HIGH PERFORMANCE, HIGH THROUGHPUT OLIGONUCLEOTIDE SYNTHESIS FOR DIAGNOSTIC AND THERAPEUTIC APPLICATIONS

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ABSTRACT

Eurofins Genomics Europe Pharma and Diagnostic Products & Services Synthesis GmbH is one of the world-leading oligonucleotide manufacturers for the industrial and research sector and a major provider of genotyping and DNA sequencing services.[1] Several different classes of unmodified and modified oligonucleotides are produced via solid phase oligonucleotide synthesis[2] in a high throughput production floor with short turnaround times (TAT). The portfolio ranges from custom DNAs and RNAs over fluorescent probes for qPCR applications to unique dual index (UDI) primer sets for state-of-the-art next generation sequencing (NGS) methods.[1]

Over the past decades, oligonucleotides have gained an important role in the field of biochemical research, diagnostics and therapeutic applications.[3] Great effort has been made to optimize and automate oligonucleotide synthesis, especially to minimize undesired by-products such as truncated sequences resulting from the large number of required individual reactions.

1. PROCESS DEVELOPMENT FOR HIGH THROUGHPUT SYNTHESIS OF OLIGONUCLEOTIDES

Since 2021, Eurofins Genomics has capitalized from next generation high throughput synthesizers, the so-called continuous flow synthesizers (CFS), which allow a simultaneous production of 6 x 384 different oligonucleotides with elevated speed reaching an excellent coupling efficiency of ≥99.6%.

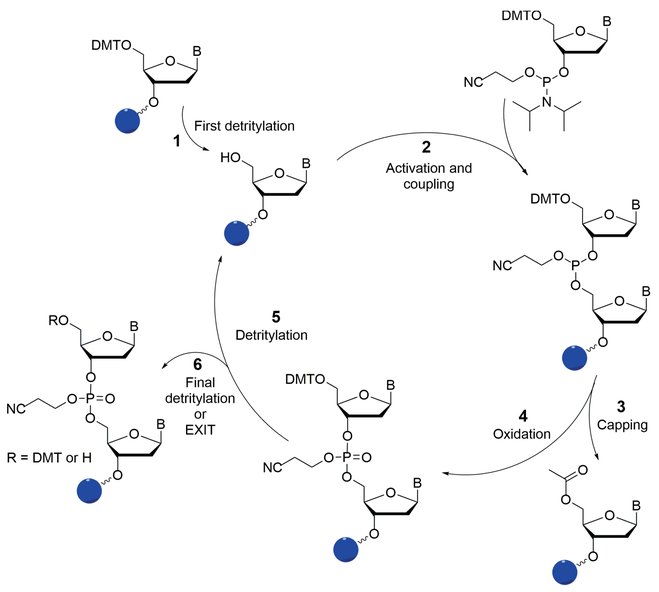

Figure 1. Periodic synthesis cycle performed during solid phase oligonucleotide production.

Oligonucleotides can be synthesized by using either polystyrene (PS) or controlled porous glass (CPG). PS has a very high loading, however, during oligo synthesis PS is swelling and therefore limits its applications. CPG is crushed glass, which gives the opportunity to synthesize longer oligos than using PS. CPG material is mixed with polyethylene for sintering frits, which are used for high throughput plate synthesizers.

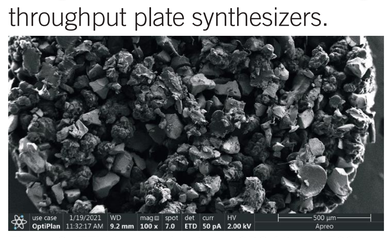

Figure 2. TEM image of sintered CPG frit, bar 500 µm.

2. METHOD DEVELOPMENT FOR SYNTHESIS OF HIGH-QUALITY OLIGONUCLEOTIDES

CFS protocols for small frits shows a homogeneous synthesis across the 384 plate with only 5% variation in yield **(Figure 3)**. New CFS protocols allows synthesis of 200mer with minimal failure sequences. UHPLC methods developed for very long oligonucleotides. ESI-MS analysis shows approx. 4% n-1 **(Figure 3)**.

