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Application Note

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PATfix® mRNA Analytical Methods for Process Development and Production

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Abstract

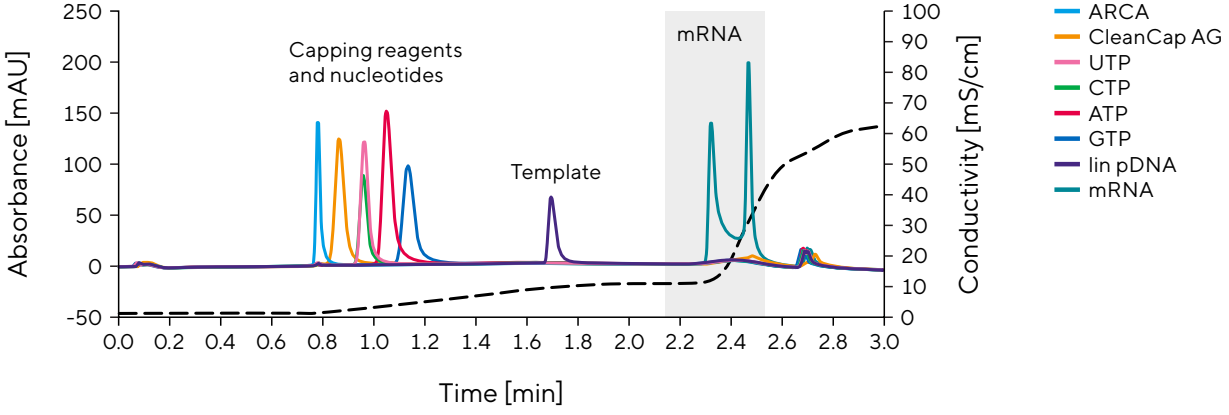
Robust and precise chromatographic analytical methods are key for the efficient development of the mRNA production process.

Three different analytical methods, which utilize three different column chemistries, are embedded in a ready-to-use PATfix® mRNA analytical platform to support mRNA process development, product quantification and characterization.

IVT Reaction Monitoring Using CIMac PrimaS™

A full at-line analysis of the IVT reaction components can be achieved using CIMac PrimaS™ column (Catalog No. 110.5118-2) with a PATfix® embedded method able to quantify the depletion of individual nucleotides, capping reagent, and generation of mRNA throughout an IVT reaction (Figure 1).

Figure 1: *PrimaS Chromatographic Method Resolves Key IVT Components.*



The chromatographic method enables rapid optimization of the IVT reaction and is critical for mRNA production, scale up, tech transfer, or other process development activities. Usually 1 µL of IVT mixture should be diluted 100x with MPA to a rough target of 10 µg/mL of mRNA. Analysis is performed at room temperature.

The method's limit of quantification (LOQ) for major IVT components is 6 ng and 12 ng for mRNA.

Two eluting peaks of mRNA, shown in Figure 1, are due to two different mRNA isoforms.

