Tiago Miguel Frasco Velasques

Licenciatura em Biologia Celular e Molecular

Development of Real Time PCR Meat detection kits and technology market transfer

Dissertação para obtenção do Grau de Mestre em Biotecnologia

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Coorientador: Joana Raquel Freitas Dias da Silva, BPMR - Produção e Desenvolvimento, Unipessoal, Lda.

Júri:

Presidente: Prof. Doutora Isabel Sá Nogueira

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Vogal: Prof. Doutora Fernanda Llussá

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Resumo

Recentemente, o número de casos de fraude alimentar tem vindo a aumentar na Europa e a consequente, confiança do consumidor na qualidade do setor alimentar tem vindo a diminuir. Desta forma, torna-se crucial a existência métodos fiáveis, rápidos e de baixo custo para detetar adulteração alimentar criminosa, nomeadamente das carnes.

Nesta tese foram desenvolvidos quatro kits de deteção DNA: frango, peru, cavalo e porco em matrizes alimentares, usando a técnica de Real Time PCR (Polymerase Chain Reaction). O desenvolvimento foi feito através da otimização dos kits de deteção BIOPREMIER Real Time PCR já existentes, produzidos e comercializados pela BPMR. A otimização consistiu em vários ensaios de Real Time PCR com diferentes concentrações de reagentes e programas de PCR de maneira a encontrar as concentrações ótimas para a deteção de cada target. Após a otimização, testes de validação foram feitos para autenticar os novos kits. Estes permitiram aferir que a performance dos quatro kits foi melhorada com indicadores de especificidade e inclusividade superiores a 98% e um limite de deteção que varia entre 1pg e 10pg.

Além do desenvolvimento dos kits, foi feito um estudo de mercado e desenhada uma estratégia de transferência de tecnologia para o mercado. A crescente preocupação pela segurança alimentar e tecnologias competidoras são fatores que têm impacto na comercialização dos kits. O mercado alvo é composto por laboratórios de análise alimentar, pequenos ou médios, localizados na EU, que possuem um termociclador de Real Time PCR com canais FAM e ROX. Os planos de marketing e de negócios permitiram a implementação dos kits no mercado, criando a linha “SUPREME Real Time PCR detection kits”.

Em suma, a proposta de valor dos SUPREME Real Time PCR detection kits consiste na entrega de testes low-cost, rápidos e qualitativos para detetar carne em amostras alimentares.

Palavras-chave: Real Time PCR, kit, otimização, marketing, fraude, alimentar

Abstract

In recent years, food fraud cases have been increasing in Europe and, consequently, the consumer trust over the food sector quality have been decreasing. Thus, the existence of reliable, fast and low-cost methods to detect criminal adulteration of food becomes crucial.

In this thesis, four meat detection kits to detect chicken, turkey, horse and swine DNA in food matrices were developed, using Real Time PCR (Polymerase Chain Reaction) technique. The development was done through optimization of the four BIOPREMIER Real Time PCR detection kits produced and commercialized by BPMR. The optimization consisted in several Real Time PCR assays with different concentration of some reagents and different PCR programs in order to find the optimal conditions to the detection of each target. After the optimization, validation tests were performed to authenticate the newly created kits. These tests assessed that the performance of all four kits increased with specificity and inclusivity indicators greater than 98% and the Limit of Detection varying between 1pg and 10pg,

Besides the development of the kits, it was done a market analysis and designed a transfer to market strategy. The increasing concern for food safety and the competing technologies are factors that impact the commercialization of the kits. The target market are small or medium laboratories of food analysis, located in the EU, with Real Time PCR thermal ccycler with FAM and ROX channels. The marketing and business plan allowed the kits implementation in the market, creating the SUPREME Real Time PCR detection kit product line.

In summary, the SUPREME Real Time PCR detection kits value proposition consists in the delivery of low-cost, quick and qualitative tests to detect meat in food samples.

Keywords: Real Time PCR, kit, optimization, marketing, food, fraud

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Abbreviations

FCT	Faculdade de Ciências e Tecnologia
EU	European Union
PCR	Polymerase Chain Reaction
ASAE	Autoridade de Segurança Alimentar e Económica
UK	United Kingdom
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
dNTPs	Deoxynucleoside triphosphates
dUTP	Deoxyuridine triphosphate
UDG	Uracil DNA-glycosylase
FAM	6-Carboxyfluorescein
ROX	5' CAL Fluor 610
IC	Internal Control

T_m	Melting Temperature
C_T	Cycle Threshold
$MgCl_2$	Magnesium Chloride

ºC	Degrees Celsius
g	gram
mg	milligram (10^{-3}g)
µg	microgram (10^{-6}g)
ng	nanogram (10^{-9}g)
pg	picogram (10^{-12}g)
L	litre
µl	microliter (10^{-6}L)
a.u.	Arbitrary Units

1. Introduction

This thesis describes the optimization of four BIOPREMIER Real Time PCR meat detection kits and the definition of a technology to market transfer strategy. Each kit has a specific target and are used to detect DNA of chicken, turkey, horse present in food matrixes. The main goal of these kits is to confirm the meat authenticity, preventing food fraud.

In this work, the optimization will be described in a scientific point of view and will be made a market analysis to study and define the best strategies to market launch the products.

The Real Time PCR detection kits were optimized over writing of the thesis in BPMR – Produção e Desenvolvimento, Unipessoal, Lda (from now on designated as BPMR). BPMR is the owner of the kits under the brand BIOPREMIER Real Time PCR Detection Kits. The optimized kits partake in a new product line named SUPREME Real Time PCR.

In October 2016, SGS acquire 70% of Biopremier, which included the NGS technology and the services provided by the company. The remaining 30% were kept by the CEO of Biopremier, Pedro Fernandes Antunes, that created BPMR as a spin-off company

The success achieved by Biopremier granted capital investment from several venture capital companies, which allowed the growth of Biopremier. This growth permitted the development of new tests, using Real Time PCR technology, these tests were focused in identify meat authenticity.

Currently, BPMR defines itself as a producer and developer of real time PCR diagnostic kits, that can be used in agri-food, environmental, clinical and veterinary sectors. The company has 62 diagnostic kits sold to testing laboratories in European Union (EU).

BPMR mission is to become a global reference in producing and developing kits for diagnostics' laboratories, in real time PCR detection kits. BPMR innovation and the capability to offer solutions to the costumers are some of the characteristics, for which BPMR hopes to become a reference in its industry So, BPMR ensures the quality of its products, based on scientific skills and experience of its professionals, intended to beat its costumers' expectations.

1.1. Food Fraud

BPMR has detection kits to pathogen, allergen, GMO and meat authenticity prevent food fraud. The Article 8 of the Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 regarding the protection of the interests of the consumers, "(…) shall prevent the fraudulent or deceptive practices, adulteration of food and any other practices which may mislead the consume (..:)".

Food fraud is, then, defined as any economical motivated deceptive practice regarding the food industry and it can be any adulteration, substitution, counterfeit, and intentionally mislabelling of food or food products (Spink & Moyer, 2011).

Despite the general definition of food fraud by the regulation, each EU country has its own set of laws regarding the number of checkpoints and the legal penalties.

In Portugal, the Autoridade de Segurança Alimentar e Económica (ASAE) is the entity responsible to inspect and ensure the enforcement of the rules defined by the European Parliament and by the Portuguese Parliament. To be deemed as food fraud, the food examined must contain at least 1% of not declared ingredient. In agreement with the Article 24 of the DL nº28/84: Infracções antieconómicas e contra a saúde pública, the sentence for whom committed any kind of food fraud can extend from a small fine to 2 years in prison.

In the remaining countries of EU, the laws regarding the food sector and the national agency responsible to enforce those laws vary according to the country. The entities of the European countries targeted by BPMR are listed in Table 1.1. The European Food Safety Authority (EFSA) is a European agency that cooperates with all the mentioned agencies and provides scientific advice on food-related risk.

Table 1.1 Target Countries of BPMR and its National Food Safety Agencies

Country	Food Safety Entity
Spain	*Agencia de Información y Control Alimentarios* (AICA)
France	*Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail* (ANSES)
Italy	*Comando carabinieri per la tutela della salute* (NAS)
Germany	*Bundesanstalt für Landwirtschaft und Ernährung* (BLE)
Belgium	*Agence Fédérale pour la Sécurité de la Chaîne Alimentaire* (AFSCA
Netherlands	Nederlandse Voedsel- en Warenautoriteit (NVWA)
UK	Department for Environment, Food and Rural Affairs (DEFRA)

The food fraud control differs of the food safety concept, since the food fraud implies every economical food related violations regardless of threating the public health or not, while the food safety concept is precautionary measures regarding the food consumption hazards (Spink & Moyer, 2011).

The 2013 Report on the food crisis, fraud in the food chain and the control thereof of the Committee on the Environment, Public Health and Food Safety (Esther de Lange, 2013) describe an increasing number of fraudulent labelling of food products and other fraud situations in the food

industry, as well as a decrease in the consumers trust in the food quality. In the same report, the main contributing factor to the increase of fraudulent practices is that the regulatory and judicial entities are more concerned to assure the food safety then its authenticity.

Although the food fraud is not necessarily a hazard to the public health, and that's why the authorities don't focus on this issue, there are some instance where the well-being of the population can be endangered. In example, if the labelling of a food product doesn't intentionally report some ingredients, it can cause a reaction in people allergic to those ingredients. These misleading practices can also affect the lifestyle of some people. For example, if a product, marketed as vegetarian, contains animal ingredients, it is disrespecting the lifestyle choice of a vegetarian.

In the meat industry, there are several common fraudulent practices, namely the substitution of the animal species, the falsification of the origin or diet of the animal, and the addition of non-meat ingredients to increase the volume, like water (Ballin, 2010). These practices are most used in food products like hamburgers, sausages, minced meat and other meat derived products. In the next paragraphs, three food fraud cases are discussed in order to illustrate how food fraud in the meet industry is relevant.

In 1986, a bovine meat supplier, distributing in New York school cafeterias, was arrested for adulteration his products with water and vegetal filling, throughout 5 years, to increase the volume of the product with less meat (Everstine, Spink, & Kennedy, 2013).

In the United Kingdom (UK), in 2009, the food safety authorities found traces of size increasing products of bovine and swine origin injected in chicken products. These products were denatured bulking agents that bind water and increase the product weight (Everstine et al., 2013).

The mediatic case, for which Biopremier became known for, happened in 2013 when Irish inspectors found horse DNA in bovine products in several Irish and British supermarkets.

The same type of products (hamburgers, lasagne and minced meat) were investigated in all the EU producers, and it was discovered that in some foodstuff there was between 80% and 100% horse meat. Due to its proportions, it was called horse meat scandal and led to an increasing demand of fast DNA detection kits.

In response to this scandal and the lack of consumer's trust, the EU created the European Union Food Fraud Network in 2013, which is a mechanism of cooperation of the competent authorities in the member nations, some non-member countries and the European Commission. This network allows the rapid communication between checkpoints and a more efficient detection and controls in cases of food fraud violations ("EU Food Fraud Network," n.d.).

There are several methods and techniques to species identification and detect food adulteration. These methods can be based in detection of specific proteins or other immunological proprieties; however, they are not efficient in highly processed foods or are not very sensitive as DNA detection techniques. Assuring an exact, specific and highly sensitive detection of the meat species is a necessity for which the BIOPREMIER Real Time PCR Detection kits arise.

In the next chapter we will address the Real Time PCR technology present in BPMR kits, and the materials and methods used in the optimization process. Chapter 2 addresses the technology

and the process of optimization of the four SUPREME Real Time PCR Detection kits. Chapter 3, it will be discussed the market analysis and strategy. Chapter 4 shows the business model of the SUPREME Real Time PCR Detection kits.

2. Food Tests: Technology involved, Validation tests, Materials and Methods

2.1. Real Time PCR

Kary Mullis, in 1985, has described an enzymatic amplification of DNA using a thermostable DNA polymerase, the Polymerase Chain Reaction (PCR) technique (Mullis & Faloona, 1987). This method is used in almost every molecular biology laboratories and it consists in the enzymatic and cyclic amplification of a DNA fragment using a thermostable DNA polymerase. The amplification results in the exponential increase of the number of copies of the wanted DNA fragment, in any sample containing DNA (Saik et al., 1988).

PCR technique can be used in molecular biology and microbiology to detect, identify or quantify the DNA of certain organism (Dooley, Paine, Garrett, & Brown, 2004; Yang & Rothman, 2004). Laboratories can also use this technology as a molecular diagnostic technique, overcoming the traditional microbiology techniques, as the time of diagnostic is shortened (Boer, Rahaoui, Leer, Montijn, & Vossen, 2015; Mirmajlessi, Destefanis, Gottsberger, Mänd, & Loit, 2015).

The PCR technique involves short heating and cooling cycles (Saik et al., 1988), which requires the use of a thermal cycler. Each thermal cycler has a thermal block capable of vary temperatures quickly enabling the reaction. The technique employs several reagents in a reaction mix, when exposed to the heating and cooling cyclers, can replicate the desired fragment (Welch, 2012).

The replication is catalysed by a thermostable DNA polymerase, often a *Taq* DNA polymerase. This enzyme is resistant to elevated temperatures since it was originally extracted and isolated from *Thermus aquaticus*, a thermophilic bacterium (Chien, Edgar, & Trela, 1976; Liu & Licata, 2014). The *Thermus aquaticus* was reported thriving in hot springs by Thomas D. Brock and Hudson Freez, and its ability to survive at such hot temperatures has led it to become the source of several thermostable enzymes (Brock & Freeze, 1969). A thermostable enzyme does not denature at the elevated temperatures reached every PCR cycle.

Since the *Taq* DNA polymerase can only add nucleotides to an existing strand of DNA, the PCR reaction mix has a set of short DNA fragments with sequence complementary to the 3' end of the amplification target, named DNA primers (Li-yeh, Yu-huei, & Yang, 2013; Saik et al., 1988). A set used in PCR is composed of a pair of primers: Primer Forward and Primer Reverse, that complement the 3' end of the antisense and sense strand, respectively. During the design of the primer set, the designer must keep in mind the melting temperature (T_m) of each primer (Welch, 2012). The T_m is the temperature in which 50% of the total DNA molecules of certain size and composition are denatured, and in both primers should be as close as possible. The primer size should be between 18 and 25 nucleotides and the GC ratio on each primer should be approximately 50%. Additionally, the sequences on both primers cannot be complimentary to prevent the formation of primer dimers (Li-yeh et al., 2013).

The reaction mix of a PCR method needs also deoxynucleoside triphosphates (dNTPs), used as DNA polymerase substrate, allowing the formation of a new DNA strand. dNTPs mix is comprised of deoxyadenosine (dATP), deoxycytidine (dCTP), deoxyguanosine (dGTP) and thymine (dTTP) with triphosphate groups.

To complete the reaction mix, it is necessary a buffer solution to regulate the solution pH, and bivalent cations that acts as a cofactor for the DNA polymerase, increasing its yield and facilitate the reaction (Erlich, 1989).

As said before, the PCR technique involves heating and cooling cycles, however some methods have a holding stage before the cycling stage. This initial step is done at temperatures ranging from 94ºC to 98ºC, during 1 to 10 min, to ensure the complete denaturation of the original DNA sample. The denaturation of the DNA molecules is the disruption of the hydrogen bond between base pairs of the nucleotides from two annealed DNA strands (Wang, Lim, & Son, 2014).

The cycling stage consists of 20 to 50 repeating cycles and each cycle is comprised of three steps held at different temperatures, during 10 to 30 seconds each. In the end of every cycle, the quantity of target DNA fragments is twice the quantity at the beginning of the cycle. The changes of temperatures of the first two cycles are shown in Figure 2.1. The final count of the wanted DNA fragment is 2^n, with "n" being the number of total cycles (Erlich, 1989). Since its development, the PCR technique has been used exhaustively because it decreases the need of original sample.

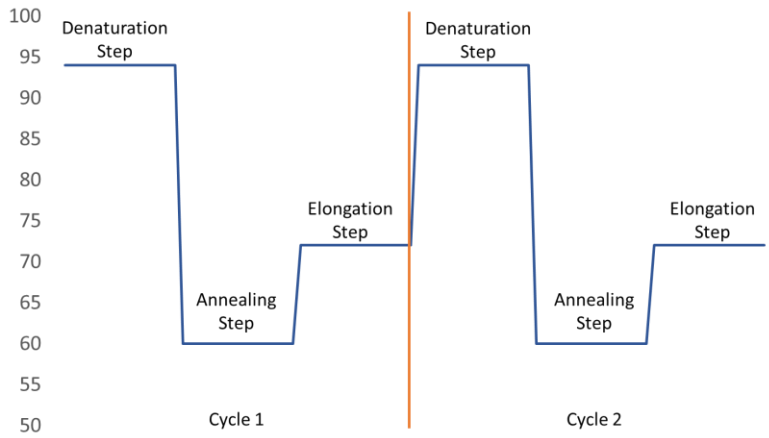

Figure 2.1 **Temperature changes in the first two PCR Cycles.** The first cycle starts with the denaturation step at temperature higher than 90ºC., the second step is the annealing step at 60ºC. and finally happens the elongation step at 72ºC. After this last step, another cycle begins repeating the denaturation, annealing and elongation steps at the same temperatures. Both cycles are separated by the orange line.

The first step is the denaturation step. This step is held for 10 to 30 seconds at temperatures above 90ºC. Despite the initial holding stage of denaturation, this step is necessary every cycle since the result of every PCR cycle is double strand DNA fragments. The high temperature will break the hydrogen bonds between the double strand, separating both DNA strands (Wang et al., 2014).

The annealing is the second step, in which the DNA primers anneal to each complementary sequence at the 3' end. The annealing temperature depends on the set of primers and is below the T_m. The temperature must be chosen as carefully as possible if the temperature is too high the annealing may not happen; if the temperature is too cold it is possible to occur non-specific hybridization (Rychlik, Spencer, & Rhoads, 1990).

The third and last step of the cycling stage is the elongation step. In this step, the DNA polymerase promotes the addition of the dNTPs to the 3' end of the previously annealed primer, creating a new DNA strand, following using the existing strand as template. The temperature of this step is dependent of the chosen DNA polymerase. Each polymerase has an optimal elongation temperature, that maximize the replication yield and minimize the probability of errors during this step. The optimal elongation temperature of the *Taq* polymerase is 72ºC (Kubista et al., 2006).

Since the development of PCR technique, have arisen other PCR-based techniques or modification to the original method. The main goals of this modifications are decrease the non-specific amplification, facilitate the analysis of PCR results or use distinct types of samples. Hot Start PCR, Reverse Transcriptase PCR and Real Time PCR are some example of these modified techniques.

The technology developed in the experimental segment of this thesis uses Real Time PCR to detect specific DNA in food matrices.

Real Time PCR was developed by Higuichi, R in 1993 as a real time monitorization of amplification reactions system (Higuchi, Fockler, Dollinger, & Watson, 1993). This system was based on the intercalation of ethidium bromide (EtBr) in the amplified DNA at every PCR cycle. The reaction took place in a modified thermal cycler, that radiate the solution with ultraviolet (UV) light, and a CCD camera detected the emitted fluorescence. The intensity of fluorescence in each cycle was proportional to the number of DNA copies.

The ease of PCR analysis, provided by the Real Time PCR technique, led to a widespread use of this technology, and consequent improvements to the original system developed by Higuchi. The precision of the current Real Time PCR thermal cyclers allowed the use of other fluorescent agents to monitor the PCR amplification. A Real Time PCR thermal cycler consists in a conventional thermal cycler with a light source to excite the fluorescent agents, a light sensor to measure the intensity of the fluorescence, and an external computer to control de thermal cycler. (Arya, Shergill, Williamson, & Gommersall, 2005; Kubista et al., 2006) Currently, there are two wide used methods for the Real Time detection of PCR products: DNA-binding fluorescent dyes and fluorophore-labelled oligonucleotides (Navarro, Serrano-heras, Castaño, & Solera, 2015; Ponchel et al., 2003; Tse & Capeau, 2003).

Both methods rely on fluorescence emission and its detection by the sensors of the thermal cycler. Fluorescence happens when an electron of a molecule relaxes to its lower-energy state, emitting a photon (Sanderson, Smith, Parker, & Bootman, 2014). In Real Time PCR, the fluorescent molecules absorb the light emitted by the thermal cycler, and its electrons are excited to the first excitatory state. The photons released by relaxation of these electrons produces light, and this process is known as fluorescence. The light emitted by the fluorescent molecules as longer wavelength than the absorbed light, meaning that the fluorescent light has a lower energy than the

absorbed light (Navarro et al., 2015; Sanderson et al., 2014). Every fluorescent molecule has a light absorption wavelength range, in which is capable of emitting fluorescence; if the light focused on the molecule is outside this range, it does not emit fluorescence because it doesn't absorb the energy.

The DNA-binding fluorescent dyes are non-specific intercalating agents that are inserted in the minor groove of the dsDNA, at the end of each PCR cycle (Navarro et al., 2015; Ponchel et al., 2003). The binding of these dyes increases its fluorescence, allowing the detection by the sensors of the thermal cycler. The main advantage of this detection method is that the use of DNA-binding dyes is cheaper than fluorophore-labelled oligonucleotides. However, such dyes are non-specific for any target, meaning that it is possible to detect non-specific PCR product or other by-products, such as primer dimers, thus biasing the PCR analysis. To minimize the effect of the non-specific DNA binding it can be done a melting curve analysis, since the specific DNA product denatures at a higher temperature than non-specific DNA and primer dimers (Ririe, Rasmussen, & Wittwer, 1997). The most common used DNA-binding dye is SYBR Green, that absorbs blue light and emits green light (Ponchel et al., 2003).

The fluorophore-labelled oligonucleotides are small single DNA strand with attached fluorophore, which is a small molecule capable of emitting fluorescence. The fluorophore molecules can be classified into donor or reporter fluorophore and acceptor or quencher fluorophore (Marras, 2006; Navarro et al., 2015). When light at a specific wavelength focus in a donor fluorophore molecule, his electrons are excited to the first energy state. The donor fluorophore can then emit fluorescence if the electrons relax to the ground state, however a can happen a Fluorescence Resonance Energy Transfer (FRET). FRET is a distance-dependent phenomenon and happens when a donor and receptor fluorophores are positioned within certain distance (10 and 100 Å) (Elangovan et al., 2003; Sekar & Periasamy, 2003). At those distances, the excited state energy is transferred from the donor fluorophore to the acceptor, that can dissipate this energy as heat (FRET-quenching), or emit as fluorescence, because the acceptor fluorophore can be a fluorescent as well. The fluorophore-labelled oligonucleotides can be sorted into three various categories: primer-probes, nucleic acid analogues, and DNA probes (Navarro et al., 2015). The Biopremier Real Time PCR detection kits uses DNA hydrolysis probes.

DNA probes are oligonucleotides coupled with a fluorophore or a set of donor and acceptor fluorophores. There are two types of DNA probes: hybridization probes and hydrolysis probes. With the hybridization probes, the fluorescence occurs when the probe anneals with the template DNA strand (Guo, Ju, & Turro, 2012; Marras, 2006).

Hydrolysis probes, also known as *Taq*Man ® probes, are design to bind to a sequence of the template DNA molecule and have a donor fluorophore at the 5'-end and an acceptor fluorophore at the 3'-end (Navarro et al., 2015) In solution and when annealed, the acceptor fluorophore FRET-quenches the fluorescence emitted by the donor fluorophore due to its proximity. However, during the extension step, the 5'-3' exonuclease activity of the DNA polymerase degrades the DNA probe separating both fluorophores, allowing the emission of fluorescence by the donor fluorophore end (Dooley et al., 2004; Navarro et al., 2015; Tse & Capeau, 2003).

Despite the wide variety of fluorophores, the real time PCR thermal cyclers are not equipped with the sensors to detect them all, meaning that is a limited number of fluorophores that can be used in real time PCR. The Biopremier Real Time PCR detection kits have DNA hydrolysis probes,

essentially, combined with 6-Carboxyfluorescein (FAM) and 5' CAL Fluor 610 (ROX). FAM is a fluorophore that has a maximum light absorption at a wavelength of 495 nm and emits fluorescence with a wavelength of 515nm (Dooley et al., 2004; Marras, 2006). While the ROX fluorophore absorbs maximum energy at a wavelength of 575 nm and emits light with a wavelength of 605 nm (Marras, 2006).

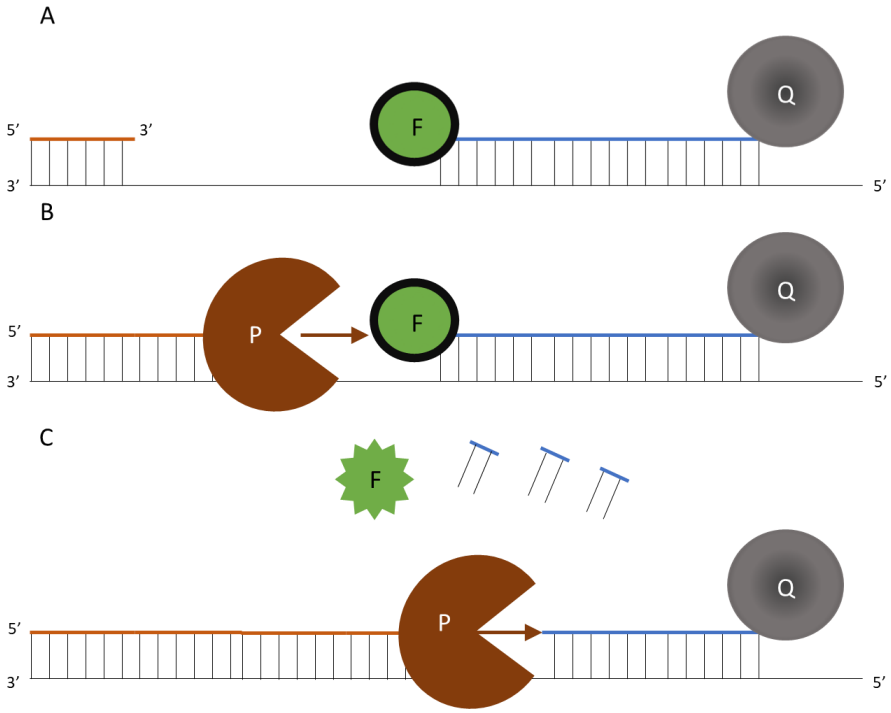

Figure 2.2 **Mechanism of action of hydrolysis probe.** [A] In the annealing step, the DNA probe (blue) and the DNA primers (orange) bind to the DNA template. The probe has two fluorophores: the donor fluorophore (F) and the acceptor fluorophore (Q). The donor fluorophore doesn't emit fluorescence due to its proximity to the acceptor fluorophore. [B] During elongation step, the DNA Polymerase (P) promotes the addiction of dNTPs to the 3' end of the DNA primer, building the new DNA strand (orange). [C] The 5'-3' exonuclease activity from the DNA polymerase degrades the probe, separating the donor fluorophore from the acceptor. Due to the increase of distance between both fluorophores, the donor fluorophore emits fluorescence, that can be detected by the real time thermal cycler sensors.

2.2. Materials and Methods

The experimental procedure of the optimization of the BIOPRIMIER Real Time PCR meat detection kits and its validation tests were done at the BPMR facilities, located in Amadora INOVA. The BPMR facilities are divided into three independent laboratories and one equipment room, which follow a unidirectional flow, to minimize the risk of contamination between steps.

The first laboratory is the DNA room. Any handling of matrices happens in this room, this includes the reception of the real matrix or the Interlaboratorial Proficiency Test Material, identification, and separation into aliquots of 200mg. Interlaboratorial Proficiency Test Material are unknown matrices provided by other laboratories to independently assess the performance of the

kits. The DNA is then extracted in this room from food matrices or microorganisms. However, it is necessary a biosafety cabinet (BSC) to protect the operator of any risk, since the microorganisms are harmful to the operator.

The next area is the PCR1 room. The room is equipped with a laminar flow cabinet and store the reagents to prepare the Master Mixes. The third chamber is the PCR2 room. The goal of this room is conclusion of the Master Mix by adding the Intern Control DNA, and the distribution of the Master Mix and sample DNA into PCR tubes, in another laminar flow cabinet. The laminar flow cabinets prevent any kind of sample contamination. Finally, the equipment room is equipped with the Applied Biosystems® 7500 Fast Real-Time PCR thermocycler.

The steps of the referred workflow follow intern protocols and regulations.

2.2.1. DNA Samples

Since the optimized kits' goal is to detect animal DNA in food matrices and to prove the labelling authenticity, the DNA must be extracted from a variety of food matrices All the food matrices were acquired in the local supermarket and comprises several types of raw meat, processed and ready-to-eat foods and vegetable and dairy products. The matrices were cut and separated into 12 aliquots of 200mg each and stored at -20ºC. Each matrix was coded with an internal Identification Code. The samples' DNA were then extracted, quantified and verified.

The detailed list of matrices used in the optimization of the BIOPREMIER Real Time PCR detection kits is in the Table 2.1.

2.2.1.1 DNA Extraction

The extraction was performed using the Nucleospin ® Food kit from Macherey-Nagel. An extraction with this kit needs 200mg of homogenized food matrix.

The Nucleospin® Food kit starts with the cell lysis, using the CF buffer and a Proteinase K, and incubated during 30 min. After incubation time, the cell lysis is complete, and it is necessary a centrifugation to separate the DNA of the cellular debris. The next step includes the mixture of the supernatant with the DNA with a binding buffer and ethanol, to ensure the conditions to the binding to the silica membrane present in extraction columns. Once bound to the silica membrane, it is washed with washing buffers CQW and C5 to remove any potential PCR inhibitors such as proteins or fats. The last step is to elute the DNA from the silica membrane, using an elution buffer. All the reagents mentioned above, minus the ethanol, and the silica membrane columns are provided in the Nucleospin® Food kit.

2.2.1.2 DNA Quantification

After the extraction, it is necessary to quantify the DNA extracted to assure the precision in the optimization process. In this thesis, all the quantification assays were done with the Invitrogen Qubit® 3.0 quantitation kit. The kit uses the Qubit® 3.0 fluorometer to detect the fluorescence of DNA binding dyes. The quantification assay consists of the addition of 1µl of sample solution to 199µl of Qubit® working solution in a 500µl microtube, the final solution is then read in the fluorometer. The instrument directly shows the concentration of DNA in the sample solution.

Table 2.1 Food Matrices and corresponding Identification Code. All the matrices were bought in the local supermarket and it were used two aliquots of 200mg of each.

Identification Code	Food Matrix
C4	Regional Sausage
C5	Horse Meat
C6	Beef
C8	Swine Meat
C9	Shredded Duck Meat
C10	Beef Meatballs
C12	Lamb Meat
C13	Duck Rice with Chorizo
C14	Pork Lasagne
C15	Turkey Meat
C16	Turkey Ham
C17	Chicken Meat
C18	Chicken Ham
C19	Ham
C20	Potato
C21	Baby Food
C23	Corn
C24	Poultry Sausages
C25	Pork Sausages
C26	Goose Foie Gras
C28	Chicken Hamburger
C29	Rabbit Meat
C30	Soy
C31	Duck Foie Gras
C32	Cat Food
C33	Goat Cheese
C51	Pork Bean Stew
C52	Pork Chorizo
C53	Mortadella
C54	Chicken Nuggets
C55	Turkey Hamburger

C56	Swine Hamburger
C57	Beef Hamburger
C60	Cow Cheese
C65	Boar Meat
C66	Vegetarian Meatballs

2.2.1.3 Sample Verification

To guarantee the authenticity of the samples, according to its label and verify the DNA in each to not mislead the optimization process, it was used the existing BIOPREMIER Real Time PCR Meat detection kits. The entire range of kits to identify meat DNA in food samples allow the detection of swine, horse, turkey, chicken, duck, cow, goat or sheep DNA.

2.2.2. PCR Reagents

The optimization process relies on several Real Time PCR's, with a wide variety of conditions, specifically different concentrations of reagents and different PCR programs.

The reagents used in all Master Mixes throughout the experimental part of this thesis were: PCR ready water, NH_4 Buffer, $MgCl_2$, dNTPs, DNA polymerase, Deoxyuridine Triphosphate (dUTP), Uracil DNA-glycosylase (UDG). The mixes also contain DNA for reaction Internal Control (IC) and its respective set of primers and probe to ensure the quality of the reaction.

In all the mixes, a set of primers and probe are present; however, the set change according with the specific target. There are two sets of primers and probe design for each target, the one already used by BPMR and a newly designed set.

The presence of any inhibitor in the reaction mix, can be translated into a false negative result. To prevent this type of result the IC is added to the mix. The IC is amplified simultaneously with the target DNA and validates any negative result.

The dUTP and UDG serve as an additional step to avoid the laboratory contamination with PCR product. The DNA polymerase will use dUTP instead of dTTP in the extension step. The probability of the substitution of dTTP by dUTP depends on the concentration of these nucleotides. The UDG recognize and cleave the PCR product where the replacement has occurred (Burkardt, 2000; Kleiboeker, 2005).

