***Highlights (for review)**

Highlights: a suitable method combining PMA sample pretreatment with qPCR has been developed to selectively detect viable cells of *Alternaria* spp. The method allowed a detection limit of 10^2 conidia/g of tomato and achieved a reduction of almost 7 cycles in the ΔCq between live and heat killed conidia.

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1 **Propidium monoazide combined with real-time quantitative PCR to quantify viable**
2 ***Alternaria* spp. contamination in tomato products**

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7

8 **Abstract.**

9 *Alternaria* is a common contaminating genus of fungi in fruits, grains, and vegetables
10 that causes severe economic losses to farmers and the food industry. Furthermore, it is claimed
11 that *Alternaria* spp. are able to produce phytotoxic metabolites, toxic to plants, and mycotoxins,
12 unsafe for human and animal health. DNA amplification techniques are being increasingly
13 applied to detect, identify, and quantify mycotoxigenic fungi in foodstuffs, but the inability of
14 these methods to distinguish between viable and nonviable cells might lead to an
15 overestimation of mycotoxin-producing living cells. A promising technique to overcome this
16 problem is the pre-treatment of samples with nucleic acid intercalating dyes, such as propidium
17 monoazide (PMA), prior quantitative PCR (qPCR). PMA selectively penetrates cells with a
18 damaged membrane inhibiting DNA amplification during qPCRs. In our study, a primer pair
19 (Alt4-Alt5) to specifically amplify and quantify *Alternaria* spp. by qPCR was designed.
20 Quantification data of qPCR achieved a detection limit of 10^2 conidia/g of tomato. Here, we have
21 optimized for the first time a DNA amplification-based PMA sample pre-treatment protocol for
22 detecting viable *Alternaria* spp. cells. Artificially inoculated tomato samples treated with 65 µM
23 of PMA, showed a reduction in the signal by almost 7 cycles in qPCR between live and heat-
24 killed *Alternaria* spp conidia. The tomato matrix had a protective effect on the cells against PMA
25 toxicity, reducing the efficiency to distinguish between viable and nonviable cells. The results
26 reported here indicate that the PMA-qPCR method is a suitable tool for quantifying viable
27 *Alternaria* cells, which could be useful for estimating potential risks of mycotoxin contamination.

28 **Keywords:** *Alternaria* spp.; Mycotoxins; Propidium monoazide; Quantitative polymerase chain
29 reaction; Tomato.

30

31 **1. Introduction**

32 *Alternaria* is a genus of fungi which includes saprophytic and pathogenic species that
33 affect field crops, reducing the yield and causing post-harvest decay of various fruits, grains,
34 and vegetables, which consequently leads to economic losses to farmers and the food industry
35 (Logrieco et al., 2003). Tomatoes are highly susceptible to fungal invasion due to their thin skin
36 and.*Alternaria* is the most common fungus found on mouldy tomatoes (Andersen and Frisvad,
37 2004; Barkai-Golan and Paster, 2008; Pitt and Hocking, 1997). More specifically, *Alternaria*
38 *alternata, A. arborescens, A. tenuissima, A. tomaticola, A. tomato,* and *A. tomatophila* (former *A.*
39 *solani*) are the primary *Alternaria* species found in raw tomatoes and tomato products
40 (Andersen et al., 2008; Somma et al., 2011; Weir et al., 1998).

41 *Alternaria* spp., besides being commonly associated with several plant diseases, play
42 an important role in the production of mycotoxins. The most relevant mycotoxins produced by
43 *Alternaria* spp. are alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TEN),
44 tenuazonic acid (TeA), altenuene (ALT), altertoxins (ATXs), stemphyltoxin III, and *Alternaria*
45 *alternata* f. sp *lycopersici* toxins (AAL-toxins), which have the potential to cause several health
46 problems in humans and animals (EFSA, 2011; Logrieco et al., 2009; Scott, 2001). Among all
47 *Alternaria* spp., *A. alternata* has been regarded as the most important mycotoxin-producing
48 species (Barkai-Golan, 2008). However, *A. arborescens*, *A. tenuissima*, *A. tomato*, *A. solani*,
49 and *A. tomatophila* are also known to produce AOH, AME, ATX-I, -II, -III, and TeA (Andersen et
50 al., 2008; Andersen and Frisvad, 2004; Andersen et al., 2002; Pollock et al., 1982).

51 When dealing with food safety and plant pathology, rapid determination of the presence
52 of fungi is essential to take the appropriate corrective actions to help the industry lower the
53 contamination levels in the final products, particularly when a post-harvest storage is required.
54 Nucleic acid-based methods are being increasingly applied to detect, identify, and quantify
55 mycotoxigenic fungi in foodstuffs (Edwards et al., 2002; Mackay, 2004; Niessen, 2007; Zur et
56 al., 1999). Polymerase chain reaction (PCR) has replaced complex and time-consuming
57 microbiological tests, based on the growth of the studied microorganism in different types of
58 culture media, for the amplification of specific genomic markers. Particularly, quantitative real-
59 time PCR (qPCR) is a technique that allows the detection, identification, and quantification of
60 DNA and RNA present in a food sample (Hayat et al., 2012; Postollec et al., 2011; Rodríguez et

61 al., 2011). However, there are still limitations in the use of nucleic acid-based techniques. One
62 of these obstacles is the inability to discriminate between the nucleic acids of viable and dead
63 microorganisms, as the DNA from dead cells can remain intact for several days or even weeks
64 (Josephson et al., 1993). Hence, the DNA from dead cells can serve as a template in PCR
65 amplification, overestimating viable *Alternaria* spp. cells, which are the potential mycotoxin
66 producers. Therefore, these techniques are not suitable for assessing the potential risk of fungal
67 contamination in foodstuffs, particularly when the raw material is stored before being processed.
68 To overcome this problem, propidium monoazide (PMA) combined with qPCR has been
69 proposed to differentiate dead and viable forms, or to detect and quantify only viable cells. PMA
70 is a nucleic acid-intercalating fluorophore that can penetrate through the damaged membranes
71 of dead cells. Once inside the cell, and after exposure to strong visible light, PMA binds to the
72 DNA of dead cells, leaving the DNA from viable cells unlabeled. The unlabelled DNA from the
73 viable cells is amplified, while the PMA bound to the DNA of dead cells inhibits the activity of the
74 polymerase and no amplification of this latter DNA occurs. Although there are several studies in
75 which PMA-qPCR is used for detecting and quantifying viable bacterial cells (Cawthorn and
76 Witthuhn, 2008; Elizaquível et al., 2012; Josefsen et al., 2010; Nocker et al., 2006; Nocker et
77 al., 2009; Pan and Breidt Jr., 2007; Zhu et al., 2012), few studies have focused this technique
78 on fungi (Vesper et al., 2008), and none on *Alternaria* spp. Here we developed a specific and
79 sensitive PMA treatment combined with qPCR in order to detect, identify, and quantify
80 *Alternaria* spp. viable cells in tomato samples.
81

