

# The Synthesis of Ribozymes using TC-RNA Chemistry

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## Introduction

Recently, 2'-O-thiomorpholine-4-carboxithioate (TC) was introduced as an alternative protection chemistry to TBDMS in RNA synthesis.<sup>1</sup> This utilises a one step simple deprotection method that removes both the 2'-TC and nucleobase protection at the same time. The 2'-TC protecting group has been designed such that it deprotects at a slower rate than the nucleobases, reducing the possibility of isomerisation during deprotection. Combined with the fact that the coupling efficiency is comparable with DNA synthesis, there is the potential to synthesise good quality long RNA such as ribozymes.

The synthesis of two ribozymes (55mer and 77mer) was carried out using TC-RNA phosphoramidite chemistry. Analysis of these was then carried out by MALDI MS, LCMS, and CGE. These were then compared to the same sequences synthesised via the more conventional TBDMS chemistry. The cleavage and ligation activity of both sets of oligonucleotides were then evaluated.

## Experimental

### Synthesis Conditions - TC RNA<sup>2</sup>

All oligonucleotides were synthesised on an ABI 394 DNA/RNA synthesiser with 5min coupling and a cap/ox/cap step. The amidites were used at 0.15M concentrations in toluene with 0.5M ETT in MeCN as the activator. Molecular sieves (4Å) were used in both the activator and the amidites. All supports used were 1000Å CPG.

### Deprotection Conditions - TC RNA<sup>2</sup>

All oligos were first treated with 20% DEA/MeCN. Deprotection was carried out with ethylene diamine/toluene 1:1 for 6h at RT and the oligonucleotides eluted from the resin with 0.1M TEAA.

### Analysis Conditions - TC & TBDMS RNA

Crude oligonucleotides synthesised with TC chemistry were analysed by LCMS using an Agilent 1200SL HPLC in-line with a 6520A QTOF MS. Dr Zoltan Timar, Agilent Technologies, Inc., Boulder, CO provided this data.

All purified oligonucleotides were analysed by CGE on a Beckman P/ACE MDQ with UV detector (254 filter), the gel and buffer are composed of Tris-Borate and urea (7M) with an untreated bare fused silica capillary (100um ID, 31cm long) and MALDI-TOF on a Bruker Ultraflex. This data was provided by EGT-SA.

### Ribozyme Cleavage Conditions

The substrate HPAS3 (TBDMS) was labelled with ATTO680, which is necessary to enable analysis with a LICOR 4200 sequencer. The following protocol was used: ribozyme 500nM; substrate HPAS3 25nM; TRIS/HCl (pH 7.5) 50mM; MgCl<sub>2</sub> 10mM; ddH<sub>2</sub>O 40µl.

Ribozyme, substrate, buffer and water were mixed and denatured at 95°C (2min), followed by incubation at 37°C (15min). The reaction was started by the addition of MgCl<sub>2</sub> at t=0. After 2, 4, 6, 8, 10, 20, 30, 60, 90 and 120min, aliquots of 1µl of the reaction mixture were added to 29µl stop mix (7M urea, 50mM EDTA). Analysis was carried out on the sequencer.

## Results & Discussion

### Oligonucleotide Synthesis

The oligonucleotide sequences synthesised for the 55mer and 77mer are shown in Figure 12. A 100mer sequence<sup>3</sup> (although not used in any application) was also synthesised.

To ensure that the synthesis was reproducible, three of each of the 77mers and 55mers were made. These were analysed by LCMS and the results are summarised in Table 1 opposite. In all

<sup>1</sup> The Development of Cost-Effective Large Scale Synthesis Process for RNA- Therapeutics, D. Dellinger, Presentation at TIDES®, May 18, 2009.

<sup>2</sup> To ensure impartiality, the oligonucleotides synthesised using TBDMS-RNA chemistry were purchased from EGT-SA and all purifications (PAGE) were carried out by EGT-SA.

