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Rapid diagnosis of *Fusarium* contamination in cereals using group-specific loop-mediated isothermal amplification (LAMP) assays

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ABBREVIATIONS

BBA	Bundesforschungsinstitut für Kulturpflanzen
BFE	Bundesforschungsanstalt für Ernährung
BIP	Backward inner primer
BLAST	Basic local alignment search tool
bp	Base pairs
CBS	Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands
d	Day/ days
DG-18	Dichloran 18% glycerol agar
DNA	Desoxyribonucleic acid
DSM	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E.	*Escherichia*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assays
EU	European Union
F.	*Fusarium*
FHB	*Fusarium* head blight
FIP	Forward inner primer
FSA	Food Standards Agency
g	G-force, gram
GC	Gas chromatography
hyd5	Hydrophobine 5
h	Hour
ha	Hectares
HPLC	High Performance Liquid Chromatography
IBT	Technical University of Denmark, Department of Biotechnology, Lyngby, Denmark
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITEM	Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy
kbp	Kilo base pairs
kg	Kilogram
l	Liter
LAMP	Loop-mediated isothermal amplification
LB	Lysogeny broth
LC	Liquid chromatography
LWL	Strains obtained from the collection of M. de Nijs, Wageningen University and Research, Wageningen, The Netherlands

m	Milli (10^{-3})
M	Molar (mol l^{-1})
M	Strain obtained from the collection of H.M.L.J Joosten, Nestlé Research Centre, Lausanne, Switzerland
MB	Mung bean agar
MBP	Maltose binding protein
MEA	Malt extract agar
min	Minute
MOPS	3-Morpholinopropanesulfonic acid
MRC	South African Medical Research Council, Tygerberg, South Africa
MW	Molecular weight
NCBI	National center for Biotechnology Information
NRRL	Northern Regional Research Laboratory
OD	Optical density
p.a.	Pro analysis
PCNB	Pentachloronitrobenzene
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEG	Polyethylene glycol
ppb	Parts per billion
ppt	Parts per trillion
s	Second
SNA	Synthetischer Nährstoffarmer Agar
spp.	Species (plural)
SYBR	Fluorogenic dye for nucleic acids (N',N'-dimethyl-N-[4-[(E)-(3-methyl-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine)
TE	Tris EDTA
TAE	Tris acetat EDTA
TLC	Thin Layer Chromatography
T_m	Melting temperatures
TMW	Technische Mikrobiologie Weihenstephan
Tris	Tris (hydroxymethyl) aminomethan
T_t	Time to threshold
UV	Ultra violet
v	Volume

v/v	Volume per volume
VLB	Versuchs- und Lehranstalt für Brauerei, Berlin, Germany
w	Weight
w/v	Weight per volume
YNB	Yeast nitrogen base
YPD	Yeast peptone dextrose
ZON	Zearalenone
μ	Micro

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1 Introduction

1.1 Habitat aspects of the genus *Fusarium* (*F.*) with special emphasis on *Fusarium* spp. in cereals

Fusarium spp. belong to a widespread group of fungi which can be found almost everywhere in the world (Nelson et al., 1983). They colonize aerial and subterranean plant parts as primary or secondary invaders (Nelson et al., 1983). According to Nelson et al. (1994) they are common in tropical and temperate regions but can also be found in desert, alpine and even in arctic environments. This widespread distribution of *Fusarium* spp. can be attributed to the ability of species to grow on divers substrates and to their efficient mechanisms for propagation and dispersal (Nelson et al., 1994). Besides colonizing plant parts, exist some common human pathogens among *Fusarium* spp. e.g. *F. solani*, *F. oxysporum*, *F. verticillioides*, *F. moniliforme* and other species. (Nucci and Anaissie, 2007). As fungal pathogens they may lead to invasive *Fusarium* infections in lung transplant patients (Carneiro et al., 2011). Other species such as *F. sacchari* are reported as a cause of exogenous fungal endophthalmitis (Chander et al., 2011). As a plant pathogen, the same species causes diseases in various plants e. g. in water melon (Martyn et al., 2012), in maize (Ostry et al., 2010), on cucumber (Qiu et al., 2012) and on many more. Especially in relation with cereals, *Fusarium* is very well-known and common. The infestation as superficial contamination and the infection of *Fusarium* in cereals are of great economic concern worldwide (Zhu et al., 1999). *Fusarium* is known as a plant pathogen as well as a producer of mycotoxins (Bernhoft et al., 2012). *Fusarium* head blight (FHB) of small grain cereals and ear rot in maize are significant diseases across the world (Parry et al., 1995). The *Fusarium* species predominantly found all over Europe and associated with FHB in wheat and other small-grain cereals are *F. graminearum, F. avenaceum* and *F. culmorum* (Bottalico and Perrone, 2002). Among the less frequently encountered species are several ones, which are less pathogenic, but also toxigenic. They include *F. poae, F. cerealis, F. equiseti, F. sporotrichioides, F. tricinctum* and, to a lesser extent, *F. acuminatum, F. subglutinans, F. solani, F. oxysporum, F. verticillioides, F. semitectum* and *F. proliferatum* (Bottalico and Perrone, 2002). *F. graminearum* and *F. culmorum* are the most important FHB pathogens of wheat because they are more pathogenic than other *Fusarium* species and produce higher amounts of mycotoxins, especially Deoxynivalenol (DON) (Edwards, 2004). The primary

inoculum for *Fusarium* infections is crop debris left on the soil surface after harvest. The most important factor is the climate prevailing during the vegetation period and especially during anthesis (Miller, 2008; Placinta et al., 1999). At less favorable weather conditions at grain harvest, cereals may become unsuited for animal fodder, especially for pigs which are particularly sensitive (Bernhoft et al., 2012). Less favorable conditions are imposed by dry weather in late spring, which promotes sporulation, and conidia are dispersed by rainfall. Under warm, wet conditions, small-grain cereals are susceptible to head infection, which means from head emergence onwards, particularly during flowering. Hooker et al. (2002) showed the importance of weather conditions during flowering by developing a model that accounted for 73 % of the variation in the concentration of DON based on weather conditions during this period. The symptoms of FHB can vary. Typical symptoms for *F. graminearum* and *F. culmorum* infections are spikelets, which show premature senescence. If also the rachis is infected, the complete head above the point of infection starts to senesce prematurely (Edwards, 2004). Grains harvested from FHB-infected spikelets are usually shriveled and are either chalky white or pink in color (tombstone grains). Grains produced in spikelets above the point of infection are usually shriveled but are not necessarily infected (Edwards, 2004). Infections result not only in reduced yield as a result of shrunken grains but also in reduced milling and malting quality and in contamination of grains with mycotoxins such as deoxynivalenol, nivalenol, T2-toxin and zearalenone (Edwards, 2004). The problem of infection by FHB has increased over time, and trends like more humid growing seasons, soil compaction through heavier machinery, specialized cash cropping and reduced soil tillage combined with herbicide spraying may contribute to explain this increase (Bernhoft et al., 2012).

1.2 Quality reduction and posed hazard of *Fusarium* in cereals

The plant disease FHB or scab of wheat and barley caused by *Fusarium* spp. results in significant economic losses by reducing seed yield and quality. In some years the disease has reached epidemic proportions in many countries of the world (Zhu et al., 1999). The estimated direct losses due to reduced yield and quality from FHB between 1991 and 1997 in the US alone were more than $1.3 billion (Johnson et al., 2003). Furthermore, infested seeds are regularly contaminated with trichothecene type mycotoxins and the estrogen analogue zearalenone (ZON) which poses a dangerous threat to animal health and food safety (Nelson et al., 1994). The existence of certain *Fusarium* species

influences the baking and malting quality of grains as well as it increases the risk of beer gushing (Lutterschmid et al., 2011).

1.2.1 Gushing

Gushing of beer displays a situation in which bottled beer foams heavily immediately upon opening of an unagitated bottle (Gjertsen, 1967; Hippeli and Elstner, 2002). The research group at Carlsberg laboratories (Gjertsen, 1967; Gjertsen et al., 1963) proposed to sub-divide gushing into two different types, e.g. "primary gushing" which occurs regularly and seems to be related to the quality of malt, and "secondary gushing" which is due to failure during beer production or due to incorrect treatment of packaged beer by the consumer (Hippeli and Elstner, 2002). Influences, which may lead to secondary gushing are diverse but are well understood and problems can be solved by technological means. Thus, research in the last 30 years has been mostly focused on primary gushing. Diverse efforts have been made which aimed at analyzing and predicting the potential of malted and unmalted brewing cereals to induce beer gushing. Several tests for gushing have been described in the past. The modified Carlsberg test (MCT) is a test, which is based on foaming activity of cereal and malt extracts. As described in Bamforth et al. (2008), the MCT is a standard method in the brewing industry to predict the potential of malted and unmalted brewing cereals to induce beer gushing. In the MCT, artificially carbonated water is used instead of beer. An extract of the malt grist is processed with cold water and then thermally concentrated. Fifty ml of the concentrated extract is added to the carbonated mineral water and agitated for 3 days. After opening of the bottles, gushing potential of a sample is ranked as follows: no gushing (loss of 0–5 g), possible gushing (loss of 5–50 g), and indicative of gushing in beer (loss of >50 g). The MCT was accepted by the "Mitteleuropäische Brautechnische Analysenkommission" (MEBAK) as a predictive test for malt induced gushing (MEBAK, 2006). However, in the experience of brewers the significance of the MCT is very low, as the risk of false negative results tends to be high (Rath, 2009). Beside the MCT gushing test, exists the Donhauser test, visual characteristics of grains, particle size distribution combined with surface charge titration, antibody reactions or MALDI TOF analysis of beer proteins are alternatives for forecasting the gushing inducing potential of malt (Christian et al., 2011; Deckers et al., 2011; Shokribousjein et al., 2011).

1.2.2 Hydrophobin gene *hyd5*

Characterization and detection of so-called “gushing factors” which are produced by fungi were described by Haikara et al. (2004) and Kleemola et al. (2001). Certain structural characteristics of these proteins are known currently. Hippeli and Elstner (2002) were the first to speculate about possible relations between the physico-chemical properties of hydrophobins and gushing. Sarlin et al. (2005) isolated and partially sequenced hydrophobins which were isolated from cultures of *Fusarium* and *Nigrospora* spp. They were able to induce gushing after addition of small amounts of purified native proteins into bottled beer. Hydrophobins of class I and class II are small fungal proteins which play an important role in hyphal growth and development of filamentous fungi. They are involved in spore formation, the formation of aerial structures or the attachment of hyphae to hydrophobic surfaces (Wessels et al., 1991). To test their effect on beer, Zapf et al. (2006) cloned different hydrophobin genes into brewing yeast and proved that only the clone expressing the gene for the class II hydrophobin FcHyd5p from *F. culmorum* resulted in a heavily gushing beer. Based on this research, Stübner et al. (2010) cloned the *fchyd5* gene in *Pichia pastoris* to produce the FcHyd5p protein for further characterization of the mechanism which leads to gushing in beer. Lateron, Sarlin et al. (2011) cloned the same gene in *Trichoderma reesei* and confirmed its status as a major factor in beer gushing. Minenko et al. (2014) recently showed that hydrophobins may have a major role in conveying surface hydrophobicity to the aerial mycelium in *F. graminearum*. Expression studies support the evidence that the class II hydrophobin FgHyd5p is produced during contact with hydrophobic surfaces (Minenko et al., 2014). Therefore is the FgHyd5p maybe an important factor during colonization of cereal by *F. graminearum*. Consequently, testing for the presence of genes coding for Hyd5p proteins may be appropriate as a marker for the presence of the class II hydrophobin and hence for the gushing potential of cereals and malt. Homologs of the *hyd5* gene have been described in *F. poae, F. graminearum, F. culmorum* and in *F. verticillioides*. Despite the fact that this gene is more related to *Fusarium* spp., homologs can also be found in the split gill fungus *Schizophyllum commune* and in other fungi (Sarlin et al., 2005). In the temperate part of Europe in addition to the cereal growing areas of the American continent, *F. graminearum* and, to a lesser extent, *F. culmorum* are the most often isolated *Fusarium* spp. from samples of cereals and malt from which gushing beer was produced. Also *F. poae* was isolated from samples of gushing malt but is more important in northern European countries.

1.2.3 Mycotoxins

A natural toxin can be defined as a substance synthesized by a plant, an animal, or by a microorganism, which is harmful to another organism (Turner et al., 2009). Mycotoxins are defined as toxic secondary metabolites produced by filamentous fungi, which are toxic to higher vertebrates after administration of small doses via natural routes of intake (modified after Bennett, 1987). They are most regularly produced by saprophytic and parasitic molds growing on a variety of foodstuffs including animal feeds (Turner et al., 2009). Within the genera *Fusarium, Aspergillus, Penicillium* and *Alternaria* many species are not only recognized as plant pathogens but are also known as sources of important mycotoxins (Abramson, 1997; Panigrahi, 1997; Placinta et al., 1999; Smith, 1997). Typically, mycotoxigenic fungi are separated into two groups: ”field” (or plant pathogenic) and ”storage” (or saprophytic) fungi (Placinta et al., 1999). For example, *Aspergillus flavus* is associated with Aspergillus ear rot and kernel rot of maize (Campbell and White, 1995). When conditions such as temperature and moisture are optimal for their growth they also colonize stored agricultural commodities (Smith, 1997). *Fusarium* mycotoxins such like the fumonisins are widely distributed, some with carcinogenic properties (Schroeders et al., 1994) and some with well-defined toxicology for farm livestock (Placinta et al., 1999). Most of the infested seeds are contaminated with trichothecene type mycotoxins and with the estrogen analogous zearalenone. They both pose a serious threat to the health of animals and to food safety (Nelson et al., 1994).

1.2.4 Trichothecenes

Trichothecenes are a large family of chemically related mycotoxins. They are produced by several fungal species of the genus *Fusarium* and related genera. Their mechanism of action involves inhibition of the eukaryotic protein biosynthesis and thereby the impairment of human and animal health (Desjardins et al., 1993). The contamination of foods and feeds is a permanent worldwide problem and the most effective way to control trichothecene mycotoxins is prevention of fungal infection and toxin production in the field and during storage (Desjardins et al., 1993). Hohn et al. (1993) demonstrated that the biosynthesis of trichothecenes is organized by a gencluster. Some genes of cluster have been analyzed for *F. sporotrichioides* by Hohn et al. (1993). The general shematic organization of the trichothecene gene cluster in *Fusarium* is demonstrated in figure 1 (Ward et al., 2002)

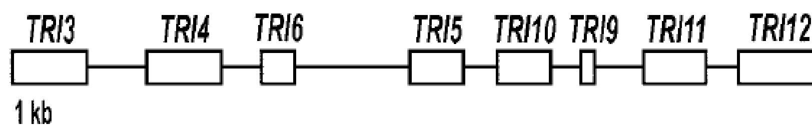


Figure 1: Shematic organization of the trichothecene gene cluster in *Fusarium* (Ward et al., 2002).

The function of each gene can be described as follows: *tri3* expresses the 15-O-acetyltransferase, *tri4* and *tri11* both code for P450 oxygenases, *tri5* encodes a trichodiene synthase, *tri6* expresses a zinc-finger containing protein which is responsible for gene activation, *tri10* is a regulatory gene, *tri12* encodes a trichothecene efflux pump protein and *tri9* expresses a protein of unknown function (Ward et al., 2002). The biosynthesis of trichothecenes proceeds from trichodiene as a ubiquitous precursor molecule which was first isolated from *Trichothecium roseum* by Machida and Nozoe (1972). All trichothecenes share the same basic structure as shown in figure 2. The epoxide moiety at positions C-12 and C-13 has been described as causative for the toxicity of trichothecenes. The chemical structures of individual members of the trichothecene family of mycotoxins can vary due to hydroxylations and esterifications ad different positions of the heterocyclic system (Desjardins et al., 1993).

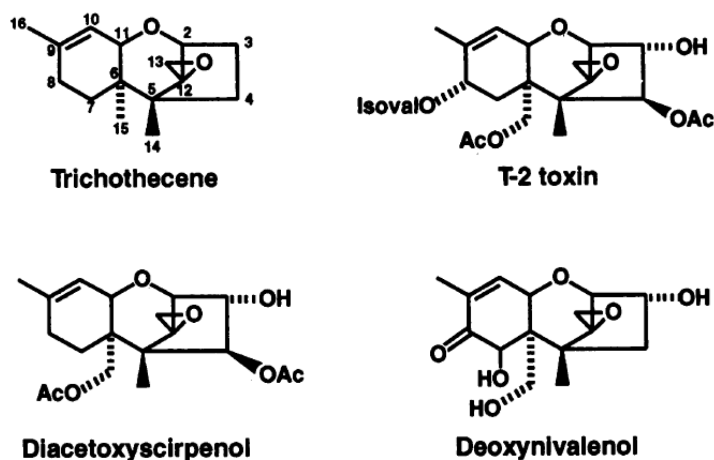


Figure 2: Structure of trichothecene, T-2 toxin, diacetoxyscirpenol and deoxynivalenol (Desjardins et al., 1993).

According to their chemical structures, more than 60 trichothecenes known to date can be divided in four subgroups A-D. Types A and B represents the most important groups of compounds: type A trichothecenes include compounds such as T-2 toxin, HT-2 toxin, neosolaniol (NEO) and diacetoxyscirpenol (DAS). The most widespread compounds in

the type B trichothecenes are deoxynivalenol (DON, vomitoxin) and its 3-acetyl- and 15-acetyl derivatives (3-ADON and 15-ADON, respectively), nivalenol (NIV) and fusarenon X (Placinta et al., 1999). The type A trichothecene production is basically found in species such as *F. acuminatum, F. equiseti, F. sambucinum, F. langsethiae, F. sporotrichioides, F. venenatum* and, in some rarer cases, in *F. poae* (Samson et al., 2010). Type B trichothecene production occurs in species such as *F. cerealis, F. culmorum, F. equiseti*, *F. graminearum* and *F. poae* (Samson et al., 2010). DON and NIV have been recognized as major food-contaminating mycotoxins. For DON, a regulatory maximum level has been established in a number of countries, including the European Union (van Egmond et al., 2007). Type B trichothecenes show a lower level of toxicity as compared to type A trichothecenes such as T-2 and HT-2 toxins, but physiological disorders observed in livestock are mostly caused by the type B group (Girish and Smith, 2008; Suzuki and Iwahashi, 2012). Furthermore, DON and NIV have a high stability during processing and storage which leads to their wide distribution in food and feed (Widestrand and Pettersson, 2001). The most commonly detected trichothecene in cereal grains in temperate regions is DON and it is commonly found at higher concentrations than NIV (Canady et al., 2001). The effect of DON on mammals was analyzed in experimental animal models. Acute DON poisoning causes emesis, whereas chronic low-dose exposure leads to growth retardation, elicits anorexia, impairs reproduction, immunotoxicity and fetal development (Pestka, 2010).

1.3 Detection of molds and mycotoxins in cereals

Most mycotoxins are chemically stable and tend to survive storage and processing even at quite high temperatures such as those prevailing during baking bread (Turner et al., 2009). Mycotoxins are notoriously difficult to remove and the best method of control is prevention. The following is a description of different techniques for the detection of molds and mycotoxins in samples.

1.3.1 Chromatographic methods for the detection of mycotoxins

Most of the chromatographic protocols published in connection with fungi were set up for the analysis of secondary metabolites, especially mycotoxins (Kamimura et al., 1981). Chromatography is the collective term for a set of laboratory techniques used for the separation of mixtures of different compounds (Turner et al., 2009). Column chromatography, planar chromatography (e.g. thin layer chromatography (TLC)), gas

chromatography (GC) and high performance liquid chromatography (HPLC) are currently the most widely used techniques. Traditionally, the most popular method used for mycotoxin analysis is TLC, which offers the ability to screen large numbers of samples economically. The use of TLC analysis for mycotoxins is furthermore popular for both quantitative and semi-quantitative purposes (Turner et al., 2009). TLC has been reported for example to be used to assess mold spoilage of *Castanea sativa* (chestnut) commercially sold in Canadian grocery stores (Overy et al., 2003). Analysis of *Fusarium* toxins in maize and wheat using TLC was done by Schaafsma et al. (1998). GC is regularly used to identify and quantify the presence of mycotoxins in food samples in order to detect volatile products (Turner et al., 2009). But most mycotoxins are not volatile and have to be derivatized for analysis using GC (Scott and Lawrence, 1995). Several techniques have been developed for the derivatization of mycotoxins. Chemical reactions such as silylation or polyfluoroacylation are employed in order to obtain volatile forms of material such like fumonisins B1 and B2 (Scott and Lawrence, 1995). HPLC has in the majority of cases taken over the role of TLC as the most important lab technology for mycotoxin analysis. In most cases it has been used in combination with other analytical tools (Turner et al., 2009). Modern analysis of mycotoxins relies heavily on HPLC employing various adsorbents depending on the physical and chemical structure of the mycotoxin (Turner et al., 2009). A number of toxins have natural fluorescence already (e.g. ochratoxin, aflatoxin, citrinin, zearalonone) and can be detected directly after HPLC separation with fluorescence detection (HPLC–FD) (Valenta, 1998). A HPLC–FD has also been used for simultaneous detection of *Fusarium* mycotoxins (ZON, NIV and DON) in cereals after derivatization (Tanaka et al., 1985). In HPLC-MS separated toxins are identified/verified by setting up a mass spectrum for each detected peak and analyzing it for the presence of a typical distribution of fragment masses. In general, physicochemical methods used for analysis of mycotoxins and their derivatives, ranging from TLC (Miller et al., 1983b) to GC (Kamimura et al., 1981), require extensive and time-consuming sample cleanup prior to analysis. Furthermore, the efforts necessary for sufficient sample preparation increase greatly with increasing requirements for specificity and sensitivity of analysis (Usleber et al., 1991).

1.3.2 ELISA assays for detection of mycotoxins

Other separation and detection techniques exist which run alongside or in place of chromatographic methods. Among these, immunochemical methods such as direct

enzyme-linked immunosorbent assays (ELISA) have become very popular due to their relatively low cost and easy application (Morgan, 1989). Commercially available ELISA kits for detection of mycotoxins are normally based on a competitive assay format that uses either a primary antibody specific for the target molecule or a conjugate of an enzyme and the required target (Morgan, 1989; Turner et al., 2009). ELISA have been established for the determination of selected trichothecenes, (Krska et al., 2001). Some ELISA were developed for one specific trichothecene, e. g. DON, 3AcDON. Others were developed for the simultaneous detection of trichothecenes in a group specific manner (Pronyk et al., 2006; Usleber et al., 1991). Not only mycotoxins can be detected but also estimation of fungal biomass during solid-state fermentation is possible (Dubey et al., 1998). The advantage of ELISA in comparison to other methods for the determination of a mycotoxin is that a cleanup and/or extraction may not be required or can be kept on a simple level (Turner et al., 2009). Sample preparation is the main time factor in analysis since it takes approximately two-thirds of the total analysis time will therefore affect the final choice of the detection procedure (Turner et al., 2009).

1.3.3 Microbiological methods

Over a long period of time molds were identified with the help of selective media. The fact that on different media the fungi show different colony forms, colors, growth or non-growth, could help to identify the fungi (Nirenberg, 1976). Many *Fusarium* species have their natural habitat on plants such as cucurbits, peas, beans, soybeans, sweet potatoes, batatas, potatoes, faba beans, barley, etc. (Smith, 2007). Complex media for growth provide the fungi with the original set of nutrients they have available when growing on plants. For many *Fusarium* species, media such as mung bean agar (MB) (Evans et al., 2000), oatmeal agar (OA) (Samson et al., 2010), potato dextrose agar (PDA) (Gams et al., 1987) or malt extract agar (MEA) are frequently used for cultivation. Furthermore, selective media exist which are ideally for the direct isolation of *Fusarium* species from plants (Nelson et al., 1983). The media are used, because they are adjusted to the needs of some *Fusarium* species or they select by inhibition of other fungi. selective Fusarium agar (SFA) (Burgess and Liddell, 1983) is an example for a selective medium frequently used. In combination of media, temperature, and light, Böhm-Schraml (1995) triggered the selective pressure by using mannit-PCNB-agar to select *F. graminearum* and mannit-malachite green-agar to select *F. culmorum.* These plates were incubated in the dark at 30 °C to complete the selective conditions for the

respective species. Although the selective action of the media used is a great advantage, it remains necessary to inspect cultures with micro morphological methods which demands for considerable knowledge of the fungal morphology and taxonomy to apply microbiological methods of identification properly.

1.3.4 Gene specific molecular detection methods

The rapid identification of mycotoxigenic fungi is desirable and early intervention steps could be applied to help limit the amounts of contaminated materials, particularly cereals and cereal-based products, gaining access to the human food chain (Edwards et al., 2002). For this a number of PCR-based methods has been developed for identification of mycotoxin biosynthetic genes in different fungal genera, together with assays developed using other genes or random amplification of polymorphic DNA (RAPD) methods for the identification of specific toxigenic fungi (Edwards et al., 2002).