82 **2. Materials and methods**

83 **2.1. Fungal isolates and culture conditions**

84 All the isolates used in this study are listed in Table 1. Fungal reference strains were
85 provided by the *Centralbureau voor Schimmelcultures* (CBS, The Netherlands) and the Spanish
86 Type Culture Collection (*CECT*, Spain). Fungal strains were stored as conidial suspensions in
87 40% glycerol at −20 ºC. Ten *Alternaria* spp. isolates obtained in our laboratory from tomatoes
88 were also included. The identification of these ten isolates was previously confirmed by
89 sequencing a beta-tubulin gene region with the Beta3-Beta4 primers (Peever et al., 2004).

90 To prepare the conidial suspensions strains were grown on Petri dishes containing
91 Potato Dextrose Agar (Biokar Diagnostics, France), at 26 °C for 6 days, in the dark. Conidia
92 were collected with a sterile solution of Tween 80 (0.005% v/v) and filtered through Miracloth
93 (Calbiochem, USA). Conidial concentration was determined using a Thoma counting chamber.

94 **2.2. DNA extraction**

95 Cultures were grown in 500 µL of Malt Extract broth (2% w/v malt extract, 0.1% w/v
96 peptone, 2% w/v glucose) for 2 days at 26 ºC. The mycelial extract was recovered after 10 min
97 of centrifugation at 17500 x g and 300 µL of DNA extraction buffer (200 mM Tris-HCl, pH 8.5,
98 250 mM NaCl, 25 mM EDTA, 0.5% w/v SDS) was added. The mycelial suspension was lysed
99 by vortexing with five 2.8 mm Precellys metal beads (Bertin Technologies, France) for 10 min.
100 After a centrifugation at 17500 x g for 10 min, 150 µL of 3 M sodium acetate (pH 5.2) was added
101 to the supernatant. The supernatant was stored at -20 ºC for 10 min and then centrifuged
102 (17500 x g, 10 min). The DNA-containing supernatant was transferred to a new tube and
103 nucleic acids were precipitated by addition of one volume of isopropanol. After a 5-minute
104 incubation time at room temperature, the DNA suspension was centrifuged (17500 x g, 10 min).
105 The DNA pellet was washed with 70% ethanol to remove residual salts. Finally, the pellet was
106 air-dried and the DNA resuspended in 50 µL of TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA).

107 **2.3. Primer design**

108 A primer pair, Alt4 (5'-CTTTTGCGTACTTCTTGTTTCC-3') and Alt5 (5'-
109 CAGGCATGCCCTTTGGATAC-3'), was designed for specific amplification of *Alternaria* spp.
110 based on sequence alignments of the internal spacer regions (ITSs) from several *Alternaria*
111 spp. strains and other related fungal co-contaminants of tomato products. Additionally, another
112 primer pair, Alt6 (5'-AACTTTCAACAACGGATCTCT-3') and Alt7 (5'-
113 ATGCTTAAGTTCAGCGGGTA-3'), was designed in a conserved ITS region, to obtain
114 amplifications of all DNA samples from *Alternaria* spp. and co-contaminants strains. Thus, DNA
115 amplification using Alt6-Alt7 primers could serve as a control of DNA integrity to prevent false
116 negative amplifications. Both pair of primers, Alt4-Alt5 and Alt6-Alt7, were designed using
117 OLIGO V5 software (http://www.oligo.net).

118 **2.4. Primer-specific PCR detection**

119 Alt4-Alt5-specific PCR assays were performed in a GeneAmp® PCR System 2700
120 (Applied Biosystems, USA). Amplification reactions were carried out in volumes of 10 µL
121 containing 10 ng of DNA, 16 mM $(NH_4)_2SO_4$, 67 mM TrisHCl pH 8.8, 0.01% (v/v) Tween 20, 1.5
122 mM $MgCl_2$, 250 µM (each) dNTP, 0.5 µM of each primer, and 0.5 U of DFS-Taq DNA
123 Polymerase (BIORON, Germany). PCR reactions were performed under the following
124 conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C
125 for 30 s, annealing at 66 ºC for 45 s, and extension at 72 °C for 60 s with a final extension of 10
126 min. PCR products were detected in 1.5% (w/v) agarose ethidium bromide gels in TAE 1X
127 buffer (40 mM Tris-acetate, 1 mM EDTA, and 20 mM acetic acid). A TrackIT 100 bp DNA ladder
128 (Invitrogen, USA) was used as the molecular size marker.

129 **2.5. Fungal detection in artificially contaminated samples**

130 Tomatoes were surface-sterilized by dipping them into a NaClO solution (0.1% w/v Cl)
131 for 5 min and then immersed in 70% ethanol for 1 min. Excess water was removed by placing
132 the tomatoes in a laminar flow bench. For tomato inoculation, fungal conidia suspensions of *A.*
133 *alternata* CECT 20560 were prepared in distilled water containing Tween 80 (0.005% v/v). Two
134 hundred grams of tomatoes were dipped into 200 ml of phosphate buffered saline (PBS), 138
135 mM NaCl, 2.7 mM KCl, 10 mM $NaH_2PO_4 \cdot 2\ H_2O$, and 2 mM KH_2PO_4, containing the conidia
136 suspensions (10^5, 10^4, 10^3, 10^2, and 10 conidia/g tomato) and homogenized in a Masticator
137 stomacher for 60 s (IUL instruments, Spain). Another two hundred grams of non-inoculated
138 tomatoes were used as the negative control. A triplicate of 50 ml aliquots were taken from each
139 stomacher bag, filtered through a Miracloth (Calbiochem, USA) and centrifuged at 15000 x g for
140 10 min. Pellets were resuspended in 2 ml of PBS, which were then treated with PMA to finally
141 perform the DNA extraction as previously detailed (2.2). Additionally, in order to see if the
142 detection limit could be improved, the artificially inoculated tomato samples were incubated at
143 26 ºC for 20 h prior DNA extraction.

