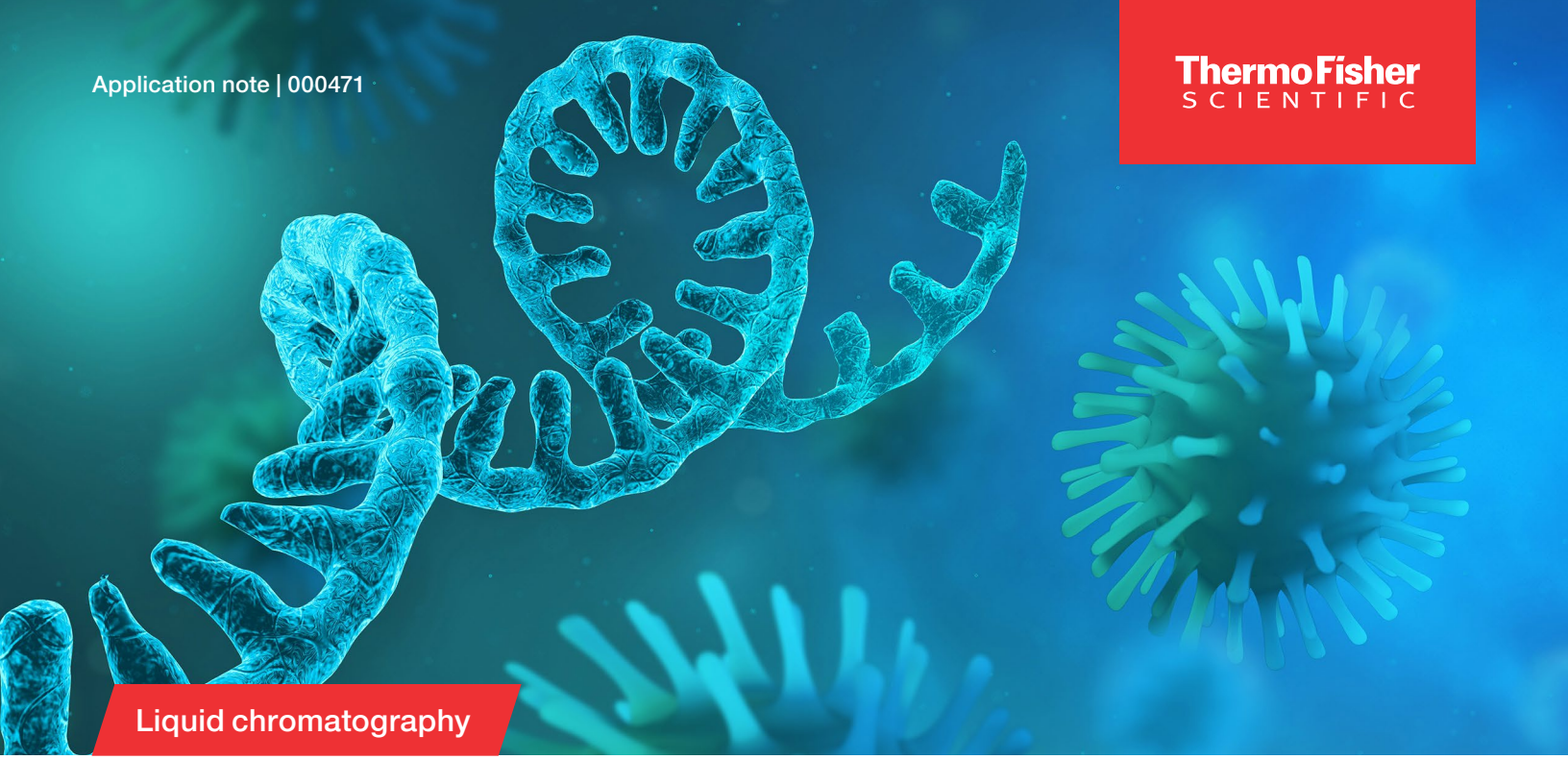

Application note | 000471

Liquid chromatography

Simultaneous reversed-phase and anion-exchange method scouting with a dual system for mRNA impurity determination

Authors

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Keywords

Vanquish Duo UHPLC, Dual-LC, mRNA, ion-exchange chromatography, ion-pairing reversed-phase chromatography, method development, solvent scouting, mRNA purification

Application benefits

- Leverage two different separation chemistries in the same instrument at the same time: ion-exchange (IEX) and ion-pairing reversed-phase (IP-RP)
- Scout up to 10 solvents for each column type to find most suitable eluent condition
- Accelerate method development
- Maximize sample knowledge
- Perform mRNA profiling and detection of post-in vitro transcription (IVT) purification impurities
- Use corrosive mobile phases, such as NaCl 1 M, with the fully biocompatible flow path of the Thermo Scientific™ Vanquish™ Duo UHPLC system

Goal

Determine the most suitable conditions for the detection of post-transcriptional impurities in mRNA with a time-effective scouting approach

Introduction

In recent years, the use of messenger ribonucleic acid (mRNA) as a new tool for scientific and pharmaceutical purposes has increased considerably and become well diversified. Some examples include vaccines, in vivo administration of mRNA to express proteins/antibodies, protein replacement, and cancer immunotherapy[1-3], reprogramming of induced pluripotent stem cells (iPS)[4], and genome editing applications[5].

