# APPLICATION NOTE

## High quality qPCR probes with improved quenching properties

**High quality qPCR probes with improved quenching properties**

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## Abstract

Diagnostic kits based on polymerase chain reaction (PCR) have become important in the diagnosis of various diseases and pathogens. Eurofins has used quantitative PCR (qPCR) probes to detect Covid 19 and helped contain the uncontrolled spread of the virus to minimize the overload of global healthcare systems. The successful design of such qPCR-based probes requires a systematic approach. Here, we describe the importance of design, synthesis, high-resolution purification and analysis of oligonucleotides and compare different designs in a SARS-CoV-2 model system. Our results show that the combination of optimized probe designs and state-of-the-art analytical methods lead to highly sensitive and optimized diagnostic assays.

## Introduction

During the past decade PCR-based probes have gained importance in several diagnostic research areas[1] and were a critical technology during the covid pandemic to slow down the spread of the virus. QPCR is a fluorescence resonance energy transfer (FRET)-based technology that can give real-time information about relative and absolute amounts of DNA present in a sample. FRET is a photophysical process, where electronic energy of an excited donor (*e.g.* fluorophore), is transferred to an acceptor (*e.g.* quencher) in its ground state ($S_0$) which consequently gets excited.[2]

Creating effective qPCR probes for diagnostic tests involves considering various factors:

**High FRET efficiency**: For successful FRET, it's crucial that the emission of the donor overlaps with the absorption spectrum of the acceptor. The donor and acceptor molecules should also be within the FRET radius, typically 10-100 Å.[3] Small changes in their distance significantly impact FRET efficiency (E), which increases as the distance decreases. The relative orientation of the molecules also plays a role.[3]

**Primer, probe, and amplicon design:** Careful planning of the locations and characteristics of primers, probes, and amplicons is essential for optimal performance.[3]

**Appropriate melting temperature (Tm):** Ensuring the right Tm helps determine the conditions for oligonucleotides to bind specifically to their target sequence. Additional requirements include: 1) optimal annealing temperature (Ta), 2) optimal GC content (35-65%) and 3) avoidance of G at the 5'-end to prevent quenching of the 5' fluorophore.[4]

**Modification of the 3' ends:** Modification prevents unspecific polymerase extension of the probe upon hybridization.[5] The 3' end of standard probes is blocked by a quencher.

**Purity of probes:** Ensuring high-purity probes, without any traces of free dyes, minimizes background fluorescence. [6]

The signalling mechanism of qPCR probes is a two-step process. During qPCR-analysis the probe hybridizes to its target DNA (**Figure 1**, **step 1**). The polymerase extends the primer and cleaves the probe from its 5'- to 3'-end resulting in a fluorescent signal (**step 2**).[3]

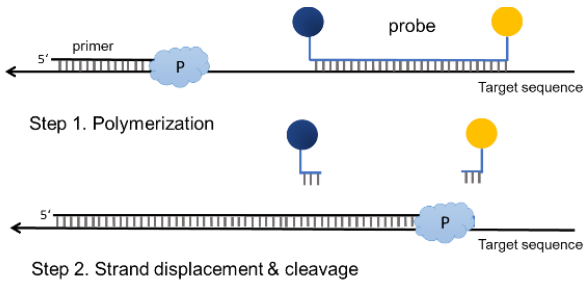


**Figure 1**. Signalling mechanism of qPCR probes. During PCR the probe hybridizes to its target DNA (**step 1**), the probe gets hydrolysed, and the nucleotide fragments are replaced from the strand. The hydrolysis of the probe leads to non-quenched, free dyes which can be detected via fluorescence (**step 2**).

To increase FRET-based quenching efficiency within probes, the dye and quencher must be in proximity. It is also possible to introduce the quencher at an internal position to optimize the FRET distance between the fluorophore and the quencher.[7] Such probes can be either double-quenched (**DQ**, internal quencher and 3'-quencher) or single internally quenched (**SIQ**) with a specific blocker at the 3' position.

