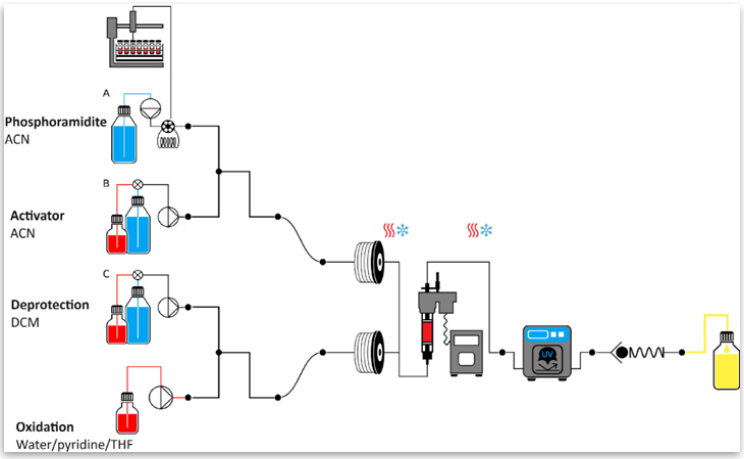


# Application Note 77: *VBFR-based continuous flow synthesis of oligonucleotides*

**Produced by Vapourtec**

## Abstract

This application note illustrates the capabilities of Vapourtec's flow chemistry systems in the field of solid-phase synthesis of oligonucleotides in continuous flow. Previous application notes have covered the synthesis of peptides and carbohydrates using polymeric resin as solid support, using a Variable Bed Flow Reactor to control swelling and growth throughout the synthesis, minimising dead volumes and eliminating channelling of reagents.

The controlled packing density and overall protocol yielded a final crude purity of ~77 % of a 9-mer oligonucleotide with an estimated isolated yield of 82 % in under 4.5 hours.

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## Introduction

Synthesis of oligonucleotides, such as DNA or RNA molecules, using solid-phase methods has been of immense importance to developing modern genetics, cell and molecular biology and drug discovery.

The development of solid phase synthesis methods for oligonucleotides started almost simultaneously alongside peptide synthesis in the 1960s and 70s and became very efficient when Caruthers et al. introduced phosphoramidite methodology in 1980s. In fact, phosphoramidite chemistry has been so successful that very little has changed in oligonucleotide synthesis since its introduction.

The original applications for synthetic oligonucleotides, such as DNA sequencing or PCR primers, needed these products in only sub-micromole scale.

Later, relatively larger quantities of these molecules were needed for antisense gene expression control, diagnostic assays, and physical studies of nucleic acids. In the last decade or so the demand on oligonucleotides and their production has increased significantly due to genetic medicines coming of age.

Various types of medicines have either been marketed or are under early or advanced clinical investigation. These include gene therapy, RNA medicines (e.g. vaccines), gene editing (e.g. CRISPR-Cas9) and RNAi therapeutics. While the first three groups are currently produced using molecular biology techniques, the last group, RNAi therapeutics are made via total chemical synthesis.

Although from a drug discovery point of view these agents are still facing major obstacles such as addressing delivery challenges, it is fair to say that their production on large scale can be considered as one of the main bottlenecks to their commercialization.

This challenge becomes more evident when we consider gradual change of target diseases from rare to more prevalent diseases. For example, while drugs used for treating hereditary transthyretin amyloidosis (hATTR) might be needed on kilogram scale annually, those used for hypertension such as zilebesiran will be needed on ton scale if they can successfully penetrate these very large markets. As such there is a significant need for increased RNA manufacturing capacity.

The current technology used for synthesis of these RNAi targets, although mature and efficient is only suitable for addressing the demand for rare diseases. The synthesis of a typical 21-mer SiRNA duplex involves more than 160 steps and yields of 50 % and purity in the region of 90 % are considered a significant achievement. Currently a typical API costs more than $1m per kg. This is due to expensive raw material but also process mass intensity (on average 5 tons of raw material to produce 1kg of API), expensive equipment and lengthy process and most importantly low yield and efficiency.

