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Development of a novel product labelling system for industrial feed additives based on DNA barcodes

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Danksagung

Nun, angekommen am Ende meines Studiums möchte ich mich bedanken…

…bei meiner ganzen Familie, die mich immer wieder ermutigt hat, weiter zu machen. Insbesondere bei meinen Eltern und Großeltern, die mir mein Studium ermöglicht haben und ganz speziell bei Mama, Omi und Jürgen die mich in guten- wie in schlechten Zeiten unterstützt haben.

…bei Babsi und Tati für die psychologische Unterstützung.

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Deutschsprachige Zusammenfassung

Ziel des Projektes war die Entwicklung eines qualitativ sowie quantitativ per real-time PCR nachweisbaren Markers, eines Barcodes auf molekularer Ebene, der in Produkte eingemischt werden soll und dadurch vor ständig steigenden Produktfälschungen schützen soll. Dieser BIOtracer besteht aus einer einzigartig designten DNA-barcode Sequenz mit einer Länge von 120 bp und soll den bisher verwendeten imitierbaren Microtracer® ersetzen.

Das Projekt gliedert sich in mehrere Phasen und startete mit der Entwicklung idealer Barcode-DNA-Sequenzen mit passenden PCR Primern und TaqMan-Sonden. Die *in silico*-Analyse sowie erste praktische real-time PCR Experimente wurden vom Projektpartner des Analytikzentrums des IFA-Tullns bewerkstelligt. Eine optimale DNA-Extraktionsmethode wurde gefunden und der qPCR-Ansatz mit Barcode-Standards zur Quantifizierung wurde etabliert. Während dieser Arbeit wurde SYBR-Green I als fluoreszierender Farbstoff in der quantitativen PCR verwendet und mit Schmelzkurvenanalysen gekoppelt um mögliche unspezifische PCR Signale zu detektieren. Positiv evaluierte Barcode-Sequenzen wurden homogen mit einem optimalen Träger (Maltodextrin G190), der hauptsächlich als Füllstoff fungiert, gemischt und mittels Gefriertrocknung (Lyophilisierung) immobilisiert. Zuvor wurde ein passendes Gefriertrocknungsgefäß von GORE™ getestet um a) vor möglichen Kreuzkontaminationen der Barcode-Sequenzen während des Trocknungsprozesses zu schützen, b) dabei optimale Trocknungsbedingungen zu gewährleisten und c) den Gefriertrockner nicht zu kontaminieren. Es wurde gezeigt, dass geringste Barcode Mengen durch die mikro-poröse Membran des GORE-Gefäßes dringen können. Mit Versuchen zur homogenen Verteilung der Barcode-Sequenzen im Tracer konnte eine geringe Wiederfindungsrate erreicht werden. Durch die multiple Standardadditionsmethode konnte gezeigt werden, dass der Rest der eingesetzten Barcode Menge nach dem Gefriertrocknungsprozess nicht mehr zur Verfügung steht, diese vermutlich im GORE-Gefäß bleibt und eine tatsächlich eingesetzte, korrigierte Wiederfindung von 99.4% erreicht werden. Einzelne Futtermittelzusätze (FMZ) wurden auf mögliche PCR Inhibitoren getestet und es stellte sich heraus, dass jeder FMZ eine bestimmte Verdünnung braucht, um messbare real-time PCR Signale zu geben und dadurch eine Barcode Quantifizierung möglich machen. Nach Einmischung des Tracers in die verschiedenen FMZ und re-Isolation der Barcode-DNA wurden die Wiederfindungsraten bestimmt. Es zeigte sich, dass die Wiederfindungsrate aus jedem Futtermittelzusatz verschieden und weitere Optimierung nötig ist. Die Stabilität des BIOtracers wurde für einen Zeitraum von sechs Monaten bestätigt.

In Zukunft werden TaqMan-Sonden zur sequenzspezifischen Detektion der Barcode-DNA verwendet die zusätzliche Schmelzkurvenanalysen überflüssig machen. Nach Abschluss der Testentwicklung im Labormaßstab wird ein Pilotmaßstab entwickelt und es folgt die Adaptierung des Nachweises für das Futter selbst (Futter gemischt mit Futtermittel-Additiven).

Abstract

Due to the raising problem of product counterfeiting on the whole market all over the world companies are forced to act. BIOMIN from the feed-industry sector reacts by developing a novel and innovative molecular-biological tool for product labeling that should displace the previously used, imitable Microtracer®. The *BIOtracer-project* aims to develop and evaluate a molecular tracer that can be qualitative as well as quantitative detected using real-time PCR technique. A unique DNA-sequence was designed by the project partner from the IFA-Tulln that acts as a barcode on molecular level. During this work SYBR-Green I was used as fluorescence dye in qPCR. Consistently Taq-Man-probes were designed and tested from the project partner for sequence specific detection in future. Maltodextrin G190 was evaluated as an optimal carrier matrix for immobilizing the barcode by lyophilisation. Special freeze-drying trays from GORE™ have been evaluated and it could be demonstrated that minimal barcode amounts can pass the tray during freeze-drying. The gained milled powder is called BIOtracer. Before mixing the tracer with several feed-additives many tests were carried out. For reliable real-time PCR results barcode-DNA-standards were optimized. Re-isolation tests of the barcode-sequence from the BIOtracer were performed leading to low recovery rates and with respect to the multiple standard addition method recovery was 99.4%. With this method it could be demonstrated that half of the initial, immobilized barcode-DNA was lost and it is expected that this happened during lyophilisation. Barcode stability within the BIOtracer (=shelf life) is checked consistently. Furthermore real-time PCR inhibitors co-extracted from the different feed-additive matrices were evaluated and by sample adequate dilutions minimized. It has to be said that all 13 tested additives behave different in qPCR inhibition. Barcode recovery rates from all different feed-additives mixed with the BIOtracer4 were analyzed and show big fluctuations depending on main composition of every additive. In consequence barcode-DNA-extraction methods must be adapted for every single feed-additive. Homogenous tracer-feed-additives mixes were produced and tested in lab scale. The IFA-Tulln consistently designs new unique barcode-DNA-sequences with corresponding primer-pairs and Taq-Man-probes.

Abbreviations

bp	base pair
cDNA	complementary DNA
c_T	threshold cycle
DEPC water	water treated with Diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ds-DNA	double-strand DNA
FRET	fluorescence resonance energy transfer
GMO	genetically modified organisms
IFA-Tulln	Interuniversitäres Forschungsinstitut für Agrarbiotechnologie in Tulln
OECD	Organisation for Economic Co-operation and Development
PCR	polymerase chain reaction
Poly(dIdC)	poly deoxyinosine deoxycytosine
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
rSD	relative standard deviation
sb	sterile and bidestilled, DEPC water
SD	standard deviation
T_a	annealing temperature
T_e	elongation temperature
T_g	glass transition temperature
T_m	melting temperature

1. **Introduction**

BIOMIN was founded in 1983 by Ernst and Margarete Erber in Austria and is now a worldwide company acting on animal nutrition. By enhancing productivity and performance potential of livestock in a natural way BIOMIN develops and produces feed additives and premixes in accordance with latest know-how and with state of the art production technology. High standards of the different products are ensured by BIOMINs experience as well as permanent quality improvements. Therefore the research and development is one of the cornerstones of BIOMIN´s successful economic development. In that account this company works in close co-operation with universities and research institutes leading to collaboration projects with more than 80 institutes worldwide over the last years. The raising demand on natural feed additives leads to an expansion and so the global BIOMIN network ranges from America to Africa, Europe, Middle East, Asia and Australia and thereby the feed-market is present in more than 100 countries. Since 1990 the head office is situated in Herzogenburg, Austria. BIOMIN earned several prizes like the Austrian export prize and the innovation prize in 2001 and 2005. The most popular product lines are found in mycotoxin inactivation and in immune activation for supporting natural resistance in cattle, pig and poultry [1]. But with the increasing demand on natural and healthy feed additives and premixes the problem of product imitation also rose. Especially in the Asian market product counterfeiting is a serious problem that constrains BIOMIN to act and gives the reason for starting the BIOtracer project for protecting BIOMIN´s products against imitation.

1.1 *Product Authenticity and Traceability*

Today, at a time when protecting the integrity of brands plays such an important role, supply chains and products are high on the international agenda. The opportunity for fraud is increasing and the need for producers to demonstrate the authenticity and safety of their products is absolutely essential. The Organisation for Economic Co-operation and Development (OECD) has pointed out in their recent report from November 2009 that counterfeit and pirated goods in world trade is estimated to have increased from 1.85% in 2000 to 1.95% in 2007 and this represents an increase up to US$250 billion worldwide (OECD-report 2009)[2]. The range of imitated goods touches almost all industries. The wide ranging problem not only includes counterfeiting, adulteration and dilution but also mislabelling or passing off inferior versions. This raises the spectre of contamination, spoilage and out-of-date products. All these facts can either have a negative effect on consumer health and severely impact producer reputation and liability. There is the possibility to use sensory and digital technologies like holograms and barcodes but these technologies alone are not enough to enhance the level of security. Therefore multi-layered devices such as hidden or covered devices that can only be identified with a sophisticated detection tool kit should be used (NGF, 2010)[3].Molecular biological tools such as DNA detection techniques especially in food and feed industry are not new. Mainly in the field of genetically modified organism (GMO) detection [4] but also in product quality control of meat [5] or wine [6] the identification of taxonomy [7] is a common tool. During this study a tracer consisting of a unique DNA-sequence has been developed, evaluated and adapted to BIOMIN´s premium feed-additives. This innovative labelling system will in future be used to trace the high quality feed-products for guaranteeing product authenticity. This new generation tracer should displace the metal particle labelling system that currently is added to BIOMIN products and this straight forward step is the company´s answer to product counterfeiting in the feed industry.

1.2 *Metal particles for product labelling*

Microtracers are small metal particles, in food industry normally iron particles of uniform size that are covered with approved food dyes. To distinguish different microtracer particles they are available in a wide range of individually selected colours. This tracer is not harmful and has a precisely defined number of particles per unit of weight. Depending on the product there are between 25 000 and 1 000 000 particles in one gram microtracer [8].

To detect these microtracers two different methods (qualitative and quantitative) can be used. For qualitative detection, also referred to as Mason Jar technique, the test-mixture has to be given into a jar where a special magnetic lid covers the top. For sample collection a filter is added on the magnetic lid (1). After shaking heavily (2) the iron particles stick to the filter and is incubated with a special reagent dyeing the tracer (4). For quantitative analyses a rotation detector is applied. Therefore it is necessary to know the quantity of tracer in the mixture. With this knowledge a defined amount of sample is taken and put into the rotation detector (Figure 1(3)). The particles are subsequently separated by a rotation magnet and sticks to a filter. Then the filter is developed by spraying a special reagent onto the filter, the coloured dots can be counted and compared to the expected value (4). To visualize this explanation please take a look onto figure 1.1.

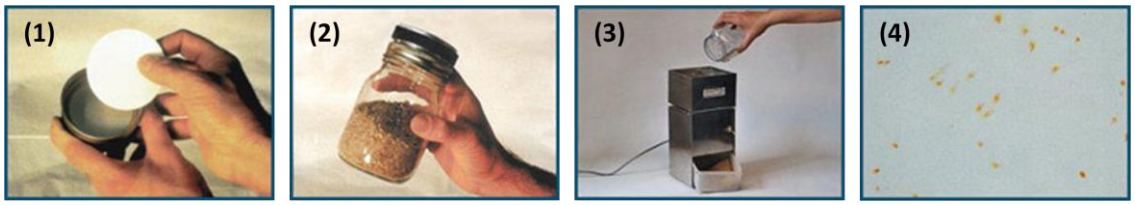

Figure 1.1: visualized flow chart of the microtracer particles for a qualitative (1, 2, 4) and quantitative (3, 4) detection. (modified from [8])

1.3 *DNA-barcoding – the BIOtracer*

To give the DNA labelling character that is required in this BIOtracer project some important facts about DNA and the application for this work have to be pointed out.

- ✓ DNA contains information
- ✓ DNA is a stable molecule
- ✓ DNA can be synthesised as designed in silico
- ✓ DNA can be immobilized – BIOtracer

Generally a DNA molecule is built up of a chain of four different nucleobases (A, T, C and G) [9]. The different arrangement of DNA-bases offers a kind of code that contains information and can be read with the polymerase chain reaction (PCR) when the individual sequence is known [9, 10]. Several scientists proved that DNA is very stable over long time periods by isolating DNA from 100 000 year old mammoth bones [11], from an about 80 000 year old Neanderthal-specimen [12] and from sediments from the Holocene and Pleistocene [13]. These detections confirm the stability of DNA. Technical acquirements in molecular biology give the option to synthesise DNA molecules of individual length and sequence leading to nearly endless possibilities of different molecules. Such stable, flexible and unique DNA sequences of about 100 to 120bp in length (1) can be immobilized

with a carrier (2) by lyophilisation (3) resulting in a so called BIOtracer (4). The principle of the BIOtracer production is illustrated in figure 1.2. The lyophilisation technique is described in chapter 1.4 and in chapter 2.4 the components of the tracer are described in a detailed manner.

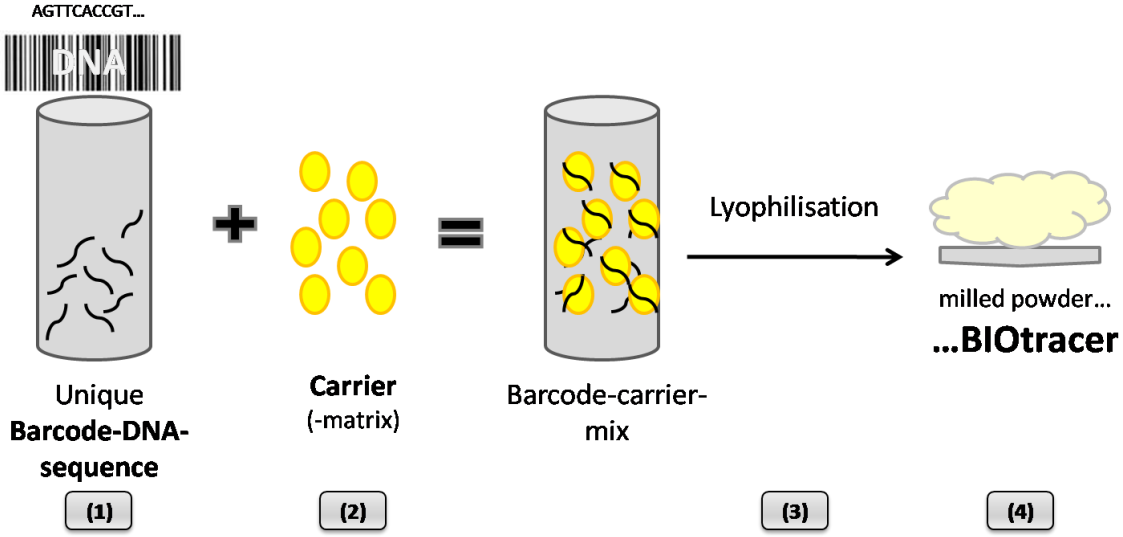

Figure 1.2: Illustration of the BIOtracer production. A unique DNA-sequence acts as a barcode on molecular level and results with a carrier after lyophilisation in the BIOtracer.

The BIOtracer will function as barcode on molecular level and by mixing it in several BIOMIN products it has a labelling character. Using a molecular biological standard technique this DNA sequence can be qualitative and quantitative detected. This technique is called real-time or quantitative PCR. It is easy to perform, provides the required accuracy and produces reliable as well as rapid quantification results [14]. The real-time PCR method will be described in detail in chapter 1.5.

Due to its unrivalled DNA-sequence more BIOtracers, each with a certain barcode-sequence will be developed and therefore many different products can be labelled by simply mixing a BIOtracer in feed or feed-additives.

1.4 *Lyophilisation*

Freeze drying or lyophilisation is a process that is based on the principle of dehydration by sublimation. It works by freezing (1) a solution or suspension, reducing the surrounding pressure (2) and heating up (3) to a certain temperature where the frozen water in the material can sublime directly from the solid phase to the gas phase. The triple point has to be passed-by and the process results in a dried, porous product a so called *lyo-cake*. A schematic drawing with points (1) to (3) is given in figure 1.3 below [15, 16, 17].

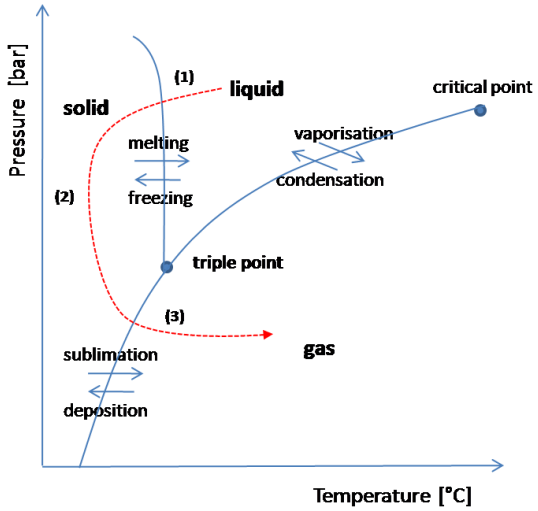

Figure 1.3: schematic drawing of the water phase diagram where the red line indicates lyophilisation process with the 3 steps (1) freezing, (2) reducing pressure and temperature increase (3).