Usually, the mRNA is produced by transcribing a plasmid DNA template via what is called in vitro transcription (IVT), mimicking the process that takes place in the cell (in vivo). For a mRNA to be mature and functional for efficient translation in eukaryotic cells, it needs to be capped (7-methylguanylate cap at the 5' end) and polyadenylated (poly-adenine(A) tail at the 3´ end). The cap and poly-A tail may be added enzymatically in a post-transcription step or co-transcriptionally (poly-A tail encoded to the DNA template).

After the synthesis, the mRNA must be purified from the remaining reaction byproducts. High pressure liquid chromatography (HPLC), oligo dT chromatography (e.g., POROS™ oligo dT purification), spin columns, and magnetic beads (e.g., Invitrogen™ Dynabeads™ MyOne™ Carboxylic Acid) are common purification methods. Purification is important because some impurities may trigger immune responses and decrease translation, affecting down-stream applications. There are various methodologies and variables that can be optimized to perform the IVT. While some impurities will remain from the raw materials, others will be more dependent on the mRNA sequence. Among the most common impurities are nucleotides, enzymes, DNA templates and fragments, abortive transcript fragments, double stranded RNA (dsRNA), and primers.

A sound method to evaluate mRNA purification is highly desired and important to ensure drug safety. Such a method should provide a reasonable separation and be sensitive to impurities with good mRNA peak shape. HPLC is beneficial for this purpose due to its high resolution, quantitative monitoring, rapid results, and small sample waste. Beyond quantifying the purity, HPLC can also perform advanced analysis to identify the impurities left after purification using standards or mass spectrometry.

The Thermo Scientific™ Vanquish™ Duo for Dual LC combined with the Method Scouting Kit offers a valuable solution for determining the most promising chromatographic conditions in a time-effective manner. With the Vanquish Duo system, two independent chromatographic chemistries (IEX and IP-RP) are scouted on the same system at the same time, which enables faster method development and requires less instrumentation. In this work, a large number of mobile phase conditions are tested with minimal preparation work. The most promising methods for each chromatography type are discussed. These methods can be further adapted to the specific type of samples. Furthermore, one stationary phase might be chosen for a particular analysis, or both might be used to maximize sample knowledge.

Experimental

Chemicals

- Deionized water, 18.2 MΩ·cm at 25 °C, Thermo Scientific™ Barnstead™ GenPure™ xCAD Plus Ultrapure Water Purification System (P/N 50136149)
- Acetonitrile, Optima™ LC/MS grade, Fisher Chemical™ (P/N A955-212)
- Triethylamine, Thermo Scientific™, 99% (P/N 157911000)
- 1-Hexylamine Alfa Aesar™, 99% (P/N A15663)
- N,N-Diisopropylethylamine, Thermo Scientific™, >99.5% (P/N 367840250)
- Acetic acid, Optima™ LC/MS grade, Fisher Chemical™ (P/N A113-50)
- Sodium chloride, Honeywell™ Fluka™, >99% (P/N 002357416)
- Sodium perchlorate (monohydrate), HPLC grade, Fisher Chemical™ (P/N S/5966/50)
- Tris(hydroxymethyl)aminomethane, Thermo Scientific™, 99.8% (P/N 424571000)
- Hydrochloric acid, fuming, 37%, Merck ACS, ISO, Reag. Ph Eur (P/N 1003171000)

Sample handling

- Fisherbrand™ Isotemp™ Stirring Hotplate (P/N S14365)
- Thermo Scientific™ Orion™ 3 Star pH Benchtop Meter (P/N 13-644-928)
- Fisher Scientific™ Fisherbrand™ Mini Vortex Mixer (P/N 14-955-152)
- Thermo Scientific™ 11 mm plastic crimp/snap top autosampler vials (P/N C4011-13)
- Cap with septum (Silicone/PTFE), Fisher Scientific™ (P/N 13-622-292)

Instrumentation

Vanquish Duo for Dual LC system consisting of:

- System Base Vanquish Duo for Dual LC (P/N VF-S02-A-02) (includes viper kit P/N 6036.2305)
- Vanquish Dual Pump F (P/N VF-P32-A)
- Vanquish Dual Split Sampler FT (P/N VF-A40-A)
- 2x Vanquish Column Compartment H (P/N VH-C10-A-02)
  - 2x Active Pre-heater, MP35N, 0.10 × 610 mm (P/N 6732.0150)
  - 2x Post-Column Cooler, MP35N, 1 µL, 0.10 × 590 mm (P/N 6732.0520)
- 2x Vanquish Diode Array Detector H (P/N VH-D10-A)
  - Standard Thermo Scientific™ LightPipe™ flow cell, biocompatible (P/N 6083.0100B) (2 µL, 10 mm, 6 MPa, fused silica)
  - High sensitivity Thermo Scientific™ LightPipe™ flow cell, biocompatible (P/N 6083.0200B) (13 µL, 60 mm, 6 MPa, fused silica)
- 2x Extension Kit for Automated Method Scouting, Vanquish Systems (P/N 6036.0100)