There are also other concerns, such as difficult scalability of the process, environmental impact of very high raw material consumption, inconsistent batch quality and low overall purity and yield of the final API. As a result, a new process that can improve any of these constraints is highly welcome and needed by the industry.

## Vapourtec's approach – use of polymeric solid support

A key challenge in the large-scale synthesis of oligonucleotides is the support on which the synthesis is performed. The ideal support should be physically robust to allow mixing to increase reaction efficiency without

damaging the matrix. This is one of the reasons why controlled pore class (CPG) or macroporous resins are used for oligonucleotide synthesis.

The drawback of using these types of solid support is that the loadings are typically very low, requiring large quantities for a scaled-up synthesis, which would impede heat and mass transfer throughout the synthesis. To increase capacity, supports have been developed with a large surface area but these materials have inherent porosity that leads to irreversible entrapment of reagents in the matrix. These polymers, when used in a conventional mixed bed reactors or the currently used static compression technology, trap the reagents in their pores which requires high volumes of solvent to wash them off. When using very large amounts of resin, even use of significant volumes of solvent, may not be enough to displace the reagents.

This trade-off between the surface and porosity has led researchers to hunt for a perfect polymeric support by studying the nature of the polymer, cross-linking level, porosity and other chemical or physical properties.

The variable-bed flow reactor (VBFR) technology developed by Vapourtec has enabled us to approach the problem differently. This patented reactor ensures a perfect packed bed reactor by constantly measuring the packing density of the media in the reactor and adjusting the reactor volume accordingly.

We envisaged that by using VBFR technology we would be able to permanently retain a perfectly packed bed reactor which can withstand a pre-defined amount of pressure. This allows us to use augmented diffusivity by convection, instead of relying on diffusion. This convective flow effectively “pushes out” the reagents from the pores resulting in the use of much smaller amounts of solvent in wash steps.

It was also hypothesised that by using the high-capacity polymers the detrimental effect of residual water on the coupling step would be reduced hence allowing us to use lower equivalents of the nucleotides and activators in the synthesis.

Finally, the flow configuration will give a better control of mass and heat transfer, leading to a more efficient synthesis protocol. For example, in a flow reactor the residence time of deprotecting reagent can be adjusted precisely in a way that all protecting groups are removed but the acid-induced depurination side reaction is avoided.

## Instrument and Reaction Setup

The oligonucleotide synthesis procedure used by our platform was based on the well-established and robust phosphoramidite approach with some modifications. A typical

cycle for solid supported oligonucleotide synthesis is shown in Figure 1:

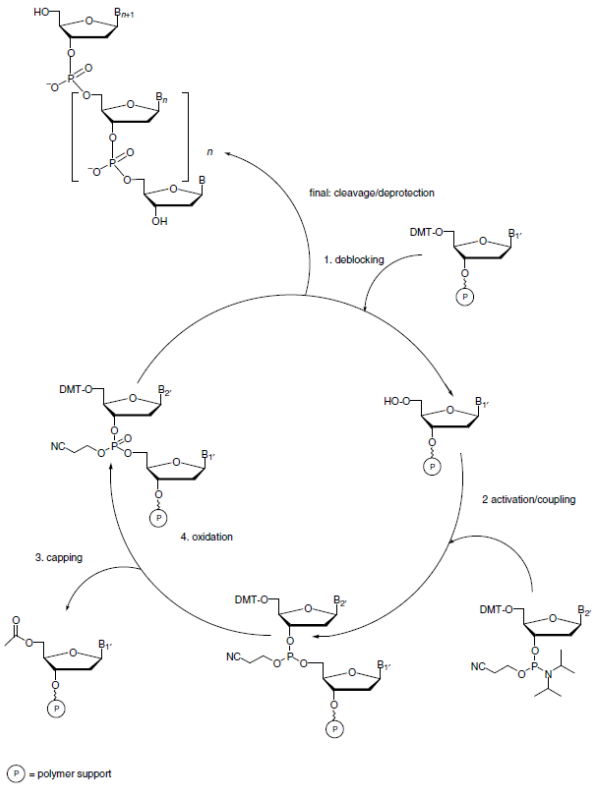


*Figure 1 - Typical cycle for solid supported oligonucleotide synthesis*

Each cycle consists of four basic steps:

1. Deblocking, which removes the DMT protecting group and hence exposing the 5'-OH on the first nucleotide which is preloaded on the support.
2. Coupling, which adds the second nucleotide to the growing chain by activating the phosphoramidite group on the 3'-side of the incoming monomer.
3. Capping, to inactivate unreacted 5'-OH and eliminate the possibility of -1 deletions.
4. Oxidation, which converts the reactive P(III) to more stable P(V).

The cycle is then repeated until the complete oligonucleotide sequence is assembled.

We followed the same basic steps with the exception that we chose a three-step instead of this common four-step cycle depicted above. The rationale was that if the coupling and washing steps are very efficient, the presence of the capping cycle will be redundant. Avoiding moisture ingress is critical, as it would reduce coupling efficiency. For that reason, reagents need to be kept under nitrogen.

As for the solid support, we chose Tentagel-N available from Rapp Polymer. We chose this resin because of its gel type nature which generally exhibits a higher activity compared to the macroporous resins. This can be attributed to the differences in active site accessibility during reaction and, hence, in resin swelling, especially when it is in contact with polar components. Tentagel resin swells almost equally well in nonpolar organic solvents as in more polar solvents (e.g. ACN). However, the behaviour of PEG-PS copolymers is dominated by the hydrophilic PEG component, and this decrease in the overall hydrophobicity of the support might be a disadvantage from the point of view of increasing the risk of moisture contamination. Another advantage of Tentagel resin, especially over CPG, is its high loading capacity. The resin we use reports 0.174 mmol/g of preloaded T-nucleotide. This

high loading is clearly advantageous when large scale synthesis is needed.

## Setup

Focusing on three basic steps of deblocking, coupling and oxidation, we thus selected an Oligo-Explorer™ as shown in Figure 2.

*Figure 2 - Vapourtec oligonucleotide synthesiser*

The platform consists of two R2C+ pump modules, each providing two piston-pumps capable of pumping up to 10 ml/min. An autosampler loads the monomer nucleotides into a sample loop, while all other reagents are directly fed in via reagent bottles.

The VBFR was set in a R4 reactor heater module, which provides the heating and temperature control. The VBFR controller measures and maintain constant packing density across the resin bed throughout the synthesis.

As Figure 3 shows, two separate reactors with 0.8 and 0.4 ml volume enable the activation and pre-heating of reagents before the reagents enter the VBFR. A UV-Vis detector, capable of monitoring up to four wavelengths monitors the solution that exits the reactor. Finally, a SF-10 pump was used as active backpressure regulator, set at 1.5 bar.

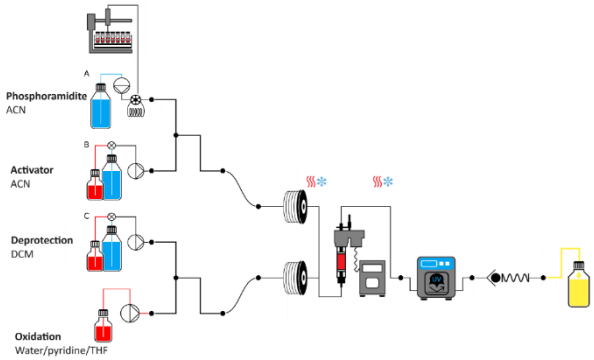


*Figure 3 - Schematic of the system used for this work*

To start the cycle, deprotection solution in pumped directly via a reagent bottle. The solution passes through a 0.4 ml reactor before entering the VBFR which contains the preloaded resin with the DMT-protected first nucleotide.

The progress of the deblocking step is monitored by the UV-Vis detector at 465 nm and other chosen wavelengths.