This drying process starts with freezing. All components are immobilized and an optimal ice-network should be built up. It has to be considered that slow freezing cause big ice crystal formation which then leads to a fast drying process because the sublimated water vapour can leave the product much easier than in the case of very small ice-crystals. In a primary drying step (sublimation) ice sublimes in vacuum after a slight increase of temperature. The last step is secondary drying also called desorption to reduce the residual water content (~20%) to a minimum (~1%) to avoid chemical reactions in the final formulation and to inhibit microorganisms and enzymes that would normally degrade the product [17].

Freeze drying of liquid or frozen products is one of the most sensitive and elegant methods to dry products and ensure stability. Therefore this process is not only used for pharmaceutical products such as enzymes, hormones, antibodies, vaccines but also for food (instant coffee, spices, astronaut food, microorganisms), taxidermy or for drying documents after water damage [17]. Nucleic acids are commonly shipped lyophilized [18].

Due to the fact that solid matter is more resistant to any form of degradation than dissolved products lyophilisation has the aim to extend stability by water dehydration. By adding the same solvent to the dried product this process is reversible [17].

1.5 *Quantitative real-time PCR*

Real-time PCR is the most sensitive technique for DNA detection and quantification currently available [19, 20, 21]. In fact, this technique is sensitive enough to enable quantification of DNA from very small amounts (10 molecules) to high concentrations of 10^{10} molecules [22] and this makes it the method of choice for detecting and quantifying the BIOtracer within the products of Biomin.

1.5.1. The polymerase chain reaction

The polymerase chain reaction, shortly named PCR, is a Nobel-Prize-winning technique developed 1984 by Kary Mullis and is used in medical and biological research labs for a variety of applications. [23, 24]

By PCR a short, target fragment of DNA is amplified exponentially and used for further analysis. This amplification process needs several components and consists of three main steps that are repeated a certain number of cycles. A basic PCR set up requires the DNA template that contains the DNA region to be amplified, two primers that are complementary to the three prime ends of each of the sense and anti-sense strand of the DNA target and the Taq-polymerase which is a thermostable DNA polymerase that is obtained from the thermophilic bacterium *Thermus aquaticus*. [23] Furthermore, Deoxynucleotide triphosphates (dNTPs) as monomers for a new DNA strand, an appropriate buffer that provides a suitable chemical environment for optimum activity and stability and co-factors such as $MgCl_2$ are also required for this reaction. The PCR is commonly carried out in small reaction tubes in a reaction volume of 10-200µl in a thermal cycler that heats and cools the reaction tubes. A schematic draw of the PCR-process is shown in Figure 1.4.

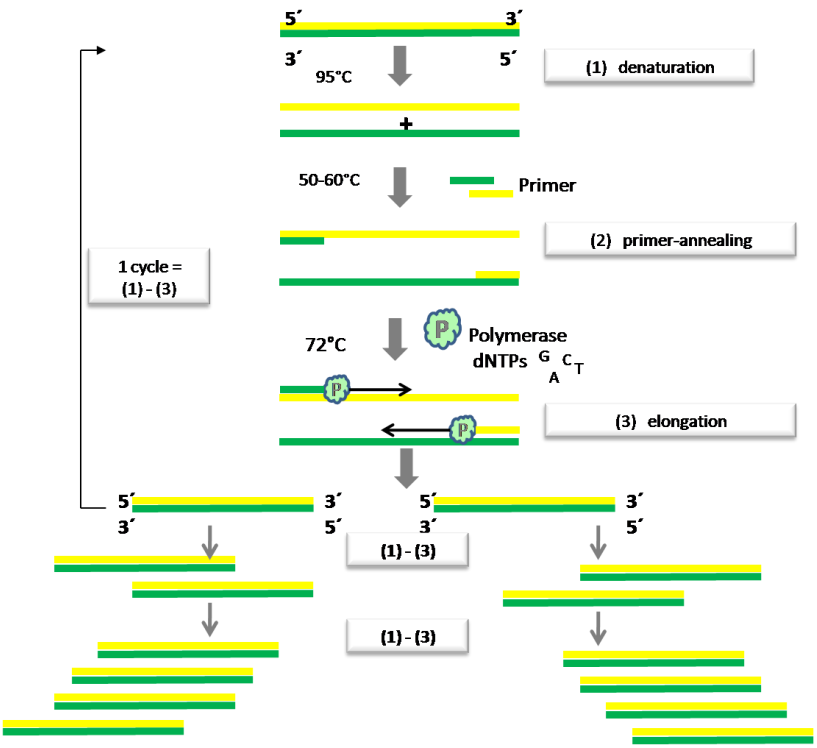

Figure 1.4: Schematic drawing oft he polymerase chain reaction. (1) Denaturation at 95°C. (2) Primer annealing at 50-65°C. (3) Elongation at 72°C, by adding dNTPs with Polymerase-enzyme. After the first cycle is completed the two resulting DNA strands serve as template for the next cycle. Thus, under optimal conditions, the amount of DNA target sequence is duplicated in each new cycle and leads to an exponential growth of this short product.

A PCR normally starts with the Initialization step at a temperature of 95°C for a few minutes. This ensures a complete denaturation of the double stranded template DNA sequence and may be required for activation of DNA polymerases [25, 26].

(1) Denaturation step: the first cycling step heats the reaction to 95°C for 20-30 seconds. It causes separation of the template DNA by denaturation of the hydrogen bonds between complementary bases. Now single stranded DNA is available for the next step.

(2) Annealing step: by decreasing temperature to 50-65°C for 20-40 seconds the annealing of the primers to the single stranded DNA templates occurs. Typically the annealing temperature is about 3-5°C below the melting temperature (T_m) of the two primers that are referred to as forward or sense primer for one strand and antisense or reverse primer for the complementary strand. Stable DNA-Primer hybrids are obtained when the primer sequence closely matches the template sequence and then the polymerase binds to the hybrid and starts DNA synthesis.

(3) Elongation step: The extension or elongation of the annealed primers is achieved by raising the temperature to the range of optimal DNA polymerase activity, usually 72°C for the Taq polymerase [27]. By adding the corresponding dNTPs to the template in 5´ to 3´ direction this step yields in a new DNA strand complementary to the DNA template strand. Under optimum conditions, if there are no limiting substrates or reagents, at each elongation step the amount of DNA target is doubled and thereby DNA is amplified exponentially.

1.5.2. Quantitative real-time PCR

Quantitative real-time PCR is a further development based on the original PCR technique and is one of the most accurate methods for detecting very low amounts of nucleic acids in biological samples [19, 20]. Nowadays this innovative DNA quantification tool is a routine technique in molecular biology [28] and molecular diagnostics as for example in the field of gene therapy, gene expression, clinical microbiology or food microbiology [29, 30, 31, 32, 33]. In contrast to conventional PCR post-reaction processing like gel electrophoresis for product detection is not necessary. The real-time PCR combines the concept of the polymerase chain reaction and fluorescence techniques for detecting product formation [19]. Fluorescence signals are proportional to the PCR-product formed and are generated by fluorescent dyes. To determine the relative concentration of DNA present in a sample during the exponential phase of the reaction the fluorescence is plotted against cycle number on a logarithmic scale. A threshold line indicates the level of fluorescence significantly above background noise. The **C_T-value**, also named as cycle threshold, is the cycle at which the fluorescence from a sample crosses the threshold [34]. The real-time PCR analysis results in an amplification curve with four different phases (Figure 1.5). In the linear ground phase the fluorescence intensity is below the lower limit of detection of the fluorescence detector. Then in the phase of growth the early exponential phase can be seen where the detected fluorescence raises significant above noise and crosses the threshold line (C_T). In the log-linear phase the product generation slows down until the plateau phase is reached where reagent limitations and loss of enzymatic activity of the Taq-polymerase leads to reaction exhaustion [35, 36].

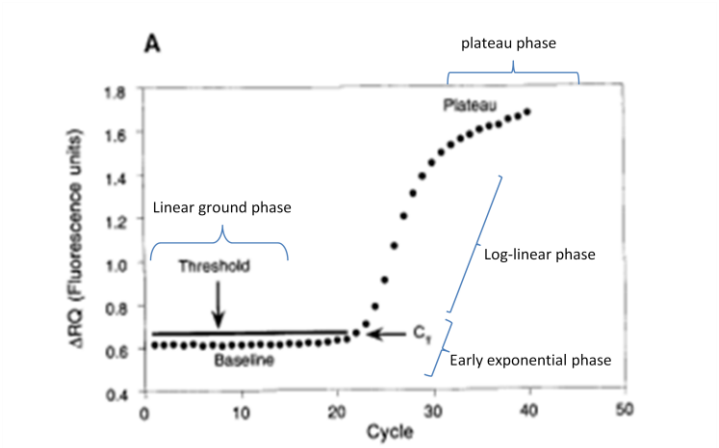

Figure 1.5: PCR product detection in real time. The fluorescence is plotted against cycle number. The C_T value is calculated by determining the point at which the fluorescence exceeds a threshold limit. Normally this is 10 times the standard deviation of the base line and around 15 cycles are needed for this determination. Exponential growth phase is then followed by a log-linear phase and a plateau phase where the reagent limitations and/or loss of Taq-Polymerase activity lead to an end of the reaction **[modified from 35 and 36].**

1.5.3. Various detection methods

Fluorescence signals can be detected either by fluorescent dyes or by sequence-specific fluorescent oligonucleotide probes. [37]

(a) **Fluorescent dyes** intercalate non-specificly in double stranded DNA and upon excitation it emits light. Thus, as a PCR product accumulates, fluorescence increases. SYBR Green is the most prominent fluorescent dye. It is inexpensive, sensitive and easy to use. However, it has to be considered that intercalation dyes bind to any double stranded DNA in the reaction, which includes primer-dimers and other non-specific reaction products, that can result in an overestimation of the target concentration. Therefore an extensive optimization and follow up assays are needed to validate results [20].

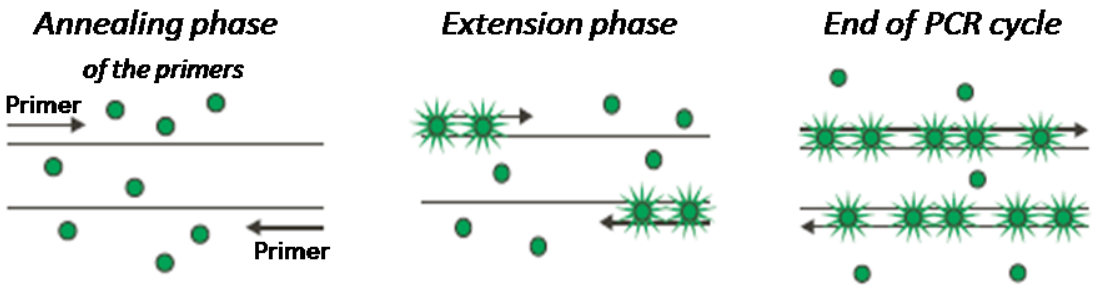

Figure1.6: principle of SYBR Green I technique in real-time PCR. Green dots indicate the fluorescent dye that binds to double-stranded DNA during extension phase. More and more SYBR Green I will bind to the PCR product resulting in an increased fluorescence and during each PCR cycle more fluorescence signal could be detected. (modified from [34])

(b) **Dual labeled oligonucleotide probes** are product specific and are based on the principle of fluorescence resonance energy transfer (FRET). FRET is a distance-dependent interaction between two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon [38]. The most popular sequence specific probes are TaqMan® probes which are also used in this work. An illustration can be seen in figure 4. These oligonucleotide-probes are 5´-terminally labeled with a reporter fluorophor and 3´-terminally labeled with a quencher [39]. The probe binds to one template strand between the two primers. During the elongation step the probe is hydrolysed by the Taq-DNA polymerase exonuclease activity and thereby fluorescence increases due to the separation of quencher and reporter [35]. The increase of fluorescence is proportional to the amount of the synthesized PCR product [40].

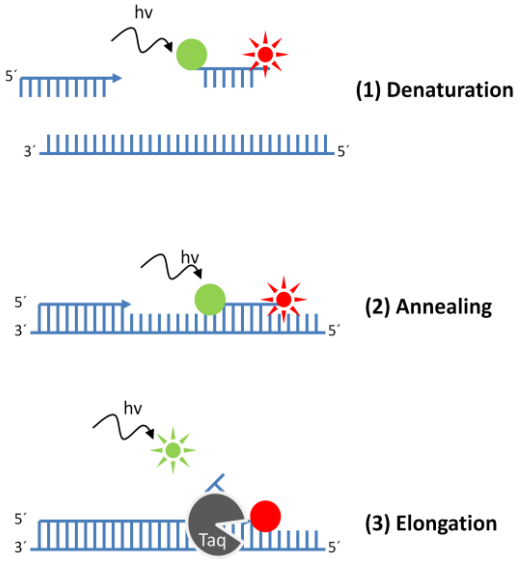

Figure 1.7: Schematic drawing of real-time PCR with TaqMan primers. (1)The green dot represents the short-wavelength fluorephore (reporter) and the red star stands for the long-wavelength fluorophor (quencher). (2)Primer and probe annealing; in the intact TaqMan probe, energy is transferred via FRET from the reporter to the quencher, quenching the short-wavelength fluorescence. (3) When the probe gets degraded by endonuclease activity of the Taq-polymerase reporter and quencher get separated, FRET is interrupted and the fluorescence from the reporter could be detected. Fluorescence from quencher decreases. [Modified from 38]

Other detection strategies for real-time PCR include hybridization probes, sunrise primers, molecular beacons and scorpion primers [37]. Theses methods are only shortly described as they are not used during this work.

Hybridization probes: Two different oligonucleotides are used. A donor with reporter fluorophore on 3´-end and the acceptor probes are 5´-terminally labeled with an acceptor fluorophore. During the annealing step these probes bind in close proximity to single stranded DNA and the excitation energy is transferred from donor to acceptor. A schematically drawing is shown in figure 1.8a.

Sunrise primers (figure 1.8b) have on their 5´-terminal end a hairpin structure that is labeled with a reporter fluorophore and a quencher. The hairpin ensures that the reporter is nearby the quencher and in the first step the sunrise primer (forward primer) gets extended. This extended product is the template for the reverse primer and in the end the polymerase opens the hairpin structure and the double-stranded PCR product is formed. Now quencher and reporter are separated and fluorescence could be detected.

Molecular beacons (figure 1.8c) are labeled with a reporter fluorophore on one end and a quencher on the other end. The middle part of the probe is complementary to the amplicon sequence whereas the terminal nucleotides (~15nts) are self-complementary. Therefore free probes form stem-loop structures where reporter and quencher are kept nearby. During annealing step the loop binds to the PCR product and is therefore opened resulting in no quenching function and the emitted fluorescence is proportional to the amount of PCR product.

Scorpion primers (figure 1.8d) are functionally and structurally related to molecular beacons but the difference is that they also serve as primers in the PCR reaction. Self-complementary sequences form a 5´-terminal stem-loop structure where the loop is complementary to the amplicon and the 3´-end serves as the primer. Furthermore the loop region is labeled with a reporter and a quencher fluorophore. First the primer is extended leading to a single-stranded template for the reverse primer in the second step. The loop opens and binds to the product, separating reporter and quencher and a fluorescence signal could be detected. In contrast to the sunrise primers, the reverse extension is blocked by a hexethylene glycol group and this ensures that the reporter of the scorpion primer is quenched in unspecific products like primer dimers.

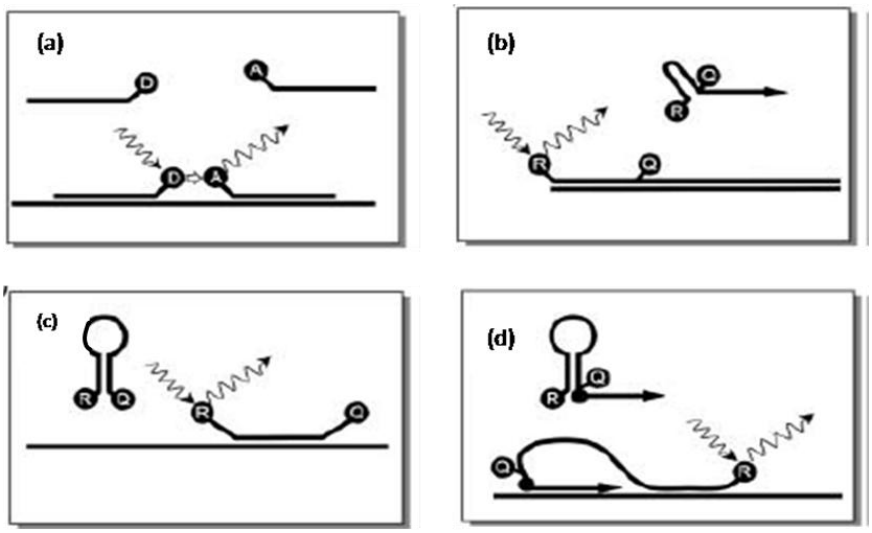

Figure 1.8: schematically drawing of (a) hybridisatoin probes, (b) sunrise primers, (c) molecular beacons and (d) scorpion primers (modified from [33])

1.5.4. Determination of real-time PCR efficiency and quantification of target DNA

The c_T -value is proportional to the initial DNA content in a sample and determination of amplification efficiency critically confirms exact and precise real-time PCR [41]. The original method for quantification is to calculate with an optimal amplification efficiency of 100% for all samples what means that after every cycle the number of templates doubles and the then exponential curves can be compared directly [42] The method was subsequently modified leading to the efficiency calculation from standard curves where many algorithms have been developed to calculate the true PCR efficiency [28, 36, 43, 44]. To estimate the efficiency objectively statistical techniques are used as the four parameter logistic model fitting algorithm (FPLM) described by Tichpad *et al.* (2003), the kinetic outlier detection (KOD) method from Bar *et al.* (2003) or the noise-resistant Real-time PCR Miner program developed by Zhao and Fernald (2005). The observed efficiency has to be determined for each new quantitative PCR assay. Dilution series of sample DNA are prepared and assays using constant volumes of template are performed [45]. A high efficiency of a PCR reaction is a crucial chain as this quarantees a low limit of detection.

