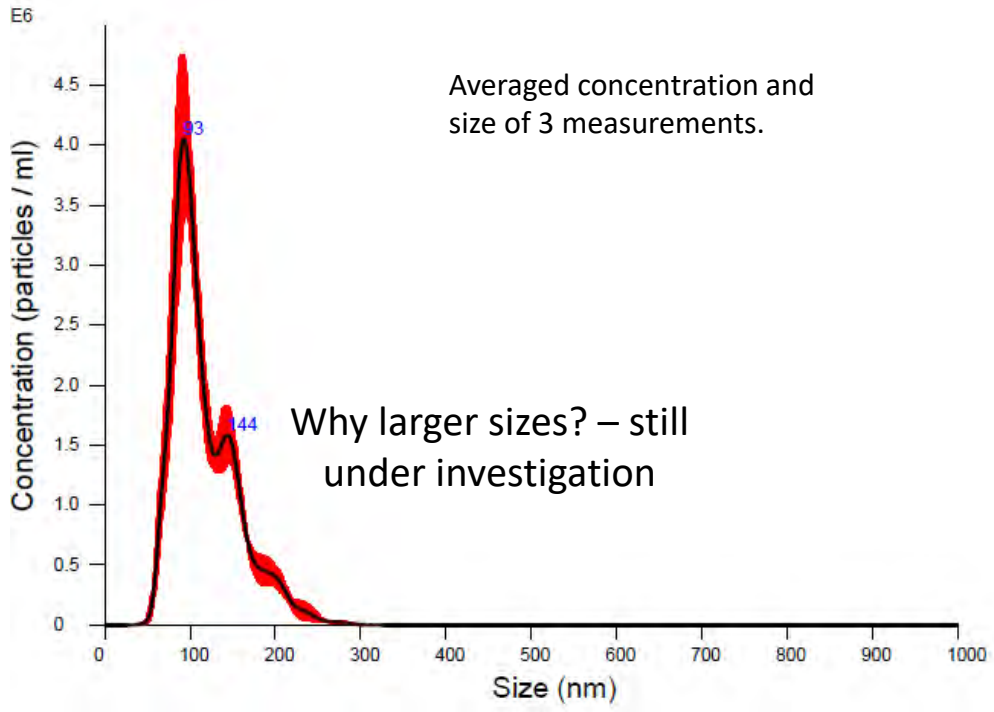
SDS-PAGE
protein detection limit: 10 ng BSA per lane



1 Protein ladder
2 Final mRNA pIVTeGFP 2.6 ug
3 Final mRNA pIVTeGFP + RnaseA
4 Oligo dT IVT153 E 0.7 ug
5 Oligo dT IVT153 E+ RnaseA
6 RnaseA
7 Protein ladder

Poly(A) mRNA cap 1 purity assessment

Nanoparticle Tracking Analysis (NTA)



Averaged concentration and size of 3 measurements.

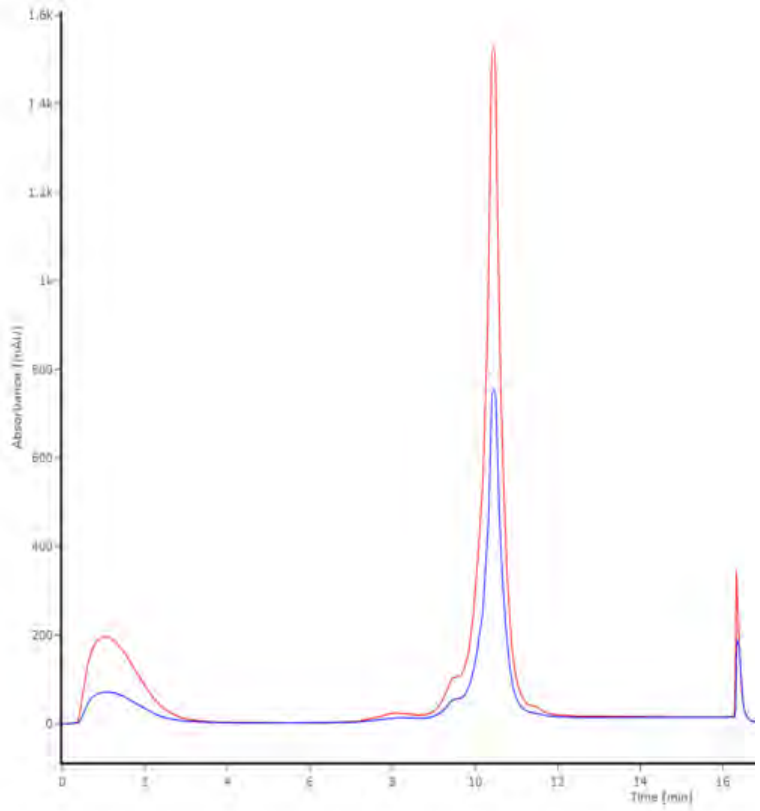
Why larger sizes? – still under investigation