The reactor is then washed by pumping a solvent such as acetonitrile (ACN) over the resin. Upon removal of the protecting group, the 5'-OH is now exposed and ready to react with the incoming second nucleotide during the coupling step. Each amidite monomer is automatically loaded via sample loop by a liquid handler.

The required amount of the activator is directly delivered from a reagent bottle. The two reagents are mixed and put through a 0.8 ml reactor where the phosphoramidite is fully activated.

The activated phosphoramidite enters the VBFR, coupling to the first amidite. Once coupling is completed, an ACN wash ensures no reagent is left in the resin.

In the third step, the oxidation solution converts P(III) to the more stable P(V). Alternatively, instead of oxidation, sulfurization can be used to prepare phosphorothioate oligonucleotides. When oxidation is completed, the resin is again washed with ACN.

After oxidation, the cycle can be repeated, gradually building the chain in a 3'-to-5' direction. The whole process is programmed and controlled by Vapourtec's R-Series Software.

We next set out to find the optimum conditions for each step of the cycle. A 9-mer DNA oligonucleotide, **3'-TTAGCCATA-5'** was chosen as the target material and its synthesis was used to develop an optimised method for the synthesis of oligonucleotide using our VBFR-based platform. LC-MS was used to confirm the structure of the desired target. This was followed by an HPLC method (see below) where the purity of the product was quantified and used for guiding our optimisation campaign.

Starting from the protocols currently in use for oligonucleotide synthesis, we looked at the type and concentration of the activator, deprotection agent and oxidation solution. Continuous parameters such as flow rates of each reagent, temperature and the stoichiometries were also optimised using the DNA 9-mer as the target.

After extensive optimisation studies, for a typical synthesis using 300 mg of Tentagel N-T-DMT (52 $\mu$ mol scale) the following protocol resulted in obtain the crude 9-mer in >80% purity as determined by HPLC.

## System Parameters

**System solvent:**

**Reagent preparation**

Resin TentaGel N-dT-DMT (300 mg) was loaded into the reactor column.

0.1 M solutions of the following 5'-DMT-protected-3'-phosphoramidite monomers were prepared in anhydrous ACN and delivered by Pump A via sample loops:

- 5'-O-DMT-thymidine 3'-CE phosphoramidite (abbreviated as T)
- 2'-Deoxy-5'-O-DMT-N2-isobutyrylguanosine 3'-CE phosphoramidite (abbreviated as G)
- N6-Benzoyl-2'-deoxy-5'-O-DMT-adenosine 3'-CE phosphoramidite (abbreviated as A)
- N4-Benzoyl-2'-deoxy-5'-O-DMT-cytidine 3'-CE phosphoramidite (abbreviated as C)

A 0.45 M solution of activator 5-(Ethylthio)-1H-tetrazole, (ETT) was made in ACN and delivered by Pump B.

A deprotection solution of Trichloroacetic acid (TCA) (4.5 % w/v) in DCM was delivered by Pump C.

A 0.1M iodine solution in water/pyridine/THF (2/20/78) was delivered using Pump D. As this solution contains water, this pump is not used for any washing step.

**Reaction protocol**

Anhydrous ACN was used to perform all the washing steps.

All reactors were set to 30 °C.

**Deblocking**: The resin was pre-wash with 4 ml of ACN at 2 ml/min. The deprotection reaction involved pumping 5 ml of 4.5 % TCA solution at 2 ml/min followed by a post-wash of 3 ml of ACN at 2 ml/min. This step resulted in complete removal of the DMT group as confirmed by the UV detector. Fig. 3 is a screen shot of R-series software for deblocking step.