Currently there are two major approaches of real-time PCR quantification in use: the absolute and the relative quantitative method [14, 28, 39, 42].

a) Absolute Quantification

The absolute quantification determines the initial copy number by relating the PCR signal to a standard curve. This standard or calibration curve is prepared of serially diluted standards of known concentrations. The linear relationship between c_T and initial DNA-amounts allows the determination of unknown samples based on their c_T values [28]. During absolute quantification the PCR efficiency is determined in each run using the calibration curve. The methodology has to be carefully validated to quarantee that the amplification efficiencies for standard and target DNA are identical [14, 25]. This method is used to determine organism DNA in samples such as GMOs or allergens [42] and in the case of this diploma thesis to quantify the absolute barcode-amount in different samples.

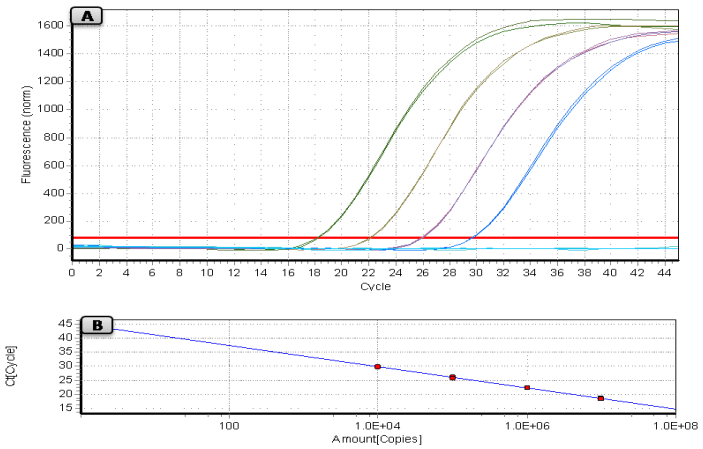

Figure 1.9: real-time PCR amplification curve (A) and corresponding regression line or calibration curve (B). The diagram in (A) shows duplicates of a 10-fold diluted barcode 4-31 DNA-standard ranging from 10^7 to 10^4 molecules per µl. The red threshold line provides the C_T values for each dilution. The regression line (B) for the determination of sample DNA is performed by plotting the copy number of each standard against the corresponding C_T. In the case of 100% PCR efficiency, the ΔC_T between two consecutive dilutions is 3.2.

The resulting c_T values are plotted against the logarithm of the template concentration and the amplification efficiency is calculated using the regression curve obtained. The dilution of one log order of magnitude leads to an optimum ΔC_T of 3.2. (Figure 1.9) For efficiency calculation equation (1) can be used.

$$Efficiency = 10^{-\frac{1}{slope}} - 1 \quad (1)$$

b) Relative Quantification

Sometimes the absolute copy number is irrelevant and a relative quantification method is therefore applied. This method is mainly used in gene expression studies. The relative quantification method relates the PCR signal of the template in a treatment group to that of an external standard or a reference sample, also known as a calibrator [28]. The calibrators are housekeeping genes and are used to normalize the data. The most common of these calibrators are ribosomal RNAs, GAPDH, cyclophilin, tubulins or albumin or other poorly regulated genes [46]. Due to the fact that efficiency is extremely important to compare the target gene with a reference gene numerous mathematical models have been developed. Their most important distinction is whether amplification efficiencies of target and reference gene are considered or not and in which way [28].

2. Aim of this work

Due to the raising problem of product counterfeiting BIOMIN has to develop a molecular-biological tool that is resistant to fraud, could tag premium feed-additives and/or high quality feed and displace the imitable metal particle system for product labeling that is now in use. This tool should act as a barcode on molecular level. The barcode should be a DNA-code of unique sequence. This sequence will only be known by BIOMIN and therefore the company has the ability to test their products and prove whether they are their own or imitations. To scan products for present barcodes real-time PCR will be used.

The barcode is a short and unique DNA sequence of about 100 to 120 bp in length that is designed, ordered and fist tested by the project partner from the IFA-Tulln. Normally such a synthetic DNA sequence is a high concentrated jelly substance. To mix the DNA-barcode homogenous into feed additives it has to be immobilized with a carrier matrix. The carrier has to be chosen very well because it must fulfill several requirements that are listed and described in detail in chapter 3.3.1. Barcode and carrier are mixed in a liquid batch and after freeze drying for immobilization a powdered, homogenous DNA-tracer or now called BIOtracer is received. Lyophilisation or freeze drying is a sensitive drying process that ensures product stability. The barcode-DNA re-isolation from the BIOtracer must be evaluated by a DNA extraction step and subsequent quantitative-PCR analysis. For absolute quantification with real-time PCR reliable barcode-standards are required and have therefore be evaluated and tested. To successfully label BIOMIN-feed and/or feed-additives with the BIOtracer first mixing and recovery tests have to be done in a lab-scale. Feed-additives must be evaluated to get an idea of possible real-time PCR inhibitors within their different matrices. The absolute detected barcode amount from the BIOtracer-feed-additive intermixes must be known. Stability tests of the BIOtracer must be performed continously.

For the future the barcode-recovery rates should be as high as possible and required extraction methods must be adapted to every different feed-additive. Reproducibility must be tested before the BIOtracer can be evaluated in production-scale. When this process works well for labeled feed additivefeed-additive augmented feed will be tested. As an aim for the whole project a lot of different barcodes and therefore many unique BIOtracers should be developed to trace any BIOMIN feed-additive and/or feed that needs product authenticity. The BIOtracer should therefore give BIOMIN the potential to prove and test whether it is their own product (qualitative detection) or not and furthermore if the product is altered or diluted (quantitative detection) or not.

3. Materials and Methods

3.1 Feed-additives

3.1.1. Feed-additives and their composition

A large variety of product lines have been developed with the aim to protect animal health. These products act in mycotoxin-deactivation (**Mycofix®** product line), increase feed palatability and improve digestion (**Biomin® P.E.P**.), preserving animal feed (**Biotronic®**), improve gut health or make chicks more resistant for pathogenic infections (**PoultryStar®**). Some product lines combine the beneficial effects of prebiotics and probiotics, have health stimulating effects and therefore they have positive influence on the gut microflora (**Biomin® C-EX, Biomin® IMBO**) [1].

Due to the confidentiality of these products no further ingredients, names or facts could be cited in this work. Therefore all analyzed feed-additives are referred to as FMZ plus number (1-13) and their different main components are listed in table 3.1.1.

Table 3.1.1: all analyzed Biomin-feed additives (FMZ) with general composition

FMZ	Composition	FMZ	composition
1	bentonite, yeast-mix	**8**	yeast
2	bentonite, yeast-mix	**9**	yeast plus carrier
3	mineral compound mixture	**10**	fish meal powder
4	probiotics	**11**	dried blood
5	mineral compound mixture	**12**	bentonite
6	mineral compound mixture	**13**	essential oils plus carrier
7	sal assortment		

Because of their first class quality these premium products need authenticity and the different and special matrices should therefore be labelled everyone with an own unique BIOtracer that protects from imitation.

3.1.2. Feed-additives and their water activity

The a_w-value is defined as the vapour pressure of water divided by that of pure water at the same temperature. This evaluation is very important in food industry because higher a_w values tend to support microorganisms growth and influences not only stability and shelf life but also taste, solubility and vitamin and protein content. Most bacteria require an aw-value of at least 0.91 and most fungi at least 0.7 and below 0.6 there is almost no microbiological growth. Enzymatic activity depends on water activity (a_w) in every sample and it is known that an a_w below 0.8 shows no or retarded enzyme activity [47, 48].

To guarantee highest stability of the BIOtracer mixed in several feed-additives (FMZ) possible DNase activity from the matrix could digest barcode DNA. Therefore it is necessary to get an idea of the water activity within the additives and within the tracer to exclude degradation due to humid environment.

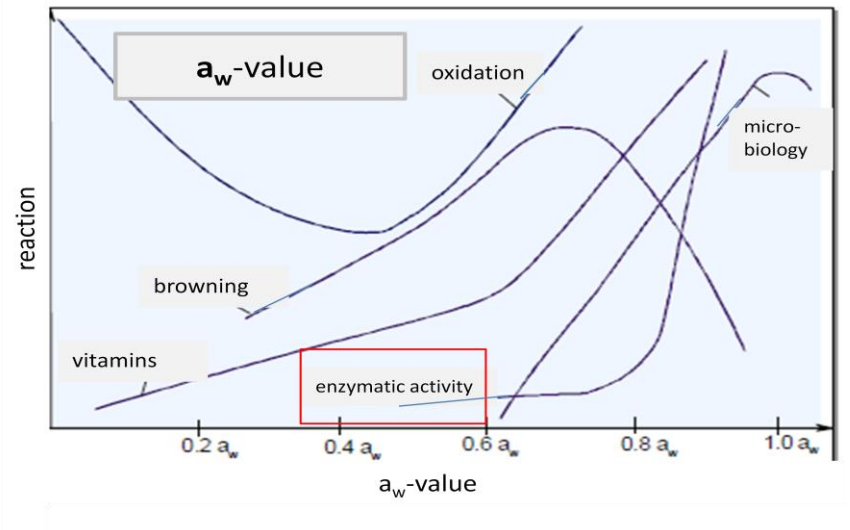

Figure 3.1.2.: The diagram shows a reaction like enzymatic activity or microbiological growth **with its corresponding a_w-value**. A a_w <0.8 shows retarded enzyme activity. [modified from 47]

For a_w measurement small disposable plastic containers were used. After a calibration step with a defined a_w-standard (0.250 +/- 0.003) the containers were bottom-filled with a FMZ or the BIOtracer itself. Samples were analyzed with the aqua lab detector (*Decagon Device Ing.*, USA).

3.2 Molecular biological standard techniques

Due to the fact that successful quantification also depends on the quality of the DNA-sample it is absolutely necessary to find the optimal extraction method to ensure high yield and quality of the DNA obtained [49].

3.2.1 DNA extraction methods

a) Barcode DNA isolation from feed-additive samples:

For barcode-DNA extraction from the different feed-additives a fast and simple extraction method is used. Therefore FMZ-samples were dissolved in a 10mM Tris buffer (pH 7.5).

1M Tris buffer pH 7.5 [100ml]**:**
12.11g Tris (MW=121.14g/mol)
250ml shott-flask
50ml falcon tube (SPL, Austria)
sterile-bidestilled water (sb H_2O)

1M Tris buffer with a pH of 7.5 was prepared. Therefore 12.11g Tris base were dissolved in 60ml dH_2O and the pH was adjusted with HCl. After the volume was filled up to 100ml with dH_2O the solution was autoclaved for 20min, at 121°C and 1 bar (Certoclav Sterilizer GmbH, Traun/Austria). A 10mM Tris-buffer was prepared with dH_2O and was again sterilized by an autoclaving process.
For extraction the required 10mM Tris-buffer amount was heated up to a temperature of 70°C within a water bath (GFL, Germany). Samples were dissolved in this pre-warmed buffer by mixing with an IKA ® VORTEX-Genius 3 mixer (Germany). To inactivate DNases the dissolved samples were incubated at 70°C for 10 minutes either within water bath or a thermo-block (Eppendorf, Germany) depending on sample size. This followed a centrifugation step at 13.000rpm in the case of 1.5ml tubes (Eppendorf, Germany) or at 3000rpm with 15ml falcon tubes and 4000rpm when 50ml falcon tubes were used as extraction flasks. 1ml of the clear supernatant was transferred into a 1.5ml tube and this was incubated at 95°C on a thermo-block for another 10 minutes to inactivate remaining DNA-degrading enzymes. Poly-dIdC was added to a final concentration of 10µg/ml to the samples and they are stored on ice till they are used for real-time PCR analysis.

b) Barcode DNA extraction from a cellulose filter

For the barcode DNA isolation from a cellulose filter a high salt solution was used.

High salt solution [50ml]**:**
1.2M NaCl
10mM Tris (MW=121.14g/mol)
100ml shott-flask
dH_2O

60.57mg Tris base were added to 70.12g NaCl in a 100ml shoff-flask. After the volume was filled up to 50ml with dH_2O the solution was autoclaved for 20min, at 121°C and 1 bar (Certoclav Sterilizer GmbH, Traun/Austria).

For the DNA extraction the cellulose filter was cut into small pieces and put into a 50ml falcon tube. 10ml of high salt solution were added and heated for 15 minutes using a 65°C water bath. After

every 5 minutes the high-salt solution-filter mix was gently mixed by hand. 1ml fractions of the clear supernatant were then transferred into six 2ml tubes. 1ml isopropanol was added to every fraction and they were incubated at -20°C over night to precipitate the DNA. After a centrifugation step of 20 minutes at 13000rpm the isopropanol was evaporated using a vacuum pump. The transparent pellet was dried at room temperature over night. Each pellet was dissolved in 60µl 10mM Tris-buffer and stored on ice till they were used for real-time PCR analysis.

3.2.2 Quantitative PCR assay

3.2.2.1. *qPCR chemistries and equipment:*

In order to avoid contaminations filter tips (starlab, Germany) and DEPC-treated DNAse free water were used.

a) DEPC (Diethylpyrocarbonate) inactivates organic substances is therefore free of DNAses and can be used for qPCR.

0.2% - DEPC-H_2O [100ml]**:**
97% DEPC, *Roth*
100ml dH_2O
200ml shoff-flask

0.2ml DEPC were added to 100ml H_2O in a 200ml shott-flask. The closed flask was slowly turned around that all inner surfaces (also the lid) were coated with the solution. After incubation over night at room temperature the DEPC-H_2O was autoclaved at 121°C, 1 bar for 20 minutes to inactivate DEPC. 1ml aliquots were stored in 1.5ml tubes at -20°C.

b) Poly-dIdC (Poly-deoxyInosinic-deoxyCytidylic) saturates the walls of the tube and therefore the template-DNA is in solution. Barcode-DNA is used in very low concentrations and poly-dIdC guarantees a homogenous distribution of DNA in solution. Every dilution for real-time PCR analysis was performed with 10µg/ml poly-dIdC. 200µl of a concentrated 50µg/ml poly-dIdC was added to 800µl DEPC-H_2O in 1.5ml tubes. The resulting 10µg/ml concentration was stored at -20°C in 1ml aliquots.

c) Primer pairs: The forward and reverse primer and barcode sequences have been previously designed by the project partner from IFA-Tulln, center of Analytical Chemistry, and were purchased from SIGMA-ALDRICH (Germany). Primers and barcode-DNA sequences were dissolved in 10mM Tris-buffer pH 7.5 by shaking on a rotary-shaker for 10 to 15 minutes at 65°C. Primers were dissolved at a final concentration of 100µM. 60µl of each primer were aliquoted in 1.5ml tubes resulting in 5 stock-solutions with 100µM primer concentration and were then stored at -20°C. Before primers could be used for real-time PCR they have to be diluted 1:16 to get a concentration of 6.25pmol/µl. 0.24µl of the 1:16 dilution were then used for the real-time PCR-mix resulting in a final primer concentration of 0.1pmol/µl.

The total PCR-reaction volume was 15µl with 13.00µl master mix and 2µl template DNA. The master mix includes the iQ™ Supermix (BioRad, CA) or KAPA-SYBR® fast (KAPABIOSYSTEMS, Republic of south Africa), $MgCl_2$, sb-water and the primers. A final $MgCl_2$ concentration of 5mM was optimized for an effective real-time PCR analysis for barcode 4-31 and this was obtained by adding an additional $MgCl_2$ stock solution of 25mM (*Promega, WI/USA*). In the case of the KAPA-Supermix no additional $MgCl_2$ was required as this Kit is already adjusted to a final concentration of 5mM. The standard pipetting scheme can be seen in table 3.2.1.

Table 3.2.1: standard pipetting scheme for a real-time PCR analysis with SYBR-Green using iQ™ Supermix or KAPA SYBR Fast**.**

reagent	volumen per reaction [15µl]	concentration in reaction [15µl]	stock concentration	
iQ™ Supermix / KAPA	7.50µl			Master mix
DEPC-H_2O	*3.82µl /5.02µl**			
$MgCl_2$	1.20µl / none*	5mM	25mM	
forward primer	0.24µl	0.1pmol/µl	6.25pmol/µl	
reverse primer	0.24µl	0.1pmol/µl	6.25pmol/µl	
template	*2µl*			
total reaction	15.00µl			

*qPCR reaction volume when KAPA-supermix is used

All reagents for the master mix are calculated with a 15% excess and this batch was prepared in the laminar-air flow or the PCR-hood to avoid contamination with DNA. The master mix was then divided into sub-mixes where the template was added and both were calculated with a 10% excess. This was done to compensate pipetting-losses. Every sample as well as a no-template control (NTC) were analysed in triplicates. The NTC was prepared with the same amount of DEPC-treated-water instead of template DNA.