144 Presence and quantitative detection of *Alternaria* spp. was performed by qPCR. PCR
145 reactions were performed in a final volume of 10 µL containing 1X SsoAdvanced™ SYBR®
146 Green Supermix (BIO-RAD, USA), 250 nM of each primer, and 4 µL of template DNA. All
147 amplifications were performed on a CFX96 TouchTM Real-Time PCR Detection System (BIO-
148 RAD, USA). The standard protocol included one cycle at 98 °C for 2 min, followed by 40 cycles

149 at 98 °C for 5 s, and 66 °C for 30 s. Reactions were done in triplicate. In all cases, a negative
150 amplification control was included using 4 µL of water instead of DNA. For the preparation of
151 standard curves five different concentrations of conidia (10^2, 10^3, 10^4, 10^5, and 10^6 conidia/g
152 tomato) were used to artificially contaminate the tomatoes as previously described. The
153 quantification cycle (Cq) value that determines the cycle number at which fluorescence
154 increases above background, was plotted against the logarithm of starting quantity of template
155 for each dilution. Amplification efficiency was calculated from the slope of the standard curve
156 ($E=10^{-1/slope}$; % Efficiency =(E−1)×100 (Kubista *et al.*, 2006)). In addition, melting curves were
157 programmed in order to check the specificity of the Alt4-Alt5 primers.

158 **2.6. Treatment of samples with PMA**

159 In order to optimize PMA treatment for quantifying viable *Alternaria* conidia, PMA
160 toxicity was tested. Aliquots of 500 µL of PBS with 10^6 conidia/ml were treated with different
161 PMA concentrations (20, 30, 40, and 50 µM). The treatment of conidia with PMA, stocks of 20
162 mM in water (BIOTIUM, USA), consisted in the addition of the reagent to the samples, an
163 incubation period of 20 min in the dark at room temperature (with occasional mixing to allow
164 reagent penetration), and 10 min of exposure to light using a photo-activation system. A closed
165 box with refractory walls and blue wavelength light-emitting diodes (LED, 6000 mcd) was
166 constructed and placed 7 cm from a 24-well microplate containing the samples. After the PMA
167 treatment, 1/100 and 1/1000 dilutions from the 10^6 conidia/ml aliquots were plated on PDA Petri
168 dishes. After a 24-hour incubation period at 26 ºC, colony forming units (CFU) were counted. To
169 determine the suitability of the PMA-qPCR technique for distinguishing viable and non-viable
170 conidia, PMA treatments were performed in three different samples of 500 µL of PBS containing
171 10^6 conidia/ml: live conidia, dead conidia (treated in a hot bath at 85 ºC for 1 h), and live:dead
172 conidia where both samples were mixed in equal proportions. Loss of cell viability for dead
173 conidia was confirmed by plating on PDA media and incubating for 24 h at 26 ºC. Each sample
174 was treated in triplicate to ensure reproducibility of the results. After PMA treatment, samples
175 were centrifuged (15000 x g, 10 min), conidia resuspended in 300 µL of DNA extraction buffer,
176 and DNA extractions were performed as previously described.

177 Additionally, to evaluate the toxicity and the efficiency of PMA in a tomato matrix, 200
178 grams of tomatoes were homogenized with PBS (1:1 dilution) using a Masticator stomacher

179 (IUL instruments, Spain) for 60 s. Aliquots of 50 ml were taken from the stomacher bag, filtered
180 through a Miracloth (Calbiochem, USA), and centrifuged at 15000 x g for 10 min as described in
181 fungal detection for tomato samples in section 2.5. For assessing efficiency, tomato pellets were
182 resuspended with 2 ml of PBS containing 10^6 conidia/ml of live conidia, dead conidia, or
183 live:dead conidia in equal proportions. For evaluating PMA toxicity in the tomato matrix, 50, 60,
184 and 65 μM of PMA concentrations were tested.

185 **2.7. Statistical analysis**

186 All statistical analyses were performed using Statgraphics Plus 5.1 (Statpoint
187 Technologies Inc., USA). One-way analysis of variance (ANOVA), with a significance level of
188 p=0.05, was carried out to determine significant differences between the means.

189

190 **3. Results and Discussion**

191 **3.1. Primer set specificity**

192 The Alt4-Alt5 primer set was designed to detect, identify, and quantify *Alternaria* spp..
193 The specificity of these primers was tested by PCR amplification of the most common *Alternaria*
194 spp., responsible of the decay of fruits and vegetables and the main co-contaminant fungi
195 present in tomatoes (Table 1). As shown in Figure 1A, amplification of all DNA samples from
196 *Alternaria* spp. (*A. alternata*, *A. arborescens*, *A. tenuissima*, *A. tomato*, *A. tomatophila*, *A.*
197 *tomaticola,* and *A. solani*) were obtained. *Ulocladium botrytis* DNA was also amplified.
198 Conversely, DNA samples from fungal co-contaminants (*Geotrichum candidum*, *Colletotrichum*
199 *dematium*, *Colletotrichum cocades*, *Colletotrichum gloesporoides*, *Botrytis cinerea*, *Fusarium*
200 *oxysporum*, *Rhizopus oryzae*, *Rhizopus microsporus* var. *rhizopodiformis, Rhizopus stolonifer,*
201 *Stemphylium eturmiunum*, and *Stemphylium lycopersici*) did not amplify any product. This
202 negative amplification was not due to low DNA integrity, since Figure 1B shows the amplification
203 of the same DNA samples but using the Alt6-Alt7 primer pair instead of the Alt4-Alt5.
204 Additionally, DNA samples from ten *Alternaria* spp. isolates from our own collection (Table 1)
205 were amplified with the Alt4-Alt5 primers (Figure 1C).

206 A close phylogenetic relationship has been established between *Alternaria* spp. and
207 *Ulocladium* spp. in some studies (Andersen and Hollensted, 2008; Chou and Wu, 2002).
208 Although it is possible to distinguish *Alternaria* spp. from *U. botrytis*, detailed morphological and

209 chemical analyzes are required. Nonetheless, it has been observed that the genus *Ulocladium*
210 contains species that are responsible for the decay of nuts, fruits, and cereals, plant pathogens,
211 and mycotoxin producers. ATX-I and TeA are two of the mycotoxin produced by *Ulocladium*
212 spp., which are also produced by *Alternaria* spp. (Andersen and Hollensted, 2008; EFSA, 2011;
213 Scott, 2001; Schlegel et al., 2001). Hence, it has not been possible to distinguish between
214 *Alternaria* spp. and *U. botrytis* using Alt4-Alt5 primers, but the joint detection of both genera
215 could be an advantage for the food industry because of their related mycotoxigenic profile.