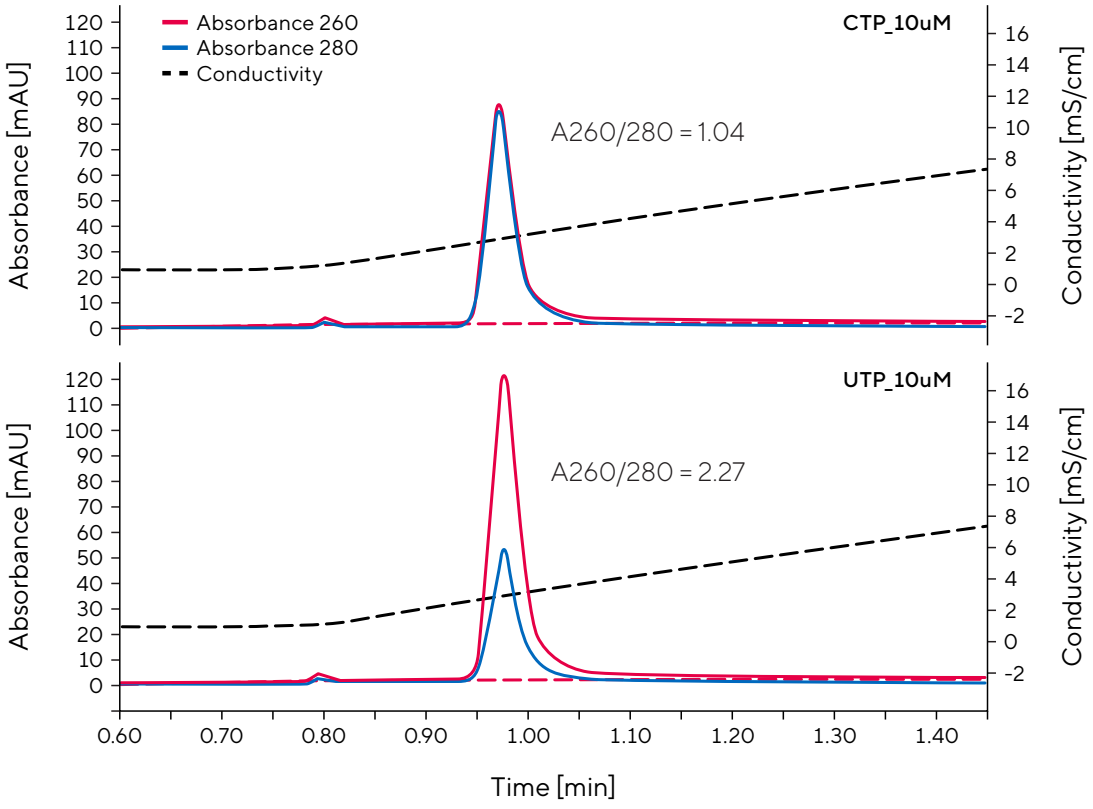
Table 1: *PATfix® IVT Reaction Monitoring Method Details (Buffers & Gradients) Using the CIMac PrimaS™ Column.*

Mobile phase A	50 mM HEPES, pH 7.0
Mobile phase B	50 mM HEPES, 100 mM $Na_4P_2O_7$, pH 8.3
Mobile phase C	100 mM NaOH, 1 M NaCl
Mobile phase D	0.5 M HEPES, pH 7.0
Detection	UV 260 nm and 280 nm, conductivity, pH
Sample amount	1 µg
System	PATfix® mRNA analytical system

Time [min]	MPA [%]	MPB [%]	MPC [%]	MPD [%]	Flow [mL/min]
0.00	100	0	0	0	2
0.10	100	0	0	0	2
1.10	55	45	0	0	2
1.80	55	45	0	0	2
1.82	0	40	60	0	2
2.50	0	40	60	0	2
2.52	0	0	100	0	2
3.40	0	0	100	0	2
3.42	0	0	0	100	2
3.52	0	0	0	100	2
3.54	100	0	0	0	2
8.00	100	0	0	0	2

PrimaS method enables quantification of individual nucleotides, including CTP and UTP that are eluted in a single peak. The method for UTP and CTP concentration estimation takes advantage of the difference in the ratio of UV absorbance areas at 260 nm and 280 nm for these two nucleotides (Figure 2).

Figure 2: *Difference in 260|280 Ratio for CTP (1.04) and UTP (2.27) Enables Quantification.*



IVT Reaction Monitoring at Optimized and Non-optimized IVT Conditions

PrimaS analytical method allows for an at-line monitoring of IVT reaction kinetics. An example of mRNA production at two different sets of IVT reaction conditions, analyzed by CIMac PrimaS™ column is presented in Figure 3 and Figure 4.

Figure 3: *IVT Reaction Monitoring at Non-optimized IVT Conditions, with 1.5 mg/mL End Point mRNA Concentration.*