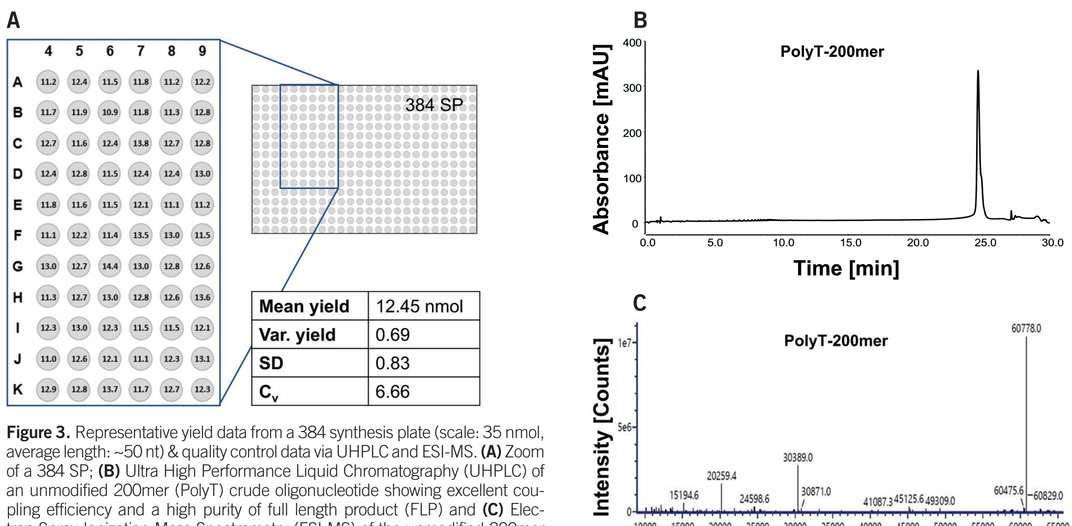

Figure 3. Representative yield data from a 384 synthesis plate (scale: 35 nmol, average length: ~50 nt) & quality control data via UHPLC and ESI-MS. **(A)** Zoom of a 384 SP; **(B)** Ultra High Performance Liquid Chromatography (UHPLC) of an unmodified 200mer (PolyT) crude oligonucleotide showing excellent coupling efficiency and a high purity of full length product (FLP) and **(C)** Electron Spray Ionization Mass Spectrometry (ESI-MS) of the unmodified 200mer (PolyT) crude oligonucleotide confirms the identity of FLP ($mass_{calc.}$ = 60776.0 Da and $mass_{found}$ = 60778.0 Da).

3. DEVELOPMENT OF EFFICIENT AND SAFE DEPROTECTION/CLEAVAGE

Cleavage / deprotection is generally performed by using a mixture of NH_3 and CH_3NH_2 in gas phase under pressure and high temperature. To avoid the transamidation side reaction that occurs with benzoyl dC and methylamine, acetyl dC is used. However, transamidation reaction does occur due to high nucleophilicity CH_3NH_2.

The gas phase deprotection is unsafe and is the reason why Eurofins Genomics Europe Pharma and Diagnostic Products & Services Synthesis GmbH developed a new deprotection method for Implementation of MW deprotection/cleavage protocols to improve TAT, safety and quality of oligonucleotides **(Figure 4)**.

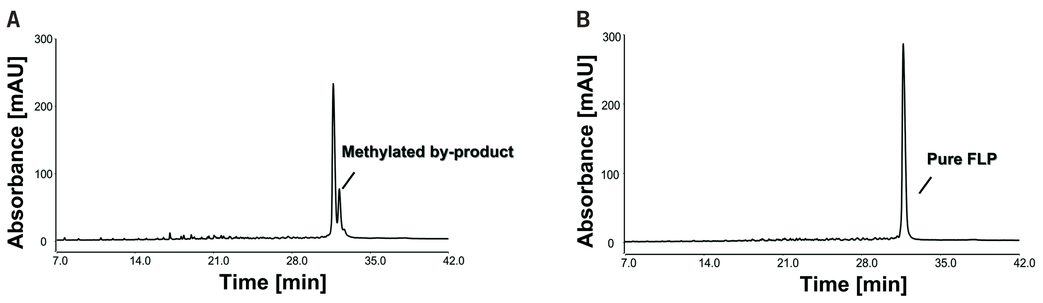

Figure 4. Representative UHPLC chromatograms of a crude oligonucleotide cleaved / deprotected by gas phase and by new deprotection method developed by Eurofins. UHPLC chromatogram of a 68mer cleaved **(A)** in gas phase using a mixture of NH_3 / CH_3NH_2 and **(B)** using MW deprotection with NH_3 & decreased to the half the deprotection time.

4. DEVELOPMENT OF HIGH-RESOLUTION UHPLC METHODS

Here, we compare commonly used chromatographic approaches (IP-RP-UHPLC, IEC) and solvent systems (triethylammonium acetate (TEAA),[5] to an internally developed high-resolution solvent system) to base-line separate the target oligonucleotide product from its impurities.