216 **3.2. Amplification efficiency and standard curves using qPCR**

217 Alt4 and Alt5 primers were also used for qPCR amplification. To test the efficiency and
218 specificity of the primers, standard curves were generated using *A. alternata* (CECT 20560)
219 DNA in a tomato matrix. As shown in Figure 2, a strong linear relationship (R^2=0.9999) was
220 found between the DNA extracted from the tomato food matrix inoculated with different
221 concentration of conidia (10^5, 10^4, 10^3, and 10^2 conidia/g) and Cq values. When Cq values were
222 plotted against conidia concentrations, a slope of -3.5708 was obtained, indicating an efficiency
223 of 1.9057 (90.57%). This efficiency is considered acceptable according to Postollec et al.
224 (2011). Furthermore, it was found that the Alt4-Alt5 primer pair, amplified all *Alternaria* spp. and
225 *U. botrytis* DNA by qPCR, confirming the results obtained by conventional PCR. Additionally, for
226 *A. solani*, *A. tomato*, and *A. tomatophila* a single melting peak (83 ºC) was observed, while for
227 the rest of positive samples the melting temperature was 82 or 82.5 ºC. Thus, primer pair Alt4-
228 Alt5 is specific and efficient and can be used for accurate quantification of *Alternaria* spp. and *U.*
229 *botrytis* contamination in tomato products.

230 **3.3. Evaluation of the detection limit using qPCR in artificially inoculated tomato samples**

231 The limit of detection (LOD) was assayed in artificially inoculated tomatoes with different
232 concentrations of *A. alternata* (CECT 20560) conidia (10^5, 10^4, 10^3, 10^2, and 10 conidia/g of
233 tomato). Additionally, the effect of pre-enrichment on contaminated tomato samples prior DNA
234 extraction was studied. Enrichment of raw inoculated tomatoes was done in PBS at 26 ºC for 20
235 h. The aim of the additional enrichment was to improve the detection and quantification limits;
236 however, a LOD of 10^2 conidia/g of tomato was obtained for both assays, with or without the
237 enrichment step (Table 2). When tomato samples were inoculated with 10^2 conidia/g of tomato
238 with no enrichment step, 6.6 x 10^2 conidia/g of tomato were detected. In contrast, 1.5x10^4

239 conidia/g of tomato was determined with a 20 h enrichment step. Thus, an inoculation of 10
240 conidia/g of tomato was not enough to produce a signal in the qPCR. No detectable signal was
241 observed in the negative controls. Our results are similar to those in previous studies focused
242 on the quantification of DNA by qPCR methods. Rodríguez et al. (2011) evaluated the
243 sensitivity of detection for different ochratoxin A producing moulds in artificially inoculated food
244 matrices, obtaining a LOD that ranged from 10 to 1 conidia/g. Similarly, Diguta et al. (2010)
245 developed a qPCR system to quantify and identify *B. cinerea*, one of the major pathogens
246 present in grapes. A LOD of 6.3 pg of DNA was obtained, corresponding to 540 conidia without
247 the enrichment step. Selma et al. (2008) established a useful qPCR protocol to detect and
248 quantify conidia in wine grapes inoculated with *Aspergillus carbonarius*, achieving a LOD of 5
249 conidia/reaction without incubation.

250 Although the LOD achieved in our study (10^2 CFU/g of food matrix) is similar to that
251 obtained with conventional culture methods, the aim of using PMA-qPCR instead of standard
252 plate counts was to reduce the analysis time, since results can be obtained in 24 hours instead
253 of the 5-7 days needed otherwise. It should be considered that *Alternaria* spp. grow slowly, so a
254 longer enrichment step might be required in order to detect lower concentrations of conidia.
255 There are no legislations regarding the presence of fungi in food products, only the level of
256 mycotoxin contamination, which must be below the value established by the law (CEC, 2006).
257 Therefore, mycotoxin analysis is undoubtedly an essential requirement. However, the detection
258 of viable cells, which have the possibility to grow and produce mycotoxins, might be useful in
259 order to apply corrective measures in an industrial context, particularly when the vegetables are
260 stored before processing.

261 **3.4. PMA sample pre-treatment combined with qPCR to detect and quantify viable**
262 ***Alternaria* conidia**

263 One of the limitations of PCR and qPCR is the inability to discriminate between live and
264 dead cells. To bypass this problem, the use of PMA has been tested in *Alternaria* spp. cells,
265 being PMA concentration a key factor to effectively discriminate between viable and non-viable
266 cells. An adequate concentration must be added to detect exclusively viable cells, but no
267 cytotoxicity should be observed. Hence, live cells from an *A. altermata* (CECT 20560) culture
268 were exposed to four different concentrations of PMA (20, 30, 40, and 50 μM), and toxicity was

269 measured as detailed above (2.6). Additionally, to study the influence of the food matrix on PMA
270 toxicity, PMA was added to artificially inoculated samples reaching final dye concentrations of
271 50, 60, and 65 µM. As shown in Figure 3A no differences in CFU counts were observed
272 between samples in PBS treated with PMA concentrations of 20, 30, and 40 µM and the
273 untreated live cells. However, a statistically significant toxic effect (p=0.05) was found when
274 conidial suspensions were treated with 50 µM of PMA. Interestingly, no cytotoxic effect was
275 observed for live cells in tomato matrices exposed to 50, 60, and 65 µM of PMA, based on plate
276 count data (Figure 3B). This finding suggests that the tomato matrix might hinder PMA entry into
277 the cell and higher PMA concentrations would be required for an efficient discrimination
278 between viable and non-viable cells. The maximum PMA concentrations with no cytotoxic
279 effects were 40 and 65 µM in PBS and food matrix respectively, thus, these were used in
280 subsequent experiments.

281 PMA specificity was obtained by comparing qPCR quantification cycle values for treated
282 live and dead cells. A signal reduction of 8.86 cycles was observed between live and heat-killed
283 conidia in PBS treated with 40 µM of PMA (Figures 4 and 5). Additionally, the subtraction of Cq
284 values of PMA-treated live cells from samples with PMA-treated live-dead (50:50) cells in PBS
285 was 0.98 cycles, which correlates with the fact that in one PCR cycle almost all the DNA is
286 duplicated (E=1.9057). Therefore, the DNA quantified in PMA-treated live-dead (50:50) cells
287 belonged to live cells, whereas the amplification of the DNA from dead cells may be inhibited
288 due to PMA activity. Conversely, when exposing fungal cells in the tomato matrix to 40 µM of
289 PMA, a difference of 3.23 Cq between live and heat-killed conidia was found (Figure 5),
290 confirming the tomato matrix effect observed in the PMA toxicity assay described above. PMA
291 efficiency was shown to increase with higher PMA concentrations, achieving a difference of
292 6.85 Cq between live and heat-killed conidia in the tomato matrix treated with 65 µM of PMA.
293 Furthermore, the subtraction of Cq values of PMA-treated live cells from samples with PMA-
294 treated live-dead (50:50) cells in tomato matrix was 1.04 cycles.