During recent years various attempts have been made to use DNA-based detection methods such as PCR, competitive PCR and real-time quantitative PCR methodologies to develop rapid diagnostic assays for *Fusarium* and other mycotoxin producing fungal species see Niessen (2008) for a review. For the identification of DON and NIV producing *F. graminearum,* Lee et al. (2001) developed a PCR for differentiating two chemotypes of *F. graminearum.* In a similar way, Bakan et al. (2002) developed a PCR for the detection of *F. culmorum* strains producing large and small amounts of DON. Edwards et al. (2001) developed a competitive PCR assay to quantify trichothecene-producing *Fusarium* based on primers derived from the trichodiene synthase gene (*tri5*). In competitive PCR, first described by Becker-André and Hahlbrock (1989), a known amount of a DNA fragment (competitor) is added to the sample. Schnerr et al. (2001) developed a real-time quantitative PCR based on PCR primers specific to the *tri5* gene, a quantitative group specific assay for *Fusarium* species producing trichothecenes. Besides PCR amplification, other techniques exist which detect genes specifically with primer-based probes. Due to the immense taxonomical diversity in fungi, true group- and species-specific detection can be difficult to achieve through selective primer-based PCR amplification alone (Wu et al., 2003). Specific oligonucleotide probes for the detection of fungal isolates can provide better specificity and sensitivity for environmental sample screening (Wu et al., 2003). Therefore, Wu et al. (2003) developed oligonucleotide probes for the detection of common airborne fungi at the genus and species levels by designing oligonucleotide probes based on the 18S rDNA

sequence variation. Another patented system has been published by Haugland and Vesper (2002). It describes specific detection of *Aspergillus nomius* based on sequences from the ITS region of the rRNA gene and was described together with a fluorescently labeled probe to be applied in a TaqMan™ quantitative real-time PCR assay (Haugland and Vesper, 2002). In general, the disadvantage of these systems are the cost of the specialized equipment that is capable of real-time fluorescent measurement of PCR reactions compared with other analysis systems. However, the need for dedicated lab equipment, time consumption and well-trained staff makes classical gene-based analysis unsuitable for rapid testing and especially for on-site testing applications.

1.3.4.1 Loop-mediated isothermal amplification (LAMP)

Notomi et al. (2000) developed a method they termed loop-mediated isothermal amplification (LAMP) of DNA. The system leads to primer initiated enzymatic amplification of DNA with high specificity, efficiency and rapidity under isothermal conditions. Since its first mention, the method has been the focus of extensive research efforts (Mori et al., 2013). The target DNA is amplified by the *Bst* DNA polymerase, a thermophilic DNA polymerase isolated from the spore-forming soil bacterium *Geobacillus (G.) stearothermophilus* (formerly *Bacillus stearothermophilus*), which shows optimum growth at temperatures around 60-65 °C. There are three different types of *Bst* polymerase available: *Bst* DNA large fragment polymerase, *Bst* 2.0 DNA polymerase, and *Bst* 2.0 WarmStart™ DNA polymerase. The large fragment of the *Bst* DNA polymerase contains the 5′–3′ polymerase activity but lacks 5′–3′ exonuclease activity. The enzyme is produced by an *E. coli* strain containing a genetic fusion of the *Bst* DNA polymerase large fragment and the maltose binding protein (MBP) of *E. coli*. MBP is used for affinity purification and is removed by cleavage of the fused proteins (Kong et al., New England Biolabs, unpublished data). *Bst* 2.0 DNA polymerase is an *in silico* designed homologue of *G. stearothermophilus* DNA polymerase I, large fragment (*Bst* DNA polymerase, large fragment). *Bst* 2.0 DNA Polymerase displays improved amplification speed, yield, salt tolerance and thermostability compared to wild-type *Bst* DNA polymerase. *Bst* 2.0 WarmStart™ DNA polymerase is also an *in silico* designed homologue of the *G. stearothermophilus* DNA polymerase I. As a modification, it has a reversibly-bound aptamer (Eaton, 1997) which inhibits polymerase activity at temperatures below 45 °C. The aptamer rapidly releases the *Bst* 2.0 WarmStart™ DNA polymerase as temperatures rise above 45 °C and therefore no special activation step is needed to activate this type

of polymerase. Identical for each of the different types of *Bst* DNA polymerase is the mode of operation when used in LAMP reactions. For the LAMP reaction shown in figure 3, besides DNA polymerase four specifically designed primers are necessary to recognize a total of six binding sites within the target DNA sequence. The mechanism shown in figure 3 is described in detail in Notomi et al. (2000). The inner primers, designated as forward inner primer (FIP) and backward inner primer (BIP), contain sequences of the sense and antisense strands of the target DNA and initiate the LAMP reaction. FIP and BIP each contain two binding sites: FIP consists of F1c and the sequences (F2) complementary to F2c; BIP consist of (B1c) complementary to B1 and B2. Additionally two outer primers exist: B3 and (F3) complementary to F3c. Starting the LAMP reaction the F2 of FIP binds to the target DNA and the *Bst* DNA Polymerase starts with the DNA amplification. The F3 initiates the strand displacement by *Bst* DNA Polymerase. The F1c and the F1 of the third strand form a loop by self-priming. B2 of BIP bind to the new third strand and initiates DNA synthesis in the same way as FIP, which leads to single strand with two loops (dumbbell structure). For the LAMP cycling FIP and BIP hybridizes to the loops. The final products are a mixture of DNA strand with loops and different lengths. To accelerate the LAMP reaction, Nagamine (2002) developed a method by using loop primers. Loop primers hybridize to the loops and prime strand displacement DNA. Since *Bst* DNA polymerase is very productive, vast amounts of high molecular weight DNA are produced within short time. The remarkably high specificity of LAMP is due to the fact that a set of four to six primers must hybridize correctly to their target sequence before DNA biosynthesis occurs. Several LAMP assays have already been developed for the detection of *Fusarium* species, e.g. for the detection of *F. oxysporum* f. sp. *cubense* race 4 (Li et al., 2013)*, F. oysporum f. sp. lycopersici* (Almasi et al., 2013) or for the detection of *F. graminearum* (Niessen and Vogel, 2010). Additionally, LAMP assays exist which could detect a whole complex of species such like the *F. tricinctum* species complex (Niessen et al., 2012). Group specific LAMP assays have as yet only been published for parasites (Kuboki et al., 2003) and for ammonia-oxidizing bacteria (Aoi et al., 2006) To date, no group specific LAMP has been developed for *Fusarium* spp.

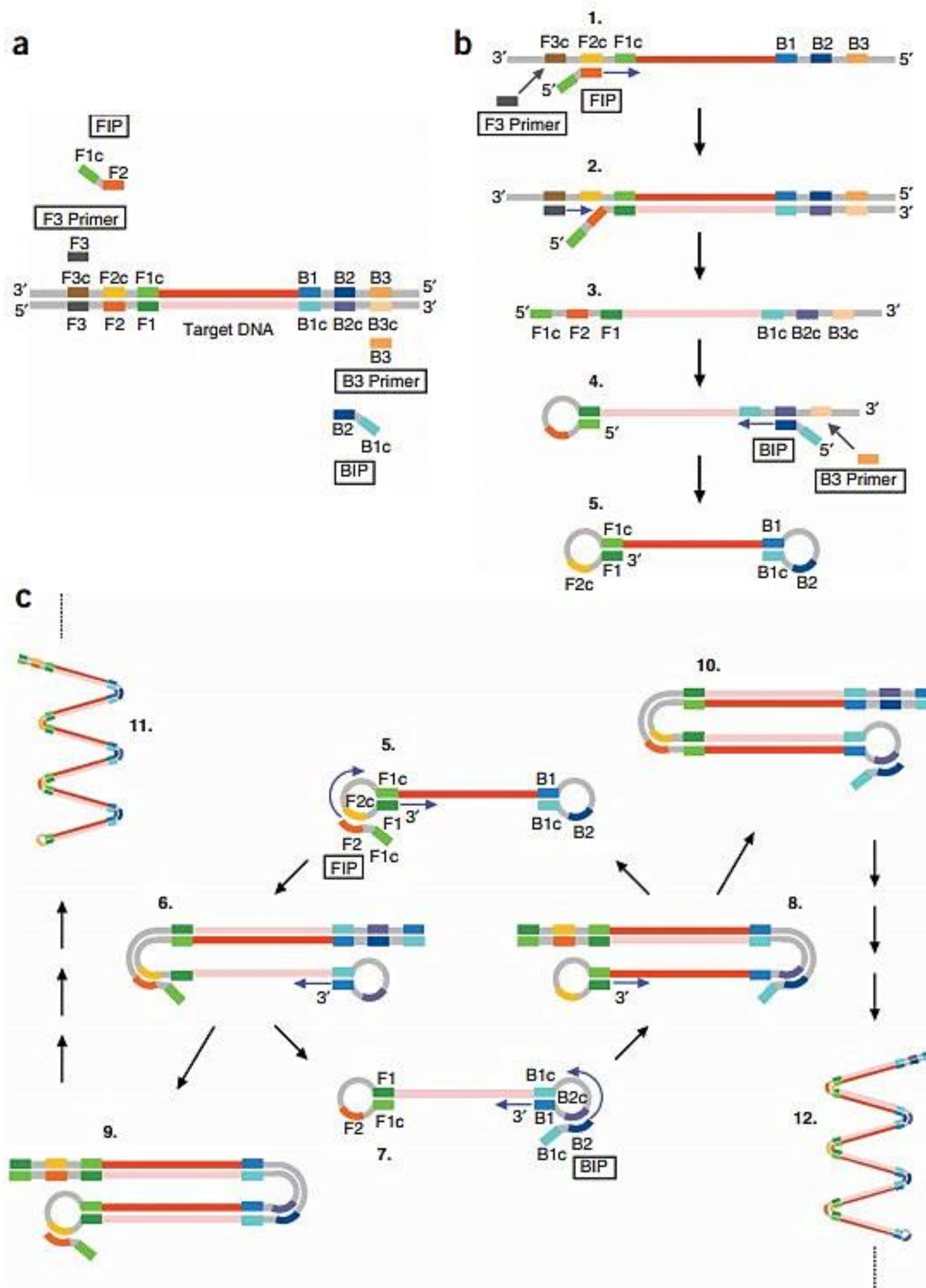


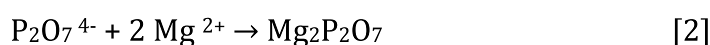
Figure 3: Schematic description of the LAMP reaction (Tomita et al., 2008).

1.3.4.2 Real time LAMP

Besides the qualitative detection of specific species by LAMP, also quantitative real-time detection is possible. The real-time measurement is possible because of the ability of the LAMP method to synthesize extremely large amounts of DNA and therefore a large

amount of by-product (Mori et al., 2001). During DNA polymerization by DNA polymerase [1], a pyrophosphate ion ($P_2O_7^{4-}$) is released from dNTP as a byproduct. When a large amount of pyrophosphate is produced, it reacts with the magnesium ion (Mg^{2+}) in the LAMP reaction buffer [2]. As by-product, magnesium pyrophosphate ions ($Mg_2P_2O_7$) are produced, which occurs as a white precipitate in the reaction mixture (Mori et al., 2001). The reaction equation is outlined below:

$$(DNA)_{n-1} + dNTP \rightarrow (DNA)_n + P_2O_7^{4-} \quad [1]$$



$$P_2O_7^{4-} + 2\,Mg^{2+} \rightarrow Mg_2P_2O_7 \quad [2]$$

(Mori et al., 2001)

The presence or absence of the white precipitate allows the distinction of whether or not nucleic acid was amplified by the LAMP method. Certain apparatus can measure an increase in the turbidity of the reaction mixture according to the production of precipitate (Mori et al., 2004). For this is the measurement of the turbidity of multiple samples simultaneously is done while maintaining constant temperature to conduct real-time measurements of the changes in the turbidity of LAMP reactions (Mori et al., 2004). The time (T_t) required for the turbidity of the LAMP reaction solution to exceed a given value is dependent on the quantity of the initial template DNA (Mori et al., 2004). Aoi et al. (2006) show as an example the application of real-time LAMP quantification of bacteria DNA. Beside turbidity measurement, the visual detection of amplification LAMP products by calcein (a fluorescent metal indicator) exists.

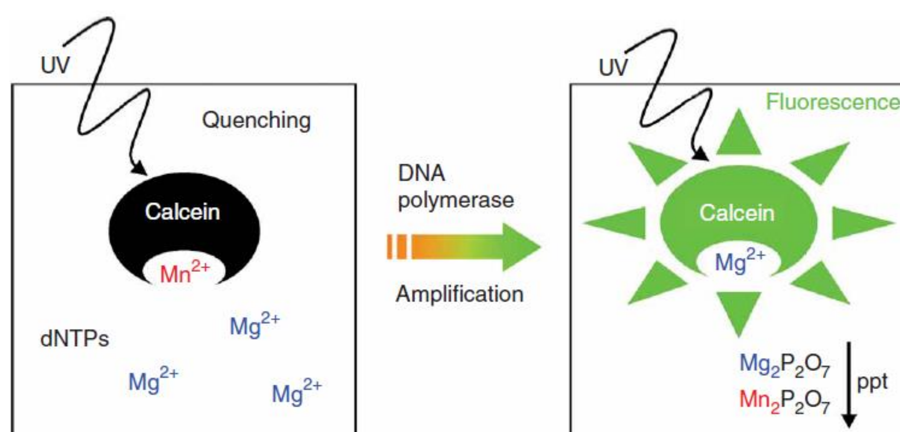


Figure 4: Principle of detection using a fluorescent metal indicator (calcein) (Tomita et al., 2008).

This reaction was first published by Tomita et al. (2008). The calcein in the reaction mixture initially is quenched with bivalent manganese ions (Mn^{2+}). When the amplification reaction proceeds, manganese ions will be deprived from the complex by pyrophosphate ions ($P_2O_7^{4-}$) generated during enzymatic in vitro DNA synthesis (Tomita et al., 2008). This de-complexation results in the emission of a bright green fluorescence by the calcein. Subsequently, the calcein combines with free magnesium ions (Mg^{2+}) present in the reaction mixture, which increases the fluorescence emission even more (Tomita et al., 2008).

The above-mentioned methods are based on indirect detection of amplification products. A direct detection is possible with DNA-stains such as V13-01184 (Dyomics GmbH, Germany). It has an absorption/emission maximum which can be detected by real-time fluorescence readers such as the Tube Scanner instrument (Denschlag et al., 2013). In Aoi et al. (2006) a similar system is applied with the same model of turbidimeter but a different type of real-time fluorescence reader and YO-PRO stain instead of V13-01184. A significant advantage of the DNA stain is the faster detection of target DNA in comparison to indirect detection methods such as calcein and turbidity measurement.

1.3.5 Objective

The current dissertation was based on the following thesis:

"Loop-mediated isothermal amplification based assays can be developed and applied as an effective and user-friendly diagnostic tool for the evaluation of mold-related quality parameters in brewing cereals and malt to improve inspection of incoming goods"

In order to reach the objectives, qualitative and semi-quantitative diagnostic assays were developed, which were based on the loop-mediated isothermal amplification (LAMP) technology, to apply these assays to the analysis of cereals and malt for the presence of fungi harboring a *hyd5* gene and genes from the trichothecene biosynthetic pathway in a group specific manner and to evaluate the correlations between assay results and quality parameters such as gushing potential and mycotoxin content. Furthermore, assays should be optimized to consume a minimum of time for sample preparation and analysis as well as for being operated under the most simple lab conditions including delivery of assays in a user-friendly storable and ready-to-use prototype format.

2 Materials and Methods

2.1 Materials

2.1.1 Fungal strains

Fungal strains used for testing specificity of the LAMP assays are listed in table 7 and table 12. DNA isolation for testing was done as described in Niessen and Vogel (2010). These fungal cultures were grown on SNAmod plates (Synthetischer Nährstoffarmer Agar, Nirenberg, 1976). Modification was done by adding double amounts of glucose and sucrose. For culture stocks, fungi were grown on porous clay granules in 3% (w/v) malt extract broth with 0.3% (w/v) soy peptone, at ambient temperature for five days. The granules were transferred into cryo vials mixed with 1 ml sterile 80% glycerol and kept frozen at -80 °C.

2.1.2 Chemicals, equipment and consumables

Table 1: Overview of used chemicals.

Chemicals	**Purity**	**Manufacturer**
Acetic acid	99 - 100 % (glacial)	Merck, Darmstadt, Germany
Agar	European agar	Difco, BD Sciences, Heidelberg
Aureomycin	for microbiology	Sigma-Aldrich, Steinheim, Germany
***Bst* DNA polymerase**	8000 U/ml	New England Biolabs, Frankfurt, Germany
***Bst* 2.0 DNA polymerase**	8000 U/ml	New England Biolabs, Frankfurt, Germany
***Bst* 2.0 WarmStart™ DNA polymerase**	8000 U/ml	New England Biolabs, Frankfurt, Germany
Calcein	p.a.	Sigma-Aldrich, Taufkirchen, Germany
Chloramphenicol	≥98,5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Chloroform	≥99 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
CTAB	≥98 %, for biochemistry	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$CuSO_4 \times 5H_2O$	p.a.	Sigma-Aldrich,

		Taufkirchen, Germany
Dextrose (Maltodextrin)	for microbiology	Sigma-Aldrich, Steinheim, Germany
Dichloran	for synthesis	Merck, Darmstadt, Germany
Dimidium bromide	≥98 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
dNTP mix (10 mm each A, T, G, C)	≥99 %	Fermentas, St. Leon-Rot, Germany
EDTA	for molecular biology	Sigma-Aldrich, Steinheim, Germany
Ethanol, absolute	≥99.8 %	VWR, Prolabo, Foutenay-sous-Bois, France
$FeSO_4 \times 7H_2O$	p.a.	Sigma-Aldrich, Taufkirchen, Germany
Glucose	for biochemical use	Merck, Darmstadt, Germany
Glycerol	99.5 %, high purity	GERBU Biotechnik, GmbH, Gaiberg, Germany
Isoamyl alcohol	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Isopropanol	≥99.5 %	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
KCl	p.a.	Merck, Darmstadt, Germany
KH_2PO_4	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$K_2HPO_4 \times 3H_2O$	p.a.	Merck, Darmstadt, Germany
KNO_3	p.a.	Merck, Darmstadt, Germany
Loading dye (for DNA, 6 x)		Fermentas GmbH, St. Leon-Rot, Germany
Malachitgreen	for microbiology	AppliChem GmbH, Darmstadt, Germany
Malt extract	microbiology grade	AppliChem, Darmstadt, Germany
Mannit	for microbiology	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
$MgCl_2$	p.a.	Merck, Darmstadt, Germany

$MgSO_4 \times 7H_2O$	p.a.	Merck, Darmstadt, Germany
$MnCl_2$	p.a.	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
MOPS **(3-Morpholinopropanesulfonic acid)**	p.a.	Merck, Darmstadt, Germany
NaCl	p.a.	Merck, Darmstadt, Germany
$NaNO_3$	p.a.	Merck, Darmstadt, Germany
$(NH_4)_2SO_4$	p.a.	Merck, Darmstadt, Germany
Oatmeal flakes	food product	Peter Kölln KGaA, Elmshorn, Germany
Peptone from casein	for microbiology	Becton, Dickinson, Le Pond de Claix, France
Primers	HPSF grade	MWG-BiotechAG, Ebersberg, Germany
Polyethylene glycol	average mol. wt. 8.000 g/mol (PEG 8K)	Sigma –Aldrich, Taufkirchen, Germany
Saccharose	p.a.	Sigma-Aldrich, Steinheim, Germany
Soy peptone	for microbiology	Oxoid, Basingstoke, Hampshire, England
Streptomycin sulfat	for microbiology	Sigma-Aldrich, Steinheim, Germany
Tris–HCl	p.a.	Merck, Darmstadt, Germany
Tween 20	p.a.	Mallinkrodt Baker B. v., Deventer, NL
Yeast extract	for microbiology	Merck, Darmstadt, Germany
$ZnSO_4 \times 7H_2O$	p.a.	Sigma-Aldrich, Taufkirchen, Germany

Table 2: Overview of used equipment.

Device	Model	Manufacturer
Autoclaves	2540 ELV	Systec GmbH, Wettenberg, Germany
	Varioklav	H + P Labortechnik, Oberschleißheim, Germany
Camera	IXUS 95	Canon, Beijing, China
Coffee grinder	MKM 6003	Robert Bosch Hausgeräte GmbH, Munich, Germany
Centrifuges	Z216MK	Hermle Labortechnik GmbH, Wehingen, Germany
	Z382K	Hermle Labortechnik GmbH, Wehingen, Germany
Counting chamber	Thoma, depth of 0.1 mm	BRAND GMBH + CO KG 97877 Wertheim Germany
DNA-quantification	Nanodrop1000	Peqlab Biotechnologie GmbH, Erlangen, Germany
Fastprep 24 bead beater	6004-500	MP Biomedicals, Illkirch, France
Microscope	Axiolab E re	Carl Zeiss, Oberkochen, Germany
Heating block	ThermoSTAR 100	Quantifoil Instruments, Jena, Germany
PCR-Cycler	Eppendorf Mastercycler gradient	Eppendorf AG, Jülich, Germany
pH determination	InLab 412, pH 0-14	Mettler-Toledo, Gießen, Germany

Pipettes	Pipetman (1 ml, 200, 100, 20, 10, and 2 µl)	Gilson-Abomed, Langenfeld, Germany
Real-time turbidimeter	Loopamp LA-320C	EIKEN Chemical Co., LTD, Tokyo, Japan
Shaking	Vortex 2 Genie	Scientific Industries Inc., Bohemia, NY, USA
Ultra sonification	UP 200S	Dr. Hielscher GmbH, Teltow, Germany
UV lamp	365 nm	MinUVIS, Desaga, Heidelberg, Germany
Vacuum drying oven	VO 400	Memmert GmbH+Co.KG, Schwabach, Germany
Water bath	Lauda BD	LAUDA Dr. D. Wobser GmbH & Co., Lauda-Königshofen, Germany
Water purification system	Euro 25 and RS 90-4/UF pure water system	SG Wasseraufbereitung GmbH, Barsbüttel, Germany
Kimtech tissue		Kimtech Science/ Kimberly-Clark Professional, Koblenz-Rheinhafen, Deutschland
Plastic bag	Microbiology Anaerocult	Merck KGaA, Darmstadt, Germany

Table 3: Overview of used consumables.

Material	Type	Manufacturer
Cryo vials	2 ml	Sarstedt, Nümbrecht, Germany
Filter pipette tips	200 µl, 100 µl, 10 µl	SafeGuard Filter Tips, PeQlab, Germany
Glass beads	5 mm, 2 mm	Carl Roth, Karlsruhe, Germany
Petri dishes	Without cams, 92×16 mm	Sarstedt, Nümbrecht, Germany
Reaction tubes	2 ml, 1.5 ml, 500 µl	Eppendorf, Hamburg, Germany
Multiply µStrip Pro 8-stripPCR tubes	200 µl	Sarstedt, Nümbrecht, Germany
Screw cap tube	1.5 ml	Sarstedt, Nümbrecht, Germany
Sea sand	extra pure	Merck, Darmstadt, Germany
Sterile Falcon tubes	15 ml, 50 ml	Sarstedt, Nümbrecht, Germany
Sterile filter	Filtropur S 0.2 (0.2 µm)	Sarstedt, Nümbrecht, Germany
Zirconia/silica beads	0.1 mm, 0.5 mm	Carl Roth, Karlsruhe, Germany
E.Z.N.A.® Bacterial DNA Kit	DNA isolation	Omega Bio-Tek Inc., Norcross, GA, USA
NucleoSpin® Plant II DNA preparation kit	DNA isolation	Macherey–Nagel, Düren, Germany

2.1.3 Oligonucleotides

Table 4: Primer list of LAMP primers and PCR primers.

LAMP primer set Tri6 ID1 (this study)	
FIP-Tri6-ID1	5’-TGGTTTGTGCTTAGACTCATG-GCTCAAGACCTACAAGAA-3’
BIP-Tri6-ID1	5’-AAGACAAGGAAGGACAACAA-CTATAGTGATCTCGCATGT-3’
F3-Tri6-ID1	5’-TTTTCTGTCGCTACTCAG-3’
B3-Tri6-ID1	5’-CAACACTTATGTATCCGC-3’
Loop B-Tri6-ID1	5’-ACCCCGGCCTTGTTATTCC-3’
LAMP primer set Tri5 ID4 (this study)	
FIP-Tri5-ID4 -Fsp	5’-AGGGACCAAAATGCCGAAGGAC-GGACGTGAACAAGCCCAT-3’
BIP-Tri5-ID4-Fsp	5’-CGCAGCACTCTGGACTGTAAGT-

	CATCATGGTCAGCCTCGG-3‘
F3-Tri5-ID4-Fsp	5’-GTACCCAACCATGGTGAACT-3’
B3-Tri5-ID4-Fsp	5’-CGATCCAGCATCCCTCGA-3’
Loop F-Tri5-ID4-Fsp	5’-TTGACGAGTGCCCACCACG-3‘
Loop B-Tri5-ID4-Fsp	5’-ACCCCGGCCTTGTTATTCC-3’
LAMP primer set Hyd5 ID4 (this study)	
FIP-Hyd5 ID4	5′-AGCACAGCACTGGGAAGTGC-GAGAAGCGACAGGCCTACA-3′
BIP-Hyd5 ID4	5′-CTTGGGTGTTGCTGACCTCGAC-GGGGCTGTTCATGTTAGCT-3′
F3-Hyd5 ID4	5′-CTTGGAGCCGTTGTCTCTG-3′
B3- Hyd4 ID4	5′-GACAGCGCTGAAGTTGTC-3′
LoopF Hyd5 ID4	5′-GTAGAGGCCACTGCAAGG-3′
LoopB-Hyd5 ID4	5′-CCGTAAGTACTCGAGTCTG-3′
PCR primers Hyd5 check (this study)	
Hyd5-check-f	5′-CGACAGGCCTACATCCCTTG-3′
Hyd5-check-r	5´-GTTAGCTTGATCTCCAATCG-3`
PCR primers Tox5 (Niessen and Vogel, 1998)	
Tox5-1	5'-GCTGCTCATCACTTTGCTCAG-3'
Tox5-2	5'-CTGATCTGGTCACGCTCATC-3'

2.1.4 Buffers and media

Unless otherwise stated, all components for the buffers were dissolved in deionized water and percentage values correspond to volume per volume (v/v) for liquid components and weight per volume (w/v) for solid components. Media were autoclaved for 15 min at 121 °C.