<sup>3</sup> UGC CCA GUC GUA CUG CCG UCC GCA CGU GCA CGU CUG GUU GCG UGU UUG GCU GGU UGG UGC UCU UGC ACC CUC GUA GCU AGU CAG GUC AUU CGA UGC CCG T

cases the desired product was present in the crude sample and the full-length concentration was consistent for each synthesis.

LCMS data for one of each length of oligonucleotide is also shown below. It is evident that as the length increases then the deprotection time needs to increase since the quantity of incomplete deprotection of 2'-TC observed increases. With the 55mer the signal due to FL+TC is small whereas with the 100mer the signal is almost as intense as that of the full-length product and FL+2TC is clearly visible. Work is in progress to determine the optimum deprotection time required for longer oligonucleotides.

Prior to analysis, it was thought that transamination may be a concern, however this was not observed in the LCMS.

Table 1. Summary of LCMS analysis on the crude oligos

Description	FL conc <sup>n</sup>	Cycle yield	Expected Mw (First/largest monoisotopic)	Found Mw (Average)
RNA 55mer	29.1%	97.7%	17909.49 / 17916.51	17918.32
RNA 55mer	29.7%	97.7%	17909.49 / 17916.51	17918.34
RNA 55mer	31.8%	97.9%	17909.49 / 17916.51	17918.37
RNA 77mer	28.5%	98.4%	25048.45 / 25057.47	25060.70
RNA 77mer	29.3%	98.4%	25048.45 / 25057.47	25060.64
RNA 77mer	26.7%	98.3%	25048.45 / 25057.47	25060.67
RNA 100mer	24.9%	98.6%	31910.01 / 31921.04	31925.50

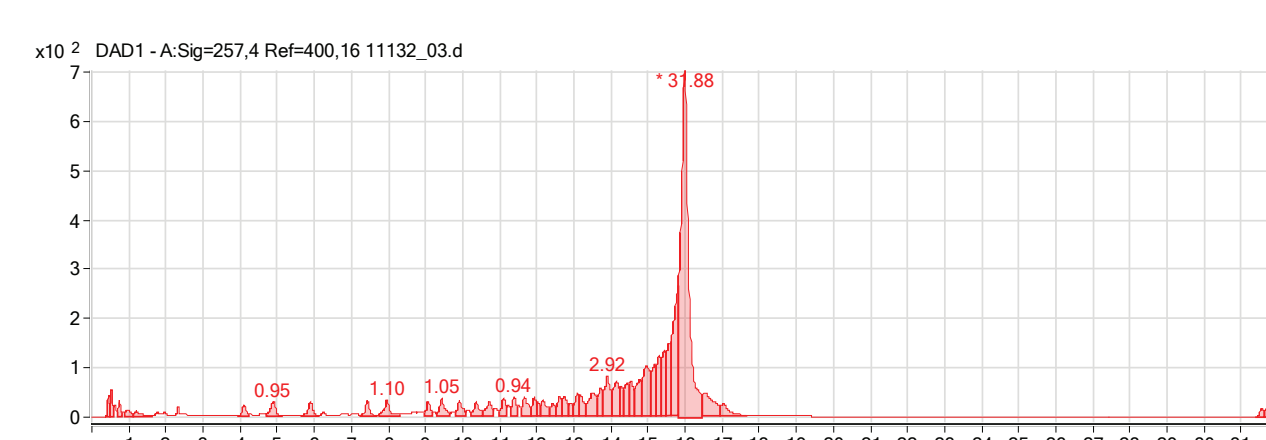


Figure 1. LCMS of HP-WTL (55mer) crude.