295 Since the first description by Nocker et al. (2006), PMA has been applied to a wide
296 variety of microorganisms including bacteria (Elizaquível et al., 2012; Kralik et al., 2010; Nocker
297 et al., 2009), yeast (Andorrà et al., 2010), virus (Fittipaldi et al., 2010; Sánchez et al., 2012), and
298 fungi (Vesper et al., 2008). PMA concentration, incubation conditions, and light source proposed

299 as optimal are highly variable depending on both the microorganism and buffers or the food
300 matrices containing the microorganisms. For instance, a 6 µM of PMA has been proposed for
301 the detection of viable yeast in wine (Andorrà et al., (2010), while 100 µM of PMA was used to
302 identify *Escherichia coli* O157:H7 in vegetables (Elizaquível et al., 2012). Consequently, the
303 efficiency of the treatment varies among samples, with a 7 to 13.6 Cq reduction between live
304 and dead cells for pure bacterial cultures (Kralik et al., 2010; Nocker et al., 2007), a 6 to 11 Cq
305 reduction for yeast in wine (Andorrà et al., 2010), a reduction that ranges from 1 to 3 Cq for
306 detecting *Vibrio parahaemolyticus* in seafood (Zhu et al., 2012), or a 1.3 to 3 reduction in $\log_{10}$
307 CFU for bacteria in vegetables (Elizaquível et al., 2012; Yáñez et al., 2011). Vesper et al. (2008)
308 have published the only study in which PMA has been used to identify viable cells in fungi.
309 These authors studied the effect of PMA in several fungal strains (*Aspergillus fumigatus*,
310 *Aspergillus flavus*, *Aspergillus terreus*, *Mucor racemosus*, *Rhizopus stolonifer*, and
311 *Paecilomyces variotii*) in air and water samples, achieving a Cq reduction of 6 to 9 cycles
312 between live and dead cells. In our study, a Cq reduction of 8.86 (a 302.9 fold reduction)
313 between live and heat-killed cells in PBS was observed for samples treated with 40 µM of PMA,
314 while a difference of 6.85 Cq (82.86 fold reduction) was found in the case of live and dead cells
315 in the tomato matrix exposed to 65 µM of PMA. Furthermore, it seems dead cells are somehow
316 *protected* by the tomato matrix, reducing PMA toxicity and the capacity to inhibit qPCR DNA
317 amplification in these cells.

318 Other useful techniques have been proposed to reveal viable cells, such as RNA
319 detection by reverse-transcriptase PCR (RT-PCR) (Kidon Sung, 2005; Sheridan et al., 1998).
320 This technique has also been assayed in food matrices, often combined with qPCR (Bleve et
321 al., 2003; Hierro et al., 2006; M. Vaitilingom, 1998; M.B. Mayoral, 2006; Pavón et al., 2012). The
322 detection of highly unstable RNA, specifically mRNA, which is only produced by metabolically
323 active cells, would allow identifying viable cells only. In contrast to highly persistent DNA that
324 can remain stable from days to three weeks (Josephson et al., 1993), RNA degrades more
325 rapidly after cell death. Nonetheless, the instability that makes RNA a suitable target for
326 detecting viable cells, would turn it to a difficult target to work with because it is prone to
327 contamination due to inadequate sample processing, unsatisfactory storage, or due to
328 contamination with RNA-degrading enzymes. All these drawbacks lead to reproducibility

329 problemsf. Furthermore, it should be considered that mRNA depends on the metabolic activity
330 of the cell, so the quantification could be overestimated when there is an active physiological
331 state or underestimated if the cells are alive but latent (Nocker and Camper, 2006).

332 The activation of PMA using blue LED photoactivation systems instead of a halogen
333 light source is another advantage initially proposed by Vesper et al. (2008), which was tested in
334 our work for *Alternaria* spp. The problem of using high-wattage halogen light sources (≥ 600 W
335 as recommended by the PMA manufacturer, BIOTIUM) is that cell membranes could be
336 damaged by the heat emitted by the lamp, making them susceptible to PMA entry. Therefore,
337 activation periods are usually < 2 minutes in PMA treatments using high-wattage halogen light
338 sources (Andorrà et al., 2010; Josefsen et al., 2010; Nocker et al., 2007). To avoid excessive
339 heating, some authors have suggested to place the sample on ice, before or after the activation.
340 Conversely, longer activation periods, from 10 to 15 minutes, which could help improve PMA
341 efficiency, have been carried out using LEDs (Elizaquível et al., 2012; Fittipaldi et al., 2010;
342 Vesper et al., 2008).

343 In this work, we have developed a fast, sensitive, and efficient technique based on the
344 pre-treatment of the sample with PMA combined with qPCR, which allows detecting DNA from
345 viable *Alternaria* spp. cells. In tomato samples, detection of DNA from dead cells is around
346 82.86-fold lower when PMA is used in comparison with live cells. This methodology can be
347 useful to the food industry as it could be employed as a preventive tool to detect the risk of
348 contamination in foodstuffs with potentially mycotoxigenic fungi as is the case of *Alternaria* spp.,
349 which are responsible of the production of mycotoxins such as AOH, AME, TEN, TeA, ALT,
350 ATXs, stemphyltoxin III, and AAL-toxins.

