

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

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For more info see
Application Note:



Abstract

Purpose: To determine the most suitable conditions for the detection of post-transcriptional impurities in mRNA with Ion Exchange (IEX) and Ion Pairing Reversed Phase Chromatography (IP-RP), simultaneously and with one system.

Methods: Purified and non-purified post transcriptional mRNA were analyzed on a Thermo Scientific™ Vanquish™ Duo for Dual LC with the solvent extension kit for automated scouting. Simultaneous chromatographic runs were performed on Thermo Scientific™ DNAPac™ RP (2.1 x 100 mm, 4 μm) and Thermo Scientific™ DNAPac™ PA200 RS (4.6 x 150 mm, 4 μm) columns.

Results: Nine conditions were scouted for IP-RP and eight for IEX at various column temperatures. Several suitable conditions were found for each separation mode. The method scouting was time-efficient and required a single instrument.

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.

Usually, mRNA is produced by transcribing a plasmid DNA template via in vitro transcription (IVT). After synthesis, the mRNA must be purified from the remaining reaction byproducts. Among the most common impurities are nucleotides, enzymes, DNA templates and fragments, abortive transcript fragments, double stranded RNA (dsRNA), and primers.

HPLC is beneficial to evaluate mRNA purification due to its high resolution, reliable quantitation, rapid results, and limited 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.

In this work, several mobile phase conditions were screened with minimal user intervention. The most promising methods for each chromatography mode are shown. These methods can be directly compared and further adapted for specific sample types.

Materials and methods

Sample preparation

The mRNA used for the analysis was approximately 2,500 nucleotides. The 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.

Table 1. Chromatographic conditions

Parameter	Setting
Column left	DNAPac RP column, 2.1 x 100 mm, 4 μm (P/N 088923)
Column right	DNAPac PA200 RS column, 4.6 x 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

Figure 1. Example of custom variables used for the IP-RP method. Custom variables simplify changing method parameters without requiring the creation of 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 be initiated to elute all remaining buffer in the tubing between the selection valve and pump.

#	UV_VIS_1	Name	Type	TCC_Seq	SSV_position	TCC_Divide	TCC_Purge	TCC_Purge	TCC_Purge
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. Flow scheme overview: Dual LC with Solvent Extension Kit for automated method scouting.