In this application note we present different designs of chemically synthesized probes and demonstrate that optimized design leads to low background fluorescence, while maintaining a high endpoint fluorescence. Furthermore, we focus on the development of chromatographic systems that allow high-resolution purification and analysis of our probes. Finally, we compare different probe designs regarding their efficiency in a SARS-CoV-2 model system.

## Results

We have synthesized different probes (**Figure 2**) named as standard probe (**STP**), double quenched probe (**DQP**) and single internally quenched probe (**SIQP**) with 5'-FAM and 5'-HEX labelling of the N gene and E gene region of SARS-CoV-2. Based on rational design we synthesized 18 qPCR probe derivatives, and we compared their efficiency in our SARS-CoV-2 model system.

Aiming for the reduction of the background fluorescence of our probes we incorporated the quenchers internally at different positions either through the backbone or attached to a base (**Figure 2**).

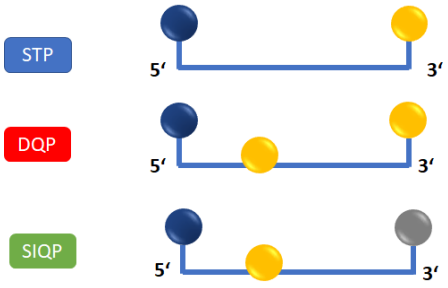


**Figure 2**. Principal design of quenched probes. Blue: standard probe (**STP**) red: double quenched probe (**DQP**) and green: single internally quenched probe (**SIQP**). Blue circle: fluorophore, yellow circle: quencher and grey circle: 3'-end blocker.

Eurofins Genomics EU has extensive experience in the synthesis of high-quality oligonucleotides. Therefore, we were able to easily overcome synthetic challenges for diagnostic probes (*e.g.* efficient dye conjugation and high coupling rates), thus achieving high-purity target sequences.[8]

To obtain high-quality probes, we utilized a novel proprietary solvent system for purifying our products via ion-pair reversed phase liquid chromatography (IP-RP-HPLC). All 18 synthesized probes underwent purification using both the commonly used HPLC buffer, triethylammonium acetate (TEAA), and our proprietary solvent system, while maintaining the same HPLC system and semi-preparative column.

Figure 3 illustrates the representative IP-RP-HPLC chromatograms of the crude SIQPs. The novel solvent system (**Figure 3B**) facilitates considerably greater resolution when compared to the conventional solvent system (**Figure 3A**). Only the proprietary solvent system allowed the detection of the impurities (as labelled with an asterix), while the conventional solvent system concealed the impurities in the main product peak. Therefore, utilizing the novel solvent system enhances the effective removal of impurities from the SIQP product.

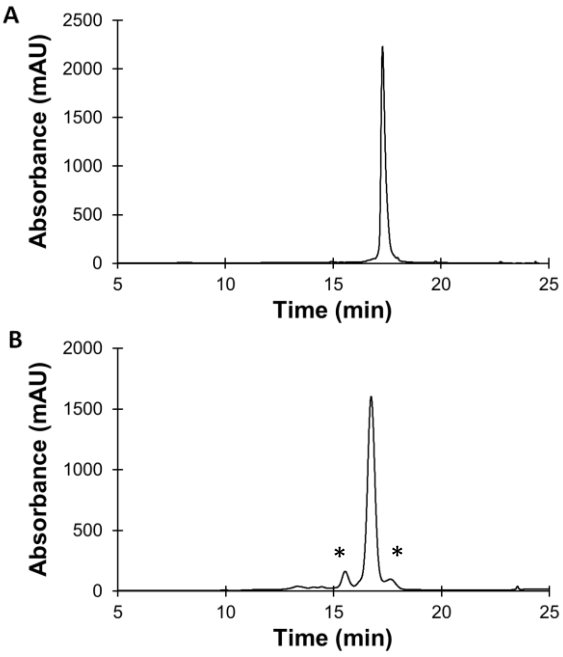


**Figure 3**. Representative semipreparative IP-RP-HPLC-chromatograms of SIQP. (A) IP-RPLC of crude **SIQP** using TEAA buffer and (B) of the same crude **SIQP** using our novel proprietary solvent system, both recorded at λ = 260 nm.