*Figure 4 - Deblocking step*

**Coupling**: (2 ml loop), 2.5 ml of the activator (ETT, 0.45M) was pumped at 0.5 ml/min while 2 ml of the monomer solution (0.1 M) ran at 0.5 ml/min with a delay of 0.5 min resulting in 0.25 ml of activator plug on either side of monomer nucleotide. The two reagents were mixed in the 0.8 ml reactor which resulted in the activation of the phosphoramidite. This activated reagent then entered the VBFR which at this stage contained the nucleotide with free 5'-OH group. After the coupling step was completed, a post wash of 3 ml of ACN at 2 ml/min was performed to remove the excess reagents. For G-amidite, a double coupling was necessary to avoid accumulation of the G-deletion by-product. Fig 4 shows the R-series parameters used for this step. Note that in concentration model section how the activator (purple line) "swamps" the amidite (orange line) to make sure the two mix quickly and efficiently.

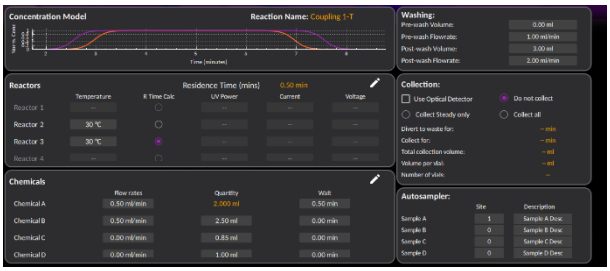


*Figure 5 - Coupling step*

**Oxidation.** Reaction involved pumping 1.5 ml of the oxidation solution at 1 ml/min. No post wash was performed to ensure minimum amount of water contamination. Fig 5 shows the R-series setting for this step.


*Figure 6 - Oxidation step*

**Cleavage of the oligonucleotide**: Once the synthesis was completed, the resin was taken out of the reactor and washed with DMF (6x3 ml) and DCM (6x3 ml) and dried under vacuum. The result was 414 mg of resin being isolated. Cleavage and global deprotection was performed by heating the resin in 28 % ammonium hydroxide (aq.) solution at 55 °C overnight. **Caution**: for safety, we used sealed microwave vials which were rated for ~30 bar.

Each cleavage was consisted of ~150 mg resin in 2.2 ml of ammonium hydroxide solution. After the reactor had cooled down, the resin

was filtered and washed with more ammonium hydroxide.

The solvent was removed to obtain 141 mg of white solid. This material was dissolved in water and extracted with ethyl acetate (x4 times) to remove the residues resulted from deprotection such as benzamide.

## Results and Discussion

Using the above conditions the synthesis of target 9-mer 3'-TTAGCCATA-5' at 52 µmol scale took 4.5 h to complete. Figure 6 shows the UV outcome of each step of the typical 3-step cycle. The synthesis starts with a deblocking step which removes the DMT group on the preloaded T nucleotide. After that a 3-step cycle starts which couples the next nucleotide, oxidises the P(III) to P(V) and deblocks the DMT protecting group on the 5'OH of the new nucleotide, ready to start the next cycle.

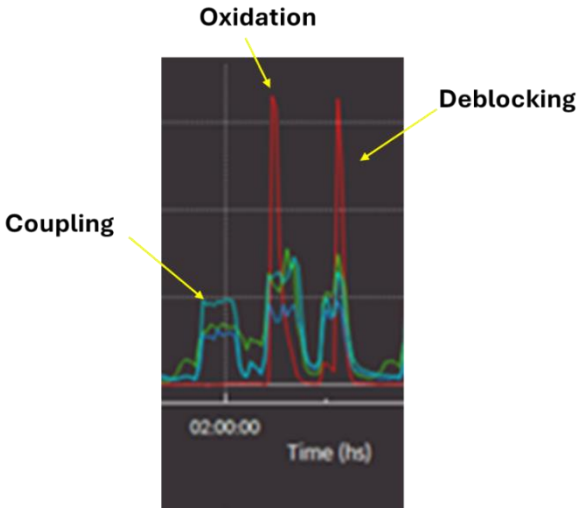


*Figure 7 - The UV trace of a typical 3-step cycle*

These three steps are repeated till the whole sequence is installed. Note that the current protocol also removes the final DMT group, however this can easily be omitted if the DMT-protected chain is required.