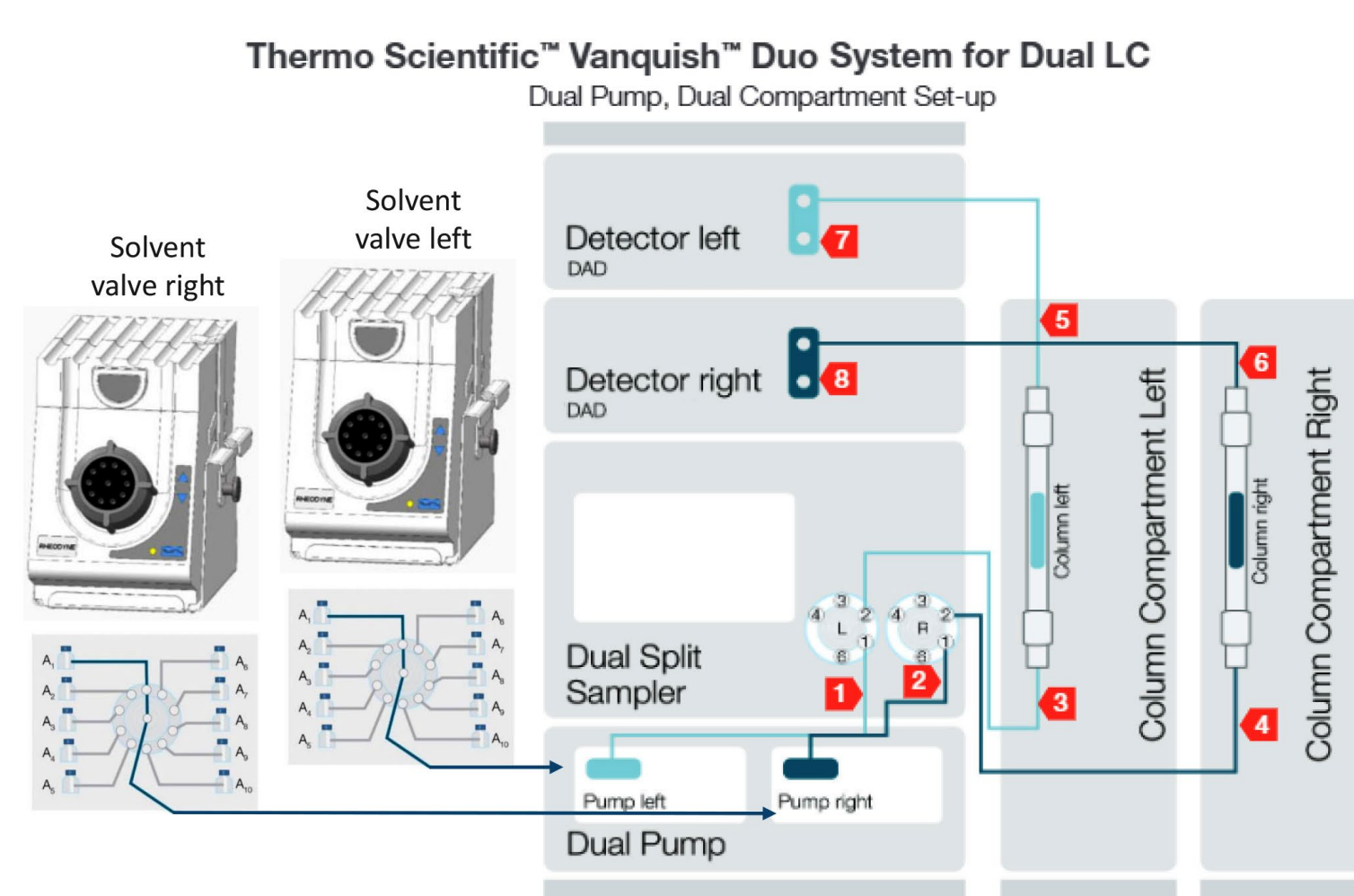


Table 2. Gradient and mobile phase conditions scouted for IP-RP method. The gradient shaded in blue matches the blue conditions and the gradient shaded in green matches the green conditions.

Time (min)	%A (Water)		%B (MeCN)		%C (Solvent selection valve)	
	20	45	5	5	75	50
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 3. Gradient and mobile phase conditions scouted for IEX method. The colors represent the eluent pairs. See below detailed information.

Time (min)	%A*		%B*		%C Solvent selection valve
	10 mM NaOH pH 11	40 mM Tris pH 9	10 mM NaOH pH 11, 10% MeCN	40 mM Tris pH 9, 10% MeCN	
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

Table 4. Relative area of the mRNA peak for 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

IP-RP chromatography

Figure 3. RP-IP example chromatograms of non-purified mRNA with different ion-pairing systems. 1 μL injection, column temperature 50 °C. 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, are shown.