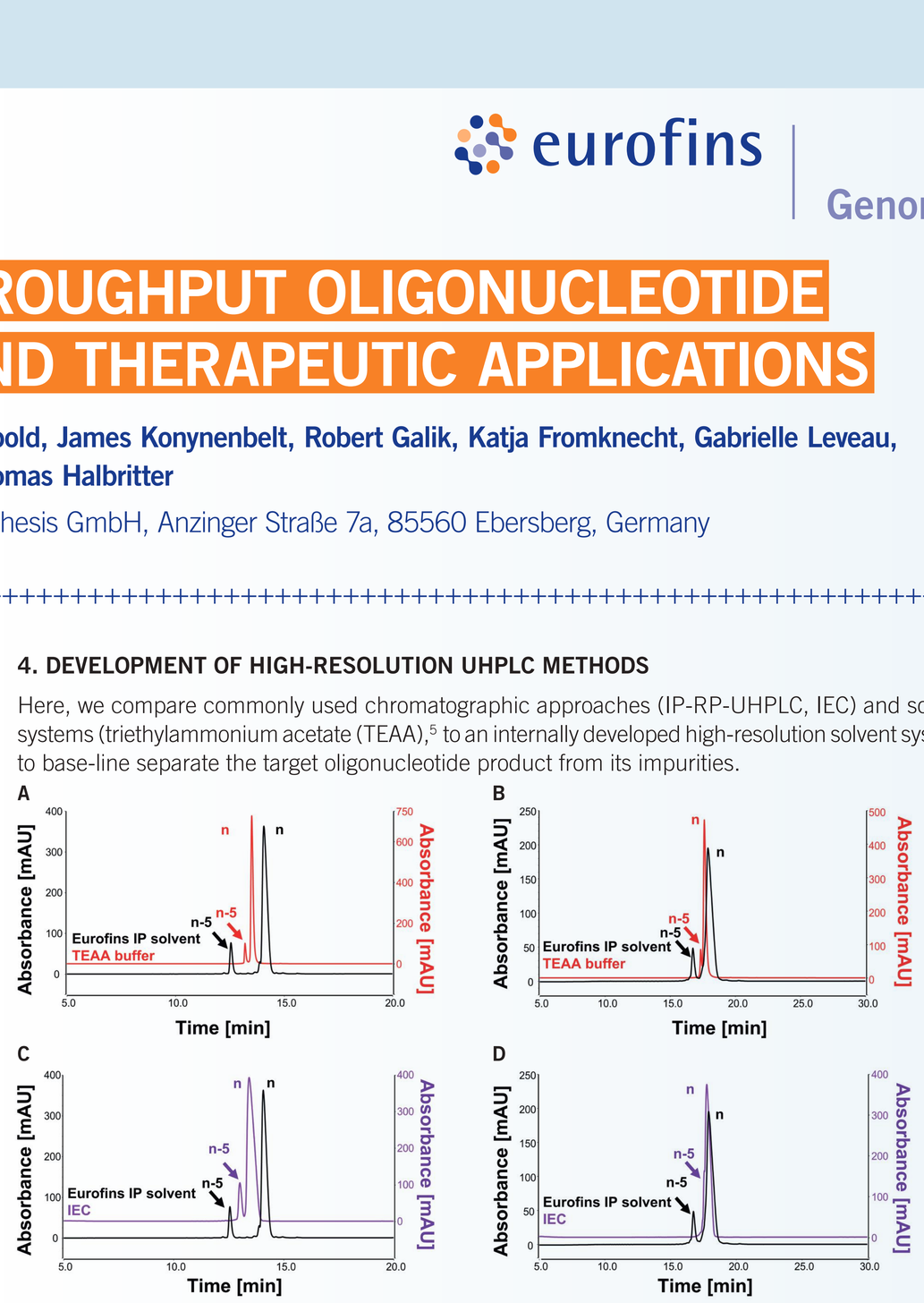

Figure 5. Comparison of UHPLC resolution using an IP-RP column with different mobile phases and IEC. **(A)** Comparison of UHPLC resolution using TEAA buffer (red) and Eurofins solvent system (black) for IP-RP to separate a mixture of PolyT 40mer (n) and 35mer (n-5) and **(B)** a mixture of PolyT 70mer (n) and 65mer (n-5) in a 1/10 ratio, respectively. **(C)** Comparison of UHPLC-resolution using Tris buffer for IEC (purple) and Eurofins solvent system (black) for IP-RP to separate a mixture of PolyT 40mer (n) and 35mer (n-5) and **(D)** a mixture of PolyT 70mer (n) and 65mer (n-5) in a 1/10 ratio, respectively.