351

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594 **Figure Captions**

595 **Fig. 1.** Specificity of *Alternaria* PCR primer set. (A) Amplification of the most common *Alternaria*
596 spp. present in fruits and vegetables and the main co-contaminant fungi present in tomatoes
597 using Alt4-Alt5 primers. (B) Control of the false negative amplifications between the most
598 common *Alternaria* spp. present in fruits and vegetables and the main co-contaminant fungi
599 present in tomatoes using Alt6-Alt7 primers. 1: *Alternaria alternata* CECT 20560; 2: *Alternaria*
600 *alternata* CBS 116.329; 3: *Alternaria alternata* CECT 2662; 4: *Alternaria alternata* CBS 119.115;
601 5: *Alternaria arborescens* CBS 109.730; 6: *Alternaria tenuissima* CBS 124.278; 7: *Alternaria*
602 *tomaticola* CBS 118.814; 8: *Alternaria tomato* CBS 114.35; 9: *Alternaria tomatophila* CBS
603 109.156; 10: *Alternaria solani* CBS 105.51; 11: *Geotrichum candidum* CBS 117.139; 12:
604 *Colletotrichum dematium* CBS 125.25; 13: *Colletotrichum cocades* CECT 21008; 14:
605 *Colletotrichum gloesporoides* CECT 21015; 15: *Botrytis cinerea* CECT 2100; 16: *Fusarium*
606 *oxysporum* CECT 2866; 17: *Rhizopus oryzae* CECT 2339; 18: *Rhizopus microsporus var.*
607 *rhizopodiformis* CBS 607.73; 19: *Rhizopus stolonifer* CECT 2344; 20: *Ulocladium botrytis* CECT
608 20564; 21: *Stemphylium eturmiunum* CBS 124279; 22: *Stemphylium lycopersici* CBS 122639; (-
609): negative control and M: molecular ladder weight of 100 bp (Invitrogen, USA). (C) Amplification
610 by PCR of *Alternaria* isolates from tomato. 1: Alt tp15; 2: Alt 09, 3: Alt 30; 4: Alt 35; 5: Alt 05; 6:
611 Alt tp13; 7: Alt tp18; 8: Alt 06; 9: Alt 3.1; 10: Alt 1.4.

612

613 **Fig. 2.** Standard curves obtained with SYBR Green I using five tomato food matrix samples
614 inoculated with *A. alternata* (CECT 20560) conidia with different concentration each sample.
615 The concentrations assayed were: 10^6, 10^5, 10^4, 10^3 and 10^2 conidia/g of tomato. After
616 inoculation the tomato sample was treated as a current sample. At the end it was carried out a
617 DNA extraction. The figure shows a strong linearity between Cq values and the concentration of
618 *A. alternata* conidia assayed (R^2=0.9999) and an efficiency of 90.57%.

619

620 **Fig. 3.** PMA toxicity to conidia suspensions of an *A. alternata* culture (CECT 20560) in PBS (A)
621 or in a tomato matrix (B). Colony forming units (CFU) were counted in PDA plates after an
622 incubation of 24 h at 26 ºC. (*) indicates statistically significant differences (ANOVA, $p < 0.05$).
623 Error bars represent standard deviations obtained from three independent replicates.

624 **Fig. 4.** Amplification curves obtained by qPCR using a 40 μM PMA treatment. Curves
625 correspond to live *A. alternata* (CECT 20560) conidia, a mix of live and heat killed conidia
626 (50:50) and heat killed conidia in PBS. Analyses were performed in triplicate. RFU: relative
627 fluorescence units.
628
629 **Fig. 5.** Effect of different PMA concentrations on Cq values. Results are presented as the
630 difference of Cq values between dead and live cells treated with PMA and the difference of Cq
631 values between dead:live (50:50) and live cells treated with PMA. Error bars represent standard
632 deviations obtained from three independent replicates.
633
634 **Table legends**
635 **Table 1.** Fungal strains used in this study indicating species and origin.
636
637 **Table 2.** Limit of detection of *A. alternata* in artificially contaminated tomato samples using the
638 primers Alt4-Alt5 for the qPCR. (A) Evaluation of detection limit without applying an enrichment
639 step to tomato inoculated samples. (B) Evaluation of detection limit applying a 20 h enrichment
640 step of raw tomatoes in PBS at 26 ºC.
641

Table 1

Table 1. Fungal strains used in this study indicating species and origin.

Species designation	Strain	Origin
Alternaria alternata	CECT 20560	Pepper-Spain
Alternaria alternata	CBS 116329	Apple-Germany
Alternaria alternata	CECT 2662	Tomato-UK
Alternaria alternata	CBS 119115	Cherry leaf-Greece
Alternaria arborescens	CBS 109730	Tomato-USA
Alternaria tenuissima	CBS 124278	Cherry-Denmark
Alternaria tomaticola	CBS 118814	Tomato-USA
Alternaria tomato	CBS 114.35	Tomato
Alternaria tomatophila	CBS 109156	Tomato leaf-USA
Alternaria solani	CBS 105.51	Tomato
Botrytis cinerea	CECT 2100	*Vicia faba*-UK
Colletotrichum cocades	CECT 21008	-
Colletotrichum dematium	CBS 12525	Leaf *Eryngium campestre*-France
Colletotrichum gloesporoides	CECT 21015	-
Fusarium oxysporum	CECT 2866	Tomato
Geotrichum candidum	CBS 117139	Tomato-Brazil
Rhizopus microsporus var. *rhizopideformis*	CBS 607.73	Cereals-Yugoslavia
Rhizopus oryzae	CECT 2339	-
Rhizopus stolonifer	CECT 2344	-
Stemphylium eturmiunum	CBS 124279	Flower, apple-Denmark
Stemphylium lycopersici	CBS 122639	Diseased leaves of tomato-China
Ulocladium botrytis	CECT 20564	Valencia, Spain
Alternaria spp.	Alt 3.1; Alt 1.4; Alt 30 Alt 35; Alt tp13; Alt tp15; Alt tp18; Alt 05 Alt 06; Alt 09	Tomato-Spain

CECT: Spanish Type Culture Collection; CBS: Centralbureau voor Schimmelcultures; Alt: strains isolated and conserved at the Applied Mycology Unit Culture Collection of the University of Lleida (Spain).

Table 2

Table 2. Limit of detection of *A. alternata* in artificially contaminated tomato samples using the primers Alt4-Alt5 for the qPCR.

Inoculated conidia/g tomato	**Detected conidia /g tomato (without incubation)**	**Detected conidia /g tomato (after 20 h incubation at 26 ºC)**
10^5	$4.4x10^5 \pm 3.1x10^5$	$3.7x10^6 \pm 3.2x10^6$
10^4	$6.4x10^4 \pm 1.6x10^4$	$2.4x10^6 \pm 1.8x10^6$
10^3	$7.1x10^3 \pm 1.1x10^3$	$5.6x10^4 \pm 6.5x10^4$
10^2	$6.6x10^2 \pm 3.4x10^2$	$1.5x10^4 \pm 1.6x10^3$
10	-	-
0	-	-

