kilobaser

DNA PROBES

SYNTHESIS AT THE PUSH OF A BUTTON IN YOUR OWN LAB

This whitepaper gives a short introduction to DNA probes, highlighting TaqMan and FISH probes, and describes how the DNA synthesis device Kilobaser enables any lab to produce their own custom probes while saving running costs and time.

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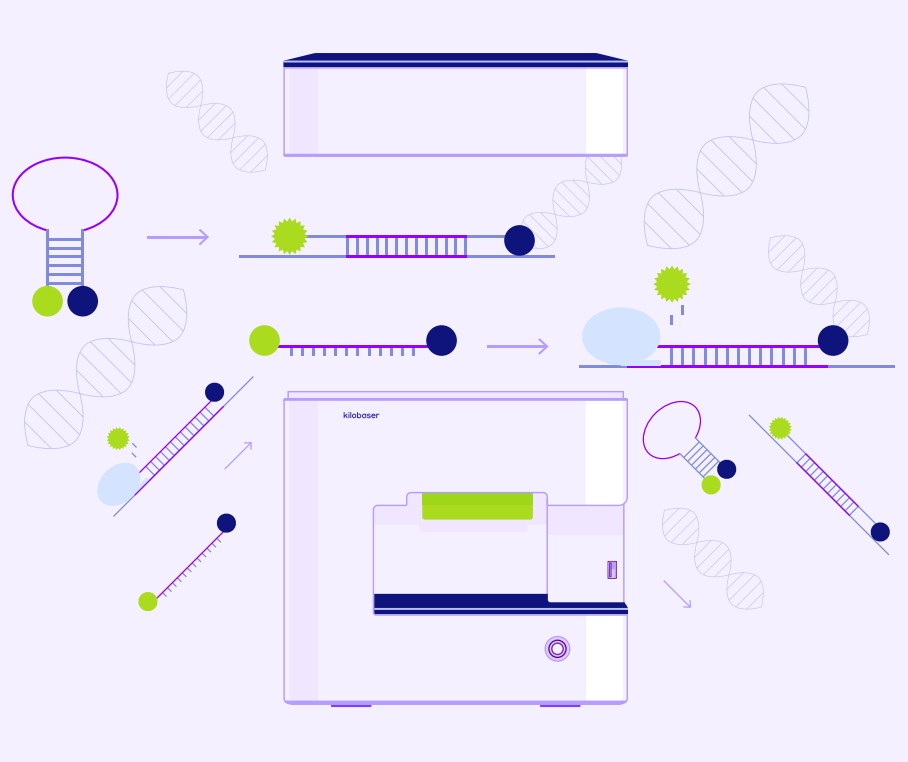


01 INTRODUCTION

01.1 DNA Probes

DNA probes are relatively short DNA strands (from 9 to 40 bases) that are modified with signal generating molecules such as fluorescent dyes or radioisotopes. With a worldwide market volume of approximately 360 million € / 435 million $ (1), they represent the second largest group among synthesized oligonucleotides next to classical DNA primers. These DNA probes are produced fully chemically, molecule by molecule in DNA synthesis devices ('DNA synthesizers').

DNA probes are used to label complementary DNA strands ('target sequence') by hybridization. Due to the modification

with, for instance, fluorescent dyes, the complementary, hybridized DNA strands can then be very specifically detected and even quantified. DNA probes are used in molecular biology research and medical diagnostics, in methods such as qPCR, Southern blot and Fluorescence-in-situ-hybridization ('FISH'). During the COVID-19 pandemic caused by the pathogen SARS-CoV-2, DNA probes reached particular importance because they were used for the highly sensitive detection of infected people by qPCR tests (2).

01.2
FISH Probes

FISH is a widely used technique to detect and localize DNA or RNA in cells. Thus this methods allows the analysis of the nuclei of single cells and metaphase chromosomes as well as the distribution of mRNA in whole embryos, sections or tissues. The detection of DNA and RNA is possible due to the sequence specificity of FISH probes. They hybridize with the targeted DNA or RNA and can then be detected microscopically by excitation with UV light. These FISH probes consist typically of a DNA oligonucleotide modified with a fluorophore; 6-carboxyfluorescein (6-FAM) (3).