The total reaction volume of 15µl was pipetted into the vials of a 96-well plate (*Eppendorf, Germany*) and was heat sealed with a sealing film. To avoid bubbles the plate was mixed for 30 seconds and centrifugated for 2 minutes at 2000rpm. The real-time PCR analysis was performed with a realplex^2 S cycler (*Eppendorf, Germany)*.

3.2.2.2. *PCR program*

The used real-time PCR program depends on the barcode-sequence of the analysed samples. During this diploma thesis two barcode-sequences were used and the corresponding real-time qPCR program is listed in table 3.2.2.

Table 3.2.2: real-time qPCR program for barcode 2-20 and 4-31.

steps	Barcode 2-20		Barcode 4-31		
	temperature	**time**	**temperature**	**time**	**cycles**
initial denaturation	95°C	3min	95°C	3min	
denaturation	95°C	10sec	95°C	10sec	
annealing	55.7°C	20sec	52°C	20sec	45
elongation	57.5°C	20sec	58°C	20sec	

When no other program is described the real-time PCR program for barcode 4-31 was used. The first strand-melting step was performed at 95°C for 3 minutes and was followed by 45 cycles with 10 seconds at 95°C denaturation time, 20 seconds at 52°C for annealing and 20 seconds at 58°C as elongation time and temperature. Both barcode sequences were analysed using 45 cycles and required a total $MgCl_2$ concentration of 5mM for optimal PCR efficiency.

3.2.2.3. *melting curve*

Using the SYBR-green fluorescence dye the detected signals usually result from barcode amplification but sometimes positive signals can result from by-products. Such artefacts can be distinguished from the desired PCR products by melting curve analysis. The melting temperature (T_m) of undesired products differs from that the target amplicon due to different GC content or length. These analyses are used to differentiate amplification products separated by less than 2°C in T_m. [50].The program started with heating up to 95°C for 15 seconds followed by a cooling step to 60°C for 15 seconds and a slow increase of temperature for 20 minutes to 95°C. The fluorescence was plotted against temperature (3.2.1a) and a strong decrease in fluorescence indicates the T_m of a product. The first deviation (-dI/dT) shows a typical melting curve profile with a peak at T_m (3.2.1b).

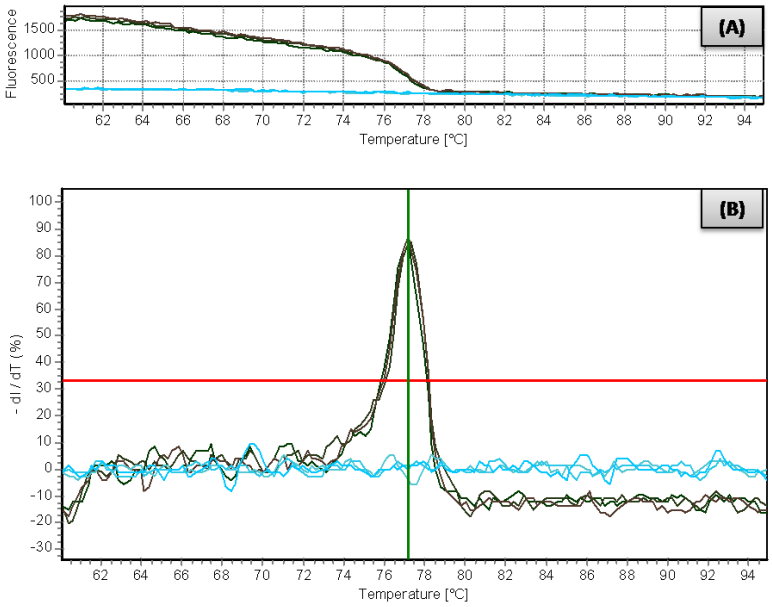

Figure 3.2.1: The melting curve profile for a barcode4-31-standard with the concentration of 10^7molecules/µl (black lines showing fluorescence**) and the no-template-control (**blue, flat lines**) are shown.** In (A) the melting curve diagram where the fluorescence is plotted against temperature can be seen. The strong decrease in fluorescence gives the T_m. The first deviation (-dI/dT, %) shows a typical peak at T_m of 77.2°C in the melting curve profile for barcode 4-31 (B).

3.2.2.4. *test for real-time qPCR inhibitors*

Real-time PCR inhibitors usually affect PCR by an interaction with DNA or interference with the thermo-stable DNA-polymerase to reduce enzyme activity. Some well known inhibiting agents are salts, like NaCl or KCl, ethanol, isopropanol, phenol, EDTA, SDS and of course complex polysaccharides (plants) and collagen [51, 52]. Inhibitors within the extracted DNA-sample reduce real-time qPCR efficiency and/or reproducibility [53]. Therefore all barcode DNA samples extracted out of feed-additives were tested for the presence of possible real-time PCR inhibitors. Thus 100mg of each sample were dissolved in 1.5ml pre-warmed 10mM Tris-buffer, incubated at 70°C for 15 minutes and spiked with barcode 2-20 DNA resulting in additive-barcode mixes with a concentration of 10^7molecules/µl. Sample 8 and 9 were dissolved in 5ml 10mM Tris-buffer to guarantee a well dissolved product. The extraction procedure is described in chapter 3.2.2. The extracted DNA was serially diluted either twofold or fourfold with 500ng/ml poly(dIdC). The original undiluted sample (1:1) and the dilution series were analyzed by qPCR. At a certain dilution PCR inhibitors are diluted and can not block the qPCR reaction anymore. A schematic drawing of sample with and without qPCR inhibitors is shown in figure 3.2.2.

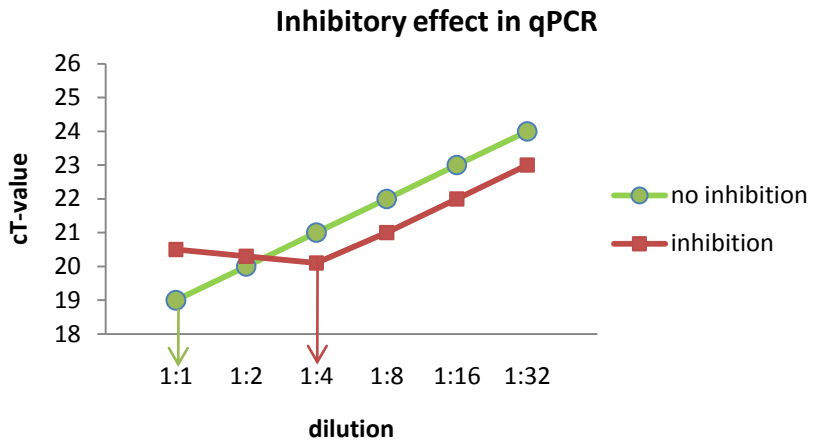

Figure 3.2.2: schematic drawing of an inhibitory effect in qPCR. The Inhibitor disturbs PCR leading to stable, less differing c_T values. Is an adequate dilution reached the blocking agent is also thinned down and c_T values differs in a constant value from each other. The dilution with lowest c_T-value that is also part of the linear slope is the concentration where the inhibition is 0 (C_{i0}).

3.2.2.5. *multiple standard addition method*

The aim of the multiple-standard-addition-method is to point out errors within the analysis method used [54]. This method follows the principle that the preparation of the sample and of the standard is two distinct procedures and standards therefore might not reflect the situation in the sample. Therefore a barcode-DNA-extraction from 20mg BIOtracer $4_{1:10}$ in 1ml 10mM Tris-buffer (chapter 3.2.1.a) was performed. This initial barcode concentration was 2.00E+08. Spiking the sample with 10µg/ml pdIdC resulted in a barcode-sample of 1.60E+08 molecules/µl (Table 3.2.3). This sample was then spiked with additional barcode amounts in the range of +0.5 fold, +1 fold, +2 fold and +4 fold of the initial expected concentration (x). A detailed list is given in table 3.2.4. After performing real-time PCR quantification the expected values are plotted against the measured values leading to an internal-standard-series (figure 3.2.3.). The formula for linear equation (equation 2) with *k* as the slope and *d* as the y-intercept was used. The y-intercept indicates the difference of measured and expected values.

$$y = kx + d \qquad (2)$$

Table 3.2.3: BIOtracer $4_{1:10}$ extraction results an initial barcode concentration of 2.00E+08 molecules per µl. The used extracted sample in the multiple standard addition method is prepared in 10µg/ml poly-dIdC leading to a concentration of 1.60E+08 barcode molecules/µl (written in bolt).

BIOtracer 4 $_{1:10}$ extract		**[molecules/µl]**
initial concentration		2.00E+08
used concentration	in [10µg/ml] pdIdC	**1.60E+08**

Table 3.2.4: Total barcode concentration [molecules/µl**] for the multiple standard addition method of the BIOtracer $4_{1:10}$ samples is shown.** Samples are spiked with additional barcode amounts in the range of 0.5 to 4 times of the expected value (x).

	sample BIOtracer 4 $_{1:10}$	**total conc.[molecules/µl]**
1	sample (x)	1.60E+07
2	sample + 0.5x	2.40E+07
3	sample + 1x	3.20E+07
4	sample + 2x	4.80E+07
5	sample + 4x	8.00E+07

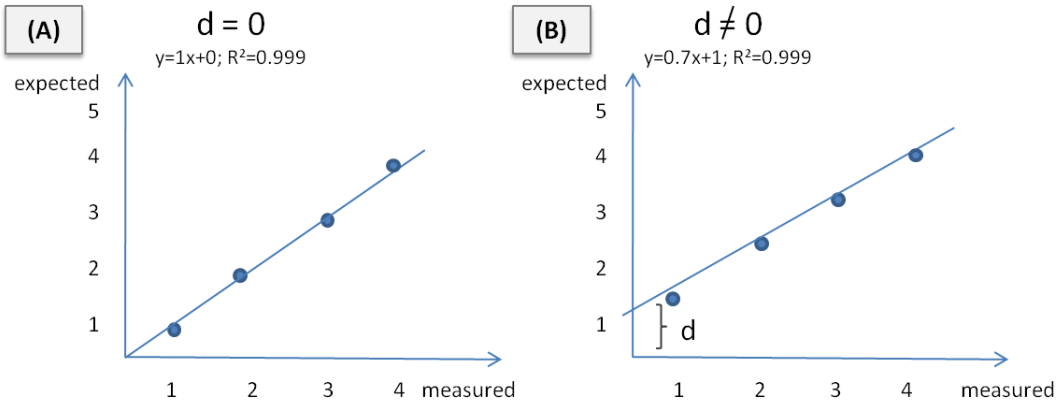

Figure 3.2.3: schematic diagram of the multiple standard addition method. By plotting expected values against measured ones and using equation (2). In contrast to diagram (A) with the assumption of a perfect analysis procedure (y=1, d=0, R^2=0.999) diagram (B) shows a difference of measured and expected values (d 0).

3.3 BIOtracer development and evaluation

Generally the BIOtracer consists of an individual, unique DNA-Sequence, referred to as barcode-sequence and a carrier material. The barcode is mixed under the carrier and a lyophilisation process is performed to dry the liquid batch. This results in a solid lyo-cake that is milled and the obtained BIOtracer can be mixed under several feed-additives for product labelling.

3.3.1. *Carrier-Matrix*

To mix the barcode-DNA sequence homogenous into several feed-additives it is essential to find the optimal carrier for the barcode-sequence. For the BIOtracer development an adequate carrier has to be found that fits following requirements:

- Plant origin (added to feed)
- No active binding of DNA
- Water soluble
- High glass transition temperature (T_g) for tracer stability
- Easy to mill after lyophilisation (homogenous distribution)
- Bulking agent

Due to the fact that in animal nutrition animalistic products are undesirable the carrier ideally is a compound found in plants. Another carrier criterion is that DNA must not bind active to it to alleviate barcode recovery out of the BIOtracer. This factor is most important in analytic because complex DNA-extraction steps can be passed by when the barcode can be easily separated from the carrier. Therefore cellulose-based matrices can be excluded. The barcode-DNA is received in a lyophilized form but for a homogenous distribution of the DNA in the carrier a fluid mixture is necessary. Therefore it is important that the carrier is water soluble. Not only these structural qualities of the carrier are important but also product characteristics during freeze drying should be

included. It has to be considered that a high glass transition temperature, T_g, where the transition from a solid "glassy" to a liquid-like "rubbery" state occurs [55, 56] is required to maximize the final product stability during storage and also leads to an easier drying [57]. Last but not least the lyophilisation process should be a standard process that is not too complex leading to a lyo-cake that can be easily milled for receiving a fine powdered tracer that can therefore be homogenously mixed under several additive products. It has to be mentioned that the main function of the carrier is to act as bulking agent for the feed-additive labelling barcode-sequence.

Due to all these specifications Maltodextrin G190 was chosen as a theoretically matching carrier and the potential has to be evaluated throughout this work. Maltodextrin is a polysaccharide built up of D-glucose units connected in chains of variable length and it can be classified as all glucose polymers by a dextrose equivalent (DE). The higher the DE value the higher the solubility, the sweetness and the shorter the glucose chains. That means that starch has a very low DE (nearby 0) and glucose has the highest value of about 100 [58]. Maltodextrin G190 that was used in this work is with a DE of 18 to 20 situated in the lower part [59, 60]. Generally maltodextrin with a DE of 20 has a T_g of about 141°C that is quite high. The higher the T_g the more stable are the sugar chains and the more heat-energy is necessary to change the glassy state into another phase like viscosity [57]. This stability ensures that during storage of the BIOtracer no clustering of the single particles can occur and less humidity can be taken up.

3.3.2. *Freeze drying of the barcode-carrier solution*

Throughout this work the freeze dryer Epsilon 2-90 from *Christ* and as product protecting tray during the lyophilisation process the GORE™ LYOGUARD® freeze-drying containers were used.

This freeze dryer is a single-chamber-system that offers a high drying capacity with high efficiency and short drying periods due to the construction of the ice condenser beside the shelves. The products have to be stored in special containers that are placed onto the food-prints of the freeze dryer. These containers must guarantee optimal heat conduction to increase product temperature and therefore an increase of sublimation and shorter drying time. The heatable shelves lead to this required energy transfer [16]. The DNA must not stick to contact surfaces of items used for the formulation preparation for a correct output analysis and therefore no glass ware could be used. GORE™-LYOGUARD® uses an expanded polytetrafluoroethylene (ePTFE) membrane that is micro-porous and Polypropylene surfaces. For lyophilisation it is essential that the DNA and the carrier remain within the tray, to receive and protect the desired product. To avoid cross contamination of different barcodes during freeze drying a `closed system´ is required where only water vapour can pass. It is known that water vapour can pass through the micro-porous membrane from GORE while water, even using a vacuum pump, can not. This membrane blocks liquid, particles and bacteria whereas gas molecules can pass through the membrane. Whether this micro-porous membrane is a barrier for the short barcode-DNA sequence has to be tested. The GORE-trays are single-use, disposable containers that can be enclosed and so essential product containment and protection features are provided. The container has a flexible, thin-film bottom which closely conforms to the dryer shelves and so ensures optimal temperature conduction [61].

The standard filling level of liquid products for freeze drying is 2cm [62]. To know how much of a Maltodextrin G190 (*SYRAL*, France) solution (10% w/w to osmotic water) as carrier is necessary for a 2cm level into the freeze drying tray a 110% solution composed of 100ml osmotic water and 10g Maltodextrin G190 was prepared. After weighting the filled tray it was known that 64g of the 110% solution are needed for the 2cm-standard filling level. Therefore 10% Maltodextrin G190 is 5.82g and 58.18g is osmotic water. Barcode DNA molecules are added per gram carrier and in the case of BIOtracer4 $5.8*10^{14}$ barcode molecules are used to augment the maltodextrin-solution that can

then be immobilized. This results in a total barcode concentration of 10^{14}molecules per gram carrier. BIOtracer2 with barcode 2-20 has a total barcode concentration of 10^{8} molecules/g (table 3.3.2). The filled GORE-tray is put onto the food-prints of the freeze dryer. The drying time depends on the amount and character of product and can be between 24h (30-40kg) and 48h when bigger volumes are dried. The freeze drying program "Christ LPC-16NT" was used. This standard drying program lasts for 24 hours and starts with freezing to minus 30°C for about 4 hours. This is followed by the primary drying step that lasts 17 hours and the pressure is reduced to 0.5mbar and the foot-prints are slowly heated up to 30°C. As a last step the secondary drying process is started and the pressure is again decreased to 0.001 mbar whereas the foot-print-temperature does not change. DNA tends to bind to cellulose and therefore a cellulose tissue is used to cover the porous membrane during freeze-drying for verifying a potential loss of barcode-DNA. After the lyophilisation cake is milled the powdered BIOtracer is stored in a closable plastic-container (Rotilabo-PVC container, *bartelt*, Austria) at 4°C.

Table 3.3.2: Immobilized barcode concentration of BIOtracer 2 and BIOtracer 4 in molecules per g carrier.

BIOtracer 2	BIOtracer 4
10^{8}/g carrier	10^{14}/g carrier

3.3.3. *Mix of tracer and feed-additives*

The BIOtracer 4 was diluted 1:10 with Maltodextrin G190 leading to a total barcode concentration of 10^{13} molecules/g carrier. The batch was mixed on an over-head-room shaker (*GFL, Germany*) at 21 rpm over night to ensure a homogenous mixture.

10g feed-additive were put into a 100ml closable plastic flask (*Lactan*, *Austria*). 100mg of the BIOtracer 4 $_{1:10}$ were added and mixed on an over-head-room shaker (*GFL*, *Germany*) at 21 rpm over night. This resulted in a FMZ-BIOtracer-mix with a total barcode concentration of 10^{11}/g.