Figure 1
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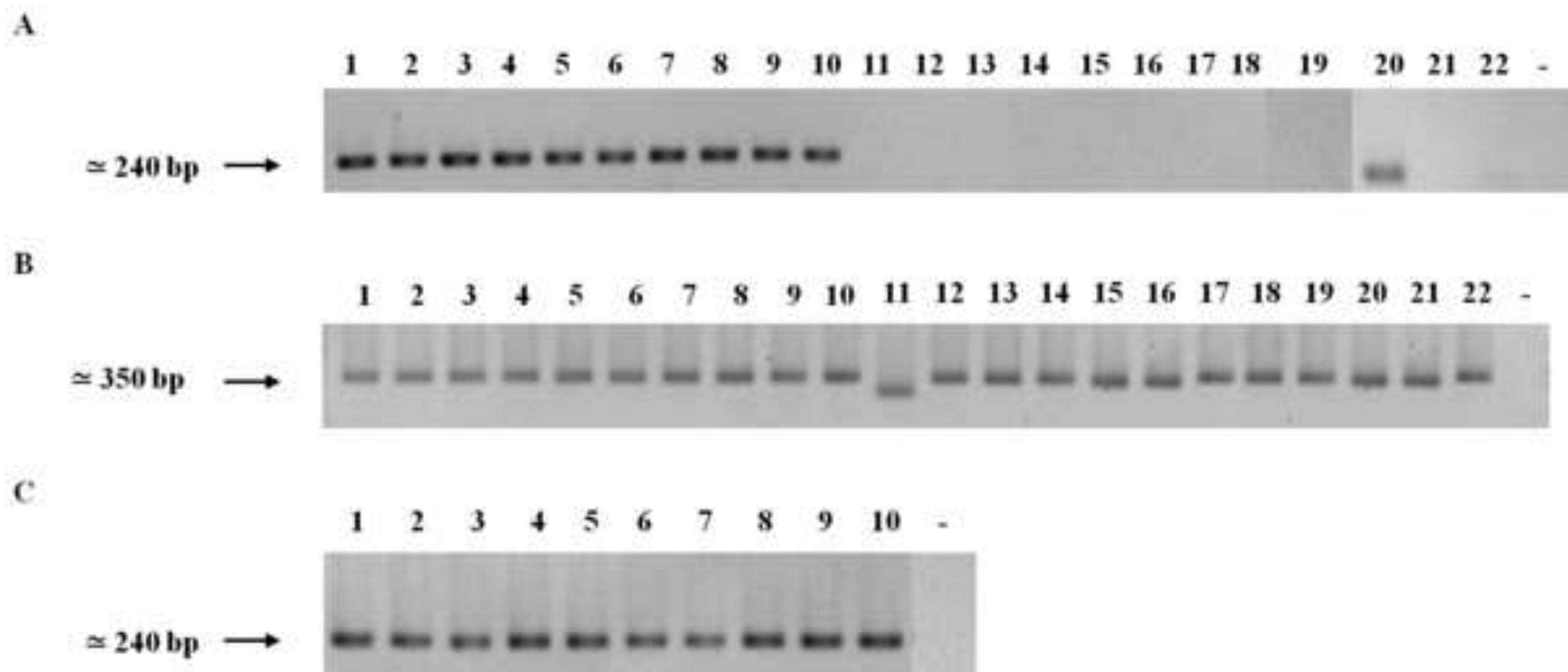

Figure 2
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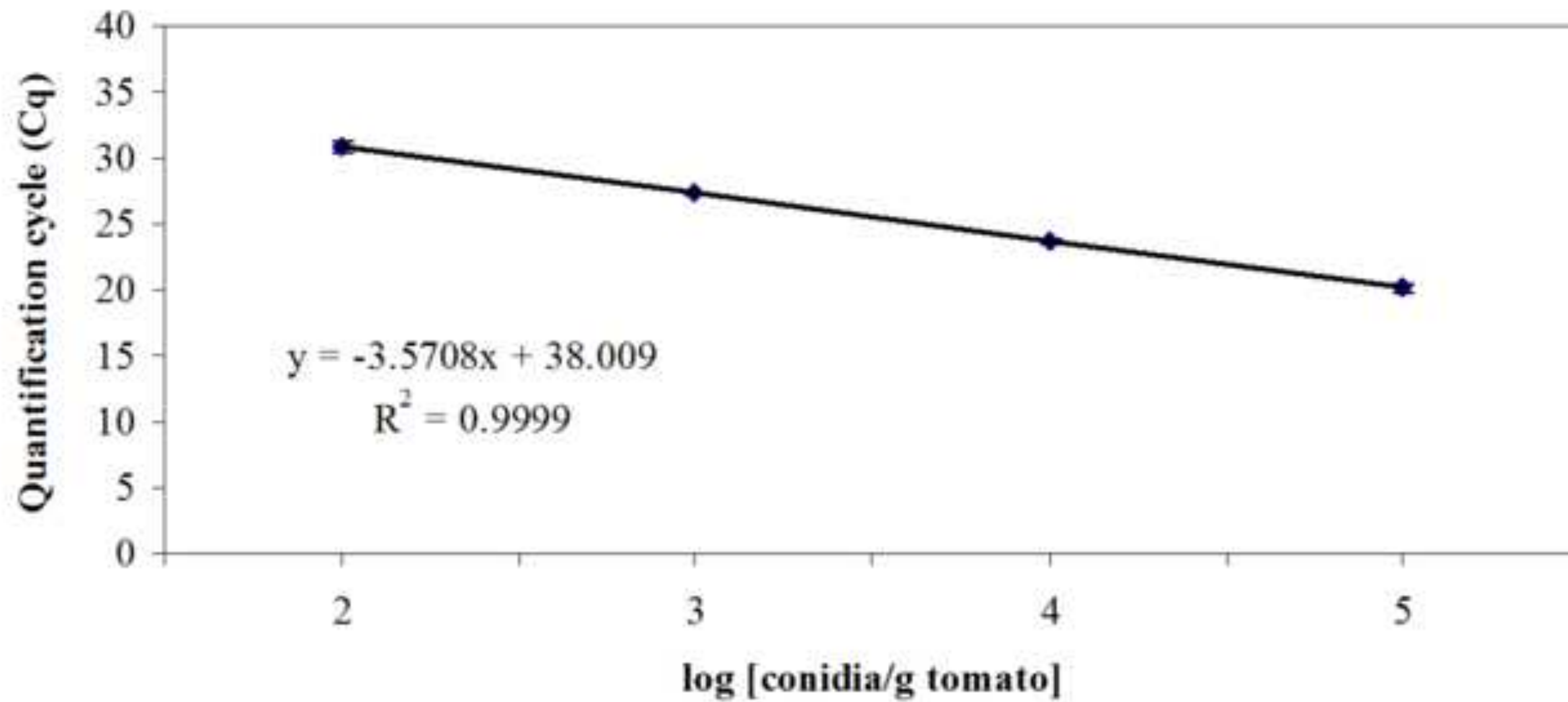