Sample preparation

The mRNA used for the analysis has a size of approximately 2,500 nucleotides.[6] The type of purification was based on magnetic beads. Non-purified mRNA is mRNA in transcription buffer (Invitrogen™ MEGAscript™ T7 Transcription Kit) at a concentration of 4.57 µg/µL, and purified is mRNA in TE Buffer pH 8.0 at 1.37 µg/µL. An Invitrogen™ Qubit™ RNA broad range (BR) assay kit was used for the quantitation.

Mobile phase preparation

For the IP-RP method, concentrated buffer was prepared in channel C to reach the indicated values in Table 3 when mixed with channels A and B. The preparation of triethylamine acetate (TEAA) and hexylamine acetate (HAA) is described in the column manual,[7] while N,N-diisopropylethylamine (DIPEA) was prepared by adding acetic acid until the desired pH was reached. For IEX, compounds were weighed or measured, and aqueous solutions were prepared using a magnetic stir bar. The pH was lowered to the desired value with HCl. Acetonitrile (MeCN) was added after the pH was set.

Chromatography Data System

The Thermo Scientific™ Chromeleon™ 7.3 CDS was used for data acquisition and analysis.

Table 1. System tubing description for Figure 1. Included in System Base Vanquish Duo for Dual LC (VF-S02-A-02). Also available separately as the Viper Kit for Vanquish Duo for Dual LC, P/N 6036.2305.

No.	Description
1, 2	Viper Capillary, 0.10 × 550 mm, MP35N (6042.2360)
3, 4	Active Pre-heater, MP35N, 0.10 × 610 mm (6732.0150)
5, 6	Post-Column Cooler, MP35N, 1 µL, 0.10 × 590 mm (6732.0520)
7, 8	Waste fluidics (included with the detector)

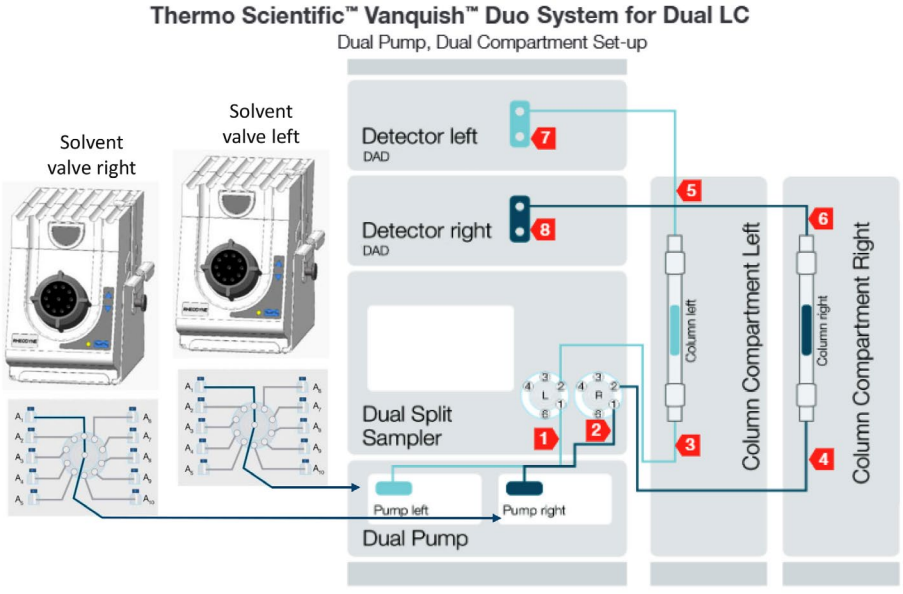

Figure 1. Flow scheme overview: Thermo Scientific Vanquish Duo for Dual LC with Solvent Extension Kits for automated method scouting. Dual pump and dual column compartment set-up.

Chromatographic conditions

Table 2. Chromatographic conditions

Parameter	Setting
Column left	Thermo Scientific™ DNAPac™ RP, 2.1 × 100 mm, 4 µm (P/N 088923)
Column right	Thermo Scientific™ DNAPac™ PA200 RS, 4.6 × 150 mm, 4 µm (P/N 082509)
Flow rate left	0.4 mL·min^{-1}
Flow rate right	1.0 mL·min^{-1}
Column and preheater temperature left	50 / 90 °C
Column and preheater temperature right	30 / 50 / 80 °C
Post-column cooler temperature	50 °C
Autosampler temperature	4 °C
Autosampler wash solvent	50/50 Acetonitrile/Water (v/v)
Injection volume	1 and 0.3 µL
Detector settings	Wavelength 260 nm, Data collection rate 10 Hz Response time 0.5 s UV 3D field 235(IEX)/240(IP-RP) – 310 nm 4 nm bunch width

Table 3. Gradient and mobile phase conditions scouted for IP-RP method. The gradient shaded in blue matches the blue conditions and the gradient shaded in green matches the green conditions.