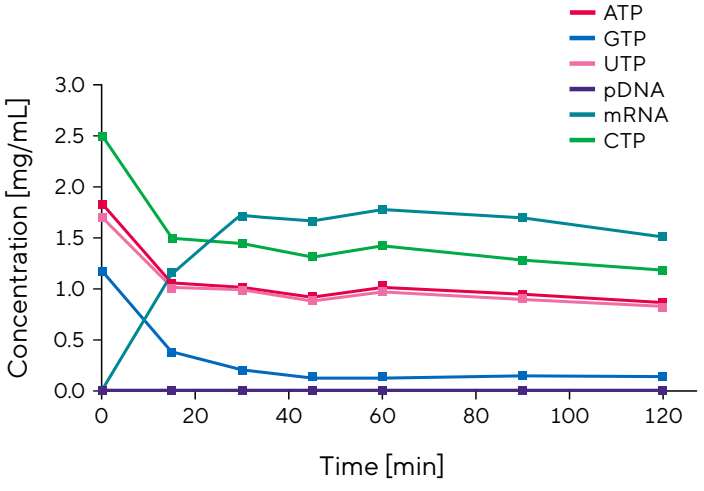
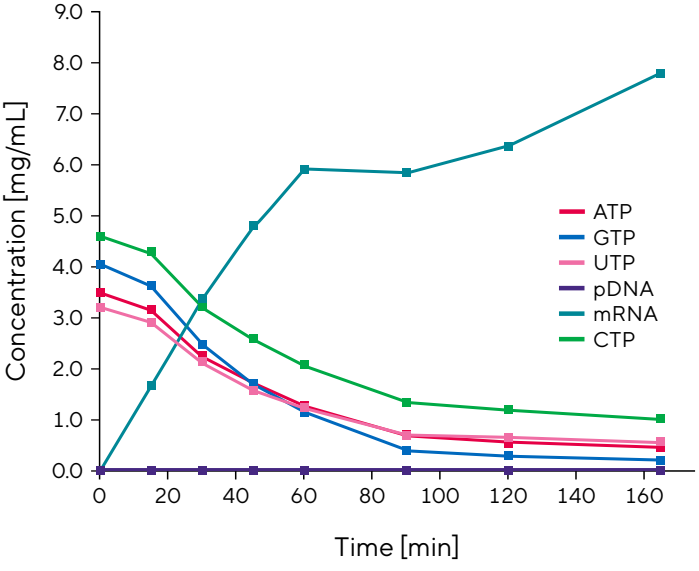


Figure 4: *IVT Monitoring at Optimized IVT Conditions, with 7.9 mg/mL End Point mRNA Concentration.*



mRNA Quantification Using CIMac™ Oligo dT

CIMac™ Oligo dT (Catalog No. 110.1219-2) is an affinity column, with dT oligonucleotides grafted to the monolith surface, binding to the polyadenylated tail (polyA) of mRNA. mRNA with a polyA tail binds to the column under high salt conditions, while all other interfering species (nucleotides, capping reagents, DNA template, enzymes) do not bind to the column and are immediately eluted, Figure 5. The LOQ for mRNA is 10 ng.

Figure 5: *Oligo dT Chromatogram of an IVT Sample. Unbound Species Such as Nucleotides, Plasmid, Capping Reagent, Enzymes, and Non-polyadenylated mRNA Are Eluted Under mRNA binding Conditions. Polyadenylated mRNA Elutes in the Elution Step.*