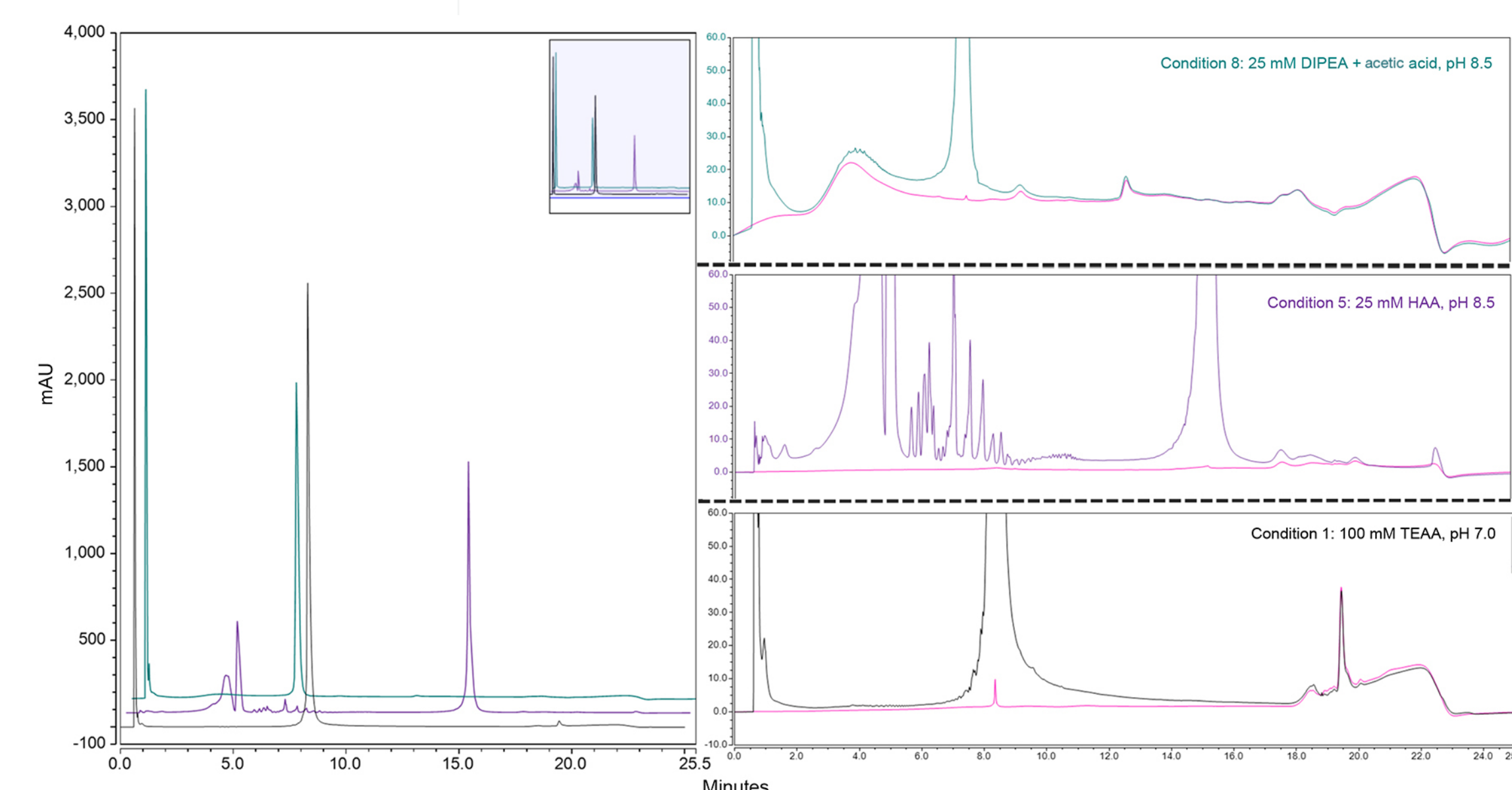