Time (min)	%A (Water)		%B (MeCN)		%C (Solvent selection valve)	
0.0	20	45	5	5	75	50
15.0	0	0	25	50	75	50
16.0	0	0	25	50	75	50
16.1	0	0	90	90	10	10
20.0	0	0	90	90	10	10
20.1	20	45	5	5	75	50
25.0	20	45	5	5	75	50

Valve channel C position	Conditions*
1	100 mM TEAA, pH 7.0
2	100 mM TEAA, pH 8.5
3	100 mM TEAA, pH 10.5
4	25 mM HAA, pH 7
5	25 mM HAA, pH 8.5
6	25 mM HAA, pH 10.5
7	25 mM DIPEA + Acetic acid to pH 7
8	25 mM DIPEA + Acetic acid to pH 8.5
9	25 mM DIPEA + Acetic acid to pH 10.5

*These conditions represent the final concentration after dilution with %A and %B in the proportioning valve (25% in the case of TEAA and 50% for HAA and DIPEA). A wash step with increased MeCN content was used from 16.1 to 20 min. Additionally, a 90% MeCN wash blank was used after each condition for 10 minutes.

Table 4. Gradient and mobile phase conditions scouted for IEX method. The color matches represent the eluent pairs. See below detailed information.

Time (min)	%A* 40mM Tris pH 9 10 mM NaOH pH 11	%B* 40mM Tris pH 9, 10% MeCN 10 mM NaOH pH 11, 10% MeCN	%C Solvent selection valve
0.0	95	95	5
15.0	0	0	100
17.0	0	0	100
17.1	95	95	5
23.0	95	95	5

Valve channel C position	Conditions
1	40 mM Tris, 1 M NaCl, pH 9
2	40 mM Tris, 1 M NaCl, 10% MeCN, pH 9
3	40 mM Tris, 0.8M $NaClO_4$, pH 9
4	40 mM Tris, 0.8M $NaClO_4$, 10% MeCN, pH 9
5	10 mM NaOH, 1 M NaCl, pH 11
6	10 mM NaOH, 1 M NaCl, 10% MeCN, pH 11
7	10 mM NaOH, 0.8M $NaClO_4$ pH 11
8	10 mM NaOH, 0.8M $NaClO_4$, 10% MeCN, pH 11
9	Water
10	MeCN

*After running the first four conditions with TRIS, eluents from channel A and B were replaced to test the next four with NaOH. Only two channels were used at the same time: either with organic or without (A with C1, C3, C5, and C7; B with C2, C4, C6, and C8). Channel 9 and 10 were used for the column wash.

Results and discussion

Initial scouting was performed using the purified sample. Although the complete impurity profile would not be observed, starting the scouting with a purified sample was preferred because the quality of the mRNA peak shape and baseline signal could be better assessed.

When analyzing nucleic acids, either single or double stranded, their spatial conformation should be considered. Even if the mRNA is single stranded, it may present hairpin loops, where the strand folds and forms base pairs with another section of the same strand, which have an effect on peak shape. Therefore, denaturing conditions where the molecule is linearized might be needed to obtain sharper peaks and possibly detect changes due to dsRNA denaturation if present. Denaturation requires either high temperature, aqueous mobile phase at around pH 12, an organic modifier, or a combination of these conditions.[10] Another option for denaturation is the addition of other substances to the mobile phase.[10] High pH (10.5 and 11) and high temperature (80 and 90 °C) were included in the method scouting for linearization of secondary nucleic acid structure.

IP-RP chromatography

Figure 3 displays chromatograms from all nine conditions tested for the IP-RP method at 50 °C. The same conditions at 90 °C did not yield any apparent improvement but caused a significant reduction in the mRNA peak signal (except DIPEA) and a slight increase in the signals of the impurities (data not shown). All three ion pairing agents at high pH (10.5) give unacceptable chromatograms, showing either poor shape of the peak assigned to mRNA (condition 6, HAA), very low retention of the main peak (condition 3, TEAA), or high background signal (condition 9, DIPEA). The remaining conditions yield mRNA as the main peak with acceptable peak shape and some impurities detected at low retention times. Some late eluting peaks are due to the gradient wash. For any ion-pairing agent, only slight differences were observed between pH 7 and 8.5.