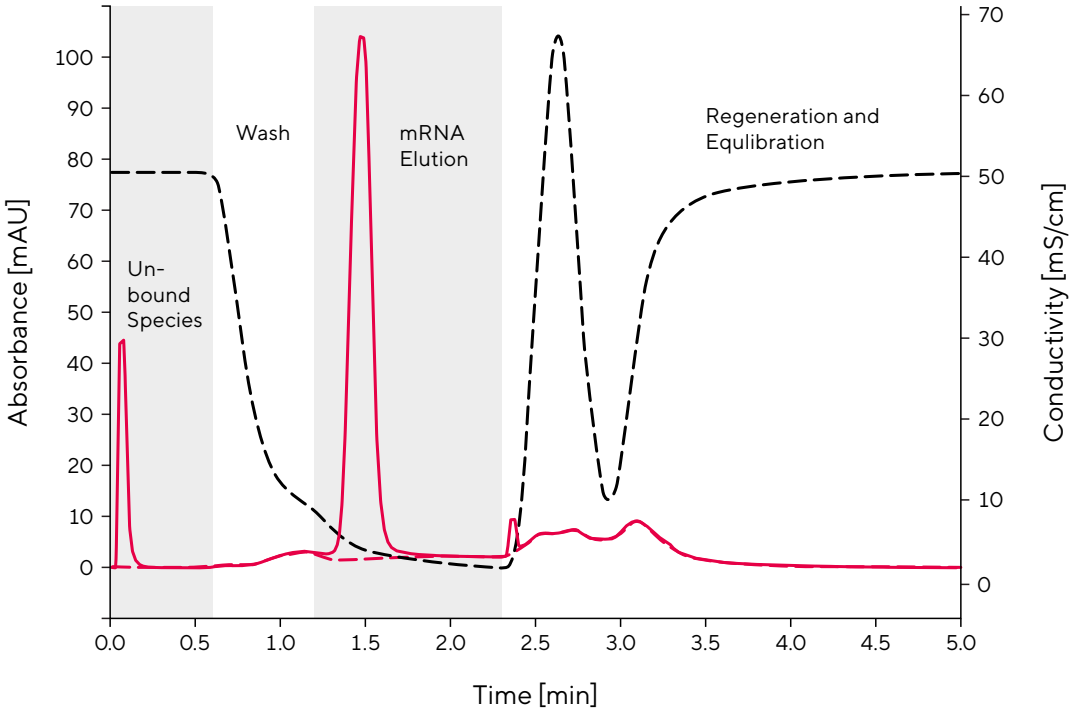


Table 2: *PATfix® Oligo dT Affinity Monitoring Method Details (Buffers & Gradients) Using the CIMac™ Oligo dT column.*

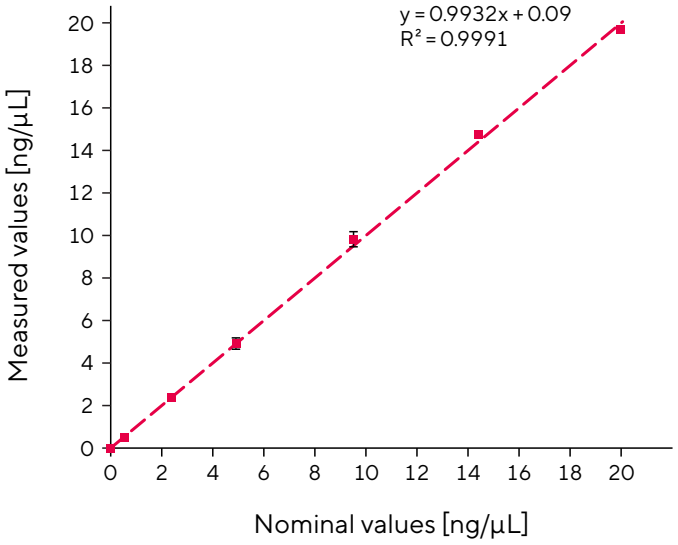
Mobile phase A	30 mM HEPES, 30 mM Na-phosphate, 0.5 M NaCl, pH 7.4
Mobile phase B	30 mM HEPES, 30 mM Na-phosphate, pH 7.4
Mobile phase C	10 mM CAPS, pH 11
Mobile phase D	0.5 M NaOH
Detection	UV 260 nm, conductivity, pH
Sample amount	0.5 µg
System	PATfix® mRNA analytical system

Time [min]	MPA [%]	MPB [%]	MPC [%]	MPD [%]	Flow [mL/min]
0.00	100	0	0	0	2
0.15	100	0	0	0	2
0.17	0	100	0	0	2
0.65	0	100	0	0	2
0.67	0	0	100	0	2
1.90	0	0	100	0	2
1.92	0	0	0	100	2
2.15	0	0	0	100	2
2.17	0	100	0	0	2
2.47	0	100	0	0	2
2.49	100	0	0	0	2
5.00	100	0	0	0	2

Method Accuracy and Precision

The analytical method using CIMac™ Oligo dT, validated following EMA and FDA guidelines, is a precise and rapid method for quantification of polyadenylated mRNA in pure and complex samples, with precision at or better than 90% (Figure 6).

Figure 6: *Method Accuracy Presented, Comparing Measured vs Nominal (Obtained by Nanodrop) Values of mRNA. Data From 6 Different Calibration Curves Were Used to the Error Bars.*



mRNA Integrity Characterization and Contaminants Detection Using CIMac™ SDVB

Analytical method using acetonitrile gradient at elevated temperature enables separation by size (Figure 7) alongside detection of impurities, with a focus on dsRNA detection (Figure 8). CIMac™ SDVB (Catalog No. BIA- 110.9001-2) column is a reverse-phase column and is used together with an ion-pairing reagent.

Figure 7: *Thermo Scientific™ RNA High Range Ladder Separation by Size on the CIMac™ SDVB Column.*