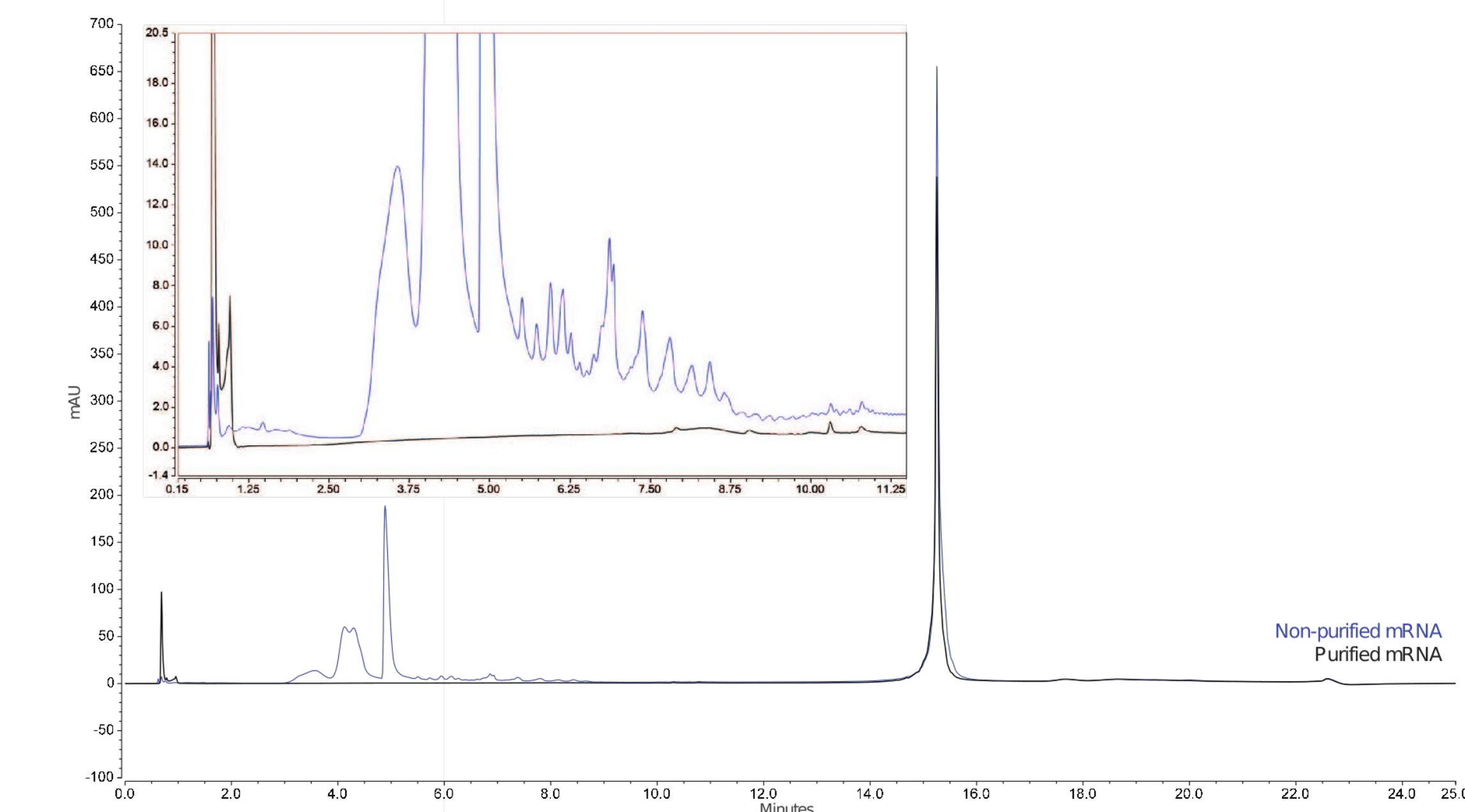


