Better purification. Faster.

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

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BPI, October 19, 2020

BIA Separations products and services

Convective Interaction Media (CIM®)

Pre-packed monolithic columns

CIMac™ Analytical and CIMmultus™ Preparative columns

Services, Process development and Technical Support

Development of processes and methods for separation/concentration/purification of large biomolecules.

Custom immobilization, product development,..

Process Analytical Technology (PATfix™)

At-line PAT HPLC suite for **faster process development** and enhanced process control

Integrated Capability from Cell Culture Production through Downstream Processing

Bioprocess scale-up from laboratory to pilot

Managing interface between upstream and downstream

Vero cell bank

Expert DSP bioprocess knowledge: royalty-free

- 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, <u>**platform process from E.coli to mRNA**</u>
- Adeno virus
- AAV **(all serotypes, > 20 tested)**
- Influenza virus (all serotypes)
- Vaccinia/MVA
- **Exosome**
- Bacteriophage
- IVIG
- IgM and many more

- **> 30 DNA, RNA, virus DSP processes tech transferred to CMOs, sponsors**
- **> 10 AAV DSP processes tech transferred to CMOs, sponsors**

BIA Separations State-of-the-Art production facility > 30M USD investment

Expansion to increase production capacity 5x before year-end 2020 and 30x before year-end 2023.

BIA separations

Testimonials

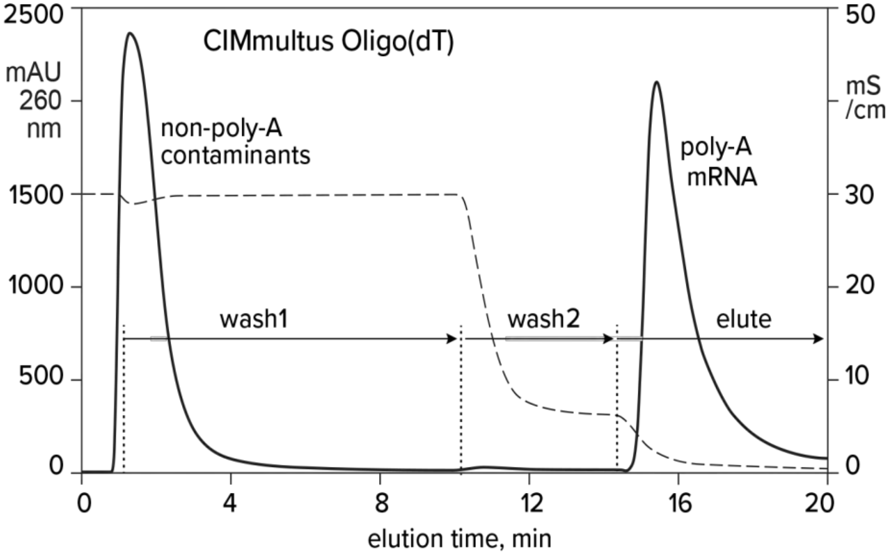
Andy Stober, Senior Vice President of Technical Operations for AveXis:

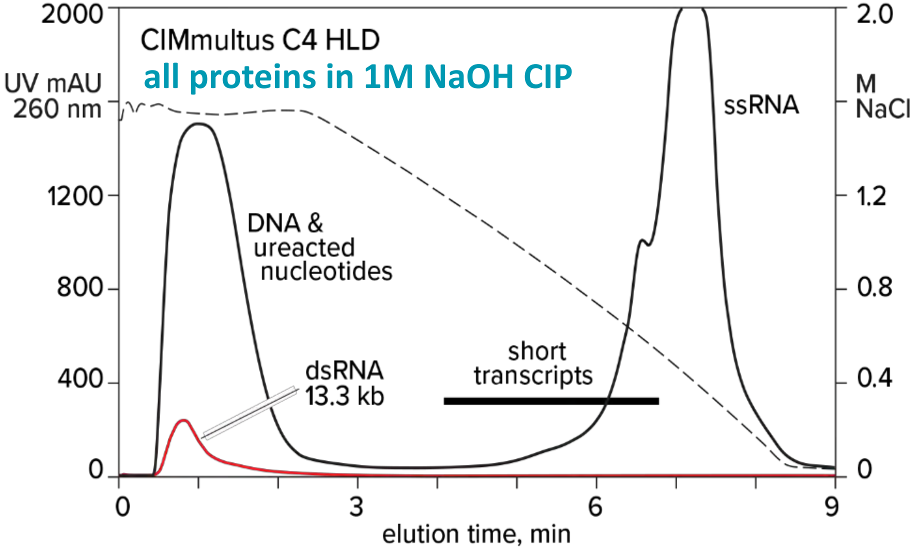
“We are especially grateful that BIA Separations shared, and operated, with the same sense of urgency we did to help bring gene therapy to the SMA community. BIA’s experience with AAV purification and its chromatographic technology were important contributions and we look forward to our continued work together.”

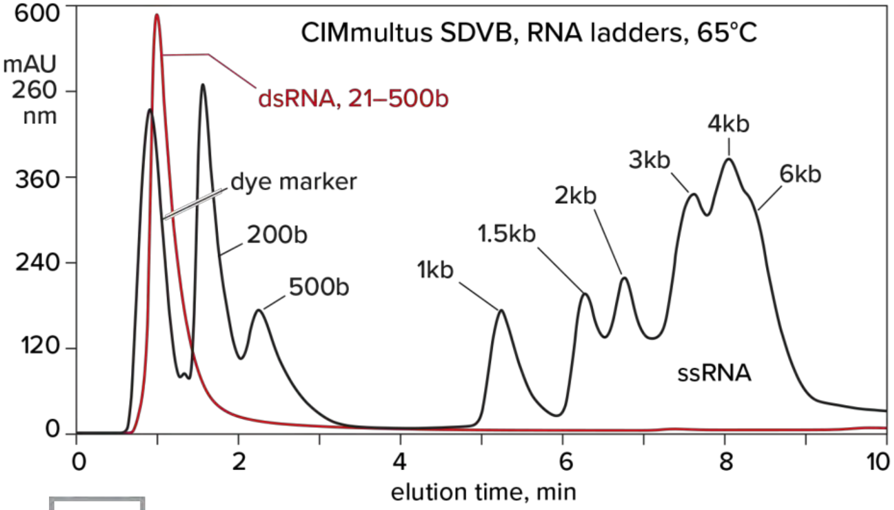
Just 15 months from the lab to manufacturing,
<u>not possible without fast analytics</u>