5. OPTIMIZED qPCR PROBES

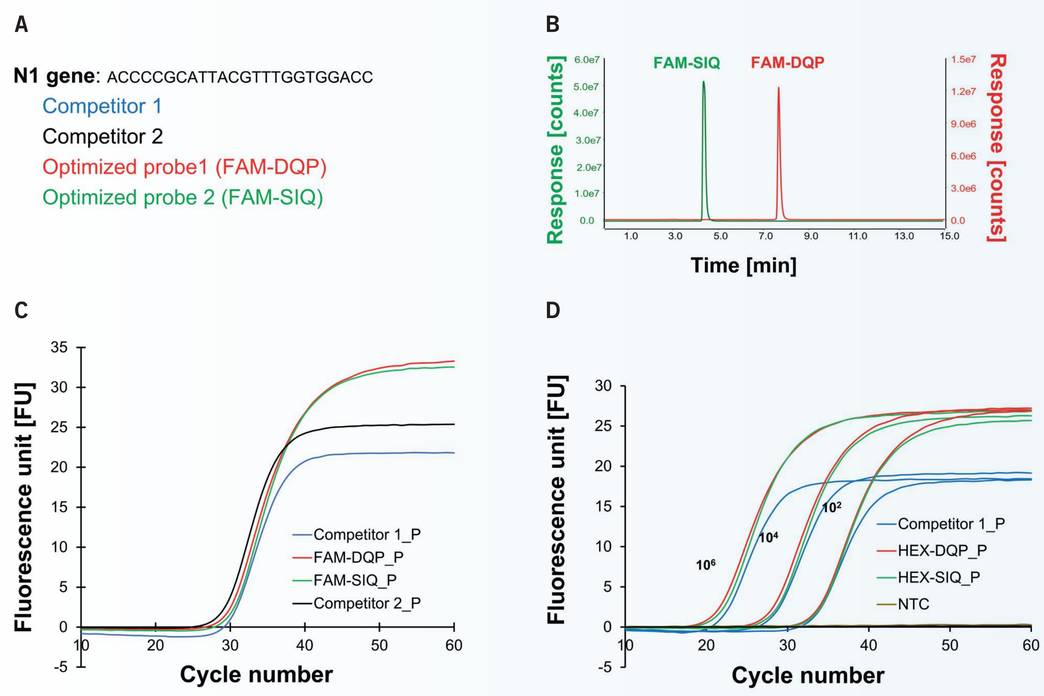

Figure 6. Design & synthesis of optimized qPCR probes targeting N1 gene and comparison to other competitors. **(A)** Design and synthesis of probes; **(B)** representative UHPLC emmission at 520 nm of FAM-SIQ (green) and FAM-DQP (red); **(C)** comparison of qPCR performance of FAM-labeled optimized probes (240 nM) at plasmid concentation 1.03×10^{-5} (ng/µL); primer (P) concentration 760nM and **(D)** comparison of qPCR performance of new HEX-labeled optimized probes (240nM) towards Competitor 1 in different plasmid dilutions; blue, red, green and brown solid lines are mean of three experiments.

6. CONCLUSIONS

CFS technology in combination with CPG material enables high coupling efficiencies leading to:

- High-purity of full-length oligonucleotides
- Advanced speed & low cost oligonucleotide production

High resolution UHPLC-IEC methods led to:

- The development of a high-resolution solvent system (Eurofins IP solvent system)
- An impressive resolution for longer oligos of up to 70mers

Design and synthesis of qPCR probes:

- Based on N1 and E gene sequences, coupled with FAM or HEX 5´end, internal quencher and/or 3'end quencher
- Exhibited extremely high purity via UPLC
- Showed impressive performance in qPCR

7. REFERENCES

1. https://eurofinsgenomics.eu/en/dna-rna-oligonucleotides/portfolio-overview, Eurofins Genomics, (2023).
2. Caruthers MH, Barone AD, Beaucage SL, Dodds DR, Fisher EF, McBride LJ, Matteucci M, Stabinsky Z, Tang JY. Chemical synthesis of deoxyoligonucleotides by the phosphoramidite method. Methods Enzymol.154, 287-313 (1987).
3. Roberts, T.C., Langer, R. & Wood, M.J.A. Advances in oligonucleotide drug delivery. Nat Rev Drug Discov. 19, 673–694 (2020).
4. McCarthy S.M & Gilar M. Hexylammonium acetate as an ion-pairing agent for IP-RP LC analysis of oligonucleotides. Waters, application note 1-7, (2016).

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