4. Results

4.1 *Optimization of the workflow to obtain barcode-DNA-standards for quantification*

For an absolute quantification serially diluted standards of known concentrations are essential to generate a standard curve [35]. The standard curve creates the linear relationship between C_T-value and initial amounts of DNA.

Before a barcode-DNA-standard can be prepared the optimal poly-dIdC concentration has to be evaluated. Poly-dIdC is generally used to saturate the reaction tube that no other DNA can stick to the surface and therefore favors the probability that the required DNA-sequence is better dissolved and distributed within a dilution. In contrast, excessive poly-dIdC would lead to increased background fluorescence or a PCR baseline shift.

4.1.1. Evaluation of the optimal poly-dIdC concentration

A barcode 4-31 concentration of 10^{10} molecule per µl was 10-fold serially diluted with 3 different poly-dIdC stocks (N1, SN1, SN2) in 3 different concentrations [1µg/ml, 5µg/ml and 10µg/ml] and these samples were then analyzed using the appropriate qPCR assay.

Table 4.1.1.: C_T-values obtained for 10-fold serial dilutions. 10^{10}/µl barcode4-31 aliquots were diluted with the different poly-dIdC concentrations. The lowest c_T-values were obtained with 10µg/ml pdIdC.

	C_T-values of the 3 different poly-dIdC stocks in different concentrations								
barcode-concentration	**N1**			**SN1**			**SN2**		
[molecules/µl]	[10µg/ml]	[5µg/ml]	[1µg/ml]	[10µg/ml]	[5µg/ml]	[1µg/ml]	[10µg/ml]	[5µg/ml]	[1µg/ml]
1.00E+07	19.00	19.36	19.59	19.13	19.32	19.53	19.00	19.24	19.48
1.00E+06	22.59	22.92	23.21	22.84	22.82	23.15	22.47	22.75	23.09
1.00E+05	26.10	26.46	26.60	26.36	26.52	26.72	26.11	26.32	26.80

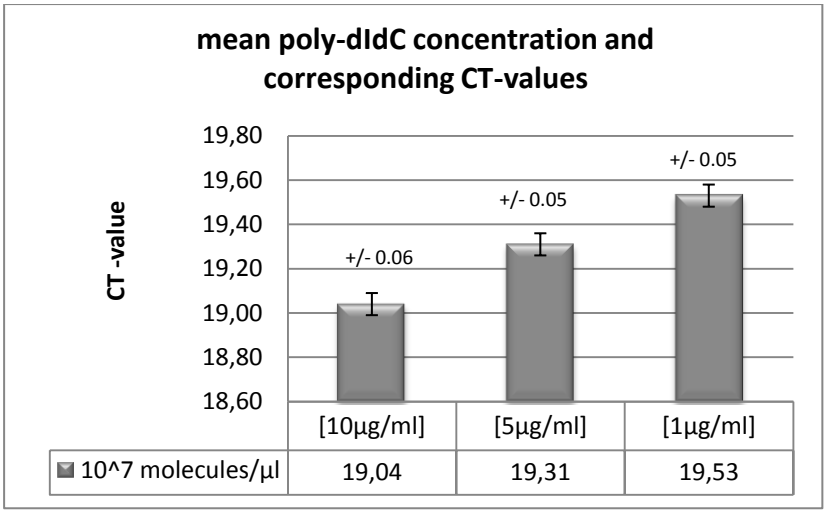

Figure 4.1.1. shows mean C_T-values of all analyzed poly-dIdC stocks (table 4.1.1.) with corresponding poly-dIdC concentrations of the 10^7 barcode-molecules/µl dilution. A high poly-dIdC concentration of 10µg/ml lead to the lowest C_T-values in all analyzed samples (n=3) (data sets for barcode concentrations of 10^6/µl and 10^5/µl are not shown).

Both, table 4.1.1. and figure 4.1.1. show lower C_T -values of qPCR analysis that results from using 10µg/ml poly-dIdC for preparation of dilutions. Therefore barcode-DNA-standard aliquots and sample dilutions for qPCR were subsequently prepared with 10µg/ml poly-dIdC.

4.1.2. influence of glycerol on qPCR results

Glycerol should be used as cryo-protector for barcode-DNA-standard aliquots of 10^{10} molecules per µl that are stored at -20°C. It is known that glycerol in concentrations >1% inhibits the PCR reaction [63].

A barcode-DNA-standard aliquot in 10% glycerol was prepared, 10-fold diluted with poly-dIdC and analyzed using the qPCR assay described in 3.2.2.2. As sample a 1% maltodextrin-osmotic water solution augmented with barcode-DNA was used and quantified once with and once without glycerol. The analyzed barcode concentrations and corresponding glycerol and maldtodextrin concentrations are listed in table 4.1.2.a whereas the results can be seen in table 4.1.2.b.

Table 4.1.2.a: barcode-DNA-standard concentration and corresponding glycerol and maltodextrin concentration. Concentrations in the range from 10^7/µl to 10^5/µl were analyzed.

barcode concentration	glycerol	maltodextirn
[molecules/µl]	[%]	[%]
1.00E+10	10%	
1.00E+09	1%	
1.00E+08	0.1%	1%
1.00E+07	0.01%	0.1%
1.00E+06	0.001%	0.01%
1.00E+05	0.0001%	0.001%
1.00E+04	0.00001%	0.0001%

Table 4.1.2.b: results of quantification of maltodextrin (MD) **samples with and without glycerol** compared to a barcode-DNA-standard with glycerol and a standard with glycerol and additional MD are shown. The ratios between samples with and without glycerin are given in percent (%) where the values <100% are marked in grey. 0.01% glycerin has no impact on qPCR efficiency.

barcode standard (+gly)					barcode standard (+gly +MD)		
sample + gly (*)		sample without gly		ratio (without/with)	sample + gly	without gly	ratio (without/with)
concentration	amount	concentration	amount		amount	amount	
(1)* 10^6	**5.1E+05**	(1) 10^6	**5.7E+05**	111.7%	**1.2E+06**	**1.4E+06**	113.3%
(1)* 10^6	**5.1E+05**	(1) 10^6	**5.7E+05**	111.7%	**1.2E+06**	**1.4E+06**	113.3%
(1)* 10^6	**5.1E+05**	(1) 10^6	**5.7E+05**	111.7%	**1.2E+06**	**1.4E+06**	113.3%
(1)* 10^5	**3.5E+04**	(1) 10^5	**4.7E+04**	134.3%	**9.5E+04**	**1.3E+05**	134.8%
(1)* 10^5	**3.5E+04**	(1) 10^5	**4.7E+04**	134.3%	**9.5E+04**	**1.3E+05**	134.8%
(1)* 10^5	**3.5E+04**	(1) 10^5	**4.7E+04**	134.3%	**9.5E+04**	**1.3E+05**	134.8%
(2)* 10^7	**8.1E+06**	(2) 10^7	**6.9E+06**	84.5%	**1.7E+07**	**1.4E+07**	84.9%
(2)* 10^7	**8.1E+06**	(2) 10^7	**6.9E+06**	84.5%	**1.7E+07**	**1.4E+07**	84.9%
(2)* 10^7	**8.1E+06**	(2) 10^7	**6.9E+06**	84.5%	**1.7E+07**	**1.4E+07**	84.9%
(2)* 10^6	**6.9E+05**	(2) 10^6	**8.4E+05**	122.8%	**1.6E+06**	**1.9E+06**	118.8%
(2)* 10^6	**6.9E+05**	(2) 10^6	**8.4E+05**	122.8%	**1.6E+06**	**1.9E+06**	118.8%
(2)* 10^6	**6.9E+05**	(2) 10^6	**8.4E+05**	122.8%	**1.6E+06**	**1.9E+06**	118.8%
(2)* 10^5	**3.7E+04**	(2) 10^5	**7.7E+04**	206.3%	**1.0E+05**	**2.0E+05**	198.0%
(2)* 10^5	**3.7E+04**	(2) 10^5	**7.7E+04**	206.3%	**1.0E+05**	**2.0E+05**	198.0%
(2)* 10^5	**3.7E+04**	(2) 10^5	**7.7E+04**	206.3%	**1.0E+05**	**2.0E+05**	198.0%
(3)* 10^7	**4.8E+06**	(3) 10^7	**5.9E+06**	124.1%	**9.8E+06**	**1.2E+07**	120.0%
(3)* 10^7	**4.8E+06**	(3) 10^7	**5.9E+06**	124.1%	**9.8E+06**	**1.2E+07**	120.0%
(3)* 10^7	**4.8E+06**	(3) 10^7	**5.9E+06**	124.1%	**9.8E+06**	**1.2E+07**	120.0%
(3)* 10^6	**5.0E+05**	(3) 10^6	**4.2E+05**	83.8%	**1.2E+06**	**9.9E+05**	83.7%
(3)* 10^6	**5.0E+05**	(3) 10^6	**4.2E+05**	83.8%	**1.2E+06**	**9.9E+05**	83.7%
(3)* 10^6	**5.0E+05**	(3) 10^6	**4.2E+05**	83.8%	**1.2E+06**	**9.9E+05**	83.7%
(3)* 10^5	**1.6E+04**	(3) 10^5	**4.8E+04**	304.5%	**4.6E+04**	**1.3E+05**	284.2%
(3)* 10^5	**1.6E+04**	(3) 10^5	**4.8E+04**	304.5%	**4.6E+04**	**1.3E+05**	284.2%
(3)* 10^5	**1.6E+04**	(3) 10^5	**4.8E+04**	304.5%	**4.6E+04**	**1.3E+05**	284.2%

The<100% ratio between samples with and without glycerol is random distributed (grey box) and not only affects the most concentrated sample. This shows that glycerol in a concentration <0.01% can be used as cryo-protector and an inhibitory effect of glycerol on qPCR reaction can be minimized by dilutions.

4.1.3. DNA-standard set up for assay preparation

Quantification was performed by interpolation in a standard regression curve of C_T -values generated from barcode-DNA of known concentrations.

To test and later guarantee constant qPCR results 10µl aliquots of a 10^{10}/µl barcode-standard in 10% glycerol and in 10µg/ml poly-dIdC were prepared, stored at -20°C and for each analysis an aliquot was diluted serially with pdIdC [10µg/ml] to final concentrations of 10^7, 10^6, 10^5 and 10^4 molecules/µl. Barcode-4-31-qPCR-assay was used and mean values with standard deviation for each barcode-concentration were calculated (figure 4.1.2.).

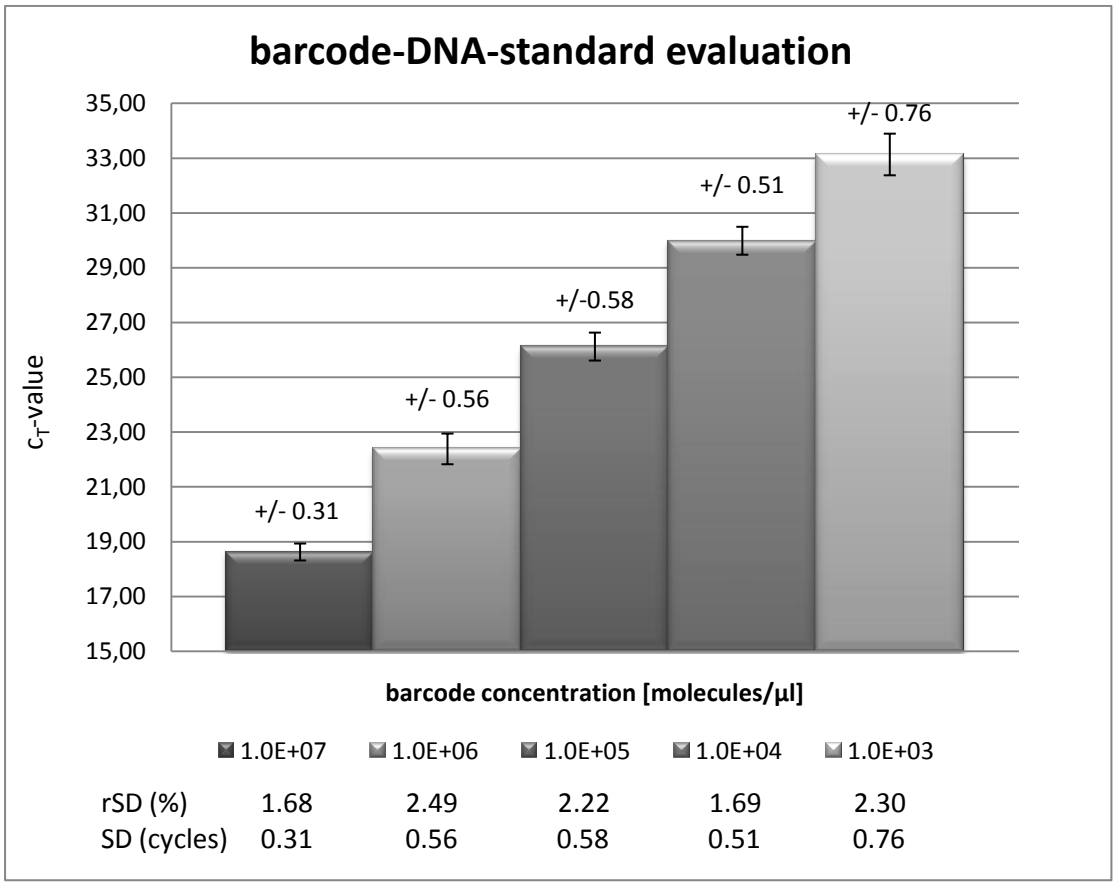

Figure 4.1.2. Barcode-4-31-DNA-standard evaluation is shown. Barcode dilutions ranging from 10^7 barcode-molecules/µl to 10^3/µl were analyzed using 4-31 qPCR assay (n=12). The higher concentrated the dilution the lower the C_T-value. The relative standard deviation (rSD) calculated for every dilution is below 2.5% and that are +/- 0.7 cycles difference.

4.2 *Evaluation of Maltodextrin G190 as a matrix to immobilize DNA*

4.2.1 Solubility and phase characteristics of Maltodextrin G190

For an effective inactivation of DNA degrading enzymes like DNases it is necessary to heat the dissolved BIOtracer up to about 65°-70°C for DNA extraction from samples (chapter 3.2.1.). For re-isolating the barcode from the carrier it must be known whether the viscosity of maltodextrin increases with temperature.

Therefore 1g Maltodextrin G190 was dissolved in 10ml osmotic water, 1ml fractions were heated up in a range of 50°C to 70°C in 5°C steps and were then cooled down to room temperature and 4°C.

Table 4.2.1: Maltodextrin G190 (10% in osmotic water**) viscosity testing** during heating and cooling down. No thickening occurred.

Fraction	Temperature	Viscosity at heating	Viscosity at 21°C	Viscosity at 4°C >10min
1	50°C	-	-	-/+
2	55°c	-	-	-/+
3	60°C	-	-	-/+
4	65°C	-	-	-/+
5	70°C	-	-	-/+

[+] stands for viscous, jelly matrix [-] means no thickening, like water [-/+] indicates low viscosity, like sirup

No thickening occurred before and after cooling down. However, after 10 minutes at 4°C a low level of viscosity was seen.

4.2.2 Influence of Maltodextrin G190 on qPCR results

It is known that some polysaccharides influence the PCR reaction in a negative manner due to the fact that they could bind to the dsDNA and make it therefore inaccessible for the *Taq*-polymerase [52]. The carbohydrate carrier was tested for its influences on quantitative PCR reactions.

Two sets of standards in the range of 10^7 to 10^4 barcode molecules per µl were used, one was spiked with maltodextrin and analyzed. The highest concentrated, spiked standard contains 0.1% of the starch sugar.

Table 4.2.2: c_T-values of the Maltodextrin G190 analysis. Standard* indicates the maltodextrin spiked standards. The differences in c_T-values are very low comparing standard with and without Maltodextrin G190. PCR-efficiency difference was 0.02.

Concentration [molecules/µl]	**c_T -value**		
	standard	standard*	**Δ cT-value**
10^7	18.96	19.02	-0.06
10^6	22.74	22.72	0.02
10^5	26.41	26.55	-0.14
10^4	29.85	30.16	-0.31
PCR efficiency	**0.87**	**0.85**	**0.02**
R^2	0.999	0.999	

The obtained results indicate no negative influence of Maltodextrin G190 on qPCR, therefore it was subsequently used to immobilize the barcode-DNA.

4.3 *Evaluation of the GORE™-LYOGUARD® freeze-drying tray to immobilize DNA in a maltodextrin matrix*

To avoid product cross contaminations during freeze-drying it was essential to find out whether the freeze-drying tray from GORE™-LYOGUARD® keeps the short barcode DNA sequence inside or looses it through the micro-porous membrane.

During lyophilisation of the BIOtracer 2 and 4 a cellulose filter was used to cover the micro-porous GORE-membrane. Evaporated barcode DNA is expected to stick on the cellulose fibres of the filter tissue. An illustration of the GORE™-tray with the filter is given in figure 4.3.1.