01.3
TaqMan Probes & qPCR

The TaqMan probes are a further development of DNA probes with fluorescent dye and have a 'quencher' at the 3' end in addition to the dye at the 5' end. Quenchers can 'swallow' emitted light from the dye as long as they are connected by the DNA strand of the probe in spatial proximity to the fluorescent dye. Thus, as long as the TaqMan DNA probe is intact, it cannot be made to glow by excitation with UV light in the qPCR instrument and is thus hardly detectable.
TaqMan probes are designed to hybridize within a DNA region amplified by a specific set of primers. As the Taq polymerase extends the primer and synthesizes the resulting strand, the 5'-to-3' exonuclease activity of the Taq polymerase degrades the hybridized probe - thereby capping the fluorescent dye-quencher linkage - the liberated fluorescent dye can now emit light when excited as shown in Figure 1. This system is used to quantify only targeted DNA and thus increases the specificity of the qPCR method (4).

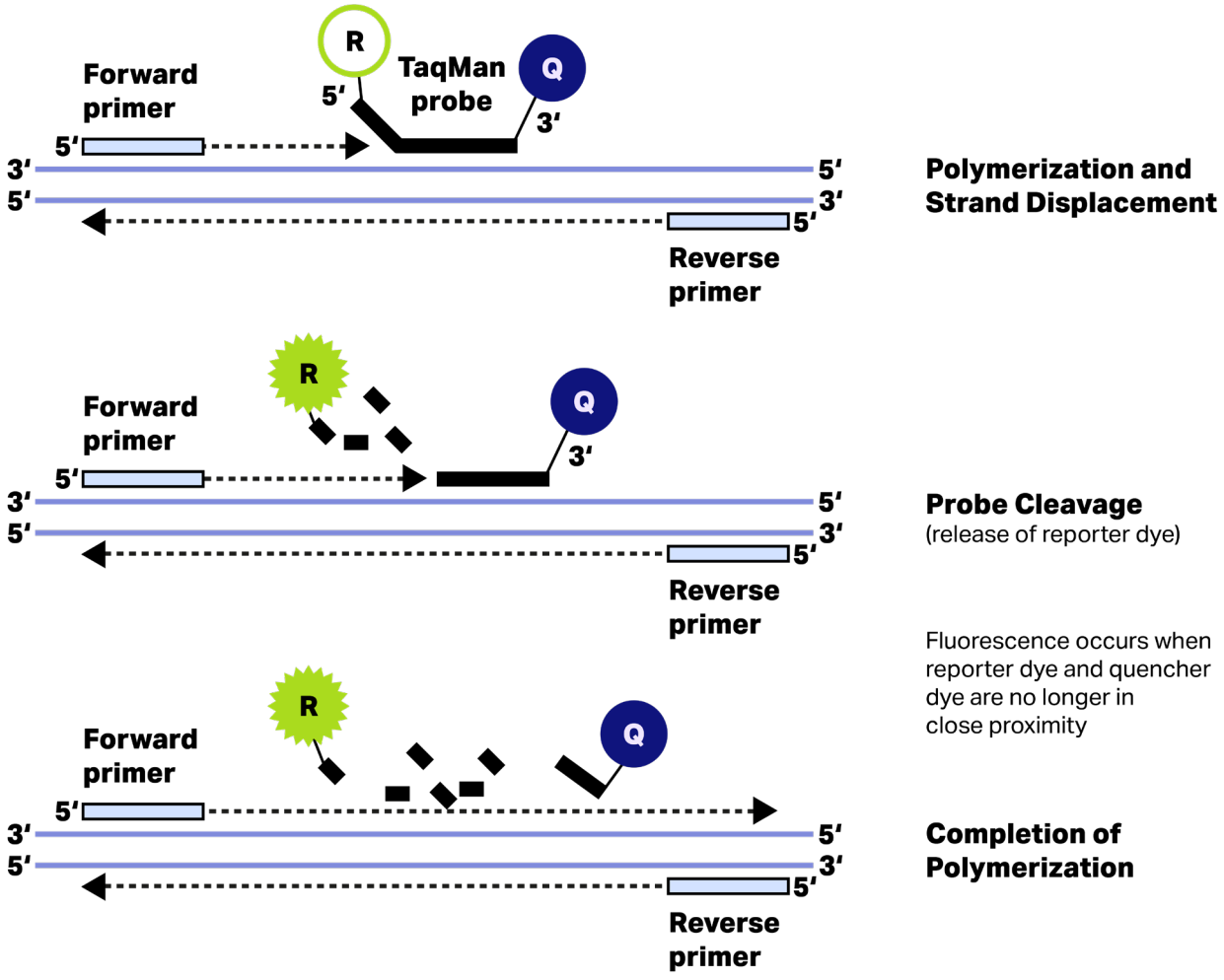


Figure 1: Principle of qPCR with TaqMan probe

Fluorophore	EX	EM	Recommended Quencher	BHQ Dye Quenching Range
FAM	495	520	BHQ-1	BHQ-1 480-580 nm
TET	521	536	BHQ-1	
VIC	538	554		
NED	546	575		
ROX	586	610	BHQ-2	BHQ-2 559-670 nm

Figure 2: Fluorophores with excitation and emission wavelengths and matching quencher.

02 PROBLEMS IN PROCUREMENT

02.1 Costs

Research with customized DNA probes is currently partly associated with high running costs. A probe with 6-FAM fluorophores, BHQ-1 quencher and only 20 DNA bases starts typically at 150€ / 180$ with all well-known manufacturers such as Eurofins, IDT DNA or Thermo Fisher. The addition of specific modifications and higher numbers of DNA bases can increase the costs significantly. The costs seem immense especially compared to the low prices of unmodified DNA oligos - such oligos with 20 bases costs only about 4€ or 4$.

02.2 Delivery times

Modifications add several days to DNA delivery times. Typically, waiting times to manufacture are 4 - 8 working days for DNA probes, shipping itself can take from 1-2 days (USA, parts of EU) to several weeks (South America, parts of Asia, Africa) This can lead to long delays, especially for building experiments that require multiple orders.