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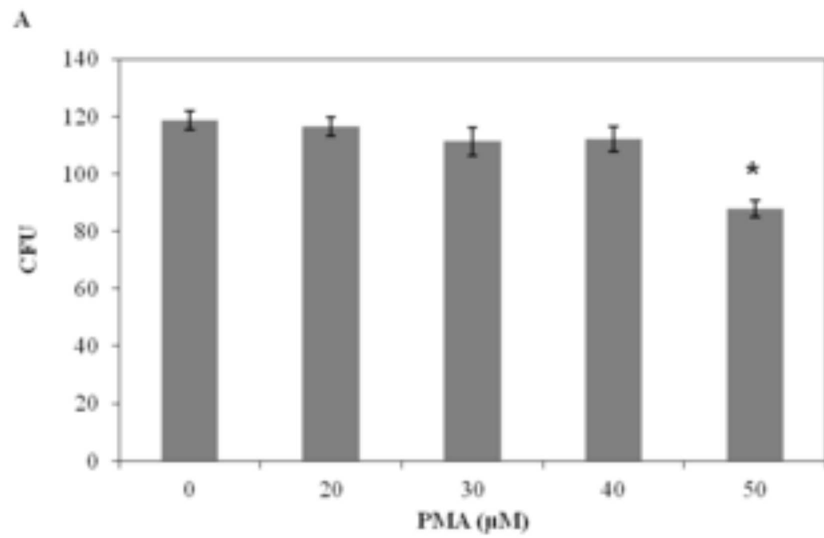

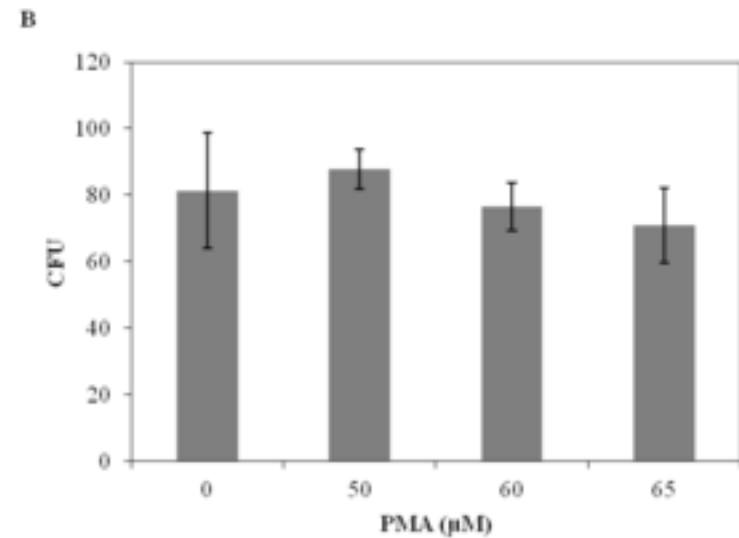

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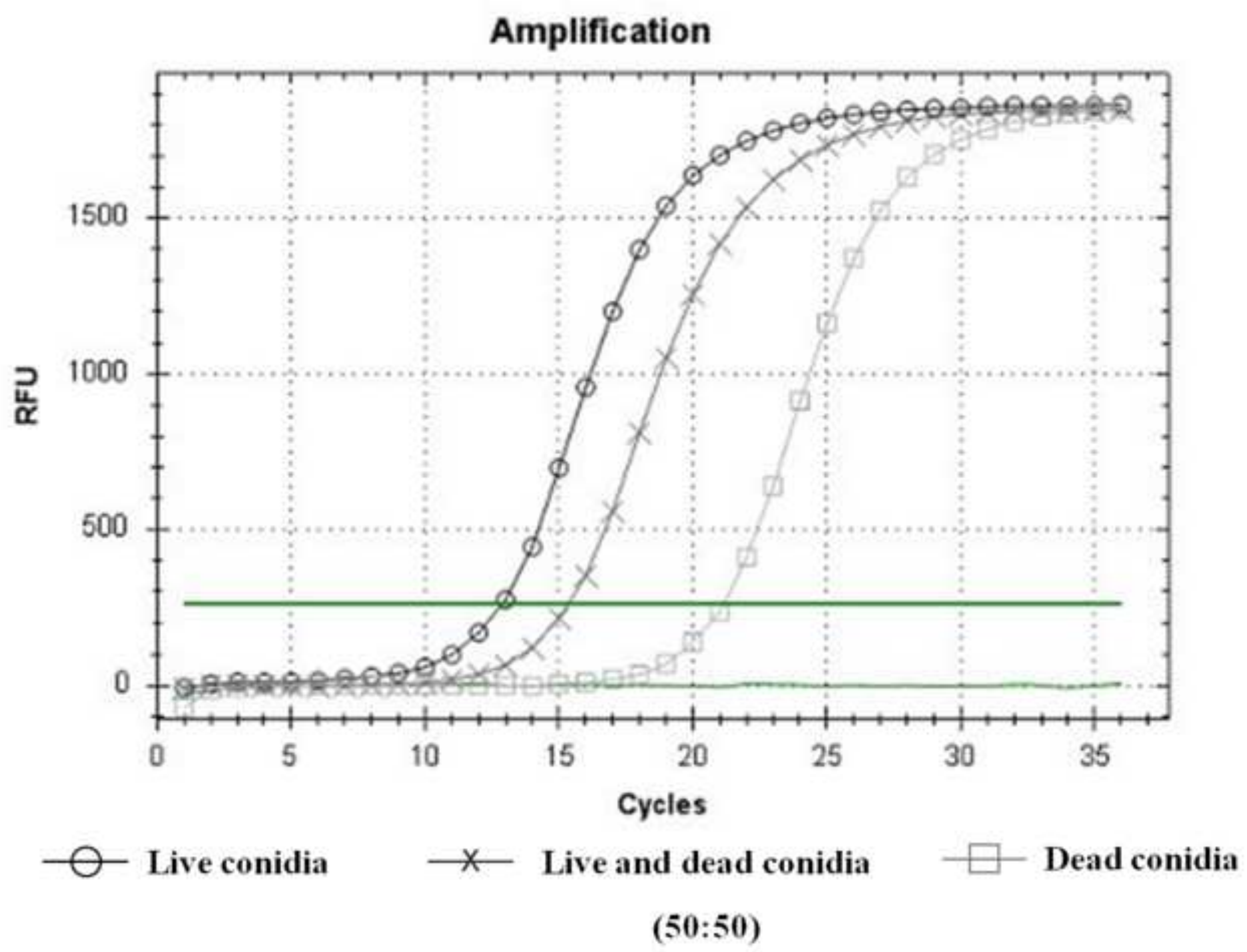

Figure 5

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