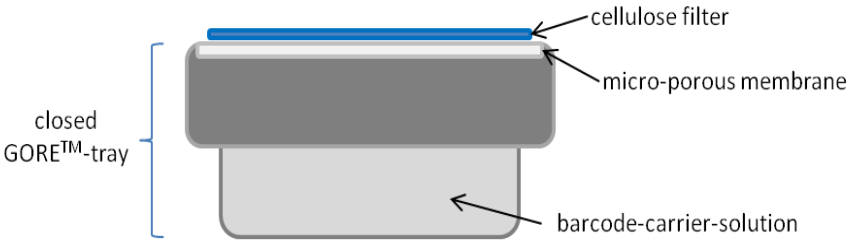

Figure 4.3.1: Illustration of the cellulose filter position during barcode immobilisation. The cellulose filter covered the micro-porous membrane of the GORE™-tray cap during the lyophilisation process. Escaped barcode molecules were then re-isolated from the filter.

DNA from the filter was extracted and subjected to qPCR analysis. Every extracted sample was diluted 2-fold resulting in 3 samples per extraction (1:1, 1:2 and 1:4). The melting curve (figure 4.3.2) shows that the detected products result from the barcode sequence and not from unspecific by-products.

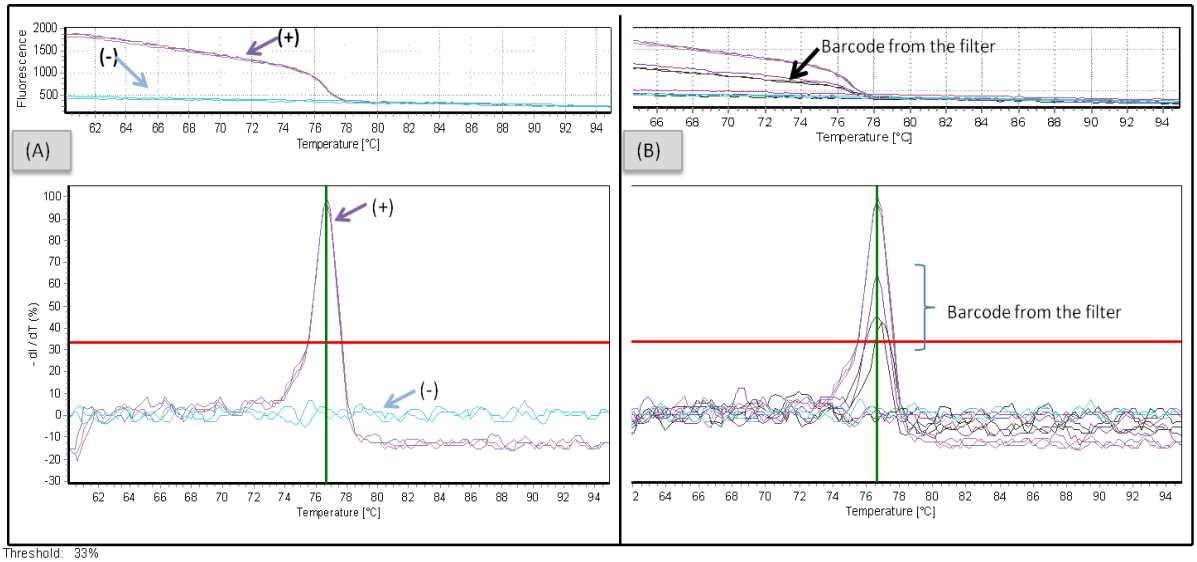

Figure 4.3.2: Melting curve analysis of extracted DNA (SYBR-detection) from the cellulose filter of BIOtracer 4 immobilisation. (A) Shows a positive control (+) of the barcode-4-31 sequence and a negative control (-) in the actually detected version (upper graph) and in the first deviation (melting curve). The peak indicates a T_m of 76.7°C. In (B) the detected barcode-4-31 sequence from the filter is shown. (data set for BC2 is not shown)

For data analysis the diluted samples are multiplied by their dilution factor to receive comparable results. The measured and calculated amounts of barcode 2-20 and 4-31 are listed in table 4.3.1.

Table 4.3.1: Detected barcode 2-20 and 4-31 molecule amounts total on the filter that covers the GORE^{TM}-membrane during lyophilisation. Both barcodes with detected concentration from 3 dilutions (1:1, 1:2, 1:4), mean values, the total detected barcode molecules on the cellulose filter and the evaporated barcode amount (%) with respect to the totally added barcode concentration in the lyophilisation batch.

barcode	detected barcode	mean value	total on the filter	totally added barcode	evaporated barcode
	[molecules/µl]	[molecules/µl]	[molecules/60µl]	[molecules]	[%]
BC 2-20	65	98	5.88E+03	5.8E+08	0.001%
	140				
	88				
BC 4-31	87	230	1.38E+04	5.8E+14	<0.001%
	103				
	500				

4.4 Determination of the recovery rate of DNA from the maltodextrin matrix

BIOtracer 4 was immobilized by lyophilisation and contains 10^{14} barcode 4-31 molecules per gram carrier. The BIOtracer was diluted 1:10 with Maltodextrine G190 receiving a total barcode concentration of 10^{13} molecules/g carrier. 20mg of the diluted tracer were extracted with 1ml 10mM Tris-buffer. The isolated barcode-molecules (templates) were analyzed in different dilutions in the range of 10^7 to 10^4molecules/µl. This analysis was performed 3 times on two different days (n=6).

Table 4.4.1: qPCR results of barcode-DNA recovery from the BIOtracer 4 $_{1:10}$ dilution. Extracted and diluted samples are shown in expected and measured concentrations. The mean barcode concentrations of each extraction (grey box) were calculated by multiplying the measured values by their dilution factors. The recovery rates and the relative standard deviation (rSD) is given for every analysis of an extracted sample. The total recovery was 41.8%.

PCR efficiency = 0.92 R^2=0.999	**BIOtracer 4 $_{1:10}$**			07.04.2010
expected concentrations	*measured concentrations*			
of the different dilutions in molecules/µl	**1**	**2**	**3**	
1.60E+07	6.48E+06	6.02E+06	8.48E+06	
1.60E+06	6.35E+05	7.15E+05	7.85E+05	
1.60E+05	5.72E+04	6.51E+04	7.54E+04	
1.60E+04	6.07E+03	6.46E+03	7.13E+03	
calculation: to a total of 1.6*10^7				
	6.48E+06	6.48E+06	6.48E+06	
*10	6.35E+06	7.15E+06	7.85E+06	
*100	5.72E+06	6.51E+06	7.54E+06	
*1000	6.07E+06	6.46E+06	7.13E+06	**total recovery**
mean value	6.16E+06	6.65E+06	7.25E+06	6.69E+06
recovery rate	38.47%	41.56%	45.31%	**41.78%**
rSD	5.47%	5.02%	8.17%	9.17%
PCR efficiency = 0.89 R^2=1.000	**BIOtracer 4 $_{1:10}$**			19.04.2010
expected concentrations	*measured concentrations*			
of the different dilutions in molecules/µl	**1**	**2**	**3**	
1.60E+07	6.23E+06	5.69E+06	7.72E+06	
1.60E+06	6.52E+05	5.82E+05	7.79E+05	
1.60E+05	6.40E+04	6.20E+04	8.77E+04	
calculation: to a total of 1.6*10^7				
	6.23E+06	6.23E+06	6.23E+06	
*10	6.52E+06	5.82E+06	7.79E+06	
*100	6.40E+06	6.20E+06	8.77E+06	**total recovery**
mean value	6.38E+06	6.08E+06	7.60E+06	6.69E+06
recovery rate	39.90%	38.02%	47.48%	**41.80%**
rSD	2.28%	3.76%	16.86%	14.27%

4.4.1. Multiple-standard-addition-method

The aim of the multiple-standard-addition-method is to point out whether the low recovery rate of 41.78% and 41.80%, respectively resulted from errors within the analysis procedure or occurred in steps before. Five samples were analyzed and four of them were spiked with additional barcode amounts in the range of 0.5 to 4 times the expected value (x) of 1.60E+06 molecules/µl. For detailed information see chapter 3.2.2.5.

Table 4.4.2: real-time PCR results of the 3 BIOtracer $4_{1:10}$ extractions of the multiple standard addition method. Every extract was spiked with additional barcode concentrations. Mean values of the extractions were calculated and compared to the expected values leading to figure 4.4.1. Value written small is not taken for calculation because it differs too much from the expected value of sample 4.

	sample	extraction (1)	extraction (2)	extraction (3)	mean value	expected
1	sample (x)	8.39E+05	6.96E+05	6.13E+05	7.16E+05	1.60E+06
2	sample + 0.5x	1.62E+06	1.45E+06	1.35E+06	1.47E+06	2.40E+06
3	sample + 1x	2.27E+06	2.15E+06	2.23E+06	2.22E+06	3.20E+06
4	sample + 2x	*2.05E+06*	4.09E+06	3.97E+06	4.03E+06	4.80E+06
5	sample + 4x	7.07E+06	6.99E+06	6.85E+06	6.97E+06	8.00E+06

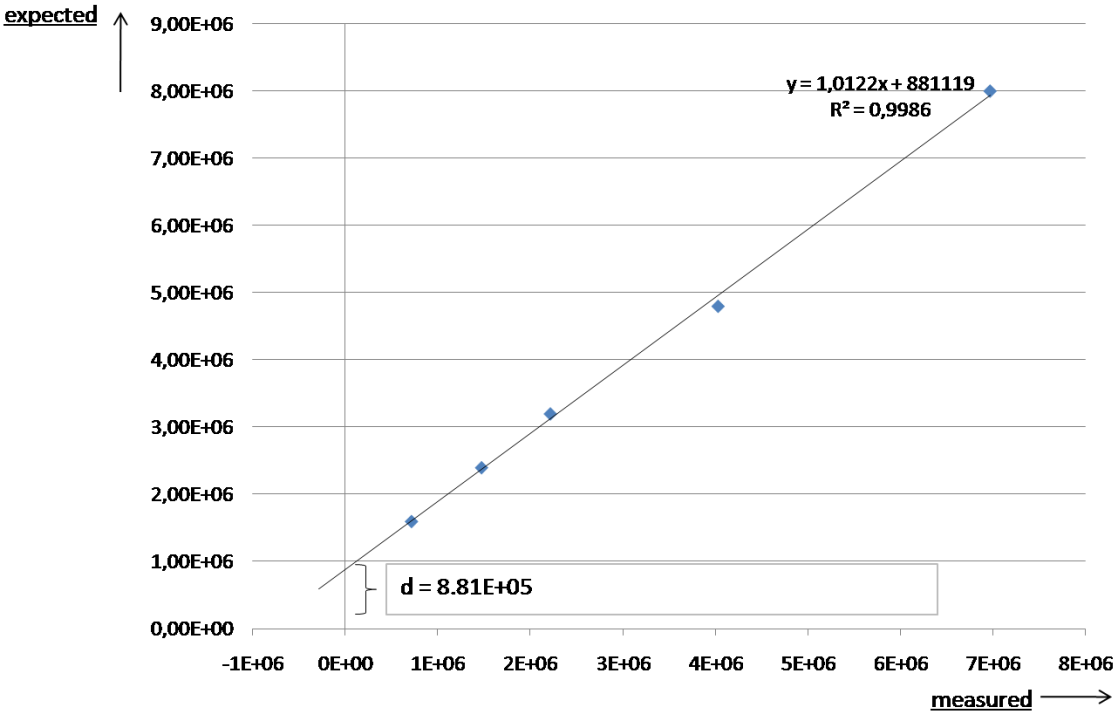

Figure 4.4.1: Diagram of the linear regression line of the multiple-standard-addition-method. 3 analyzed BIOtracer $4_{1:10}$ extractions that have been spiked with additional barcode concentrations were analyzed and expected values were plotted against measured mean values. For calculating d that is $8.81*10^5$ barcode molecules/µl the formula for linear function (y=kx+d) was used. The slope of 1,0 and the R^2 of 0.999 demonstrate the high accuracy of the analysis.

Table 4.4.2 shows the total corrected barcode recovery rate from BIOtracer $4_{1:10}$ by including the detected error of 8.81E+05 barcode molecules per µl. The expected value minus the error gives the actual expected value of 99.4% recovery.

Table 4.4.2: Corrected recovery rate of BIOtracer 4 $_{1:10}$ with respect to the detected error of 8.81E+05 barcode molecules/µl.

	Barcode [moleculae/µl]	Recovery [%]
expected	1.60E+06	
- error	8.80E+05	
actual expected	7.20E+05	100%
measured	7.16E+05	**99.4%**

4.5 Testing the different feed-additive matrixes

For a quantification of barcode molecules extracted from different feed-additives two crucial points were tested:

a) Degradation of the BIOtracer due to enzymatic activity (4.5.1.) and

b) Presence of PCR inhibitory compounds in the different feed-additives (4.5.2.).

4.5.1 Water activity of the feed-additives and the BIOtracer $4_{1:10}$

Generally a_w-values below 0.8 show no enzymatic activity and and this ensures tracer stability. For a_w-value determination all feed-additives and the BIOtracer 4 in a 1:10 diluted form (BT $4_{1:10}$) were analyzed as described in chapter 3.1.1.

Table 4.5.1.: measured water activity (a_w-values) with corresponding temperature of all analyzed feed additives (FMZ) and the BIOtracer $4_{1:10}$. A_w-values <0.8 have retarded enzymatic activity [47].

FMZ	aw_1	aw_2	mean aw	temperature
1	0.376	0.372	0.374	24.9°C
2	0.422	0.416	0.419	24.9°C
3	0.436	0.432	0.434	24.9°C
4	0.208	0.215	0.212	25.0°C
5	0.364	0.362	0.363	24.8°C
6	0.529	0.522	0.526	24.9°C
7	0.369	0.366	0.368	25.0°C
8	0.329	0.328	0.329	24.9°C
9	0.296	0.296	0.296	24.9°C
10	0.550	0.550	0.550	24.9°C
11	0.268	0.271	0.270	25.0°C
12	0.449	0.446	0.448	24.9°C
13	0.528	0.527	0.528	24.9°C
BT $4_{1:10}$	0.337	0.332	0.335	24.9°C

4.5.2. Test of feed-additives for the presence of putative PCR inhibitors

Feed additives were tested for their real-time PCR inhibitory nature.

The different additives were spiked with barcode 2-20 DNA (3.2.2.4) and the DNA was then extracted (3.2.1.a) and serially diluted. The dilution with lowest c_T -value but still on the linear slope (figure 4.5.1) is the desired concentration with no inhibitory effect (C_{i0}).

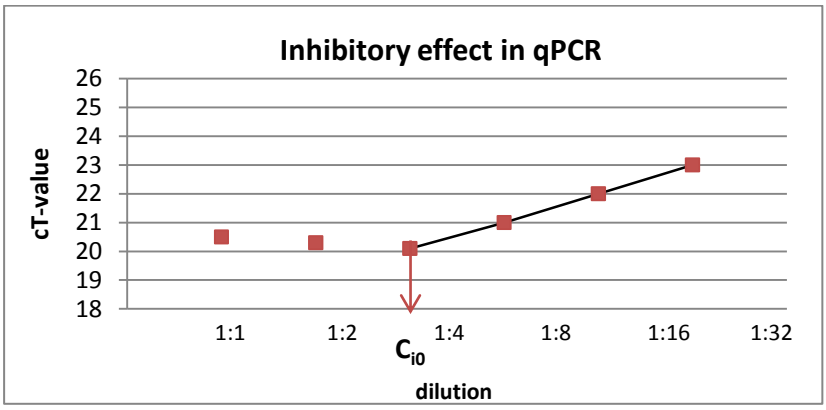

Figure 4.5.1.: Schematic diagram of a PCR inhibitor that can be handled with by adequate dilution. With every dilution the Δc_T –value is one cycle. The dilution with lowest cT-value that is also part of the linear slope is the concentration where the inhibition is 0 (C_{i0}). This was evaluated for every feed-additive.

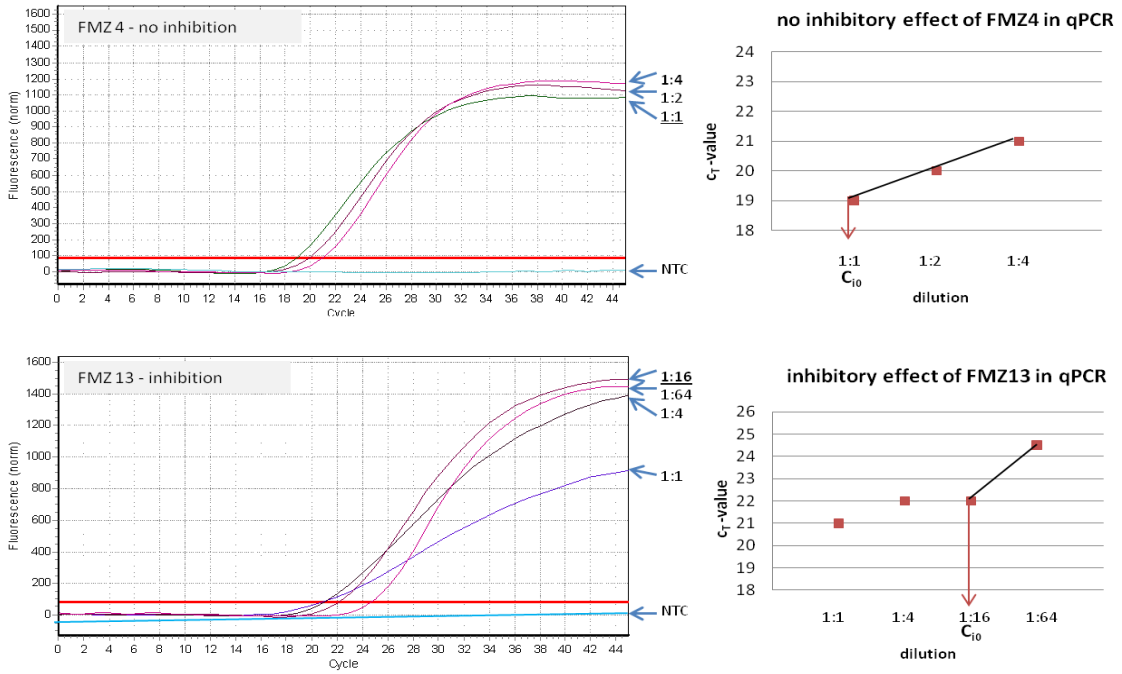

Figure 4.5.2.: real-time PCR diagram of inhibitor analysis of FMZ4 and FMZ13 with corresponding C_{i0} definition with respect to c_T and linear slope. The fluorescence is plotted against cycle number, the cycle-threshold is indicated with a red line and the negative control (NTC) is under the threshold line. Dilutions of extracted barcode-DNA show different slopes, C_T -values and fluorescence due to inhibitory effects (FMZ13). FMZ4 showed no inhibitory effect, ΔC_T – values of dilutions were 1 cycle. FMZ13 needs a dilution ratio of 1:16 to reach a minimal inhibitory effect (C_{i0}).