02.3 Dependency

With few exceptions, most laboratories worldwide depend on custom synthesis mailorder companies. DNA primers as well as DNA probes are routinely ordered online by researchers, and then custom made and delivered by these synthesis companies.

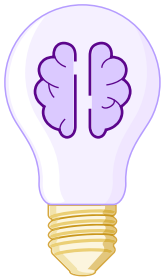
The conventional DNA synthesizers available on the market can hardly compete against the custom synthesis companies due to several factors mentioned as follows:

- **High acquisition costs** (starting at about 35 000€ / 40 000$ for a device)
- **Extensive infrastructure** (ventilation, exhaust fume extraction)
- **High operating costs** due to the high consumption of expensive reagents especially for modified DNA
- **Complex operating requiring** specially trained personnel

Because of these factors, laboratories currently equipped with their own DNA synthesizer are rather a marginal phenomenon.

02.4 Intellectual Property Risk

To the best of our knowledge, the few DNA synthesizers in use by end customers today are primarily operated by in-house departments in corporations. One example is the BASF group, whose research departments are not allowed to order DNA from contract synthesis companies, at least in part, in order to protect valuable intellectual property. The risk of data theft potentially exists both when the data is transmitted via the Internet, as well as by the order taker and ultimately also by the messenger services.



03
ALTERNATIVE FOR DNA PROBES: KILOBASER

03.1
Synthesis Platform Kilobaser Overview

The Synthesis Platform consists of the actual synthesis device Kilobaser and two consumables - DNA Cartridges & Fluidic Chips. For operation, both consumables must be inserted into the device. A DNA cartridge contains all necessary synthesis reagents (especially the 4 base building blocks for adenine, guanine, cytosine, thymine in the form of phosphoramidites) for a total of 200 bases. The instrument uses argon gas pressure to transfer the reagent required for each synthesis step from the cartridge into the Fluidic Chip.