Some mixes also have Dimethyl sulfoxide (DMSO) or Betaine. This two compounds serve as PCR enhancers which increase the specificity and the yield of the PCR amplification, more specific in DNA with high GC content (Chakrabarti & Schutt, 2001; Henke, Herdel, Jung, Schnorr, & Loening, 1997; Ralser, Querfurth, Lehrach, Yaspo, & Krobitsch, 2006)

2.2.3. Optimization of PCR conditions

The optimization of the conditions of the Real Time PCR comprises changes to the concentration of some reagents and changes in the PCR program. The changes' goal is to improve the sensibility, specificity and yield of the PCR. The reagents which concentration was altered were $MgCl_2$, dNTPs, dUTP, primers set specific for the target and IC DNA. The enhancer's presence, DMSO or betaine, were also tested. The optimal annealing temperature and the specific time of each PCR step were tested after the concentrations of the reagents were defined.

Since it's the optimization of four separated BIOPREMIER Real Time PCR Meat detection kits, four different sets of reactions, each aiming a distinct target, were completed. It was used different samples in each set, in order to test the specificity and sensibility of the kit.

The set of samples used in the optimization of each kit is listed in the Table 2.2.

Table 2.2 Samples used in the kit optimization for each target

Target	Sample	Identification Code
Swine	Horse Meat	C5
	Pork Lasagna	C14
	Ham	C19
	Rabbit Meat	C29
Horse	Horse Meat	C5
	Beef	C6
	Pork Meat	C8
	Beef Meatballs	C10
	Pork Lasagna	C14
Chicken	Shredded Duck Meat	C9
	Turkey Meat	C15
	Chicken Meat	C17
	Chicken Ham	C18
Turkey	Shredded Duck Meat	C9
	Turkey Meat	C15
	Turkey Ham	C16
	Chicken Meat	C17

Additionally, all PCR assays must have at least a positive and negative control. The negative control is PCR water and the positive control depends on the target. It's from a sample that it's confirmed to have the targeted DNA. The samples used as positive controls in the assays for each target are listed in Table 2.3.

Table 2.3 Positive Controls for each target

Target	Positive Control	Identification Code
Swine	Ham	C19
Horse	Horse Meat	C5
Chicken	Chicken Ham	C18
Turkey	Turkey Ham	C16

2.2.4. Analysis of Results

The optimal conditions were chosen by comparing the results of Real Time PCR assays and selecting the conditions with the best results. The results are shown in an amplification curve traced in a plot of fluorescence intensity by cycle. The fluorescence intensity is the strength of the fluorescence signal. Since it depends of the molecules excitation by the instrument, the results are shown in Arbitrary Units (a.u.) normalized to the base value. The intensity is proportional to the quantity of target DNA in the solution. The interpretation of the results of a Real Time PCR assay is associated with two variables: the fluorescence's maximum intensity and the Cycle Threshold (C_T). These indicators are associated with the higher concentration of initial DNA. The maximum intensity of fluorescence usually is the fluorescence intensity at the last cycle. The C_T is the cycle in which the fluorescence intensity surpasses the fluorescence threshold and is associated to the start of the exponential amplification phase. A threshold is the value of intensity for which the fluorescence surpasses the background fluorescence intensity. The C_T correspond to the cycle where the amplification curve intersects the threshold line. If the amplification curve isn't visible or doesn't intersect the threshold line, there was no DNA amplification, or the initial DNA concentration were lower than the limit of detection of the kit ("Real-time PCR : Understanding Ct," 2011).

The C_T and intensity necessary to consider an assay positive or not, varies from assay to assay. BIOPREMIER Real Time PCR meat detection kits, an assay is considered positive if has a C_T lower than 37 and a fluorescence intensity higher than 50000

As said in session **2.1 Real Time PCR,** the BIOPREMIER Real Time PCR meat detection kits have probes labelled with two fluorophores, the DNA probe for each target is labelled with FAM fluorophore and the IC probe is labelled with ROX fluorophore. The combination of these two probes allow a more thorough analysis.

The assay must be validated through the analysis of the controls. Independently of the target, the controls must have the results described in Table 2.4. If the controls don't match the following results the assay cannot be valid, and the assay must be repeated.

Table 2.4 positive and negative control results

	Target Detection (FAM)	IC Detection (ROX)
Positive Control	Positive	Not Significant
Negative Control	Negative	Positive

If the controls match Table 2.4 results, the results of the samples can be interpreted as said in Table 2.5. A positive sample result means that this sample has targeted DNA. A negative sample result means that there is no targeted DNA present. An invalid result means that the reaction hasn't occurred correctly; this result can be due to lack of some reagent or sample driven inhibition.

Table 2.5 Interpretation of the sample results

Target Detection (FAM)	IC Detection (ROX)	Interpretation
Positive	Positive	Positive Sample
Positive	Negative	Positive Sample
Negative	Positive	Negative Sample
Negative	Negative	Invalid

2.2.5. Validation Tests

The validation tests are extremely important in order to calculate the kits performance, and necessary to market launch the kits.

The set of validation tests comprises exclusivity, inclusivity, sensibility and robustness tests (Broeders et al., 2014). To ensure the validity of the kits, there should be done specificity validation tests and sensibility tests.

2.2.5.1 Exclusivity Tests

The exclusivity tests are a set of Real Time PCR assays with non-target samples, meaning that the matrices used have no target DNA. The exclusivity tests infer the affinity of the primers and consequential specificity of the kits. The kits' specificity indicator is the percentage of true negative results in all the specificity results, including the true negative results and the false positive results, and must 95% or higher to validate the tests (Broeders et al., 2014; Kawamura, 2002). To ensure the specificity of the kits, the assays used non-target samples with elevated DNA concentrations, from 1ng to 5ng. The specificity indicator is calculated through equation 1 (Kawamura, 2002).

$$Specificity\ Indicator = \frac{100 * True\ Negative\ Results}{True\ Negative\ Results + False\ Positive\ Results} \quad (1)$$

The exclusivity tests for each of the four targets used different samples. The lists of the samples used in each exclusivity test is available in the Appendix 1.

2.2.5.2 Inclusivity and Sensibility Tests

The inclusivity and sensibility tests are a set of Real Time PCR assays with samples with target DNA. The inclusivity tests ensure the kits detect the target DNA in all positive samples. The inclusivity tests allow the calculation of the kits' inclusivity indicator. This indicator is the percentage of true positive results in all the inclusivity test results, including the true positive results and the false negative results, and must be 95% or higher. (Broeders et al., 2014; Kawamura, 2002). The inclusivity indicator is calculated through equation 2 (Kawamura, 2002). The sensibility tests use samples with target DNA at concentration of 100pg, 10pg, 5pg and 1pg to infer the limit of detection (LoD). The LoD is the lowest DNA concentration to having positive results in all samples.

$$Inclusivity\ Indicator = \frac{100 * True\ Positive\ Results}{True\ Positive\ Results + False\ Negative\ Results} \quad (2)$$

The inclusivity and sensibility tests for each of the four targets used different samples. The lists of the samples used in each inclusivity and sensibility test is available in Table 2.6.

Table 2.6 Samples used in each kit inclusivity test

Target	Sample	Identification Code
Swine	Regional Sausage	C4
	Swine Meat	C8
	Duck Rice with Chorizo	C13
	Ham	C19
	Pork Sausage	C25
	Pork Chorizo	C52
	Swine Hamburger	C56
Horse	Horse Meat	C5
	Beef + Horse Meat	C10+C5

	Pork Lasagna + Horse Meat	C14+C5
	Beef Hamburger + Horse Meat	C57+C15
Chicken	Regional Sausage	C4
	Poultry Sausage	C24
	Chicken Meat	C17
	Chicken Hamburger	C28
	Chicken Nuggets	C54
Turkey	Turkey Meat	C15
	Turkey Ham	C16
	Poultry Sausage	C25
	Turkey Hamburger	C55

2.2.5.3 Robustness Tests

The robustness tests are exclusivity and inclusivity tests, to infer the performance of the kits in adverse conditions. This method guarantees the performance of the tests assuming that the user's equipment is not calibrated or shows temperature shifts (Broeders et al., 2014).

The robustness exclusivity tests comprise of regular exclusivity tests with as decrease the annealing temperature by 2ºC, to facilitate the primer annealing and facilitate the reaction.

The robustness inclusivity tests comprise of regular inclusivity tests with an increase the annealing temperature by 2ºC, to difficult the primer annealing and difficult the reaction

2.3. Results and Discussion

The results and subsequent discussion will be shown by target species. The results correspond to the outcomes of the Real Time PCR and were obtained directed through fluorescence readings by the Real Time thermal cycler. The results are displayed in order according with the conditions previously defined.

The results are shown in plots of intensity of fluorescence by cycle number and each plot is composed by amplification curves: target amplification curve and IC amplification curve. Both curves were analysed and compared between different assays, simultaneously. The curves were assessed according with the C_T and the intensity of fluorescence, bearing in mind that higher intensity comprises a greater contamination risk.

All assays contained a positive and negative controls to evaluate the efficiency of the tests by monitor potential contaminations, DNA degradations or operator's mistakes.

Each PCR assay is labelled with a number that correspond to its BPMR code.

2.3.1. Chicken Detection Kit

2.3.1.1 Optimization of PCR Conditions

The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined to the chicken detection kit.

To guarantee the performance of the optimized solution and subsequent amplification of target DNA, preventing non-specific amplification, matrices with and without chicken DNA were used as testing samples. All samples were tested in a concentration of 1ng/µl. The results and subsequent analysis will highlight a sample with the targeted sequence (C18), and a sample without the target sequence (C9). Amplification is not expected in sample C9 because it is from duck meat. If an amplification is shown in sample C9, the PCR conditions must be reconsidered, because it means the lack of specificity of the test. To define the C_T and consider an amplification positive, the amplification curve must have an intensity of fluorescence greater than the threshold of 50000.

In Table 2.7, it is shown the order of the conditions tested, as well as the PCR assays performed and the respective BPMR code.

To facilitate the comparison between different assays, plots were standardized and a maximum of 500 000 units of fluorescence intensity was defined.

Table 2.7 List of ordered and optimized PCR conditions of the Chicken Detection kit and its BPMR Code. The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined.

Order	Test Conditions	BPMR Code
1	Type of primers and probe	PCR 84 and 85
2	Concentration of $MgCl_2$ and dNTPs	PCR 102, 103 and 104
3	Presence of DMSO	PCR 102 and 110
4	Concentration of primers and probe	PCR 110, 111 and 112
5	Annealing Temperature	PCR 110, 132 and 133
6	Duration of each cycle phase	PCR 110, 152, 153 and 154
7	Concentration of dUTP	PCR 153 and 174

2.3.1.1.1 Type of primers and probe

The first condition optimized in the chicken detection kit was the type of primers and probe. The analysis was done to two different sets of primers and probe. The first set is already used by BPMR in the current BIOPREMIER Chicken detection kit and the second is a new set of primers and probe newly designed and never used in BPMR laboratories. Figure 2.3 shows the results obtained using the current set [A] and the new set [B] with the same samples. The samples represented are Chicken Ham, C18 (blue continuous line); Duck Meat, C9 (purple dotted line); Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

As expected, the sample C9 didn't show amplification in any PCR, meaning that neither set is unspecific and doesn't detect duck.

Since the sample C18 have chicken DNA in it, it shows an amplification curve in both PCR. Analysing the C_T, both curves pass the threshold near the 22nd cycle. However, the peak of fluorescence intensity of the sample C18 in PCR 84 is greater than the peak in PCR 85. This means that the current set of primes have a better affinity to the target DNA, than the new set, facilitating the annealing and consequently the DNA amplification.

The IC of the negative sample shows an amplification curve in both PCR with a similar C_T of 29/30. The intensity of fluorescence is greater in the curve of PCR 85 than the one of PCR 84. The IC of the positive sample just shows a significant amplification curve in PCR 85. In PCR 84, the IC of the positive sample only amplified in the last two cycles, not reaching the threshold. The quantity of target DNA in the reaction mix is greater comparing with the IC DNA. The difference of DNA concentration causes a competition for the active centres of the DNA polymerases, resulting in the amplification of the target DNA in favour of the IC. Since the PCR 84 amplified more target DNA, the IC curve is almost null (Markoulatos, Siafakas, & Moncany, 2002; Schrader, Schielke, Ellerbroek, & Johne, 2012).

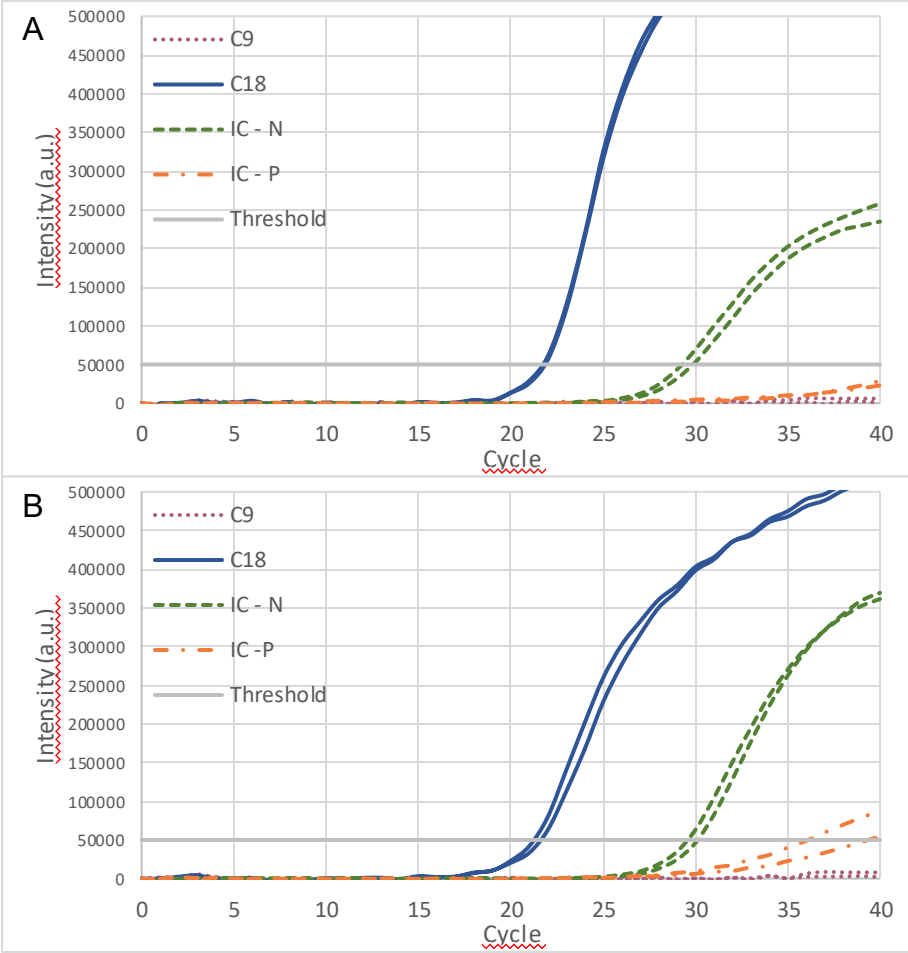

Figure 2.3 **Graphic representation of the PCR results:** PCR 84 with current set of primers and probe [A] and PCR 85 with the new set of primers and probe [B]. The samples represented are C18 (blue continuous line), C9 (purple dotted line), Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The primers and probe defined for the SUPREME Real Time Chicken detection kits are the current ones, because the peak of intensity of fluorescence of C18 is greater in PCR 84 and the set of primers and probes doesn't have an effect to the intensity of the fluorescence of the curve of the IC negative samples.

2.3.1.1.2 Concentration of $MgCl_2$ and dNTPs

The concentration of $MgCl_2$ and dNTPs was defined after the set of primers and probe were chosen. The optimal concentration of these reagents was chosen by analysing the PCR 102, 103 and 104. The PCR 102 had the lowest concentration of both reagents, while PCR 104 used the highest concentration. The PCR 103 maintained the low concentration of $MgCl_2$ of PCR 102 but used the high concentration of dNTPs of PCR 104. Figure 2.4 shows the PCR assays with the lowest concentration of $MgCl_2$ and dNTPs [A], the highest concentration of the reagents [B] and with the mixture between both previous assays [C]. All assays were done using the same samples. The

samples represented are Chicken Ham, C18 (blue continuous line); Duck Meat, C9 (purple dotted line); Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

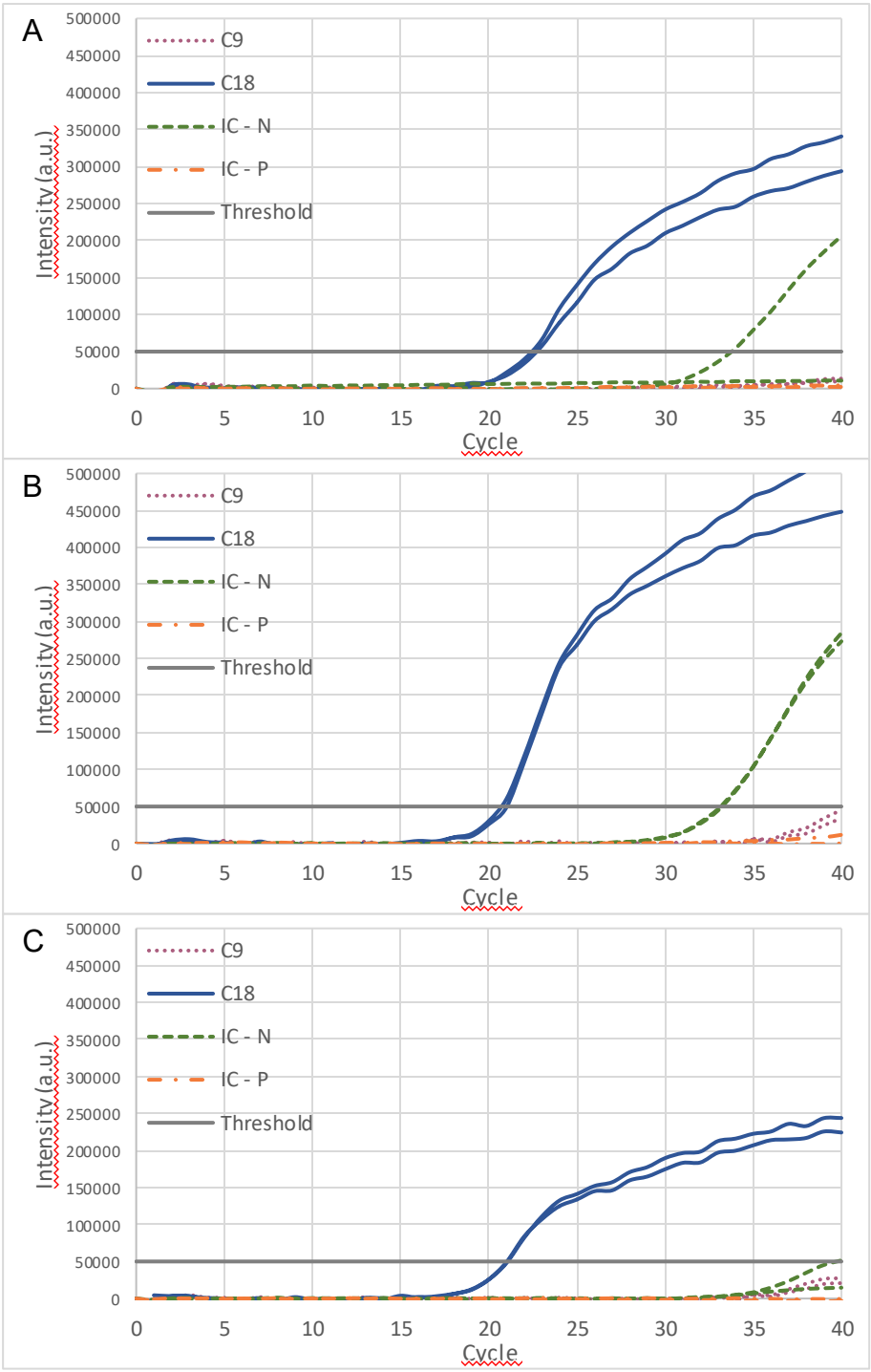

Figure 2.4 **Graphic representation of the PCR results:** PCR 102 with lowest concentration of both reagents [A], PCR 104 with the highest concentration of both reagents [B] and PCR 103 with low concentration of $MgCl_2$ and high concentration of dNTPs [C]. The samples represented are C18 (blue continuous line), C9 (purple dotted line), Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey co ntinuous flat line.

In PCR 102 (A), the results are as expected. The sample C9 didn't amplify because it doesn't have chicken DNA. The sample C18 with chicken DNA shows an amplification curve with a C_T, approximately, in the 23rd cycle and a maximum intensity of fluorescence between 300000 and 350000. The IC of the negative sample also shows an amplification curve with a C_T in the 34th cycle and with the intensity of fluorescence reaching the 200000. The IC of the positive sample didn't amplify due to the competition between the target DNA and the IC for the reagents.

Although the positive sample C18, in PCR 104 (B), has a higher maximum intensity of fluorescence (around 500000) and a lower C_T cycle (21st cycle) than the same sample in PCR 102, the sample C9 in PCR 104 shows amplification. The sample C9 doesn't have chicken DNA in it and it mustn't amplify as non-specific amplification undermines the specificity of the kit.

The amplification of the negative sample and the better amplification curve of the sample C18 can be explained due to the increase in the concentration of $MgCl_2$. The Mg^{2+} is a cofactor of the DNA polymerase by complexing with dNTPs forming the substrate of the enzyme. The ion also facilitates the base pairing between two strands of DNA increasing the stability of the primer annealing. In high concentrations of Mg^{2+}, the substrate for the DNA polymerase is widely available and the high stability of primer annealing increase the probability of mispairing bases, diminish the specificity of the reaction (Markoulatos et al., 2002; Montgomery & Wittwer, 2014).

In PCR 103, the negative sample C9 didn't amplify and the positive sample C18 have a good amplification curve with a C_T in the 21st cycle and the lowest intensity of fluorescence, reaching 250000. However, the IC of the negative sample have a low and late amplification. The C_T of this curve is almost in the last cycle and the intensity of fluorescence barely reach the 50000. The absence of a good amplification curve for the IC of the negative sample and the low intensity of fluorescence in the sample C18 amplification curve means that the conditions of this assay are not appropriate for meat detection.

When comparing with the PCR 102, the only condition changed was the concentration of dNTPs, which is greater in PCR 103. As said before, the Mg^{2+} ions complex with the dNTPs available forming the substrate that the DNA polymerase can use, however if there is too much free dNTPs it will cause a Mg^{2+} depletion and a consequent inhibition of the reaction, as verified in PCR 103 (Markoulatos et al., 2002).

After the analysis of the three PCR assays, the PCR 102 have the best combination of $MgCl_2$ and dNTPs concentration. This assay used the lowest concentration of both reagents not compromising the specificity and not inhibiting the reaction. These conditions were selected for the SUPREME Real Time PCR Chicken Detection kit.

2.3.1.1.3 Presence of DMSO

The optimization of the chicken detection kit counted with a step to evaluate the usage of DMSO. The PCR 102 which contained DMSO was analysed against the PCR 110 that not used DMSO nor another PCR enhancer. Figure 2.5 shows the PCR 102 with DMSO [A] and PCR 110 without DMSO [B]. All assays were done using the same samples. The samples represented are Chicken Ham, C18 (blue continuous line); Duck Meat, C9 (purple dotted line); Internal Control of the

negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

Neither assay has non-specific amplification as the sample C9 didn't amplify. The IC of positive sample also didn't amplify, due to the competition for the reaction reagents as it can be verified with the high intensity of the target amplification curve.

Sample C18 shows an amplification curve in both PCR assays. The intensity of fluorescence in PCR 102 is between 300000 and 350000, which is lower when compared with the intensity of fluorescence in PCR 110, that reached 500000. The C_T of the amplification curve is higher in PCR 102 (23rd cycle) than the C_T of the amplification curve of the same sample in PCR 110 (21st cycle).

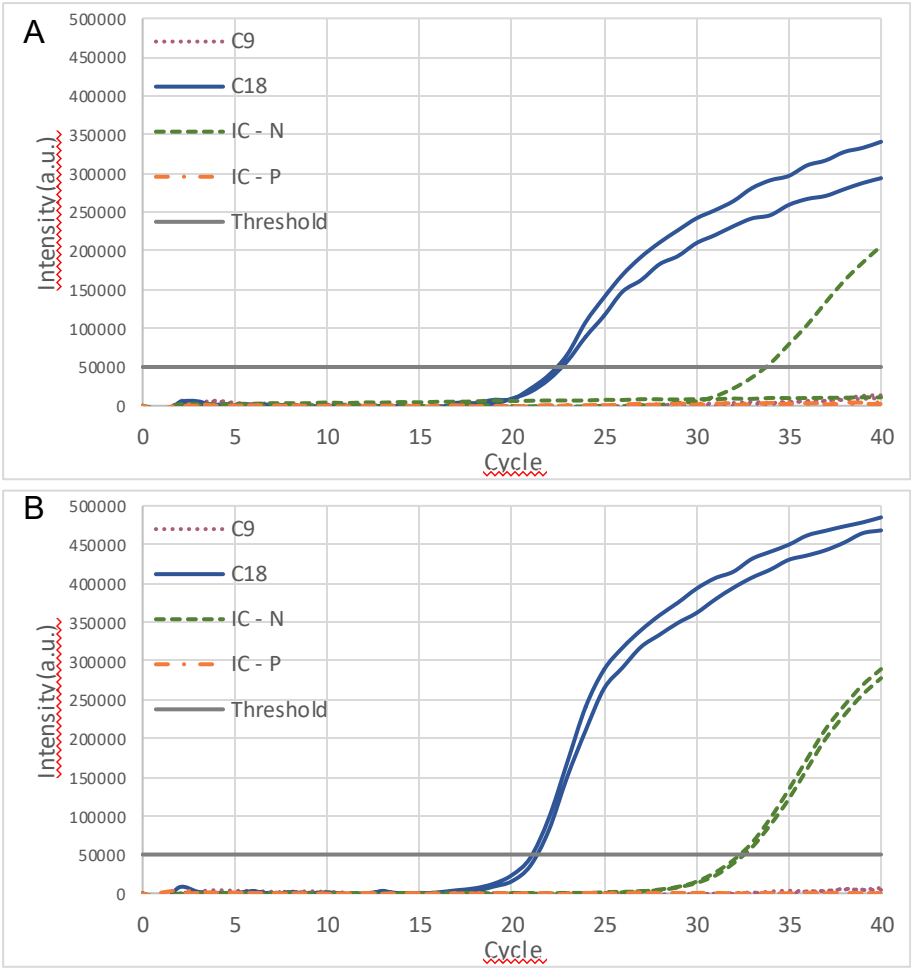

Figure 2.5 **Graphic representation of the PCR results:** PCR 102 with DMSO [A] and PCR 110 without DMSO [B]. The samples represented are C18 (blue continuous line), C9 (purple dotted line), Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

This tendency is confirmed with the IC from the negative sample. In PCR 102, the amplification curve of this sample has a C_T in the 34th cycle and the intensity of fluorescence reaches the 200000. While, in PCR 110, the amplification curve is better, having a C_T in the 32nd cycle and the intensity of fluorescence almost reaches 300000.

This results are as expected because DMSO is an enhancer which increase the specificity the PCR by lowering the T_m (Chakrabarti & Schutt, 2001; Henke et al., 1997; Montgomery, Rejali, & Wittwer, 2014; Ralser et al., 2006). The lower T_m prevents the occurrence of secondary DNA structures and non-specific binding, because the lower the T_m, the more difficult is to the primer to bind to the template. This thermodynamic behaviour increases the specific annealing, since the correct base pairing is more favourable. However, by lowering the T_m, the primer has difficulty binding to the template causing a decrease in the yield of the reaction when comparing positive samples with assays without DMSO. As so, the amplification curve of C18 and the IC of the negative sample in PCR 110 is better than the amplification curve of these two samples in PCR 102.

Therefore, the SUPREME Real Time PCR will not contain DMSO as the PCR 110 have better amplification curve in the sample C18 and in the IC of negative samples, without compromising the specificity in sample C9. However, in the exclusivity tests it will be confirmed the specificity of the reaction and if the DMSO is necessary.

2.3.1.1.4 Concentration of primers and probe

The concentration of primers and probe was the fourth condition optimized in the chicken detection kit. So, were analysed the PCR 110, 111 and 112, with different concentrations of primers and probe. The assays were done using increasing concentrations of this reagents: the PCR 111 using the lowest concentration, the PCR 112 using the highest concentration of primers and probes and PCR 110 using a concentration in the middle. Figure 2.6 shows the results of PCR 110 with a medium concentration of primers and probe [A], PCR 111 with low concentration of this reagents [B] and PCR 112 with the highest concentration [C]. All assays were done using the same samples. The samples represented are Chicken Ham, C18 (blue continuous line); Duck Meat, C9 (purple dotted line); Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

PCR 110 have the results as expected with amplification in the positive sample C18 and no amplification in the negative sample C9. Both IC also have an expected behaviour.

In contrast, PCR 111 and 112, although the positive sample C18 and the IC of negative sample showed a slightly better amplification curve, both assays have a small amplification in the negative sample C9. This amplification means a loss in the specificity of the assay with low and high concentrations of primers and probe. Therefore, both assays must be discarded.

Assays with lower concentrations of primers and probe expected lower amplification of positive sample and an increase in specificity (Ruiz-Villalba, Pelt-verkuil, Gunst, Ruijter, & Hoff, 2017), however in PCR 111 that is not the case. A possible explanation for this occurrence is that with low concentrations of primers, there is more Mg^{2+} ion disponible to stabilize the primers, facilitating the annealing to the template, increasing the PCR product, specific and non-specific.

Higher concentrations of primers and probe, as in PCR 112, promote non-specific annealing as well as the occurrence of primer dimers, due to the excess of this DNA molecules (Markoulatos et al., 2002; Ruiz-Villalba et al., 2017). This effect explains the amplification of the sample C9 in PCR 112.

The medium concentration of primers and probe is the optimal concentration, as seen in PCR 110. This concentration is, therefore, defined to the SUPREME Real Time PCR Chicken Detection kit.

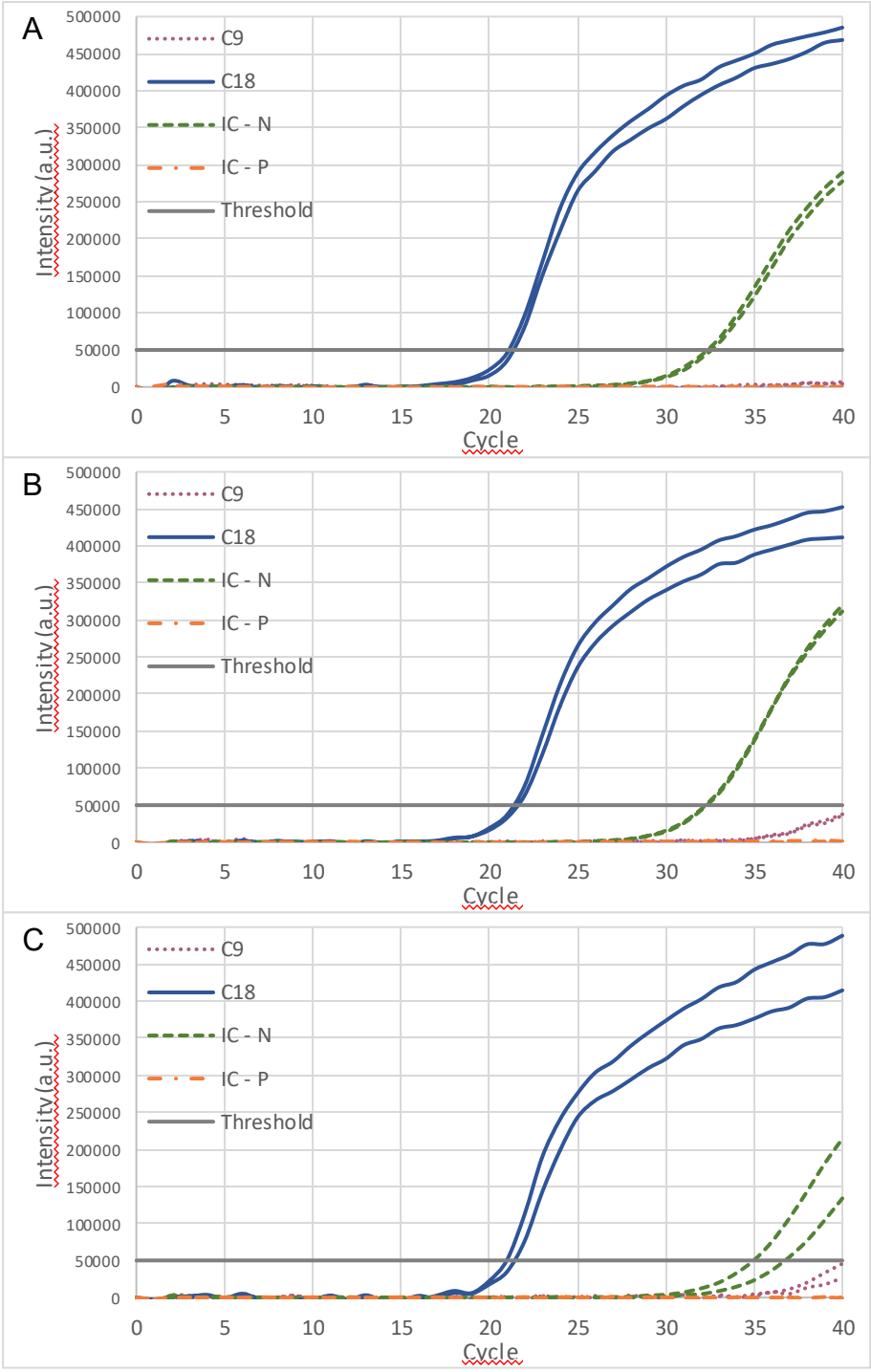

Figure 2.6 **Graphic representation of the PCR results:** PCR 110 with a medium concentration of primers and probe [A], PCR 111 with low concentration of this reagents [B] and PCR 112 with the highest concentration [C]. The samples represented are C18 (blue continuous line), C9 (purple dotted line), Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

2.3.1.1.5 Annealing Temperature

The annealing temperature is a defining step in the optimization of a PCR kit. The PCR 110, 132 and 133 used different temperatures as means to test its effects in the yield and specificity of the kit. PCR 110 was done with an annealing temperature of 60ºC, PCR 132 dropped the annealing temperature to 58ºC and the annealing temperature of PCR 133 was 62ºC. Figure 2.7 shows the results of PCR 110 with an annealing temperature of 60ºC [A], PCR 132 with lower annealing temperature [B] and PCR 133 with the annealing temperature at 62ºC [C]. All assays were done using the same samples. The samples represented are Chicken Ham, C18 (blue continuous line); Duck Meat, C9 (purple dotted line); Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

The PCR 110, as previously analysed, shows expected results. The sample C18 have an amplification curve with a maximum intensity of fluorescence of 500000 and a C_T in the 21st cycle and the IC of negative sample with an amplification curve almost reaching the 300000 of intensity of fluorescence and a C_T in the 32nd cycle. The sample C9 didn't amplify, as expected.