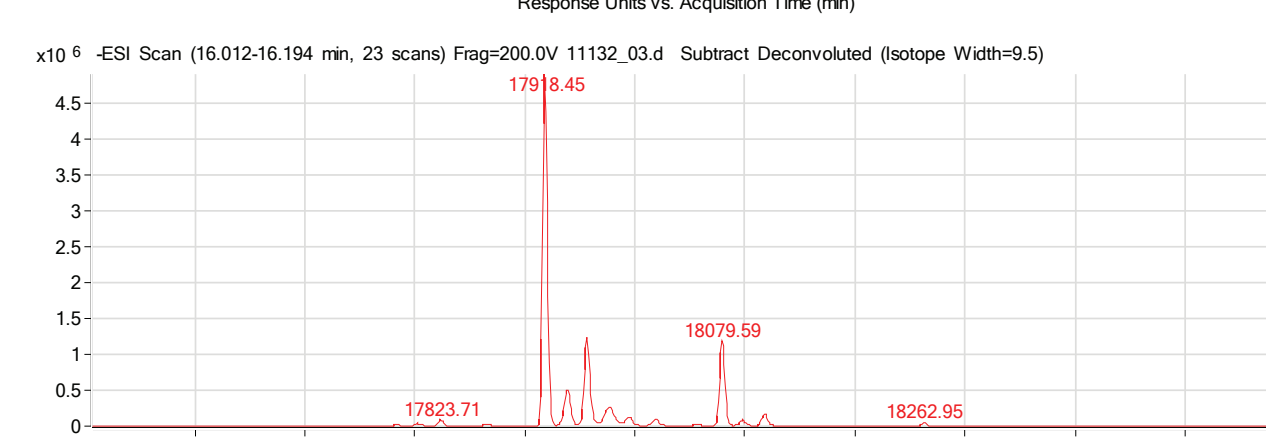


Figure 2. LCMS of HPAR2 (77mer) crude.

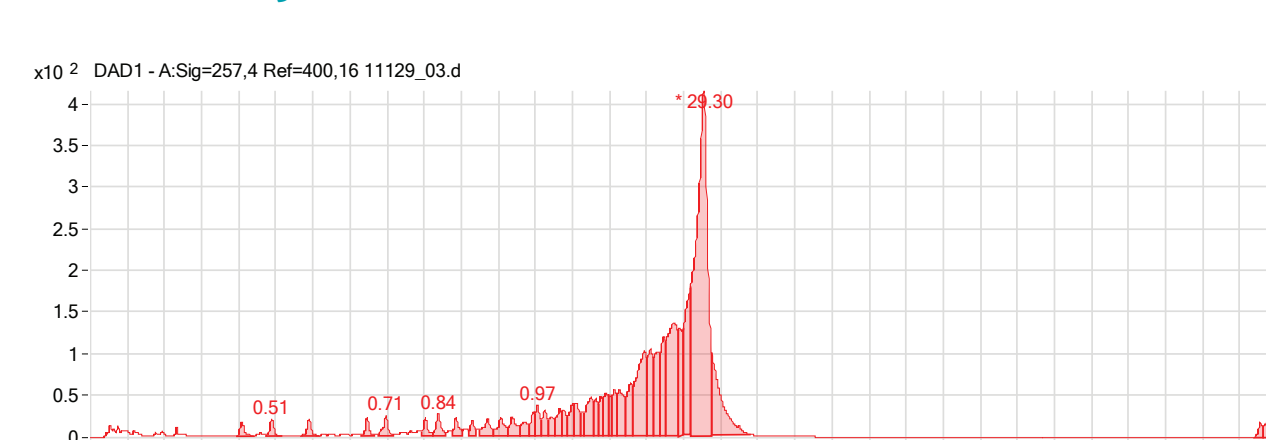


Figure 3. LCMS of 100mer crude.