#	UV_VIS_1	Name	Type	*PCC_temp	*SSV_position	*TCC_Oven_temp	*TCC_Preheater_temp	*Buffer_Name
1	None	Blank - equilibration&"purge"	Blank	50	1	50	50	100mM TEAA pH 7
2	None	Blank	Blank	50	1	50	50	100mM TEAA pH 7
3	None	Blank	Blank	50	1	50	50	100mM TEAA pH 7
4	None	mRNA_Condition_1	Unknown	50	1	50	50	100mM TEAA pH 7
5	None	mRNA_Condition_1	Unknown	50	1	50	50	100mM TEAA pH 7
6	None	mRNA_Condition_1	Unknown	50	1	50	50	100mM TEAA pH 7
7	None	Blank	Blank	50	1	50	50	100mM TEAA pH 7
8	None	WASH	Blank	50	1	50	50	100mM TEAA pH 7

Figure 2. Example of custom variables used for the IP-RP method. These method parameters can be set in the sequence table rather than creating multiple instrument methods. “SSV_position” refers to the solvent selection valve position. When changing solvents, the purge via autosampler command may be used or a 100% C blank run can simply be initiated to elute all remaining buffer in the tubing between selection valve and pump. Users can find custom variable tutorials as the top search results for “create custom variables” and “use custom variables” in Chromeleon’s help function. Refer to References 8 and 9 for more details about custom variables and method scouting options.

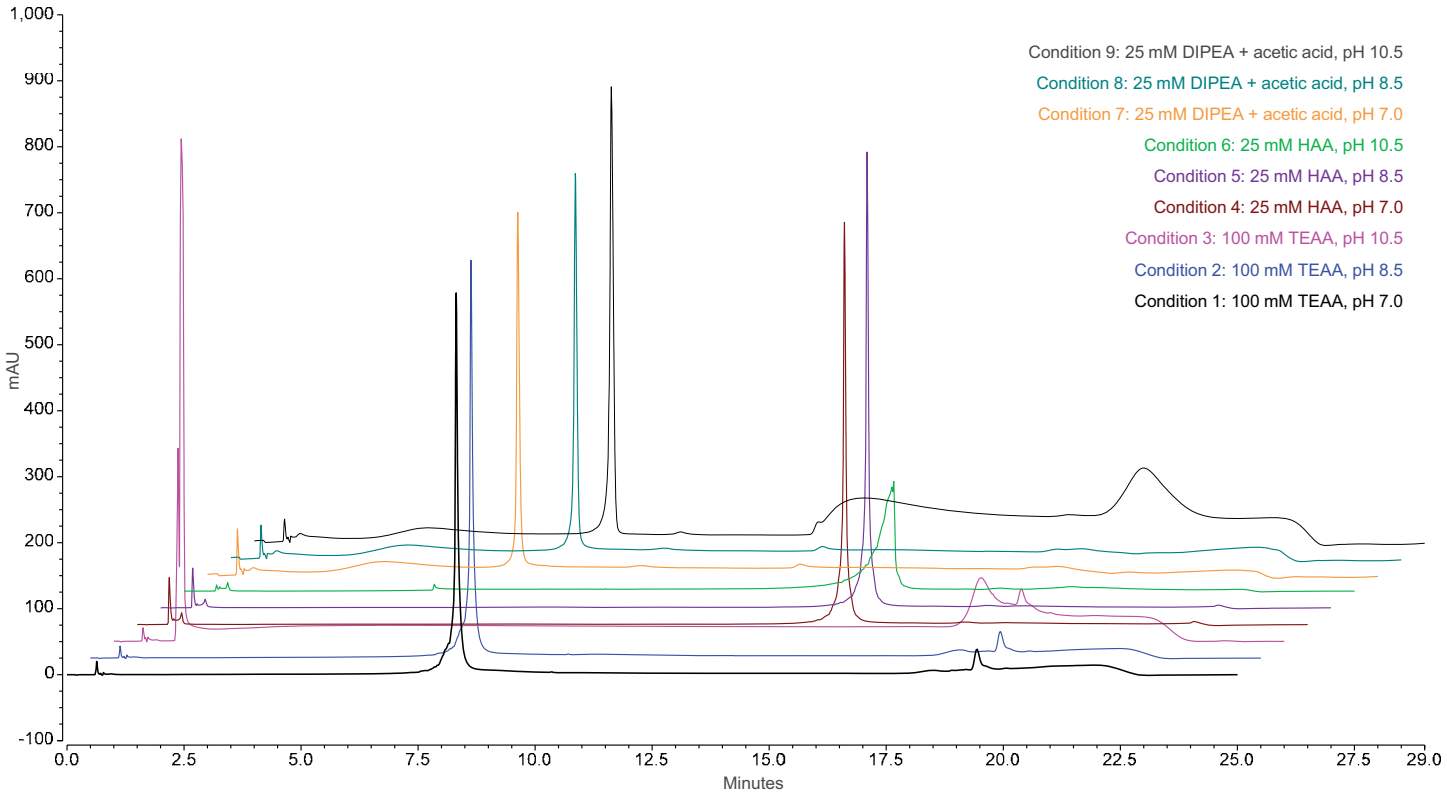

Figure 3. IP-RP purified mRNA scouting conditions overlay at 50 °C. 1 µL injection. mRNA is the most intense peak.