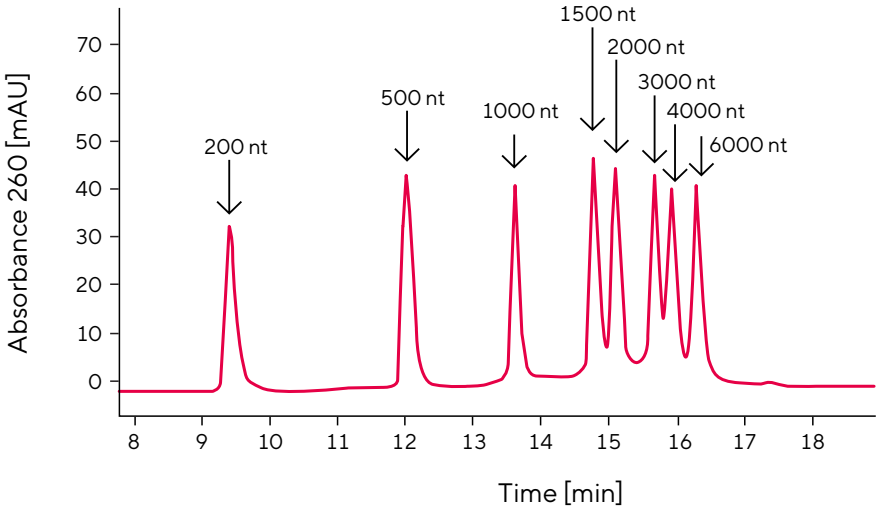


Table 3: *PATfix® Reverse Phase Product QC Method Details (Buffers & Gradients) Using the CIMac™ SDVB column.*

Mobile phase A	50 mM TEAA, 7.5% Acetonitrile, pH 7.0
Mobile phase B	50 mM TEAA, 18% Acetonitrile, pH 7.0
Mobile phase C	50 mM TEAA, 75% Acetonitrile
Temperature	60°C
Detection	UV 260 nm
Sample amount	0.5 µg
System	PATfix® mRNA analytical system

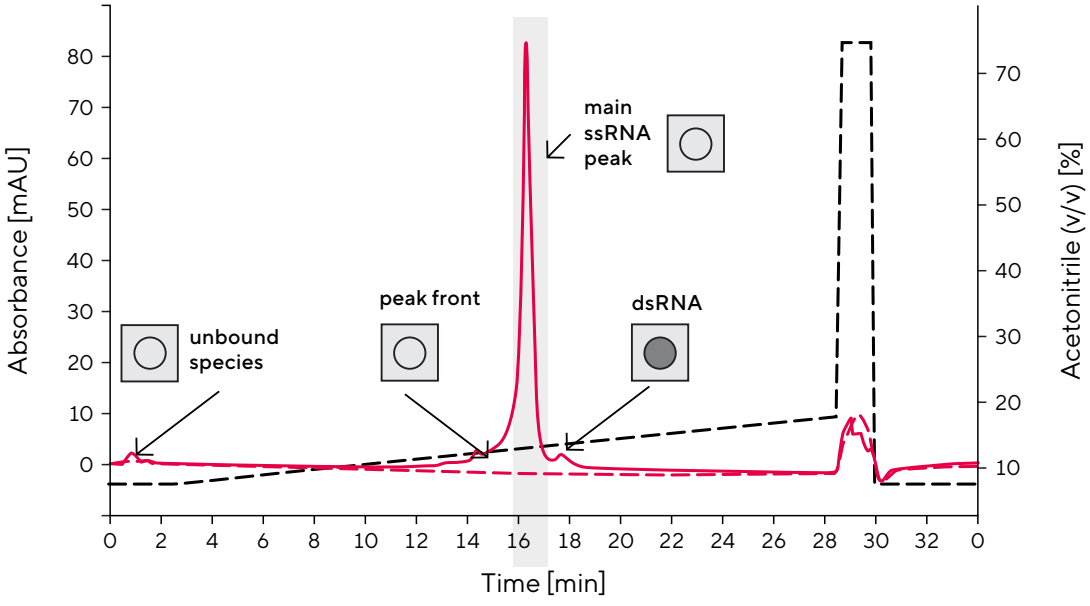
Time [min]	MPA [%]	MPB [%]	MPC [%]	MPD [%]	Flow [mL/min]
0.00	100	0	0	0	1
0.20	100	0	0	0	1
26.45	0	100	0	0	1
26.65	0	0	100	0	1
27.86	0	0	100	0	1
28.00	100	0	0	0	1
33.00	100	0	0	0	1

Detecting dsRNA Impurities Using SDVB Analytical Method

In the final mRNA product, one of the most critical impurity is dsRNA. The SDVB column analytical method can separate this impurity in a single chromatographic run (Figure 8).

The separation of dsRNA was confirmed with J2 dot-blot immunoassay.