Table 5: Buffers used in this study.

Name	**Composition**
TAE buffer	2.0 M Tris, 1.0 M acetic acid, 0.1 M EDTA, pH 8.2
10×LAMP buffer	200 mm MOPS, 100mM KCl, 100 mm (NH4)2SO4, pH 8.8

Table 6: Media used in this study.

Medium	**Composition**
Mannit-malachitgreen-agar	2 g mannit, 20 g agar, ad 1 l aqua dest., after autoclaving add sterile at 60 °C 15.62 mg/l malachitgreen, 30 mg/l aureomycin, 30 mg/l streptomycinsulfat
Mannit-PCNB-agar	2 g mannit, 20 g agar, ad 1 l aqua dest., after autoclaving add sterile at 60 °C 2 mg/l dichloran (2.6-dichloro-4-nitroanilin), 30 mg/l aureomycin, 30 mg/l streptomycinsulfate
MB	Green mung beans (4 % w/v) were heated in water until boiling. After 5 min of boiling the broth was filtered off and agar (2 % w/v) was added.
MEA	2 % malt extract broth, 0.2 % soy peptone,15 g agar, ad 1 l aqua dest., pH 5.4 ± 0.2
OA	30 g oatmeal-flakes, 0.01 g $ZnSO_4$ x 7 H_2O, 0.005 g $CuSO_4$ x 5 H_2O, 15 g agar, ad 1 l aqua dest.
PDA	200 g scrubbed and diced potatoes boiled in 1 l water for 1 h, pass through a fine sieve, add 20 g dextrose and 15 g agar and autoclave
SFA	20 g glucose, 0.5 g KH_2PO_4, 2 g $NaNO_3$, 0.5 g $MgSO_4$ x 7 H_2O, 1 g yeast extract, 1 % $FeSO_4$ x 7 H_2O (1 g/l H_2O), 20 g agar, ad 1 l aqua dest.; after autoclaving: 1 % 2.6-Dichloro-4nitro-anilin (0.005 g/l H_2O), 0.1 g streptomycin sulfate, 0.01 g aureomycin dissolved in 70 % EtOH
SNA	Salt solution, autoclave separately: 1.0 g KH_2 PO_4 , 1.0 g KNO_3, 0.5 g KCl add salt solution to agarmedium after autoclaving; Agarmedium: 0.5 g $MgSO_4$ x 7 H_2O, 0.2 g glucose, 0.2 g sucrose, 20.0 g agar, ad 1.0 l aqua dest.
SNAmod	SNA modified by addition of double amounts of glucose and sucrose
YES	20 g yeast extract, 150 g sucrose, 0.5 g $MgSO_4$ x 7 H_2O, 0.01 g $ZnSO_4$ x 7 H_2O, 0.005 g $CuSO_4$ x 5 H_2O, 20 g agar, ad 1 l aqua dest.
YPD	1 % yeast extract, 2 % peptone, ad 1 l aqua dest.; after autoclaving: 2 % dextrose (sterile filtered through 0.2 µm filter)
YPG	5 % yeast extract, 10 % peptone from casein, 20 g D (+) glucose, 15 g agar; ad 1.0 l aqua dest.

2.2 Methods

2.2.1 DNA preparation

Preparation of highly purified DNA from fungal cultures was done as described in Niessen and Vogel (2010). Small scale DNA samples were prepared from fungal cultures according to the following method. Fungal cultures were grown for 5–7 days at ambient temperature on a rotary shaker (120 rpm) in sterile 15 ml plastic tubes (Sarstedt, Nümbrecht, Germany) containing 7 ml of 2 % (w/v) malt extract broth (AppliChem, Darmstadt, Germany) with 0.2 % (w/v) soy peptone (Oxoid, Hamshire, England). Mycelia were centrifuged at 5000 × g for 10 min. The supernatant was discarded and mycelia were washed twice with 7 ml of sterile tap water. If not used immediately, mycelia were stored at −20 °C for up to several weeks. For DNA isolation the mycelium, 100 mg of sterile sea sand (Merck, Darmstadt, Germany) and glass beads (1mm diameter) were transferred into a sterile 1.5 ml reaction tube with screw cap (Sarstedt, Nümbrecht, Germany). 300 µl of Yeast Cell Lysis Solution as contained in the MasterPure™ yeast DNA purification kit (Epicentre, Oldendorf, Germany) were added to each microtube. Tubes were treated for cell disruption in a FastPrep®-24 cell disruptor (M.P. Biomedicals, Eschwege, Germany) at 6.0 m/s for 60 s at ambient temperature. Following maceration, tubes were centrifuged for 5 min at 8000 × g. Supernatant was transferred to a fresh microtube. The latter step was repeated if sand was still visible. DNA was prepared from the cell free supernatant according to the manufacturer's recommendations. DNA was re-suspended in 35 µl of sterile deionized water. For barley DNA isolation, grains of barley (*Hordeum vulgare,* variety "Marthe") were grown on a wet sterile paper towel in a petri dish at ambient light and temperature. After 6 to 7 days, 14 leaves were removed and macerated using a mortar and pestle with glass beads added (content of E.Z.N.A Stool DNA Isolation Kit). Complete destruction of the leaf structure was monitored using a microscope. DNA extraction from macerated leaves was done according to the manufacturer's recommendations using the E.Z.N.A Stool DNA Isolation Kit (Omega Biotek, Norcross, GA 30071, USA).

For extraction of total DNA from barley, 10 g of grains were ground in a Bühler Universal lab grinder (Bühler GmbH, Braunschweig, Germany) with a 200 µm milling gap. Two gram of the flour were mixed with 1 g of sterile glass beads (1.25–1.65 mm

diameter, Carl Roth GmbH, Karlsruhe, Germany), 0.5 g acid washed sea sand (Merck, Darmstadt, Germany) and 3 ml sterile deionized water in a sterile 15 ml reaction tube with screw cap (Sarstedt, Nümbrecht, Germany). Tubes were treated for cell disruption in a FastPrep®-24 cell disruptor (M.P. Biomedicals, Eschwege, Germany) at 6.5 m/s for 60 s at ambient temperature. One gram of the resulting slurry was mixed with 400 µl of cell lysis buffer PL1 from the NucleoSpin® Plant II DNA preparation kit (Macherey–Nagel, Düren, Germany) in a fresh 2 ml reaction tube and incubated at 65 °C for 1 h in a water bath. Tubes were centrifuged for 2 min at 11.000 × g. The supernatant was transferred to a NucleoSpin® Filter provided with the NucleoSpin kit. All following steps were done according to the manufacturer's instructions. Elution of the DNA was done with 50 µl H_2O heated to 65 °C. DNA concentration of samples was assessed using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA) and samples were stored at 4 °C. Pure fungal DNA used as positive control was prepared as described in Niessen and Vogel (2010). Purified genomic DNA from barley was isolated from fresh grown surface disinfected barley leaves (Denschlag et al., 2012). For the extraction of total DNA from wheat it was done as described with barley, but 5 g of grains were used instead of 10 g.

2.2.2 Growth conditions and maintenance of fungal cultures

The general growth conditions for fungal cultures were 7 to 10 days at 16-20 °C under $UV_{360\ nm}$-light. For direct analysis of fungal cultures, each of the fungal strains given in table 8 was cultivated on 8 different agar media: MB (Evans et al., 2000), OA (Samson et al., 2010), PDA (Gams et al., 1987), SFA (Burgess and Liddell, 1983), SNAmod, YES (Samson et al., 2010), YPG (Samson et al., 2010), MEA.

For specific analysis of numbers of colony forming units (cfu) of *F. culmorum* and *F. graminearum*, respectively, in wheat samples, the selective media described by Böhm-Schraml et al. (1993) and by Böhm-Schraml (1995) were used. 1 ml of dilutions 10^{-1}-10^{-3} from a 10-fold serial aqueous dilution of wheat grains ground as previously described was plated on each of two different selective media. Mannitol-PCNB-agar was used for the selection of *F. graminearum*. For selective growth of *F. culmorum* mannitol-malachite green-agar was applied. Plates were incubated in the dark at 30 °C for 3 to 5 days until growth of fungal colonies occurred which produced a water soluble cherry red pigment. For the production of spore suspensions from cultures of *F. culmorum* DSM 62191 and *F. graminearum* DSM 4529 the method described by Evans et al. (2000) was used with

minor modifications. MB was produced using 30 g of mung beans instead of 40 g. Cultures were incubated at 20 °C for 10 days until sporulation occurred. Serial dilutions of spore suspensions of *F. graminearum* and *F. culmorum* had initial spore counts of 1240/µl and 1500/µl, respectively. Suspensions were diluted 10^{-1} to 10^{-5} and 10^{-1}- 10^{-8} in autoclaved tap water to result in serial dilutions for *F. graminearum* and *F. culmorum*, respectively.

2.2.3 Molecular detection methods

DNA amplification was done using the loop-mediated isothermal amplification (LAMP) technology (Notomi et al., 2000) and PCR. Modifications to the original protocol of LAMP were done as described in Niessen and Vogel (2010).

2.2.3.1 Loop-mediated isothermal amplification (LAMP)

Two different LAMP assays were developed in this study: Hyd5 ID4 LAMP assay and Duplex LAMP assay. For the Hyd5 ID4 LAMP assay calcein fluorescence was used for indirect in-tube detection of DNA amplification during the reaction (Tomita et al., 2008). The master mix for the *hyd5* gene specific LAMP reaction was prepared containing per 25 µl reaction: 2.5 µl 10×LAMP buffer (200 mm MOPS, 100mM KCl, 100 mm $(NH_4)_2SO_4$, pH 8.8 (all chemicals from Sigma-Aldrich, Taufkirchen, Germany)), 3.5 µl dNTP mix (10 mm each, Fermentas, St. Leon-Rot, Germany), 1 µl 200 mm MgCl2, 2.6 µl primer mix (1.6 µM FIP-Hyd5 ID4, 1.6 µM BIP-Hyd5 ID4 5, 0.2 µM F3-Hyd5 ID4, 0.2 µM B3- Hyd4 ID4, 0.8 µM LoopF-Hyd5 ID4, 0.8 µM LoopB-Hyd5 ID4, EurofinsMWG Operon, Ebersberg, Germany) (for sequences see table 4), 8 U (1.0 µl) *Bst* DNA polymerase, large fragment (8000 U/ml,New England Biolabs, Frankfurt, Germany), 1 µl calcein reagent (see in Niessen and Vogel, 2010). Sterile deionized water was added to result in a 25 µl total reaction volume, including sample DNA. Primers for the *hyd5* gene-specific LAMP assay were designed using the PrimerExplorer V4 software tool freely accessible on the Eiken Genome site (http://primerexplorer.jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). Four different primer sets were generated from a 392 bp fragment of the *F. culmorum hyd5* gene precursor sequence (GenBank accession number DQ449530).

All sets were tested *in silico* against the NCBI nucleotide sequence database for similarities with sequences of other organisms using the nucleotide BLAST algorithm for highly similar sequences search tool (Altschul et al., 1990). In order to increase the concentration of amplification product during the LAMP reaction, a pair of loop primers was designed manually (Nagamine, 2002). The binding sites of each primer within the

nucleotide sequence of *hyd5* are shown in figure 6. In order to determine the optimum incubation temperature for the Hyd5 ID4 LAMP reaction, primers were tested with highly purified DNA of *F. graminearum* DSM 4527 in a temperature gradient (65 °C±5 °C). Samples were incubated at the respective constant temperature in the heating block of a PCR machine (Mastercycler gradient, Eppendorf, Hamburg, Germany) for 30–60 min. Highly purified DNA from cultures of *F. graminearum* DSM 4527 or *F. culmorum* DSM 62191 was used as positive control (80 ng/reaction). A hand held digital camera Digital IXUS 95 (Canon) was used for documentation as described in Niessen and Vogel (2010).

For the Duplex LAMP a master mix was prepared containing the following per 25 µl reaction: 2.5 µl 10×LAMP buffer (200 mm MOPS, 100 mm KCl, 100 mm $(NH_4)_2SO_4$, pH 8.8 (all chemicals from Sigma-Aldrich, Taufkirchen, Germany)), 3.5 µl dNTP mix (10 mm each, Fischer Scientific, St. Leon-Rot, Germany), 1 µl 200 mm $MgCl_2$, 2.2 µl primer mix Tri6 ID1 (1.6 mm FIP-Tri6-ID1, 1.6 mm BIP-Tri6-ID1, 0.2 mm F3-Tri6-ID1, 0.2 mm B3-Tri6-ID1, 0.8 mm Loop B-Tri6-ID1 or /and 2.6 µl primer mix Tri5 ID4 (1.6 mm FIP-Tri5-ID4-Fsp, 1.6 mm BIP-Tri5-ID4-Fsp, 0.8 mm LoopF-Tri5-ID4-Fsp, 0.8 mm LoopB-Tri5-ID4-Fsp, 0.2 mm F3-Tri5-ID4-Fsp, 0.2 mm B3-Tri5-ID4-Fsp (Eurofins MWG Operon, Ebersberg, Germany) (for sequences see table 4), 8 U (1.0 µl) *Bst* DNA polymerase, large fragment (8000 U/ml, New England Biolabs, Frankfurt, Germany), 1 µl calcein reagent (used only to test optimum reaction temperature, see Niessen and Vogel (2010)). The binding sites of each primer within the nucleotide sequence of *tri5* and *tri6* are shown in figure 18. Sterile deionized water was added to result in a 25 µl total reaction volume, including sample DNA. The primer sets Tri5 ID4 and Tri6 ID1 were tested in separate reactions as well as in combination (Duplex LAMP) with water added as appropriate. Primers for the *tri5* and *tri6* gene-specific LAMP assay were designed using the PrimerExplorer V4 software freely accessible on the Eiken Genome site (http://primerexplorer.jp/e/) provided by Eiken Chemical Co., Ltd. (Tokyo, Japan). The optimum reaction temperature was tested in a temperature gradient using the heating block of a Mastercycler gradient thermal cycler (Eppendorf, Hamburg, Germany.

2.2.3.2 Real-time LAMP reaction

Possible quantification with the LAMP reaction was tested in real-time for the Hyd5 ID4 LAMP assay on two different platforms: on the Loopamp LA-320C real-time

turbidimeter (Eiken Chemical Co., LTD, Tokyo, Japan) and on the ESEQuant TS95 tube scanner (Qiagen Lake Constance GmbH, Konstanz, Germany). Reactions were incubated at a constant temperature of 62.5 °C for 60 min in a Loopamp LA-320C real-time turbidimeter (Eiken Chemical Co., LTD, Tokyo, Japan). The LA-320CE software package (Eiken Chemical Co., LTD, Tokyo, Japan) was used for control of the turbidimeter and real-time measurement. Turbidity was monitored at 650 nm in a maximum of 32 parallel reactions in 4 independent heating blocks. Measurement was done in intervals of 6 s. A threshold of 0.05 for the first derivative of turbidity against time was set to measure time to threshold (T_t) throughout all experiments (see figure 5).

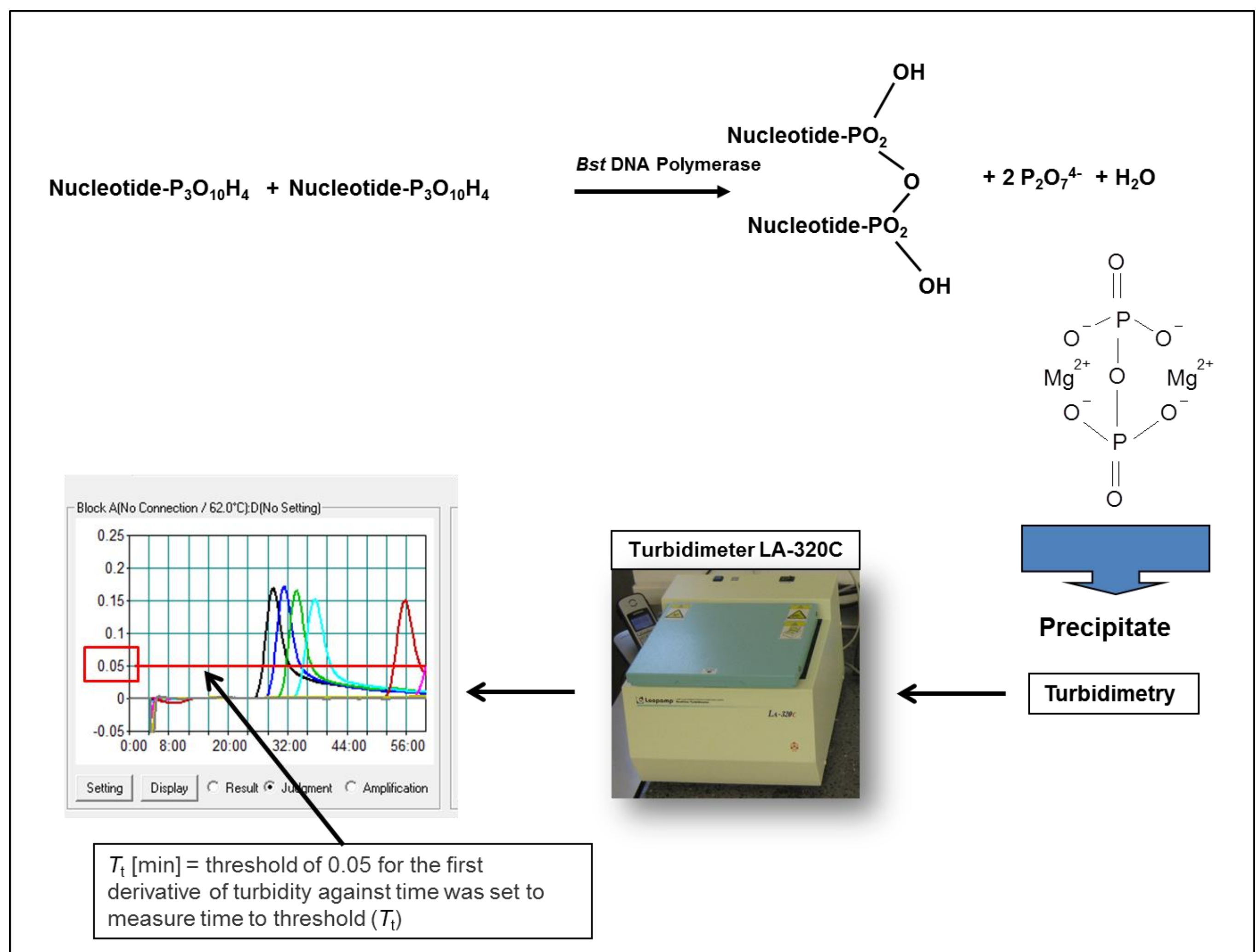


Figure 5: Indirect detection of LAMP products using real-time turbidimetry.

As an alternative platform, the ESEQuant TS95 tube scanner (Qiagen Lake Constance GmbH, Konstanz, Germany) was used to assess fluorescence of an intercalating dye during DNA amplification in real-time. The tube scanner Studio™ software package

(Qiagen Lake Constance GmbH, Konstanz, Germany) was used to control the device and for the documentation of results. Excitation intensity was set to 80 [AU]. Reactions were incubated at a constant temperature of 65 °C for 60 min. For result evaluation the baseline validation mode was used between 0 and 1 min. The slope validation mode with lower and upper limits of 100 mV and 750 mV, respectively, in a time frame between 1 and 20 min and a lower slope limit of 30 mV/s was applied for automated differentiation of positive from negative results. Period point setting was set to 4. Fluorescence was measured using the 6-FAM-channel of the tube scanner device with an excitation maximum of 494 nm and emission maximum of 515 nm. The LAMP reaction master mix was basically the same as described above, but with 1 µl of V13-01184 stain (Dyomics GmbH, Germany) added per reaction (see figure 15 as an example). V13-01184 stock solution was prepared by dissolving 1 mg of the stain in 1 ml dimethylformamide (DMF). This solution was diluted 1:100 in ultrapure water and sterile-filtered (filter pore size 0.2 µm) and used as working solution. V13-01184 stock solution was stored at 4 °C. Working solutions were stored at −20 °C. Working solutions were stored further at 4 °C after their first removal from the freezer.

Sensitivity of the Hyd5 ID4 real-time LAMP assay was analyzed with a serial dilution of genomic DNA isolated from pure cultures of *F. culmorum* strain TMW 4.0548. Gene copy numbers were calculated under the assumption that *hyd5* is a single copy gene (http://www.uri.edu/research/gsc/resources/cndna.html) and on the basis of the genome size of *F. graminearum* PH-1 (36.45 Mbp) (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/Genome Stats.html). The reactions were repeated three times and the average value was used for further calculations. The reaction time after which the deviation of turbidity (OD 600 nm) as a function of time (dturb./dt) reached a threshold level of 0.05 (time to threshold, T_t) was plotted against DNA concentration. Based on exponential regression of the data a standard curve was calculated and used to determine the DNA concentration of unknown samples from their T_t value. The exponential regression was calculated according to $y = m \bullet e^{(b \bullet x)}$. Parameters m and b were calculated with MS-Excel version 2010 using the form provided by Ernst-Albrecht Boergener (http://www.excelformeln.de/formeln.html?welcher=276). Using the formula, concentrations were calculated from the T_t values of unknown samples. A Standard curve for real-time LAMP performed with the tube scanner instrument was prepared using a serial dilution of *F. culmorum* DNA TMW 4.0548. Gene copy numbers were

plotted against the reaction time at which the increase of fluorescence intensity of the V13-01184 stain over time exceeded a threshold level of 30 mV/sec. In order to evaluate the influence of background DNA on both real-time assays, purified genomic DNA from barley leafs was mixed with *F. culmorum* DNA to result in an up to 1000-fold excess of the background DNA over the target DNA. Mixtures were used as template in LAMP reactions using the Hyd5 ID4 primer set and results compared with the results of a LAMP reaction performed without the background DNA.
For real-time Duplex LAMP reactions the assay described above was used. The reactions were incubated for 60 min at a constant temperature of 64 °C in a Loopamp LA-320C real-time turbidimeter (Eiken Chemical Co., LTD, Tokyo, Japan). The rest was done as previously described for the Hyd5 ID4 assay.

2.2.3.3 Polymerase chain reaction (PCR) for verification

Conventional PCR was used to verify the identity of the Hyd5 ID4 LAMP product. Sequence specific primers for the *hyd5* gene were designed from the sequence published in GenBank under accession number AY158024. Primers were chosen to bind to positions downstream and upstream of the 3´-ends of primers FIP-Hyd5 ID4 and BIP-Hyd5 ID4. A LAMP reaction was separated on a 2 % agarose gel (figure 9) and the smallest fragment was cut out and purified using the E.Z.N.A Cycle Pure Kit (Omega Biotek, Norcross, GA 30071). Purified DNA was used as PCR template in a master mix containing per 25 µl reaction: 2.5 µl of 10xamplification buffer containing 1.5 mm MgCl2 (MP Biomedicals, Freiburg, Germany), 0.5 µl dNTP's (MP Biomedicals, Freiburg, Germany), 0.25 µl Taq polymerase (250 U/µl, MP Biomedicals, Freiburg, Germany), 1 µl 50 pmol/µl primer Hyd5-check-f (5′-CGACAGGCCTACATCCCTTG-3′), 1 µl 50 pmol/µl primer Hyd5-check-r (5´-GTTAGCTTGATCTCCAATCG-3`) (Eurofins MWG Operon, Ebersberg, Germany), 1 µl DNA sample. Sterile deionized water was added to result in a 25 µl total reaction volume. Samples were preheated at 95 °C for 5 min. Subsequently, 40 cycles of 1 min 95 °C, 1 min 60 °C, 8 s 72 °C were run. A short amplification period of 8 s was chosen in order to favor amplification of short PCR-products. PCR products were separated on 1 % agarose gels (figure 9). After purification of the product from the gel using the E.Z.N.A Cycle Pure Kit (Omega Biotek, Norcross, GA 30071,USA), DNA was sequenced by GATC Biotech AG (Konstanz, Germany) using the Hyd5-check primers.