In the next step, we analysed our newly developed probes after their purification via analytical ion-pair reversed phase ultra-high performance liquid chromatography (IP-RP-UHPLC) to assure highest purity. Representative examples of **SIQP** and **DQP** are shown in **Figure 4**.

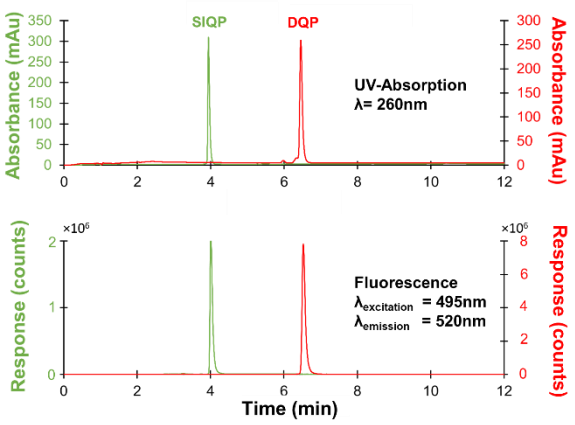


**Figure 4**. Representative IP-RP-UHPLC chromatograms of newly developed **SIQP** (green) and **DQP** (red) after purification with the novel solvent system and recorded at λ = 260 nm (upper panel) and the corresponding emission chromatograms at λ = 520 nm (lower panel).

Both IP-RP-UHPLC chromatograms confirm the high quality of our probes and thus avoid negative effects that could be attributed to any contamination or impurity, *e.g.* free dye.

Next, we investigated the effects in terms of quenching efficiency and fluorescence enhancement of our newly developed **SIQP** and **DQP** towards our **STP** and a competitor probe (different **DQP**). All probes were labelled with 5'-FAM and both regions of the N and E genes (data not shown) were tested in our SARS-CoV-2 plasmid DNA based model system (**Figure 5**).

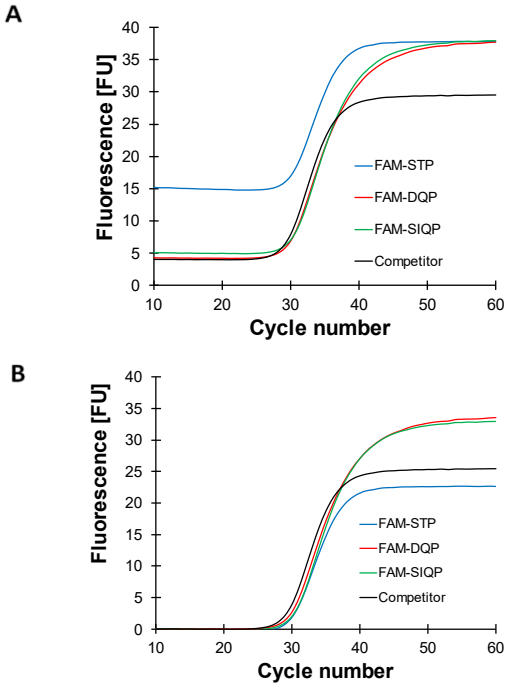


**Figure 5**. Amplification curves of the N gene qPCR assay for dilution level 6 ($10^4$ target copies). (A) Amplification plot without background correction (raw data), (B) amplification plot with background correction (normalized data). Blue line (**STP**); red line (**DQP**); green line (**SIQP**) and black line competitor (mean of three experiments).

Finally, we were interested in the performance of 5'-HEX-**SIQP** and 5'-HEX-**DQP** of the N and E target region, respectively, in qPCR assays. Therefore, the probes were used to read signal amplification across a dilution series of the plasmid; representative data are shown in **Figure 6**. The resulting curves are plotted after baseline subtraction to better illustrate the probe efficiency.