Figure 8: *Chromatogram of an mRNA Sample Containing a Detectable Amount of dsRNA, Which Was Corroborated With Dot Blot Using J2 Antibody.*

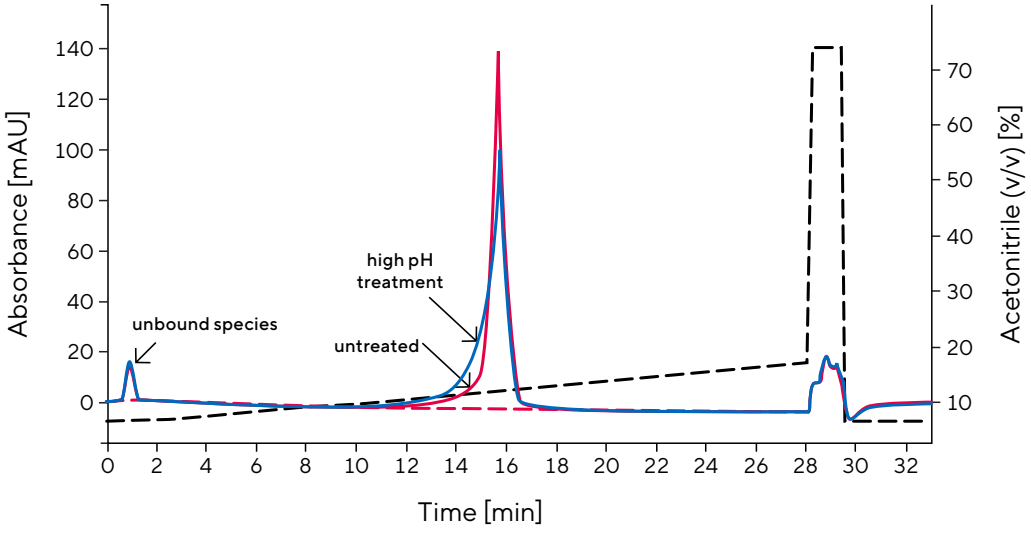


SDVB is a Stability-indicating Method for mRNA

mRNA integrity could be studied using CIMac™ SDVB column, where the fronting of the main peak could indicate mRNA degradation fragments or abortive transcripts from the IVT reaction.

In Figure 9, a forced degradation study (pH 11, 60 min) of mRNA (4000 nt) is presented. Fronting of the main peak, due to the emergence of shorter fragments eluting before the main, intact mRNA can be observed.

Figure 9: *Example of mRNA Forced Degradation Study Using Strong Base Treatment.*



Notice

PATfix® mRNA platform enables:

- At-line quantitative monitoring of the IVT reaction components using CIMac PrimaS™ column.
- Precise and fast polyadenylated mRNA quantification in pure and complex samples with CIMac™ Oligo dT column.
- Size, integrity and dsRNA impurity detection using CIMac™ SDVB column.

For best results the system flowpath should be RNase-free.

FAQ

What is the recommended protocol for IVT sample preparation for analysis on CIMac PrimaS™?

- Crude IVT aliquots should be quenched immediately after sampling using an EDTA-containing buffer.
- For best performance, the quenched sample should be diluted at least 100x with MPA to a rough target of 10 µg/mL of mRNA.

Can the methods described here be operated at ambient temperatures?

- CIMac™ SDVB method is performed with a column thermostat set to 60 °C. CIMac PrimaS™ and Oligo dT analytics are performed without a column thermostat and at room temperature.

Can we use the columns and methods on UPLC?

Yes, however the method needs to be optimized for your setup | system. This is true for any UPLC | HPLC system besides PATfix®. Additionally, on UPLC systems, special care should be taken to not exceed maximum CIMac™ pressures. Please refer to the specific column Product Sheet and Instruction Manual document for details.

What is the difference between C6 and C12 linker on the OdT column?

The starting column of choice for PATfix® analytics is the CIMac™ Oligo dT with the C12 linker. We offer both C6 and C12 columns because some customers prefer one over the other, but from our point of view performance is comparable between the two. Performance can be further evaluated if desired by testing both.

Why does the mRNA on CIMac PrimaS™ elute in two peaks (as shown in Figure 1)?

Our separate analysis of the two peaks with orthogonal methods, show that the two eluting peaks are a result of two different mRNA isoforms having a different chromatographic behaviour. Structurally they are the same mRNA.

Additional Literature

ebook
High-Selectivity Biochromatography mRNA Analytics

Technical note
Exploring CIMac PrimaS™ for Analysis of mRNA

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