From PCR 132 forward, it was used a 5-fold higher concentration of IC DNA to guarantee a well-defined IC curve. Since the increase in the IC DNA concentration is so low that doesn't affect the amplification of target DNA, an optimization step is not necessary. However, in the current optimization step it isn't possible comparing the IC samples with the PCR 110.

PCR 132 shows a result as expected., bearing in mind the decrease in the annealing temperature. The sample C18 amplification curve is better than the amplification curve of PCR 110, having a C_T in the 22nd cycle but an intensity of fluorescence surpassing the 500000 in the 28th cycle. However, the sample C9 shows an amplification curve in the final cycles, revealing a non-specific annealing. This result can be explained due to the lowering of the annealing temperature. Low annealing temperatures facilitate the formation of hydrogen bonds between primer and template, producing a more stable annealing (Montgomery et al., 2014; Rychlik et al., 1990). The easy and stable annealing facilitate the specific amplification but also increase the probability of non-specific amplification.

In contrast, sample C18 of PCR 133 shows an amplification curve of with a C_T in the 22nd cycle and an intensity of fluorescence similar to PCR 110. The sample C9 didn't amplify. PCR 133 have a higher annealing temperature than PCR 110 and high annealing temperatures are closer to T_m, disturbing the formation of hydrogen bonds and consequent primer annealing (Montgomery et al., 2014; Rychlik et al., 1990). However, it is not the case, as both amplification curves of the sample C18 are very similar, and neither specificity is compromised. Concluding, that this chicken detection assay has a wide range of optimal annealing temperature, that includes 60ºC and 62ºC.

It's worth remembering that this optimization process has a commercial goal. So, when defining the SUPREME Real Time PCR Chicken Detection kits annealing temperature, it is vital to bear in mind the kit ease of use. The customer, regularly, uses several Detection kits simultaneously, so it's an advantage to have all the kits with the same annealing temperature. Taking that into consideration, the SUPREME Real Time PCR Chicken Detection kits annealing temperature is set

to 60ºC, as it's an efficient to chicken DNA detection and it's the optimal temperature of some of other BPMR kits.

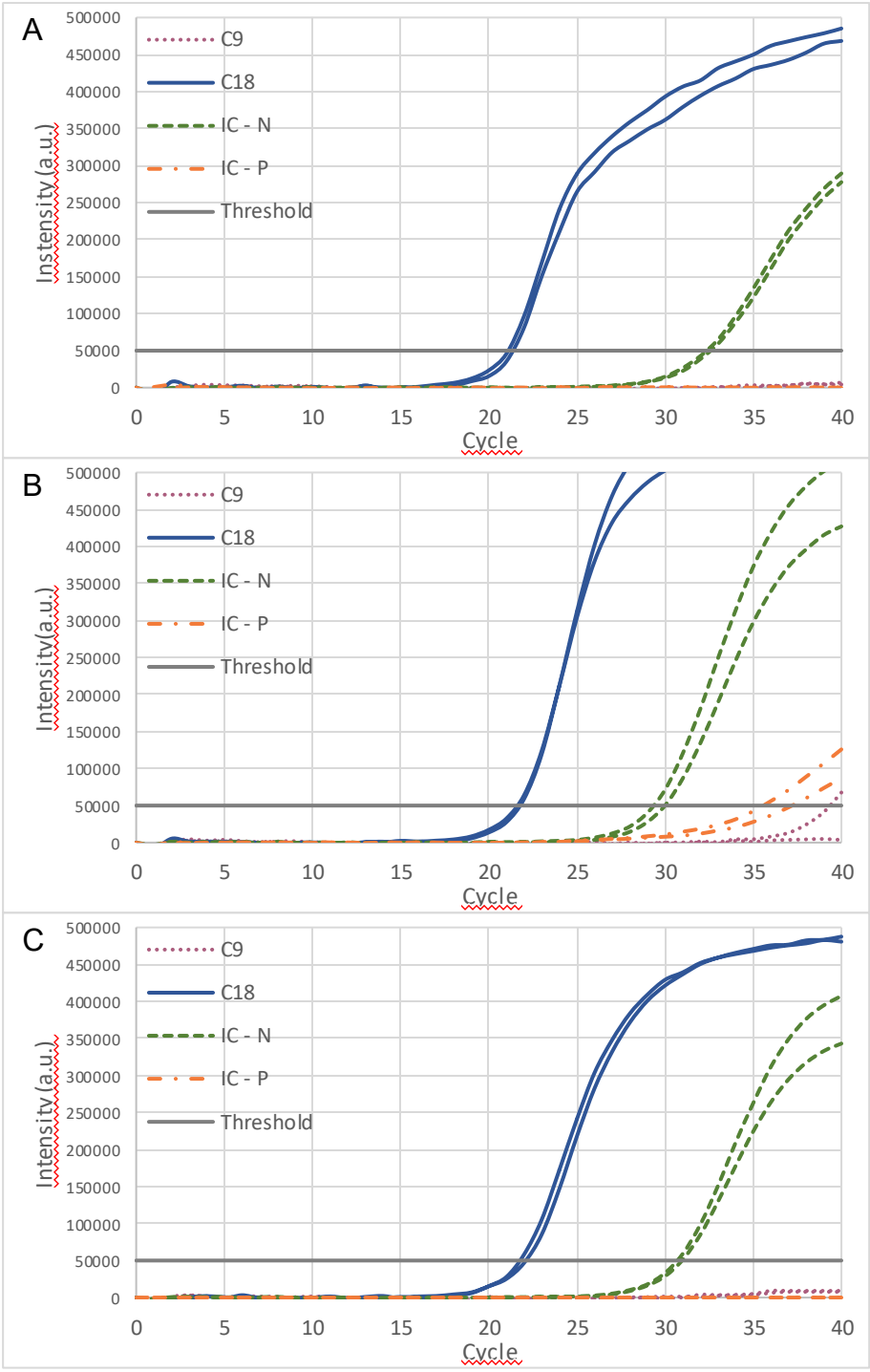

Figure 2.7 **Graphic representation of the PCR results:** PCR 110 with an annealing temperature of 60ºC [A], PCR 132 with lower annealing temperature [B] and PCR 133 with the annealing temperature at 62ºC [C]. The samples represented are C18 (blue continuous line), C9 (purple dotted line), Internal Control of the nega 1tive sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

2.3.1.1.6 Duration of each cycle phase

The next step of the optimization of the Chicken detection kit is the definition of the duration time of each cycle phase. The denaturation phase of PCR 110 is 30 seconds, the annealing phase lasts 30 seconds and the extension phase also last 30 seconds. In PCR 152, each cycle phase lasts 20, 30 and 20 seconds respectively. In PCR 153, each cycle phase lasts 15, 30 and 15 seconds respectively. In PCR 154, each cycle phase lasts 15, 30 and 10 seconds respectively. Figure 2.8 shows the results of PCR 110 [A], PCR 152 [B], PCR 153 [C] and PCR 154 [D]. All assays were done using the same samples. The samples represented are Chicken Ham, C18 (blue continuous line); Duck Meat, C9 (purple dotted line); Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The negative sample C9 didn't amplify in any assay, meaning that the duration of the cycle doesn't compromise the specificity of the kit.

The positive sample C18 have similar amplification curves in all assays, with C_T varying between the 21st and 22nd cycle and an intensity of fluorescence being always around 500000, being slightly greater in PCR 152 and 153.

The only difference can be found in the IC of the negative sample, which as a better curve in PCR 110. In PCR 110, the IC of the negative sample have a C_T in the 32nd cycle and an intensity of fluorescence reaching 300000. In contrast, the remaining assays, the C_T is in the same cycle and the intensity of fluorescence varies between 200000 and 250000. This slight decrease in the intensity of fluorescence can be explained due to the shorter cycle duration, namely the elongation phase. In this phase, *Taq* Polymerase catalyse the addition of nucleotides in to the newly formed DNA strand. If the duration of this phase is too short, the polymerase can't complete the new DNA strand, undermining the amplification. However, the different is not significant enough.

One of the optimization goals is to reduce the waiting time without compromising the kit performance. So, to reduce the cycle duration and, since there is none significant difference between assays with different durations, the SUPREME Real Time PCR Chicken detection kit will have a denaturation phase of 15 seconds, an annealing phase lasting 30 seconds and an extension phase of 15 seconds.

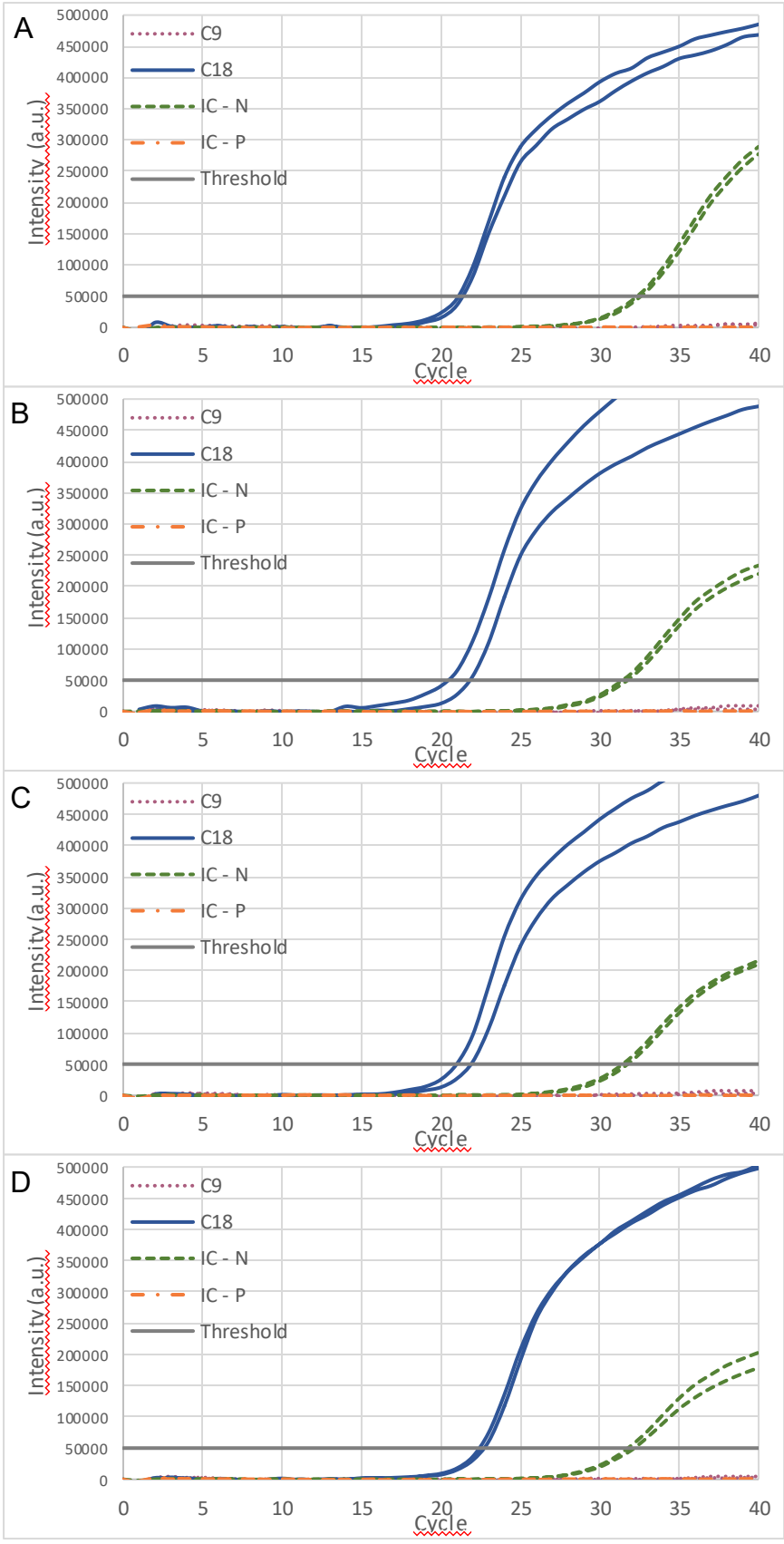

Figure 2.8 **Graphic representation of the PCR results:** PCR 110 [A], PCR 152 [B], PCR 153 [C] and PCR 154 [D]. The samples represented are C18 (blue continuous line), C9 (purple dotted line), Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

2.3.1.1.7 Concentration of dUTP

As an optional step of the optimization of the BIOPREMIER Real Time PCR kit is defining the concentration of dUTP. The PCR 153 with a higher dUTP concentration and the PCR 174 with a lower concentration of dUTP were analysed. Figure 2.9 shows the results of PCR 153 with higher dUTP concentration [A] and of PCR 174 with lower concentration [B]. All assays were done using the same samples. The samples represented are Chicken Ham, C18 (blue continuous line); Duck Meat, C9 (purple dotted line); Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The sample C9 didn't amplify in any assay with the change of the concentration of dUTP, meaning that the reaction is already robust enough.

Sample C18 in PCR 174 has an amplification curve with a C_T between the 21st and 22nd cycle, similarly to PCR 153. However, the intensity of fluorescence is slightly lower in PCR 174, not even reaching the 500000.

The IC of the negative sample have a higher C_T in PCR 174 (34th cycle) and an intensity of fluorescence not reaching the 200000. These values reveal a slightly worse curve than the amplification curve of the same sample in PCR 153. However, since the difference is not significant.

The SUPREME Real Time PCR Chicken Detection kit will use the concentration used in PCR 153, due the minor the difference of the results of both PCR and the fact that a higher concentration of dUTP will prevent more efficiently PCR product contamination, as the UDG will have more substrate available. (Burkardt, 2000).

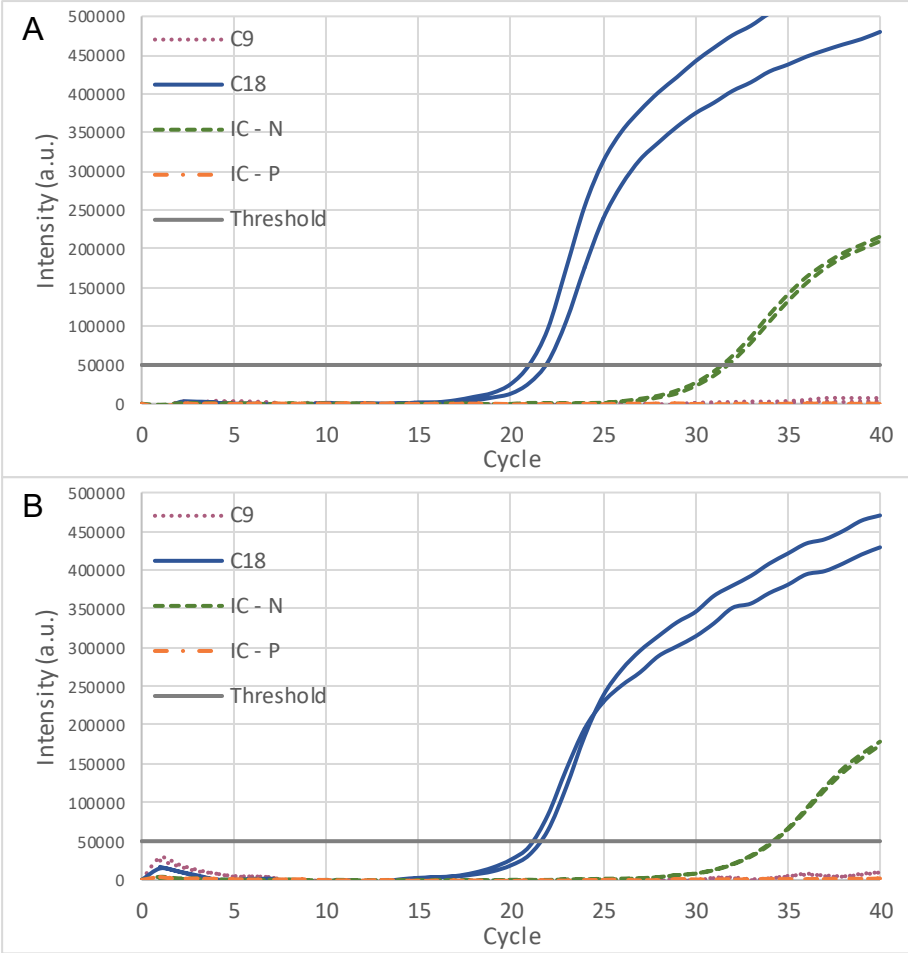

Figure 2.9 **Graphic representation of the PCR results:** PCR 153 with higher dUTP concentration [A] and of PCR 174 with lower concentration [B]. The samples represented are C18 (blue continuous line), C9 (purple dotted line), Internal Control of the negative sample C9 (green dashed line) and Internal Control of the positive sample C18 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.1.2 Validation Tests

The validation tests are a necessary tool to confirm the legitimacy of the kit as well as assess the performance of the kit (Broeders et al., 2014). Assessing these parameters is required to commercialize the kits.

The performance of the kit is measured through the calculation of the specificity, LoD and Inclusivity and robustness.

2.3.1.2.1 Exclusivity Test

The exclusivity test guarantees that the previously chosen conditions didn't compromise the specificity of the new kit, assuring that any non-target sample doesn't amplify. This test enables the calculation of the specificity indicator, through equation 1 (in session 2.2.5.1 Exclusivity Tests). The exclusivity test was done using 10 replicates off 14 non-chicken samples with 5ng of sample DNA.

The list of samples used in the exclusivity test is available in the Appendix 1. Figure 2.10 shows the results of the chicken detection kit exclusivity test with all non-chicken samples (blue dotted line) and respective IC (pink line).

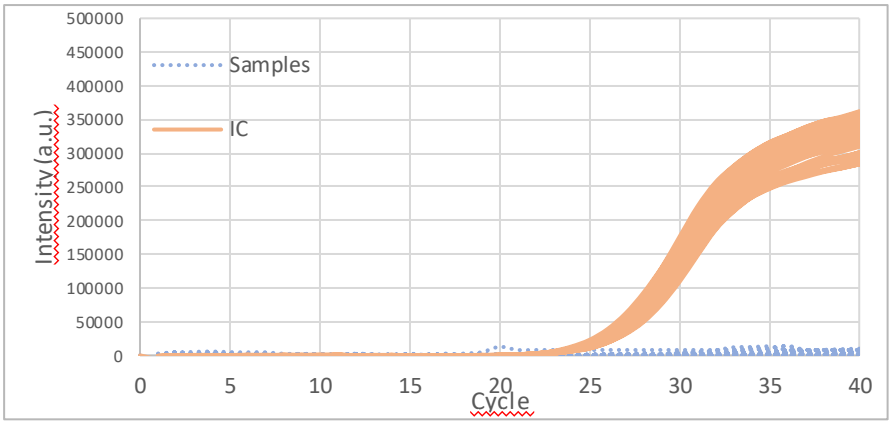

Figure 2.10 **Graphic representation of the PCR results**: chicken detection kit exclusivity test with 14 non-chicken samples (blue dotted line) and their IC (pink line)

The IC confirm the validity of the test and the lack of amplification of any non-target samples proves the specificity of the kit, with an indicator of 100%.

2.3.1.2.2 Inclusivity and Sensibility Tests

The sensibility test allows to determine the lowest amplifiable target DNA quantity. The sensibility test was done using 1ng, 100pg, 10pg, 5pg and 1pg of chicken DNA. The inclusivity test guarantees that the previously chosen conditions can detect target DNA in every chicken matrix. This test enables the calculation of the inclusivity indicator, through equation 2 (in session 2.2.5.2 Inclusivity and Sensibility Tests). The inclusivity test was done using 10 replicates of 5 different chicken samples at the LoD. Figure 2.11 shows a sensibility test with sample C17 at 1ng (darkest blue line with diamonds), 100pg (dark blue line with squares), 10pg (blue line with circles), 5pg (light blue line with stars) and 1pg (lightest blue line with triangles). The threshold is the grey flat line.

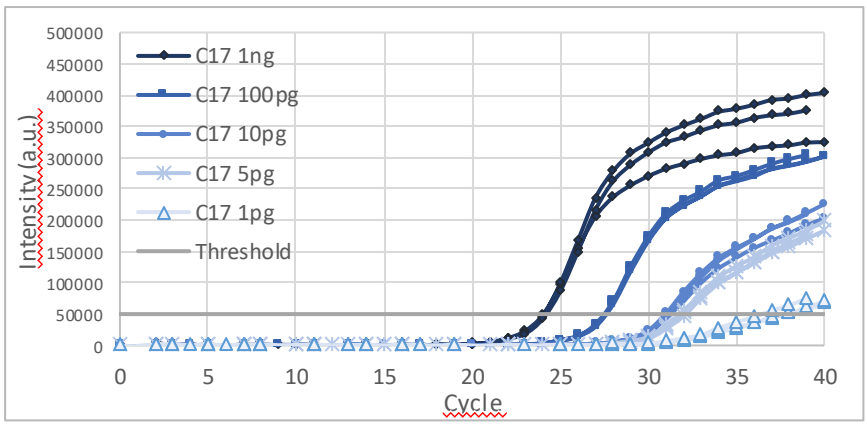

Figure 2.11 **Graphic representation of the PCR results:** the chicken detection kit sensibility test. It was used the sample C17 at 1ng (darkest blue line with diamonds), 100pg (dark blue line with squares), 10pg (blue line with circles), 5pg (light blue line with stars) and 1pg (lightest blue line with triangles). The threshold is the grey flat line.

Analysing the results, all DNA concentrations show amplification. Logically, as the concentration of DNA is decreasing, the amplification curve starts in later cycles and has a lower intensity of fluorescence. The lower DNA quantity amplified was 1pg with a C_T in the 37th cycle and an intensity of fluorescence almost reaching 100000. Although it's a low intensity of fluorescence value, the curve surpasses the threshold, setting the LoD of 1pg. Figure 2.12 shows the inclusivity test with 5 chicken matrices with 1pg of DNA. The samples are C4 (dotted orange line), C24 (green continuous line), C28 (purple dashed and double dotted line), C54 (blue dashed and dotted line) and C17 (lightest blue line with triangles). The threshold is the grey flat line.

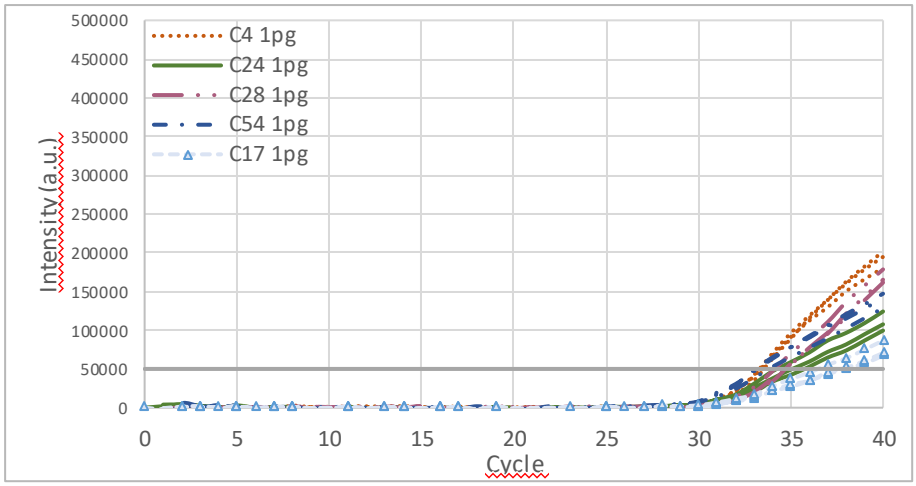

Figure 2.12 **Graphic representation of the PCR results:** the chicken detection kit inclusivity test. The samples are C4 (dotted orange line), C24 (green continuous line), C28 (purple dashed and double dotted line), C54 (blue dashed and dotted line) and C17 (lightest blue line with triangles). The threshold is the grey flat line.

All 5 different samples show amplification with a C_T ranging from the 33rd to the 37th cycle and an intensity of fluorescence reaching 200000. This result confirms the LoD of 1pg and the amplification of every target samples proves the inclusivity of the kit, with an indicator of 100%.

2.3.1.2.3 Robustness Tests

The robustness tests are exclusivity and inclusivity tests, to infer the performance of the kits in adverse conditions. This method guarantees the performance of the tests assuming that the user's equipment is not calibrated or shows temperature shifts (Broeders et al., 2014).

The exclusivity robustness test was done decreasing the annealing temperature 2ºC, facilitating the reaction. In these conditions none sample showed amplification, guaranteeing the specificity of the test.

The inclusivity robustness test was done increasing the annealing temperature 2ºC, disturbing the reaction. In these conditions, every sample showed amplification, guaranteeing the LoD and specificity of the test.

2.3.2. Turkey Detection Kit

2.3.2.1 Optimization of PCR Conditions

The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined to the turkey detection kit.

To guarantee the performance of the optimized solution and subsequent amplification of target DNA, preventing non-specific amplification, matrices with and without turkey DNA were used as testing samples. All samples were tested in a concentration of 1ng/µl. The results and subsequent analysis will highlight a sample with the targeted sequence (C16), and a sample without the target sequence (C17). Amplification is not expected in sample C17 because it is from chicken meat. If an amplification is shown in sample C17, the PCR conditions must be reconsidered, because it means the lack of specificity of the test. To define the C_T and consider an amplification positive, the amplification curve must have an intensity of fluorescence greater than the threshold of 50000.

In Table 2.8, it is shown the order of the conditions tested, as well as the PCR assays performed and the respective BPMR code.

Table 2.8 List of ordered and optimized PCR conditions of the Turkey Detection kit and its BPMR Code. The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined.

Order	Test Conditions	BPMR Code
1	Type of primers and probe	PCR 86 and 87
2	Presence of DMSO	PCR 105 and 113
3	Concentration of primers and probe	PCR 113, 114 and 115
4	Annealing Temperature	PCR 115, 134 and 135
5	Duration of each cycle phase	PCR 115, 155, 156 and 157
6	Concentration of dUTP	PCR 156 and 175
7	Concentration of $MgCl_2$ and dNTPs	PCR 156 and 252

To facilitate the comparison between different assays, plots were standardized and a maximum of 500 000 units of fluorescence intensity was defined.

2.3.2.1.1 Type of primers and probe

The first condition optimized in the turkey detection kit was the type of primers and probe. The analysis was done to two different sets of primers and probe. The first set is already used by BPMR in the current BIOPREMIER Turkey detection kit and the second is a new set of primers and probe newly designed and never used in BPMR laboratories. Figure 2.13 shows the results of PCR 86 with current set of primers and probe [A] and PCR 87 with the new set of primers and probe [B]. The samples represented are Turkey Ham, C16 (blue continuous line); Chicken Meat, C17 (purple dotted line); Internal Control of the negative sample C17 (green dashed line) and Internal Control of

the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

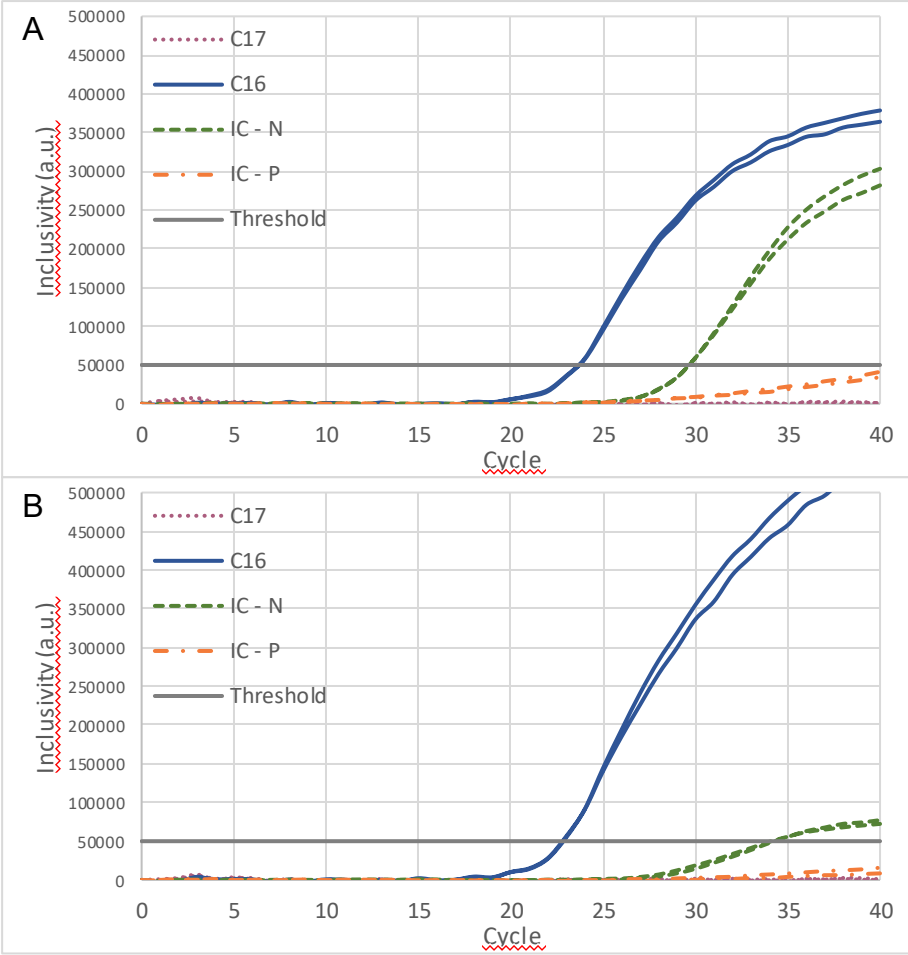

Figure 2.13 **Graphic representation of the PCR results:** PCR 86 with current set of primers and probe [A] and PCR 87 with the new set of primers and probe [B]. The samples represented are C16 (blue continuous line), C17 (purple dotted line), Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

As expected, the sample C17 didn't show amplification in any PCR, meaning that neither set is unspecific and doesn't detect turkey.

Since the sample C16 have turkey DNA in it, it shows an amplification curve in both PCR. Analysing the C_T, the amplification curve of PCR 86 passes the threshold near the 24th cycle, while in PCR 87 the C_T is in the 23rd cycle. Additionally, the peak of fluorescence intensity of the sample C16 in PCR 87 is greater than the peak in PCR 86. This means that the new set of primes have a

better affinity to the target DNA, than the current set, facilitating the annealing and consequently the DNA amplification.

However, the amplification curves of the IC of the negative sample are significantly different. In PCR 86, the curve has a C_T in the 29th cycle and an intensity of fluorescence of 300000. Whereas, in CR 87, the curve has a C_T in the 34th cycle and an intensity of fluorescence not reaching. This huge difference between both curves means an inhibition of the IC amplification by the new set of primers

The IC of the positive sample just shows a significant amplification curve in PCR 87. In PCR 86, the IC of the positive sample only amplified in later cycles, not reaching the threshold. The quantity of target DNA in the reaction mix is greater comparing with the IC DNA. The difference of DNA concentration causes a competition for the active centres of the DNA polymerases, resulting in the amplification of the target DNA in favour of the IC (Markoulatos et al., 2002; Schrader et al., 2012).

The primers and probe defined for the SUPREME Real Time Turkey detection kit are the current ones, because, even though the amplification curve of C16 is better with the new set, this set inhibits the IC amplification, making the set not valid.

2.3.2.1.2 Presence of DMSO

The optimization of the turkey detection kit counted with a step to evaluate the usage of DMSO. The PCR 105 which contained DMSO was analysed against the PCR 113 that not used DMSO nor another PCR enhancer. Figure 2.14 shows results of PCR 105 with DMSO [A] and PCR 113 without DMSO [B]. The samples represented are Turkey Ham, C16 (blue continuous line); Chicken Meat, C17 (purple dotted line); Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

Neither assay has non-specific amplification as the sample C17 didn't amplify. The IC of positive sample also didn't amplify, due to the competition for the reaction reagents as it can be verified with the high intensity of the target amplification curve.