Stats: Mean +/- Standard Error	
Mean:	116.9 +/- 1.8 nm
Mode:	91.2 +/- 3.0 nm
SD:	38.5 +/- 1.2 nm
D10:	76.3 +/- 1.3 nm
D50:	105.0 +/- 2.3 nm
D90:	169.1 +/- 4.6 nm
Concentration (Upgrade):	2.48e+008 +/- 1.09e+007 particles/ml
	39.0 +/- 1.3 particles/frame
	47.2 +/- 1.7 centres/frame

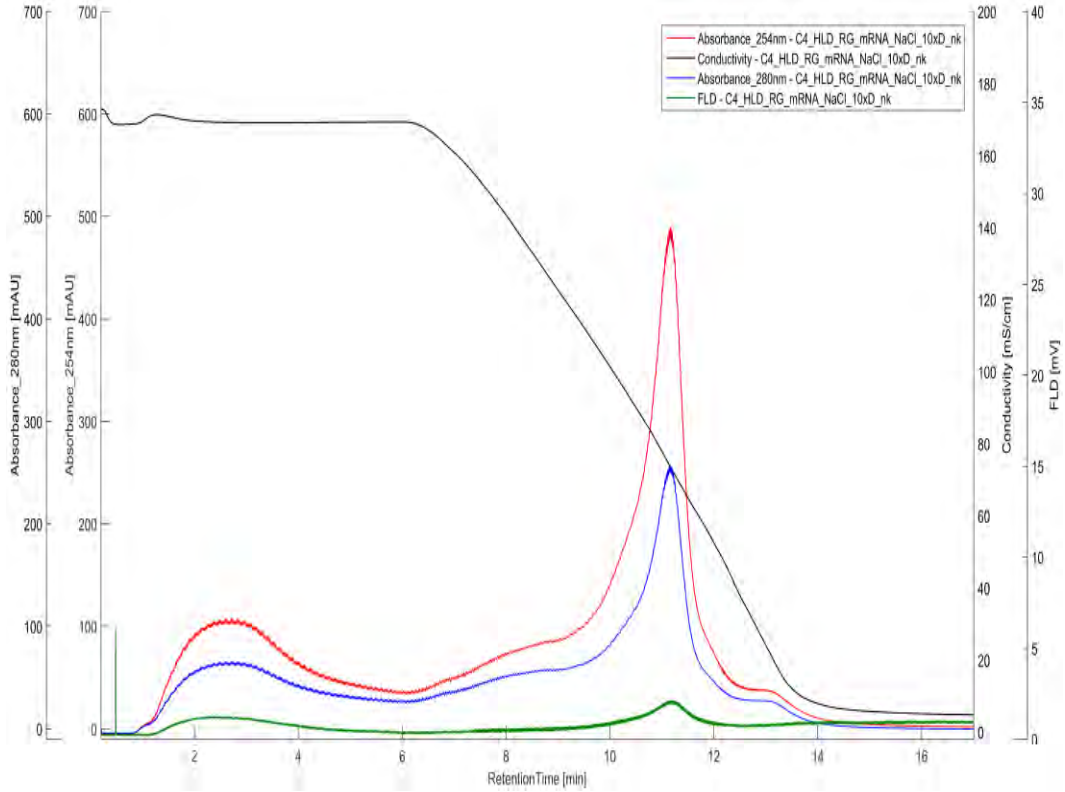
The concentration of total particles is approximately 2.5E8/mL, 90 nm is the most abundant particle size