Table 4.5.2.: All analyzed feed-additives with their corresponding dilution needed to reach inhibition free conditions (C_{i0}). Results demonstrate that almost all additives show inhibitory effects but adequate sample dilutions resolves the problem.

FMZ	C_{i0}
1	1:32
2	1:64
3	1:512
4	1:1
5	1:128
6	1:32
7	1:256
8	1:16
9	1:4
10	1:4
11	1:16
12	1:64
13	1:16

4.6 Recovery of barcode-DNA from different feed additives

It was tested whether the BIOtracer could be distributed homogenously in the different additives and how much of the initial barcode amount could be re-isolated. 1g of the FMZ-BIOtracer-intermix (chapter 3.1.3.) was dissolved in 15ml pre-warmed 10mM Tris-buffer and DNA-extraction was performed as described in chapter 3.2.1.a. FMZ-BIOtracer-samples 8 and 9 were dissolved in 40ml 10mM Tris-buffer before barcode-DNA was extracted to guarantee a well-dissolved solution. This evaluation was performed 3 times (n=3). Excel sheet tables showing the results for all feed-additives are shown in chapter 7 - *appendix*.

Table 4.6.1.: Barcode recovery rates from all analyzed feed additives (FMZ) and their main components. Recovery rates <10% were gained from bentonite-mixes and <5% from mineral compound mixtures. High barcode recoveries were got from probiotic-mixes (>90%). The relative standard deviation (rSD) from 3 extractions (n=3) of every FMZ are given in %.

FMZ	main components	recovery rate [%]	rSD [%]
1	bentonite, yeast-mix	8.2	18
2	bentonite, yeast-mix	1.7	21
3	mineral compound mixture	51.9	24
4	probiotics	107.8	2
5	mineral compound mixture	0.1	65
6	mineral compound mixture	10.7	17
7	sal assortment	0.5	97
8	yeast	84.6	31
9	yeast plus carrier	34.8	8
10	fish meal powder	43.9	11
11	dried blood	9.8	12
12	bentonite	1.1	7
13	essential oils plus carrier	52.0	13

4.7 Stability tests for the BIOtracer (shelf life tests)

Long term stability of immobilized DNA is a crucial factor. Feed-additives have expiration dates and tracer stability must be guaranteed for this period of time.

20mg of the BIOtracer $4_{1:10}$ were dissolved in 1ml pre-warmed 10mM Tris-buffer and barcode-DNA extraction was performed as described in chapter 3.2.1.a. The barcode amount was quantified and the recovery rate was determined.

Table 4.7.1: measured and calculated barcode amount of BIOtracer $4_{1:10}$ shelf-life tests part I. The same analysis procedure was performed on 2 different days. Measured amounts were multiplied by their dilution factor and mean values, recovery rates and relative standard deviations (rSD) from every analyzed sample was calculated. The total recovery rate is marked grey and was calculated from the values in the bold border. Dilution errors are marked in light grey. (n=5 per day)

qPCR Efficiency = 0.86 R^2=1.000	sample					40 days after lyophilisation
measured concentrations	**1**	**2**	**3**	**4**	**5**	
of the different dilutions in molecules/µl						
$2*10^7$	1.54E+07	1.46E+07	1.71E+07	1.20E+07	1.60E+07	
$2*10^6$	1.45E+06	1.65E+06	1.85E+06	1.37E+06	1.78E+06	
$2*10^5$	1.34E+05	1.33E+05	5.96E+04	1.21E+05	1.21E+05	
$2*10^4$	1.34E+04	1.21E+04	7.22E+03	9.62E+03	5.98E+03	
calculation: to a total of $2*10^8$						
*10	1.54E+08	1.46E+08	1.71E+08	1.20E+08	1.60E+08	
*100	1.45E+08	1.65E+08	1.85E+08	1.37E+08	1.78E+08	
*1000	1.34E+08	1.33E+08	5.96E+07	1.21E+08	1.21E+08	*recovery*
*10000	1.34E+08	1.21E+08	7.22E+07	9.62E+07	5.98E+07	10^7+10^6
mean value	1.42E+08	1.41E+08	1.22E+08	1.19E+08	1.30E+08	**1.56E+08**
recovery rate	70.88%	70.63%	60.98%	59.28%	64.85%	**78.05%**
rSD	6.82%	13.34%	53.44%	14.18%	40.34%	**12.69%**

qPCR Efficiency = 0.84 R^2=0.999						48 days after lyophilisation
measured concentrations	**1**	**2**	**3**	**4**	**5**	
of the different dilutions in molecules/µl						
$2*10^7$	1.43E+07	1.41E+07	1.40E+07	1.51E+07	1.58E+07	
$2*10^6$	1.49E+06	1.39E+06	1.50E+06	1.64E+06	6.47E+05	
$2*10^5$	1.03E+05	9.57E+04	8.66E+04	5.80E+04	4.35E+04	
$2*10^4$	3.72E+04	8.10E+03	7.12E+03	6.60E+03	3.79E+03	
calculation: to a total of $2*10^8$						
*10	1.43E+07	1.41E+07	1.40E+07	1.51E+07	1.58E+07	
*100	1.49E+07	1.39E+07	1.50E+07	1.64E+07	6.47E+06	
*1000	1.03E+07	9.57E+06	8.66E+06	5.80E+06	4.35E+06	*recovery*
*10000	3.72E+07	8.10E+06	7.12E+06	6.60E+06	3.79E+06	$2*10^7+2*10^6$
mean value	1.92E+07	1.14E+07	1.12E+07	1.10E+07	7.60E+06	**1.47E+07**
recovery rate	95.88%	57.09%	55.98%	54.88%	38.01%	**73.56%**
rSD	63.57%	26.65%	34.74%	50.56%	73.47%	**5.65%**

Table 4.7.2: measured and calculated barcode amount of of BIOtracer $4_{1:10}$ shelf-life testing part II. The same analysis was performed on 4 different days. Measured amounts were multiplied by their dilution factor and mean values, recovery rates and relative standard deviations (rSD) from every analyzed sample was calculated. The total recovery rate of each day was determined with respect of all gained results (n=3 per day) and is marked grey.

qPCR Efficiency = 0.86 R^2=1.000	sample			89 days after lyophilisation	qPCR Efficiency =0.93 R^2=0.996			97 days after lyophilisation
measured concentrations	1	2	3		1	2	3	
molecules/µl								
$2*10^7$	2.01E+07	2.19E+07	1.95E+07		3.10E+07	2.99E+07	2.57E+07	
$2*10^6$	2.22E+06	2.27E+06	1.99E+06		2.83E+06	2.55E+06	2.55E+06	
$2*10^5$	1.28E+05	1.43E+05	1.41E+05		2.41E+05	2.25E+05	2.29E+05	
$2*10^4$	1.26E+04	7.27E+03	1.35E+04		*Not analyzed*			
calculation: to a total of 2*10^8								
*10	2.01E+08	2.19E+08	1.95E+08		3.10E+08	2.99E+08	2.57E+08	
*100	2.22E+08	2.27E+08	1.99E+08		2.83E+08	2.55E+08	2.55E+08	
*1000	1.28E+08	1.43E+08	1.41E+08		2.41E+08	2.25E+08	2.29E+08	
*10000	1.26E+08	7.27E+07	1.35E+08	**total recovery**	*Not analyzed*			**total recovery**
mean value	2.12E+08	2.23E+08	1.97E+08	**2.11E+08**	2.78E+08	2.60E+08	2.47E+08	**2.62E+08**
recovery rate	105.75%	111.50%	98.50%	**105.25%**	139.00%	129.83%	123.50%	**130.78%**
rSD	7.02%	2.54%	1.44%	**6.51%**	12.51%	14.33%	6.32%	**11.42%**

qPCR Efficiency =0.85 R^2=0.995	sample			103 days after lyophilisation	qPCR Efficiency =0.85 R^2=0.998			189 days after lyophilisation
measured concentrations	1	2	3		1	2	3	
in molecules/µl								
$2*10^7$	2.14E+07	2.04E+07	2.35E+07		1.39E+07	1.27E+07	1.70E+07	
$2*10^6$	2.16E+06	2.47E+06	2.44E+06		1.59E+06	1.42E+06	1.88E+06	
$2*10^5$	2.25E+05	2.51E+05	2.44E+05		1.72E+05	1.65E+05	2.31E+05	
$2*10^4$	1.85E+04	2.36E+04	2.08E+04		*Not analyzed*			
calculation: to a total of $2*10^8$								
*10	2.14E+08	2.04E+08	2.35E+08		1.39E+08	1.27E+08	1.70E+08	
*100	2.16E+08	2.47E+08	2.44E+08		1.59E+08	1.42E+08	1.88E+08	
*1000	2.25E+08	2.51E+08	2.44E+08		1.72E+08	1.65E+08	2.31E+08	
*10000	1.85E+08	2.36E+08	2.08E+08	**total recovery**	*Not analyzed*			**total recovery**
mean value	2.10E+08	2.35E+08	2.33E+08	**2.26E+08**	1.57E+08	1.45E+08	1.96E+08	**1.66E+08**
recovery rate	105.00%	117.25%	116.38%	**112.88%**	97.92%	90.42%	122.71%	**103.68%**
rSD	8.26%	9.08%	7.32%	**9.09%**	10.61%	13.23%	15.96%	**18.62%**

Table 4.7.3: The recovery rates from BIOtracer $4_{1:10}$ shelf-life testing from 2.12.2009 till 19.04.2010 is shown in this table. The same analysis procedure was performed on 6 different days (table 4.7.2 and 4.7.3) and here the mean recovery rate of 100% was determined.

date	**day after lyophilisation**	**recovery rate** [%]
02.12.2009	40	78
10.12.2009	48	74
19.01.2010	89	105
27.01.2010	97	131
01.02.2010	103	113
19.04.2010	189	104
mean recovery		**100.8**
Standard deviation		+/- 21.6

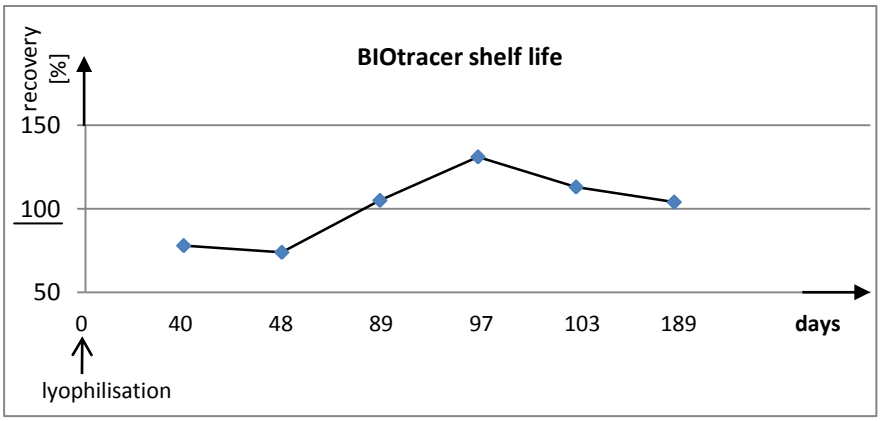

Figure 4.7.1: The BIOtracer shelf life over a period of 6 months is shown. Barcode recovery rates were 100% +/- 21,6% and were obtained after certain periods of time.

5 Discussion and outlook

Due to the raising problem of product counterfeiting BIOMIN decided to develop a novel and innovative system that acts as a barcode on molecular level for labelling their feed-additives. This BIOtracer should displace the imitable previously used tracing system consisting of small metal particles in the field of product labelling.

Comparison of the metal-particle-labelling system and the BIOtracer:

An advantage of the BIOtracer in contrast to the microtracer is specificity and inimitability. The individual barcode sequences are only known by BIOMIN. Without the specific primer pair for each BIOtracer the barcode can not be read by PCR. For imitations of these barcode sequences they have first to be isolated, separated and distinguished form other co-extracted DNA (yeast, diverse probiotics, plants) followed by a complex sequencing procedure. Furthermore the barcode concentration must be found out and inhibitory effects or assay optimisation including DNA standards for quantification have to be accounted for. Not only this high effort but also the equipment will not value imitation. Although assay optimisation is a time consuming step and not only includes optimisation of the PCR-program for maximum efficiency but also barcode-DNA-standard preparation the analysis itself does ot take much time. Normally a PCR-run for 9 different samples in 3 dilutions, a barcode-standard-series consisting of 4 standards for quantification can be analyzed within about 1 hour. A main disadvantage of the Microtracer is that feed-additives are mixed under feed and sometimes feed-mills have magnets to separate iron particles like nails or other metal material from the feed. During this process also some microtracer-particles removed what leads to wrong quantitative evaluations from feed-additive augmented feed. Furthermore, it could happen that during a quantitative analysis two, three or even more microtracer-dots stick together onto the filter and after developing the filter some big colored dots arise. Therefore it is not possible to count every single marked dot and this leads to a roughly quantification estimate. Using the BIOtracer and specific primers for quantitative PCR an amplification signal will only occur if one particular barcodewas used to trace the product. Furthermore, knowing the intermix-ratio a well defined c_T -value for each sample is expected and therefore the quantification results are more precise.

From September 2009 to February 2010 there were approximately 750 barcodes evaluated *in silico* by the project partner IFA-Tulln. About ten of them did show the desired properties (short, unique and almost 100% PCR efficiency) and were tested *in vitro* leading to two barcodes (BC2 and BC4) as good candidates for functionally BIOtracers. These numbers give a rough estimation of the difficulty of barcode-design. Nevertheless, two barcodes have been immobilized to BIOtracers (BIOtracer2 and BIOtracer4) and many tests using qPCR have been performed. However, SYBR Green I detects specific but sometimes also non specific PCR products and therefore the assay requires careful optimization.

Performing reproducible barcode-DNA-standard-series for quantification

The problem of high dilutions is that there are only few molecules per reaction tube and they may stick together or on the tube walls. Therefore poly-dIdC is used to saturate the reaction tube walls and to facilitate homogenous DNA distributions. Low concentrations lead to dilution problems and high c_T-values and in contrast, excessive poly-dIdC results in increased background fluorescence. Experiment 4.1.1 shows that a poly-dIdC concentration of 10µg/ml leads to earliest c_T -values compared to other concentrations (5 and 1µg/ml) and is therefore the most effective one for preparing barcode-standard and sample dilutions. To produce barcode-standards barcode aliquots of a certain concentration were frozen in glycerol as a cryo-protector. Table 4.1.2.b shows that an inhibitory effect of glycerol on qPCR reaction is minimized by dilutions and therefore is negligible.

For barcode quantification frozen barcode-standard aliquots (10µl) with concentrations of 10^{10}molecules/µl in 10% glycerin and in 10µg/ml pdIdC were prepared and tested for reproducibility. It is absolutely necessary for qualification that a standard of a certain concentration gives same c_T -values in every single run. Analyses of 12 real-time PCR runs each with a barcode-standard-series prepared from another frozen aliquot demonstrated that the barcode-standard aliquots are reproducible. This means that they have low relative standard deviations leading to differences in c_T -values of +/-0.5 cycles within one dilution (figure 4.1.2.). This difference is high for quality control reasons and needs therefore further optimisation. However, for the here described experiments these standard aliquots were considered as sufficiently accurate. For barcode-standard optimization it should be tested whether 4°C storage at large volume would be more effective than -20°C storage of small aliquots. Also it should be tested whether larger standard storage volumes than 10µl aliquots could improve reproducibility. A negative aspect in high-volume storage is the fact that contamination with DNA-degrading enzymes can easily occur and therefore careful and exact working is even more necessary.

Maltodextrin G190 valuation concerning viscosity and effects on qPCR

Polysaccharides can negatively influence the PCR reaction by making DNA-strands inaccessible for the *Taq*-polymerase [52]. Maldex G190 - a maltodextrin (MD) - was used for immobilization of the barcodes and therefore functions as the carrier. The difference in PCR-efficiency of two standards should be <0.03 [63]. In table 4.2.2 where standards with and without MD were analyzed a difference in efficiency of 0.02 was detected and that let one conclude that Maldex G190 does not negatively influence the qPCR reaction. Concerning viscosity it was tested that no thickening occurred before and after cooling down what is perfect for barcode-extraction. After these positive evaluations (4.2.1 and 4.2.2) Maltodextrin G190 appeared as the optimal carrier matrix for the BIOtracer project.