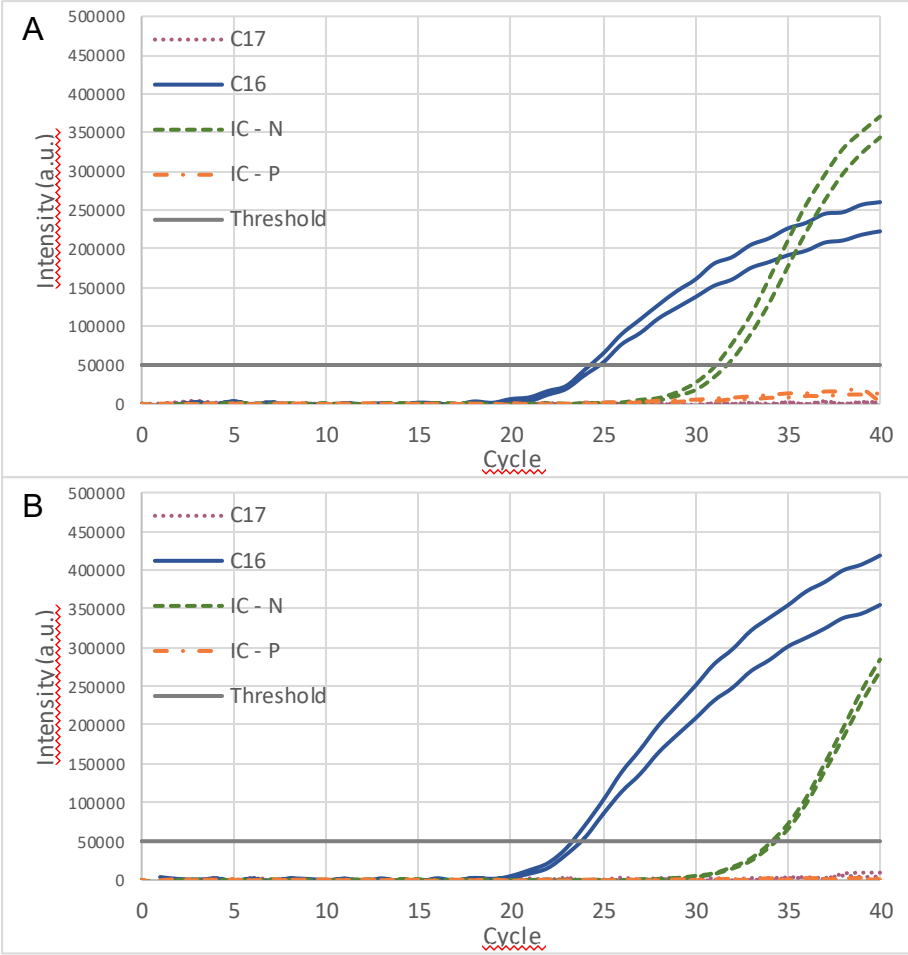

Figure 2.14 **Graphic representation of the PCR results:** PCR 105 with DMSO [A] and PCR 113 without DMSO [B]. The samples represented are C16 (blue continuous line), C17 (purple dotted line), Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The sample C16 shows an amplification curve in both PCR assays. The intensity of fluorescence in PCR 105 is between 210000 and 360000, which is lower when compared with the intensity of fluorescence in PCR 113, that reached 400000. The C_T of the amplification curve is higher in PCR 105 (24th cycle) than the C_T of the amplification curve of the same sample in PCR 113 (23rd cycle).

Adversely, the IC from the negative sample, in PCR 105, the amplification curve of this sample has a C_T in the 32nd cycle and the intensity of fluorescence reaches the 350000. While, in PCR 113, the amplification curve is better, having a C_T in the 34th cycle and the intensity of fluorescence almost reaches 300000. This difference is a minor difference explained with operator errors, not significant enough to discard the assay.

This results are as expected because DMSO is an enhancer which increase the specificity the PCR by lowering the T_m (Chakrabarti & Schutt, 2001; Henke et al., 1997; Ralser et al., 2006). The lower T_m prevents the occurrence of secondary DNA structures and non-specific binding, because the lower the T_m, the more difficult is to the primer to bind to the template. This thermodynamic behaviour increases the specific annealing, since the correct base pairing is more favourable. However, by lowering the T_m, the primer has difficulty binding to the template causing a

decrease in the yield of the reaction when comparing positive samples with assays without DMSO. As so, the amplification curve of C16 in PCR 113 is better than the amplification curve of this sample in PCR 105.

Therefore, the SUPREME Real Time PCR will not contain DMSO as the PCR 113 have better amplification curve in the sample C16, without compromising the specificity in sample C17. However, in the exclusivity tests it will be confirmed the specificity of the reaction and if the DMSO is necessary.

2.3.2.1.3 Concentration of primers and probe

The concentration of primers and probe was the fourth condition optimized in the chicken detection kit. So, were analysed the PCR 113, 114 and 115, with different concentrations of primers and probe. The assays were done using increasing concentrations of this reagents: the PCR 114 using the lowest concentration, the PCR 115 using the highest concentration of primers and probes and PCR 113 using a concentration in the middle. Figure 2.15 shows the results of PCR 113 with a medium concentration of primers and probe [A], PCR 114 with low concentration of this reagents [B] and PCR 115 with the highest concentration [C]. All assays were done using the same samples. The samples represented are Turkey Ham, C16 (blue continuous line); Chicken Meat, C17 (purple dotted line); Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

All three assays have the results as expected with amplification in the positive sample C16, with a similar C_T in the 24th cycle. The intensity of fluorescence is a little lower in PCR 114, rounding 300000, in contrast with the intensity of fluorescence of the curves in PCR 113 and 115, that reach the 350000. There is no amplification in the negative sample C17 in any assay. Both IC also have an expected a similar behaviour in all three PCR.

Assays with lower concentrations of primers and probe expected lower amplification of positive sample and an increase in specificity (Markoulatos et al., 2002; Ruiz-Villalba et al., 2017), as seen in PCR 114. Higher concentrations of primers and probe, as in PCR 113 and 115, will facilitate the availably of primer and, consequently, increasing the amplification (Ruiz-Villalba et al., 2017).

Since PCR 113 and 115 have similar results, the concentration of primers and probe defined in any of these assays is optimal to the kit. Therefore, the concentration set to the SUPREME Real Time PCR Turkey Detection kit was the concentration of PCR 115, since it has a cleaner amplification curve.

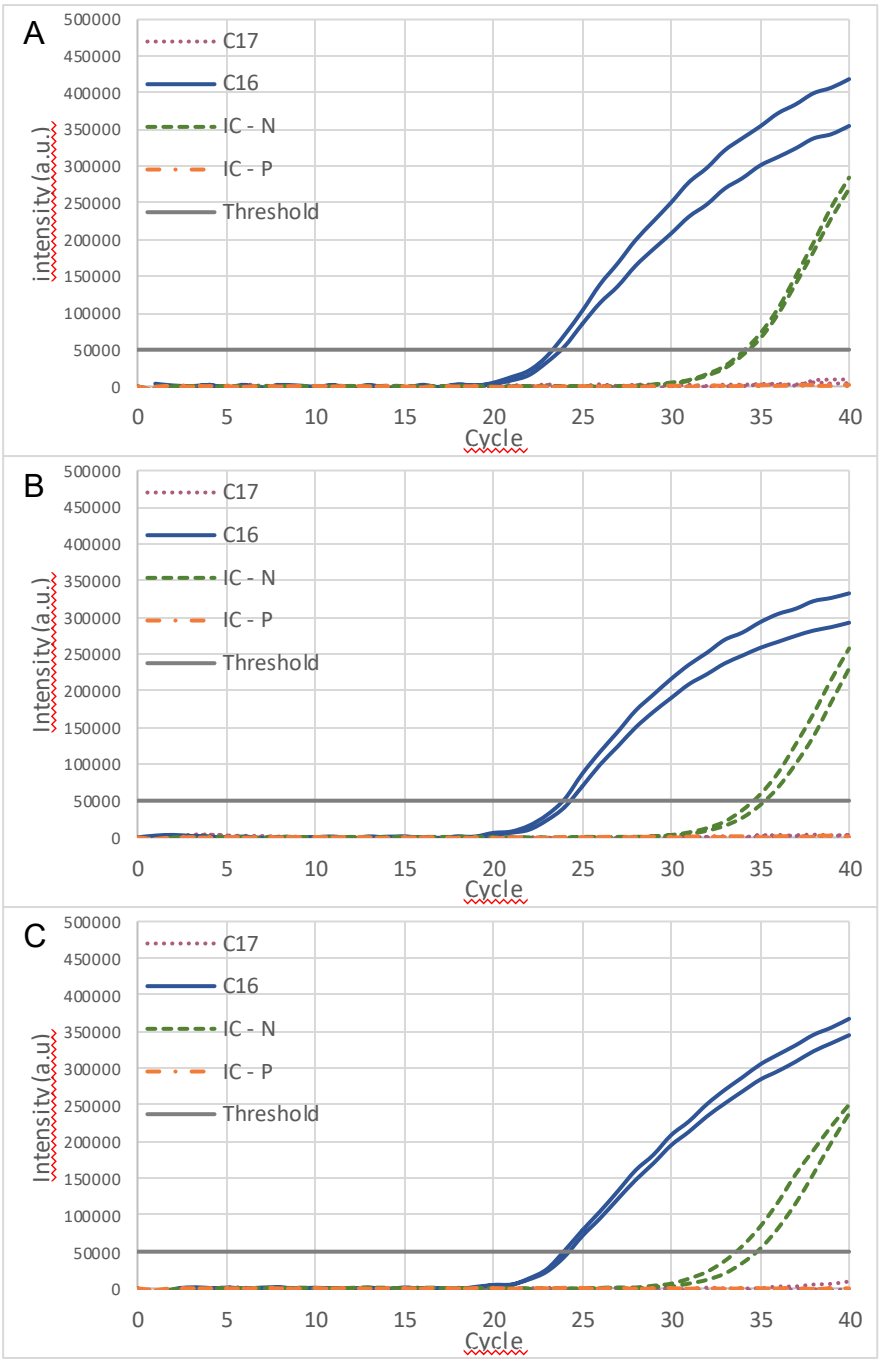

Figure 2.15 **Graphic representation of the PCR results:** PCR 113 with a medium concentration of primers and probe [A], PCR 114 with low concentration of this reagents [B] and PCR 115 with the highest concentration [C]. The samples represented are C16 (blue continuous line), C17 (purple dotted line), Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.2.1.4 Annealing Temperature

The annealing temperature is a defining step in the optimization of a PCR kit. The PCR 115, 134 and 135 used different temperatures as means to test its effects in the yield and specificity of

the kit. PCR 110 was done with an annealing temperature of 60ºC, PCR 134 dropped the annealing temperature to 58ºC and the annealing temperature of PCR 135 was 62ºC. Figure 2.16 shows the results of PCR 115 with an annealing temperature of 60ºC [A], PCR 134 with lower annealing temperature [B] and PCR 135 with the annealing temperature at 62ºC [C]. All assays were done using the same samples. The samples represented are Turkey Ham, C16 (blue continuous line); Chicken Meat, C17 (purple dotted line); Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

PCR 115, as previously analysed, shows expected results. The sample C16 have an amplification curve with a maximum intensity of fluorescence of 350000 and a C_T in the 24th cycle and the IC of negative sample with an amplification curve almost reaching the 250000 of intensity of fluorescence and a C_T in the 34th cycle.

PCR 134 shows a result as expected., bearing in mind the decrease in the annealing temperature. The sample C16 amplification curve is better than the amplification curve of PCR 115 and 135, having a C_T in the 23rd cycle but an intensity of fluorescence surpassing the 500000 in the 30th cycle. This result can be explained due to the lowering of the annealing temperature. Low annealing temperatures facilitate the formation of hydrogen bonds between primer and template, producing a more stable annealing (Montgomery et al., 2014; Rychlik et al., 1990). The easy and stable annealing facilitate the amplification as seen in PCR 134. The IC of the negative sample have a similar, although a little lower, amplification curve as PCR 115 and PCR 135

In contrast, sample C16 of PCR 135 shows a worse amplification curve with a C_T in the 26th cycle and an intensity of fluorescence of 150000. This is supported with the higher annealing temperature of PCR. High annealing temperatures are closer to T_m, disturbing the formation of hydrogen bonds and consequent primer annealing (Montgomery et al., 2014; Rychlik et al., 1990).

Sample C17 didn't amplify in any assay, meaning that this range of temperatures doesn't affect the specificity of the turkey detection kit.

The optimal temperature for the turkey detection kit is 58ºC, although It's worth remembering that this optimization process has a commercial goal. So, when defining the SUPREME Real Time PCR Turkey Detection kit annealing temperature, it is vital to bear in mind the kit ease of use. The customer, regularly, uses several Detection kits simultaneously, so it's an advantage to have all the kits with the same annealing temperature. As previously defined, the SUPREME Real Time PCR Chicken Detection kits annealing temperature is 60ºC, and since, the same temperature is also efficient in to detect turkey DNA, the annealing temperature set for SUPREME Real Time PCR Turkey Detection kit is 60ºC.

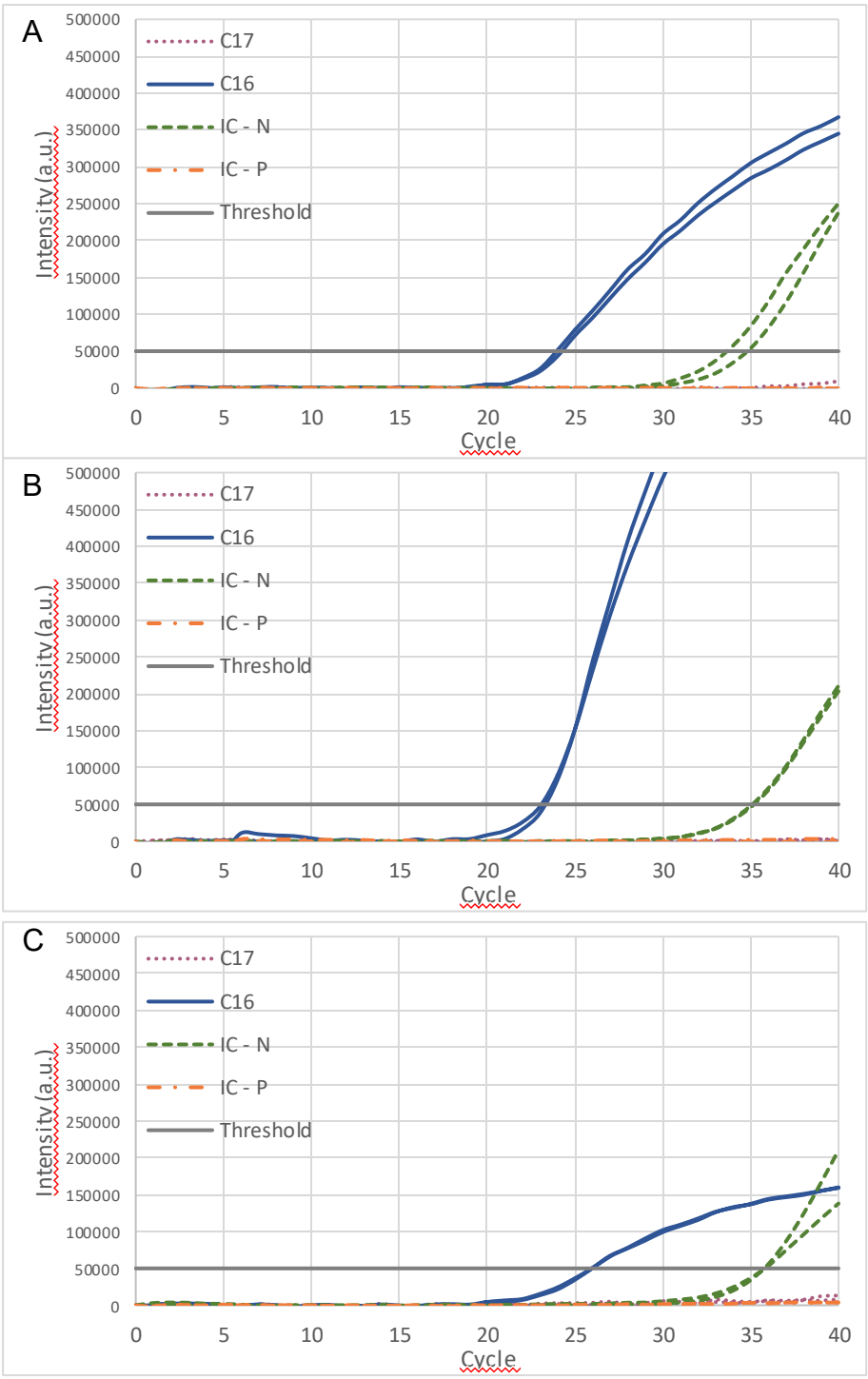

Figure 2.16 **Graphic representation of the PCR results:** PCR 115 with an annealing temperature of 60ºC [A], PCR 134 with lower annealing temperature [B] and PCR 135 with the annealing temperature at 62ºC [C]. The samples represented are C16 (blue continuous line), C17 (purple dotted line), Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.2.1.5 Duration of each cycle phase

The next step of the optimization of the turkey detection kit is the definition of the duration time of each cycle phase. The denaturation phase of PCR 115 is 30 seconds, the annealing phase lasts 30 seconds and the extension phase also last 30 seconds. In PCR 155, each cycle phase lasts 20, 30 and 20 seconds respectively. In PCR 156, each cycle phase lasts 15, 30 and 15 seconds respectively. In PCR 157, each cycle phase lasts 15, 30 and 10 seconds respectively. Figure 2.17 shows the results of PCR 115 [A], PCR 155 [B], PCR 156 [C] and PCR 157 [D]. All assays were done using the same samples. The samples represented are Turkey Ham, C16 (blue continuous line); Chicken Meat, C17 (purple dotted line); Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The negative sample C17 didn't amplify in any assay, meaning that the duration of the cycle doesn't compromise the specificity of the kit.

The positive sample C16 have similar amplification curves in all assays, with C_T varying between the 24^{th} and 25^{th} cycle and an intensity of fluorescence ranging between 350000 in PCR 115 and 450000 in PCR 155 and 156. In PCR 157, the intensity of fluorescence is approximately 400000

From PCR 155 forward, it was used a 5-fold higher concentration of IC DNA to guarantee a well-defined IC curve. Since the increase in the IC DNA concentration is so low that doesn't affect the amplification of target DNA, an optimization step is not necessary. However, in the current optimization step it isn't possible comparing the IC samples of PCR 155, 156 and 157 with the PCR 115.

One of the optimization goals is to reduce the waiting time without compromising the kit performance. So, to reduce the cycle duration and, since there are none significant differences between assays with different durations, the SUPREME Real Time PCR Turkey detection kit will have a denaturation phase of 15 seconds, an annealing phase lasting 30 seconds and an extension phase of 15 seconds.

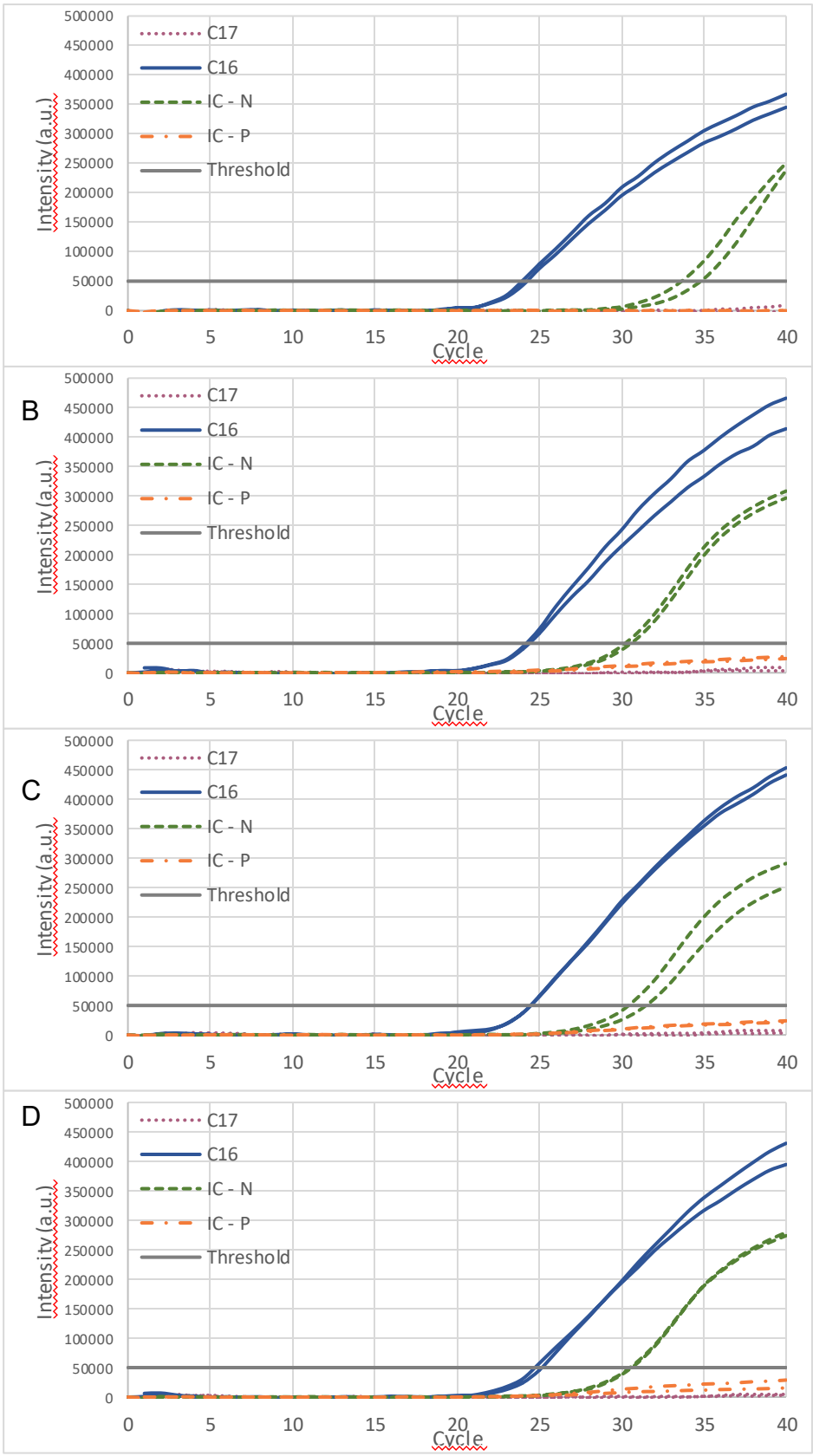

Figure 2.17 **Graphic representation of the PCR results:** PCR 115 [A], PCR 155 [B], PCR 156 [C] and PCR 157 [D]. The samples represented are C16 (blue continuous line), C17 (purple dotted line), Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.2.1.6 Concentration of dUTP

As an optional optimization step of the BIOPREMIER Real Time PCR kit, the concentration of dUTP is defined. The PCR 156 with a higher dUTP concentration and the PCR 175 with a lower concentration of dUTP were analysed. Figure 2.18 shows the results of PCR 156 with higher dUTP concentration [A] and of PCR 175 with lower concentration [B]. All assays were done using the same samples. The samples represented are Turkey Ham, C16 (blue continuous line); Chicken Meat, C17 (purple dotted line); Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

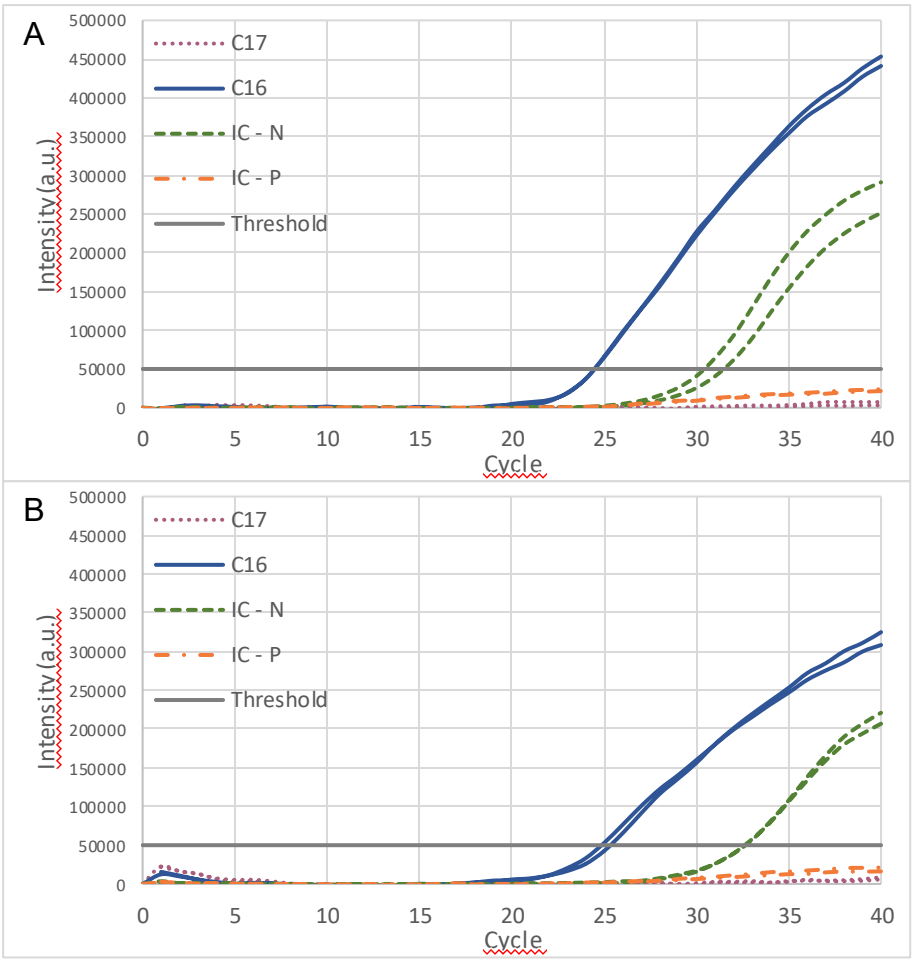

Figure 2.18 **Graphic representation of the PCR results:** PCR 156 with higher dUTP concentration [A] and of PCR 175 with lower concentration [B]. The samples represented are C16 (blue continuous line), C17 (purple dotted line), Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The sample C17 didn't amplify in any assay with the change of the concentration of dUTP, meaning that the reaction is already robust enough.

Sample C16 in PCR 175 has an amplification curve with a C_T in the 25th cycle, higher than the C_T of PCR 156. The intensity of fluorescence is lower in PCR 175, reaching 300000 while the intensity of fluorescence in PCR 156, that reaches 450000.

The IC of the negative sample also have a higher C_T in PCR 175 (32nd cycle) and an intensity of fluorescence barely reaching 200000. These values reveal a slightly worse curve than the amplification curve of the same sample in PCR 156 with a C_T in between 30th and 32nd cycles and an intensity of fluorescence of 250000 to 300000.

The greater the concentration of dUTPs, the more likely is its inclusion in the DNA strand. When the dUTPs are present in the DNA strand, the UDG enzyme cleave the strand in the dUTP residues (Burkardt, 2000; Kleiboeker, 2005). This should decrease the intensity of fluorescence, which is not the case.

The SUPREME Real Time PCR Turkey Detection kit will use the concentration used in PCR 156, as the conditions of this assays guarantee a better amplification curve and the fact that a higher concentration of dUTP will prevent more efficiently PCR product contamination, as the UDG will have more substrate available (Burkardt, 2000).

2.3.2.1.7 Concentration of $MgCl_2$

The last optimization step of the BIOPREMIER Real Time PCR kit is defining the concentration of $MgCl_2$. The optimal concentration of this reagent was chosen by analysing the PCR 156 and 252. 102 had the lowest concentration of $MgCl_2$, while PCR 252 used the highest concentration. Figure 2.19 shows the results of PCR 156 with the lowest concentration of $MgCl_2$ [A] and PCR 252 with the highest concentration of the reagent [B]. All assays were done using the same samples. The samples represented Turkey Ham, C16 (blue continuous line); Chicken Meat, C17 (purple dotted line); Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

In PCR 156, as previously analysed, the results are as expected. The sample C17 didn't amplify because it doesn't have turkey DNA. The sample C16 with turkey DNA shows an amplification curve with a C_T, approximately, in the 24th cycle and a maximum intensity of fluorescence of 450000. The IC of the negative sample also shows an amplification curve with a C_T between 30th and 32nd cycles and with the intensity of fluorescence reaching the 3000000. The IC of the positive sample didn't amplify due to the competition between the target DNA and the IC for the reaction reagents.

The positive sample C16, in PCR 252, has a higher maximum intensity of fluorescence surpassing 500000 in the 28th cycle and a C_T in the 24th cycle. The IC of the negative sample also shows a better amplification curve with a C_T in the 27th cycle and with the intensity of fluorescence reaching the 500000 in the 32nd cycle. Unlike the previously analysed assays, this PCR has a good amplification of the IC of the positive sample with a C_T in the 27th cycle and with the intensity of fluorescence reaching the 450000.

The better amplification curve of the sample C16 and of both IC is supported due to the increase in the concentration of $MgCl_2$. The Mg^{2+} is a cofactor of the DNA polymerase by complexing with dNTPs forming the substrate of the enzyme. The ion also facilitates the base pairing between two strands of DNA increasing the stability of the primer annealing. In high concentrations of Mg^{2+}, the substrate for the DNA polymerase is widely available and the high stability of primer annealing increase the probability of mispairing bases, diminish the specificity of the reaction (Markoulatos et al., 2002; Montgomery & Wittwer, 2014). However, the specificity of this kit is not affected, since the sample C17 didn't amplify, despite the increasing in $MgCl_2$ concentration.

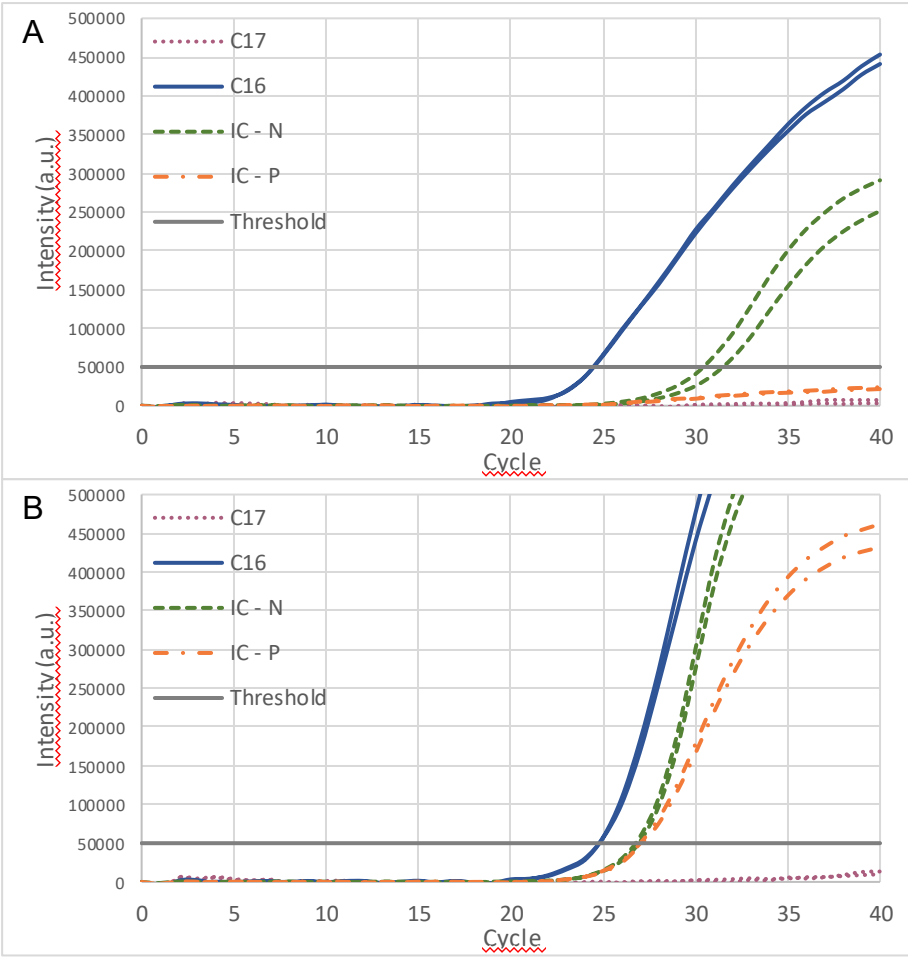

Figure 2.19 **Graphic representation of the PCR results:** PCR 156 with the lowest concentration of $MgCl_2$ [A] and PCR 252 with the highest concentration of the reagent [B]. The samples represented are C16 (blue continuous line), C17 (purple dotted line), Internal Control of the negative sample C17 (green dashed line) and Internal Control of the positive sample C16 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

After the analysis of all PCR assays, the PCR 252 have the optimal concentration of $MgCl_2$ for the SUPREME Real Time PCR Turkey Detection kit. This assay used the high concentration of both reagents not compromising the specificity and not inhibiting the reaction. All the conditions used in PCR 252 were selected for the SUPREME Real Time Turkey Detection kit.

2.3.2.2 Validation Tests

The validation tests are a necessary tool to confirm the legitimacy of the kit as well as assess the performance of the kit (Broeders et al., 2014). Assessing these parameters is required to commercialize the kits.