Figure 4. IP-RP method condition 5 (HAA). Overlay of purified versus non-purified mRNA at 50 °C. Injection volumes of 1 μL and 0.3 μL, respectively, were used to have approximately the same amount of mRNA injected.



IEX chromatography

Figure 5. 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.

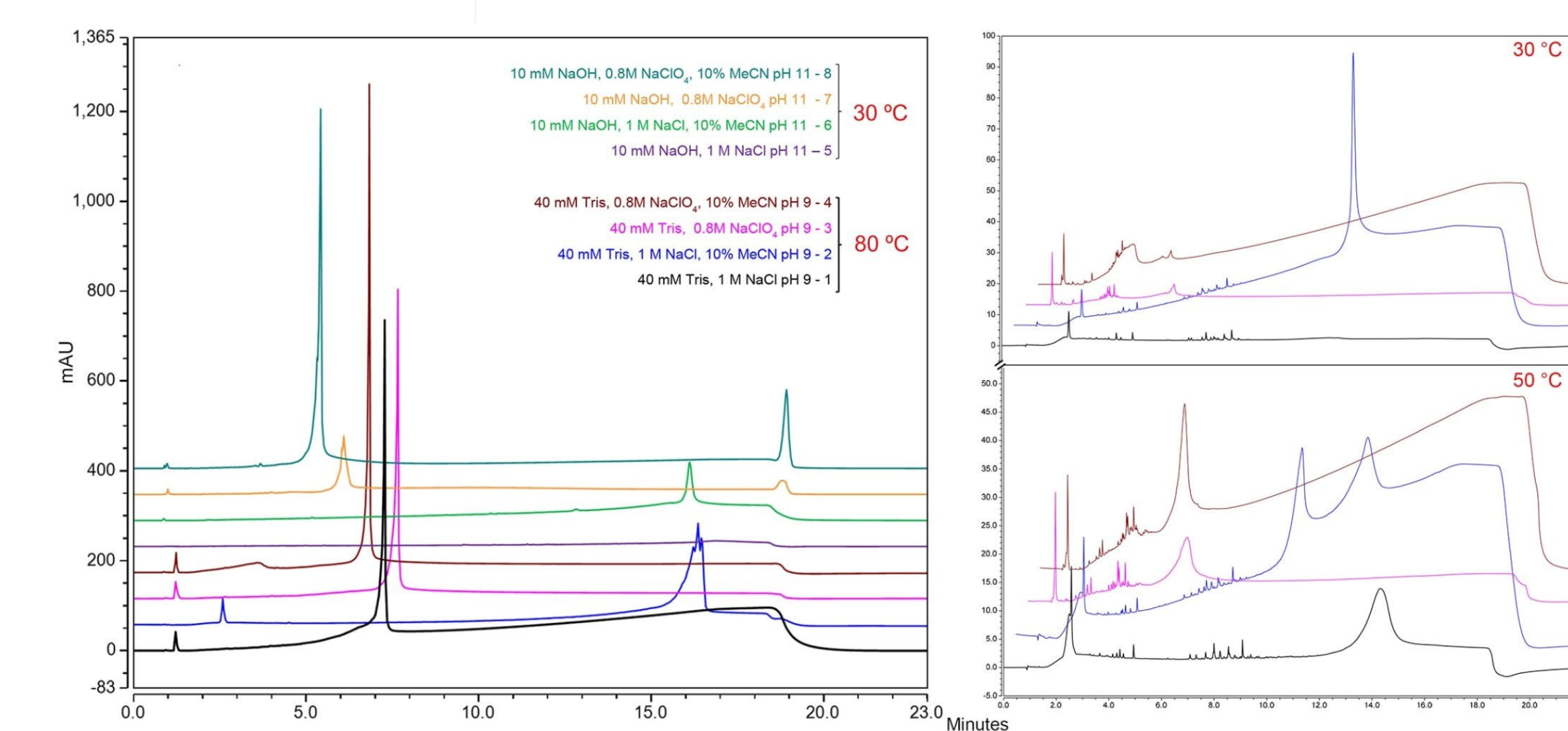
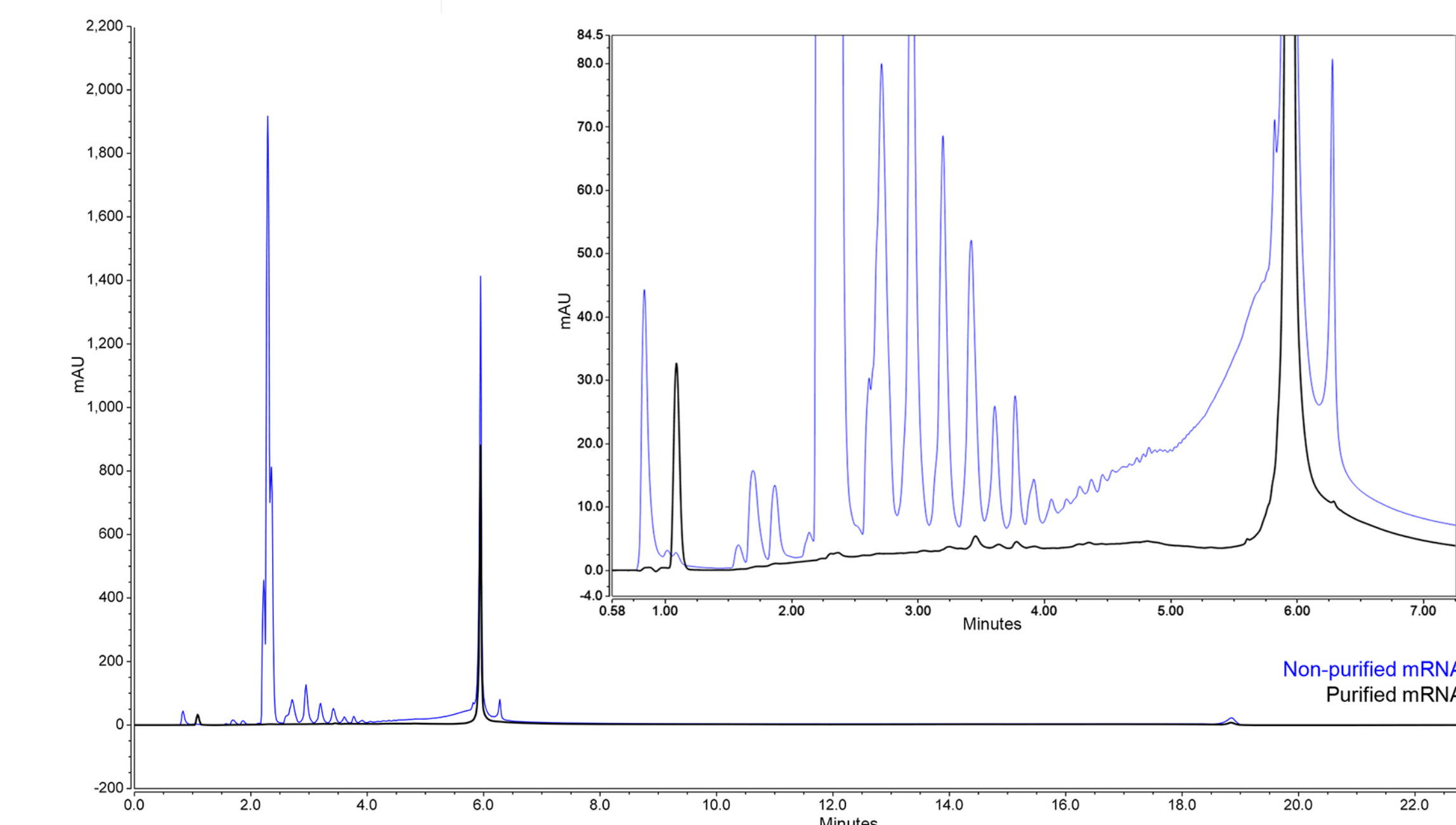


Figure 6. IEX method condition 4 slightly optimized by increasing the organic proportion to 20%. Overlay of purified versus non-purified mRNA at 80 °C. Injection volumes of 1 μL and 0.3 μL, respectively, were used to have approximately the same amount of mRNA injected.



Conclusions

- The Vanquish Duo for Dual LC system extended with the Method Scouting Kit enables simultaneous scouting of columns with different chemistries, thereby greatly reducing the time investment for complex method development tasks.
- The most suitable conditions were selected based on the shape of mRNA peak, the impurities content, and the level of details of the impurity profiles.
- Suitable method conditions for the detection of mRNA and impurities were selected: 25 mM HAA for IP-RP and 40 mM Tris/0.8 M perchlorate/20% MeCN for IEX.
- The selected AEX and RP-IP delivered similar estimation of mRNA purity (Table 4). The AEX provided a more detailed impurity profile

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