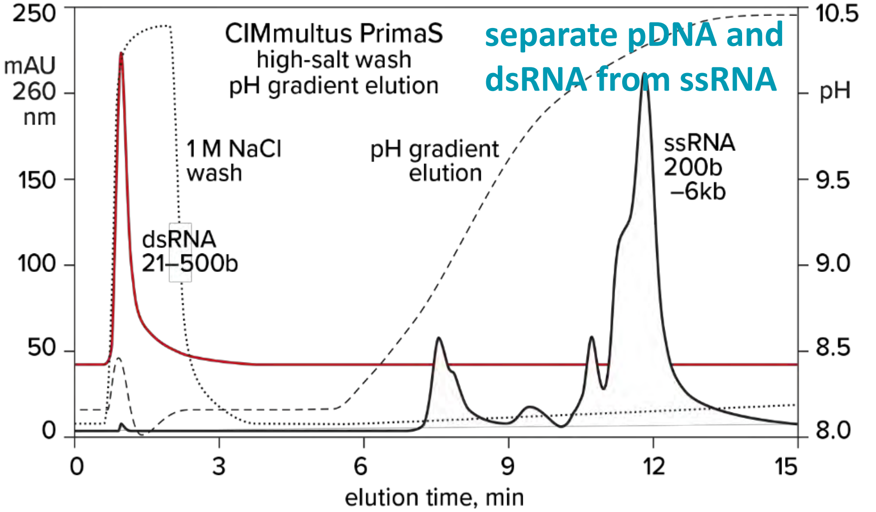
from E.coli to pure mRNA

BIA Separations chromatographic tools for mRNA analytics and purification




all proteins in 1M NaOH CIP




separate pDNA and dsRNA from ssRNA

From E. coli to mRNA

E. coli culture with pDNA

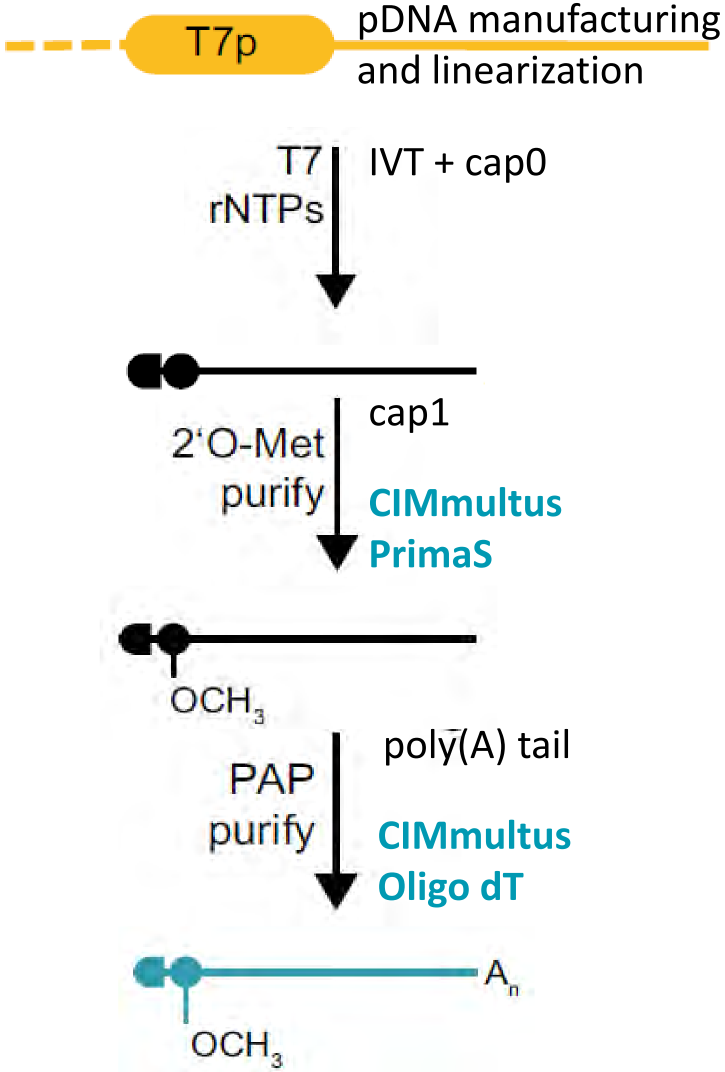
Step 1: pDNA isolation

Step 2: pDNA linearization

Step 3: mRNA production

Step 4: mRNA purification

Case study 1. - poly(A) tail enzymatically added to the mRNA after IVT and capping



pDNA manufacturing
and linearization

IVT + cap0

cap1

**CIMmultus
PrimaS**

poly(A) tail

**CIMmultus
Oligo dT**

Ref. to Schlake et al. Cellular and Molecular Life Sciences (2019) 76:301–328

Preparative LC methods conditions

Linear pDNA purification:

Column: **CIMmultus C4 HLD 1mL**

Method: 10 min MPA, 20 min step 100% MPB

- 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

mRNA purification:

Column: **CIMmic PrimaS 0.1 mL**

Method: 3 min MPA, 5 min step 100% MPB

- MPA: 20mM MES, 20mM Tris, 20mM BTP, 20 mM Gly, 1M NaCl pH 6.0
- MPB: 20mM MES, 20mM Tris, 20mM BTP, 20 mM Gly, 1M NaCl pH 10

Column: **CIMmic Oligo dT18 C12 0.1 mL**

Method: 15 min 100% MPA, 10 min 100% MPB, 7 min MPC

- MPA: 50mM Na-phosphate, 250mM NaCl, 2mM EDTA, pH 7.0
- MPB: 50mM Na-phosphate, 2mM EDTA, pH 7.0
- MPC: 10mM Tris, pH 7.2

HPLC analytical methods conditions

pDNA analytics:

Column: **CIMac pDNA 0.3 mL** analytical column

Method: 25% - 100% of MPB in 25 min

- MPA: 50 mM HEPES, 1 % Tween, pH 7.5
- MPB: 50 mM HEPES, 1 M guanidineHCl, 1 % Tween, pH 7.5

mRNA IEX analytics:

Column: **CIMac PrimaS 0.1 mL** analytical column

Method: 0% - 100% of MPB in 8 min

- MPA: 50 mM MES pH 6.0
- MPB: 50 mM MES pH + 200 mM Na or K pyrophosphate, pH 6.0

mRNA Reverse phase analytics:

Column: **CIMac SDVB 0.1 mL** analytical column

Method: 7.5% ACN to 22.5% ACN in 5 min

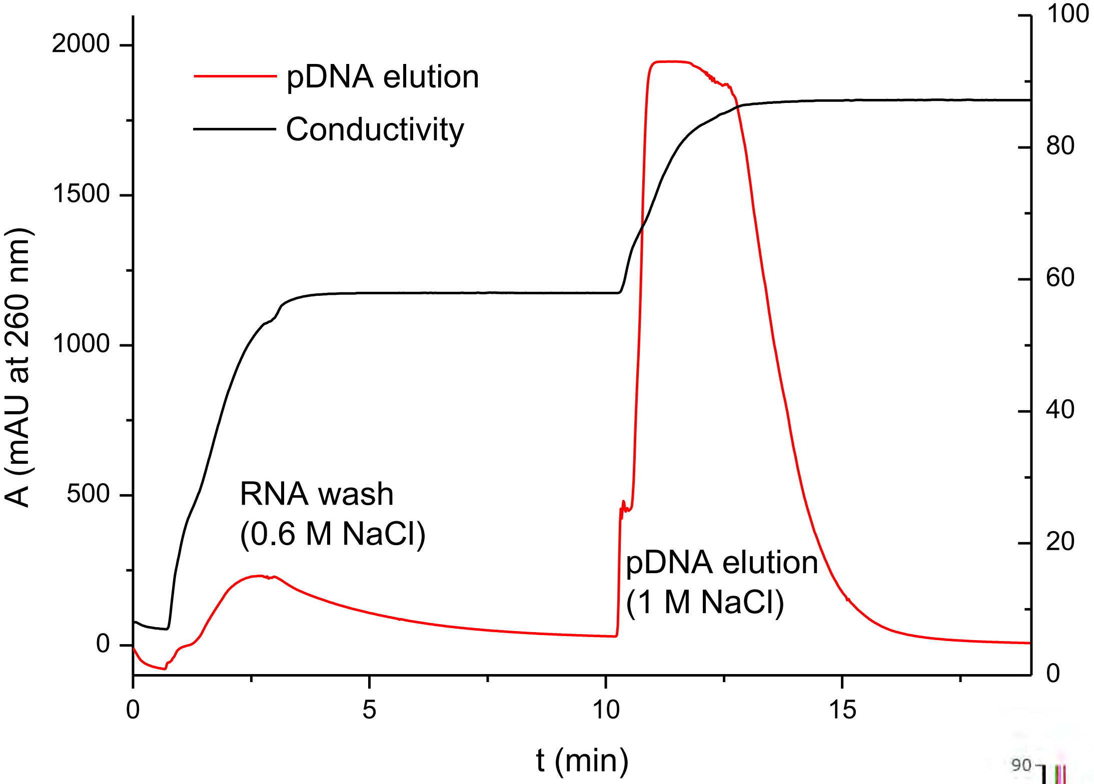
- MPA: 0.1 M TEAA + 7.5% ACN pH 7.0
- MPB: 0.1 M TEAA + 22.5% ACN pH 7.0