The performance of the kit is measured through the calculation of the specificity, LoD and Inclusivity and robustness.

2.3.2.2.1 Exclusivity Test

The exclusivity test guarantees that the previously chosen conditions didn't compromise the specificity of the new kit, assuring that any non-target sample doesn't amplify. This test enables the calculation of the specificity indicator, through equation 1 (in session 2.2.5.1 Exclusivity Tests). The exclusivity test was done using 10 replicates of 14 non-turkey samples with 5ng of sample DNA. The list of samples used in the exclusivity test is available in the Appendix 1. Figure 2.20 shows the results of the turkey detection kit exclusivity test with all non-turkey samples (blue dotted line) and respective IC (pink line).

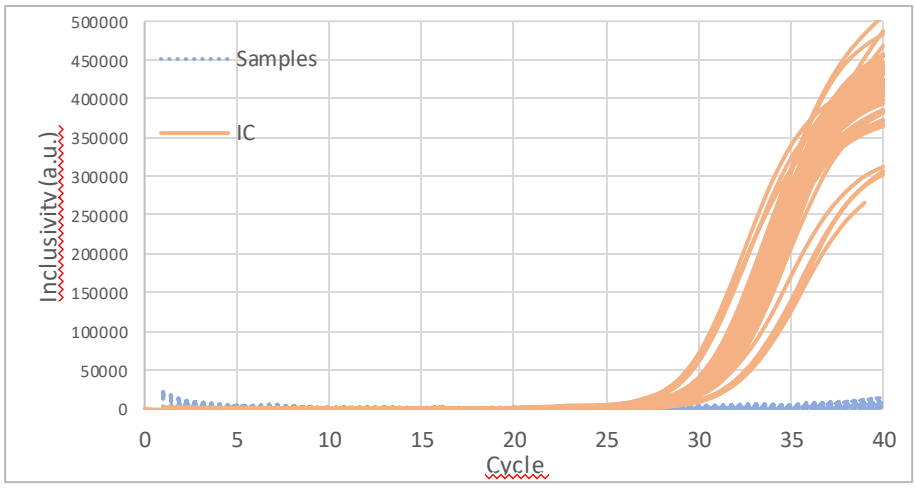

Figure 2.20 **Graphic representation of the PCR results:** turkey detection kit exclusivity test with 14 non-turkey samples (blue dotted line) and their IC (pink line)

The IC confirm the validity of the test and the lack of amplification of any non-target samples proves the specificity of the kit, with an indicator of 100%.

2.3.2.2.2 Inclusivity and Sensibility Tests

The sensibility test allows to determine the lowest amplifiable target DNA quantity. The sensibility test was done using 1ng, 10pg, 5pg and 1pg of turkey DNA. The inclusivity test guarantees that the previously chosen conditions can detect target DNA in every turkey matrix. This test enables the calculation of the inclusivity indicator, through equation 2 (in session 2.2.5.2 Inclusivity and Sensibility Tests). The inclusivity test was done using 10 replicates of 4 different turkey samples at the LoD. Figure 2.21 shows a sensibility test with the sample C16 at 1ng (darkest blue line with

diamonds), 10pg (blue line with circles), 5pg (light blue line with stars) and 1pg (lightest blue line with triangles). The threshold is the grey flat line.

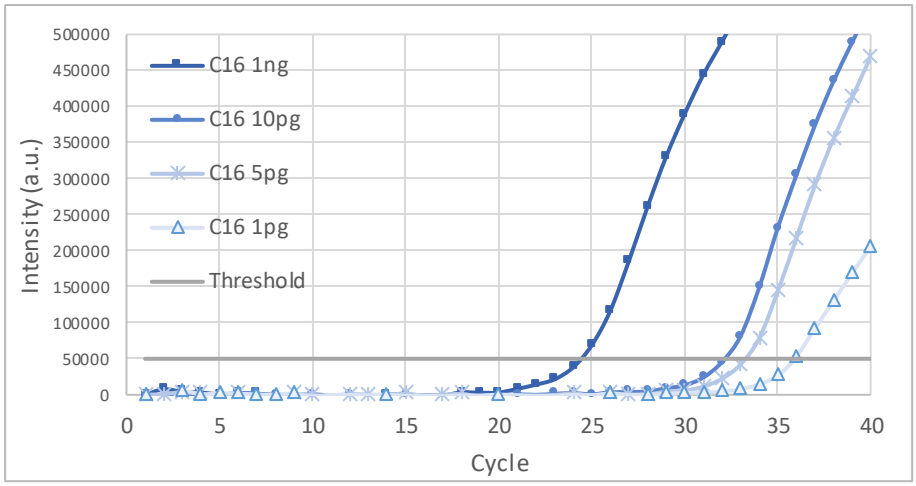

Figure 2.21 **Graphic representation of the PCR results:** turkey detection kit sensibility test. It was used the sample C16 at 1ng (darkest blue line with diamonds), 10pg (blue line with circles), 5pg (light blue line with stars) and 1pg (lightest blue line with triangles). The threshold is the grey flat line.

Analysing the results, all DNA concentrations show amplification. Logically, as the concentration of DNA is decreasing, the amplification curve starts in later cycles and has a lower intensity of fluorescence. The lower DNA quantity amplified was 1pg with a C_T in the 35th cycle and an intensity of fluorescence almost reaching 250000, setting the LoD of 1pg.

Figure 2.22 shows the inclusivity test with 4 turkey matrices with 1pg of DNA. The samples are C15 (dotted orange line), C24 (green continuous line), C55 (purple dashed and double dotted line) and C17 (lightest blue line with triangles). The threshold is the grey flat line.

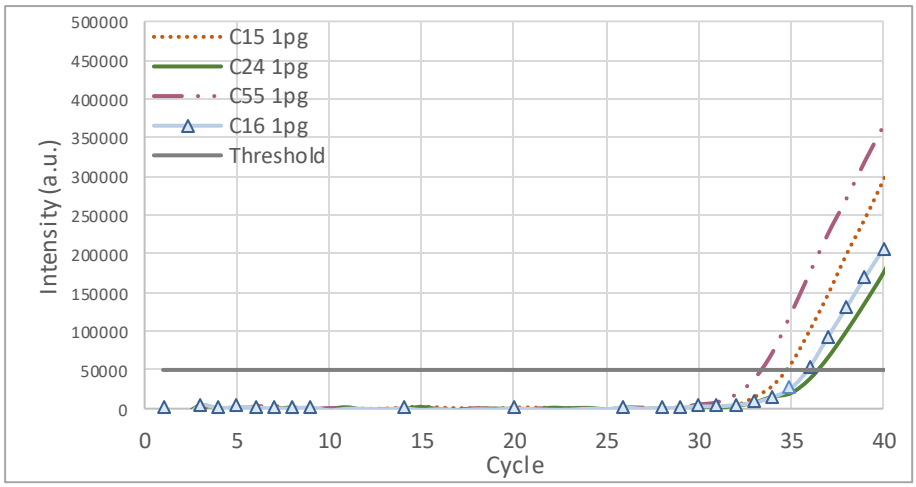

Figure 2.22 **Graphic representation of the PCR results:** turkey detection kit inclusivity test. The samples are C15 (dotted orange line), C24 (green continuous line), C55 (purple dashed and double dotted line) and C17 (lightest blue line with triangles). The threshold is the grey flat line.

All 5 different samples show amplification with a C_T ranging from the 32nd to the 35th cycle and an intensity of fluorescence reaching 400000. This result confirms the LoD of 1pg and the amplification of every target samples proves the inclusivity of the kit, with an indicator of 100%.

2.3.2.2.3 Robustness Tests

The robustness tests are exclusivity and inclusivity tests, to infer the performance of the kits in adverse conditions. This method guarantees the performance of the tests assuming that the user's equipment is not calibrated or shows temperature shifts (Broeders et al., 2014).

The exclusivity robustness test was done decreasing the annealing temperature 2ºC, facilitating the reaction. In these conditions none sample showed amplification, guaranteeing the specificity of the test.

The inclusivity robustness test was done increasing the annealing temperature 2ºC, disturbing the reaction. In these conditions, every sample showed amplification, guaranteeing the LoD and specificity of the test.

2.3.3. Horse Detection Kit

2.3.3.1 Optimization of PCR Conditions

The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined to the horse detection kit.

To guarantee the performance of the optimized solution and subsequent amplification of target DNA, preventing non-specific amplification, matrices with and without horse DNA were used as testing samples. All samples were tested in a concentration of 1ng/µl. The results and subsequent analysis will highlight a sample with the targeted sequence (C5), and a sample without the target sequence (C10). Amplification is not expected in sample C10 because it is from beef meat. If an amplification is shown in sample C10, the PCR conditions must be reconsidered, because it means the lack of specificity of the test. To define the C_T and consider an amplification positive, the amplification curve must have an intensity of fluorescence greater than the threshold of 50000. In Table 2.9, it is shown the order of the conditions tested, as well as the PCR assays performed and the respective BPMR code.

Table 2.9 List of ordered and optimized PCR conditions of the Horse Detection kit and its BPMR Code. The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined.

Order	Test Conditions	BPMR Code
1	Annealing Temperature	PCR 116, 138 and 139
2	Duration of each cycle phase	PCR 116, 161, 162 and 163
3	Type of primers and probe	PCR 255 and 257
4	Concentration of primers, $MgCl_2$ and dNTPs,	PCR 257, 259, 266 and 278

To facilitate the comparison between different assays, plots were standardized and a maximum of 500 000 units of fluorescence intensity was defined.

2.3.3.1.1 Annealing Temperature

The annealing temperature is a defining step in the optimization of a PCR kit. The PCR 116, 138 and 139 used different temperatures as means to test its effects in the yield and specificity of the kit. PCR 116 was done with an annealing temperature of 60ºC, PCR 138 dropped the annealing temperature to 58ºC and the annealing temperature of PCR 139 was 62ºC.

Figure 2.23 shows the results of PCR 116 with an annealing temperature of 60ºC [A], PCR 138 with lower annealing temperature [B] and PCR 139 with the annealing temperature at 62ºC [C]. All assays were done using the same samples. The samples represented are Horse Meat, C5 (blue continuous line); Beef Meatballs C10 (purple dotted line); Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

PCR 116 shows expected results. The sample C5 have an amplification curve with a maximum intensity of fluorescence between 200000 and 300000 and a C_T between the 28th cad 30th cycles and both IC with amplification curves surpassing the intensity of fluorescence of 500000 and C_T in the 28th cycle.

PCR 138 shows a result as expected., bearing in mind the decrease in the annealing temperature. The sample C5 amplification curve is better than the amplification curve of PCR 116, having a C_T in the 26th cycle and an intensity of fluorescence reaching the 450000. This result can be explained due to the lowering of the annealing temperature. Low annealing temperatures facilitate the formation of hydrogen bonds between primer and template, producing a more stable annealing (Montgomery et al., 2014; Rychlik et al., 1990). The easy and stable annealing facilitate the amplification as seen in PCR 138. The IC of the negative sample have a similar, although a little lower, amplification curve as PCR 116 and PCR 139. In contrast, sample C5 of PCR 139 didn't amplify due to the higher annealing temperature of PCR. High annealing temperatures are closer to T_m, disturbing the formation of hydrogen bonds and consequent primer annealing (Montgomery et al., 2014; Rychlik et al., 1990). The PCR 139 must be discarded, due to the no amplification of the positive sample.

Sample C10 didn't amplify in any assay, meaning that this range of temperatures doesn't affect the specificity of the turkey detection kit.

The optimal temperature for the horse detection kit is 58ºC, although It's worth remembering that this optimization process has a commercial goal. So, when defining the SUPREME Real Time PCR Horse Detection kit annealing temperature, it is vital to bear in mind the kit ease of use. The customer, regularly, uses several Detection kits simultaneously, so it's an advantage to have all the kits with the same annealing temperature. Taking that into consideration, the SUPREME Real Time PCR Horse Detection kits annealing temperature is set to 60ºC, as it's an efficient to horse DNA detection and it's the optimal temperature of some of other BPMR kits.

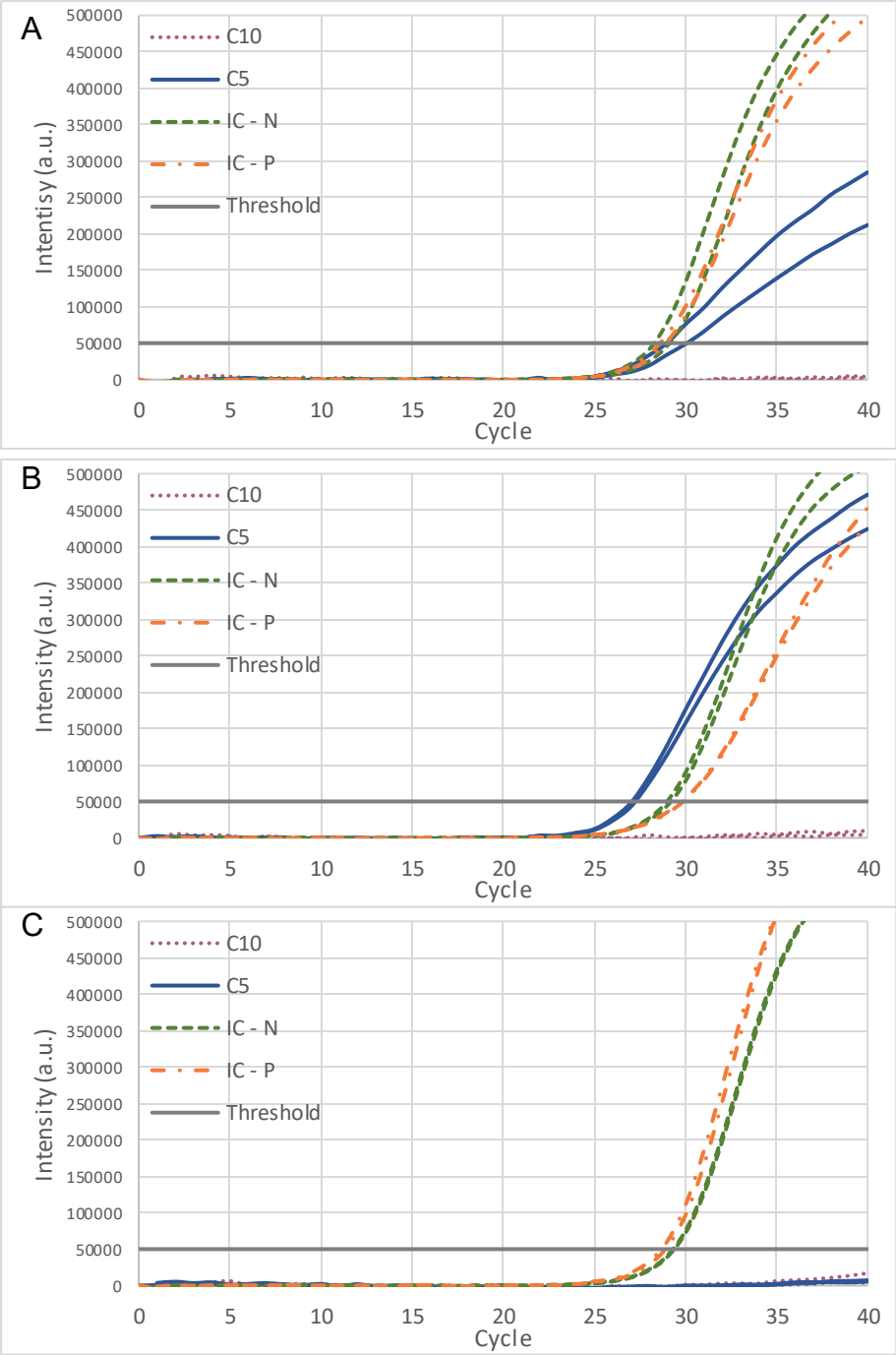

Figure 2.23 **Graphic representation of the PCR results:** PCR 116 with an annealing temperature of 60ºC [A], PCR 138 with lower annealing temperature [B] and PCR 139 with the annealing temperature at 62ºC [C]. The samples represented are C5 (blue continuous line), C10 (purple dotted line), Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.3.1.2 Duration of each cycle phase

The next step of the optimization of the horse detection kit is the definition of the duration time of each cycle phase. The denaturation phase of PCR 116 is 30 seconds, the annealing phase

lasts 30 seconds and the extension phase also last 30 seconds. In PCR 161, each cycle phase lasts 20, 30 and 20 seconds respectively. In PCR 162, each cycle phase lasts 15, 30 and 15 seconds respectively. In PCR 163, each cycle phase lasts 15, 30 and 10 seconds respectively. Figure 2.24 shows the results of PCR 116 [A], PCR 161 [B], PCR 162 [C] and PCR 163 [D]. All assays were done using the same samples. The samples represented are Horse Meat, C5 (blue continuous line); Beef Meatballs C10 (purple dotted line); Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

The negative sample C10 didn't amplify in any assay, meaning that the duration of the cycle doesn't compromise the specificity of the kit. The positive sample C5 have similar amplification curves in all assays, with C_T varying between the 28^{th} and 30^{th} cycle and an intensity of fluorescence ranging between 200000 in PCR 161, 162 and 163 and 300000 in PCR 116. These minor differences are due to the shorter extension phase of PCR 161, 162 and 163, causing a slight decrease in the intensity. The IC of the negative and positive samples also didn't have a significant change between the four assays, PCR 116 having the better amplification curves.

One of the optimization goals is to reduce the waiting time without compromising the kit performance. So, to reduce the cycle duration and, since there are none significant differences between assays with different durations, the SUPREME Real Time PCR Horse detection kit will have a denaturation phase of 15 seconds, an annealing phase lasting 30 seconds and an extension phase of 15 seconds.

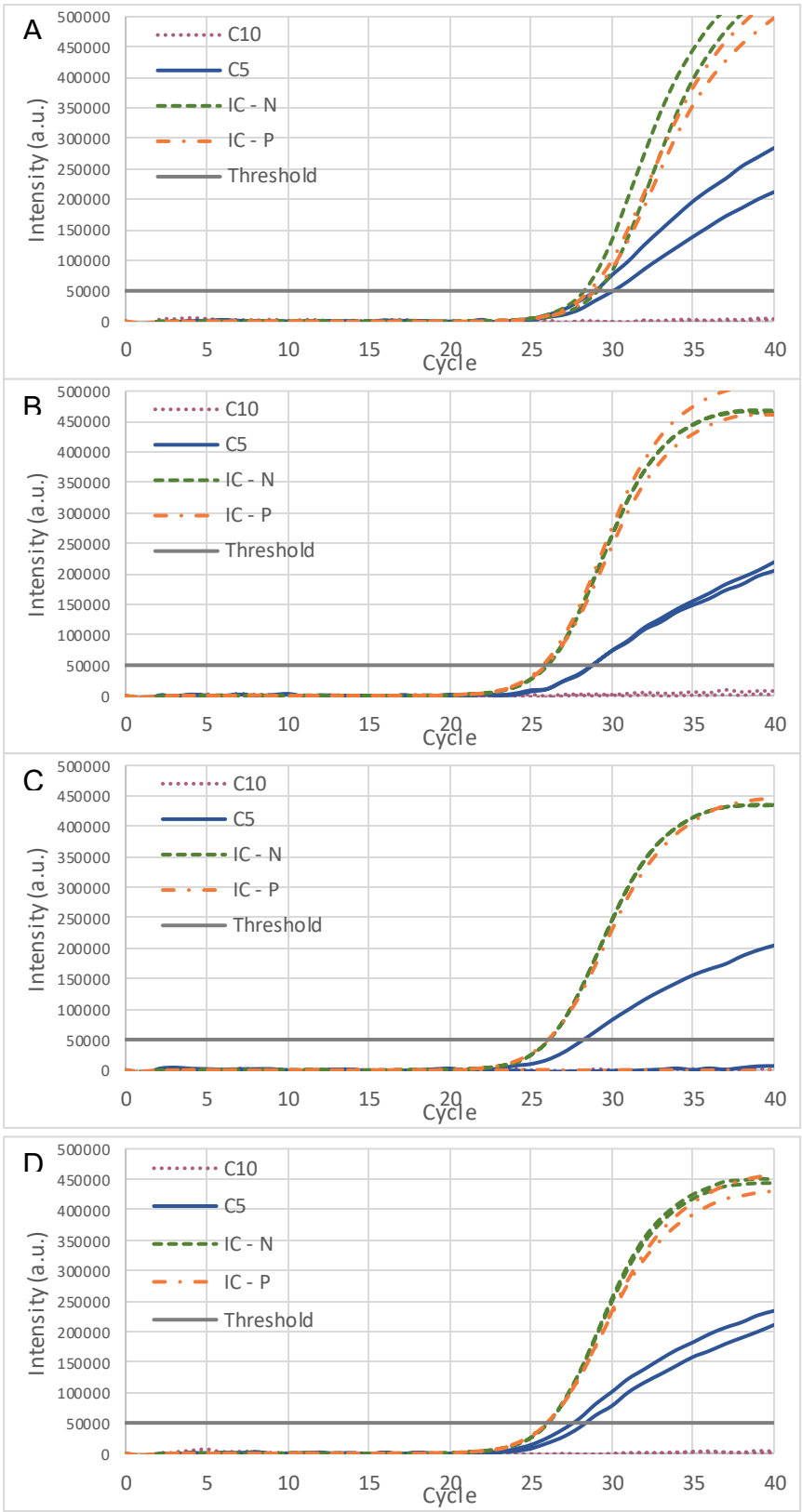

Figure 2.24 **Graphic representation of the PCR results:** PCR 116 [A], PCR 161 [B], PCR 162 [C] and PCR 163 [D]. The samples represented are C5 (blue continuous line), C10 (purple dotted line), Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line

2.3.3.1.3 Type of primers and probe

The chosen annealing temperature was 60ºC, however, there was no positive amplification at 62ºC. Even though the amplification at the chosen temperature occurs, the no amplification at 62ºC can jeopardize the optimization if the kit fails in the robustness tests. In that case, the next optimization steps will be conducted with an annealing temperature of 62ºC. The defined temperature for this kit will be tested with the conditions closed in the next steps in the Inclusivity and Exclusivity Tests.

The next condition optimized in the horse detection kit was the type of primers and probe. The analysis was done to two different sets of primers and probe. The first set is already used by BPMR in the current BIOPREMIER Horse detection kit (PCR 257) and the second is a new set of primers and probe newly designed and never used in BPMR laboratories (PCR 255). Figure 2.25 shows the results of PCR 255 using the new set [A] and PCFR 257 with the current set [B] with the same samples. The samples represented are Horse Meat, C5 (blue continuous line); Beef Meatballs C10 (purple dotted line); Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

As expected, the sample C10 didn't show amplification in any PCR, meaning that neither set is unspecific and doesn't detect horse.

Since the sample C5 have horse DNA in it, it must show an amplification curve in both PCR. However, in PCR 255 that is not the case, as there is no positive amplification. In contrast, in PCR 257, the amplification curve is not a good curve, as the C_T is in the 38th cycle and the intensity of fluorescence doesn't reach 100000. The low amplification curve in PCR 257 is explained due the high annealing temperature, disturbing the reaction.

The amplification curves of both IC are very similar with a C_T around the 30th cycle and an intensity always reaching 500000.

The primers and probe defined for the SUPREME Real Time Horse detection kit are the current ones, because, even though the amplification curve of C5 is very little in PCR 257, there is no amplification in PCR 255, so this assay must be discarded.

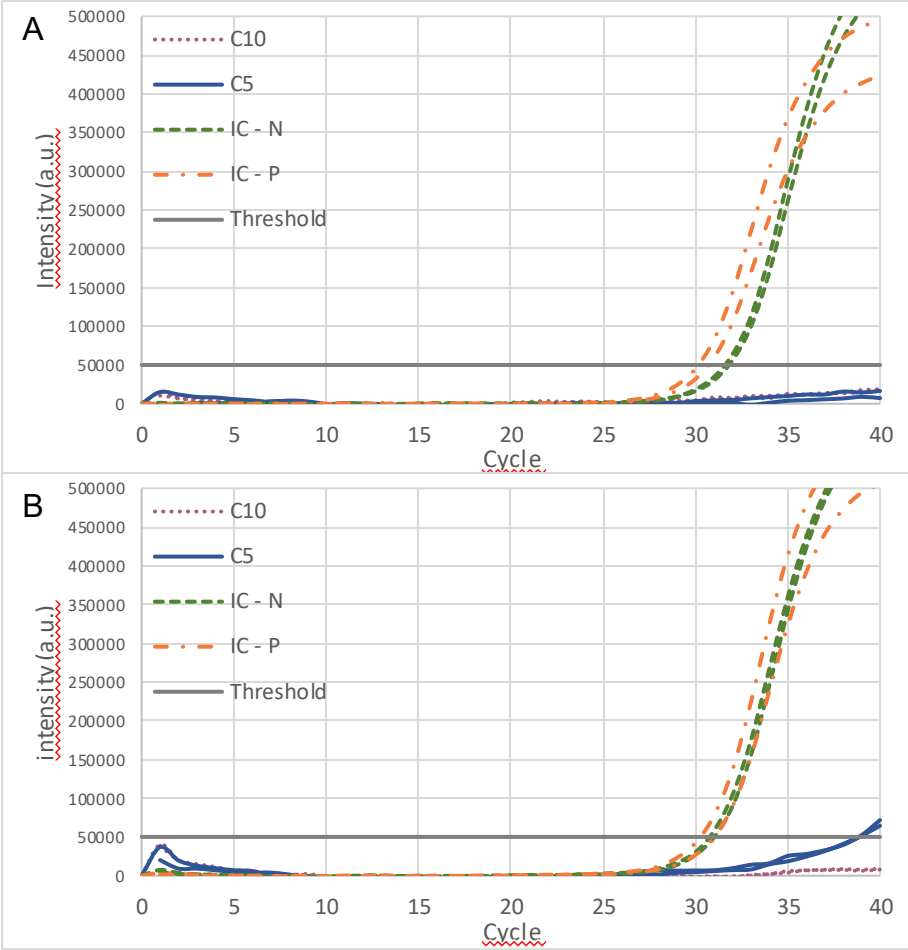

Figure 2.25 **Graphic representation of the PCR results:** PCR 255 using the new set [A] and PCFR 257 with the current set [B]. The samples represented are C5 (blue continuous line), C10 (purple dotted line), Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.3.1.4 Concentration of primers, $MgCl_2$ and dNTPs

The concentration of $MgCl_2$ and dNTPs was defined after the set of primers and probe were chosen. The optimal concentration of these reagents was chosen by analysing the PCR 257, 259 and 266 and 278. The PCR 257 had the lowest concentration of primers, but the highest concentration of $MgCl_2$ and dNTPs. The PCR 259 had the lowest concentration of $MgCl_2$, but the highest concentration of primers and dNTPs. The PCR 266 had the highest concentration of primers, but the lowest concentration of $MgCl_2$ and dNTPs. Finally, PCR 278 had the lowest concentration of dNTPs, but the highest concentration of $MgCl_2$ and primers. Figure 2.26 shows the results of PCR 257 [A], PCR 259 [B], PCR 266 [C] and PCR 278 [D]. All assays were done using the same samples. The samples represented are Horse Meat, C5 (blue continuous line); Beef Meatballs C10 (purple dotted line); Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The amplification curves of both IC are very similar in PCR 257 and 259 with a C_T around the 30th cycle and an intensity always reaching 500000. In PCR 266 and PCR 278, the amplification curves are better, having a C_T in the 28th cycle and an intensity of fluorescence reaching 500000 in

the 30th cycle. The sample C10 didn't amplify any PCR as expected because it doesn't have horse DNA. The sample C5 in PCR 257 presented a low amplification curve, as previously analysed. The same sample in PCR 259 have a similar amplification curve. In PCR 266 and 278, the amplification of the sample C5 have a significant improvement. Both curves have a C_T in the 32nd cycle and an intensity of fluorescence surpassing 500000 in the 38th cycle (PCR 278) and in the 39th (PCR 266).

This sudden increase in the intensity of fluorescence coincides with the lower concentrations of dNTPs. The Mg^{2+} ions complex with the dNTPs available forming the substrate that the DNA polymerase can use, however if there is too much free dNTPs it will cause a Mg^{2+} depletion and a consequent inhibition of the reaction, as it happens in PCR 257 and 259.

The increasing in the concentration of $MgCl_2$, should improve the amplification curves, as the Mg^{2+} is a cofactor of the DNA polymerase by complexing with dNTPs forming the substrate of the enzyme. The ion also facilitates the base pairing between two strands of DNA increasing the stability of the primer annealing. In PCR 278, with the increase of the concentration of $MgCl_2$, the amplification curve has slightly higher intensity of fluorescence. Despite the improvement, the concentration of $MgCl_2$ and of primers is not as significant as the concentration of dNTPs in the horse detection kit (Markoulatos et al., 2002; Montgomery & Wittwer, 2014; Ruiz-Villalba et al., 2017).

After the analysis of the four PCR assays, the PCR 278 have the optimal concentration of primers, $MgCl_2$ and dNTPs. This assay used the lowest concentration of dNTPs and the highest concentration of $MgCl_2$ and primers not compromising the specificity and not inhibiting the reaction. These conditions were selected for the SUPREME Real Time PCR Horse Detection kit.

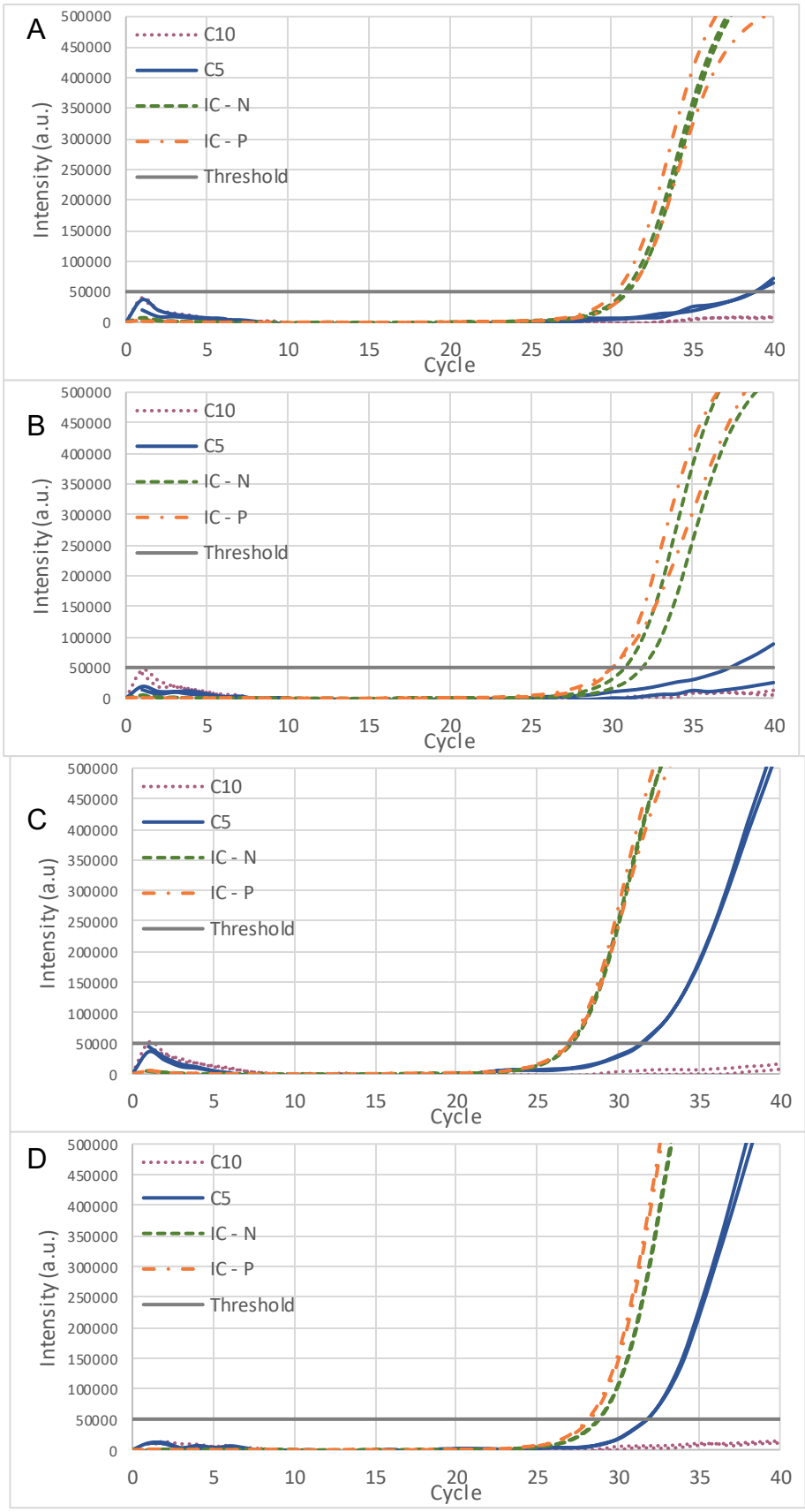

Figure 2.26 **Graphic representation of the PCR results:** PCR 257 [A], PCR 259 [B], PCR 266 [C] and PCR 278 [D].The samples represented are C5 (blue continuous line), C10 (purple dotted line), Internal Control of the negative sample C10 (green dashed line) and Internal Control of the positive sample C5 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.3.2 Validation Tests

The validation tests are a necessary tool to confirm the legitimacy of the kit as well as assess the performance of the kit (Broeders et al., 2014). Assessing these parameters is required to commercialize the kits.