For verification of Duplex LAMP results the PCR described by Niessen and Vogel (1998) using a *tri5* gene specific primer pair was used with an annealing temperature of 66.5 °C according to the author's description.

2.2.3.4 Vacuum drying of Hyd5 ID4 LAMP master mix

For vacuum drying of master mixes, a vacuum drying oven (VO 400) was preheated at 60 °C together with an aluminum block. PCR stripes with the Hyd5 ID4 LAMP master mix were incubated in the aluminum block at a pressure of 85 mbar. After the vacuum drying the stripes were either stored in a plastic bag which was filled with a silica pad (2 g silica gel in Kimtech tissue) and welded or directly used. For utilization of the Hyd5 ID4 LAMP master mix after vacuum drying the lyophilisate was rehydrated with 20 µl water. Rehydrated master mixes with 5 µl template DNA added were incubated in the turbidimeter for 60 min at 63.5 °C. Ten nanogram per microliter of purified DNA from *F. graminearum, F. culmorum*, and *F. cerealis* was used as template for testing the reaction of the Hyd5 ID4 LAMP after vacuum drying. The sensitivity for detection of *F. graminearum, F. culmorum*, and *F. cerealis* DNA after vacuum drying was tested with a serial dilution of the respective DNA starting from 50 ng per reaction to 0.00005 ng per reaction. For testing of stability under long time storage conditions, vacuum dried Hyd5 ID4 LAMP master mix was stored in welded plastic bags with silica pads added for desiccation. Samples were stored at different temperatures and repeatedly inspected for their activity after 0-72 h of storage. Samples stored at 4 °C were inspected for up to 888 h (37 days).

2.2.4 Application of LAMP assays

Robustness of the Hyd5 ID4 LAMP assay was tested with fungal mycelia as well as different growth media added directly to the master mix. For analysis a 5 x 5 mm piece was removed from the cultures and mixed with 200 µl sterile deionized water, a small lab spoon of sterile sea sand and one glass bead (1 mm diameter) in a 0.5 ml reaction tube. After incubation at 95 °C for 5 min and vortexing for 10 s at maximum speed, 2 µl of the supernatant were added as template into the LAMP master mix. Blank media without mycelial growth were tested for their effect on the LAMP reaction by heating portions of 80 mg in 200 µl sterile water at 95 °C for 5 min before vortexing for 10 s at maximum speed and adding 2 µl of the supernatant into the LAMP master mix together with positive control DNA (*F. graminearum*). Performance of the reactions with and without addition of culture media was compared. Experiments were repeated three

times with *F. venenatum, F. trichothecioides, F. heterosporum, F. graminearum* (7 isolates)*, F. equiseti, F. culmorum* (3 isolates)*, F. cerealis* (4 isolates) and *Alternaria mali*, respectively, on all the media given under chapter 2.1.2 and table 8.

For testing sample material with the Hyd5 ID4 LAMP, the barley samples used during the current study were grown under field conditions and inoculated with *F. culmorum* spore suspension (10^5 spores per mL) with the help of a knapsacksprayer equipped with a spraybar at the time of flowering. Infection levels of *F. culmorum* and other fungi present in the inoculated barley and in the uninfected control barley were analyzed by plating 100 grains per sample to SNAmod plates (5 grains per plate) after surface disinfection as described in Niessen and Vogel (2010). The identification of the *Fusarium* spp. growing on the grains was done according to the taxonomy of Nelson et al. (1994) and Gerlach and Nirenberg (1982). Infected barley was mixed (w/w) with non-infected barley to result in different percentages of infection. 50 samples (32 gushing malt, 18 non-gushing malt) of malted barley were tested for their gushing potential with the MCT (Anger, 2006) (MEBAK: http://www.mebak.org/de/home.html) as reference method. Tests were performed by the staff of a German brewery and the results were used for statistical analysis during the current work.

Wheat samples analyzed with the Duplex LAMP assay were collected from the 2012 harvest from fields at locations all over the Bavarian state of Germany. Samples were analyzed for DON using HPLC (data provided by J. Rieder, Bayerische Landesanstalt für Landwirtschaft (LfL), Freising, Germany). For LAMP analysis, 5 µl of total DNA isolated from wheat samples was diluted 1:20 with sterile distilled water before addition to LAMP reactions as template. For testing of the same samples using a simpler and more rapid method, 20 g of seeds was covered with distilled water in 50 ml plastic tubes (Sarstedt, Nümbrecht, Germany) and shaken vigorously by hand for 1 min. After shaking, 500 µl of the supernatant was removed and transferred to a 1.5 ml reaction tube. Samples were cooled to 4 °C and 5 µl was added to 20 µl of the Duplex LAMP master mix previously described. Reactions were incubated for 1 h at 64 °C in a Loopamp LA-320C real-time turbidimeter as previously described.

3 Results

In the following chapters, the results of the current thesis are presented. The chapters are structured as follows: The first chapter describes development of the Hyd5 ID4 LAMP assay with primer design, application of the test with gushing inducing fungi and barley samples, and quantification of target DNA in real-time. The second chapter deals with the development of a Duplex LAMP assay for potential trichochecene producers among *Fusarium* spp. dealing with primer design and testing of specificity and sensitivity of assays as well as with its application to the analysis of samples. The third and final chapter is dedicated to experiments aimed at providing LAMP assays in a user-friendly and ready-to-use format.

3.1 Development and application of a gene specific LAMP assay for detection and identification of hydrophobin 5 producing *Fusarium* species

3.1.1 Primer design

For the *hyd5* gene-specific LAMP assay the primer set Hyd5 ID4 (figure 6) was chosen for further experiments because *in silico* hybridization with the primer set against GenBank revealed highly similar sequences only in *F. cerealis, F. crookwellense* (syn. *F. cerealis*), *F. culmorum F. graminearum* and *F. lunulosporum.* In the literature, *F. lunulosporum* has been termed an insufficiently documented species (Gerlach and Nirenberg, 1982; Nelson et al., 1983). *F. lunulosporum* is assumed to be synonym of *F. graminearum* (NCBI database alpha-tubulin). Since introns often show a high degree of sequence variation between related species it was important for the study that primers hybridize exclusively to exons of the *hyd5* gene, as the aim of the study was to set up a group specific assay. Therefore, only gene regions with a high degree of conservation between homologous genes in the target species were selected for primer design. According to the results of *in silico* hybridization experiments the primers were thus anticipated to be highly specific for detection of the target sequence in gushing relevant fungal species.

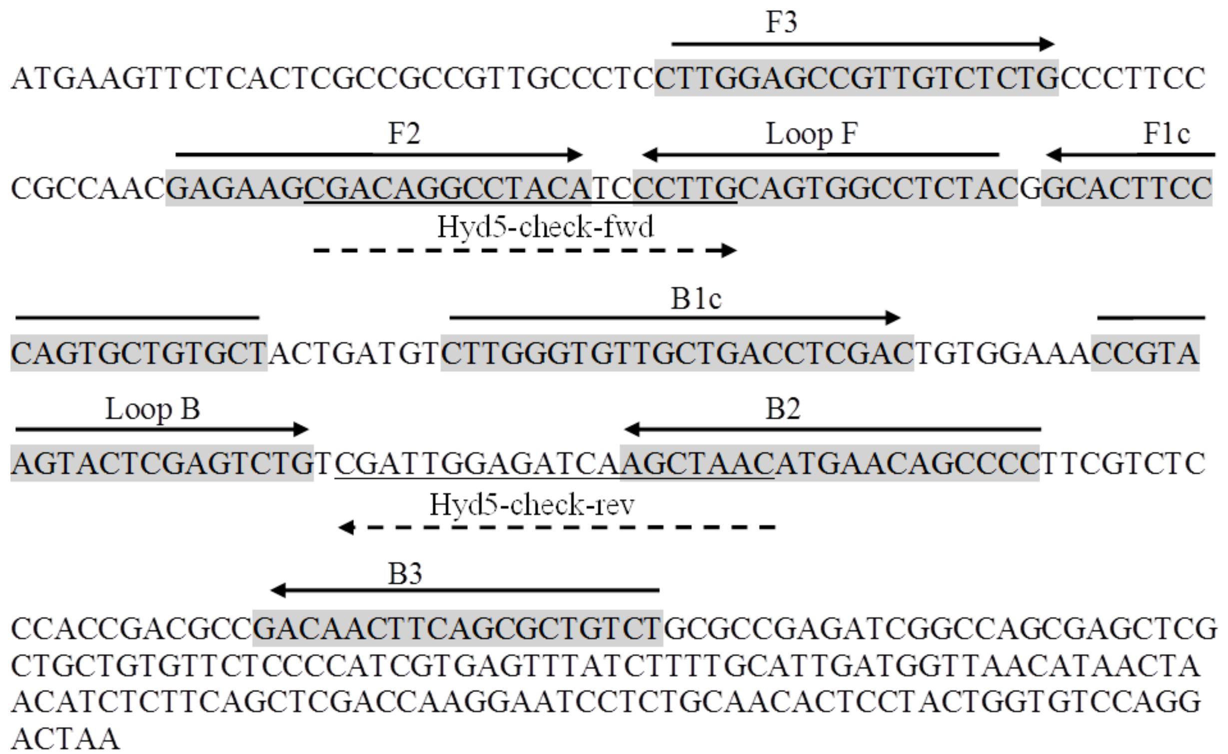


Figure 6: Binding sites of LAMP primer set Hyd5 ID4 and the Hyd5-check primer pair within the nucleotide sequence of the *F. culmorum hyd5* gene (accession number DQ449530).

3.1.2 Optimization of LAMP assay conditions

The optimum isothermal incubation temperature for the LAMP reaction with primers Hyd5 ID4 was found to be 63 °C after running the reaction in a temperature gradient using calcein fluorescence for signal detection (see figure 7).

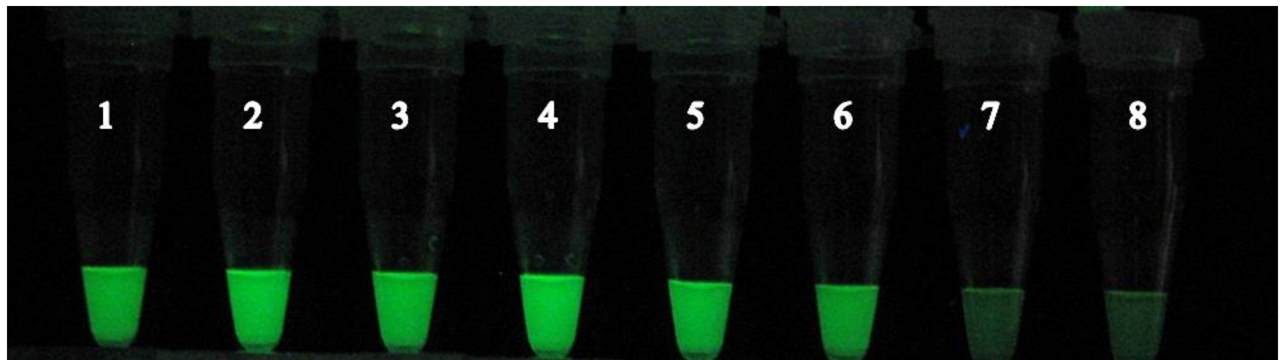


Figure 7: LAMP reaction running in a temperature gradient with genomic DNA from *F. graminearum* DSM 4527 using primer set Hyd5 ID4. Calcein fluorescence under UV366nm light. 1 = 60.7 °C, 2 = 61.6 °C, 3 = 62.8 °C, 4 = 64.1 °C, 5 = 65.5 °C, 6 = 66.9 °C, 7 = 68.1 °C, 8 = 69.2 °C. The contrast of the picture was modified for better visibility of results

Following optimization of the incubation temperature, sensitivity of the reaction was tested by addition of 2 µl of a serial dilution of genomic DNA purified from *F. graminearum* DSM 4527 as template to the LAMP master mix before incubation for 60 min at the optimum temperature of 63 °C. As shown in figure 8, a bright green fluorescence was still visible under $UV_{366\ nm}$ light in a LAMP reaction to which 0.74 pg of template DNA were added. A minimum of 0.74 pg per reaction was detected within 30 min of incubation without any post assay manipulations necessary. Incubation of the reactions for >30 min did not improve sensitivity. In order to verify the results, 10 µl of each LAMP reaction shown in figure 9 were separated on a 2 % agarose gel and stained with dimidium bromide. Results showed that the reaction produced the typical ladder-like assemblage of DNA fragments, the smallest of which was 150 bp. This size was in accordance with the number of nucleotides separating the 5´ and 3´ ends of the binding sites of primers F2 and B2 given in figure 6.

Following sensitivity testing, the specificity of the developed assay was analyzed by challenging LAMP primers with genomic DNA isolated from 133 different fungal isolates representing 99 fungal species from 13 genera. The test array comprised most species frequently occurring on cereals and malt (see table 7). DNA isolated from wheat and barley was also tested in order to make sure no interference with background DNA would occur when analyzing sample materials. As a result, a positive LAMP reaction was observed only when DNA isolated from cultures of *F. cerealis* (syn. *F. crookwellense, F. lunulosporum*), *F. culmorum,* and *F. graminearum* was used as template. In the test, the culture assigned to *F. crookwellense* gave a positive result, but as the name is invalid, isolates should be assigned to *F. cerealis* as basonym. Also the isolate of *F. lunulosporum* resulted in a positive LAMP reaction. Comparison of the translation elongation factor-1alpha gene (*tef1α*) sequence of the respective strain with GenBank showed that it had 100 % sequence identity with the *tef1α* gene of *F. cerealis* (GenBank accession number AF212467). Also in the literature, *F. lunulosporum* has been termed an insufficiently documented species (Gerlach and Nirenberg, 1982). The fact that only one isolate of the species exists would also argue in favor of *F. lunulosporum* being a synonym of *F. cerealis. F. cerealis sensu stricto* is most closely related to *F. culmorum* and *F. graminearum* and it is therefore highly probable that the species has a *hyd5* homolog sharing a high sequence similarity with both of the former species. Results obtained showed that the amplification reaction was highly specific for a set of target species.

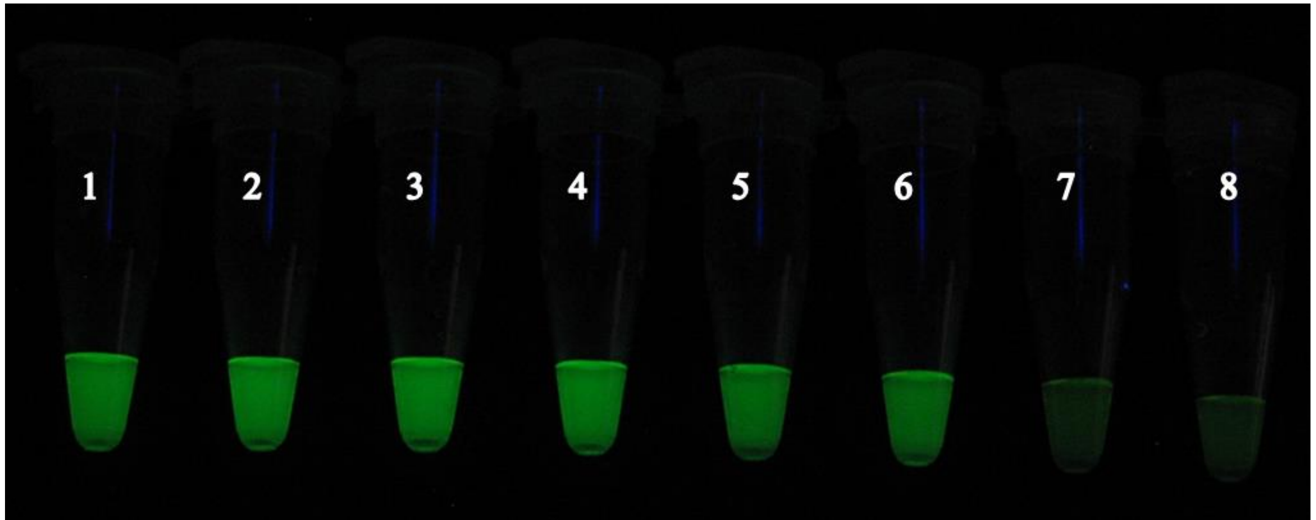


Figure 8: LAMP reaction with a serial dilution of highly purified genomic DNA from *F. graminearum* DSM 4527 using primer set Hyd5 ID4. Calcein fluorescence under UV_{366nm} light. 1 = 74 ng/reaction, 2 = 7.4 ng/reaction, 3 = 0.74 ng/reaction, 4 = 0.074 ng/reaction, 5 = 0.0074 ng/reaction, 6 = 0.00074 ng/reaction, 7 = 0.000074 ng/reaction, 8 = negative control (water instead of DNA). The contrast of the picture was modified for better visibility of results.

Table 7: Fungal cultures used for specificity testing of the Hyd5 ID4 LAMP assay and results of their LAMP reactions after 30 min reaction time at 63 °C.

Genus	Species	Strain	Clone	LAMP result
Alternaria	*alternata*		[g] TMW 4.0438	-
Aspergillus	*alliaceus*	[c] DSM 813	TMW 4.1077	-
	carbonarius	[l] M 324	TMW 4.1512	-
	clavatus	[b] CBS 513.65	TMW 4.1512	-
	flavus	CBS 113.32	TMW 4.1085	-
	fumigatus	CBS 113.55	TMW 4.0623	-
	niger	CBS 101698	TMW 4.1068	-
	ochraceus	CBS 263.67	TMW 4.0706	-
	parasiticus	CBS 126.62	TMW 4.1768	-
	terreus	CBS 377.64	TMW 4.1060	-
	tubingensis	[e] ITEM 4496	TMW 4.2008	-
	versicolor	CBS 245.65	TMW 4.1600	-
Drechslera	*teres*	CBS 378.59	TMW 4.0558	-
Epicoccum	*nigrum*		TMW 4.0483	-
Fusarium	*anguioides*	CBS 172.32	TMW 4.0460	-
	annulatum	CBS 258.54	TMW 4.0461	-
	anthophilum	CBS 222.76	TMW 4.0490	-

	armeniacum	CBS 485.94	TMW 4.0701	-
	aquaeductum	CBS 837.85	TMW 4.0914	-
	arthrosporioides	CBS 173.32	TMW 4.0463	-
	avenaceum	DSM 62161	TMW 4.0140	-
	beomiforme	[a] BBA 69406	TMW 4.0513	-
	buharicum	CBS 796.70	TMW 4.0627	-
	camptoceras	ITEM 1235	TMW 4.0359	-
	caucasicum	CBS 179.35	TMW 4.0492	-
	cavispermum	CBS 172.31	TMW 4.0491	-
	cerealis	CBS 589.93	TMW 4.0406	+
	chlamydosporum	CBS 145.25	TMW 4.0404	-
	coeruleum	CBS 836.85	TMW 4.0494	-
	compactum	CBS 466.92	TMW 4.0433	-
	concolor	CBS 183.34	TMW 4.0556	-
	crookwellense	CBS 623.85	TMW 4.0407	+
	culmorum	DSM 62191	TMW 4.0149	+
			TMW 4.0111	+
		[k]LWL F 64-2	TMW 4.0539	+
		LWL F 85-5	TMW 4.0548	+
			TMW 4.0102	+
			TMW 4.0539	+
		LWL F 64-4	TMW 4.0545	+
		LWL F 64-3	TMW 4.0540	+
	decemcellulare	CBS 113.57	TMW 4.0493	-
	detonianum	CBS 736.79	TMW 4.0557	-
	dimerum	CBS 175.31	TMW 4.0626	-
	dlamini	[f] MRC 3024	TMW 4.0571	-
	equiseti	CBS 406.86	TMW 4.0477	-
	eumartii	DSM 62809	TMW 4.0303	-
	fujkuroi	DSM 63217	TMW 4.0336	-
	globosum	MRC 6646	TMW 4.0584	-
	graminearum	DSM 4529	TMW 4.0175	+
			TMW 4.0169	+

			TMW 4.0157	+
		DSM 4527	TMW 4.0208	+
			TMW 4.0238	+
			TMW 4.0185	+
			TMW 4.0173	+
		[i]E50	TMW 4.0170	+
			TMW 4.0169	+
			TMW 4.0159	+
			TMW 4.0157	+
			TMW 4.0155	+
			TMW 4.0151	+
			TMW 4.0150	+
			TMW 4.0148	-
			TMW 4.0143	+
			TMW 4.0133	+
			TMW 4.0204	+
			TMW 4.0127	+
			TMW 4.0124	+
			TMW 4.0122	+
		VLB 20/2	TMW 4.0447	+
		VLB 67/1	TMW 4.0450	+
		VLB 63/2	TMW 4.0449	+
		BBA 62182	TMW 4.0111	+
	graminum	DSM 62224	TMW 4.0209	-
	heterosporum	DSM 62231	TMW 4.0224	-
	inflexum	DSM 63203	TMW 4.0423	-
	javanicum	DSM 62233	TMW 4.0215	-
	kyushuense	BBA 70812	TMW 4.1053	-
	langsethiae	[d] IBT 9956 [T]	TMW 4.0072	-
	larvarum	CBS 783.79	TMW 4.0434	-
	longipes	CBS 739.79	TMW 4.0350	-
	lunulosporum	CBS 636.76	TMW 4.0414	+
	macroceras	CBS 146.25	TMW 4.0419	-

	melanochlorum	CBS 202.65	TMW 4.0625	-
	merismoides var. chlamydosporum	DSM 62256	TMW 4.0247	-
	musarum	ITEM 1294	TMW 4.0361	-
	napiforme	BBA 67629	TMW 4.0510	-
	nelsonii	ITEM 1681	TMW 4.0363	-
	neoceras	CBS 147.25	TMW 4.0349	-
	nygamai	BBA 67375	TMW 4.0512	-
	oxysporum	DSM 62292	TMW 4.0163	-
	poae	DSM 62376	TMW 4.0134	-
	proliferatum	DSM 62261	TMW 4.0236	-
	reticulatum	DSM 62395	TMW 4.0305	-
	robustum	CBS 637.76	TMW 4.0418	-
	sacchari var. subglutinans	CBS 215.76	TMW 4.0408	-
	sambucinum	CBS 185.29	TMW 4.0165	-
	scirpi	CBS 448.84	TMW 4.0410	-
	semitectum var. majus	DSM 63310	TMW 4.0191	-
	setosum	CBS 574.94	TMW 4.1059	-
	solani	DSM 62416	TMW 4.0255	-
	sporotrichioides	CBS 412.86	TMW 4.0475	-
	subglutinans	BBA 63621	TMW 4.0947	-
	sublunatum var. sublunatum	CBS 189.34	TMW 4.0417	-
	succisae	DSM 63162	TMW 4.0309	-
	sulphureum	DSM 62433	TMW 4.0310	-
	tabacinum	DSM 2125	TMW 4.0284	-
	torulosum	BBA 64465	TMW 4.0437	-
	trichothecioides	CBS 136.73	TMW 4.0427	-
	tricinctum	CBS 410.86	TMW 4.0476	-
	tumidum	CBS 486.76	TMW 4.0413	-
	udum	DSM 62451	TMW 4.0258	-

	venenatum	CBS 458.93	TMW 4.0462	-
	verticillioides	CBS 218.76	TMW 4.0409	-
	xylarioides	CBS 258.52	TMW 4.0436	-
Microdochium	*majus*		TMW 4.0496	-
	nivale		TMW 4.0495	-
Mucor	*mucedo*	DSM 809	TMW 4.0441	-
Myrothecium	*roridum*	CBS 331.51	TMW 4.0668	-
Penicillium	*camemberti*	DSM 1233	TMW 4.0442	-
	commune	CBS 311.48	TMW 4.1088	-
	corylophilum	CBS 321.48	TMW 4.1598	-
	expansum	DSM 62841	TMW 4.0466	-
	griseofulvum		TMW 4.1543	-
	nordicum	[j]BEF 487	TMW 4.2213	-
	purpurescens	CBS 223.28	TMW 4.1082	-
	roquefortii	CBS 221.30	TMW 4.1599	-
	verrucosum	IBT 12935	TMW 4.0925	-
Stachybotrys	*chartarum*	CBS 485.48	TMW 4.0663	-
Trichoderma	*harzianum*	IBT 9471	TMW 4.1502	-
Trichothecium	*roseum*	CBS 567.50	TMW 4.0691	-
Verticillium	*sp.*		TMW 4.0424	-
Plant DNA				
Hordeum vulgare cf. Marthe				-
Triticum aestivum				-

[a] BBA = Julius Kühn Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, Germany.
[b] CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
[c] DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, Germany.
[d] IBT = Technical University of Denmark, Department of Biotechnology, Lyngby, Denmark.
[e] ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy.
[f] MRC = South African Medical Research Council, Tygerberg, South Africa.
[g] TMW = Lehrstuhl für Technische Mikrobiologie, Freising, Germany.
[h] VLB = Versuchs- und Lehranstalt für Brauerei, Berlin, Germany.
[i]E = Collection of Dr. G. Engelhard, Landesanstalt für Ernährung, Munich, Germany.

[j]BEF = Max Rubner Institute, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany.
[k]LWL = strains obtained from the collection of M. de Nijs, Wageningen University and Research, Wageningen, The Netherlands.
[l]M = strain obtained from the collection of H.M.L.J Joosten, Nestlé Research Centre, Lausanne, Switzerland.