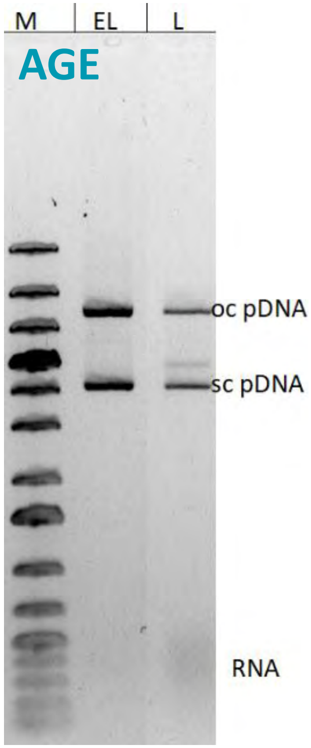
Step 1: pDNA isolation workflow

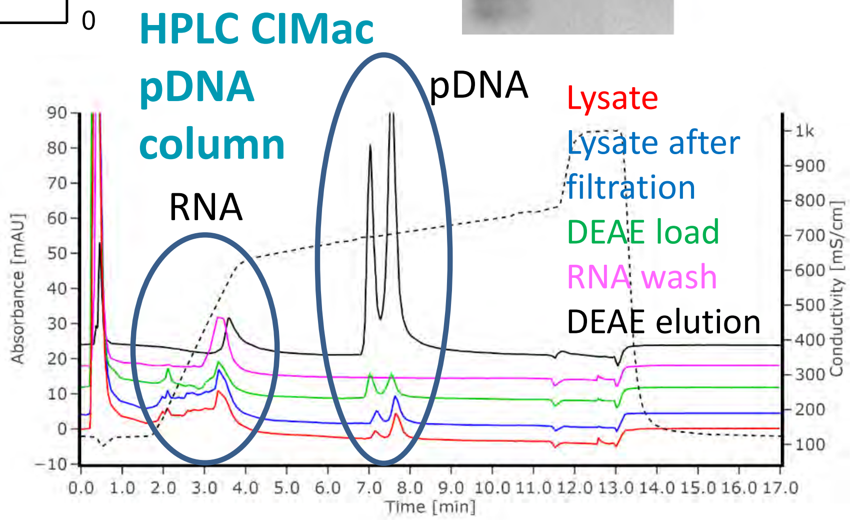
Plasmid pAL101 provided by Addgene, size 6409 bp

1. Alkaline lysis steps:
    - E. coli biomass lysed with 0.1 M NaOH
    - Precipitation with 0.75 M $CaCl_2$ for impurities
2. Two step filtration ($CaCl_2$ improves the filterability)
3. DEAE isolation (lysate diluted with water to 35 mS/cm)

Purification of pDNA (6.4 kbp) using CIMmultus DEAE column







Parameters – elution fraction	
pDNA recovery in elution (%)	83
RNA removal (%)	92
oc pDNA (%)	31

Step 1: pDNA isolation summary

BIA Separations process

E. coli culture with plasmid pAL101 size 6409 bp

Cell harvest

Alkaline lysis + $CaCl_2$ RNA precipitation

Clarification
(two step filtration)

CIMmultus DEAE monolith
83% recovery

Buffer exchange
95% recovery

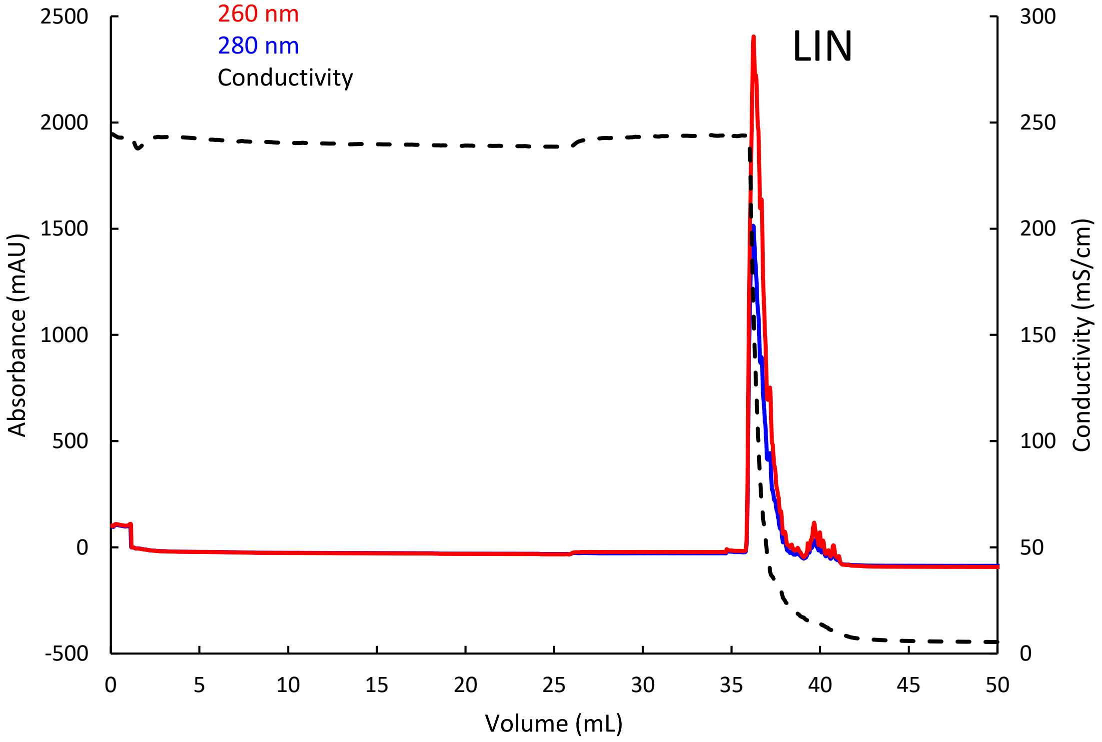
plasmid DNA **including oc form and dimers**

Step 2: pDNA linearization workflow

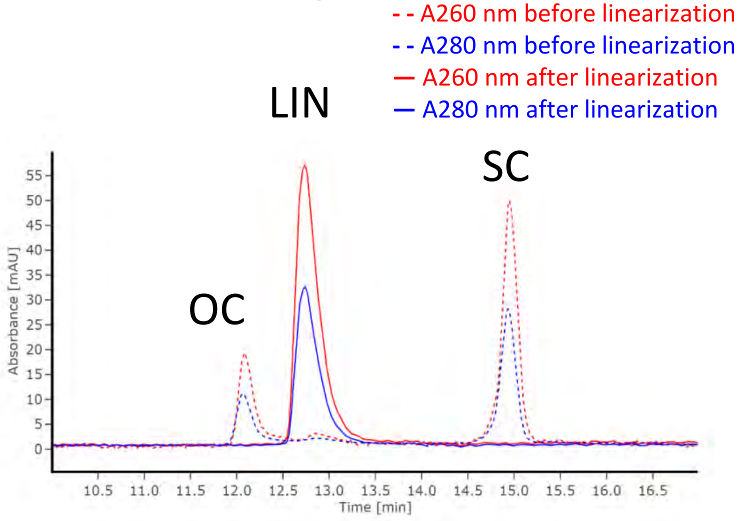
1. Linearization of pDNA (6409 bp) with restriction enzyme and buffer without BSA, 25°C, 4 h)
2. Purification on CIMmultus C4 HLD using step elution from 2.5 M to 0 M Ammonium sulphate (powerful protein removal)
3. Buffer exchange: Amicon 3 kDa (RC membrane)