The performance of the kit is measured through the calculation of the specificity, LoD and Inclusivity and robustness. The validation tests were done with an annealing temperature of 60ºC to confirm the specificity and sensibility of the kit, as it weren't tested at this temperature in the last steps of the optimization process.

2.3.3.2.1 Exclusivity Test

The exclusivity test guarantees that the previously chosen conditions didn't compromise the specificity of the new kit, assuring that any non-target sample doesn't amplify. This test enables the calculation of the specificity indicator, through equation 1 (in session 2.2.5.1 Exclusivity Tests). The exclusivity test was done using 10 replicates of 16 non-horse samples with 5ng of sample DNA. The list of samples used in the exclusivity test is available in the Appendix 1. Figure 2.27 shows the results of the horse detection kit exclusivity test with all non-horse samples (blue dotted line) and their IC (pink line).

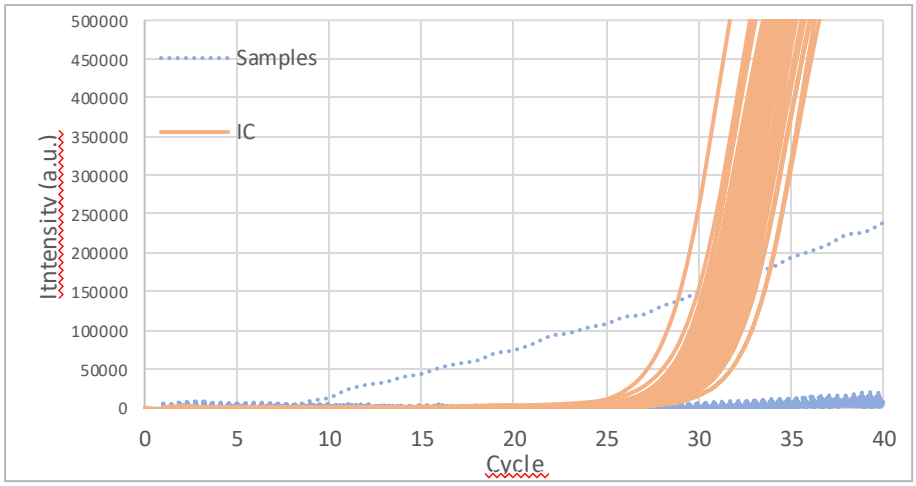

Figure 2.27 **Graphic representation of the PCR results:** horse detection kit exclusivity test with 16 non-horse samples (blue dotted line) and their IC (pink line)

The IC confirm the validity of the test and only one sample had amplification. However, it is not a well-defined curve, as it must be the result of some operator's error. Nonetheless, it counts as one false positive replicate, setting the specificity indicator at 99,4%, proving the specificity of the kit.

2.3.3.2.2 Inclusivity and Sensibility Tests

The sensibility test allows to determine the lowest amplifiable target DNA quantity. The sensibility test was done using 1ng, 100pg 10pg, 5pg and 1pg of horse DNA. The inclusivity test guarantees that the previously chosen conditions can detect target DNA in every horse matrix. This test enables the calculation of the inclusivity indicator, through equation 2 (in session 2.2.5.2

Inclusivity and Sensibility Tests). Since there is other type of horse meat available in the market, the inclusivity test was done it three different samples with 50ng of DNA with the LoD quantity of horse DNA. The samples mixed with horse DNA were, C10, beef meatballs, C14, Pork Lasagne and C57, beef hamburger. Figure 2.28 shows a sensibility test with the sample C5 at 1ng (darkest blue line with diamonds), 100pg (dark blue line with squares), 10pg (blue line with circles), 5pg (light blue line with stars) and 1pg (lightest blue line with triangles). The threshold is the grey flat line.).

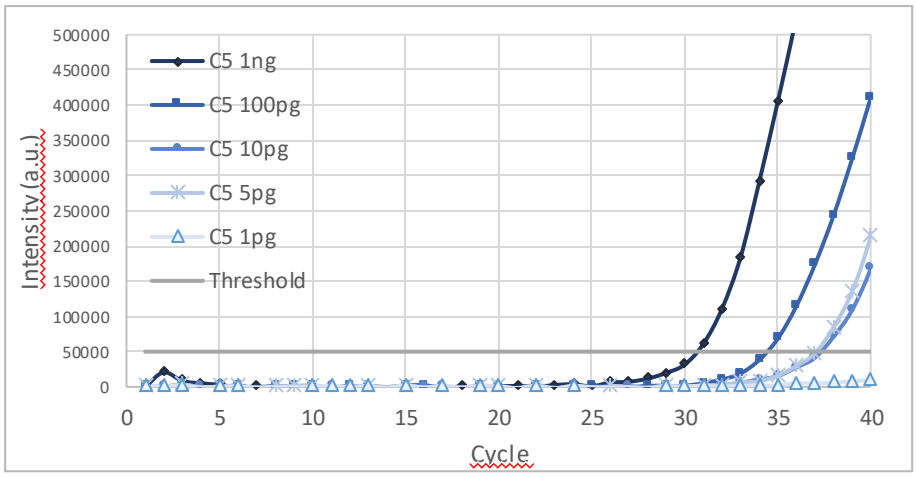

Figure 2.28 **Graphic representation of the PCR results:** horse detection kit sensibility test. It was used the sample C5 at 1ng (darkest blue line with diamonds), 100pg (dark blue line with squares), 10pg (blue line with circles), 5pg (light blue line with stars) and 1pg (lightest blue line with triangles). The threshold is the grey flat line.

Analysing the results, all DNA concentrations show amplification. Logically, as the concentration of DNA is decreasing, the amplification curve starts in later cycles and has a lower intensity of fluorescence. The lower DNA quantity amplified was 5pg with a C_T in the 36th cycle and an intensity of fluorescence almost reaching 250000. However, due to the previously problems with the increase of temperature, to avoid the failing in the robustness test, it was chosen the LoD of 10pg.

Figure 2.29 shows the inclusivity test with the horse meat sample and the 3 mixed samples with 10pg of DNA. The samples are C10+C5 (dark blue), C14+C5 (orange), C57+C5 (yellow) and C5 (light blue).

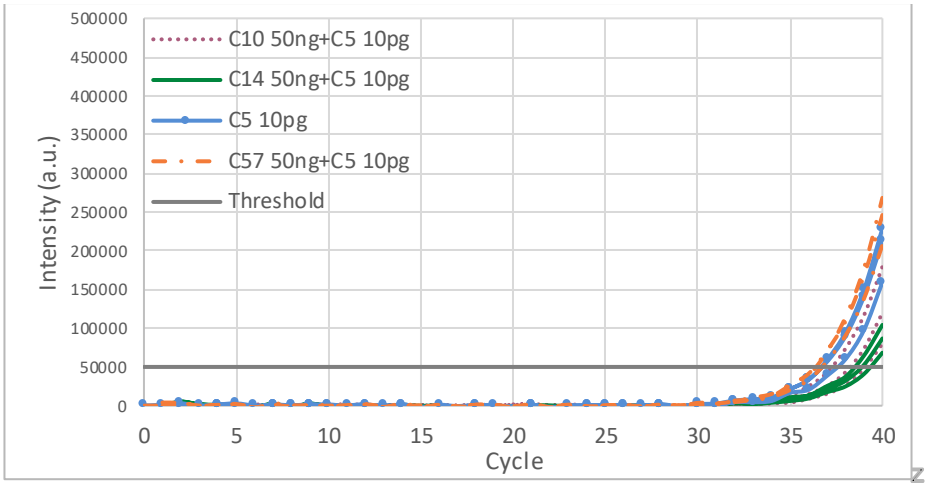

Figure 2.29 **Graphic representation of the PCR results:** horse detection kit inclusivity test. The samples are 50ng of C10 plus 10pg of C5 (dotted orange line), 50ng of C14 plus 10pg of C5 (green continuous line), C5 (blue line with circles), 50ng of C57 plus 10pg of C5 (orange dashed and dotted line) and C17 (lightest blue line with triangles). The threshold is the grey flat line.

All 4 different samples show amplification with a C_T ranging from the 35th to the 39th cycle and an intensity of fluorescence reaching 250000. This result confirms the LoD of 10pg and the amplification of every target samples proves the inclusivity of the kit, with an indicator of 100%.

2.3.3.2.3 Robustness Tests

The robustness tests are exclusivity and inclusivity tests, to infer the performance of the kits in adverse conditions. This method guarantees the performance of the tests assuming that the user's equipment is not calibrated or shows temperature shifts (Broeders et al., 2014).

The exclusivity robustness test was done decreasing the annealing temperature 2ºC, facilitating the reaction. In these conditions none sample showed amplification, guaranteeing the specificity of the test.

The inclusivity robustness test was done increasing the annealing temperature 2ºC, disturbing the reaction. In these conditions, every sample showed amplification, guaranteeing the LoD and specificity of the test.

2.3.4. Swine Detection Kit

2.3.4.1 Optimization of PCR Conditions

The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined to the swine detection kit.

To guarantee the performance of the optimized solution and subsequent amplification of target DNA, preventing non-specific amplification, matrices with and without swine DNA were used as testing samples. In PCR 100 and 125 the positive sample, C19 was tested in a concentration of 10ng. In all the remaining assays, all samples were tested in a concentration of 1ng/µl. The results and subsequent analysis will highlight a sample with the targeted sequence (C19), and a sample without the target sequence (C29). Amplification is not expected in sample C29 because it is from rabbit meat. If an amplification is shown in sample C29, the PCR conditions must be reconsidered, because it means the lack of specificity of the test. To define the C_T and consider an amplification positive, the amplification curve must have an intensity of fluorescence greater than the threshold of 50000.

In Table 2.10, it is shown the order of the conditions tested, as well as the PCR assays performed and the respective BPMR code.

Table 2.10 List of ordered and optimized PCR conditions of the Swine Detection kit and its BPMR Code. The PCR conditions comprises the kind and concentration of the master mix reagents and the PCR program defined.

Order	Test Conditions	BPMR Code
1	Presence of DMSO	PCR 100 and 125
2	Annealing Temperature	PCR 125, 136 and 137
3	Duration of each cycle phase	PCR 125, 158, 159 and 160
4	Type of primers and probe	PCR 303 and 304
5	Concentration of primers and $MgCl_2$	PCR 304, 305/306 and 307/308

To facilitate the comparison between different assays, plots were standardized and a maximum of 750 000 units of fluorescence intensity was defined.

2.3.4.1.1 Presence of DMSO

The first step in the optimization of the swine detection kit is evaluation of the usage of DMSO. The PCR 100 which contained DMSO was analysed against the PCR 125 that not used DMSO nor another PCR enhancer. Figure 2.30 shows the results of PCR 100 with DMSO [A] and PCR 125 without DMSO [B]. All assays were done using the same samples. The samples represented are Ham, C18 (blue continuous line); Rabbit Meat, C29 (purple dotted line); Internal Control of the negative sample C29 (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

Neither assay has non-specific amplification as the sample C29 didn't amplify. The IC of positive sample has an amplification curve in both assays, however, in PCR 100 it barely reaches the intensity of fluorescence of 50000 and has a C_T in the 38th cycle. In PCR 125, the IC has a C_T in the 36th cycle and an intensity of fluorescence of 100000

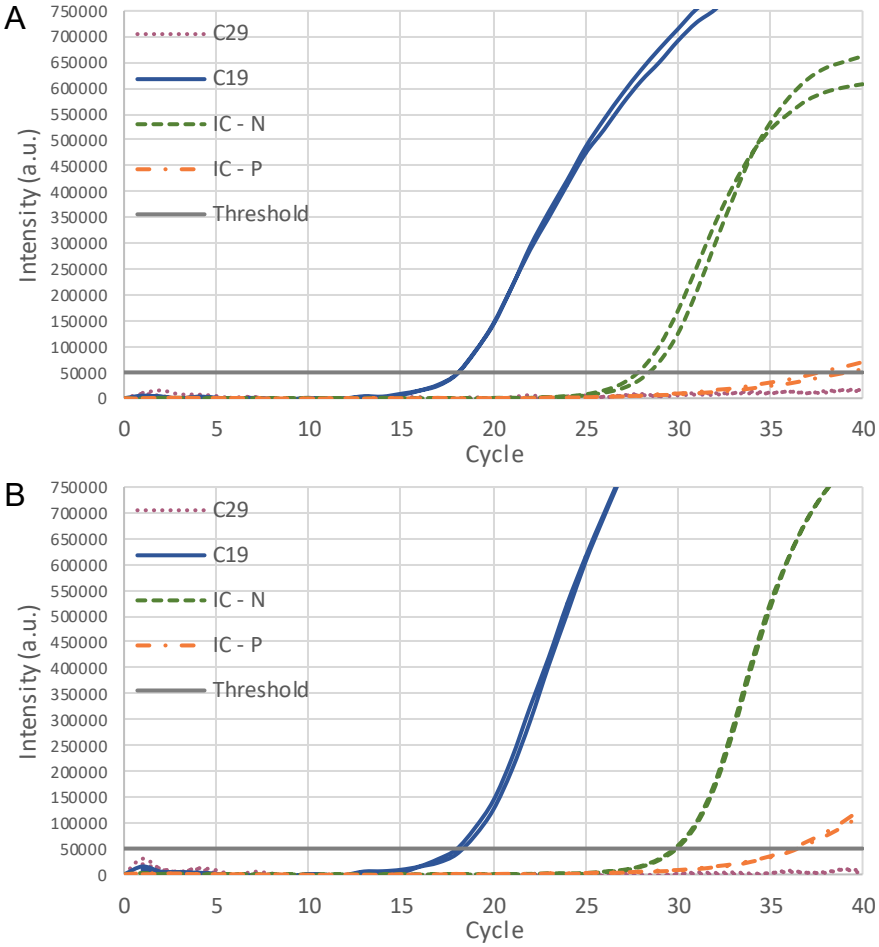

Figure 2.30 **Graphic representation of the PCR results:** PCR 100 with DMSO [A] and PCR 125 without DMSO [B]. The samples represented are C19 (blue continuous line), C29 (purple dotted line), Internal Control of the negative sample C29 (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

Sample C19 shows an amplification curve in both PCR assays, as expected. The intensity of fluorescence of the amplification curve of sample C19 in PCR 100 surpasses the 750000 in the 32nd cycle and has a C_T in the 18th cycle. The same sample has a similar CT; however, the intensity of fluorescence reaches the 750000 earlier, in the 27th cycle.

The IC from the negative sample in PCR 100 has an amplification curve of this sample with a C_T in the 28th cycle and the intensity of fluorescence reaching 650000. While, in PCR 125, the amplification curve is better, having a C_T in the 30th cycle and the intensity of fluorescence almost surpassing 750000.

This results are as expected because DMSO is an enhancer which increase the specificity the PCR by lowering the T_m (Chakrabarti & Schutt, 2001; Henke et al., 1997; Ralser et al., 2006). The lower T_m prevents the occurrence of secondary DNA structures and non-specific binding, because the lower the T_m, the more difficult is to the primer to bind to the template. This thermodynamic behaviour increases the specific annealing, since the correct base pairing is more favourable. However, by lowering the T_m, the primer has difficulty binding to the template causing a decrease in the yield of the reaction when comparing positive samples with assays without DMSO.

As so, the amplification curves of C19 and the both IC in PCR 125 are better than the amplification curves of these sample in PCR 100.

Therefore, the SUPREME Real Time PCR will not contain DMSO as the PCR 125 have better amplification curve in the sample C19, without compromising the specificity in sample C29. However, in the exclusivity tests it will be confirmed the specificity of the reaction and if the DMSO is necessary.

2.3.4.1.2 Annealing Temperature

The annealing temperature is a defining step in the optimization of a PCR kit. The PCR 125, 136 and 137 used different temperatures as means to test its effects in the yield and specificity of the kit. PCR 125 was done with an annealing temperature of 60ºC, PCR 136 dropped the annealing temperature to 58ºC and the annealing temperature of PCR 137 was 62ºC. Figure 2.31 shows the results of PCR 125 with an annealing temperature of 60ºC [A], PCR 136 with lower annealing temperature [B] and PCR 137 with the annealing temperature at 62ºC [C]. The samples represented are Ham, C18 (blue continuous line); Rabbit Meat, C29 (purple dotted line); Internal Control of the negative sample C29 (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

Sample C29 didn't amplify in any assay, meaning that this range of temperatures doesn't affect the specificity of the turkey detection kit.

PCR 125, as previously analysed, shows expected results. The sample C19 have an amplification curve with an intensity of fluorescence surpassing 750000 in the 27th cycle and a C_T in the 18th cycle and the IC of negative sample with an amplification curve almost reaching the 750000 of intensity of fluorescence and a C_T in the 30th cycle.

PCR 136 shows a result as expected., bearing in mind the decrease in the annealing temperature. The sample C19 have an amplification curve with a C_T in the 24th cycle but an intensity of fluorescence reaching the 650000. In contrast, sample C19 of PCR 137 shows an amplification curve of with a C_T in the 25th cycle and an intensity of fluorescence of 450000. These results can be explained due to the variations of the annealing temperature. Low annealing temperatures, as in PCR 136, facilitate the formation of hydrogen bonds between primer and template, producing a more stable annealing. The easy and stable annealing facilitate the specific amplification but also increase the probability of non-specific amplification (Montgomery et al., 2014; Rychlik et al., 1990). PCR 137 have a higher annealing temperature than PCR 110 and high annealing temperatures are closer to T_m, disturbing the formation of hydrogen bonds and consequent primer annealing. The amplification curves of IC of the negative sample in PCR 136 and 137 are similar.

On the contrary, the IC of the positive sample in PCR 137 have a greater amplification than in PCR 136. The quantity of target DNA in the reaction mix is greater comparing with the IC DNA. The difference of DNA concentration causes a competition for the active centres of the DNA polymerases, resulting in the amplification of the target DNA in favour of the IC. Since the PCR 136 amplified more target DNA, the IC curve barely reaches the threshold.

Since the sample C19 was used in the different concentration in PCR 125, it cannot be compared it the remaining assays. There was no need to an additional assay to replace 125, since the goal of the step defining the annealing temperature of the kit, besides discover the optimal temperature, is to assess if the temperature of annealing affects significantly the amplification of positive samples and the specificity of the kit. If these conditions met, as they were, the defined annealing temperature must be the same as the other kits, hence 60ºC was the temperature set for the SUPREME Real Time PCR Swine Detection kit.

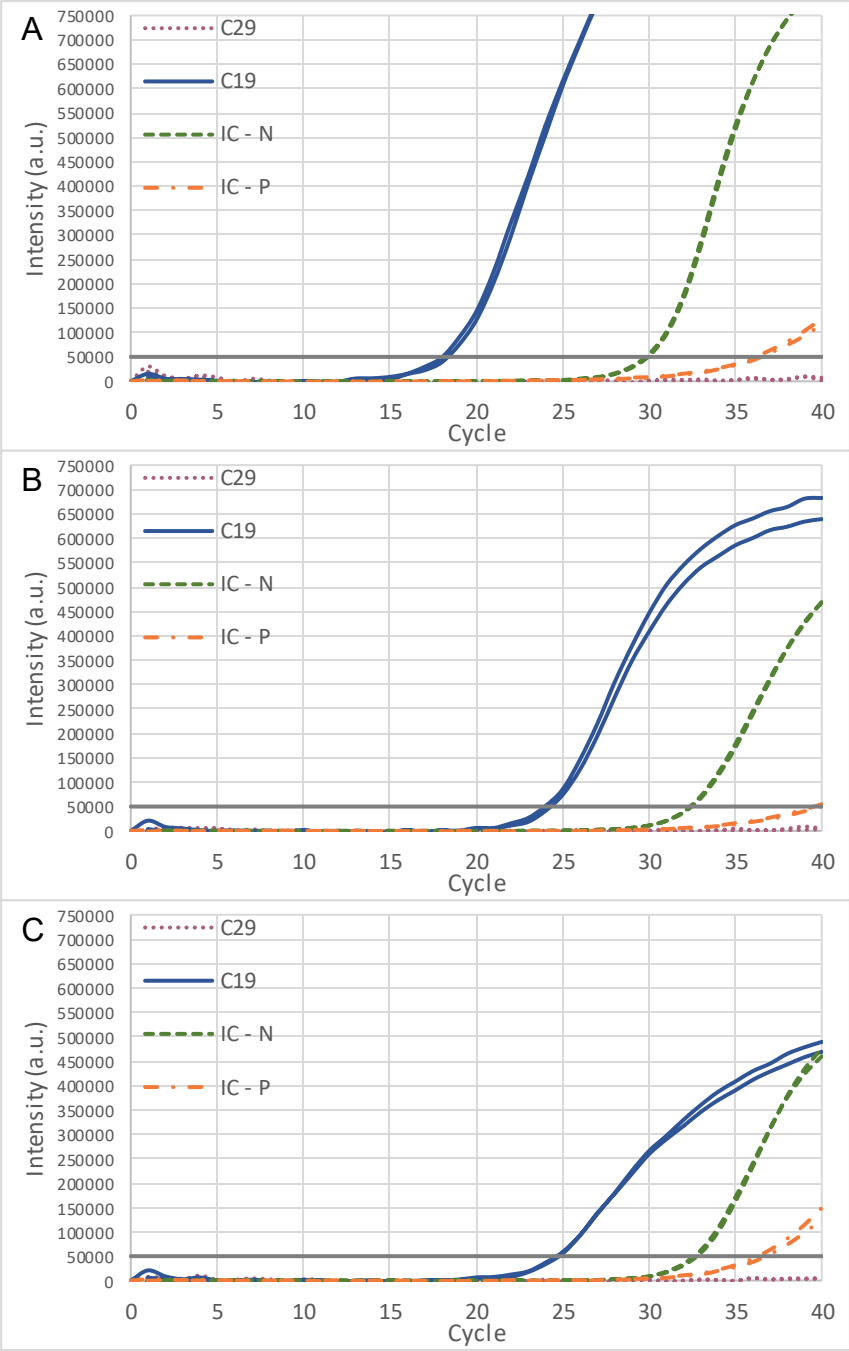

Figure 2.31 **Graphic representation of the PCR results:** PCR 125 with an annealing temperature of 60ºC [A], PCR 136 with lower annealing temperature [B] and PCR 137 with the annealing temperature at 62ºC [C]. The samples represented are C19 (blue continuous line), C29 (purple dotted line), Internal Control of the negative sample C29 (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.4.1.3 Duration of each cycle phase

The next step of the optimization of the swine detection kit is the definition of the duration time of each cycle phase. The denaturation phase of PCR 125 is 30 seconds, the annealing phase lasts 30 seconds and the extension phase also last 30 seconds. In PCR 157, each cycle phase lasts 20, 30 and 20 seconds respectively. In PCR 159, each cycle phase lasts 15, 30 and 15 seconds respectively. In PCR 160, each cycle phase lasts 15, 30 and 10 seconds respectively. Figure 2.32 shows the results of PCR 125 [A], PCR 158 [B], PCR 159 [C] and PCR 160 [D]. The samples represented are Ham, C18 (blue continuous line); Rabbit Meat, C29 (purple dotted line); Internal Control of the negative sample C29 (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The negative sample C29 didn't amplify in any assay, meaning that the duration of the cycle doesn't compromise the specificity of the kit.

The positive sample C19 have similar amplification curves in all assays, except in PCR 125, with C_T in the 25^{th} cycle and an intensity of fluorescence ranging between 650000 and a little over 750000. These minor differences are due to the shorter extension phase of PCR 158, 159 and 160, causing a slight decrease in the intensity. The IC of the negative and positive samples also didn't have a significant change between these four assays, with a C_T in the 26^{th} cycle with an intensity of fluorescence of 450000.

Since the sample C19 was used in the different concentration in PCR 125, it cannot be compared it the remaining assays. There was no need to an additional assay to replace 125, since the goal of the step defining the duration of each cycle phase of the kit, besides discover the cycle duration, is to assess if the duration of each cycle can be reduced without compromising the kit performance. If these conditions met, as they were, the defined duration of each cycle phase must be the same as reduced as possible and the same as the other kits, hence the SUPREME Real Time PCR Swine detection kit will have a denaturation phase of 15 seconds, an annealing phase lasting 30 seconds and an extension phase of 15 seconds.

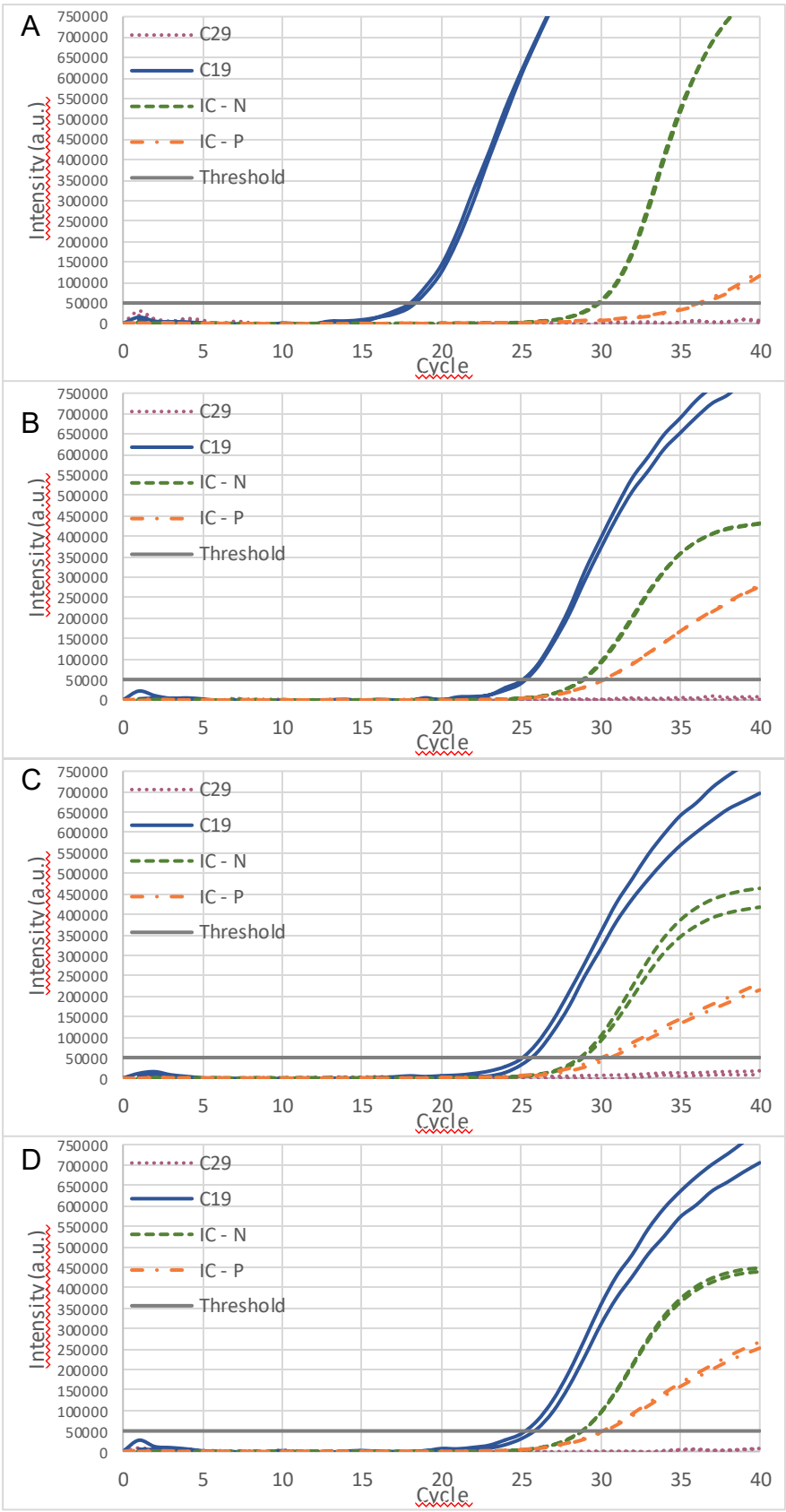

Figure 2.32 **Graphic representation of the PCR results:** PCR 125 [A], PCR 158 [B], PCR 159 [C] and PCR 160 [D]. The samples represented are C19 (blue continuous line), C29 (purple dotted line), Internal Control of the negative sample C29 (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

2.3.4.1.4 Type of primers and probe

The next condition optimized in the swine detection kit was the type of primers and probe. The analysis was done to two different sets of primers and probe. The first set is already used by BPMR in the current BIOPREMIER Swine detection kit (PCR 303) and the second is a new set of primers and probe newly designed and never used in BPMR laboratories (PCR 304). Figure 2.33 shows the results of PCR 303 using the current set [A] and PCR 304 the new set [B] with the same samples. The samples represented Ham, C18 (blue continuous line); NegativeControl (purple dotted line); Internal Control of the negative control (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

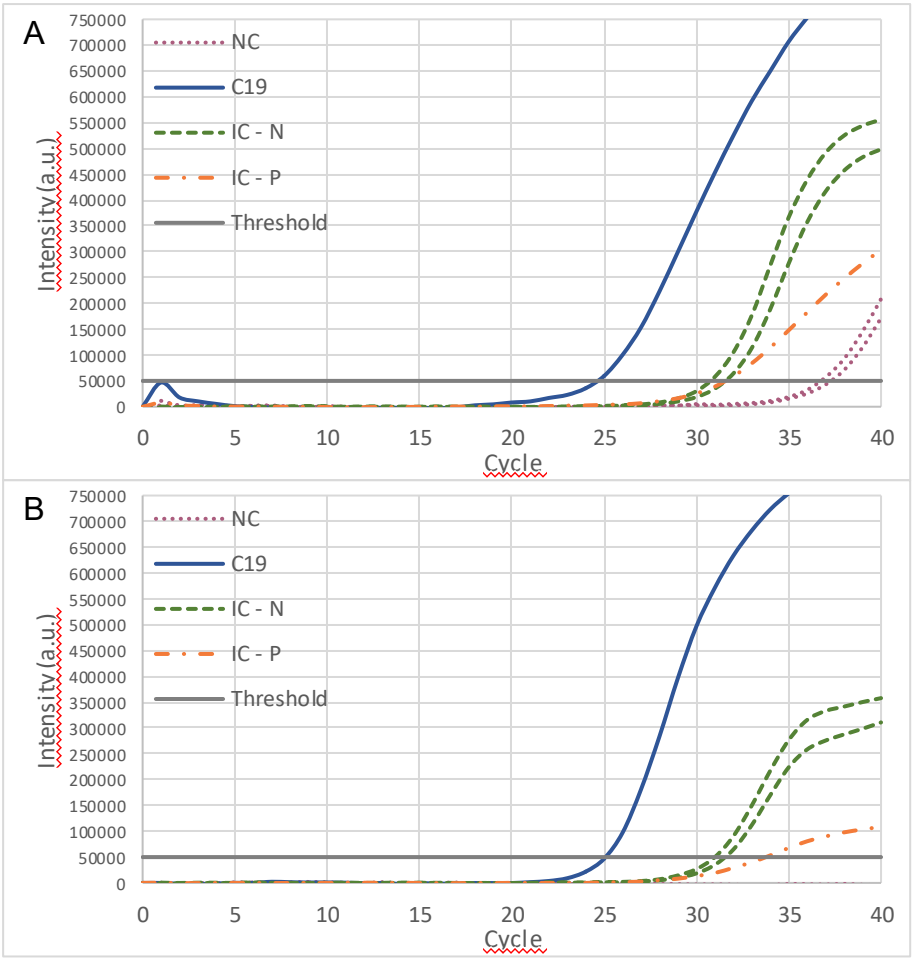

Figure 2.33 **Graphic representation of the PCR results:** PCR 303 using the current set [A] and PCR 304 the new set [B]. The samples represented are C19 (blue continuous line), Negative Control (purple dotted line), Internal Control of the Negative Control (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

As of the PCR 303, assays done with the same conditions were amplifying, even negative control samples. In order to ensure the integrity and the specificity of the kit, the next optimization steps will be conducted with the Negative Control instead of a negative sample. The negative samples will be tested with final the conditions in the Exclusivity Test.

The IC of the negative control amplify in both PCR with an amplification curve with a C_T between the 31st and 32nd cycle and with the intensity of fluorescence ranging between 350000 in PCR 304 and 500000 in PCR 303. The IC of the positive sample has an expected result.

Although the positive sample C19, in PCR 303, has an intensity of fluorescence reaching 750000 in the 36th cycle and a C_T in the 25th cycle, the PCR 303 must be discarded as the negative control shows amplification. The negative control can never amplify.

In contrast, in PCR 304, the results are as expected. The negative control didn't amplify and the sample C19 shows an amplification curve with a C_T, approximately, in the 25th cycle and a maximum intensity of fluorescence surpassing 750000 in the 36th cycle.

Therefore, the primers and probe defined for the SUPREME Real Time Swine detection kit are the new ones as the PCR 304, because there is amplification of the negative control in the assay, discarding the current set of primers and probe. The amplification of a negative control can mean the formation of primer dimers or the contamination of the reagents, consumables or equipment with PCR product originated from the previous assays. The new set of primers can target a different portion of Swine DNA, avoiding the possible contamination or the set could have been designed to avoid primer dimers.