3.1.3 Verification of the LAMP product

In order to verify the identity of the amplification product, the smallest DNA fragment was excised from the gel and purified in order to use it as template in a PCR with the Hyd5-check pair of primers (see chapter 2). The ca. 100 bp PCR product was sequenced using the Hyd5-check primers. Sequence analysis revealed 100 % identity with the *hyd5* gene sequences of *F. culmorum* (GenBank accession number DQ449530.1) and *F. graminearum* (GenBank accession number FN668637.1) at nucleotide positions 115 to 200.

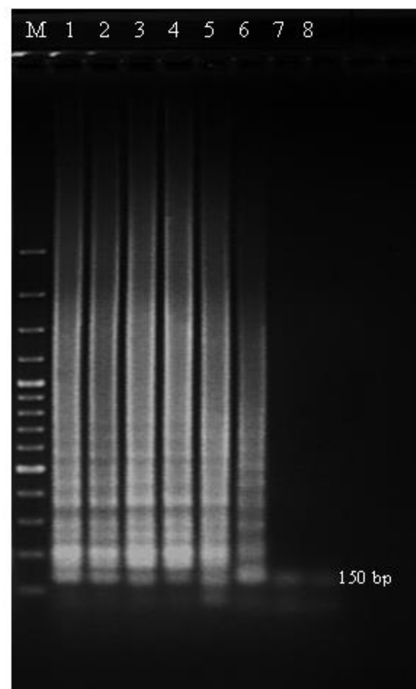


Figure 9: Separation of LAMP products obtained with primer set Hyd5 ID4 and a serial dilution of *F. graminearum* DSM 4527 DNA as template on a 2 % agarose gel. M = size marker, 100 bp ladder (MP Biomedicals), 1 = 74 ng/reaction, 2 = 7.4 ng/reaction, 3 = 0.74 ng/reaction, 4 = 0.074 ng/reaction, 5 = 0.0074 ng/reaction, 6 = 0.00074 ng/reaction, 7 = 0.000074 ng/reaction, 8 = negative control (water instead of DNA). The contrast of the picture was modified for better visibility of results.

3.1.4 Direct testing of fungal cultures with the Hyd5 ID4 LAMP assay

Usefulness of the Hyd5 ID4 LAMP assay was demonstrated by testing the reaction with mycelia, which were directly applied as template without any previous DNA extraction. Cultures of 13 different *Fusarium* spp. as well as *Epicoccum nigrum* and *Alternaria mali* were grown on 8 different agar media (see legend to table 8). In order to analyze a possible influence of the agar media used on the LAMP reaction, blank pieces of medium were tested by comparison of the reactions after addition to the LAMP reaction with and without template DNA as previously described. MB added to the LAMP assay resulted in complete inhibition of the amplification reaction. Also adding mycelia directly to the LAMP reaction, none of the cultures grown on MB showed a positive LAMP reaction. When mycelia from cultures grown OA, PDA, SFA, SNAmod, YES and MEA were used as template, only *F. cerealis* and *F. culmorum* showed positive LAMP reactions with cultures grown on all the media tested (table 8). In the case of *F. graminearum*, addition of mycelia from a culture grown on OA gave no LAMP signal whereas mycelia from all other media resulted in a positive LAMP signal. Addition of mycelia from cultures of *F. equiseti, F. heterosporum, F. poae, F. langsethiae, F. scirpi* and *F. sporotrichioides* did not result in a positive signal in the Hyd5 ID4 LAMP assay independently from the medium they were grown on. Cultures of *F. sambucinum, F. trichothecioides, F. venenatum, E. nigrum* and *A. mali* showed fluorescent LAMP signals upon growth on some of the media but were negative upon growth on others. With respect to the spectrum of species and their involvement as regards gushing, direct addition of SNAmod grown mycelia gave the most reproducible results because no false positive signals were observed in this study.

Table 8: Reactions of the Hyd5 ID4 LAMP assay after addition of mycelia from cultures of 12 different fungal species after growth on 8 different media.

Species	Isolate	Media*							
		A	B	C	D	E	F	G	H**
F. camptoceras	CBS 193.65	-	+	+	-	-	-	-	-
F. cerealis	CBS 589.93	+	+	+	+	+	+	+	-
F. culmorum	DSM 62191	+	+	+	+	+	+	+	-
F. equiseti	CBS 406.86	-	-	-	-	-	-	-	-

F. graminearum	DSM 4527	-	+	+	+	+	+	+	-
F. heterosporum	DSM 62231	-	-	-	-	-	-	-	-
F. poae	DSM 62376	-	-	-	-	-	-	-	-
F. langsethiae	IBT 9956	-	-	-	-	-	-	-	-
F. scirpi	CBS 448.84	-	-	-	-	-	-	-	-
F. sambucinum var. sambucinum	CBS 185.29	-	-	-	-	-	+	+	-
F. sporotrichioides	CBS 412.86	-	-	-	-	-	-	-	-
F. trichothecioides	CBS 136.73	-	-	+	-	-	-	-	-
F. venenatum	CBS 458.93	-	+	-	-	-	-	-	-
Eppicocum nigrum	TMW 4.0483	+	+	-	-	-	-	-	-
Alternaria mali	CBS 106.24	-	+	-	-	+	+	+	-

*Media: A: Oatmeal agar (OA); B: Potato dextrose agar (PDA); C: Selective Fusarium Agar (SFA); D: Synthetischer Nährstoffarmer Agar (SNA); E: Yeast extract sucrose agar (YES); F: Yeast peptone glucose agar (YPG); G: Malt extract agar (MEA); H: Mung bean agar (MB) ** with mycelia grown on plain mung bean agar no positive results could be obtained.

3.1.5 Application of the Hyd5 ID4 LAMP assay for detection of hydrophobin Hyd5p producing fungi in barley grains

In order to demonstrate the usefulness of the developed LAMP assay, barley grains were directly tested for the presence of fungi carrying the Hyd5p encoding gene. For setting up a sample preparation protocol and testing its sensitivity, a model sample containing 50 % (number/number) of grains infected with *F. culmorum* was mixed with a *Fusarium*-free barley sample to result in various different infection levels ranging from 0.5 % to 50 %. In a preliminary experiment, samples were mixed with distilled water and the supernatant was directly used as template in the Hyd5 ID4 LAMP assay. Results obtained revealed high sensitivity of the method, but repeatability was unacceptably poor. This problem was overcome by heating samples after mixing them with sterile tap

water. For sample preparation the water used must not necessarily be sterile because the supernatant is used immediately as template in the LAMP reaction and there is no incubation time in which contaminants from the water might multiply. Moreover, sterilization of water does not destroy potentially contaminating DNA. Heating up the samples was necessary in order to disrupt fungal cell membranes and to set the DNA free for analysis (Tournas, 1994). Results revealed that the time needed to set free detectable concentrations of target DNA depended on the ratio of volumes of grains and water applied. Applying a grain-water ratio of 1:1.75, a bigger amount of water and grains needed to be heated up for a longer period of time. 40 g of grains with 70 ml of distilled water needed to be treated in a boiling bath for 10 min, while 200 g of grains with 350 ml distilled water needed to be treated for at least 20 min. Using supernatants from grain samples treated as described above as template in the LAMP assay revealed that 0.5 % of *F. culmorum* infected grains were reproducibly detected as contamination in an uninfected model barley sample (figure 10).

The Hyd5 ID4 LAMP assay was tested with MEBAK standards because many German breweries today rely on standards set up by the MEBAK Commission (MEBAK, 2006) as reference for quality control of their raw materials (Daniel Schock, German Brewers Association, personal communication, October 18th, 2011). According to the regulations, the limit for the number of malt grains with a typical red to violet discoloration in brewing malt samples is supposed to be 5/200 g. In order to analyze samples, 200 g of grains are analyzed visually grain by grain using a magnification glass. In the current work it was tested whether the Hyd5 ID4 LAMP assay can be used to detect grains infected with potential gushing inducing *Fusarium* spp. in a malt sample with sensitivity comparable to that of the standard MEBAK method. Wheat grains were infected with *F. graminearum* DSM 4527 during the malting process to result in heavily contaminated malt with masses of grains showing the typical deep red discoloration. Wheat grains were used because infection has a better visibility as compared to barley. Infected grains can easily be differentiated by their red to violet discoloration, often with visible mycelium on the grain's surface. Batches of 200 g of brewing barley grains previously found to be uncontaminated by *Fusarium* spp. after microbiological analysis on SNAmod medium were mixed with different numbers of *F. graminearum* contaminated wheat malt grains and the complete batch was mixed with 350 ml distilled water and treated in a boiling water bath for 20 min. Using the supernatant directly as template in the LAMP

assay showed that a minimum of 3 infected grains in 200 g of brewing barley were reproducibly detected (figure 11).

Results indicated that the LAMP-based detection method may provide an alternative for cumbersome manual counting of discolored grains in malt and cereal samples in the brewing industry. Using this simple protocol it was possible to detect fungal infection when the proportion of infected grain was as low as 0.5 % (w/v). Even lower proportions were detected but results were not always fully reproducible. Addition of single visibly infected wheat malt grains to 200 g portions of uninfected barley revealed that the assay fulfills the quality requirements adopted by many European breweries in their raw material contracts according to which the number of visibly infected ("relevant") grains should be five or less in 200 g of brewing barley (MEBAK, 2006). The detection limit of the current assay was three grains in 200 g of non-infected barley malt grains. Addition of five grains of wheat, which were heavily infected with *F. poae* during malting (Zapf et al., 2006), to 200 g of uninfected barley did not result in detection of a LAMP signal when samples were treated as described previously. This result was in accordance with the results obtained with pure DNA and also with culture material of the fungus. Although Sarlin et al. (2005) demonstrated that the addition of small concentrations of a hydrophobin purified from *F. poae* induced gushing in beer, the fungus cannot be assumed relevant for gushing induction. According to results published by Niessen et al. (1992), the fungus neither caused excessive foaming of beer produced from grains contaminated with it during malting nor was it found in higher frequencies on grains of cereals samples which were tested positive in a gushing prediction test according to Donhauser et al. (1990). However, Northern European climate conditions are more suitable for *F. poae* infections in the field so that the fungus may occur more frequently and occasionally cause problems with gushing there.

Results presented here show that the LAMP-assay based on detection of the *hyd5* gene in fungi typically associated with gushing of beer can be used as a highly group specific and easy to use tool for quality control in the brewing industry. Application of the assay in cereals and brewing malt revealed comparable or even higher sensitivity as compared to visual detection of *Fusarium* contaminated grains in brewing cereals and malt. Therefore, it may provide breweries, malting companies, grain suppliers and grain producers with a powerful new tool for quality control.

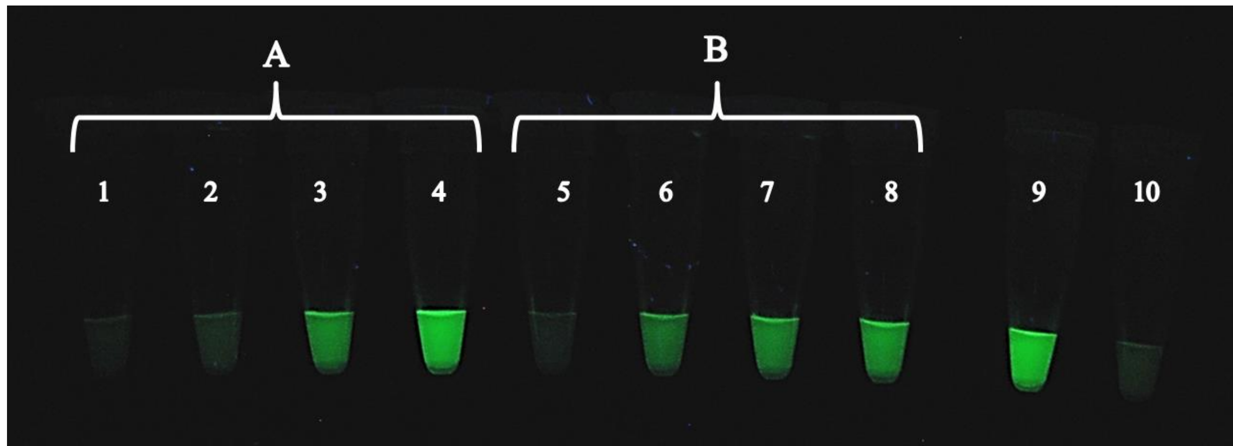


Figure 10: LAMP reactions using primer set Hyd5 ID4 with 2 µl supernatant from model barley samples spiked with 2 %, 1 %, 0.5 % and 0 % (number/number) of *F. culmorum* infected grains, respectively, after treatment in a boiling water bath for 5 min (A) and 10 min (B). Calcein fluorescence under UV_{366nm} light. 1 = 0 %, 2 = 0.5 %, 3 = 1 %, 4 = 2 %, 5 = 0 %, 6 = 0.5 %, 7 = 1 %, 8 = 2 %, 9 = positive control (pure DNA of *F. graminearum* DSM 4527), 10 = negative control (water instead of DNA). The contrast of the picture was modified for better visibility.

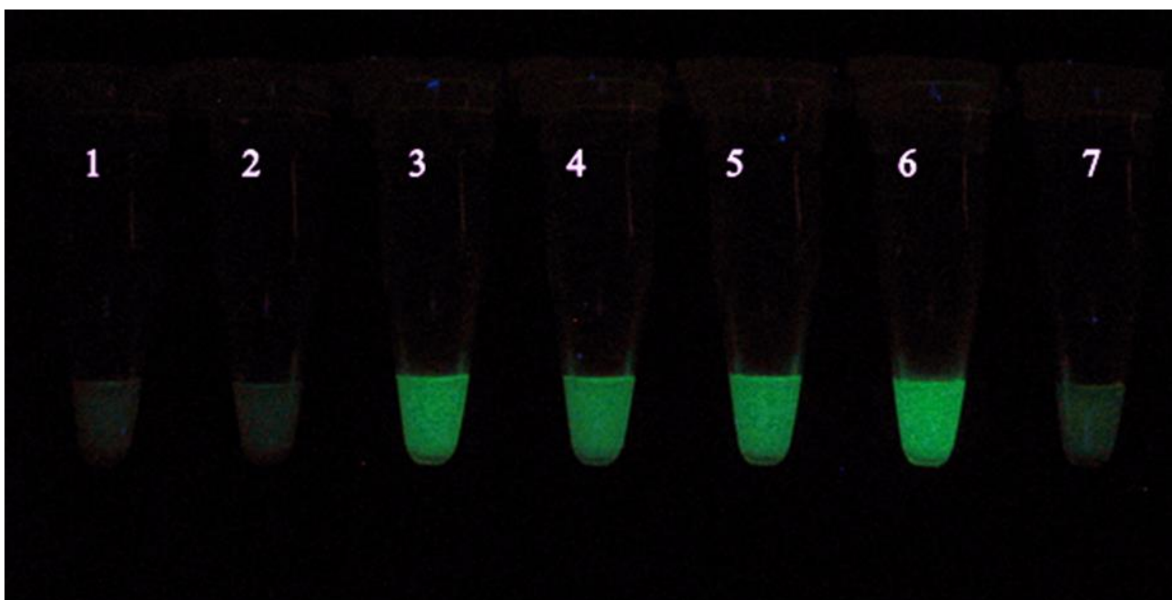


Figure 11: LAMP reactions using primer set Hyd5 ID4 with 2 µl supernatant from 200 g uninfected barley malt containing 0, 1, 3, 5, 8 of *F. graminearum* TMW 4.0175 infected wheat malt grains, respectively, after treatment in a boiling water bath for 20 min. Calcein fluorescence under UV_{366nm} light. 1 = 0, 2 = 1, 3 = 3, 4 = 5, 5 = 8 infected grains added to 200 g of uninfected grains, 6 = positive control (pure DNA of *F. graminearum* DSM 4527), 7 = negative control (water instead of DNA). The contrast of the picture was modified.

3.1.6 Optimization of real-time Hyd5 ID4 LAMP assay conditions with the turbidimeter and tube scanner device

Using the LA-320c real-time turbidimeter, the Hyd5 ID4 LAMP assay showed optimum performance at 62.5 °C. Specificity of the assay was assessed using DNA isolated from the same array of tester strains (see table 7). The specificity as previously established

was fully verified. In order to increase the effectiveness of the LAMP reaction, the influence of chemically inert water-soluble polymers on the performance of the LAMP reaction was tested. Addition of polyethylene glycol (PEG) had a highly increasing effect on the speed of the LAMP reaction. Several different types of PEG with different average molecular weights were tested. PEG 8000 (PEG 8K) revealed to have the highest effect on the speed of the LAMP reaction. Optimization of the concentration of PEG 8K in the master mix revealed that addition of 5.7 % (w/v) PEG 8K resulted in a 16 % reduction of T_t in a reaction containing 62.5 ng/reaction of *F. culmorum* TMW 4.0548 DNA as compared to the same reaction performed without PEG 8K in the master mix. Figure 12 depicts the results and shows that the effect was even more considerable (35 %) when the reaction was run in the tube scanner instrument with fluorescence detection of DNA amplification.

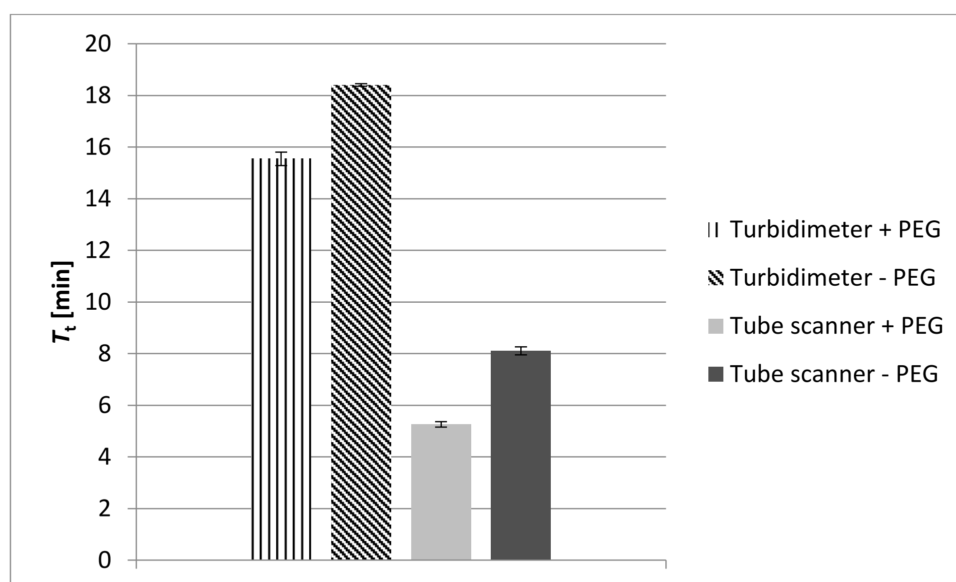


Figure 12: Effect of 5.7 % (w/v) PEG 8K on time to threshold (T_t [min]) in real-time LAMP reactions run in a real-time turbidimeter and in the tube scanner device. Reactions were run using the Hyd5 ID4 primer set and 62.5 ng/reaction of *F. culmorum* TMW 4.0548 DNA as template. Reactions with and without PEG 8 K added were run in parallel quadruples on both platforms.

In order to further characterize and optimize the turbidimeter based real-time LAMP assay, influence of loop primers on the performance of the LAMP reaction was analyzed. To determine the range of the standard curve and the detection limit of the assay, a fivefold serial dilution of DNA from *F. culmorum* TMW 4.0548 was used as template in the turbidimeter based real-time LAMP assay to find the optimum range for the standard curve. The standard curve was tested with loop primers (see figure 13, lower graph) and without loop primers (see figure 13, upper graph). The standard curves were generated by plotting the T_t (time to threshold) versus $\log_5$ of genome copy numbers per

reaction. The range of highest reproducibility of both standard curves was between 2.17 x 10^6 and 695 genome copies per reaction with an average standard deviation of 3 % with loop primers and 4.1 % without loop primers. Both assay variants displayed a high correlation between parameters (R^2 = 0.94 and 0.985). The lower limit of detection of the assay was determined to be 27 gene copies. DNA concentrations below this lower limit did not result in a measurable signal within 1 h of reaction time.

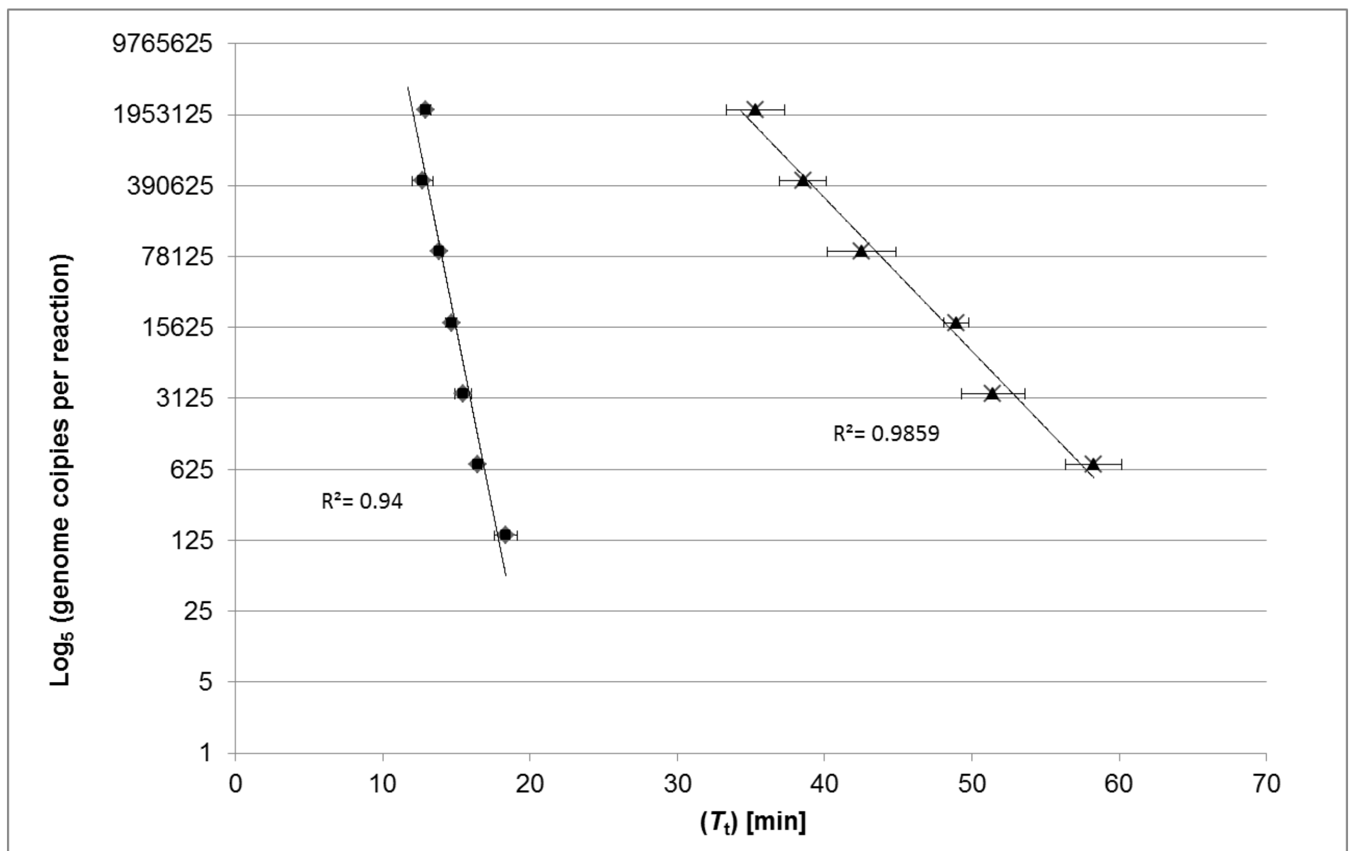


Figure 13: Standard curves for the turbidimeter-based real-time LAMP for determination of copy numbers of the *hyd5* gene in gushing inducing fungi using primer set Hyd5 ID4 with loop primers (■) and without loop primers (▲). Log_5 dilution of *F. culmorum* TMW 4.0548 DNA was plotted against the time after which the first derivative of turbidity against time reached a threshold of 0.05 (T_t) [min].

Results shown in figure 13 demonstrate that with loop primers added the reaction started about 20 min earlier as compared to the reaction involving no loop primers. Moreover, with loop primers added, the exponential regression of the data revealed a much steeper slope with nearly no standard deviation between replicates. In contrast to this, the curve resulting from the LAMP performed without loop primers added was less steep. In this curve the standard deviation was much higher as compared to the standard deviation involving loop primers. However, the fit between the curve and the data points was better when loop primers were used. In order to analyze which of the

two standard curves would be most suitable for DNA quantification, seven DNA solutions from *F. culmorum* TMW 4.0548 were prepared to cover the concentrations between the data points of the standard curve and used as templates in either of the two turbidimeter based real-time LAMP assays. DNA concentrations in the three replicate samples per data point were calculated from the respective calibration curves (see figure 13) and measured quantities (calibration curve in figure 11) were plotted against predicted quantities (photometer) for either assay. The results are shown in figure 14. The equations calculated for both regressions showed a slope of 0.84 for the curve generated from measurement with loop primers whereas the slope of the curve generated without loop primers was only 0.41 demonstrating that the calibration curve generated with loop primers represents a nearly optimum correlation between measured vs. predicted DNA concentrations.