Purification of linear pDNA using CIMmultus C4 HLD column

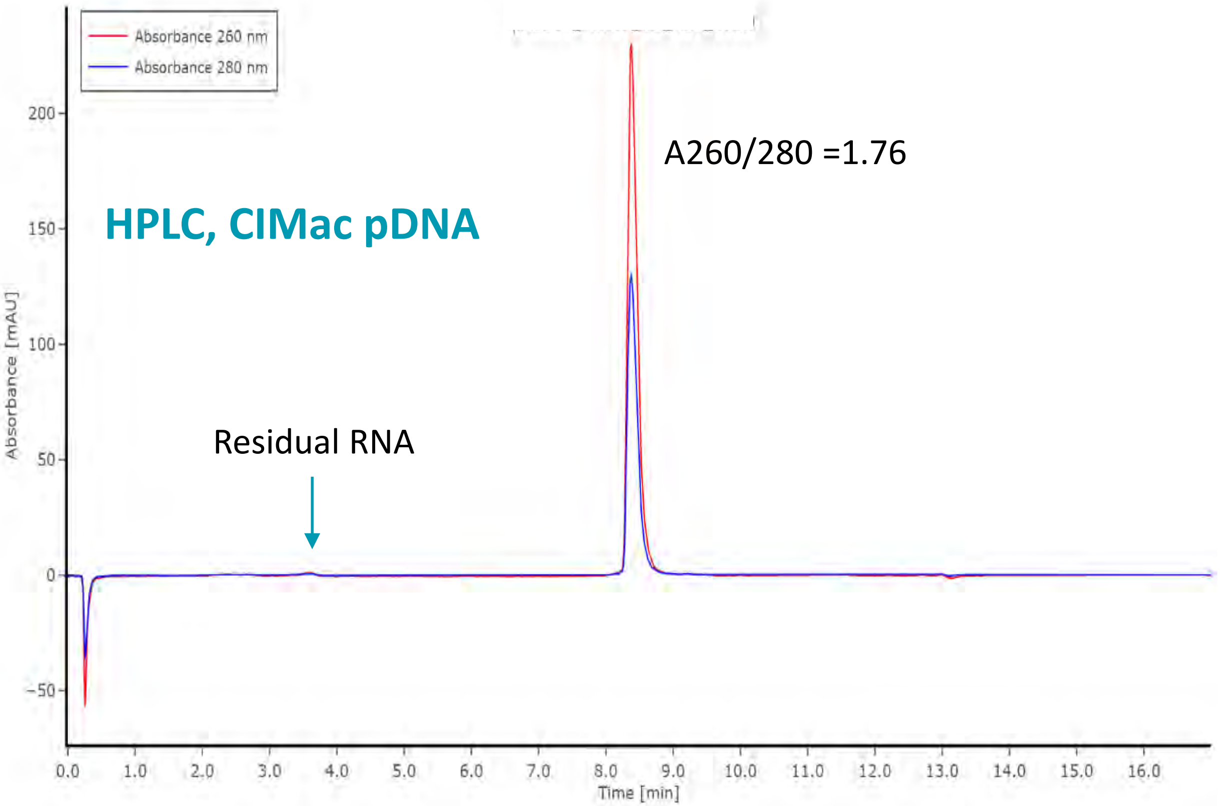
Preparative CIMmultus C4 HLD. <u>All proteins elute in 1M NaOH (CIP step)</u>



HPLC CIMac pDNA



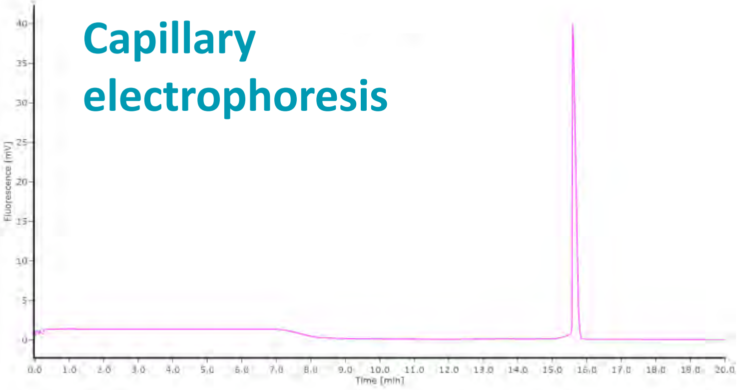
Purity check of linearized pDNA (6.4 kbp)



HPLC, CIMac pDNA

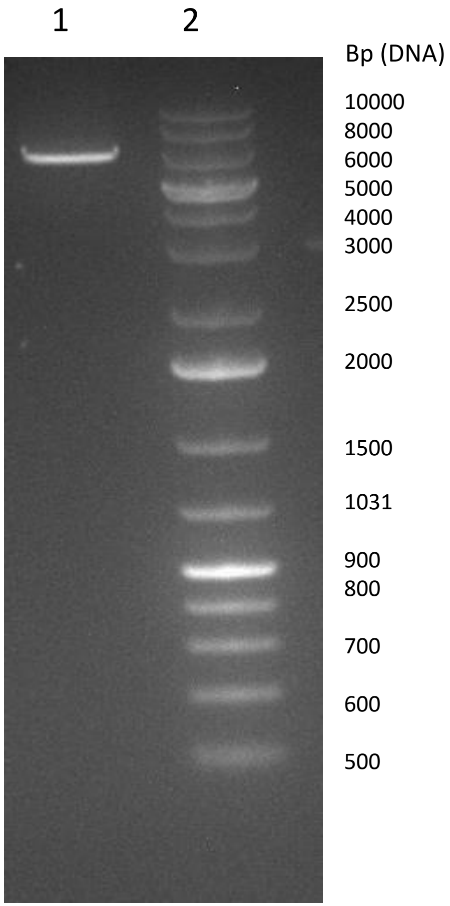
A260/280 =1.76

Residual RNA



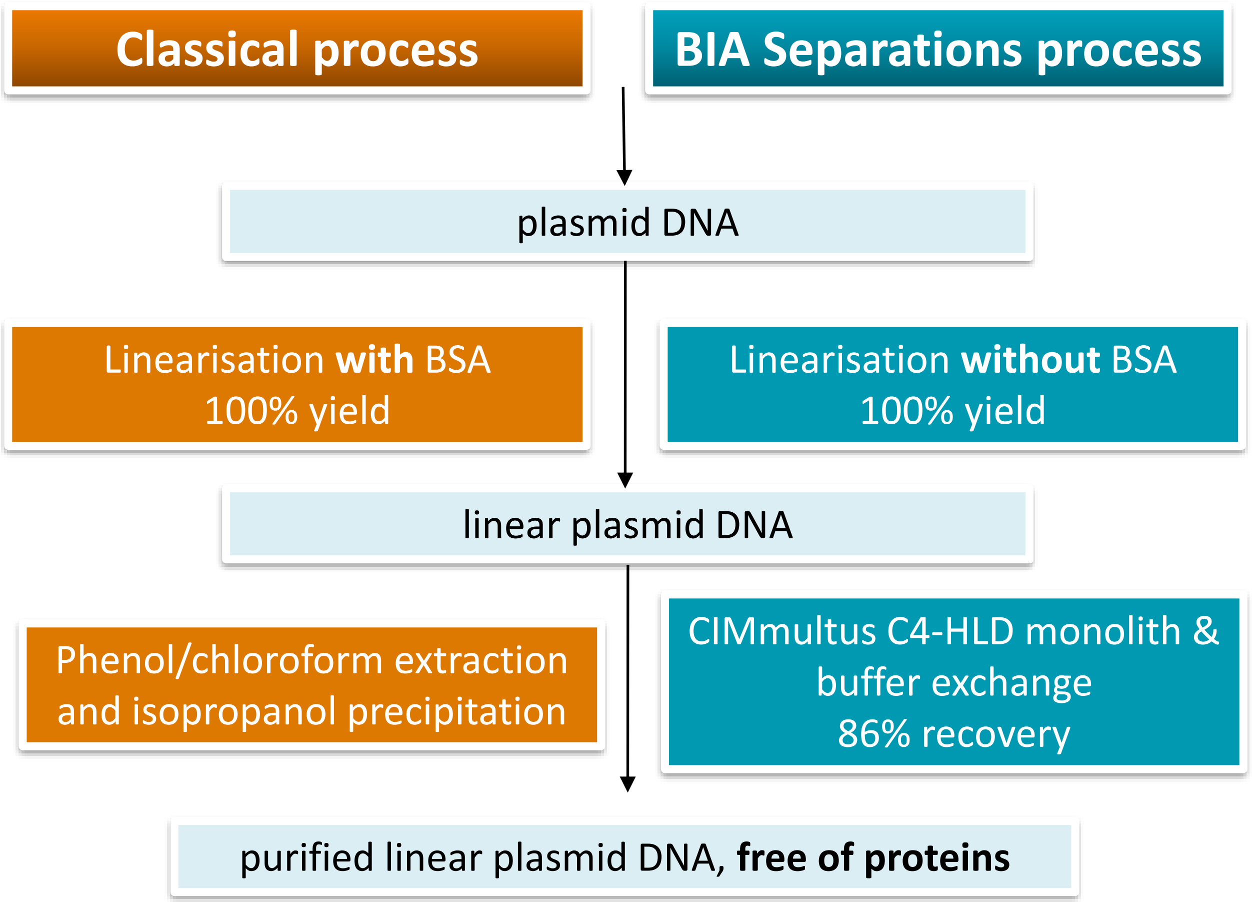
Capillary electrophoresis

AGE (agarose electrophoresis)