2.3.4.1.5 Concentration of primers and $MgCl_2$

The concentration of $MgCl_2$ and dNTPs was defined after the set of primers and probe were chosen. The optimal concentration of these reagents was chosen by analysing the PCR 304, PCR 305/306 and PCR 307/308. The PCR 304 have the lowest concentration of primers and $MgCl_2$. The PCR 305 and PCR 306 have the same conditions and the lowest concentration of primers, but the highest concentration of $MgCl_2$. Finally, PCR 307 and 308 have the same conditions and the highest concentration of $MgCl_2$ and primers. Figure 2.34 shows the results of PCR 304 [A], PCR 305/306 [B] and PCR 307/308 [C]. All assays were done using the same samples. The samples represented are Ham, C18 (blue continuous line); Negative Control (purple dotted line); Internal Control of the Negative Control (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

The negative control didn't amplify any PCR, as expected. The sample C19 in PCR 304 presented an amplification curve, as previously analysed. The curve has a C_T, approximately, in the 25th cycle and an intensity of fluorescence surpassing 750000 in the 36th cycle. The same sample in PCR 305 has a C_T, approximately, in the 24th/25th cycle and an intensity of fluorescence surpassing 750000 in the 32nd cycle. In PCR 307/308, the sample C19 have the better amplification curve, with a C_T, approximately, in the 25th cycle and an intensity of fluorescence surpassing 750000 before the 30th cycle.

The IC of the negative control follows a similar tendency, except in the PCR 305/306. In that assay, the IC of the negative control have an intensity of fluorescence reaching the 200000 and a C_T in the 32nd cycle. In contrast, in PCR 304 the C_T of the amplification curve of the IC of the negative control is in the 32nd cycle and the intensity of fluorescence is 350000. In PCR 307/308, the amplification curve of the IC of negative control have a C_T in the 28th cycle and an intensity of

fluorescence ranging between 500000 and 650000. The low amplification in PCR 305/306, can be explained through degradation of IC DNA.

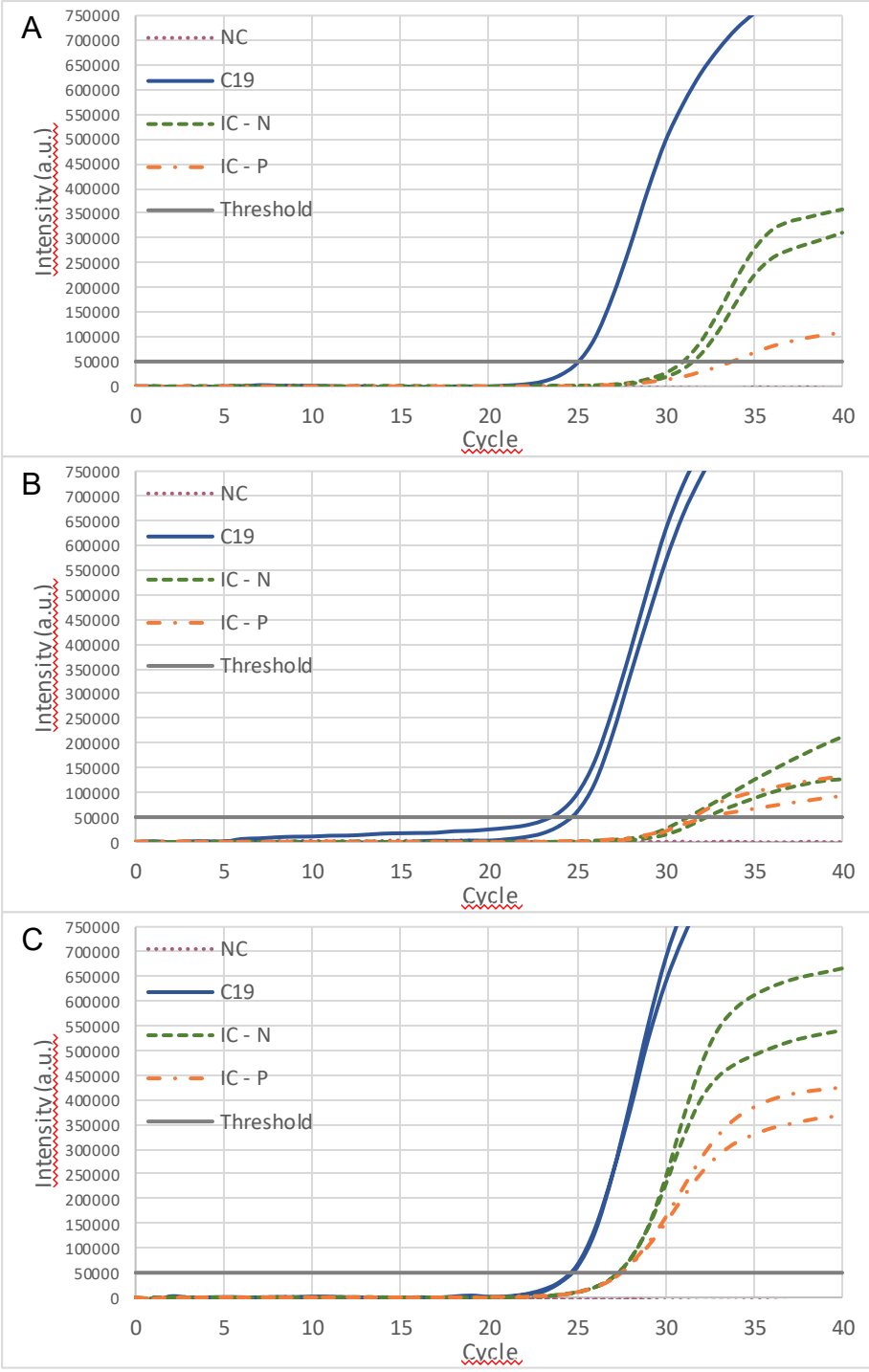

Figure 2.34 **Graphic representation of the PCR results:** PCR 304 [A], PCR 305/306 [B] and PCR 307/308 [C]. The samples represented are C19 (blue continuous line), Negative Control (purple dotted line), Internal Control of the Negative Control (green dashed line) and Internal Control of the positive sample C19 (orange dotted and dashed line). The threshold is represented by the grey continuous flat line.

In PCR 304 and PCR 305/306, the IC of the positive sample has a low amplification curve, explained with the competition for the DNA polymerase. In PCR 307/308, the IC of the positive

sample has an amplification curve with a C_T in the 28th cycle and an intensity of fluorescence reaching 400000, although the amplification of the target DNA.

PCR 305/306 and PCR 307/308 have better amplification curves than PCR 304 due to the increase in the concentration of $MgCl_2$, as the Mg^{2+} is a cofactor of the DNA polymerase by complexing with dNTPs forming the substrate of the enzyme. The ion also facilitate the base pairing between two strands of DNA increasing the stability of the primer annealing. (Markoulatos et al., 2002; Montgomery & Wittwer, 2014)

Comparing PCR 305/306 with PCR 307/308, the later has a higher intensity of fluorescence. This supported with the higher concentration of primers and probe. High concentrations of primers and probe, as in PCR 307/308, will facilitate the availably of primer and, consequently, increasing the amplification (Markoulatos et al., 2002; Ruiz-Villalba et al., 2017).

After the analysis of the PCR assays, the PCR 307/308 have the optimal concentration of primers and $MgCl_2$. This assay used the highest concentration of $MgCl_2$ and primers not compromising the specificity and not inhibiting the reaction. The conditions of this assay were selected for the SUPREME Real Time PCR Swine Detection kit.

2.3.4.2 Validation Tests

The validation tests are a necessary tool to confirm the legitimacy of the kit as well as assess the performance of the kit (Broeders et al., 2014). Assessing these parameters is required to commercialize the kits.

The performance of the kit is measured through the calculation of the specificity, LoD and Inclusivity and robustness.

2.3.4.2.1 Exclusivity Test

The exclusivity test guarantees that the previously chosen conditions didn't compromise the specificity of the new kit, assuring that any non-target sample doesn't amplify. This test enables the calculation of the specificity indicator, through equation 1 (in session 2.2.5.1 Exclusivity Tests). The exclusivity test was done using 10 replicates of 11 non-swine samples with 5ng of sample DNA. The list of samples used in the exclusivity test is available in the Appendix 1. Figure 2.35 shows the results of the horse detection kit exclusivity test with all non-horse samples (blue dotted line) and their IC (pink line)

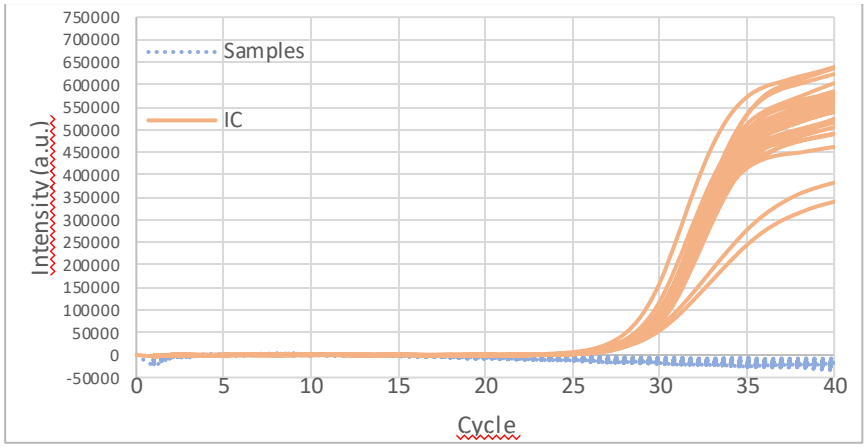

Figure 2.35 **Graphic representation of the PCR results**: swine detection kit exclusivity test with 11 non-swine samples (blue dotted line) and their IC (pink line)

The IC confirm the validity of the test and the lack of amplification of any non-target samples proves the specificity of the kit, with an indicator of 100%.

2.3.4.2.2 Inclusivity and Sensibility Tests

The sensibility test allows to determine the lowest amplifiable target DNA quantity. The sensibility test was done using 1ng and 5pg of swine DNA. The sensibility test was only with these two concentrations of DNA as the LoD was estimated based in the LoD of the other SUPREME Real Time PCR Detection kits, in order to save reagents. The inclusivity test guarantees that the previously chosen conditions can detect target DNA in every horse matrix. This test enables the calculation of the inclusivity indicator, through equation 2 (in session 2.2.5.2 Inclusivity and Sensibility Tests). The inclusivity test was done using 10 replicates of 7 different pork samples at the LoD. Figure 2.36 shows a sensibility test with the sample C19 at 1ng (darkest blue line with diamonds) and 5pg (dark blue line with squares).

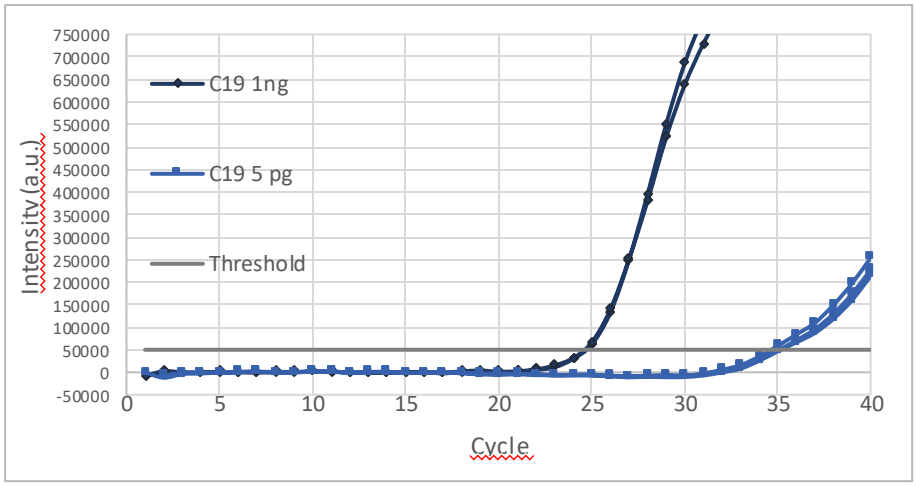

Figure 2.36 **Graphic representation of the PCR results:** swine detection kit sensibility test. It was used the sample C19 at 1ng (darkest blue line with diamonds) and 5pg (dark blue line with squares). The threshold is the grey flat line.

Analysing the results, all DNA concentrations show amplification. Logically, as the concentration of DNA is decreasing, the amplification curve starts in later cycles and has a lower

intensity of fluorescence. The lower DNA quantity amplified was 5pg with a C_T in the 35th cycle and an intensity of fluorescence reaching 250000, setting the LoD of 5pg.

Figure 2.37 shows the inclusivity test with 7 swine matrices with 5pg of DNA. The samples are C25 (dotted orange line), C52 (green continuous line), C56 (purple dashed and double dotted line), C4 (green dashed and dotted line), C8 (lightest blue line with triangles), C13 (red line with circles) and C19 (blue line with squares). The threshold is the grey flat line.

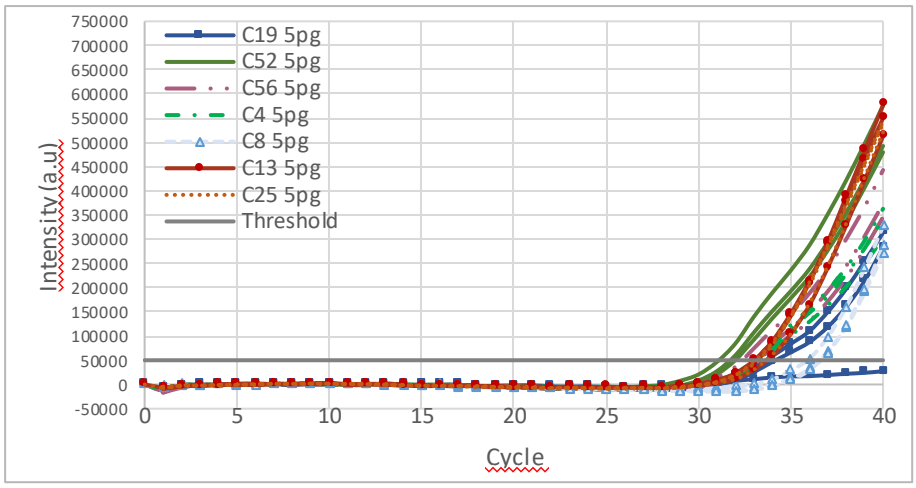

Figure 2.37 **Graphic representation of the PCR results:** swine detection kit inclusivity test**.** The samples are C25 (dotted orange line), C52 (green continuous line), C56 (purple dashed and double dotted line), C4 (green dashed and dotted line), C8 (lightest blue line with triangles), C13 (red line with circles) and C19 (blue line with squares). The threshold is the grey flat line.

All 7 different samples show amplification with a C_T ranging from the 31st to the 37th cycle and an intensity of fluorescence reaching 550000. This result confirms the LoD of 5pg and the amplification of every target samples, but one proves the inclusivity of the kit, with an indicator of 98,5%.

2.3.4.2.3 Robustness Tests

The robustness tests are exclusivity and inclusivity tests, to infer the performance of the kits in adverse conditions. This method guarantees the performance of the tests assuming that the user's equipment is not calibrated or shows temperature shifts (Broeders et al., 2014).

The exclusivity robustness test was done decreasing the annealing temperature 2ºC, facilitating the reaction. In these conditions none sample showed amplification, guaranteeing the specificity of the test.

The inclusivity robustness test was done increasing the annealing temperature 2ºC, disturbing the reaction. In these conditions, every sample showed amplification, guaranteeing the LoD and specificity of the test.

3. From the technology to the market

The kits developed in this dissertation are Real Time PCR kits used in the detection of swine, horse, chicken and turkey DNA in food matrices to test its authenticity and prevent food fraud.

The objective is the commercialization of a more sensitive and accurate DNA detection solution kit in order to detect food fraud in swine, horse, chicken and turkey in food matrices.

In the next sessions a market analysis is discussed in order to design the best-selling strategy.

3.1. Market Analysis

3.1.1. SWOT analysis

The market situation can be studied resorting to strategic techniques, as the SWOT matrix. A SWOT analysis consists of the study of a company Strengths and Weaknesses, as well as the market Opportunities and Threats. The strategies can be traced by using the Opportunities to reinforce the Strengths and remedy the Weaknesses and using the Strengths to protect the company to the market Threats. The SWOT matrix is traced in Table 3.1.

Table 3.1 SWOT Matrix. The SWOT matrix is a market analysis technique, that cross-reference the Strength and Weakness of BPMR and SUMPREME Real PCR detection kits with the Opportunities and Threats of the market situation.

		Strengths (S)	**Weaknesses (W)**
		- High Quality/Price ratio - Qualified human resources - 60 Real Time PCR with high sensibility to DNA detection in food matrices - Certified Company by the ISO 9001 to the quality management system	- Microbusiness - Small customer base - Young company
Opportunities (O)	-Increasing concern for food safety - More strict regulation in food sector in the EU -Emerging usage of Real Time PCR -Reduced Time-to-Market	**SO** - Quality/price ratio defined to attract customers - Design personalized selling strategies to each customer - Development of new kits to address concerns related to food safety	**WO** - Create job opportunities to increase the production capacity - Partner with Food Regulation Agencies around the world.
Threats (T)	- Direct Competition dominating the market - Existing other techniques that can prove food authenticity - Specific and necessary instruments to perform the tests are expensive - Tighter customs control	**ST** - Optimize the already existing kits - Competition-based pricing strategies - Partnerships with Real Time PCR instruments manufacturers - Develop new accommodation conditions	**WT** - Guarantee the loyalty of the old clients - Partnerships with distribution companies

In the next session we will discuss the market situation and the strategies traced in the SWOT analysis.

3.1.1.1 Strengths (S)

High Quality/Price ratio:

The SUPREME Real Time PCR detection kits have a high quality, which entails a high specificity and sensibility. The high quality of the detection kit is proven in through the validation tests, in session 2.3 Results and Discussion. Besides, these kits are low-cost compared to competitors as shown in Table 3.3 in session 3.1.1.4 Threats

Qualified human resources:

Being a biotechnology company, it is necessary that its employees have scientific background as well as a profound knowledge about the techniques and reagents used in the production and development of the SUPREME Real Time PCR detection kits. Besides, the management and sales team have corporate background and ensure a competent company management and marketing.

60 Real Time PCR with high sensibility to DNA detection in food matrices

BPMR sells 60 real time PCR under the brand “BIOPREMIER Real Time PCR detection kits”. The kits can be used in food matrices and are divided into 5 categories according to the detection target: pathogenic microorganism detection, non-pathogenic microorganism detection, meat DNA detection, genetic modified organism (GMO) detection and allergen detection.

BPMR is also optimizing some of the current kits and developing a new product line named “SUPREME Real Time Detection kits”.

Certified Company by the ISO 9001 to the quality management system

BPMR is a certified company by the ISSO 9001 standard. This standard attests the implementation of a quality management system, which ensures a better productivity and higher company credibility and visibility.

3.1.1.2 Weaknesses (W)

Microbusiness

According to the Comission Recomendation of 6 May 2003 concerning the definition of micro, small and medium-sized enterprises, a company can be classified regarding its dimension. The classification is mostly through two criteria, the number of employees and the company’s turnover. If a company has less than 250 employees and its annual turnover is lower than 50 million

euros, it is a micro, small or medium-sized company (SME). If a SME has more than 50 employees and if its annual turnover exceeds 10 million euros, the company is named medium-sized business. However, if a SME has more than 10 employees and an annual turnover of more than 2 million euros, it is a small company. At last, if a SME doesn't surpass the 10 employees and the annual turnover of 2 million euros, it is considered a microbusiness. Giving this definition, BPMR is a microbusiness, since has less than 10 employees, namely the CEO, Pedro Antunes, responsible for the management of the company; the technical coordinator, Joana Freitas, who oversees the production and development; the Business development manager, Sérgio Loureiro, who oversees the marketing and sales; and a two-person technical team.

Young Company

BPMR was created in November 2016, less the two years ago. Although, it's a spin-off company, the short time of BPMR can be seen as having a lack of experience.

Small customer base

The customers are an essential part in every business. A customer is an individual or business that purchase or consume products or services provided and has the ability to choose between different products and suppliers.

The customers of BPMR are food testing laboratories and distribution companies that buy the SUPREME Real Time PCR Detection kits. However, due to its early age, BPMR have a small customer base.

3.1.1.3 Opportunities (O)

Increasing concern for food safety

Food safety is the set of regulations for the food production, transportation and storage, to ensure that a food is suitable for human consumption. The food safety concept must guarantee that the producers follow several norms when1 handling food. The natural growth of the society entails several factors that affect the food safety and arouse some concern for what is consumed. The globalization is one of those factors; the globalization enlarges the distribution chain and increase the possibility of contamination or adulteration. Other factors linked the concern about food safety are the food trends and new consumption habits. Pre-cooked foods, processed foods and food served raw (like sushi) have a greater risk of contamination, motivating the people to a more informed consumption ("10 facts on food safety," 2016). Due to this growing contamination and adulteration risk, some organizations, like the European Commission and the World Health Organization (WHO) created programs to alert the consumers and producers about the risks and to teach preventing measures to ensure a better and flawless food safety.

More strict regulation in food sector in the EU

The regulation in food sector has been reinforced year after year to prevent food fraud incidents. In 2013, the EU launched the Food Fraud Network (FFN), a network for prevention and

rapid detection of food fraud incidents. This network is comprised of governmental entities from a member country and a contact in the European Commission, in constant communication to allow a fast creation of an alert state in cases of international food fraud. In Table 3.3, are listed the contact points of FFN.

Table 3.2 Contact Points of FFN

Country	Entity	Contact
Austria	Federal Ministry of Health Department Consumer Health	ffcp@bmg.gv.at
Belgium	FOD Economie DG de l'inspection économique – Direction A – Contrôles qualité et sécurité des produits	eco.inspec.cdc@economie.fgov.be
Bulgaria	Department Food Safety Animal Health and Food Safety Directorate Ministry of Agriculture and Food	AAC-FF.Bulgaria@mzh.government.bg
Croatia	Veterinary and Food Safety Directorate Ministry of Agriculture	food.fraud-CRO@mps.hr
Cyprus	Health Services, M&PHS, Ministry of Health	rasffcy@mphs.moh.gov.cy
Czech Republic	Ministry of Agriculture of the Czech Republic, Director General of Food Production Section – Food Authority	aacs@szpi.gov.cz
Denmark	Ministeriet for Fødevarer, Landbrug og Fiskeri Fødevarestyrelsen	34@fvst.dk
Estonia	Veterinary and Food Board	AAC@vet.agri.ee
Finland	Finnish Food Safety Authority Evira	food.fraud@evira.fi
France	Direction générale de la concurrence, de la consommation et de la répression des fraudes	contactfraude@dgccrf.finances.gouv.fr
Germany	BVL - Federal Office of Consumer Protection and Food Safety - Unit 104 " Crisis Management, Rapid Alert Systems "	LM-Betrug@bvl.bund.de
Greece	Ministry of economy - Development and Tourism - General Secretary of commerce and consumer protection	sykap@gge.gr
Hungary	National Food Chain Safety Office	
Ireland	Food Safety Authority of Ireland	aacfoodfraud@fsai.ie
Italy	Ministry of Agriculture Foodstuff and Forestry Policies: Central Inspectorate for Quality Controls and Antifraud of Foodstuff and Agricultural Products	Icqrf.capodipartimento@politicheagricole.it
Latvia	Food and Veterinary Service,	
Lithuania	Veterinary Sanitary Department SFVS of Lithuania	AAC@VMVT.LT

Luxembourg	Organisme pour la Sécurité et la Qualité de la Chaine alimentaire	foodfraud-LU@osqca.etat.lu
Malta	MFH-Environmental Health	
Netherlands	Netherlands Food and Consumer Product Safety Authority	
Poland	Agricultural and Food Quality Inspection Main Inspectorate	cpfoodfraud@ijhars.gov.pl
Portugal	Economic and Food Safety Authority National Unit of Enforcement	uno@asae.pt
Romania	National Sanitary Veterinary and Food Safety Authority	aac-ff@ansvsa.ro
Slovakia	State Veterinary and Food Administration of the Slovak Republic	Contact-Fraude@svps.sk
Slovenia	The Administration of the Republic of Slovenia for Food safety, Veterinary sector and Plant protection	Foodfraud.uvhvvr@gov.si
Spain	Ministry of Agriculture, Food and Environment	control.laboratoriosalimentarios@mapama.es
Sweden	National Food Agency	ffcp@slv.se
United Kingdom	Food Standards Agency	NFCU@foodstandards.gsi.gov.uk
Norway	Norwegian Food Safety Authority	aac@mattilsynet.no
Switzerland	Federal Food Safety and Veterinary Office Division of Food and Nutrition	food-fraud@blv.admin.ch
Iceland	Office of import and export	
European Commission	DG SANTE/G5	Sante-food-fraud@ec.europa.eu

Real Time PCR market growing

According to a report published by the American consultancy company, Grand View Research, the Real Time PCR market was estimated to be 3 billion USD in 2016. It is expected an annual growth of 8,7% until 2025, reaching the 6,3 billion USD at that point (*Real time PCR (qPCR) and digital PCR (dPCR) market analysis and segment forecasts to 2025*, 2018)

This estimated growth is a result of the publication of the Minimum Information for publication of quantitative Real Time PCR (MIQE) guidelines, that guide the laboratories and other entities in the treatment and publication of Real Time PCR data (Bustin et al., 2009). The increasing number of molecular biology laboratories, due to the precision and sensibility of Real Time PCR, is also a key factor in the market growth.

Reduced time-to-market

Time-to-market is the amount of time that a company takes to launch a product in the market, including its development. Usually, in biotechnology products directed to human usage, the time-to-market is extensive, sometimes taking more than 10 years. Most of the biotechnology industry

products and services are for the health care market. Thus, they are likely to carry a considerable risk to the public health if there is no regulation. The biotech health products also need several clinical trials to test for safety conditions. The extensive regulations and the need for clinical trials result in extended time-to-market for most biotech products.

On the other hand, the SUPREME Real Time PCR Detection kits are used in food matrixes corresponding to a lower risk in case of default, and consequently to a reduced number of regulation steps and time-to-market.

3.1.1.4 Threats (T)

Direct competitors dominating the market

The direct competitors of BPMR are the companies that develop and sell Real Rime PCR DNA detection kits directed to food matrices. However, developers, producers and distributors of meat DNA detection kits uses other techniques. Most of these companies are multinational businesses whose focus isn't exclusively the development and commercialization of food detection kits, having high turnovers and being well-known in the market. Bio-rad is an example of one of BPMR direct competitors. The turnover of Bio-Rad in 2016 was larger than two billion dollars (*Bio-Rad Laboratories: Annual Report*, 2016). A performance map with competitors' comparison is shown in Table 3.3. The parameters compared are the number of kits available in the market, the number of reactions per kit, the price per reaction and the minimum assay time.

Table 3.3 Performance Map.

Company	Usage	Meat Detection kits available	Reactions per Kit	Price per reaction	Minimum assay time	Sensibility
BPMR	Liquid Master mix	8	100	4,50 €	62 min	1 to 10 pg
ThermoFisher	Freeze-dried Master Mix	7	96	7,81 €	40 min	5 pg
Eurofins	Liquid Master Mix new set each utilization	12	96	-	100min	10 pg
Biotecon Diagnostics	Freeze-dried Master Mix	7	96	-	63 min	1 pg to 66 pg
4LabDiagnostics	Liquid Master mix	2	100	7,07 €	-	3,7pg
Qiagen	Liquid Master mix	9	96	6,04 €	72 min	37pg
Genesig	Freeze-dried Master Mix Own brand equipment	23	150	4,81 €	60 min	<300 pg

Other molecular biology techniques to detect meat in food matrices.

Biopremier Real Time PCR DNA detection kits, as its name indicate, are based in Real Time PCR. However, it isn't the only technique that can be used in the detection of meat in food matrices. Besides, Real Time PCR technique, the most used methods to test meat authenticity are enzyme-linked immunosorbent assay (ELISA) and Next Generation Sequencing (NGS). ELISA is a protein detection technique that use enzyme-linked antibodies that in the presence of the target antigen, provokes a colour change in the solution. NGS is a type of massive DNA sequencing, that identify the percentage of a species DNA in a sample. There are several other techniques to detect meat in food matrices, however they are expensive or complicated to use, in Table 3.4 is showed a visual comparison between detection techniques.

Table 3.4 Comparison between detection techniques.

	Real Time PCR	NGS	Sequencing	Microbiology	ELISA
Aplication	All Biological	All Biological	All Biological	Microorganism	Protein
Price	✓✓	✗	✗	✓✓✓	✓✓
Sensivity	✓✓✓	✓✓✓	✓✓✓	✓✓	✓
Contamination risk	✓✓✓	✓✓✓	✓✓✓	✗	✓
Analysis	✓✓✓	✓	✓	✓	✓✓
Easy to use	✓✓✓	✓✓	✓✓	✓✓✓	✓✓
Time	✓✓	✓	✓	✗	✓✓✓

Real Time PCR is a relatively low-cost and quick technique, with high sensitivity, low contamination risk, easy analysis and to use, and is applicable to all biological samples. NGS and standard Sequencing have a sensitivity and contamination risk similar to Real Time PCR, however are much more expensive, and a have a more difficult handling and analysis. Microbiology can only detect microorganism, takes much more time and have an elevated contamination risk; sensitivity isn't as good as the other techniques, however its much cheaper and easier to use. Finally, ELISA can only detect proteins but has a reduced sensibility and consequently a reduced price; it is the faster technique.

Specific and necessary instruments are expensive

As said in session 2.1, Real Time PCR, to do a PCR assay, it is necessary a thermal cycler, yet a Real Time PCR assay needs thermal cycler capable of amplify DNA and detect the fluorescence. There are several Real Time PCR thermal cyclers available in the market, however the price ranges from 10000€ to 50000€. The high price can be a decisive factor in the buying decision by the molecular biology laboratories. Some laboratories may not be able to make the investment, meaning that they will not buy BIOPREMIER Real Time PCR detection kits, as they are unusable without the specific equipment.

Tighter customs control

BPMR is a Portuguese company and, due to the conditions of the European Single Market, the exportation SUPREME Real Time PCR detection to the acceding countries is facilitated. However, some of the biggest Real Time PCR markets, like the United States of America (USA), India and China, don't have free trade agreements. This means that, the kits sold by BPMR have tighter customs control, which increase the cost for the company and the delivery time. This factor diminishes the interest of the laboratories from this market in the SUPREME Real Time PCR detection kits.

Additionally, the transportation of SUPREME Real Time PCR detection kits needs to be, at most, 4ºC, to guarantee its function meaning that it is more difficult to ensure the quality of the product, the further is the delivery destination.

In order to minimize the transportation temperature SUPREME Real Time PCR detection kits must be shipped in an expanded polystyrene (XPS) box with dry ice to keep the kits cold. However, some countries like India have strict regulations against the importation of products accommodated in dry ice, making it difficult and expensive the shipment of SUPREME Real Time PCR detection kits to such countries.

3.1.1.5 Possible Strategies

3.1.1.5.1 Strengths x Opportunities (SO)

Quality/price ratio defined to attract customers

The increase in food safety concerns around the globe implies in a larger number of analysis and tests in food samples. This means that the quantity of laboratories willing to acquire DNA detection kits in food matrices is also increasing given the augment of clients demands.

The SUPREME Real Time PCR detection kits' high sensibility and specificity are desirable attributes in a DNA detection kit. The SUPREME Real Time PCR detection kit can be sold at a very low price, when compared it the competitors with similar sensibility and specificity, to penetrate the market and attract clients.

Customized solutions to each client

The analysis of food sample can have some variations depending on the laboratory. These variations can go from the quantity, quality or availability of the sample to the target selected or quantity of reactions needed. Since BPMR has highly qualified personnel, it can be performed a specialized consultancy service to find the solution who best fit each customer needs.

Development of new kits

Overall, BPMR has 8 kits to detect animal species in food matrices to prove the meat authenticity However, the eight animal species detected by the kits sold by BPMR don't cover all the kinds of meat and fish we consume. Besides, the people willing to commit food fraud can also use other types of meat or fish to substitute the original. So, BPMR can develop more kits that target different animal species and widen the range of food fraud detected.

3.1.1.5.2 Weaknesses x Opportunities (WO)

Partner with Food Regulation Agencies around the world.

The EU Food Regulation Agencies must perform several audits to companies in the food industry, either restaurants, food producers or retailers. These audits have chemical and biological analysis to these entities to determine its composition, detect pathogenic microorganisms or assure the food authenticity. The tighten of the regulation in the EU and the creation of the FFN increased the number of audits preformed. BPMR can establish partnerships with the Regulation Agencies and be the exclusive supplier of kits to test the food authenticity.

Create job opportunities to increase the production capacity

The SUPREME Real Time PCR detection kits will be produced as they are requested by the clients, and due to the elevated cost of production and the six-month validity, BPMR only have a few kits in stock. If there is a sudden increase in orders, due to its small dimensions, BPMR is not capable of responding to all. In that case, the company must open job vacancies to a laboratory technician, to take care exclusively of the production of the SUPREME Real Time PCR detection kits.

3.1.1.5.3 Strengths x Threats (ST)

Optimize the already existing kits

The BIOPREMIER Real Time PCR kits are a high quality and low-cost real time PCR kits used to detect pathogenic, meat species, and others in food matrices. The BPMR direct competitors have real time PCR detection kits used in samples of food matrices with similar quality. To differ itself from the competition, BPMR can optimize the already existing kits, improving its sensibility and specificity, or adding some new features, for example, adding UDG that minimize the post PCR contamination. This strategy is already being followed by BPMR with the SUPREME Real Time PCR detection kits.