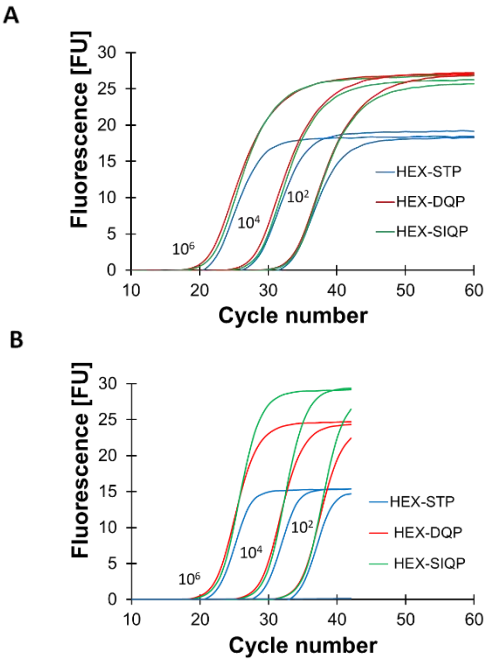


**Figure 6**. Comparison of **STP** and newly developed **SIQP** and **DQP**. (A, B) Amplification curves of qPCR performance of HEX-labelled probes (**SIQP**, **DQP** and **STP)** in different plasmid dilutions ($10^2$, $10^4$ and $10^6$ target copies) of N (A) and E gene regions (B); blue, red and green lines are mean of three experiments.

The results (**Figure 5** and **Figure 6**) demonstrate a significant improvement in signal response with each assay, particularly in the case for **SIQP** tested against the N and E gene.

## Summary

We show that internally quenched probes lead to a significant improvement in the quenching of the background fluorescence in diagnostic assays due to a stronger FRET effect. In addition, our newly developed IP-RP HPLC solvent systems enable high-purity and high-performing oligonucleotides due to a baseline separation of the target oligonucleotide from its byproducts and allow state-of-the-art analysis of diagnostic probes. Moreover, the new probes showed significant improvements regarding their quenching efficiencies in a SARS-CoV-2 model system. In particular, **SIQP** showed higher endpoint fluorescence after normalization and often earlier $C_q$ values compared to **STP**. Consequently, the robustness of qPCR

experiments is increased. Our results demonstrate the importance of an optimal oligonucleotide design yielding in high-performance diagnostic assays. In addition to **SIQP**'s improved performance over **DQP**, **SIQPs** are more cost-effective in producing sensitive assays because only one quencher needs to be incorporated in the oligonucleotides.

## Experimental

**Preparation of probes**. All probes and primers were synthesized at Eurofins Genomics EU under ISO13485 certified quality management dissolved in $H_2O$ and adjusted to a concentration of 100.00 ± 3.75 µmol/L.

**Semi-preparative chromatography**. Purification of the synthesized oligonucleotides was performed on a semi-preparative HPLC (Agilent 1260 Infinity II) equipped with a quaternary pump, a multiple wavelength UV-VIS detector (MWD), an autosampler and a C18 column. The flow rates were 4 mL/min. The UV absorbance was monitored at 260 nm and at the absorbance maximum of the dyes FAM/HEX (495/520 nm), respectively.

**Analytical chromatography**. IP-RP UHPLC was performed on a Vanquish Horizon Duo system from ThermoFisher Scientific equipped with a binary pump, a diode array (UV / visible) detector (DAD), fluorescence detector (FD) with dual photomultiplier tube (PMT) and using a C18 column. The flow rates were 0.5 mL/min. The UV absorbance was monitored at 260 nm. Furthermore, an in-house developed high-resolution IP solvent system was used for the IP-RP-UHPLC experiments. Thermo Scientific™ Chromeleon™ Chromatography Data system (7.2.0 ES) was used for data acquisition and analysis.

**qPCR-experiments**. All reactions were performed using a Roche LightCycler 480. The data analysis was performed using the LightCycler 480 software (cf. Roche, 2008). All collected $C_q$ values were calculated automatically by the software using the second derivative maximum method.

## Learn more

These newly developed SIQ probes are available for your diagnostic assays as Ultimate Precision Probes. For more information and contact information please visit:

https://eurofinsgenomics.eu/ultimate-precision-probes/

## References

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