1 linear plasmid DNA
2 Massruler DNA ladder mix

Step 2: pDNA linearization summary



Classical process

BIA Separations process

plasmid DNA

Linearisation **with** BSA
100% yield

Linearisation **without** BSA
100% yield

linear plasmid DNA

Phenol/chloroform extraction and isopropanol precipitation

CIMmultus C4-HLD monolith & buffer exchange
86% recovery

purified linear plasmid DNA, **free of proteins**

Step 3: mRNA production – IVT process workflow

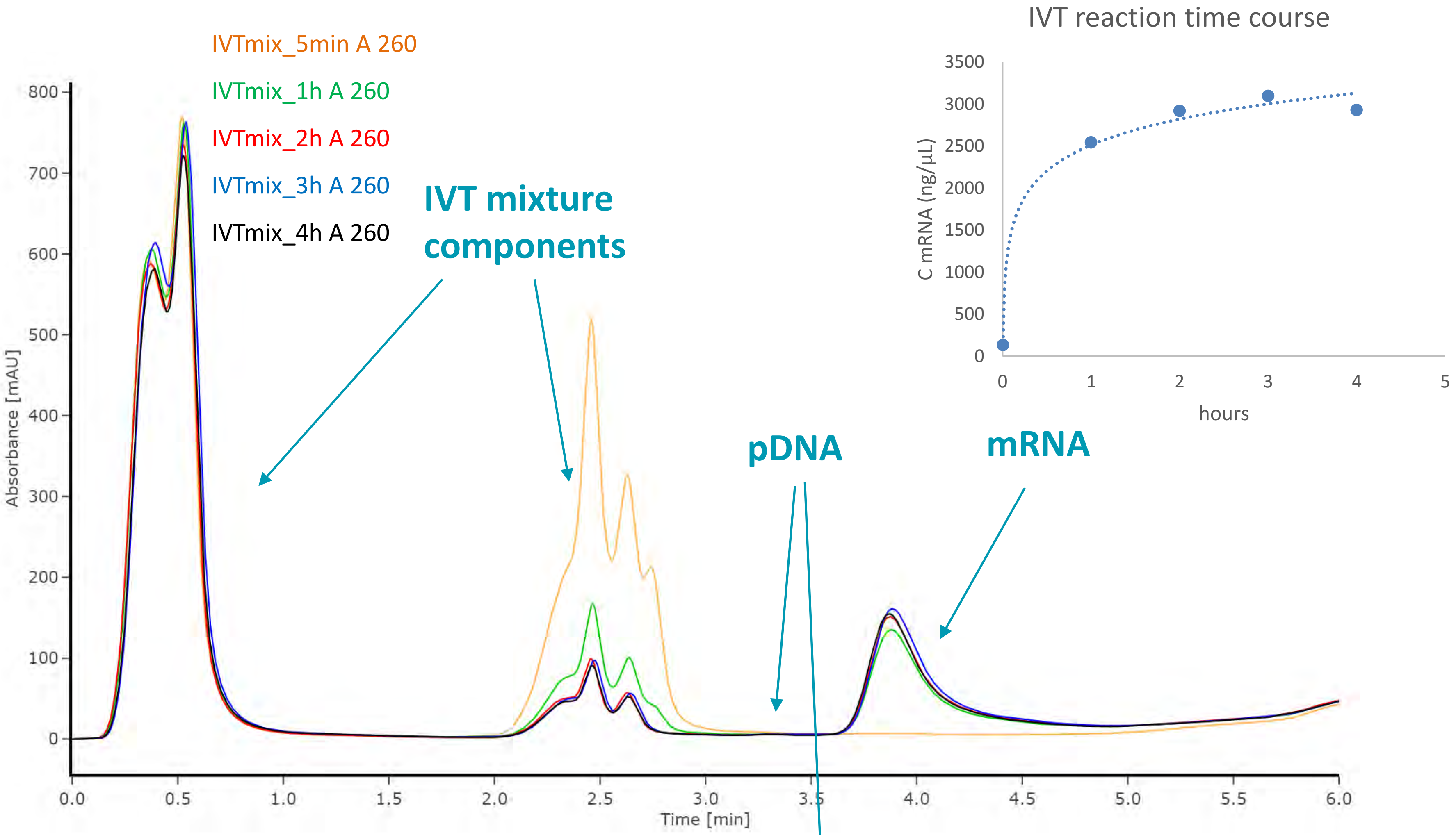
IVT reaction:

- T7 RNA polymerase (NEB), pyrophosphatase (NEB), RNAse inhibitor (NEB), and capping reagent ARCA (NEB); incubation 37°C, 3h; inactivation at 70°C, 10 min; subsequent O-methylation (cap1 formation) and polyadenylation (tail length greater than 100 bp), expected mRNA size: 1367 bp

LC purification:

- **CIMmultus PrimaS:** removal of process components before capping 1 reaction (plasmid DNA, nucleotides, buffer components, proteins) and removal of product related mRNA impurities (dsRNA)