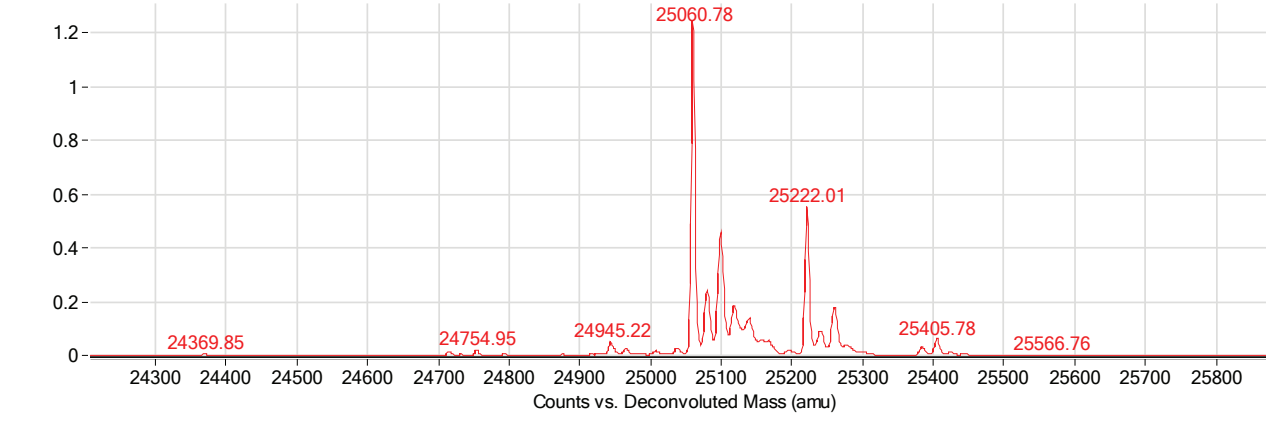


Table 2. Summary of analysis on the PAGE purified oligos

Description	MW calc.	MW obs.	% Purity by CGE
55mer-TC	17918	17946	78.2
55mer-TBDMS	17918	17932	89.4
77mer-TC	25060	25165	97.3
77mer-TBDMS	25060	25106	61.9

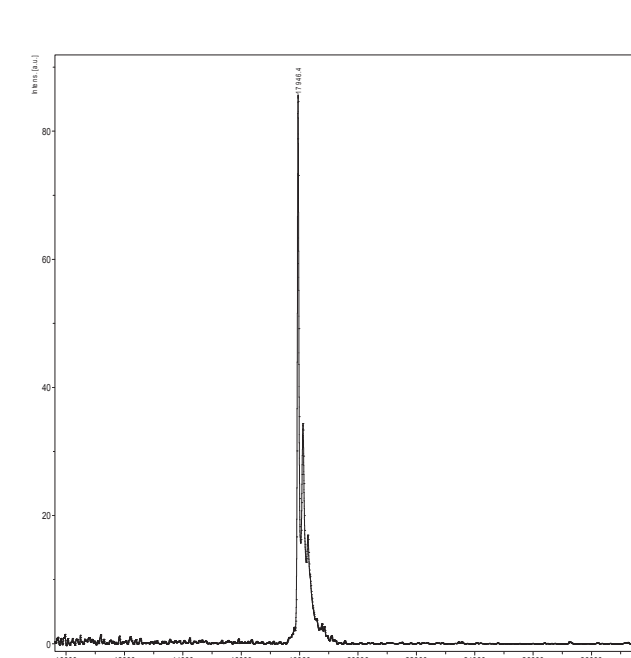


Figure 4. MALDI MS of 55mer-TC after PAGE purification.