The Fluidic Chip has two main functions: 1. The tiny fluid valves on it control the flow of reagents; 2. In the micro-synthesis chamber (classically known as 'columns'), the actual solid-phase synthesis of the DNA oligos takes place. To synthesize a DNA oligo with 20 bases, Kilobaser needs less than 1.5 hours, and the yield is around 300 picomoles.

DNA Probes	
Synthesis Time	2.5 minutes per base + one-time 25 minutes post-processing
Final Yield	300 picomol (0.3 nanomol)
Stepwise-Yield	99,5%
Cartridge Content	100 bases

03.2
Product Launch

From February 2021, the company Kilobaser will start selling a complementary product for their same named DNA synthesizer Kilobaser: 6-FAM DNA cartridges and Quencher Fluidic Chips.

This gives every laboratory the simple and efficient possibility to produce DNA probes like TaqMan probes and FISH probes in their own laboratory.

In a first step, cartridges for 6-FAM labeling and Fluidic Chips for BHQ-1 labeling are available.

The probe cartridges contain standard reagents for 100 DNA bases as well as 6-FAM, which is coupled to the 3'-end of the DNA. The BHQ-1 chips contain the synthesis column with already coupled BHQ-1 quencher, which is coupled to the 5' end of the DNA.

The consumables can be combined with each other as desired, allowing various options:

• The 6-FAM cartridges and DNA Fluidic Chips can be used to produce one or more DNA probes labeled at the 5' end 6-FAM with a total of up to 100 bases, also known as FISH (fluorescence in situ hybridization) probes.

• Using the BHQ-1 Fluidic Chips and a standard DNA cartridge, multiple DNA primers labeled at the 3' end BHQ-1 can be prepared with a total of up to 200 bases.

• Combining the 6-FAM probe cartridge and BHQ-1 Fluidic Chips for synthesis, one or more TaqMan DNA probes with a total of up to 100 bases can be produced, also known as TaqMan probes.

03.3
Costs

6-FAM cartridge: A 6-FAM cartridge with 100 bases content costs 200€.

BHQ-1 Chips: A pack of 5 pieces BHQ-1 chips costs 200€.

A starter set thus costs 400€ and allows, for example, the synthesis of 5x FAM-BHQ1 DNA probes with 20 arbitrary bases each and yields of 300pmol each. In addition to the speed advantage, this also results in significant cost advantages.

Alternatively, the synthesis of 2x FAM-BHQ-1 DNA probes with 50 bases each would also be possible, for example. The remaining 3 chips can then be used with the next cartridge.

03.4
Shelf Life

6-FAM cartridge: The shelf life of 6-FAM cartridges inserted into the instrument is limited. After the first synthesis, the cartridge can be used for syntheses for 7 more days and then must be replaced.

The short shelf life results from the instability of 6-FAM in solutions. Unopened cartridges have a shelf life of at least one year, as 6-FAM is stored dry in them. We recommend reagent cartridges be stored at 4°C.

BHQ-1 chip: The chips have a shelf life of at least one year. A chip is used for the synthesis of an oligo and must then be disposed of as the solid phase column is consumed.

The fluidic chips should be stored in the dark at room temperature and in a dry place.

03.5
Quality & Sequence Length

Kilobaser has a percentage yield per added DNA base ('Stepwise-yield') of about 99.5% and thus achieves comparable results to custom synthesis companies. The quality is equivalent to that of 'standard desalted' probes. For applications where researchers usually order and use 'standard desalted' probes, the probes synthesized with Kilobaser can be used directly.

Basically, probes without further purification are contaminated by synthesis with unbound fluorophore. This is not relevant for FISH, because unbound molecules including the free fluorophore are removed in washing steps.

TaqMan probes can usually be ordered exclusively HPLC purified from service providers, as the unbound fluorophores can lead to interfering background noise in qPCR instruments - after all, these are not suppressed by a 'tacked-on' quencher at the very start of the process.

Most applications actually don't require purification (5), since the slight background noise doesn't disturb the analysis and can be canceled via qPCR software. Still Kilobaser offers columns for manual purification of TaqMan probes.