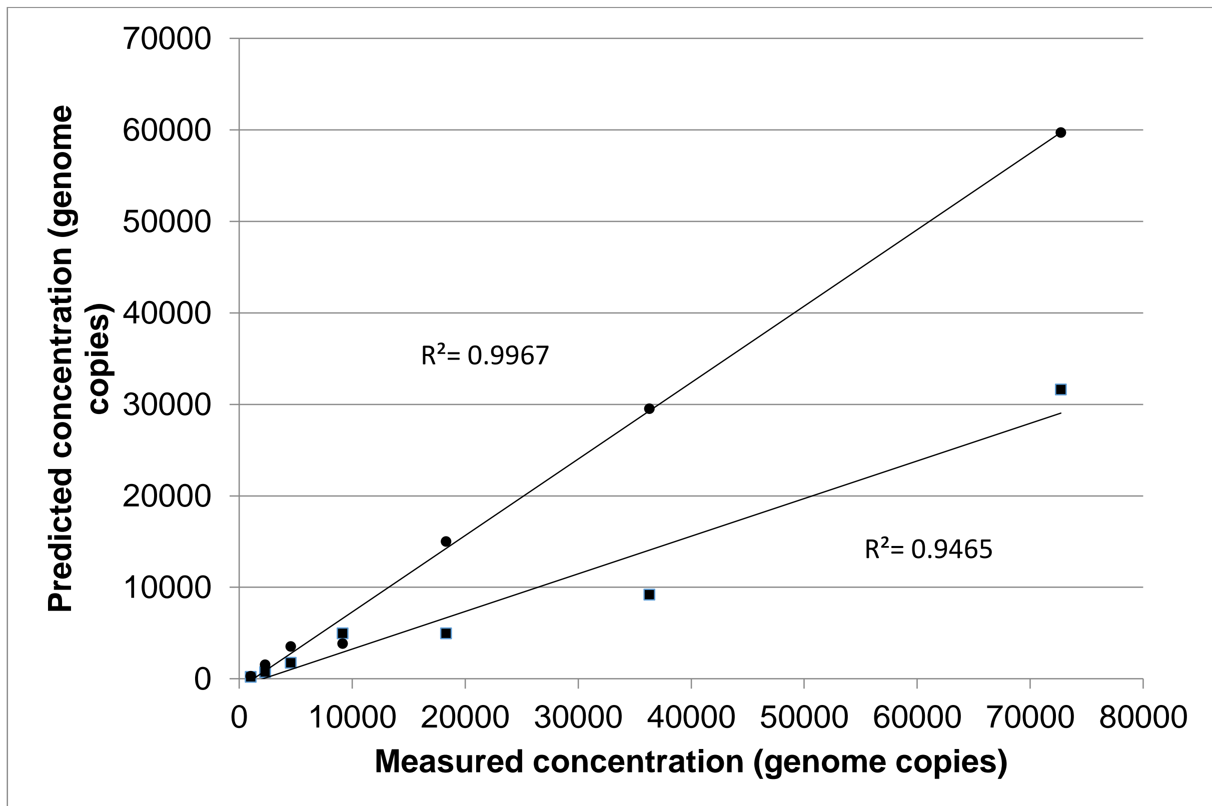


Figure 14: Correlation between predicted DNA concentrations (NanoDrop ND1000 spectrophotometer) and DNA concentrations calculated from standard curves generated in the real-time LAMP assay on a turbidimeter (● = with loop primers; ■ = without loop primers).

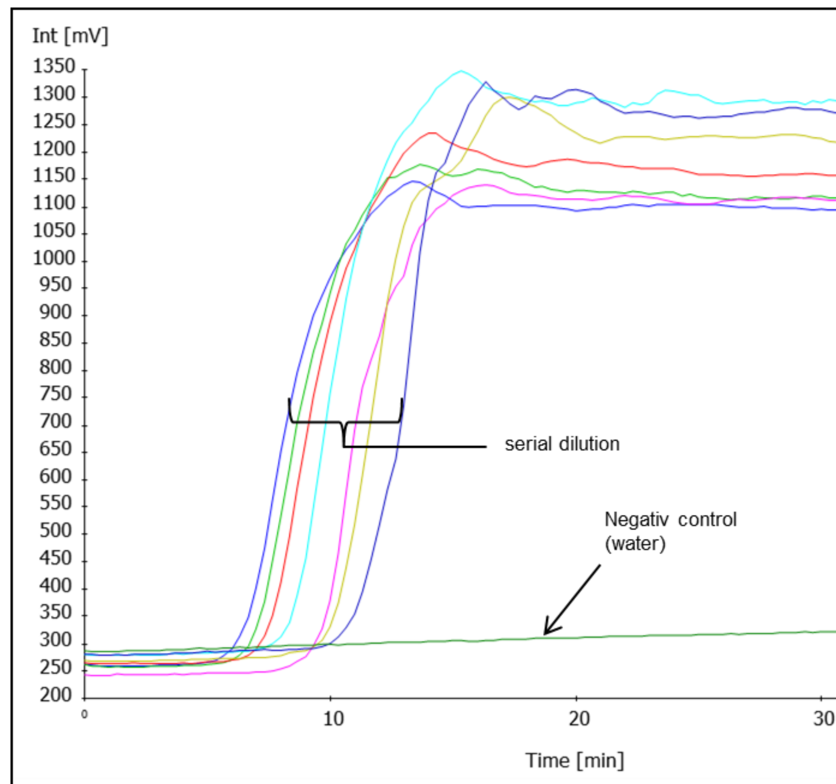


Figure 15: Fluorescence signal of V13-01184 dye (mV) over time (min) for the tube scanner based real-time LAMP using a fivefold serial dilution of DNA from *F. culmorum* TMW 4.0548 as template with the primer set Hyd5 ID4 (see also figure 16).

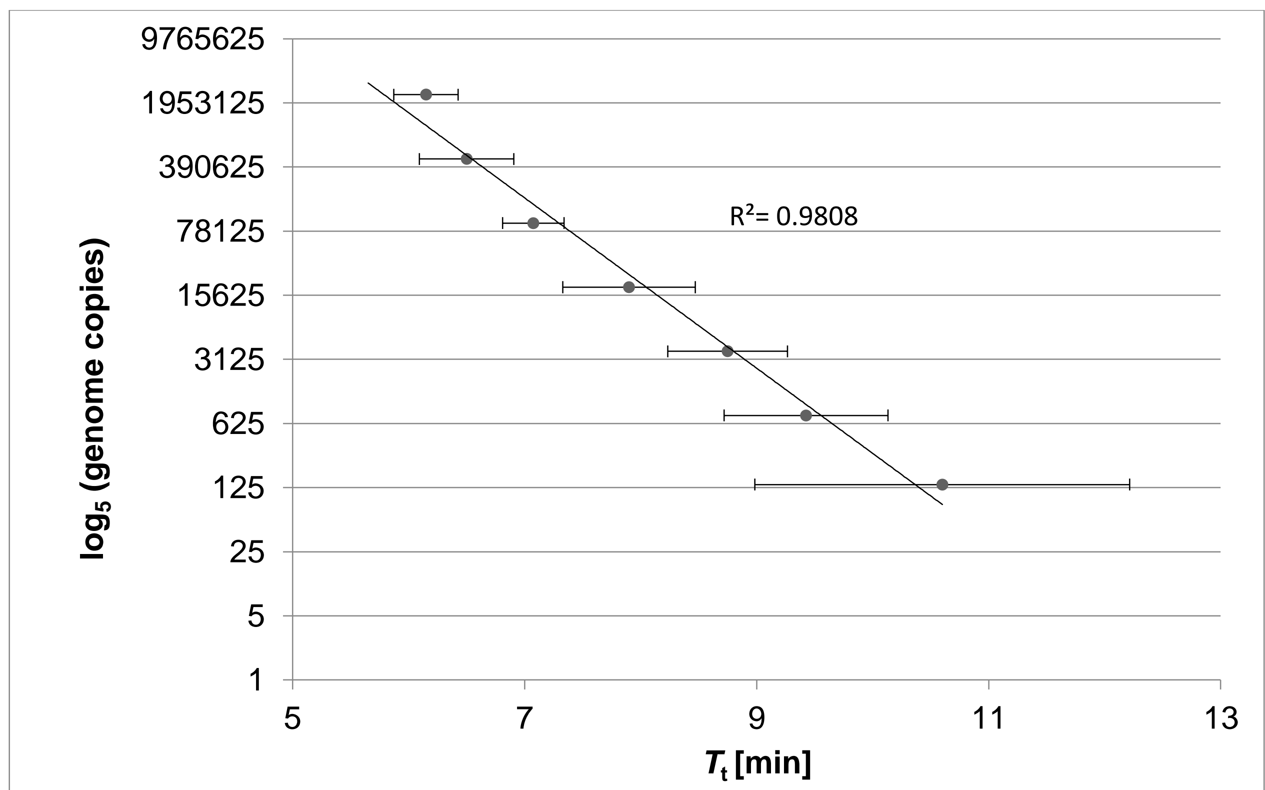


Figure 16: Standard curve for the tube scanner based real-time LAMP of a fivefold serial dilution of DNA from *F. culmorum* TMW 4.0548. Time to threshold (T_t) [min] was plotted against $\log_5$ of genome copies.

As with the turbidimeter, the optimum temperature had to be re-adjusted for the tube scanner, and was found to be 65 °C. Also for reactions run in the tube scanner, 5.7 % (w/v) PEG 8K was added and the effect was similar to the one shown in figure 12 for the turbidimeter-based assay. Addition of PEG resulted in a 35 % increase of speed of the LAMP reaction run in the tube scanner device (see figure 12). A fivefold serial dilution of DNA from *F. culmorum* TMW 4.0548 covering the range between 2.4 x 10^6 and 1.35 x 10^2 genome copies per reaction was used as template in a tube scanner based real-time LAMP with primer set Hyd5 ID4, including loop primers to analyze the detection range of the assay (figure 15 and figure 16). The standard curve was generated by plotting T_t (time to threshold 30 mV/sec) against the $\log_5$ of genome copies per reaction. Parameters showed a linear correlation between 2.4 x 10^6 and 135 genome copies per reaction and a good fit of the calculated regression (R^2 = 0.9808) was found. As for the turbidimeter-based assay, the lower limit of detection was also 27 genome copies per reaction. However, standard deviations found for T_t were generally high for measurements in the tube scanner device.

3.1.7 Application of real-time Hyd5 ID4 LAMP assay by estimation of *F. culmorum* infection levels in model barley sample materials

The field inoculated barley used here contained 80 % of *F. culmorum* infected grains. The barley used as uninfected control was found to be virtually free of any *Fusarium* infection. In order to obtain different ratios between uninfected barley and *F. culmorum* field inoculated barley, materials were mixed to result in 1 %, 5 %, 10 %, 30 %, 40 % (w/w) of infection levels**.** Total DNA was prepared three times from each mixture and from the uninoculated control in independent experiments and used as template in real-time LAMP reactions with loop primers under the optimized conditions described previously on both platforms. Figure 17 shows the results obtained in the turbidimeter-based assay for samples containing 10 % to 40 % (w/w) of infected barley.

As shown in figure 17, standard deviations found between individual measurements became high towards the lower end of the scale of infection levels demonstrating that quantification of lower infection levels is not possible with high accuracy. However, since signals of a positive LAMP reaction were reproducibly obtained with model samples at lower infection levels on both analytical platforms, it appeared to be worthwhile to determine the lower detection levels of infection. Both the turbidimeter-

and the tube scanner-based assays showed that samples with a minimum of 1 % (w/w) infected barley were detected within a reaction time of 40 min and 20 min, respectively. If no positive reaction is observed before the end of the reaction time, a sample can be supposed to contain less than 1 % of infected barley grains.

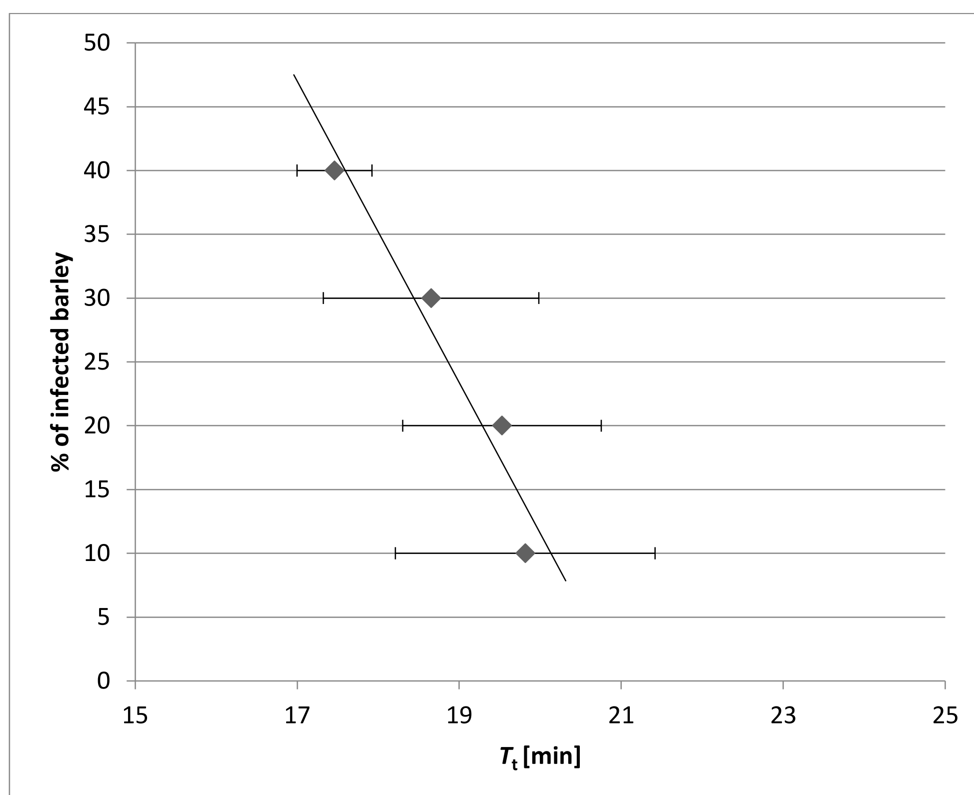


Figure 17: Turbidimeter-based real-time LAMP assay for the *hyd5* gene using total DNA extracted from model barley samples containing 10 % to 40 % (w/w) of infected barley. Five µl of extracted DNA per assay were used as template.

3.1.8 Correlation between Hyd5 ID4 LAMP results and gushing data in barley and malt

In order to analyze a possible correlation between infection levels of the model barley samples described previously and their potential to induce gushing, the mixtures of uninfected and *F. culmorum* infected barley were analyzed with the MCT. Results presented in table 9 show that all samples with infection levels between 10 % and 40 % (w/w) were positive in the MCT meaning that the addition of an extract to carbonated water resulted in an excessive foaming volume of at least 5 ml from a 330 ml bottle. No strict correlation occurred between infection levels and gushing volumes. The uninfected barley as well as the samples with infection levels below 10 % (w/w) were

gushing negative. In contrast to this, real-time LAMP assays were positive in all samples except from the negative control.

Table 9: Gushing inducing potential (MCT test) of model barley samples infected with *F. culmorum* to result in different infection levels.

Percentage of *F. culmorum* infected barley (w/w)	MCT gushing volume [ml]	
	average	standard deviation
40 %	77	10
30 %	151	9
20 %	73	19
10 %	37	7
5 %	0	0
1 %	0	0
0 %	1	1

After testing barley with defined infection levels, barley malt samples from the market were tested in order to analyze for possible correlations between their gushing potential (MCT) and the results of the real-time LAMP assays described here. Malt was defined gushing positive when the gushing volume of carbonated water was in excess of 5 ml (personal communication K. Pieczonka, quality management Privatbrauerei Erdinger Weißbräu Werner Brombach GmbH, Erding, Germany, November 19th, 2012). DNA was isolated from 50 barley malt samples and tested with the LAMP assay on the turbidimeter and the tube scanner platforms with three repetitions for each sample. Since no strict correlation was found between gushing volume in the MCT and T_t values in neither of the real-time LAMP assays, only a possible positive/negative correlation for both parameters was analyzed. LAMP results were termed positive if the LAMP signal occurred before 40 min in the turbidimeter or before 20 min in the tube scanner assays, respectively, in all three replications of the reaction. The LAMP results and gushing activities of corresponding samples are listed in table 10 for turbidimeter and the tube scanner based assays. Altogether, 32 of the malts were MCT positive and 18 were tested negative. LAMP results obtained on the different platforms were in good agreement in most samples. Only in 4 of the samples the result of the measurement differed between the two assays. Both the turbidimeter assay after 40 min and the tube scanner assay after 20 min indicated 9 positive and 41 negative results. Comparison of the LAMP

results obtained on both platforms with the MCT results showed that 32 of the 50 malt samples were indicated to have a gushing inducing potential. A positive correlation of gushing potential and LAMP signal was found in only nine of the samples. Consistence between negative MCT result and negative result in the LAMP assays was found in 17 of the malts. Altogether, consistence of results between MCT and LAMP was found in 50 % of analyzed samples.

Table 10: Comparison of the MCT results of 50 barley malts with the results obtained from LAMP-based measurement on the tube scanner and the real-time turbidimeter.

Result			Number of samples (percentage)
MCT	**LAMP turbidimeter**	**LAMP tube scanner**	
+	+	+	6 (12 %)
+	-	-	22 (44 %)
+	-	+	4 (8 %)
+	+	-	0 (0 %)
-	+	+	1 (2 %)
-	-	-	17 (34 %)
-	-	+	0 (0 %)
-	+	-	0 (0 %)

3.2 Development and application of a Duplex LAMP assay for the group specific detection of important trichothecene producing *Fusarium* species

3.2.1 Primer design

Two sets of LAMP primers were generated from the 694 bp trichodiene synthase (*tri5*) gene sequence (GenBank accession number DQ676573) of *F. sporotrichioides* strain UAMH 5334 and from the 657 bp *tri6* gene sequence coding for a regulatory protein in the biosynthesis of trichothecenes (GenBank accession number AB514437) of *Gibberella zeae* strain MAFF 240562. All sets suggested by the program were tested by *in silico* hybridization against the NCBI nucleotide sequence database for similarities with sequences of other organisms using the nucleotide BLAST algorithm for highly similar sequences (Altschul et al., 1990). Primer sets were selected according to the *in silico* results showing hybridization with the broadest possible spectrum of established trichothecene producing *Fusarium* spp. *In silico* analysis revealed broad and non-overlapping spectra of detected species for primer sets Tri6 ID1 and Tri5 ID4. Primer binding sites within the corresponding gene sequence for each of the primers used are shown in figure 18.

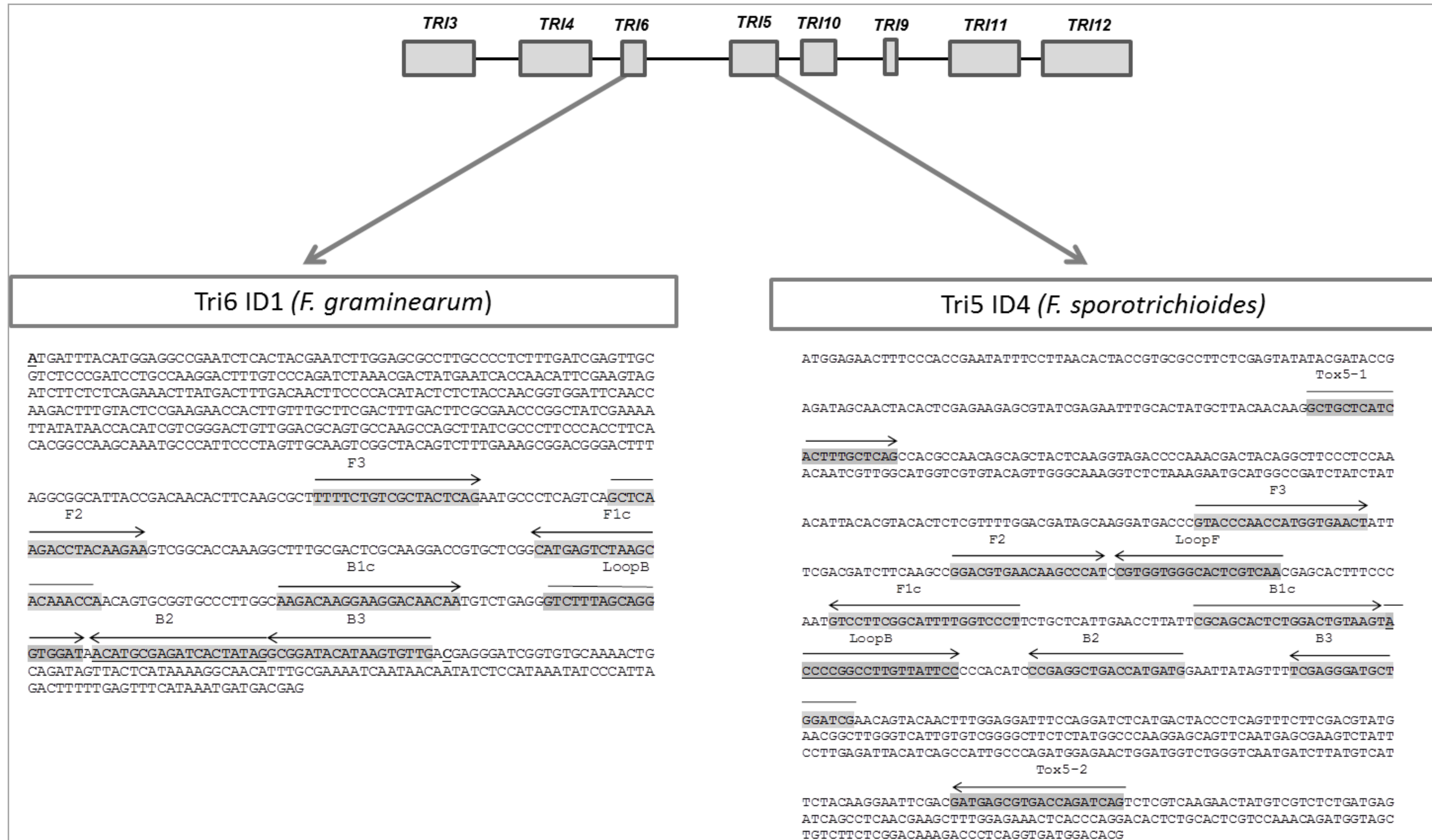


Figure 18: Schematic organization of the *tri* gene cluster in *Fusarium* spp. and positions of LAMP and PCR primers in *tri6* and *tri5* genes selected as LAMP targets.

In table 11, the sequences of the primers are compared with the corresponding sequences found in several strains which gave a positive LAMP result with the either of the two primer sets. Nucleotides differing from the primer sequence are marked in grey. Analysis showed that especially the sequences of *F. poae* strains displayed a great difference when compared to the sequences of the Tri5-ID4 primer set. This effect was also shown when results given in table 12 were analyzed. Multiple strains of all LAMP positive target species were tested in order to evaluate the sensitivity of the LAMP assays for the different species and possible variation of results. In the experiment all tested isolates strains reacted positive except for *F. poae*, where only one strain out of six tested gave a positive signal in the Tri5 ID4 assay as well as in the combined LAMP assay. Morphological revision of the non-reacting *F. poae* strains revealed that they were clearly assigned to that species.

Table 11: List of primers used in LAMP and PCR experiments. LAMP primers used during the study are marked in bold. Primer binding sites in the detected species are shown for comparison. Nucleotides differing from primer sequence are marked in grey.