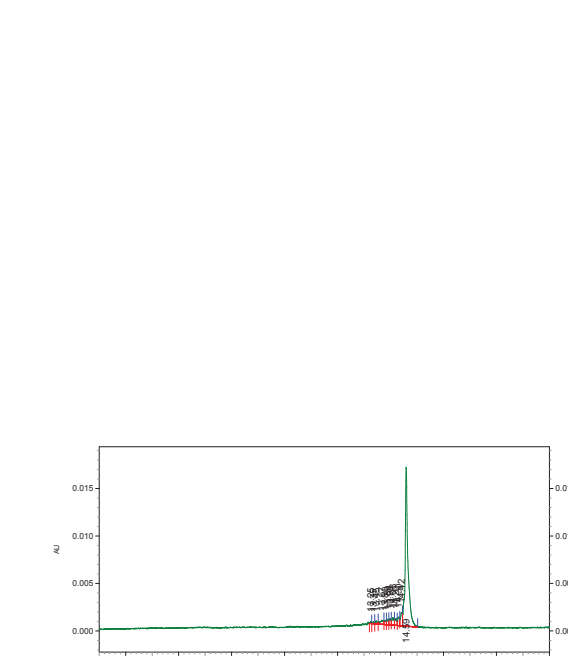


Figure 5. CGE of 55mer-TC after PAGE purification.

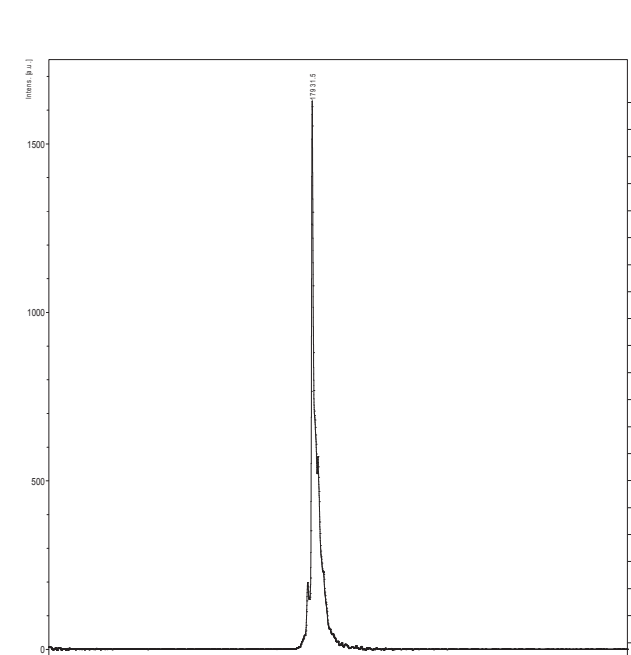


Figure 6. MALDI MS of 55mer-TBDMS after PAGE purification.

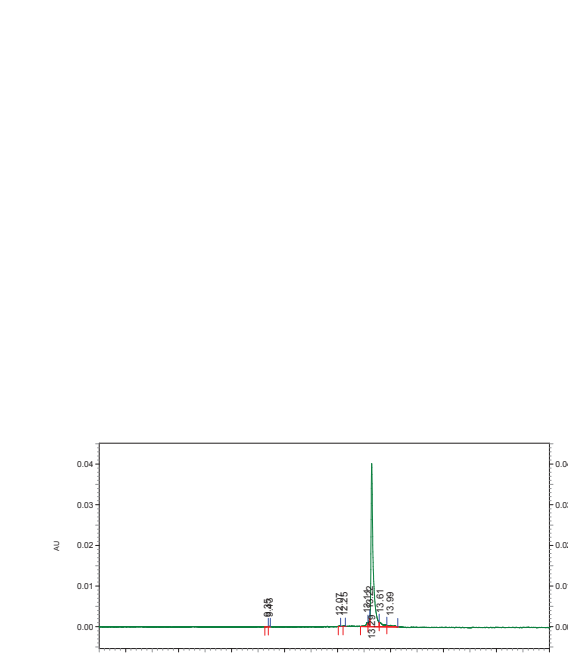


Figure 7. CGE of 55mer-TBDMS after PAGE purification.