In the next step, the method assessment was continued with the non-purified sample. In Figure 4, the chromatograms of the non-purified mRNA from one condition for each ion pairing agent are compared. With DIPEA and TEAA, most impurities co-elute in a large split peak, which is poorly retained. Nevertheless, at 90 °C both display a small rider peak on the mRNA tail that is not observed with HAA (data not shown). Despite that, HAA is the only tested IP agent with acceptable retention and separation of most impurities; therefore, this method was deemed the most suitable for impurity profiling.

Chromatograms showing condition 5 with HAA at pH 8.5 for purified and non-purified mRNA are compared in Figure 5. The method was suitable for controlling the effectiveness of the purification process. Most of the impurities, observed at a retention time lower than the main peak in the non-purified sample, were eliminated or substantially diminished by the purification process. It is interesting to note that the first eluting peak of the purified sample is bigger than the non-purified, which suggests that some changes might have taken place in the sample.

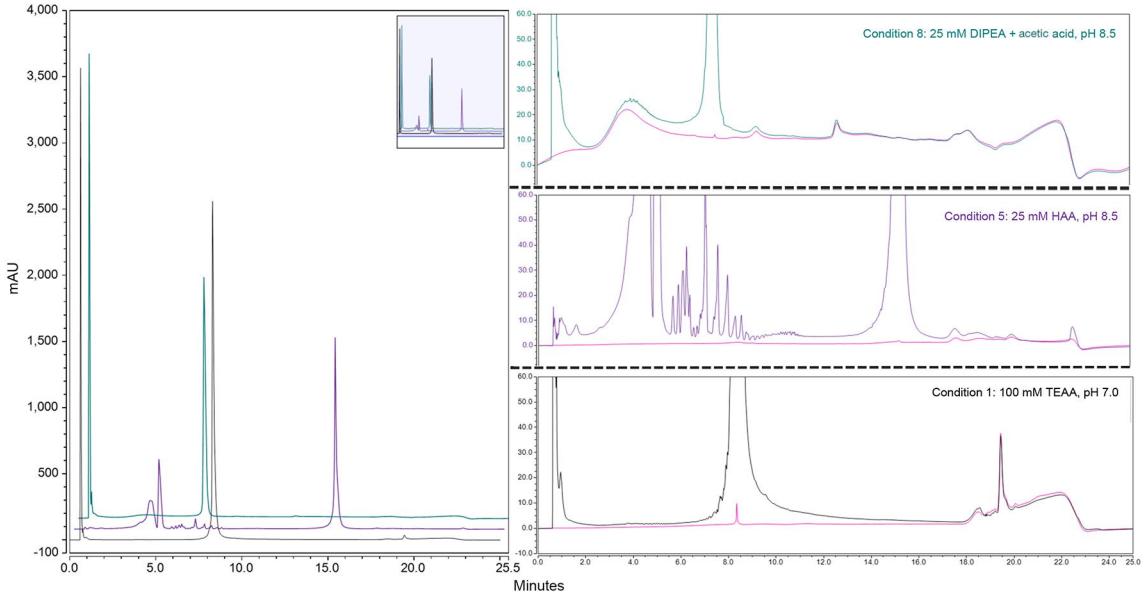

Figure 4. IP-RP of non-purified mRNA scouting at 50 °C. 1 µL injection. On the left panel, the overlaid chromatogram shows results from the three IP agents. On the right panels, an expanded view of the same chromatograms, with overlaid blank chromatograms in pink, is shown.