The yield of a DNA probe with a defined length can be roughly calculated as follows:
0.995 (% stepwise yield) to the power of the number of bases = percent full length product.

For a probe with 20 DNA bases in length, the equation is:
0.995 ^ 20 = 0.905 = 90.5%.
50 DNA bases:
0.995 ^ 50 = 0.778 = 77.8%.
100 DNA bases:
0.995 ^ 100 = 60.5%.

In general, we recommend to produce a maximum length of 50 DNA bases. While many applications are not critical, special applications may require purification steps such as PAGE or HPLC after synthesis to remove truncated oligos.

04
MEASUREMENT DATA

To demonstrate the functionality of by Kilobaser synthesized TaqMan probes, they were applied in qPCRs and compared with external ordered probes by Eurofins Genomics. Thereby, two differently treated TaqMan probes by Kilobaser were applied:
1. not treated probes, which were directly applied after synthesis and
2. desalted probes, from which, among others, unbound FAM was removed prior to qPCR.

In Figure 3 representative amplification plots of qPCRs with the mentioned probes are shown. By subtracting the baseline signal from the detected fluorescence signal, as seen in Figure 3a, the PCR amplification of the target DNA is clearly visible and occurred in case of all probes similar.

This result proves that Kilobaser probes even without purification can perform sufficient for DNA detection. The effect of purification can be observed in Figure 3b, in which the original fluorescence signal is shown. The difference in total signal is significant due to unbound and thus unquenched FAM as background signal in the probe obtained directly from the synthesis device. However, a simple desalting of these probes already decreased the background signal significantly.