Primer list		Source
LAMP primer set Tri6 ID1		this study
FIP-Tri6-ID1	**5’-TGGTTTGTGCTTAGACTCATG-GCTCAAGACCTACAAGAA-3’**	
F. graminearum	5’-TGGTTTGTGCTTAGACTCATG-GCTCAAGACCTACAAGAA-3’	
F. cerealis	5’-TGGTTTGTGCTTAGACTCATG-GCTCAAGACCTACAAGAA-3’	
F. culmorum	5’-TGGTTTGTGCTTAGACTCATG-GCTCAAGACCTACAAGAA-3’	
BIP-Tri6-ID1	**5’-AAGACAAGGAAGGACAACAA-CTATAGTGATCTCGCATGT-3’**	
F. graminearum	5’-AAGACAAGGAAGGACAACAA-CTATAGTGATCTCGCATGT-3’	
F. cerealis	5’-ACGACCAGGAAGGACAACAA-CTATAGTGATCTCGCATGT-3’	
F. culmorum	5’-AAGACAAGGAAGGACAACAA-CTATAGTGATCTCGCATGT-3’	
F3-Tri6-ID1	**5’-TTTTCTGTCGCTACTCAG-3’**	
F. graminearum	5’-TTTTCTGTCGCTACTCAG-3’	
F. cerealis	5’-TTTTCTGTCGCTACTCAG-3’	
F. culmorum	5’-TTTTCTGTCGCTACTCAG-3’	
B3-Tri6-ID1	**5’-CAACACTTATGTATCCGC-3’**	
F. graminearum	5’-CAACACTTATGTATCCGC-3’	
F. cerealis	5’-CAACCCTTGTGTATCCGC-3’	
F. culmorum	5’-CAACACTTATGTATCCGC-3’	
Loop B-Tri6-ID1	**5’-ACCCCGGCCTTGTTATTCC-3’**	
F. graminearum	5’-ACCCCGGCCTTGTTATTCC-3’	
F. cerealis	5’-ACCCCGGCCTTGTTATTCC-3’	
F. culmorum	5’-ACCCCGGCCTTGTTATTCC-3’	
LAMP primer set Tri5 ID4		
FIP-Tri5-ID4	**5’-AGGGACCAAAATGCCGAAGGAC-GGACGTGAACAAGCCCAT-3’**	
F. sporotrichioides	5’-AGGGACCAAAATGCCGAAGGAC-GGACGTGAACAAGCCCAT-3’	
F. poae	5‘-AGGGACCAAAATGTCGAAGGAC-GGACGAGAACAGGCTCAC-3‘	

F. langsethiae	5‘-AGGGACCAAAATGCCGAAGGAC-GGACGTGAACAAGCCCAT-3’	
BIP-Tri5-ID4	**5’-CGCAGCACTCTGGACTGTAAGT-CATCATGGTCAGCCTCGG-3‘**	
F. sporotrichioides	5’-CGCAGCACTCTGGACTGTAAGT-CATCATGGTCAGCCTCGG-3‘	
F. poae	5’-CGCAGCACTCTGGACTGTAAGT-CATTCTTCACATCCTTGG-3‘	
F. langsethiae	5’-CGCAGCACTCTCGACTGTAAGT-CATCATTGTCAGCTTCGG-3‘	
F3-Tri5-ID4	**5’-GTACCCAACCATGGTGAACT-3’**	
F. sporotrichioides	5’-GTACCCAACCATGGTGAACT-3’	
F. poae	5‘-ATATCCAGCCATGATGAACT-3‘	
F. langsethiae	5’-GTACCCAACCATGGTGAACT-3’	
B3-Tri5-ID4	**5’-CGATCCAGCATCCCTCGA-3’**	
F. sporotrichioides	5’-CGATCCAGCATCCCTCGA-3’	
F. poae	5‘-CGATCCAGCATCCCTCAA-3‘	
F. langsethiae	5’-CGATCCAGCATCCCTCGA-3’	
Loop F-Tri5-ID4	**5’-TTGACGAGTGCCCACCACG-3‘**	
F. sporotrichioides	5’-TTGACGAGTGCCCACCACG-3‘	
F. poae	5‘-TTGACGAGTGCCCACCACG-3‘	
F. langsethiae	5‘-TTGACGAGTGCCCACCACG-3‘	
Loop B-Tri5-ID4	**5’-ACCCCGGCCTTGTTATTCC-3’**	
F. sporotrichioides	5’-ACCCCGGCCTTGTTATTCC-3’	
F. poae	5’-ACACTGGATACATTGTTTC-3’	
F. langsethiae	5’-ACCCCGGCCTTGTTATTCC-3’	
PCR primer pair Tox5		Niessen and Vogel (1998)
Tox5-1	5'-GCTGCTCATCACTTTGCTCAG-3'	
Tox5-2	5'-CTGATCTGGTCACGCTCATC-3'	

3.2.2 Optimization of Duplex LAMP assay conditions with the turbidimeter

Temperature optima for the LAMP reactions both with primer sets Tri6 ID1 and Tri5 ID4 were found to be at 64 °C when the Loopamp LA-320C real-time turbidimeter was used for incubation and measurement. The LA-320CE software package (EIKEN Chemical Co., LTD, Tokyo, Japan) was used to control the turbidimeter and the real-time measurement. Turbidity of LAMP reactions was monitored at 650 nm in a maximum of 32 parallel reactions in 4 independent heating blocks. Measurement of turbidity in each individual reaction was done in intervals of 6 s. A threshold of 0.05 for the first derivative of turbidity against time was set to measure time to threshold (T_t) throughout all experiments. Testing for specificity of the Tri6 ID1 and Tri5 ID4 primer sets was done using gDNA isolated from 127 fungal isolates representing 112 different fungal species within 13 genera. Also gDNA isolated from wheat was tested in order to make sure no interference with background DNA would occur when analyzing sample materials. The time to threshold method was used to decide whether a LAMP signal was positive or negative. For a positive signal the time needed for the second derivative of turbidity over time to reach a value of 0.05 was below 60 min. Both primer sets were tested in separate assays as well as in an assay in which both sets were combined. As shown in table 12, the LAMP assay using the Tri5 ID4 primer set resulted in T_t values <60 min when gDNA isolated from cultures of *F. langsethiae, F. sporotrichioides* and *F. poae* was added as template. Using the Tri6 ID1 primer set, T_t values <60 min occurred with gDNA isolated from pure cultures of *F. graminearum, F. cerealis* and *F. culmorum*. When both primer sets were combined in a Duplex LAMP assay, analysis of specificity resulted in a combined set of species detected (see table 12). For all other species tested there was either no signal at all or the time for the signal to reach the threshold value was >60 min in single set assays or in the Duplex set up.

Table 12: Fungal cultures used during the current study and their reaction with *tri5* and *tri6* specific LAMP assays as well as in a Duplex LAMP assay. Positive reactions (T_t [min] < 60 min) marked with (+). Negative reactions (T_t [min] >60 min) marked with (-).

Genus	*Species*	*Strain*	*Clone*	*LAMP result*		
				Tri5 ID4	**Tri6 ID1**	**Duplex: Tri5 ID4+ Tri6 ID1**
Alternaria	*alternata*		[g] TMW 4.0438	-	-	-
Aspergillus	*alliaceus*	[c] DSM 813	TMW 4.1077	-	-	-
	carbonarius	[l]M 324	TMW 4.1512	-	-	-
	clavatus	[b] CBS 513.65	TMW 4.1512	-	-	-
	flavus	CBS 113.32	TMW 4.1085	-	-	-
	fumigatus	CBS 113.55	TMW 4.0623	-	-	-
	niger	CBS 101698	TMW 4.1068	-	-	-
	ochraceus	CBS 263.67	TMW 4.0706	-	-	-
	parasiticus	CBS 126.62	TMW 4.1768	-	-	-
	terreus	CBS 377.64	TMW 4.1060	-	-	-
	tubingensis	[e] ITEM 4496	TMW 4.2008	-	-	-
	versicolor	CBS 245.65	TMW 4.1600	-	-	-
Drechslera	*teres*	CBS 378.59	TMW 4.0558	-	-	-
Epicoccum	*nigrum*		TMW 4.0483	-	-	-
	acutatum	BBA 69580	TMW 4.0835	-	-	-
Fusarium	*anguioides*	CBS 172.32	TMW 4.0460	-	-	-
	annulatum	CBS 258.54	TMW 4.0461	-	-	-
	anthophilum	CBS 222.76	TMW 4.0490	-	-	-
	anthophilum	BBA 63270	TMW 4.0880	-	-	-
	aquaeductum	CBS 837.85	TMW 4.0914	-	-	-
	armeniacum	CBS 485.94	TMW 4.0701	-	-	-
	arthrosporioides	CBS 173.32	TMW 4.0463	-	-	-
	avenaceum	DSM 62161	TMW 4.0140	-	-	-
	bactridioides	CBS 177.35	TMW 4.0464	-	-	-
	begoniae	BBA 69131	TMW 4.1019	-	-	-

	beomiforme	[a] BBA 69406	TMW 4.0513	-	-	-
	brevicatenulatum	BBA 69197	TMW 4.1020	-	-	-
	buharicum	CBS 796.70	TMW 4.0627	-	-	-
	bulbicola	BBA 63620	TMW 4.0946	-	-	-
	caucasicum	CBS 179.35	TMW 4.0492	-	-	-
	cavispermum	CBS 172.31	TMW 4.0491	-	-	-
	cerealis	CBS 589.93	TMW 4.0406	-	+	+
	cerealis		TMW 4.0128	-	+	+
	chlamydosporum	CBS 145.25	TMW 4.0404	-	-	-
	circinatum	BBA 69855	TMW 4.0864	-	-	-
	coeruleum	CBS 836.85	TMW 4.0494	-	-	-
	compactum	CBS 466.92	TMW 4.0433	-	-	-
	concentricum	BBA 69720	TMW 4.0853	-	-	-
	concolor	CBS 183.34	TMW 4.0556	-	-	-
	culmorum	DSM 62191	TMW 4.1049	-	+	+
	culmorum		TMW 4.0539	-	+	+
	culmorum		TMW 4.0540	-	+	+
	decemcellulare	CBS 113.57	TMW 4.0493	-	-	-
	denticulatum	BBA 65244	TMW 4.0980	-	-	-
	detonianum	CBS 736.79	TMW 4.0557	-	-	-
	dimerum	CBS 175.31	TMW 4.0626	-	-	-
	diversisporum	VLB 67/1	TMW 4.0450	-	-	-
	dlamini	[f] MRC 3024	TMW 4.0571	-	-	-
	equiseti	CBS 406.86	TMW 4.0477	-	-	-
	equiseti	DSM 62202	TMW 4.0164	-	-	-
	eumartii	DSM 62809	TMW 4.0303	-	-	-
	globosum	MRC 6646	TMW 4.0584	-	-	-
	graminearum	DSM 4529	TMW 4.0185	-	+	+
	graminearum		TMW 4.0173	-	+	+
	graminearum		TMW 4.0169	-	+	+
	graminum	DSM 62224	TMW 4.0209	-	-	-

	guttiforme	BBA 69661	TMW 4.0844	**-**	**-**	**-**
	lactis	BBA 68590	TMW 4.1004	**-**	**-**	**-**
	langsethiae	[d] IBT 9956 [T]	TMW 4.0072	**+**	**-**	**+**
	langsethiae		TMW 4.0077	**+**	**-**	**+**
	langsethiae		TMW 4.0766	**+**	**-**	**+**
	larvarum	CBS 783.79	TMW 4.0434	**-**	**-**	**-**
	longipes	CBS 739.79	TMW 4.0350	**-**	**-**	**-**
	macroceras	CBS 146.25	TMW 4.0419	**-**	**-**	**-**
	melanochlorum	CBS 202.65	TMW 4.0625	**-**	**-**	**-**
	merismoides var. chlamydosporum	DSM 62256	TMW 4.0247	**-**	**-**	**-**
	musarum	ITEM 1294	TMW 4.0361	**-**	**-**	**-**
	napiforme	BBA 67629	TMW 4.0510	**-**	**-**	**-**
	nelsonii	ITEM 1681	TMW 4.0363	**-**	**-**	**-**
	neoceras	CBS 147.25	TMW 4.0349	**-**	**-**	**-**
	nygamai	BBA 67375	TMW 4.0512	**-**	**-**	**-**
	oxysporum		TMW 4.0225	**-**	**-**	**-**
	phyllophilum	BBA 63639	TMW 4.0795	**-**	**-**	**-**
	poae	ITEM 3778	TMW 4.1056	**+**	**-**	**+**
	poae	DSM 62376	TMW 4.0134	**-**	**-**	**-**
	poae		TMW 4.0714	**-**	**-**	**-**
	poae	IBT-1876	TMW 4.0789	**-**	**-**	**-**
	poae		TMW 4.0092	**-**	**-**	**-**
	poae		TMW 4.0043	**-**	**-**	**-**
	proliferatum	DSM 62261	TMW 4.0236	**-**	**-**	**-**
	pseudoanthophilum	BBA 70128	TMW 4.0807	**-**	**-**	**-**
	pseudocircinatum	BBA 69598	TMW 4.0842	**-**	**-**	**-**
	pseudonygamai	BBA 69551	TMW 4.0829	**-**	**-**	**-**
	ramigenum	BBA 68592	TMW 4.1006	**-**	**-**	**-**
	reticulatum	DSM 62395	TMW 4.0305	**-**	**-**	**-**
	robustum	CBS 637.76	TMW 4.0418	**-**	**-**	**-**

	sacchari	BBA 69863	TMW 4.0872	-	-	-
	sacchari var. subglutinans	CBS 215.76	TMW 4.0408	-	-	-
	sambucinum	CBS 185.29	TMW 4.0165	-	-	-
	scirpi	CBS 448.84	TMW 4.0410	-	-	-
	semitectum var. majus	DSM 63310	TMW 4.0191	-	-	-
	setosum	CBS 574.94	TMW 4.1059	-	-	-
	solani	DSM 62416	TMW 4.0255	-	-	-
	sporotrichioides	CBS 412.86	TMW 4.0475	+	-	+
		DSM 62425	TMW 4.0273	+	-	+
		ITEM 194	TMW 4.1685	+	-	+
	subglutinans	BBA 63621	TMW 4.0947	-	-	-
	sublunatum var. sublunatum	CBS 189.34	TMW 4.0417	-	-	-
	succisae	DSM 63162	TMW 4.0309	-	-	-
	sulphureum	DSM 62433	TMW 4.0310	-	-	-
	tabacinum	DSM 2125	TMW 4.0284	-	-	-
	thapsinum	BBA 69581	TMW 4.0836	-	-	-
	torulosum	BBA 64465	TMW 4.0437	-	-	-
	trichothecioides	CBS 136.73	TMW 4.0427	-	-	-
	tricinctum	CBS 410.86	TMW 4.0476	-	-	-
	tumidum	CBS 486.76	TMW 4.0413	-	-	-
	udum	DSM 62451	TMW 4.0258	-	-	-
	venenatum	CBS 458.93	TMW 4.0462	-	-	-
	verticillioides	CBS 218.76	TMW 4.0409	-	-	-
	xylarioides	CBS 258.52	TMW 4.0436	-	-	-
Microdochium	*majus*		TMW 4.0496	-	-	-
	nivale		TMW 4.0495	-	-	-
Mucor	*mucedo*	DSM 809	TMW 4.0441	-	-	-
Myrothecium	*roridum*	CBS 331.51	TMW 4.0668	-	-	-
Penicillium	*camemberti*	DSM 1233	TMW 4.0442	-	-	-
	commune	CBS 311.48	TMW 4.1088	-	-	-

	corylophilum	CBS 321.48	TMW 4.1598	-	-	-
	expansum	DSM 62841	TMW 4.0466	-	-	-
	griseofulvum		TMW 4.1543	-	-	-
	nordicum	[j]BEF 487	TMW 4.2213	-	-	-
	purpurescens	CBS 223.28	TMW 4.1082	-	-	-
	roquefortii	CBS 221.30	TMW 4.1599	-	-	-
	verrucosum	IBT 12935	TMW 4.0925	-	-	-
Stachybotrys	*chartarum*	CBS 485.48	TMW 4.0663	-	-	-
Trichoderma	*harzianum*	IBT 9471	TMW 4.1502	-	-	-
Trichothecium	*roseum*	CBS 567.50	TMW 4.0691	-	-	-
Verticillium	*sp.*		TMW 4.0424	-	-	-
Wheat DNA				-	-	-

[a] BBA = Julius Kühn Institut, Bundesforschungsinstitut für Kulturpflanzen, Berlin, Germany.
[b] CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.
[c] DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen, Darmstadt, Germany.
[d] IBT = Technical University of Denmark, Department of Biotechnology, Lyngby, Denmark.
[e] ITEM = Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy.
[f] MRC = South African Medical Research Council, Tygerberg, South Africa.
[g] TMW = Lehrstuhl für Technische Mikrobiologie, Freising, Germany.
[h] VLB = Versuchs- und Lehranstalt für Brauerei, Berlin, Germany.
[i]E = Collection of Dr. G. Engelhard, Landesanstalt für Ernährung, Munich, Germany.
[j]BEF = Max Rubner Institute, Federal Research Institute of Nutrition and Food, Karlsruhe, Germany.
[k]LWL = strains obtained from the collection of M. de Nijs, Wageningen University and Research, Wageningen, The Netherlands.
[l]M = strain obtained from the collection of H.M.L.J Joosten, Nestlé Research Centre, Lausanne, Switzerland.

Sensitivity of the Duplex LAMP assay was tested by addition of 5 µl DNA from a serial dilution of gDNA of *F. cerealis* CBS 589.93, *F. culmorum* DSM 62191, *F. graminearum* DSM 4529, *F. langsethiae* IBT 9956, *F. poae* TMW 4.1056 and *F. sporotrichioides* CBS 412.86 in separate reactions. The detection limit was defined as the smallest amount of template DNA which resulted in a T_t value just below 60 min for all three replicates. As shown in table 13, detection limits for the different species varied between 0.004 ng per reaction for *F. graminearum* and 15.74 ng per reaction for *F. poae*.

Table 13: Detection limits (ng/reaction) of purified *Fusarium* spp. DNA in the Duplex LAMP assay.

Species	Strain	LAMP detection limit (ng DNA/reaction)
F. cerealis	CBS 589.93	13.74
F. culmorum	DSM 62191	0.022
F. graminearum	DSM 4529	0.004
F. langsethiae	IBT 9956	0.62
F. poae	TMW 4.1056	15.72
F. sporotrichioides	CBS 412.86	0.005

3.2.3 Application of the Duplex LAMP assay for detection of DON producing *Fusarium* spp. in wheat grains

In order to analyze whether the results obtained with the Duplex LAMP assay were correlated to the DON content of naturally contaminated materials, 100 wheat samples were analyzed for both parameters and data were compared. Total DNA extracted from wheat samples was used as template in the LAMP assay run in a LA-320c real-time turbidimeter (Denschlag et al., 2014). Extraction was done in triplicate. Each extract was tested three times and averages were used for further analysis of results. The average of the T_t values from three parallel measurements was plotted against DON concentrations found in corresponding samples (see table 14). As shown in figure 19, statistical analysis of data did not reveal a strict correlation between DON concentrations and LAMP results in corresponding samples ($R^2 = 0.1433$). However, despite the poor correlation between parameters, further analysis revealed that data were useful to define a threshold T_t value applicable for the quality analysis of samples (figure 19). Thresholds were defined for this study. It appeared that using a threshold T_t value of 47 min enabled correct identification of 95 % of samples at a DON concentration threshold of ≥ 163 ppb for positive-negative LAMP results with a high degree of accuracy. Under these conditions, the LAMP assay produced 2 % false positive and 3 % false negative results relative to the DON-HPLC data. Testing the same samples using vigorous shaking of seeds in water as the only sample preparation step and using the washing liquid directly as template, the LAMP results could be used to set up a threshold T_t value of 39 min and a threshold DON concentration ≥ 1000 ppb with similarly high accuracy (see figure 20). With this method, 6 % false positive and 3 % false negative results relative to the DON-HPLC data were observed.

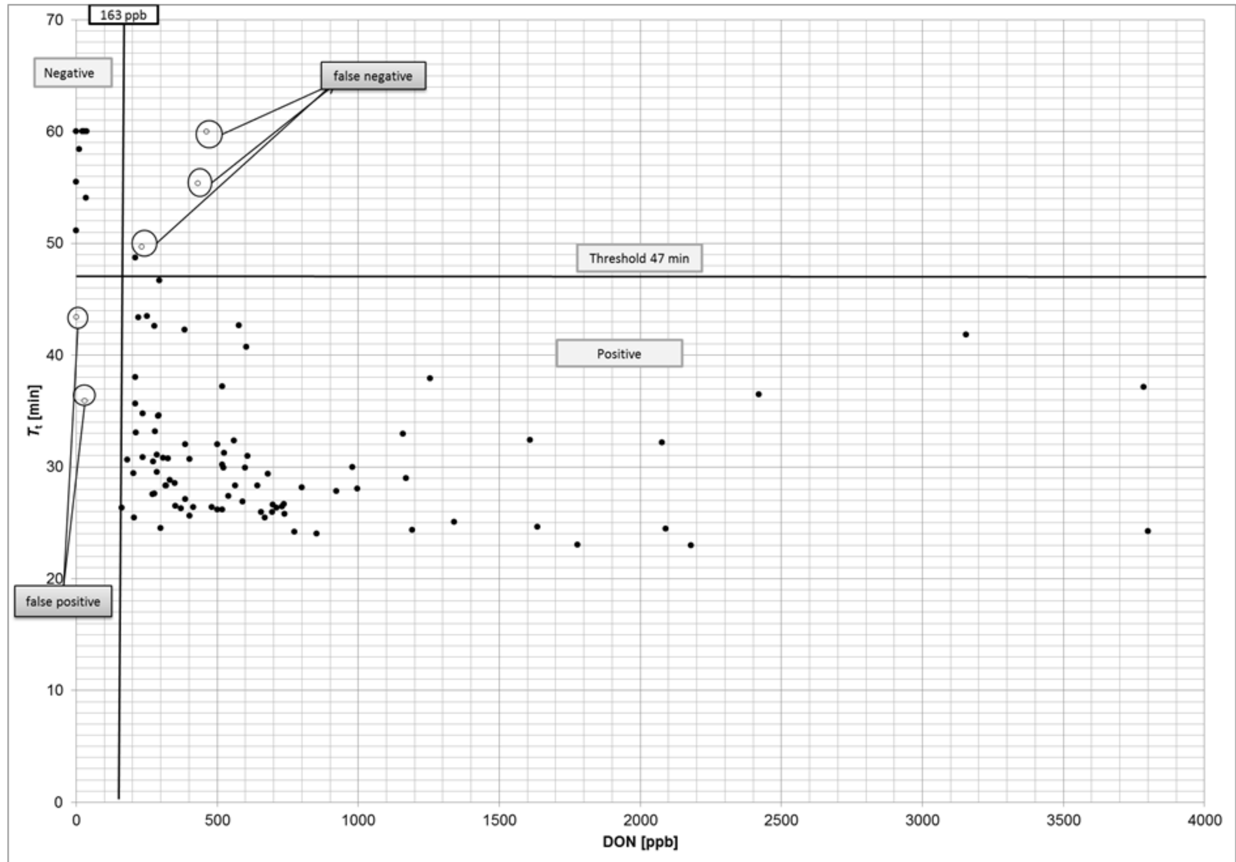


Figure 19: Mean values (3 repetitive measurements) of Duplex LAMP (T_t [min]) using DNA extracted from wheat grains as template. For the purpose of clearer presentation of data, only samples are shown which have a DON (ppb) content <4000 ppb.

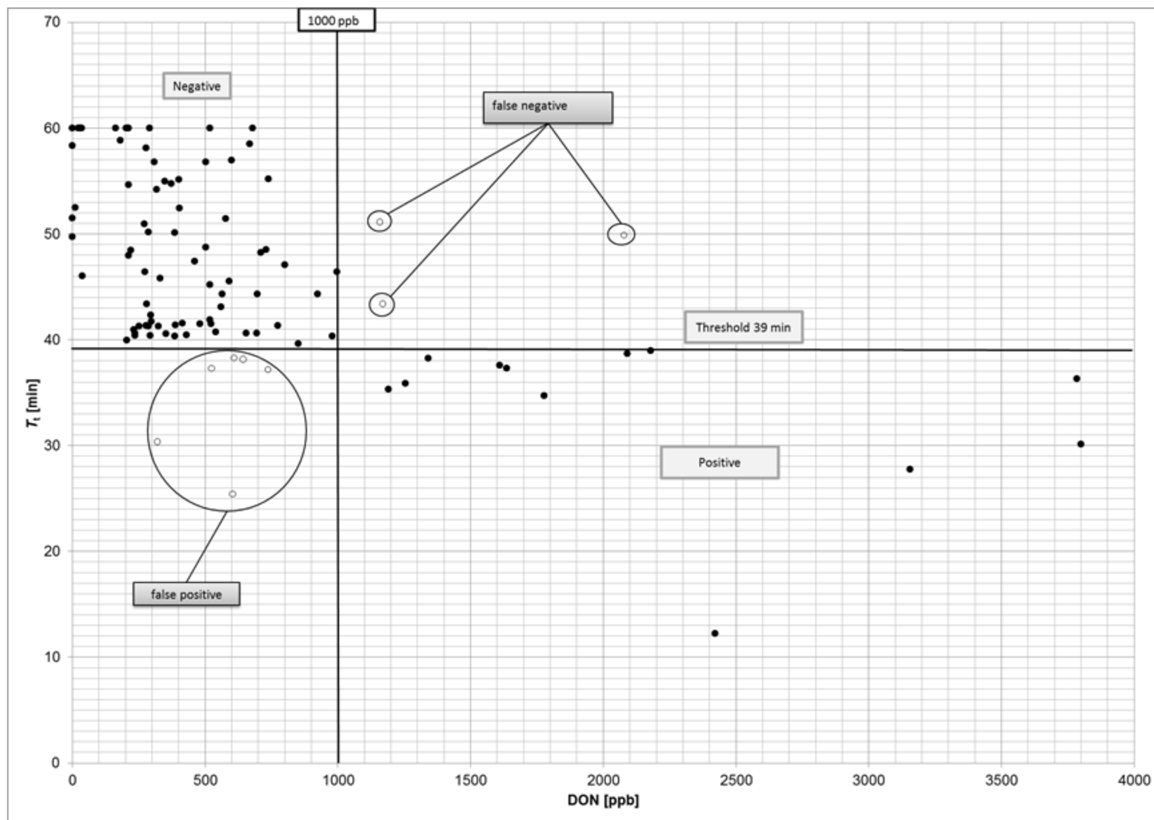


Figure 20: Mean values (3 repetitive measurements) of Duplex LAMP (T_t [min]) using total supernatant from wheat grains after shaking as template. For the purpose of clearer presentation of data, only samples are shown which have a DON (ppb) content <4000 ppb.