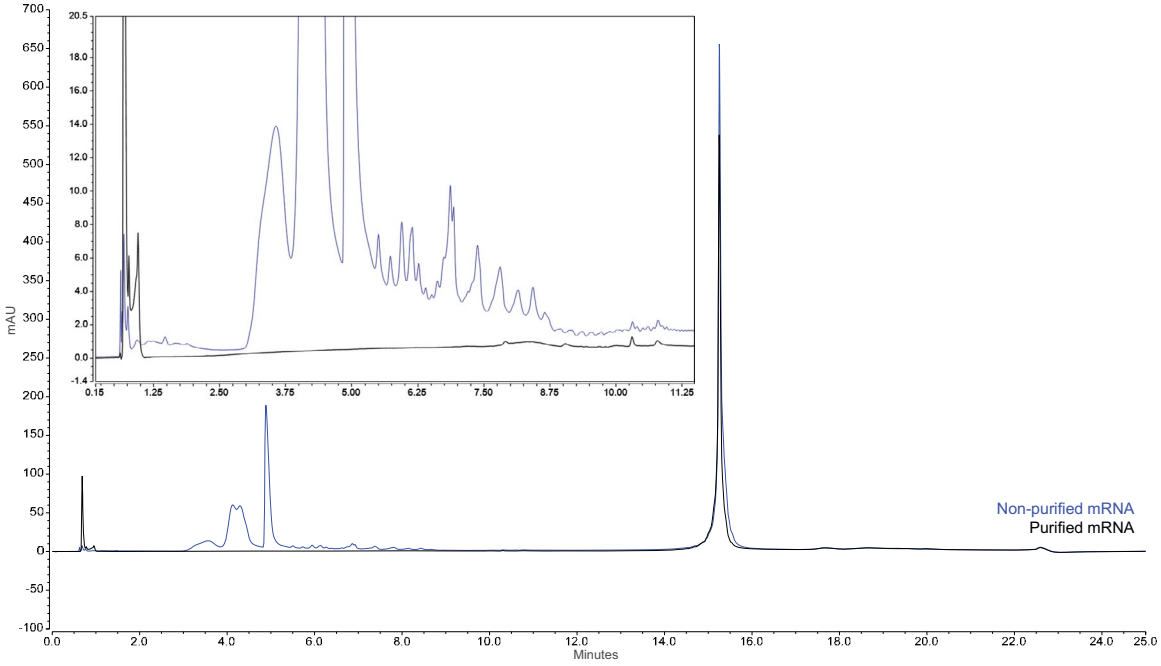

Figure 5. IP-RP method condition 5 (HAA). Overlay of purified versus non-purified mRNA at 50 °C. Injection volumes of 1 µL and 0.3 µL, respectively, were used to have approximately the same amount of mRNA injected.

Because TEAA is widely used in the literature, further optimization was performed starting the gradient from 0% MeCN. Although better retention and separation of some impurities was obtained, most eluted in a single split peak at low retention (data not shown).

IEX chromatography

In Figure 6, the chromatograms of the conditions for the IEX method are overlaid. Tris buffer with perchlorate in MeCN at 80 °C (condition 4) was the only condition capable of eluting the RNA and which gave reasonable peak shape. The gradient with 1 M NaCl did not result in sufficient mobile phase strength to elute the mRNA.

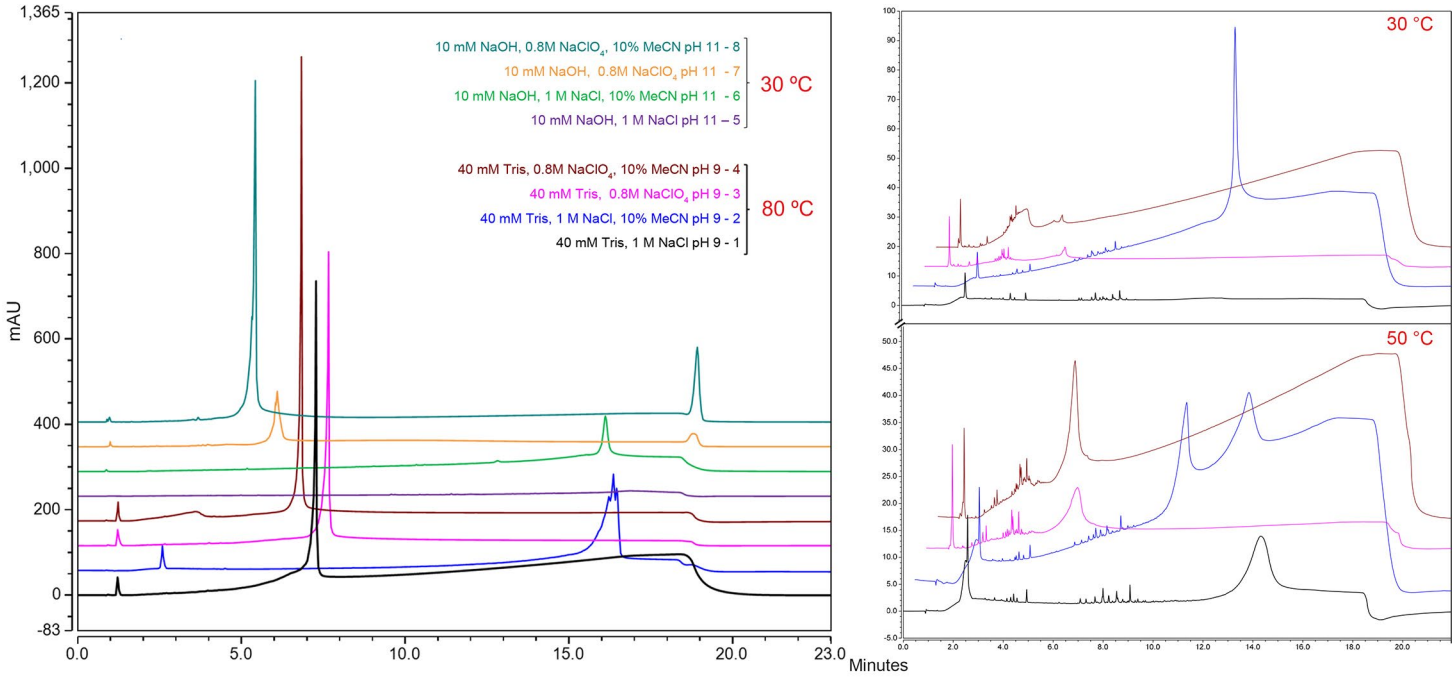

Figure 6. IEX purified mRNA scouted buffers and temperatures. Conditions 1–4 were analyzed at 80 °C (left chromatograms); 30 °C and 50 °C (right chromatograms). Conditions 5–8 were analyzed only at 30 °C.