HPLC in-process control using CIMac PrimaS column

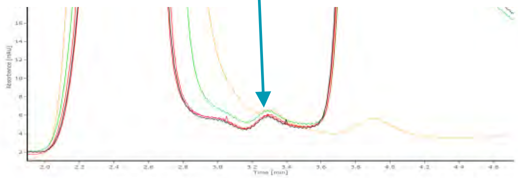


IVT mixture components

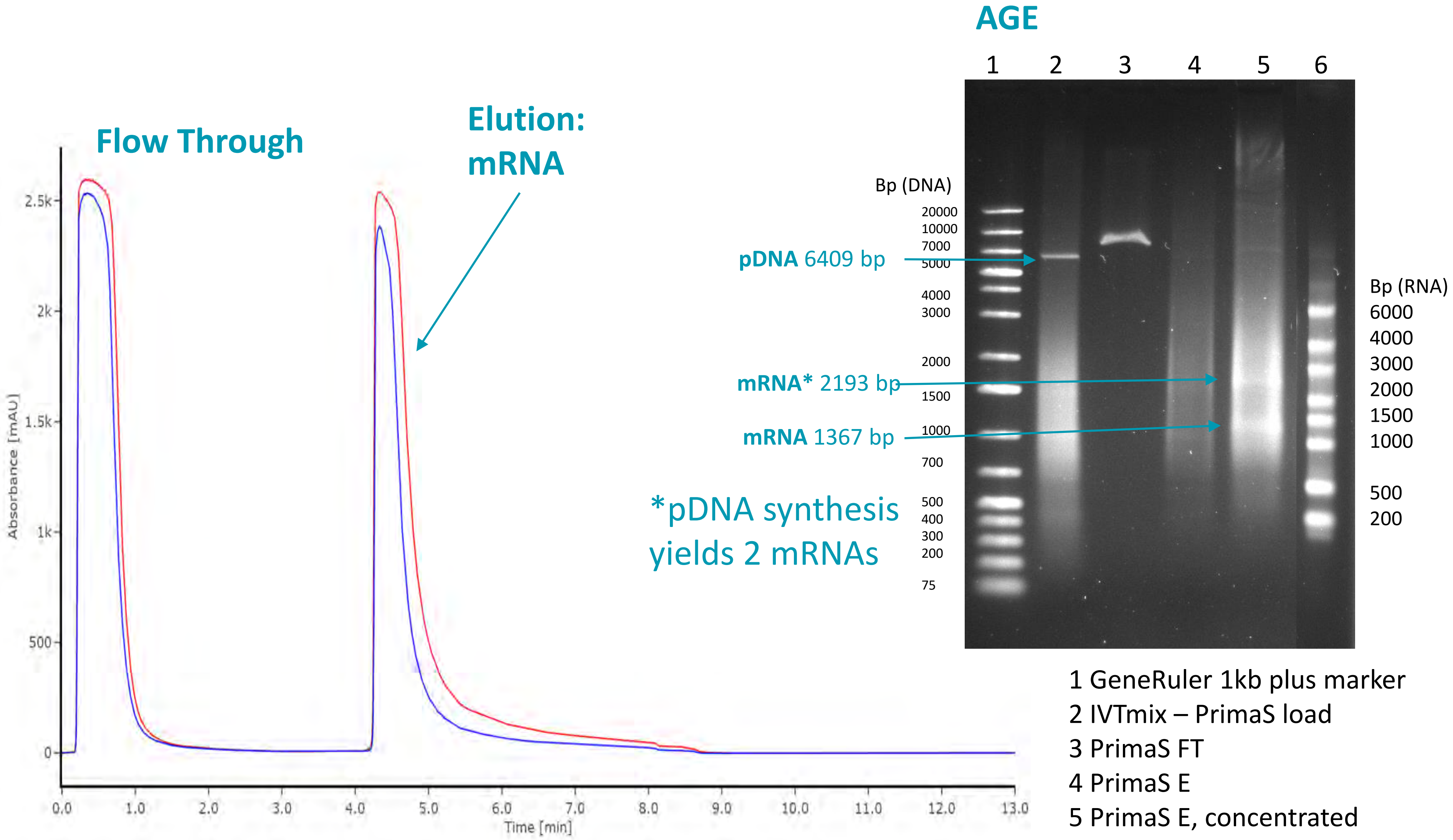
pDNA

mRNA

zoom-in



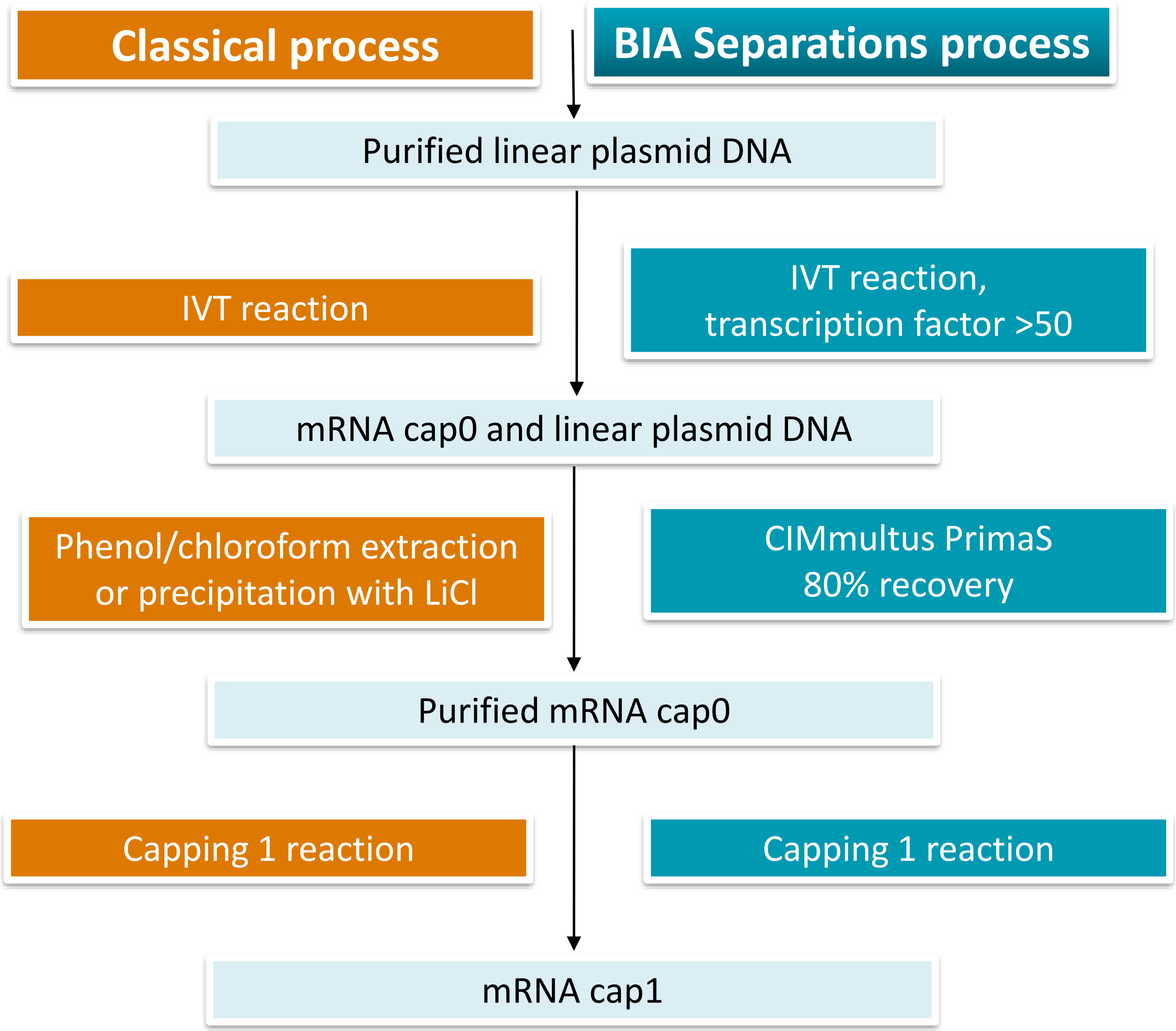
Purification of mRNA cap0 using CIMmultus PrimaS



*pDNA synthesis yields 2 mRNAs

1 GeneRuler 1kb plus marker
2 IVTmix – PrimaS load
3 PrimaS FT
4 PrimaS E
5 PrimaS E, concentrated
6 RiboRuler

Step 3: mRNA production – IVT process summary



Classical process

BIA Separations process

Purified linear plasmid DNA

IVT reaction

IVT reaction,
transcription factor >50

mRNA cap0 and linear plasmid DNA

Phenol/chloroform extraction
or precipitation with LiCl

CIMmultus PrimaS
80% recovery

Purified mRNA cap0

Capping 1 reaction

Capping 1 reaction

mRNA cap1

Step 4: mRNA poly(A) synthesis and purification workflow

mRNA poly(A) tail synthesis:

- Polyadenylation by adding ATP and poly(A) polymerase;

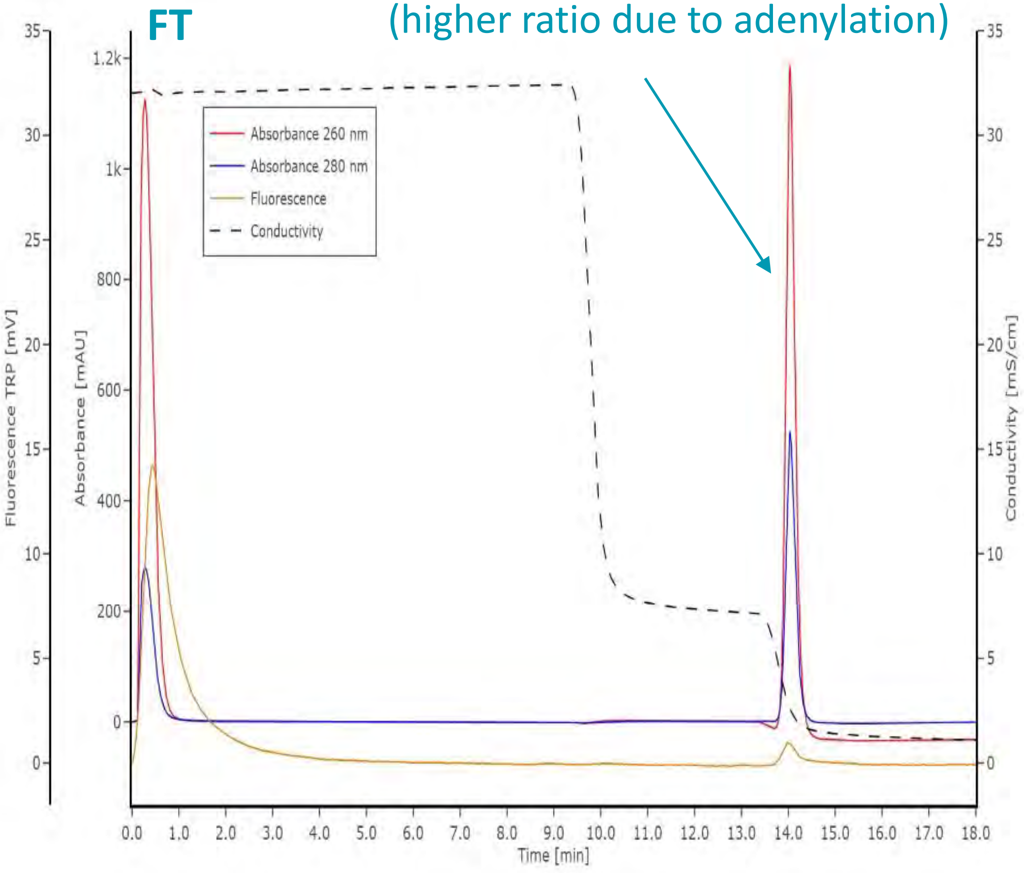
incubation at 37°C for 30 min

LC purification:

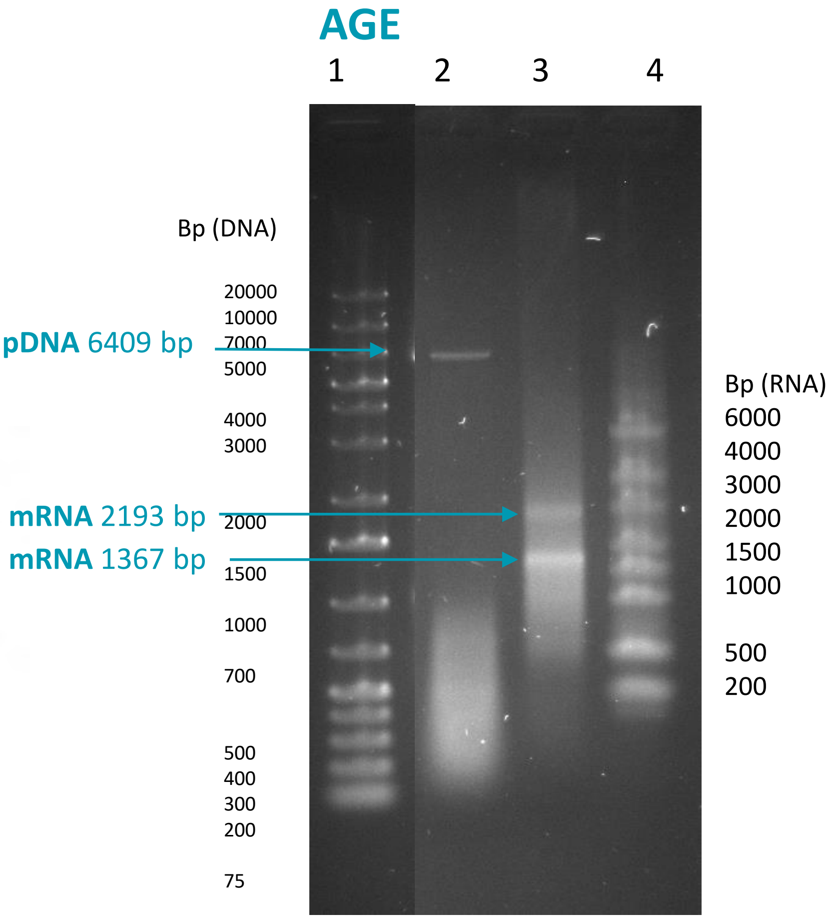
- **CIMmultus Oligo dT:** removal of components without poly(A) tail

Purification of cap1 polyadenylated mRNA using CIMmultus Oligo dT

Elution E: poly(A) mRNA
A 260/280 = 2.2
(higher ratio due to adenylation)