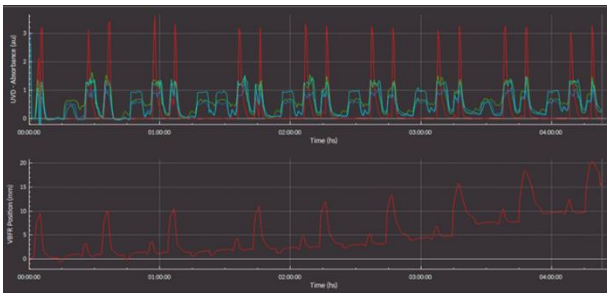

*Figure 8 - VBFR volume change (orange) and UV (blue) data over time for the synthesis*

As can be seen in Figure 8, addition of G to the chain (the third cycle) is achieved by a double coupling. This is because reaction of activated G-phosphoramidite is slower than other nucleotides, most likely due to more prominent steric hinderance.

Based on the process explained above, when the synthesis is performed on 300 mg of resin (52 µmol scale) each reagent is used in excess compared to the resin as reported in table 1:

| Reagent | Stoichiometry (vs resin) |
|---|---|
| Nucleotide monomers T, A and C | x3.8eq |
| Nucleotide monomer G | x7.2eq |
| Activator | x21.6eq |
| TCA | x26.5eq |
| $I_2$ | x2.9eq |

*Table 1 - Reagents stoichiometry compared to the resin loading*

Within the synthesis, the UV-Vis trace of each deblocking step at 465 nm (red) shows a constant height of ~3 A.U. pointing to the fact that each coupling is close to 100 % efficient. If the efficiency of a coupling step is less than perfect, then it is expected that a lower

absorbance its registered when its DMT group is released via deprotection. However, it should be noted that because the detector is operated close to the saturation of its dynamic range, accurate quantification of each deprotection may not be possible.

Figure 7 shows that the VBFR also behaves as expected. With addition of each nucleotide to the chain, the reactor expands. By the time the 9$^{th}$ nucleotide is installed the reactor's length would increase by ~10 mm. This ensures that the porosity of the resin remains high throughout the synthesis providing access to reactive sites.

After the synthesis was completed, 150 mg of resin was cleaved, obtaining 117 mg of the crude 9-mer **3'-TTAGCCATA-5'** product as a white solid. This material was initially analysed by LC-MS to confirm the structure of the desired product. Th parent mass of the desired product is 2697 and the main peak clearly showed [M+2]/2 = 1349.5, [M+3]/3=900.9 and [M+4]/4=675.3 as seen in Figure 8:

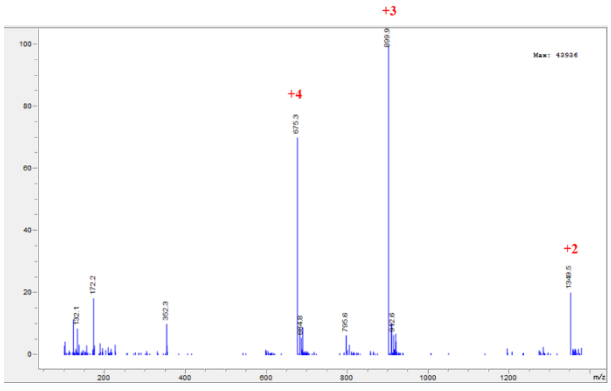


*Figure 9 - MS charge state of the desired crude 9-mer*

Ion-paired reverse phase HPLC was used to determine the purity of the crude. A 4.6x100 mm AdvanceBio Oligonucleotide C18 columns (Agilent) was used. A 10 minute method with a flow rate of 1 ml/min was developed to analyse the crude sample. Solvent A was an aqueous solution of 100 mM triethylammonium acetate (TEAA) and solvent B was 100 mM TEAA/ACN. The method gradient is seen in Table 2:

| Time [min] | A [%] | B [%] | Flow [mL/min] | Max. Pressure Limit [bar] |
|---|---|---|---|---|
| 0.00 | 93.0 | 7.0 | 1.000 | 400.00 |
| 4.00 | 93.0 | 7.0 | 1.000 | 400.00 |
| 4.05 | 90.0 | 10.0 | 1.000 | 400.00 |
| 9.80 | 90.0 | 10.0 | 1.000 | 400.00 |
| 10.00 | 93.0 | 7.0 | 1.000 | 400.00 |