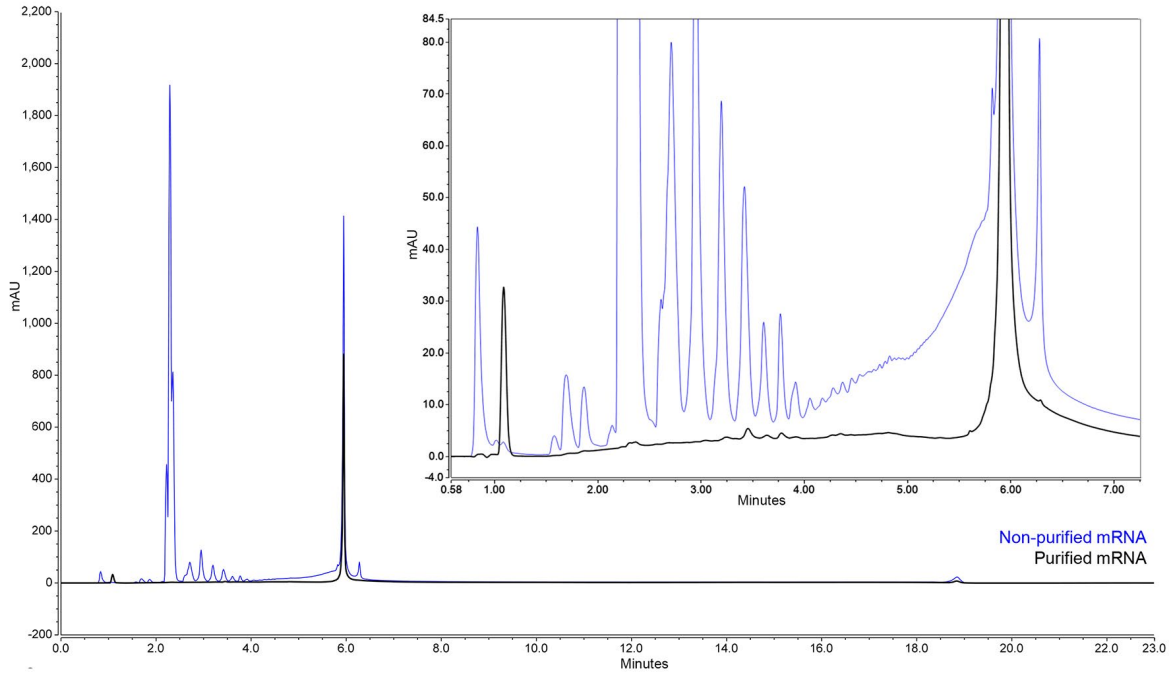

Figure 7. IEX method condition 4 slightly optimized by increasing the organic proportion to 20%. Overlay of purified versus non-purified mRNA at 80 °C. Injection volumes of 1 µL and 0.3 µL, respectively, were used to have approximately the same amount of mRNA injected.

The mobile phases based on a combination of perchlorate and acetonitrile were the most promising, specifically condition 4 (pH 9, 80 °C) and condition 8 (pH 11, 30 °C). In the next step, although condition 8 gave good separation of impurities (data not shown), condition 4 with MeCN increased to 20% resulted in better mRNA peak elution and baseline signal (Figure 7 on page 7). A small peak after the mRNA can be observed in the non-purified sample. It is hypothesized that it corresponds to a DNA template or longer RNA sequence. Also, as for the IP-RP, a bigger peak of impurity (1.15 min) appears in the purified sample than in the non-purified.

A rough estimation of the purity from the chromatograms based on the IP-RP and IEX selected methods was calculated (Table 5). The selected methods provided a comparable estimation of the purity of the mRNA sample. Contrasting IP-RP condition 5 with IEX condition 4, some low-level impurity peaks remaining in the purified sample and the assumed impurity after the mRNA are better detected with IEX condition 4.

Table 5. Relative area of the mRNA peak of the purified sample, calculated using a blank injection as reference for the integration

	IP-RP condition 5	IEX condition 4
Purified mRNA relative area (%)	92.70	90.97

Conclusion

- Two methods were developed for the assessment of purification efficiency of mRNA and for purity profiling of purified and non-purified mRNA.
- The DNAPac RP and DNAPac PA 200RS columns deliver high selectivity and efficiency for the separation of mRNA impurities.
- The Vanquish Duo for Dual LC system extended with the Method Scouting Kit is a valuable solution that enables simultaneous scouting of columns with different chemistries, thereby, greatly reducing the time investment for complex method development tasks.
- Suitable method conditions for the detection of mRNA and impurities were found to be 25 mM HAA for IP-RP and 40 mM Tris/0.8 M perchlorate/20% MeCN for IEX.

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