C4 HLD preparative run - comparison of the classical and new mRNA processes

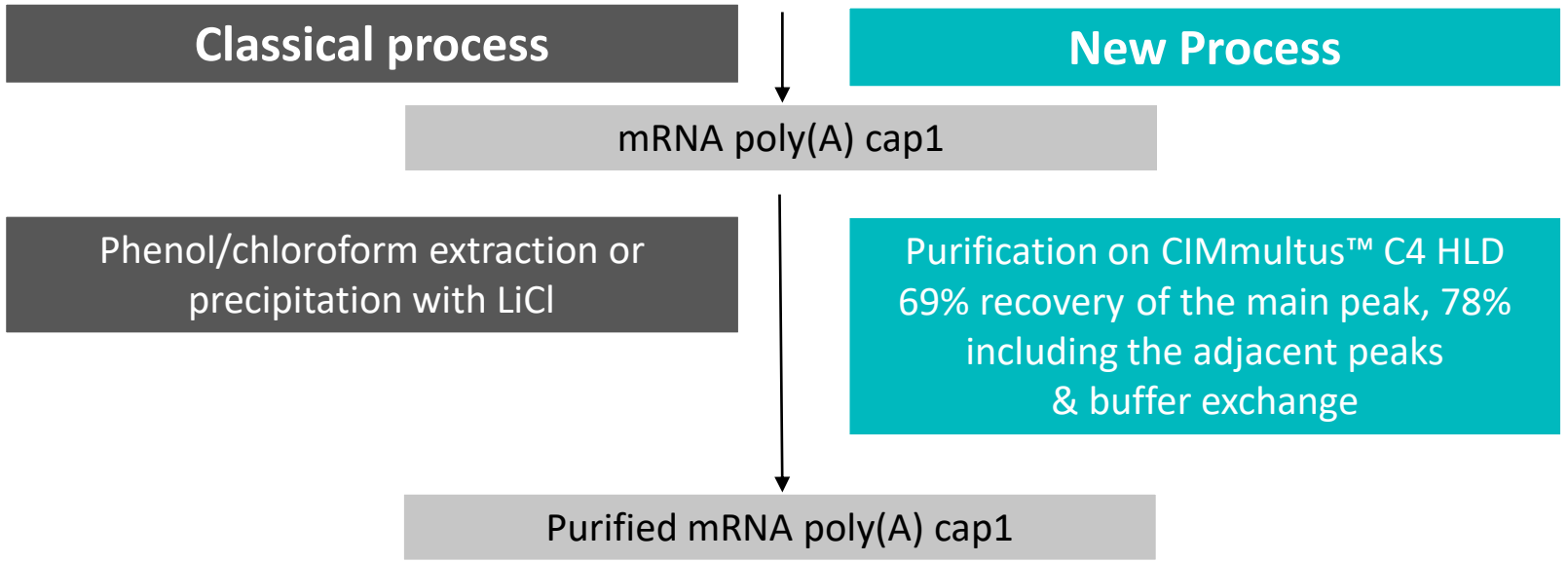
New method – much lower amount of "truncated" mRNA



Classical method – high amount of "truncated" mRNA and other impurities



Step 4: mRNA purification summary



Comparison of mRNA production platforms

Step	Platform 1: pAL101* PrimaS capture Polyadenylation Oligo dT polish	Platform 2: pIVTeGFP Oligo dT capture C4 HLD polish
Capture step recovery	80%	95%
Polishing step recovery	100%	69% (78%)

*protocol on request

Conclusions

- For the efficient and economic process of mRNA production the pDNA production should be part of the mRNA process flow (oc and multimers of the pDNA can be linearized, proteins should be removed before entering the IVT synthesis - not before the linearization).
- High purity of the raw materials and HPLC in-process control of the IVT process allow for high transcription number (proteins complex with the mRNA as soon it is formed), much purer mRNA and lower consumption of expensive reagents.
- Proper management of the IVT reaction allows for high yield and purer product with less purification steps. Most important, for cheaper manufacturing costs.
- In-process control using HPLC allows for faster process development and more robust process.