AGE



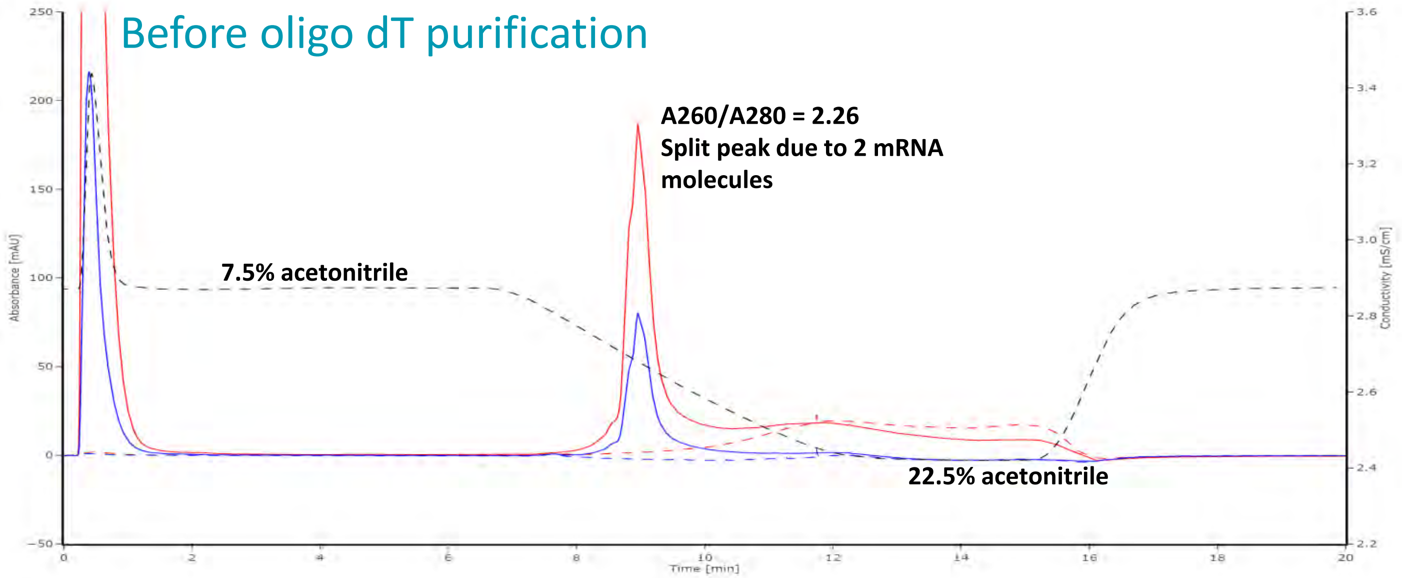
pDNA 6409 bp

mRNA 2193 bp

mRNA 1367 bp

1 GeneRuler 1kb plus marker
2 Oligo dT FT
3 Oligo dT E
4 RiboRuler

HPLC in-process control using CIMac SDVB column

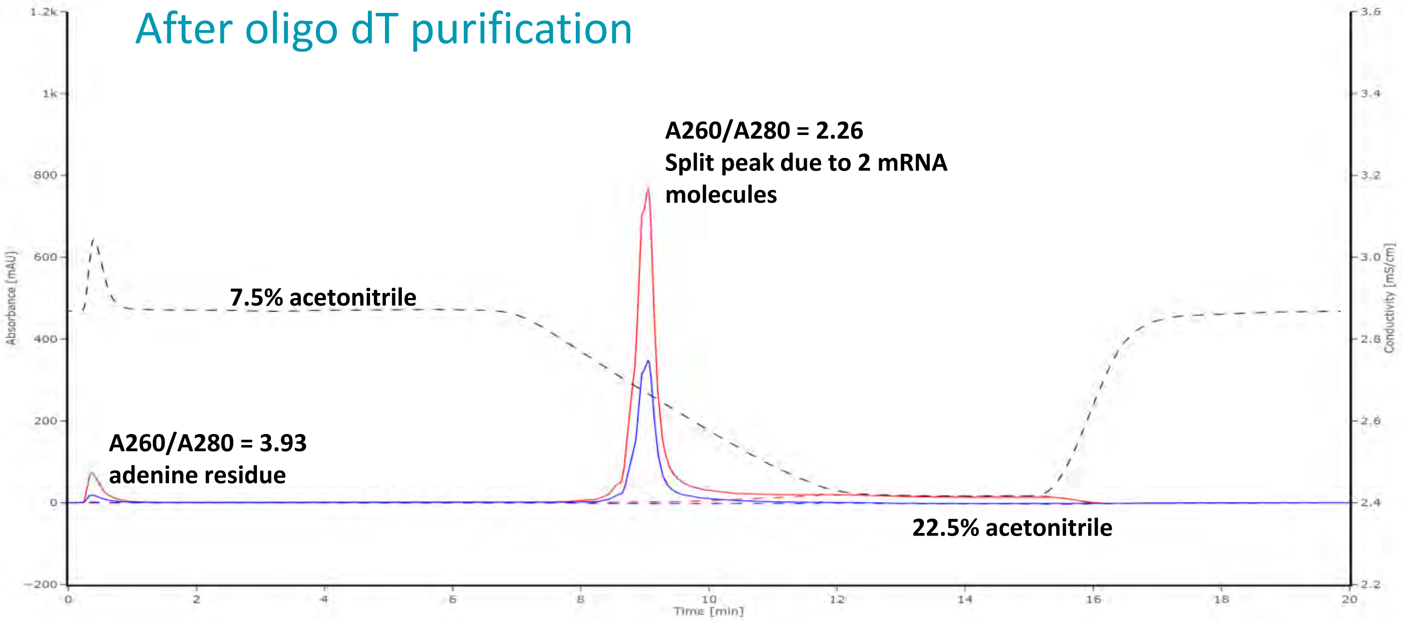


Before oligo dT purification

A260/A280 = 2.26
Split peak due to 2 mRNA molecules

7.5% acetonitrile

22.5% acetonitrile



After oligo dT purification

A260/A280 = 2.26
Split peak due to 2 mRNA molecules

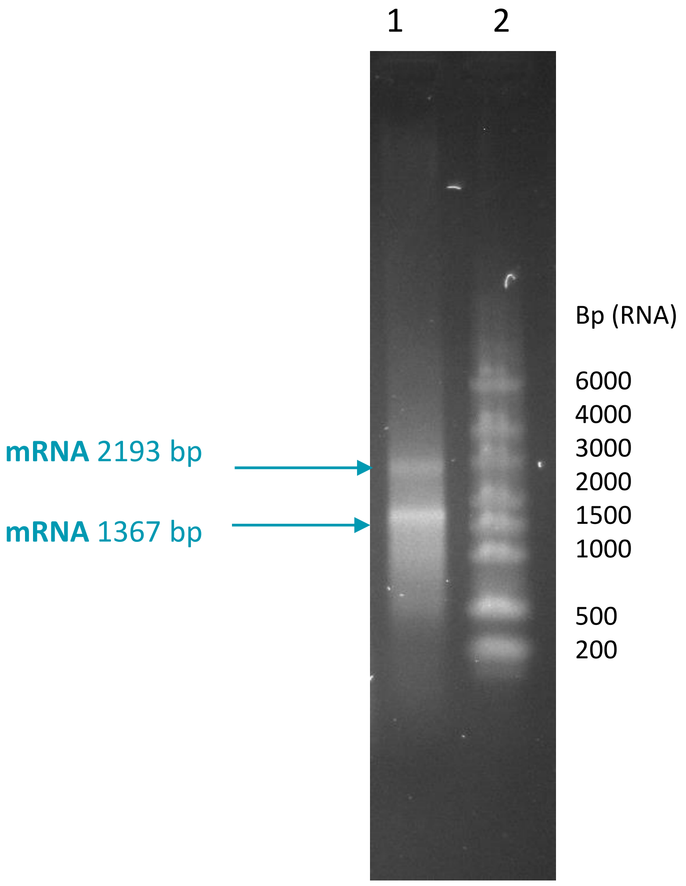
7.5% acetonitrile

A260/A280 = 3.93
adenine residue

22.5% acetonitrile

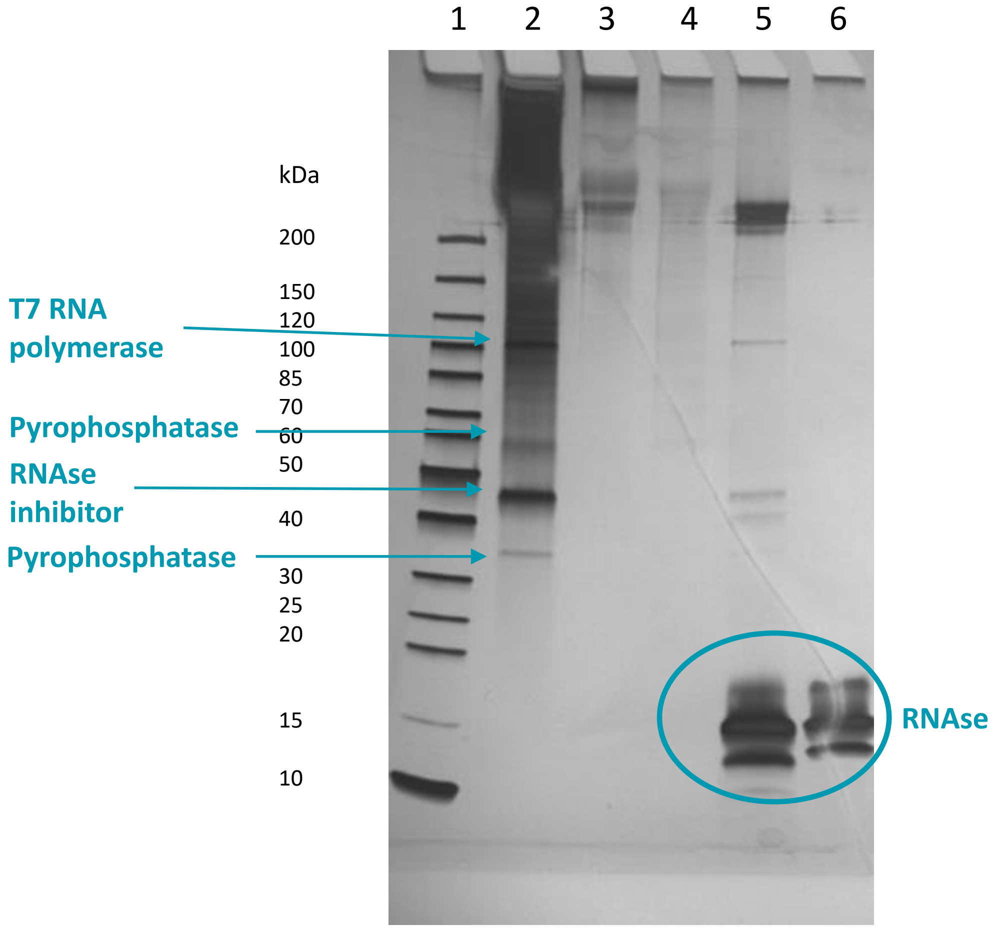
Poly(A) mRNA purity assessment

AGE



1 Purified mRNA
2 RiboRuler HR

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



1 Protein ladder
2 IVT mix
3 Oligo dT Elution

4 Oligo dT FT
5 IVT mix, treated with RNAse
6 Oligo dT E, treated with RNAse

Step 4: mRNA purification summary

Classical process

BIA Separations process

purified mRNA cap1

Poly(A) Polymerase

Poly(A) Polymerase

poly(A) mRNA

Phenol/chloroform extraction or precipitation with LiCl

Purification on CIMmultus
Oligo dT
88 % recovery

purified poly(A) mRNA cap1

Conclusions

- Monoliths support represent rapid, **high capacity and high yield** purification platform of pDNA and mRNA.
- To reduce the pDNA production costs plasmid DNA purification **should not remove the oc isoform and dimers**.
- Process impurities, especially the **proteins should be removed as early in the process as possible.**
- CIMmultus Oligo dT, C4 HLD, PrimaS and SDVB allow for **streamline mRNA purification** without any precipitation step – easier scale-up, higher yield.
- Chemistries for preparative purification are available in CIMac **analytical HPLC formats to support raw material characterization**, process development, validation, in-process monitoring, and characterization of the final product.

BIA separations