Cost and Competition-based pricing strategies

Each company has its own pricing strategy. To gain some advantage on the competitors, BPMR can define a cost and competition-based pricing strategy. A cost-based pricing defines the price according with the production cost and a competition-based pricing strategy define the price according with the selling price of the competitors (see session 3.2.2.2 Price).

Partnerships with Real Time PCR instruments manufacturer

The elevated price of the Real Time PCR thermal cyclers needed to use the BIOPREMIER Real Time PCR kits is a decisive factor in buying this type of equipment by the laboratories. Some clients couldn't use BIOPREMIER Real Time PCR kits if they have no financial conditions to acquire a Real Time PCR thermal cycler.

BPMR can team up with Real Time PCR thermal cyclers manufacturers to facilitate the acquisition of the equipment by the laboratories and subsequent purchase of SUPREME Real Time PCR detection kits. BPMR could formulate a plan of a regular purchase of the kits with the thermal cycler as an offer.

Develop new accommodation conditions

SUPREME Real Time PCR detection kits are composed with several reagents, including DNA primers and DNA probes, which need to be stored and transported in cold. This is not a problem when the kits are shipped to the countries of the European Single Market. However, countries outside this agreement are farther and have tighter customs control, meaning that the time to destination is longer. So, the longer the delivery, the greater the difficult to keep the kits in the ideal temperature. SUPREME Real Time PCR detection kits are transported inside a XPS box and accommodated in dry ice, that maintain the ideal temperature during a week. If the arrival at destination takes more time than a week, the kit starts to lose its function. To ensure a longer travel without the loss of function, BPMR can develop and test new accommodation and transport conditions, such as freeze drying the kits, to eliminate the need of dry ice and the need of refrigeration in the transportation.

3.1.1.5.4 Weaknesses x Threats (WT)

Guarantee the loyalty of the old clients

The number of clients it's one of the key growth factors of the company. Due to the recognition of the competitors, some of the old BPMR's clients could change its supplier of kits. BPMR can create loyalty contracts, promotions and give some benefits to the clients who choose to keep purchasing at BPMR. This works as an action to guarantee that a client always chooses BPMR as the main supplier of Real Time PCR kits to food analysis.

Partnership with distribution companies

To generate revenue, it's needed an elevated number of clients on a regular basis, and without the human resources available and the high pressure of the competitors, the customer recruitment becomes very difficult. To raise the number of customers and ensure a high sales volume, BPMR can partner with scientific distribution companies to contact probable clients. The distributors can facilitate the order to both, BPMR and the client, while raising awareness about SUPREME Real Time PCR detection kits, by presenting the kits to the laboratories.

3.2. Marketing Plan

3.2.1. Marketing Strategy

3.2.1.1 Market Segmentation

Market segmentation is a strategic process to divide the potential customers in subgroups with similar characteristics named market segments. These segments are standardised, and each contain a subgroup of potential customers with some similar characteristics. The goal of the market segmentation is to easily identify the group of customers with favourable features to the product/service purchase in order to design the most appropriate marketing strategy. However, the segmentation criteria differ among companies according to their strategies.

BPMR strategy is to sell the kits to companies or governmental organizations. Thus, the segmentation strategy is Business-to-Business (B2B).

The B2B market is characterized by a reduced number of customers with a higher sales volume per each customer. The purchase process is typically more complex and longer than the purchase process of business to consumers market (B2C) and there are differences in the segmentation criteria used in each of these markets.

In BPMR, the market segmentation takes into consideration the objective characteristics and the goal of a work group, instead of the psychological traits of an individual. The purchase decision is well thought and conscious and it is not an impulsive one.

Consequently, the segmentation criteria for the SUPREME Real Time PCR kits for detection of food fraud in the meat industry are the following:

a) **Area of activity**

b) **Equipment availability**

c) **Dimension of the laboratory**

d) **Geographic location**

3.2.1.2 Targeting

The target market is the segment or subgroup of customers defined as the addressable market.

There are three strategies to define the target market: mass marketing (or undifferentiated marketing strategy) which doesn't separate the different segments, targeting the largest number of

customers; differentiated marketing strategy which differentiate the segments and acts accordingly in each; and the concentrated marketing strategy (niche marketing) which focus in a specific segment, allowing a more thorough analysis of this specific subgroup.

The most suitable strategy for BPMR it's the concentrated one because it focuses in a specific subgroup of scientific laboratories.

According with the segmentation criteria defined in previous session, the target market for the SUPREME Real Time PCR Detection kits contains:

a) According with the sector of activity, the consumers of Real Time PCR kits are:

   I. Governmental agencies for food fraud detection, i.e. ASAE

   II. Laboratories of food safety analysis

   III. Retail companies with own brands who have laboratories.

b) The type of equipment available should be able to perform Real Time PCR and allow a fluorescence reading in FAM and ROX channels.

c) The dimension of the laboratory is measured by the number of workers. The small and medium laboratories are the target segment. The big laboratories use more advanced and expensive techniques, like NGS, allowing several samples tested at a time.

d) Although the geographic location is not an exclusive criterion, BPMR has a preference for laboratories in Portugal or in EU. The loose customs control allows an easier order of the kits and consequent delivery.

In conclusion, the kits have as its target market small or medium laboratories of food analysis, located in the EU, with Real Time PCR thermal cycler with FAM and ROX channels.

3.2.1.3 Positioning

Positioning is how the product/service is seen by its customers, which allows its distinction in a universe of similar products. The BIOPREMIER Real Time PCR kits developed in this work have the following positioning statement:

"The SUPREME Real Time PCR detection kits are Real Time PCR to detect meat DNA in food samples with a very high sensibility and a precise specificity, for laboratories of food analysis".

To define the positioning, it is necessary to describe two complementary factors: identification and differentiation. Identification is the category we want the customers to associate our product. Differentiation is the distinctive characteristics associated to the product.

The identification of the SUPREME Real Time PCR detection kits is in the category of Real Time PCR kits for DNA detection in food samples.

The SUPREME Real Time PCR detection kits differ from the other Real Time PCR kits due to its high sensibility, accurate specificity and reduced price per reaction. The SUPREME product line is more sensible than BIOPREMIER Real Time PCR and include an anti-contamination technology. These characteristics show that the differentiation axis of the SUPREME Real Time PCR detection kits is the association of objective characteristics of the product. The differentiation axis definition is a marketing strategy to positioning the product. There are three types of differentiation axis: association of objective characteristics to the product, suitability to a specific user type and the symbolic characteristics that confer identity to the product.

3.2.2. Marketing Mix

3.2.2.1 Product

The product characterized in this marketing mix is SUPREME Real Time PCR Detection kits. The SUPREME Real Time PCR detection kits are qualitative, meat DNA detection tests in food samples with high quality.

There are four SUPREME Real Time PCR Detection Kits and target: chicken, turkey, horse and swine meat, respectively. The high quality of the kits entails a high sensibility, meaning that the kits detect fraud even when the food sample total DNA have less than 1% of target DNA., and a high specificity, assuring the detection of target DNA with a confidence coefficient higher than 95%. These factors are assured in session 2.3 Results and Discussion.

The kits aim to be used by food analysis laboratories, governmental entities of food regulation and retail companies with own brands who have laboratories, to assure the meat authenticity.

Every kit is comprised of 100 Real Time PCR assays. There is the possibility of acquiring a sample version of the kit with only 10 assays to test the kit before buying

Each kit is presented in four different microtubes, as seen in Figure 3.1 (A, B, C, D). The Master Mix Tube, containing the buffer solution, DNA polymerase, dNTPs, $MgCl_2$, UDG, dUTP and the Internal Control DNA needed for the reaction; the Assay Mix Tube, containing both sets of primers and fluorescent probes, to the target DNA and to Internal Control DNA; the Positive Control Tube, which contain target DNA that will be detected; and Negative Control Tube, which contain just water or buffer solution that will not be amplified.

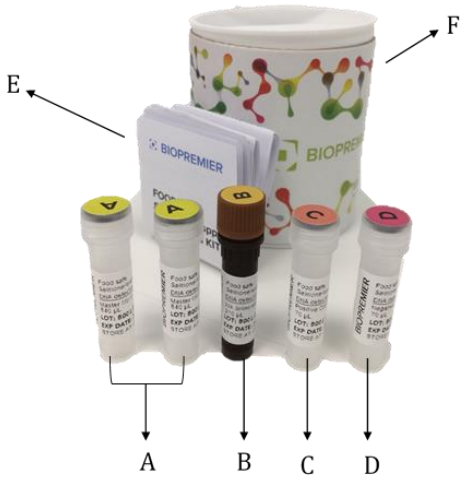

Figure 3.1 **SUPREME BIOPREMIE Real Time PCR kit.** A. Master Mix Tube, containing the buffer solution, DNA polymerase, dNTPs, $MgCl_2$, UDG, dUTP and the Internal Control DNA needed for the reaction. B. Assay Mix Tube, containing both sets of primers and fluorescent probes, to the target DNA and to Internal Control DNA. C. Positive Control Tube, which contain target DNA that will be detected. D. Negative Control Tube, which contain just water or buffer solution that will not be amplified. E. Technical Sheet. F. SUPREME Real Time PCR Cardboard Package.

The SUPREME Real Time PCR detection kits are stored at -20ºC to ensure the stability of all the reagents. The storage must be protected from the light, because an excessive exposure to light can affect the fluorescent probes. The kits have a shelf life of 6 month, if the right conditions are applied.

In terms of package, each kit is disposed in a cylindric cardboard box, accommodated by a sponge. Each package has a label with the name of the product, the target of the kit, the number of reactions, the storage conditions, the shelf life and the batch information. In Fig. 4 (F), is an example of a SUPREME Real Time PCR kit cardboard package.

Besides the tubes with the kit, each package has a technical sheet with information about the respective kit. The technical sheet consists of the description of the kit and the Real Time PCR technology, content and storage information, procedure to follow, material required and not supplied, precautions and recommendations, data interpretation guidelines and specificity and sensibility information

The after-sale service provided includes returns until 14 days after the delivery of the product. However, the return cost is paid by the customer. BPMR provides technical support, if the customer needs help with the use of the kits or with data interpretation.

3.2.2.2 Price

The SUPREME Real Time PCR detection kits are to be sold at 4,5€ per reaction. There are several strategies to set the price of a product. The price chosen for the SUPREME Real Time PCR

detection kits was attributed using a combined strategy based in the production cost, competitors' price and the value given by the customer.

To avoid economic loss, the product price must be at least 5 times greater than the production cost. The production cost is calculated through the sum of two types of costs: variable and fixed costs. The variable costs vary according to the quantity of produced goods; some examples of this type of costs are reagents, packages, and consumables costs. Using SUPREME Real Time PCR detection kits as an example, the production of 10 kits will spend ten times more reagents, packages and consumables then the production of just one kit. The fixed costs are the costs that don't vary, independently of the production quantity. Salary, rent, electricity, and other types of services are some examples of fixed costs.

The choice of the pricing strategy for SUPREME Real Time PCR detection kits must bear in mind the quantity of kits produced and sold, to compensate the fixed costs of BPMR. The fewer kits produced, the bigger the share of fixed cost per each kit. However, with the increase of kits' sales, the fixed costs will be spread over a larger number of kits, decreasing the total production cost.

The production costs set as a reference for the price value of the kits. Also, the competitors' price and the value perceived by the customer should be taken into consideration. If the final price is set too low, the customer, probably, will doubt about the quality of the kit. If the price is set to high, they will search for cheaper alternatives. The price of SUPREME Real Time PCR detection kits was set bearing in mind the price of the kits of the competitors, as shown in Table 3.5.

Table 3.5 Price Comparison between BPMR and its competitors

Competitor	Reactions per Kit	Price per reaction	Total Price
BPMR	100	4,50 €	450€
Eurofins	96	-	-
Biotecon Diagnostics	96	-	-
4LabDiagnostics	100	7,07 €	707€
Qiagen	96	6,04 €	579,84€
Genesig	150	4,81 €	721,5€

Finally, the SUPREME Real Time PCR detection kits' price has into consideration the value attributed by the customer. The greater the value perceived by the customer, the more willing he is to pay a higher amount for the product.

Since the SUPREME Real Time PCR detection kits have high specificity and sensibility and are better than the standard BIOPREMIER Real Time PCR kits, the customer should valorise the optimized kits. Despite the good perception of the SUPREME product line, the price must not surpass the competitors' price, since the high-quality kits at low price are part of the positioning of this product line.

3.2.2.3 Place

The distribution politic of SUPREME Real Time PCR detection kits is selecting direct and indirect distribution channels.

BPMR takes directly orders from food analysis laboratories and governmental entities, being a direct distribution channel. The orders are taken via BPMR website or by email, through filling an order form. In this type of distribution channel, the transport of the kits is assured by the client. However, if the order is greater than five kits, BPMR assures the transportation. The transportation is done by a specialized transport company, TNT. Since the kits need to be transported at cold temperature, the transportation is done in XPS box filled with dry ice.

As indirect distribution channels, some scientific distributors were contacted. This distributor companies can oversee the transportation of the kits and the contact with the client. This way, BPMR can focus in the production and development of kits.

There are three kinds of distribution: exclusive, selective and mass distribution. A producer that have exclusive distribution only have one distributor, allowing a more tighten control over the distribution of its product. In cases of selective distribution, the manufacturer chooses the more adequate distributors to sell its product. Mass distribution is the selling of a product throughout the greatest number of intermediates as possible.

The SUPREME Real Time PCR detection kits are distributed through a selective distribution. BPMR can choose the best fitted scientific distributors to sell the kits. The distributors selected by BPMR differ by the geographic location (Europe) and the large sales area. The distributors which have or had contract with BPMR are described in Table 3.6.

Table 3.6 Scientific Distributors of SUPREME Real Time PCR detection kits.

Distributor	Number of Suppliers	Sales Area	Contact
Cymit química	9	Spain	Santander, 42-48, Nave 2 08020 Barcelona Spain Tel: +34 93 241 29 27 info@cymitquimica.com
Gentaur	149	Europe	Voortstraat 49 1910 Kampenhout Belgium Tel: 0032 16 58 90 45 lieven@gentaur.com
Caltag Medsystems Ltd	42	UK	11 Little Balmer Buckingham, UK MK18 1TF Tel: +44 (0)1280 827460 sales@caltagmedsystems.co.uk
Prodottigianni	-	Italy	Via Quintiliano, 30 20138 Milano Italy Tel: +39 02 509 7 571 bondurri@prodottigianni.com

The partnership contracts with distributors include quantitative and qualitative sales goals, and can involve exclusivity in a certain region, within a given period. The contract can be extended according the completion of the goals.

3.2.2.4 Promotion

The promotion mix includes four variables, which importance vary according to the type of customer. Since BPMR market is B2B, the mix importance is (from the most important to the least important one): sales force, public relations, sales promotion and advertising.

Sales force is the process of individuals persuading to buy the product/service. Sérgio Loureiro is responsible for the commercial area in BPMR. He is in charge to stablish a network of potential customers and distributors. He promotes the SUPREME Real Time PCR with the aid of a slide presentation with the characteristics of the kits and a brief technology explanation. A brochure is also used to promote BPMR kits. The brochure contains all BPMR kits with all the kits available. Personal Selling is the main strategy used by BPMR to acquire new customers to get them to know the SUPREME Real Time PCR detection kits, as it provides knowledge about the brand directly to the laboratories. During the writing of this thesis, more than 900 laboratories and more than 100 distributors were contacted. The CEO, Pedro Antunes as well as Sérgio Loureiro are responsible for the company's public relations. They oversee the direct contact with other companies and new partnerships opportunities.

In terms of sales promotions, BPMR assures a discount of 20% off in all available kits to the clients who sign a contract of multiple orders. Additionally, as of the Summer of 2018, BPMR has a discount of 10 to 20% off in all orders if it was done by August 31st. Since it is a biotechnology company, BPMR can participate in several events and exhibitions related with technology development or the agro-food sector. These exhibitions bring together food producers, technology developers and investors to seek out business opportunities and make knowledge of the brands. As of January of 2018, the exhibitions and congresses available to BPMR show its kits are shown in Table 3.7.

Table 3.7 Exhibitions and Products shows related with food fraud and food safety in 2018, in Europe

Name	Start of the exhibition	Duration	Location	Stand Cost
Food Safety Kongress	20/02/2018	2 days	Berlin	-
Food Fraud Conference	22/02/2018	1 day	London	3 050,00 €
Food Safety Europe 2018	22/02/2018	1 day	London	-
Food Fraud: A global insight	01/03/2018	1 day	London	-

Anuga Food Tech	20/03/2018	4 days	Cologne	5 409,00 €
Food Safety Congress	03/05/2018	2 days	Istanbul	1 750,00 €
8th International Conference on Food Safety & Regulatory Measures	11/06/2018	2 days	Barcelona	1 650,00 €
Innovations in Food Analytics- An International Conference & Expo	19/09/2018	3 days	Munich	-
International Conference On Agricultural Engineering and Food Security	12/11/2018	2 days	Frankfurt	2 410,00 €

At last, advertising isn't the focus of the company to promote SUPREME Real Time PCR detection kits, due to the strict specific target market. However, BPMR has a website (www.biopremier.com) that allows the customer to get to know the company and the kits and to place orders directly. BPMR has, also, a LinkedIn account (www.linkedin.com/company/bpmr-production-and-development/), which allows to extend the contacts network.

3.3. Implementation

To implement SUPREME Real Time PCR detection kits in the market, BPMR need to set a Market strategy.

3.3.1. Market Entry and Expansion Strategy

A market entry strategy is necessary for a company to enter a market not already covered. The SUPREME Real Time PCR detection kits are a new product line, newly developed, meaning that a strong market entry strategy is needed.

There are several market entry strategies, though the best suited for SUPREME Real Time PCR detection kits is Exportation. Exportation can be defined as the selling in other countries the goods produced in the origin country (Carter, 1997). BPMR manufacture the kits in Portugal and market them in other countries. However, since the SUPREME product line shares the same niche market has the standard BIOPREMIER Real Time PCR kits, BPMR just needs to adapt the marketing to the new product line.

The exporting measures done by BPMR can be considered direct and aggressive exporting. Aggressive exporting happens when the company has planned the marketing strategy and is actively letting the market know the product. Direct exporting use direct contact or a distributor in the foreign country to marketing the products (Carter, 1997).

After the market entry strategy is defined, a company needs to increase the number of sales. To do that, a company needs to set a market expansion strategy.

The BPMR market expansion strategy for the SUPREME Real Time PCR detection kits comprises the strategic operations to increase the number of clients. The number of clients can increase by actively search and contact food analysis laboratories. The contacts are done by the commercial consultor with a Personal Selling strategy, promoting the kits to each laboratory. The more laboratories are contacted, the greater the knowledge about BPMR brands and the chances of a new client place an order.

The commercialization of the kit outside the European Union will require the study of local regulation, exchange rates, trade barriers, product certifications among other issues. Thus, in an initial phase we will commercialize the kits in the EU countries.

To facilitate the growth, BPMR can form partnerships with scientific distributors. The distributors will contact food analysis laboratories and promote the SUPREME Real Time PCR 1detection kits. The distributors can also act as an intermediary to the laboratories, placing orders and aiding in the transportation.

4. Business Model

A business model is a description of how said company works (Joan Magretta, 2002). The business model includes the value of the company, how the value is seen by its customers and partners, how the company is generating revenues and the associated costs.

4.1. Business Model Canvas

To facilitate the creation of a business model, Alexander Osterwalder developed a Business Model Canvas in 2008.

The Business Model Canvas (BMC) is a template in the format of a visual chart with several elements to describe the business model of a company. Figure 4.1 shows the Business Model Canvas implemented to the SUPREME Real Time PCR detection kits. In the next sessions each element of the BMC is explained.

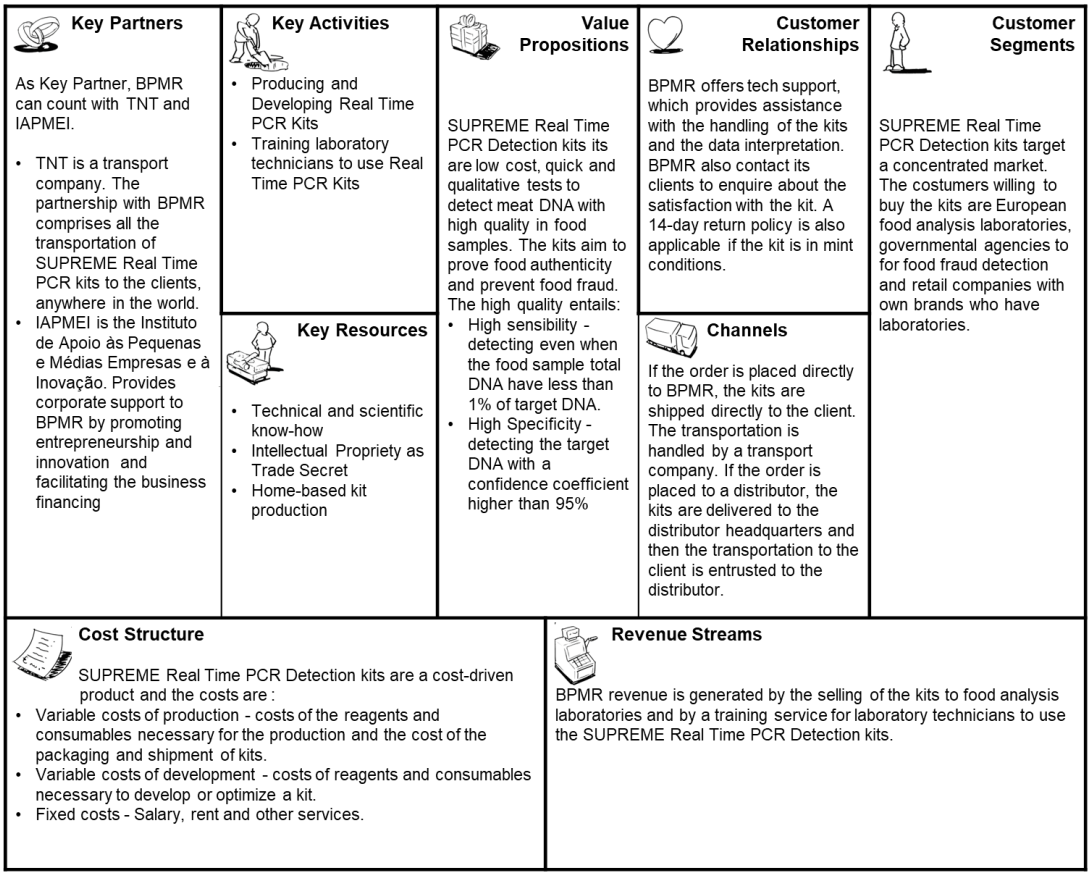

Figure 4.1 **Business Model Canvas for SUPREME Real Time PCR detection kits.** Its elements are Value Propositions, Customer Segments, Customer Relationships, Channels, Revenue Streams, Key Partners, Key Activities, Key Resources and Cost Structure

4.1.1. Value Propositions

It is how the product, or the company is valorised. Comprises the product defined characteristics and the problem it aims to solve. The Value Propositions of SUPREME Real Time PCR detection kits are:

"SUPREME Real Time PCR detection kits are low cost, quick and qualitative tests to detect meat DNA with high-quality in food samples. The kits aim to prove food authenticity and prevent food fraud. The high-quality entails:

- High sensibility → detecting even when the food sample total DNA have less than 1% of target DNA.
- High Specificity → detecting the target DNA with a confidence coefficient higher than 95%"

4.1.2. Customer Segments

SUPREME Real Time PCR detection kits target is a concentrated market. The customers are the European food analysis laboratories, governmental agencies in food fraud detection and retail companies that have their own laboratories (See Section 3.2.1.2 Targeting).

4.1.3. Customer Relationships

It is how the company relates with the customer segments and what kind of relationship they establish.

BPMR offers tech support, which provides assistance with the handling of the kits and the data interpretation.

BPMR also contact its customers to enquire them about their satisfaction with the kit. A 14-day return policy is also applicable if the kit is in mint conditions.

4.1.4. Channels

Ensure how the value propositions is delivered to customers. SUPREME Real Time PCR kit are channelled to the customers by a combination of two strategies:

- If the order is placed directly to BPMR, the kits are shipped directly to the client. The transportation is handled by a transport company.
- If the order is placed to a distributor, the kits are delivered to the distributor headquarters and then the transportation to the client is entrusted to the distributor.

4.1.5. Revenue Streams

Define the way the company generate profits. BPMR has two revenue streams:

- Sales of SUPREME Real Time PCR detection kits to food analysis laboratories
- Training service for laboratory technicians to use the SUPREME Real Time PCR detection kits.

4.1.6. Key activities

Comprises the central activities done by the company to assure the value propositions. BPMR Key Activities are:

- Producing and Developing Real Time PCR Kits
- Training laboratory technicians to use Real Time PCR Kits

4.1.7. Key Resources

Defines the important assets of the company to create the value propositions. BPMR Key Resources are:

- Technical and scientific know-how
- Intellectual Propriety as Trade Secret
- Home-based kit production

4.1.8. Key Partners

These are the partnerships formed to help the thriving of the company. As Key Partner, BPMR can count with:

- TNT - a transport company. The partnership with BPMR comprises all the transportation of SUPREME Real Time PCR detection kits to the clients, anywhere in the world.
- IAPMEI - the Instituto de Apoio às Pequenas e Médias Empresas e à Inovação. Provides corporate support to BPMR by promoting entrepreneurship and innovation and facilitating the business financing.

4.1.9. Cost Structure

Defines which are the financial costs of the business model. Since, the business model for SUPREME Real Time PCR detection kits delivers a low-cost set of kits to the laboratory, the business

model is cost-driven with the costs decreasing with the amount of kits ordered. The main sources of expenditures are:

- Variable costs of production - costs of the reagents and consumables necessary for the production and costs of the packaging and shipment.
- Variable costs of development - costs of reagents and consumables necessary to develop or optimize a kit.
- Fixed costs - Salary, Rent and other services.

5. Conclusions and Future Perspectives

As the customers awareness about what they eat increases, the EU took steps to regulate the food sector to prevent food fraud cases. From the need of more efficient and accurate methods to detect food fraud, more specifically, meat adulteration, arise the need to optimize the current BIOPREMIER Real Time PCR meat detection kits into SUPREME Real Time PCR meat detection kits.

The optimization of all detection kits comprised the addition of dUTPs and UDG to avoid PCR product contamination and it was assessed by comparison of the amplification of target DNA and IC, and the no amplification of the non-target DNA. The optimization consisted in assays to evaluate the type of primers and probe used, the concentration of $MgCl_2$ and dNTPs, the presence of DMSO, the concentration of primers and probe and the concentration of dUTPs. The annealing temperature and duration of each cycle phase were also tested.

PCR 153 defined the final SUPREME Real Time PCR chicken detection kit conditions. The kit has the current set of primers and probe, the lowest $MgCl_2$ and dNTPs concentration, the medium concentration of primers and probe and the highest concentration of dUTPs. The kit hasn't DMSO and it is done with a denaturation phase of 15 seconds, an annealing phase during 30 seconds with a temperature of 60ºC and an extension phase of 15 seconds.

The performance of the kit was then evaluated in the validation tests, through the calculation of the specificity and inclusivity indicators and the LoD. The kit didn't amplify any non-target sample corresponding to a specificity indicator of 100% and the kit amplified all the target samples corresponding to an inclusivity indicator of 100%, as well. The LoD was set at 1pg.

The final conditions of the SUPREME Real Time PCR turkey detection kit were defined in PCR 252. The kit has the current set of primers and probe and the highest concentration of $MgCl_2$, dNTPs, primers and probe and dUTPs. The kit hasn't DMSO and it is done with a denaturation phase of 15 seconds, an annealing phase during 30 seconds with a temperature of 60ºC and an extension phase of 15 seconds.

The performance of the SUPREME Real Time PCR turkey detection kit was then evaluated in the validation tests, through the calculation of the specificity and inclusivity indicators and the LoD. The turkey detection kit didn't amplify any non-target sample corresponding to a specificity indicator of 100% and the kit amplified all the target samples corresponding to an inclusivity indicator of 100%, as well. The LoD was set at 1pg.

PCR 278 defined the final SUPREME Real Time PCR horse detection kit conditions. The kit has the current set of primers and probe, the lowest dNTPs concentration and the highest concentration of $MgCl_2$ and primers. The kit is done with a denaturation phase of 15 seconds, an annealing phase during 30 seconds with a temperature of 60ºC and an extension phase of 15 seconds.

The performance of the horse detection kit was then evaluated in the validation tests, through the calculation of the specificity and inclusivity indicators and the LoD. This kit just amplified one non-target sample corresponding to a specificity indicator of 99,4% and but amplified all the target samples corresponding to an inclusivity indicator of 100%. The LoD was set at 10pg.

The final conditions of the SUPREME Real Time PCR swine detection kit were defined in PCR 307/308. The kit has the new set of primers and probe and the highest concentration of $MgCl_2$ and primers and probe. The kit hasn't DMSO and it is done with a denaturation phase of 15 seconds, an annealing phase during 30 seconds with a temperature of 60ºC and an extension phase of 15 seconds.

The performance of kit was then evaluated in the validation tests, through the calculation of the specificity and inclusivity indicators and the LoD. The swine detection kit didn't amplify any non-target sample corresponding to a specificity indicator of 100%. However, didn't amplify one target sample corresponding to an inclusivity indicator of 98,5%. The LoD was set at 5pg.

All the SUPREME Real Time PCR detection kits passed the robustness tests. The kits amplified all the target in the LoD with an annealing temperature of 62ºC and didn't amplify the non-target samples with an annealing temperature of 58ºC.

The BIOPREMIER Real Time PCR detection kits have a LoD of 10pg to 100pg. Since the newly optimized SUPREME Real Time PCR detection kits have a LoD of 1pg to 10pg, it may be concluded that the optimization of the Real Time PCR detection kits was successful and since, they passed the validation tests, they are ready to be commercialized.

The SUPREME Real Time PCR detection kits marketing premise is that they are high specificity and sensibility meat detection kits, sold at the low cost of 4,5€ per reaction. The kits are directed to laboratories of food safety analysis and governmental entities and food producers with own laboratories. These laboratories must have Real Time PCR equipment and should be located in Europe. The awareness of the kits is raised through sales force to new and old connections and the opening of distribution channels with the scientific distributors.

When compared with the available kits in the market, the SUPREME Real Time Detection kits detect the lowest quantity of DNA and has a lesser cost per reaction, raising its attractiveness in the market.

The SUPREME Real Time PCR detection kits have a low LoD, allowing the thorough analysis of food samples. However, each kit only detects the giving target. Future works by BPMR can focus on the development of Real Time PCR detection kits with other common food fraud targets, like cat and dog meat. BPMR can also focus in the development of a multiplex kit to detect several targets in one assay, minimizing the reagents and time spent and a more complex analysis of a food sample. To increase sales, BPMR can focus on the partnership with distribution companies and direct contact with laboratories, in all countries in Europe. An agreement with customs of several key countries outside EU can also be beneficial to the selling of SUPREME Real Time PCR.

In conclusion, the development of the SUPREME Real Time PCR was successful. Their low limit of detection and low cost are some of the characteristics that capture the attention of the market.

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Appendix 1 Tables listing the samples used the exclusivity test of each kit. Table [A] is comprised of samples of the chicken detection kit exclusivity test. Table [B] is comprised of samples of the turkey detection kit exclusivity test. Table [C] is comprised of samples of the horse detection kit exclusivity test. Table [D] is comprised of samples of the swine detection kit exclusivity test.

A

Identification Code	Food Matrix
C5	Horse Meat
C13	Duck Rice with Chorizo
C17	Chicken Meat
C20	Potato
C23	Corn
C26	Goose Foie Gras
C28	Chicken Hamburger
C29	Rabbit Meat
C30	Soy
C31	Duck Foie Gras
C33	Goat Cheese
C57	Beef Hamburger
C60	Cow Cheese
C66	Vegetarian Meatballs

B

Identification Code	Food Matrix
C5	Horse Meat
C13	Duck Rice with Chorizo
C19	Ham
C20	Potato
C23	Corn
C26	Goose Foie Gras
C29	Rabbit Meat
C30	Soy
C31	Duck Foie Gras
C33	Goat Cheese
C55	Turkey Hamburger
C57	Beef Hamburger
C60	Cow Cheese
C66	Vegetarian Meatballs

C

Identification Code	Food Matrix
C9	Shredded Duck Meat
C14	Pork Lasagne
C17	Chicken Meat
C20	Potato
C23	Corn
C25	Pork Sausages
C26	Goose Foie Gras
C28	Chicken Hamburger
C29	Rabbit Meat
C30	Soy
C31	Duck Foie Gras
C33	Goat Cheese
C57	Beef Hamburger
C60	Cow Cheese
C65	Boar Meat
C66	Vegetarian Meatballs

D

Identification Code	Food Matrix
C5	Horse Meat
C9	Shredded Duck Meat
C17	Chicken Meat
C20	Potato
C21	Baby Food
C23	Corn
C28	Chicken Hamburger
C29	Rabbit Meat
C30	Soy
C31	Duck Foie Gras
C33	Goat Cheese
C60	Cow Cheese
C66	Vegetarian Meatballs