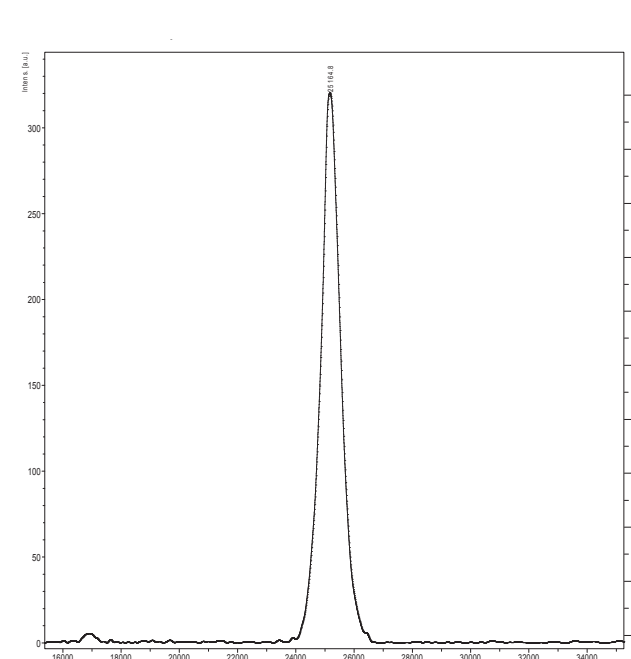


Figure 8. MALDI MS of 77mer-TC after PAGE purification.

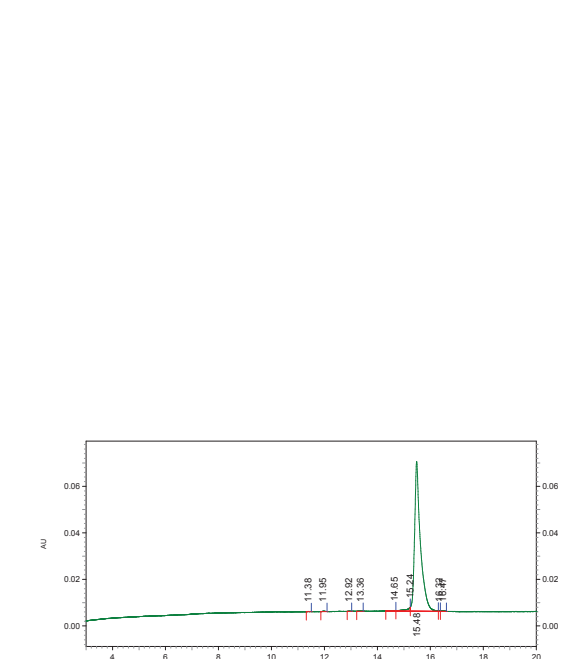


Figure 9. CGE of 77mer-TC after PAGE purification.

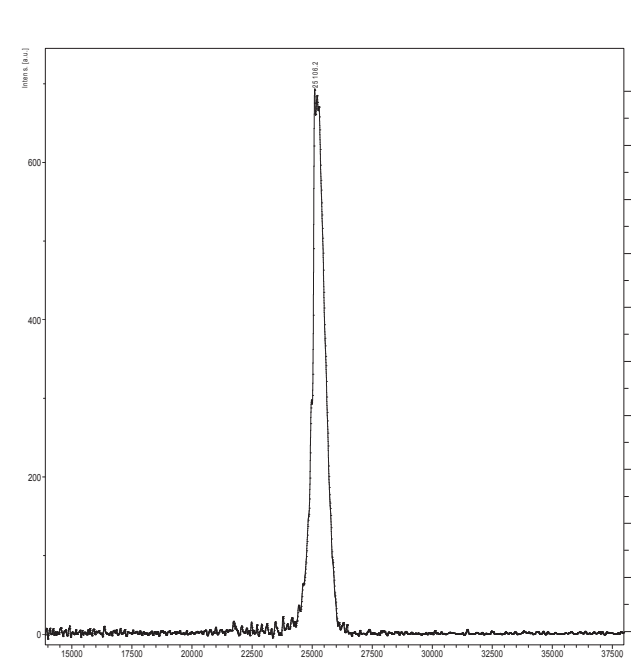


Figure 10. MALDI MS of 77mer-TBDMS after PAGE purification.