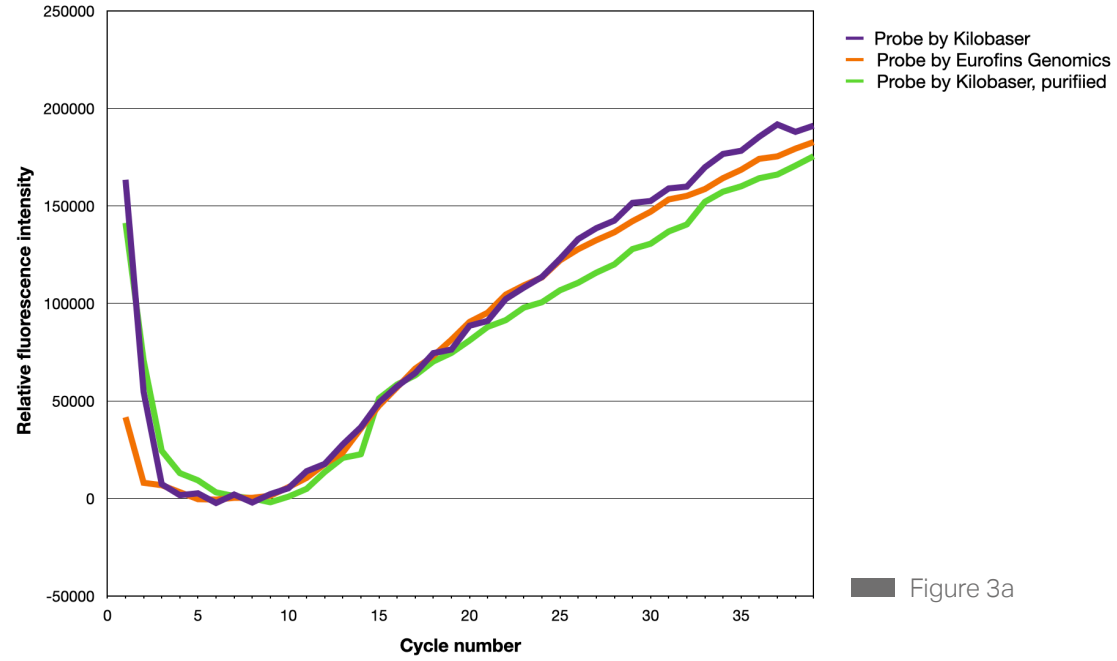


Figure 3a

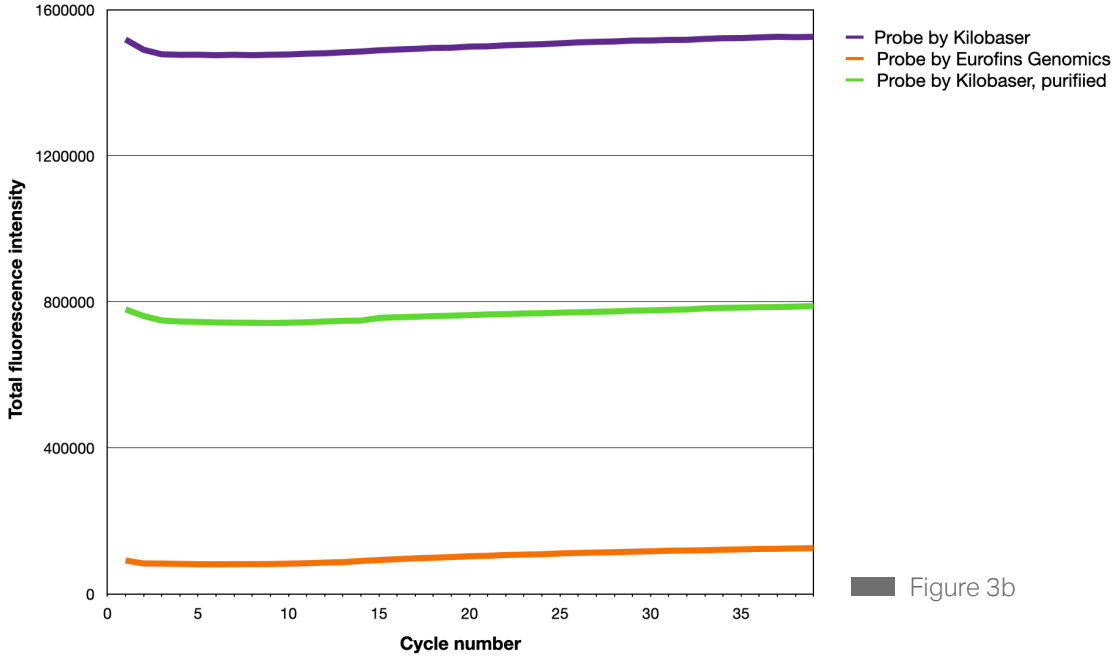


Figure 3b

Figure 3: Signal amplification of qPCR with different probes plotting (a) relative fluorescence signal without background signal and (b) total fluorescence signal vs. cycle number.

05
CONCLUSION & OUTLOOK

05.1
Conclusion

Currently DNA synthesis services are primarily outsourced because conventional DNA synthesis devices are cost and time intensive.

With low acquisition costs, simple infrastructure, a maximum of user friendliness, Kilobaser provides an innovative kind of DNA synthesis, that allows every lab to produce manifold applicable DNA probes fast and for low costs.

The first application of Kilobaser probes demonstrate that they perform similar good as other probes even without purification as the in HPLC found contaminants are not affecting the standard qPCR.

05.2
Outlook

Several other additions are currently in development for the Kilobaser Platform, including:

- BHQ-2 chips
- Various fluorophor cartridges
- Cartridges for aptamer synthesis
- RNA synthesis
- Multi column chips

(1) MarketsAndMarkets - Oligonucleotide Synthesis Market - Forceasts to 2021

(2) https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html (02.02.2021)

(3) J.M. Levsky, R.H. Singer: Fluorescence in situ hybridization: past, present and future; Journal of Cell Science 116: 2833-2838; (2003) doi: 10.1242/jcs.00633

(4) P.M. Holland et. al.: Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of Thermus aquaticus DNA polymerase; PNAS 88:7276-7280 (1991) doi: 10.1073/pnas.88.16.7276

(5) A.T. Yeung et. al.: Evaluation of dual-labeled fluorescent DNA probe purity versus performance in real-time PCR; BioTechniques 36:266-275 (2004) doi: 10.2144/04362RR01