GORE™-LYOGUARD® freeze-drying tray evaluation

The freeze-drying tray from GORE™-LYOGUARD® was tested whether the short barcode sequence of about 100 to 120bp in length can pass the micro-porous membrane or functions as a kind of sterile barrier. Not only to avoid product cross contaminations during freeze-drying but also to protect the product itself and the freeze dryer from cross contamination a cellulose filter covered the micro-porous membrane during lyophilisation. It can be seen in table 4.3.1. that although the BIOtracer 4 consists of more barcode molecules per µl (+10^6/µl) as the BIOtracer 2 not much more membrane-passing molecules were detected on the filter. It could be demonstrated that minimal barcode amounts (0.001%) can pass the micro-porous membrane of GORE^{TM} but due to the vacuum effect during the lyophilisation process the suction goes out of the tray barcode cross contaminations are avoided. As additional contamination protection of the freeze drying equipment cellulose filters should be used for every drying process to adsorb the passing barcode molecules at least partially.

Recovery of barcode-DNA from the maltodextrin matrix

A first barcode re-isolation from the BIOtracer$4_{1:10}$ showed that 42% could be detected (table 4.4.1.). This low recovery rate not only results from the barcode amount found in the cellulose filter. However, before going ahead and starting recovery tests from FMZ-BIOtracer mixes the undetected or lost barcode amount had to be found. Therefore the multiple standard addition method was used. Extracted barcode samples were additionally spiked with a defined barcode amount leading to an internal standard series. Using the formula for linear function, that is defined as y=kx+d where *k* is the slope and *d* indicates the error, so a possible analysis error can be found out. It was demonstrated that about 55% of the initial barcode concentration were somehow lost in other steps than isolation or analysis. With respect to the multiple standard addition method a total barcode recovery rate of 99.4% was calculated. It is expected that the loss of barcode molecules occurred during lyophilisation. Barcode DNA may stick to the surfaces of the lyophilisation tray (membrane, walls, bottom...) throughout the freeze drying process and remains within the GORE-

tray. To solve this problem after every single lyophilisation event the barcode amount of the obtained BIOtracer must be measured and can then be adjusted to a certain concentration. Another possibility is to add a high excess of non-specific DNA to the barcode-DNA solution before freeze drying. This unspecific DNA could be poly-dIdC or maybe salmon sperm DNA (cheaper) as these compounds are known from molecular-biological standard experiments like yeast-transformation protocols or southern-blotting where non-specific DNA is required [64, 65]. This DNA should function as inert background, lead to a homogenous mixture, saturate the surfaces and therefore increase the probability that most barcode-DNA gets immobilized with the carrier.

Influence of different feed-additives on PCR analysis

Before quantification of barcode molecules from different feed-additives it was necessary to a) get an idea whether the BIOtracer could get degraded within the feed-additives due to possible enzymatic activity and b) whether there are any real-time PCR inhibiting substances within the different additive matrices. Due to the low a_w-values (<0.5) DNA-degrading enzyme activity can be excluded and this positively affects the barcode- and therefore the BIOtracer stability. Activity of DNases is known to be problematic above an a_w-value of 0.8. Anyhow, the BIOtracer´s stability is contionously been tested after a certain time period to know whether the tracer is still stable. The different feed-additive structures that covers the range of very fine powdered to coarse grained and also the diverse composition of every additive makes careful tests indispensable. Evaluation of several feed additives revealed the presence of PCR inhibitors in nearly every FMZ. However, experiments pointed out that adequate dilution of the additive-samples solves the problem. But it has to be noticed that every single feed additive is different in these experiments and needs to be optimized for itself.

Barcode recovery out of different feed additives

The next step was to test whether the BIOtracer could be distributed homogenously in the different additives and how much of the barcode could be re-isolated (recovery rate). These experiments pointed out that the tracer was successfully and homogenous mixed under most additives and as expected the recovery rate differs in every single FMZ. A rSD of more than 25% indicate sampling errors within the 3 extractions. The highest rSD were detected in FMZ 7 – the sal assortment-mix and FMZ 5 a mineral-compound mixture. It can be seen in table 4.6.1. that exact these two additives show lowest recovery rates, too. The highest recovery rates were gained from FMZ 4 (probiotics, 107%), FMZ 8 (yeast-mix, 85%) and FMZ 13 (essential oils plus carrier, 52%). The bentonit-mixes (FMZ 1, 2, 12) the mineral compound mixtures (FMZ 5, 6) and the sal-assortment-mix (FMZ 7) show lowest barcode recovery of less than 10%. This low recoveries plus high rSD could result from tracer-clustering where the tracer-particles stick to single drops of the especially coarse additives. This problem can be solved by grinding the tracer-additive mix by a mortar in future. In the case of mineral-compound mixtures and sal-assortment mix high salt concentrations lead to low pH-values that inhibit PCR reaction. To adjust the pH-value of all feed additives dissolved in i) dH_2O and ii) in different Tris-buffer concentrations (e.g. 10mM as used during this work, 100mM and 1M) may solve the problems. But it has also to be tested whether a high buffer concentration of 1M Tris does negatively influence the qPCR reaction. Concerning the bentonite-mixes FMZ 1, 2 and 12 it should be pointed out that bentonite belongs to the group of mineral silicates with the property of adsorbing large amounts of DNA molecules from aqueous solutions [66]. Generally silica-membranes were used in spin columns for DNA purification. The DNA binds under high salt concentrations and can be eluted under low salt concentrations [67]. In the case of the bentonite-FMZs the barcode-DNA could probably bind to the bentonite and by the centrifugation step the barcode is centrifuged down with the bentonite. Therefore it can not/hardly be detected within the supernatant. With respect to the described “bind-and-elute” fact it should be tested whether the recovery rates and rSD-values could be improved by dissolving the samples in low-salt (<10mM Tris-buffer) solutions.

Stability testing of the BIOtracer

The shelf life of the BIOtracer was continuously tested from December 2009 till April 2010. Thereby 3 to 5 extractions were analyzed on 6 different days and with respect to all analysis (table 4.7.1 and 4.7.2) a total barcode recovery from the BIOtracer4$_{1:10}$ was calculated with 100.8% (table 4.7.3). This result shows that no barcode-DNA was degraded by DNases and the complete immobilized barcode can be recovered from the BIOtracer. Recovery rates of 130% are of course not realistic and it is suggested that these high as well as the low rates of about 74% results from DNA standard inconsistency and this needs further optimisation. However, the stability of the BIOtracer has to be observed for longer time periods to guarantee a reliable labelling system.

Outlook

The aim of this project is to develop different tracers and thereby different barcodes for many feed-additives and make the analysis method as sensitive as possible. Therefore in parallel to the barcode design and evaluation novel *TaqMan* probes are tested by the project partner for specific detection. These dual labelled probes ensure specificity and thereby make additional melting curves dispensable. Other barcode sequences have already been designed by the IFA-Tulln that allow in first evaluations detection limits of about 10 molecules and quantification limits of about 100 molecules from the BIOtracer itself but this has to be tested for analysis of traced feed-additives as well. The next step following the analysis of FMZ-tracer mixes will be the adaption of the analysis procedure to feed-additive augmented feed. Recovery rates must be evaluated and also other extraction methods could be necessary because of the high polysaccharide content of different feed (maize, corn, wheat,...). Next to this evaluation the optimized lab scale procedure has to be brought to a production scale what also includes the evaluation of bigger lyophilisation trays. For quality control reasons special criteria must be defined. This includes perfect barcode-standards and also external positive controls (EPC) that should give information whether a run can be accounted for analysis or not. This EPC could be a FMZ-tracer extract that works well and where the exact c_T -value in defined solution is known. Also internal positive controls (IPC) could be a topic as it is described by Diane L.Eisler, 2004 [68] or Amaresh Das et at, 2006 [69]. Anyhow, external or internal positive controls must be evaluated carefully but once established they will ensure quality control on a high level in the BIOtracer project.

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7 Appendix

Table 7.1: Detailed real-time PCR results of barcode recovery tests from the FMZ-BIOtracer4$_{1:10}$-intermixes (chapter 4.6.). 3 extractions from every FMZ-mix were performed (3.2.1.a), analyzed with qPCR assay for barcode 4-31 and by multiplying the measured values by their dilution factor mean values, recovery rates and rSD were calculated. rSD[a] values were calculated from C_{i0} 1-3 and values marked with (*) were error-corrected with respect to the multiple standard addition method (mSAM).

			FMZ1	**27.04.2010**			
			samples with pdIdC				
	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0=1:32	6.10E+03	6.70E+03	8.60E+03	7.13E+03	1.66E+05	7.47E+04	18%
Ci0 1:2	3.00E+03	3.80E+03	4.90E+03	3.90E+03	8.32E+04		
Ci0 1:4	5.90E+01	7.90E+01	2.50E+03	2.50E+03	4.16E+04		
calculated	6.10E+03	6.70E+03	8.60E+03				
1:1	6.00E+03	7.60E+03	9.80E+03				
1:1	2.36E+02	3.16E+02	1.00E+04				
mean value	**6.15E+03**						
recovery rate	3.70%	**8.2%** `*					
rSD	24.50%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

			FMZ 2	**04.04.2010**			
			samples with pdIdC				
Ci0=1:64	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	5.79E+02	8.23E+02	5.79E+02	6.60E+02	8.34E+04	3.75E+04	21%
Ci0 1:2	3.44E+02	4.26E+02	2.61E+02	3.44E+02	4.17E+04		
Ci0 1:4	1.50E+02	1.44E+02	1.64E+02	1.53E+02	2.09E+04		
calculated	5.79E+02	8.23E+02	5.79E+02				
1:1	6.88E+02	8.52E+02	5.22E+02				
1:1	6.00E+02	5.76E+02	6.56E+02				
mean value	**6.53E+02**						
recovery rate	0.78%	**1.7%** `*					
rSD total	17.68%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

			FMZ3	**25.04.2010**			
			samples with pdIdC	10mM extraction			
Ci0=1:512	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	2.80E+03	1.70E+03	2.50E+03	2.33E+03	1.00E+04	4.50E+03	24%
Ci0 1:2	1.60E+03	9.00E+02	1.00E+03	1.17E+03	5.20E+03		
calculated	2.80E+03	1.70E+03	2.50E+03				
1:1	3.20E+03	1.80E+03	2.00E+03				
mean value	**2.33E+03**						
recovery rate	23.33%	**51.9%** `*					
rSD total	25.67%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

			FMZ 4	**19.04.2010**		
Ci0=1:2	1	2	mean value	expected	actual expexted*	rSD[a]
Ci0	3.03E+06	3.10E+06	3.07E+06	5.34E+06	2.40E+06	2%
Ci0 1:2	1.15E+06	1.31E+06	1.23E+06	2.67E+06		
Ci0 1:4	5.33E+05	5.89E+05	5.61E+05	1.33E+06		
calculated	3.03E+06	3.10E+06				
1:1	2.30E+06	2.62E+06				
1:1	2.13E+06	2.36E+06				
mean value	**2.59E+06**					
recovery rate	48.50%	**107.8%** `*				
rSD	15.48%					

`*error corr.from mSAM

[a] = compared Ci0 1-3

				FMZ 5	**04.04.2010**		
	samples without pdIdC						
Ci0=1:128	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	7.89E+00	3.19E+01	1.62E+01	1.87E+01	4.17E+04	1.88E+04	65%
Ci0 1:2	2.15E+00	2.82E+00	5.25E+00	3.41E+00	2.08E+04		
Ci0 1:4	1.73E+00	1.95E+01	1.62E+00	7.62E+00	1.04E+04		
calculated	7.89E+00	3.19E+01	1.62E+01				
1:1	4.30E+00	5.64E+00	1.05E+01				
1:1	6.92E+00	7.80E+01	6.48E+00				
mean value	**1.86E+01**						
recovery rate	0.04%	**0.1%** `*					
rSD	65.39%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

				FMZ6	**25.04.2010**		
	samples with pdIdC			10mM extraction			
Ci0=1:32	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	6.50E+03	9.00E+03	7.40E+03	7.63E+03	1.66E+05	7.47E+04	17%
Ci0 1:2	3.20E+03	5.00E+03	3.60E+03	3.93E+03	8.34E+04		
Ci0 1:4	2.00E+03	2.50E+03	1.80E+03	2.10E+03	4.17E+04		
calculated	6.50E+03	9.00E+03	7.40E+03				
1:1	6.40E+03	1.00E+04	7.20E+03				
1:1	8.00E+03	1.00E+04	7.20E+03				
mean value	**7.97E+03**						
recovery rate	4.80%	**10.7%** `*					
rSD	17.44%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

	FMZ 7 30.04.2010						
	samples with pdIdC						
Ci0=1:256	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	1.03E+02	1.86E+01	2.40E+01	4.85E+01	2.09E+04	9.41E+03	97%
Ci0 1:2	1.04E+01	1.90E+01	1.65E+01	1.53E+01	1.04E+04		
Ci0 1:4	1.86E+01	3.64E+00	1.84E+01	1.35E+01	5.20E+03		
calculated	1.03E+02	1.86E+01	2.40E+01				
1:1	2.08E+01	3.80E+01	3.30E+01				
1:1	7.44E+01	1.46E+01	7.36E+01				
mean value	**4.44E+01**						
recovery rate	0.21%	**0.5%** `*					
rSD total	70.65%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

	FMZ 8 30.04.2010						
	samples with pdIdC						
Ci0=1:4	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	6.50E+04	3.40E+04	5.70E+04	5.20E+04	1.25E+05	5.63E+04	31%
Ci0 1:2	2.80E+04	1.50E+04	2.60E+04	2.30E+04	6.25E+04		
Ci0 1:4	1.50E+04	7.60E+03	1.10E+04	1.12E+04	3.13E+04		
calculated	6.50E+04	3.40E+04	5.70E+04				
1:1	5.60E+04	3.00E+04	5.20E+04				
1:1	6.00E+04	3.04E+04	4.40E+04				
mean value	**4.76E+04**						
recovery rate	38.08%	**84.6%** `*					
rSD	28.17%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

	FMZ9 30.04.2010						
	samples with pdIdC						
Ci0=1:4	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	8.20E+04	9.20E+04	5.40E+04	8.70E+04	5.00E+05	2.25E+05	8%
Ci0 1:2	3.80E+04	4.30E+04	4.00E+04	4.03E+04	2.50E+05		
calculated	8.20E+04	9.20E+04	5.40E+04				
1:1	7.60E+04	8.60E+04	8.00E+04				
mean value	**7.83E+04**						
recovery rate	15.67%	**34.8%** `*					
rSD	16.74%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

	FMZ 10			10.04.2010			
Ci0=1:4	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	3.33E+05	2.84E+05	2.74E+05	2.97E+05	1.34E+06	6.03E+05	11%
Ci0 1:2	1.34E+05	1.17E+05	1.26E+05	1.26E+05	6.68E+05		
Ci0 1:4	6.60E+04	5.40E+04	6.40E+04	6.13E+04	3.34E+05		
calculated	3.33E+05	2.84E+05	2.74E+05				
1:1	2.68E+05	2.34E+05	2.52E+05				
1:1	2.64E+05	2.16E+05	2.56E+05				
mean value	**2.65E+05**						
recovery rate	19.74%	**43.9%** `*					
rSD	12.46%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

	FMZ 11			17.04.2010			
Ci0=1:16	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	1.20E+04	1.50E+04	1.50E+04	1.40E+04	3.34E+05	1.50E+05	12%
Ci0 1:2	6.80E+03	8.50E+03	7.90E+03	7.73E+03	1.67E+05		
calculated	1.20E+04	1.50E+04	1.50E+04				
1:1	1.36E+04	1.70E+04	1.58E+04				
mean value	**1.47E+04**						
recovery rate	4.41%	**9.80%** `*					
rSD	11.82%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

	FMZ 12			17.04.2010			
Ci0=1:64	1	2	3	mean value	expected	actual expexted*	rSD[a]
Ci0	3.95E+02	4.38E+02	3.84E+02	4.06E+02	8.34E+04	**3.75E+04**	7%
Ci0 1:2	2.28E+02	1.45E+02	1.80E+02	1.84E+02	4.17E+04		
Ci0 1:4	1.02E+02	1.11E+02	1.00E+02	1.04E+02	2.08E+04		
calculated	3.95E+02	4.38E+02	3.84E+02				
1:1	4.56E+02	2.90E+02	3.60E+02				
1:1	4.08E+02	4.44E+02	4.00E+02				
mean value	**3.97E+02**						
recovery rate	0.48%	**1.06%** `*					
rSD	12.74%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

	FMZ13			17.04.2010			
Ci0=1:16	1	2	3	mean value	expected	actual expected*	rSD[a]
Ci0	8.90E+04	7.60E+04	6.90E+04	7.80E+04	3.34E+05	1.50E+05	13%
Ci0 1:2	3.70E+04	3.80E+04	4.00E+04	3.83E+04	1.67E+05		
Ci0 1:4	2.20E+04	2.00E+04	1.80E+04	2.00E+04	8.34E+04		
calculated	8.90E+04	7.60E+04	6.90E+04				
1:1	7.40E+04	7.60E+04	8.00E+04				
1:1	8.80E+04	8.00E+04	7.20E+04				
mean value	**7.82E+04**						
recovery rate	23.42%	**52.04%** `*					
rSD	8.69%						

`*error corr.from mSAM

[a] = compared Ci0 1-3

8 Curriculum vitae

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