*Table 2 - HPLC method used to analyse the sample*

The sample was analysed using this bespoke method with a shallow gradient of 7-10 % as seen in Table 2. The chromatogram of HPLC analysis of the crude 9-mer oligonucleotide at 264 nm is reported in Figure 10.

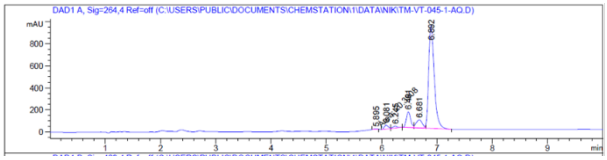

*Figure 10 - HPLC analysis of the crude DNA 9-mer*

The peak at 6.8 min corresponds to the desired product with the purity estimated to be ~77 % with an estimated isolated yield of 82 %.

The main impurity according to the LC-MS studies was an A-deletion by-product which we assume corresponds to the peak at 6.4 min and is estimated to be around 9 %. This

impurity can easily be omitted by a double coupling for nucleotide A.

Due to the fact that there are three A nucleotides in the sequence, further studies are needed to determine which one is causing the main impurity and then a doubling can be used to improve the efficiency coupling at that particular position, instead of performing a double addition for all A positions which will be wasteful.

| Initial resin | Resin Isolated | Product Isolated | Product isolated after W/U | Yield | Purity |
|---|---|---|---|---|---|
| 300 mg | 415 mg | 141 mg | 117 mg | 82 % | 77 % |

*Table 3 - results summary for the synthesis*

## Conclusion

This preliminary work has shown the benefits of applying the VBFR technology in the efficient synthesis of oligonucleotides using single pass continuous flow. We believe that this efficiency is mainly due to presence of the perfect packed bed reactor which allows us to balance the high loading of the resin versus its porosity.

The flow configuration of the system allowed a precise control over mass and heat transfer and efficient wash the resin. As the synthesis progressed, the reactor volume is automatically adjusted maintaining a constant packing density across the resin bed throughout the process, unlike traditional reactors.

An example DNA 9-mer was synthesised at 52 $\mu$mol scale resulting in isolating the crude target material in good purity (77 %) and yield (82 %). More importantly this was achieved by using few equivalents of amidite monomers. It was also demonstrated that a 3-step cycle is as effective as the currently 4-step cycle which includes a capping step; a fact that can save significant time and reagents for large scale preparation of oligonucleotides.

We believe that by increasing the scale of the reaction and using a more concentrated solutions for each reagent we will be able to reduce the excess reagents even further. This is because at higher concentrations, the detrimental effect of the residual water becomes less important. The efficient wash step also means that for the synthesis of the 9-mer product on >100 mg scale we used less than 100 ml of ACN for washing the resin/reactor (10 ml per step). Both reagent stoichiometries and solvent volumes in our process compare very favourably with what is reported in the literature. As the platform is highly chemically inert, other solvents, such as DCM, could be used for resins that are more hydrophobic.

The synthesis cycle is also fast, considering the relatively large scale of the experiment (~ 30 min per cycle). This time can be reduced further by increasing the flow rates and unlike the current mixed-bed reactors will likely not increase much further at higher scales. Another advantage is that the system is flexible with regards to the reagents used. For example, while we chose to use ETT as the activator, other tetrazole-based reagents (such as BTT or tetrazole) or dicyanoimidazole (DCI) can also be used, although the solubility

of these reagents should be taken into account when designing the process.

## References

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2. Vapourtec Ltd. *Application Note 50: Rapid Mixing Reactor for Biphasic Reaction Scale-up*. (2017).