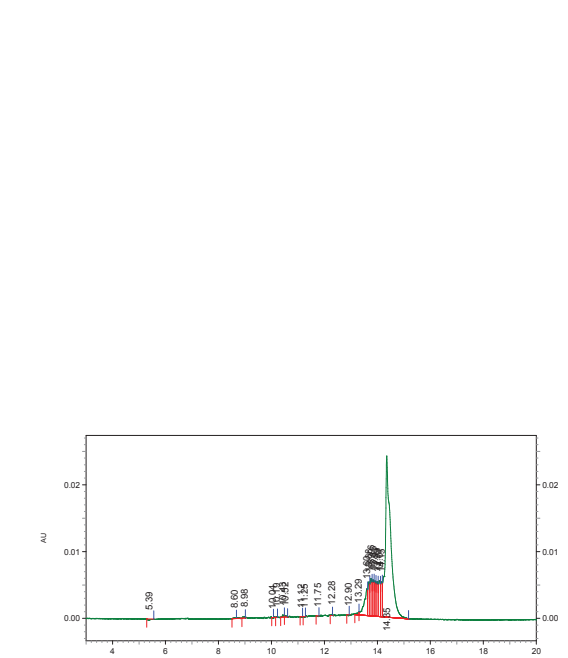


Figure 11. CGE of 77mer-TBDMS after PAGE purification.

### Ribozyme Activity

Preliminary results show the cleavage activity of RNA synthesised with TC chemistry is higher than that synthesised with TBDMS chemistry. This may be linked to the purity of the RNA as seen by comparing the CGE of the two 77mers where the activity difference is most dramatic. The results are shown in Figure 12.

## Conclusions

TC-chemistry provides a means of synthesising long-RNA oligonucleotides with comparable yields to TBDMS chemistry - but with higher purity. Some work still needs to be carried out to optimise the deprotection time in order to avoid incomplete deprotection.

## Further Information

Any queries regarding this work should be directed to Dr Catherine McKeen, Product Manager, Link Technologies Ltd.

[www.linktech.co.uk](http://www.linktech.co.uk)

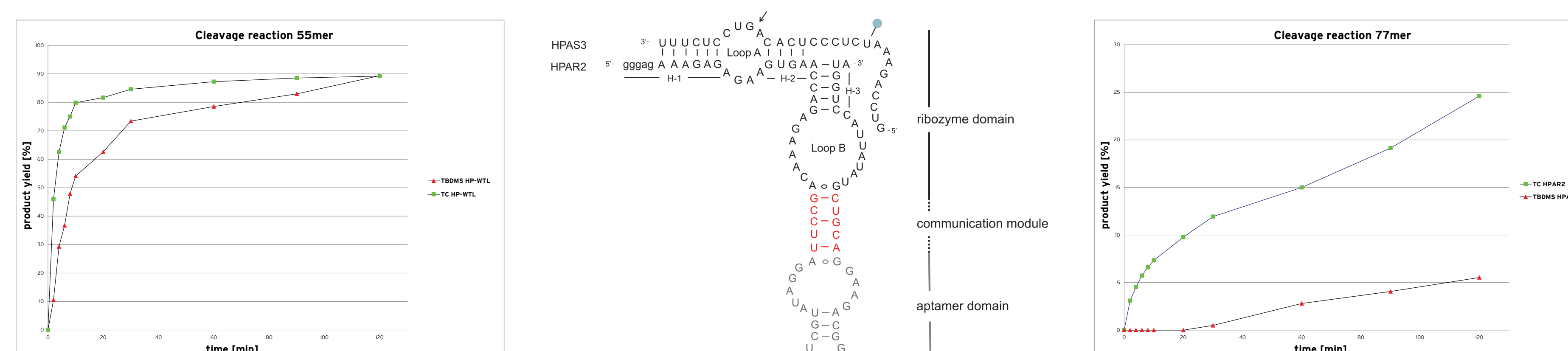


Figure 12. Cleavage activity.