3.2.4 Verification of Duplex LAMP results in wheat samples

In order to verify false positive and false negative results with the total DNA extraction method found in the 100 samples with the Duplex LAMP assay relative to HPLC results, the respective samples were analyzed using the *tri5* gene specific PCR assay published by Niessen and Vogel (1998) as well as by plating a serial 10 fold dilution of the ground samples to two different growth media selective for *F. graminearum* and *F. culmorum*, respectively. Results given in table 14 showed that two samples with a false positive result were verified to contain *Fusarium* spp. carrying a *tri5* gene and also the presence of *F. graminearum* was verified in the sample. Three of the samples tested had no LAMP signal but contained DON (table 14 and table 15) at levels well above the threshold corresponding to the lower detection level of the LAMP assay. PCR verified the presence of *Fusarium* spp. *tri5* gene target but growth of neither *F. graminearum* nor *F. culmorum* was detected in any of the samples. In order to verify false positive and false negative results obtained with shaking as rapid sample preparation method for the LAMP analysis, these results were compared to the results obtained in the respective samples

using total DNA of samples as the LAMP template. All false positive samples (6 %) were found to be also positive when total sample DNA of those samples was used as template. Only one sample which was false positive according to the shaking method showed a positive LAMP result when extracted total DNA was used as template. The sample was false positive according to the shaking method in regard to its DON content of 603 ppb, which was below the 1000 ppb threshold value. This sample was verified by plating 100 grains per sample on SNAmod plates (5 grains per plate) after surface disinfection as described in Niessen and Vogel (2010). Results revealed that 5 % of grains were contaminated with *F. graminearum*. All false negative results (3 %) according to the shaking method had positive results when the DNA was extracted from the seeds of the respective samples.

Table 14: Results of a *Fusarium* spp. *tri5* gene specific PCR and microbiological analysis of wheat samples showing false positive and false negative results in the Duplex LAMP assay relative to DON HPLC analysis

	Sample no.	NIV ppb	DON ppb	LAMP [min]	Growth of *F. culmorum* or *F. graminearum*	Tri5 PCR product
False positive	135	0	0	43.44	+	+
	110	0	30	35.93	+	+
False negative	126	0	431	55.39	-	+
	139	0	232	49.69	-	+
	146	0	461	60.00	-	+

Table 15: DON concentrations vs. raw data of Duplex LAMP assay using extracted DNA and total supernatant from shaking grains as template, respectively, in corresponding samples. Thresholds are marked with a bold line.

DON ppb	Extracted DNA from grains	Supernatant from water shaking grains
	T_t [min]	T_t [min]
0	55,47	49,73
0	43,44	51,51
0	51,14	58,35
0	60,00	60,00
12	58,39	52,49
23	60,00	60,00
29	60,00	60,00
30	35,93	60,00
36	54,04	60,00
38	60,00	46,02
163	26,36	60,00
182	30,62	58,85
203	29,42	60,00
205	25,47	39,95
210	38,04	60,00
211	35,63	54,67
213	33,05	60,00
220	43,36	48,46
232	49,69	40,95
236	30,85	40,40
236	34,77	40,63
250	48,73	47,97
252	43,47	41,26
271	27,57	50,93
273	30,45	46,40
278	42,61	41,32
279	27,61	58,12
280	33,20	43,40
286	31,11	50,18

287	29,55	41,26
292	34,57	60,00
293	34,59	40,40
295	46,69	42,32
299	24,53	41,69
308	30,83	56,81
318	28,30	54,18
320	28,34	30,38
325	30,77	41,26
332	28,79	45,83
349	28,53	54,97
353	26,51	40,57
373	26,30	54,73
386	42,26	40,32
387	32,01	50,12
388	27,13	41,38
402	25,61	55,12
403	30,67	52,45
415	26,40	41,53
431	55,39	40,46
461	60,00	47,41
482	26,40	41,51
502	32,01	56,81
502	26,15	48,73
519	26,20	45,22
519	30,18	60,00
519	37,18	41,87
522	29,91	41,51
524	31,22	37,30
541	27,36	40,75
560	32,32	43,08
564	28,34	44,34
578	42,67	51,45

591	26,89	45,55
600	29,95	56,95
603	40,73	25,43
609	30,95	38,30
643	28,32	38,17
656	25,95	40,63
669	25,45	58,49
680	29,38	60,00
695	25,97	40,61
697	26,59	44,34
710	26,34	48,24
730	26,47	48,51
737	26,69	37,20
739	25,81	55,18
774	24,20	41,36
800	28,16	47,08
852	24,05	39,61
924	27,81	44,34
980	29,99	40,36
998	28,03	46,40
1158	32,95	51,14
1169	28,97	43,40
1192	24,34	35,30
1256	37,91	35,89
1340	25,07	38,22
1610	32,38	37,57
1636	24,61	37,30
1778	23,01	34,71
2077	32,20	49,91
2091	24,45	38,67
2180	22,99	38,97
2421	36,49	12,22

3156	41,85	27,77
3784	37,12	36,30
3800	24,22	30,12
5397	38,38	31,14
10142	24,09	38,26
12839	24,89	35,47

3.3 Conservation of Hyd5 ID4 LAMP master mix by vacuum drying

As preliminary analysis the compatibility of Hyd5 ID4 LAMP master mix with sorbitol (sugar alcohol) and sucrose (disaccharide) in different percentages was tested with *Bst*, *Bst* 2.0 and *Bst* WarmStart™ DNA polymerase. For the vacuum drying different quantities of sorbitol and sucrose were tested. The optimum in quantity and type of sugar in the Hyd5 ID4 LAMP master mix was 9.4 µl (15 % (w/v)) sucrose solution (see table 16). Tubes were weighed before drying and afterwards. The Hyd5 ID4 LAMP master mix was prepared without DNA before vacuum drying.

Table 16: Hyd5 ID4 LAMP master mix for vacuum drying.

Reagents	**1x quantity [µl]**
Magnesiumchlorid ($MgCl_2$)	1
10x MOPS buffer	2.5
dNTP	2.6
Primer mix	3.5
Polymerase	1
Sucrose	9.4 (15 % (w/v))
Template (DNA)	5 (10 ng/µl)

The drying period was depending on the number of tubes. It took for 1-3 stripes 35 min, for 4-6 stripes 45 min and for 7-12 stripes 50 min. *Bst, Bst* 2.0 and *Bst* WarmStart™ DNA polymerase were tested for their robustness. Table 17 shows that *Bst* 2.0 and *Bst* WarmStart™ DNA polymerases are 3 to 4 min shorter in the T_t value than the *Bst* DNA polymerase. After 1.5 h storage at 20 °C they showed the same T_t value. Only the *Bst* DNA polymerase needed 2 min longer after 1.5 h storage at 20 °C. According to this, the *Bst* WarmStart™ DNA polymerase was used for further studies. Since the *Bst*

WarmStart™ DNA polymerase did not show any amplification activity after vacuum drying without any protective substances, different sugars were tested as protectants.

Table 17: Three different *Bst* polymerases tested directly and after 1.5 h storage at 20 °C.

Polymerase	T_t [min]	T_t [min] after 1.5 h storage at 20 °C
Bst DNA polymerase	18.52	21.52
Bst 2.0 DNA polymerase	14.33	15.20
Bst 2.0 WarmStart™ DNA polymerase	15.00	15.15

The *Bst* WarmStart™ DNA polymerase was tested with different percentages of sucrose and sorbitol, respectively (see figure 21 and figure 22). The smallest T_t [min] with 28.22 min resulted after addition of 15 % (w/v) sucrose whereas the smallest T_t [min] with 36.42 min resulted after addition of 16 % (w/v) sorbitol.

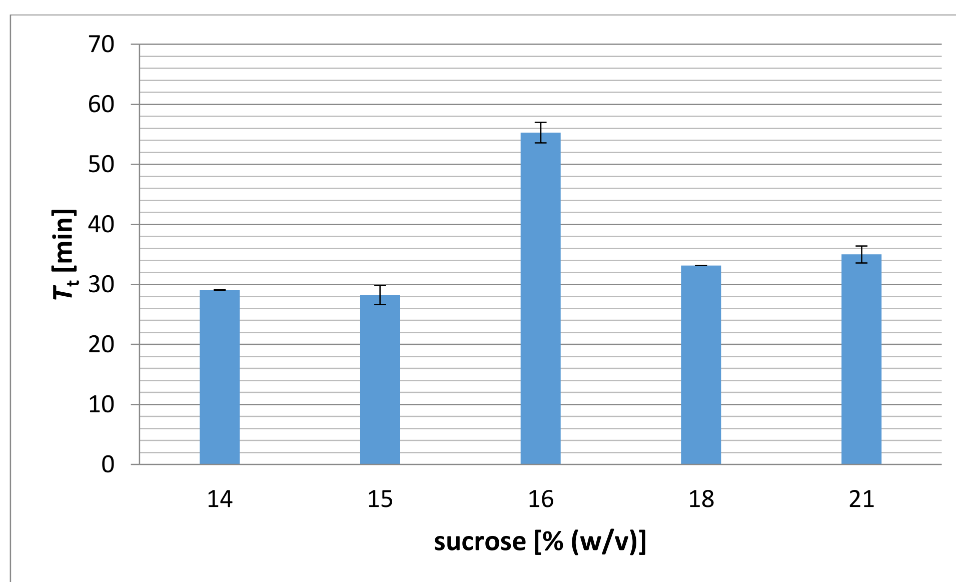


Figure 21: Results of Hyd5 ID4 LAMP reaction with standard deviation tested with *Bst* WarmStart™ DNA polymerase with different percentage of sucrose after vacuum drying.

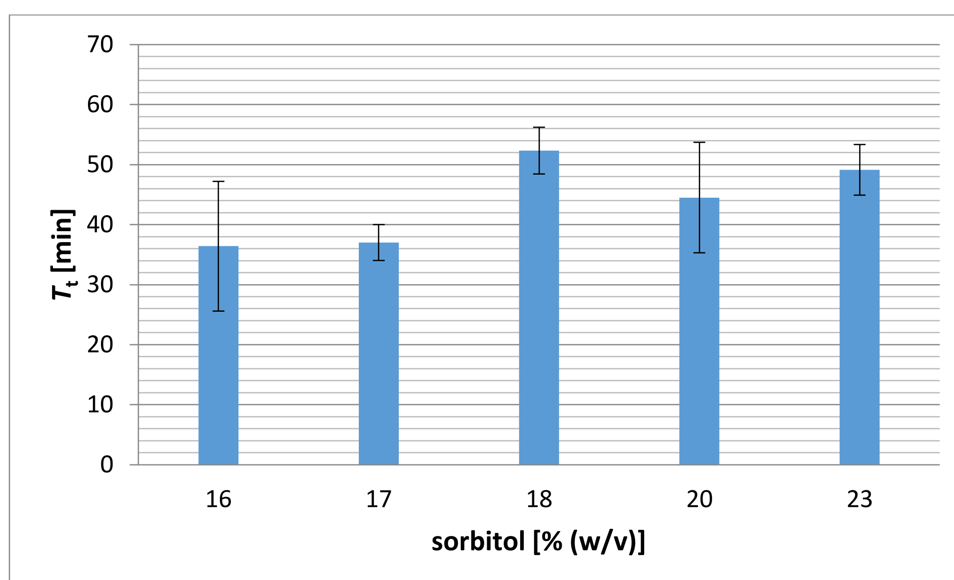


Figure 22: Results of Hyd5 ID4 LAMP reaction with standard deviation tested with *Bst* WarmStart™ DNA polymerase with different percentage of sorbitol after vacuum drying.

Despite the protective effect of sucrose as an additive it was also important to keep the vacuum drying period at a minimum in order to impose not too much stress on the DNA polymerase enzyme used. Variation of air pressure and temperature during drying revealed optimum conditions at 85 mbar/60 °C. After optimization of sucrose content and drying conditions, shelf life studies were done in order to evaluate the influence of storage conditions on the activity of the dried master mixes. Samples dried under optimized conditions at 85 mbar/60 °C were stored at -20 °C, 4 °C, 20 °C, 30 °C and 37 °C for 72 h. During the storage period, aliquots of samples were removed after different periods of time and rehydrated with water and template DNA was added. Reactions were run under standard conditions on the real-time turbidimeter. Samples stored at 4 °C were tested for a prolonged period of 37 days (888 h) in order to simulate standard storage conditions. Results given in figure 23 showed that T_t values were independent from storage conditions within a 72 h storage period. Prolonged storage at 4 °C revealed that under these conditions the *Bst* WarmStart™ DNA polymerase remained active for at least 37 d.

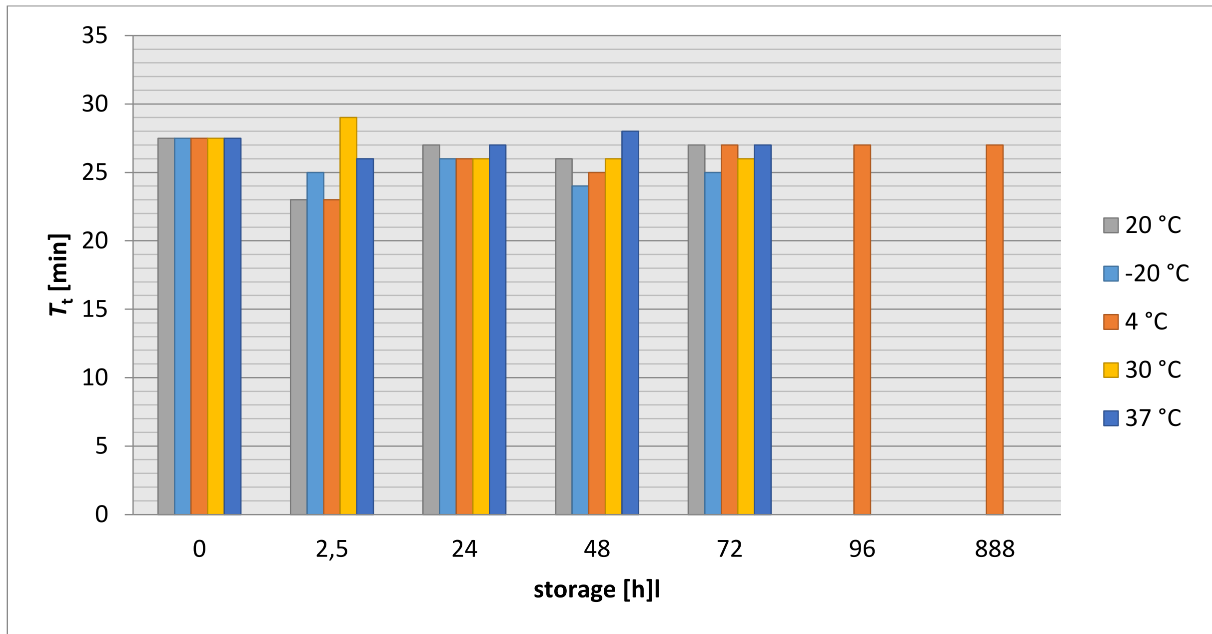


Figure 23: Activity of the vacuum dried Hyd5 ID4 LAMP master mix after storage at different temperatures and periods of time.

4 Discussion

Many fungi are known to contaminate food raw materials. Some are harmless saprophytes or parasites but some produce toxic secondary metabolites which causes a risk to humans and animals. *Fusarium* spp. represent a group of molds which can produce mycotoxins in food raw materials and reduce the general quality of food stocks. Many of the species are in connection with cereal grains and grain based food. The detection of *Fusarium* spp. and their mycotoxins, e.g. DON, in grains is still a highly demanding analytical problem. Since the first application of PCR for the detection of a fungus (*Phoma trecheiphila*, (Rollo et al., 1990)), amplification-based molecular methods have been established as a promising tool for the detection of molds in cereals and many other agricultural commodities. Meanwhile, PCR-based detection has been described for nearly all mycotoxin producing fungi of concern in food commodities (see Niessen, 2008 for a review). Despite its importance as a generally accepted laboratory technology, PCR has some disadvantages which do render it unfit for broader application in on site testing situations in which no dedicated lab equipment is available, e.g. during testing of incoming goods in the food and beverage industry. Moreover, sensitivity of the method is often not high enough to enable direct testing of samples without previous enrichment of contaminants and it has been found to be quite sensitive to inhibition by compounds regularly present in natural sample materials. LAMP provides an alternative amplification technology, which avoids many of the disadvantages of PCR and offers high specificity and sensitivity in combination with high robustness and ease of use. Its robustness makes the LAMP method attractive for direct testing of samples as demonstrated in the current work. The advantage of its high specificity simultaneously turns into a disadvantage of the LAMP method since it may hamper detection of marker genes which provide certain properties to a group of different species such as production of gushing inducing hydrophobins or trichothecene mycotoxins.

The work presented here describes the development and application of LAMP-based assays as a useful and novel alternative method for the rapid diagnosis of *Fusarium* spp. in barley and wheat as well as in malts made thereof, which are a serious problem to the quality of those commodities since they induce gushing in beer and – even more critical – produce mycotoxins. For application of LAMP tests concerning fungal contaminations see review of Niessen et al. (2013). To the authors knowledge this is the first report of using LAMP for gene specific diagnosis in a group of fungal species and also the first

report of combining two primer sets with a non-overlapping spectrum of detected species to result in a fully combined species spectrum.

4.1 Design of optimum LAMP primers

In this work two LAMP assays are presented which were developed and optimized for the detection and identification of gushing inducing *Fusarium* spp. as well as for typical producers of trichothecene mycotoxins among *Fusarium* spp. For the design of a test, which detects gushing inducing *Fusarium* spp. it was important to know that hydrophobins are discussed as an important gushing inducing factor in recent publications (Deckers et al., 2011; Niessen et al., 1992; Sarlin et al., 2012; Shokribousjein et al., 2011). Especially the class 2 hydrophobin hyd5p plays an important role in triggering the gushing effect in beer (Sarlin et al., 2012). For the design of LAMP primers the *hyd5* gene was chosen as target for the Hyd5 ID4 LAMP assay. Setting up the primer set used, importance was given to the fact that the primers hybridize to exon regions of the gene. This is important here since the objective of the study was to set up group specific assays. It was therefore important for primer design to use nucleotide positions with a high degree of conservation between the target species. Results obtained in the presented work showed that the amplification reaction was highly specific for a set of target species. The Hyd5 ID4 LAMP assay was tested with genomic DNA isolated from 133 different fungal isolates, most of them representing species, which occur on cereals or on malt. Of these fungal isolates only those *Fusarium* spp. showed a positive reaction, which have been suspected to induce gushing in beer. For verification of results, the smallest amplification product obtained by LAMP amplification of target DNA was sequenced and compared to published *hyd5* sequences. Identity between the LAMP product obtained and the target gene was fully verified showing that the correct target was amplified during the process. Various authors (e.g. (Fang et al., 2010; Njiru et al., 2008; Yano et al., 2007) have used restriction analysis in order to prove that the DNA amplified during their LAMP reactions corresponds with the target sequence. However, in the case of a gene specific LAMP reaction like the current one, variation may occur within the nucleotide sequence between different target species, which renders restriction analysis an insufficient tool to prove the identity of the LAMP product. Specificity testing with the Hyd5 ID4 LAMP assay demonstrated that important gushing inducing *Fusarium* spp. such as *F. cerealis, F. culmorum* and *F. graminearum* can be readily detected.

The second LAMP assay developed during the current study focused on the detection of the group of the most important trichothecene producing *Fusarium* spp. In order to find suitable target sequences displaying a sufficiently high degree of similarity among different species, sequence alignments of all the genes within in the trichothecene cluster (see figure 1 and Ward et al., 2002) were analyzed to allow for the design of primers which picked up several of the representatives within the group of trichothecene producing species. As a result, no particular gene could be identified with a sufficiently conserved sequence to allow for the establishment of a gene specific set of LAMP primers. Instead, the *tri6* and *tri5* genes proved to be suitable targets for LAMP primer sets, each of which detecting a group of three species within the type A and type B trichothecene producing *Fusarium* species, respectively. Interestingly, both genes are not restricted to the respective group of species they detect but are common to all trichothecene producing *Fusarium* spp. The *tri6* gene encodes a peptide with regions similar to Cys_2His_2 zinc finger DNA-binding proteins and is involved in the regulation of trichothecene biosynthesis (Proctor et al., 1995). The *tri5* gene encodes trichodiene synthase, an enzyme catalyzing the first specific step in the biosynthesis of all trichothecene mycotoxins (Proctor et al., 1995). LAMP assays for the detection of the two genes were developed independently from each other and primer sets Tri6 ID1 and Tri5 ID4 each detected a non-overlapping array of species where each group represented commonly encountered producers of A trichothecenes (i.e. *F. langsethiae, F. poae*, and *F. sporotrichioides*) and B trichothecenes (i.e. *F. cerealis, F. culmorum* and *F. graminearum*). Testing of DNA extracted from multiple isolates of each of the species showed high consistency of results for most species. Only some of the *F. poae* strains tested were not picked up by the Tri5 ID4 primer set indicating that they might not contain a *tri5* gene or have truncations in the sequence of that gene. Sequence variation in the genome of *F. poae* was also shown in the publication of Yli-Mattila et al. (2004), supporting the results obtained during the current study. During tests for assay optimization it turned out that both LAMP reactions ran optimally at the same incubation temperature. Therefore, both primer sets were combined in one Duplex LAMP reaction which also showed a combined spectrum of the species detected (*F. culmorum, F. cerealis, F. graminearum, F. langsethiae, F. poae* and *F. sporotrichioides*). Within this group, *F. sporotrichioides, F. graminearum,* and *F. culmorum* were detected with the highest sensitivity. This is in agreement with the group of species of highest importance as producers of trichothecenes of types A and B in a variety of different

geographic regions (Placinta et al., 1999). Differences within the detection limits found between the six species picked up by the assay may be due to minor interspecific sequence variations between the target genes. Sequence variations in the respective binding sites between primers and genes are shown in table 11. The relatively high degree of variation between primers and gene sequence in *F. cerealis* may explain the lower sensitivity of the assay. In this case primer binding would be relatively weak, especially if the nucleotides differ in the 5' prime region of the primer since this is the binding region of the *Bst* polymerase. The lower sensitivities of the assay for detection of *F. langsethiae* and *F. poae* can also be explained by such sequence variations. The extent of sequence variation between primers and binding sites was assumed to be correlated to the sensitivity of the assay for the detection of the respective species. This became particularly obvious in the case of *F. poae*, in which variation was especially high, even to an extent at which some *F. poae* strains did not show a LAMP reaction at all. As *tri5* gene sequences were not available for all *F. poae* strains tested in the study, it cannot be conclusively decided whether variation in that gene would match the observation of two distinct phylogenetic taxa in *F. poae* as suggested by Yli-Mattila et al. (2004). However, also with the Duplex assay, comparison of different strains of each species revealed that some *F. poae* strains did not react for the reason discussed previously.

4.2 Application of LAMP for the detection of gushing inducing *Fusarium* spp. in pure cultures, cereals and malts

Apart from the development of primers useful for the LAMP-based detection of purified DNA of gushing-relevant *Fusarium* spp., setting up robust and user-friendly assays as well as the demonstration of their usefulness was a core objective of the current study. This was demonstrated by using the method for screening of pure fungal cultures for identification and grain samples for detection of target species. Preparation and purification of DNA from infected samples or from fungal pure cultures can be time consuming and is a frequent source of trouble when working with molecular biological assays. Due to its high sensitivity and tolerance against inhibitors, LAMP has a potential of being applied to relatively impure sample materials (Jayawardena et al., 2007). Besides the Hyd5 ID4 LAMP assay exists other methods like the MCT and the applicability of dynamic light scattering technique as method to characterize the primary gushing potential of raw materials (Deckers et al., 2011). But for both methods is it necessary to prepare samples in a time-consuming way with expensive equipment.

In order to demonstrate the usefulness of the Hyd5 ID4 LAMP assay, mixtures of mycelia and spores from 12 different fungal species were used as template by introducing the materials directly into the master mix without any previous sample preparation. Parallel investigations using addition of eight different commonly used mycological agar media together with pure target DNA revealed that only the use of mung bean agar (MBA) resulted in complete inhibition of the LAMP reaction. Some inhibitors of the LAMP reaction have been described recently (Kaneko et al., 2007). One common inhibitor found in the study was polyphenols, which are present in high concentrations in mung bean seeds. It can therefore be assumed that polyphenols of the mung beans also appeared to inhibit the LAMP reaction during the current study. The remaining seven agar media were used as growth medium for all 12 fungal species and tested systematically. Among the seven media tested, only SNAmod resulted in the detection of the same spectrum of species as compared to the use of purified DNA as template. Results obtained correspond well with results published by Niessen and Vogel (2010) who used a *F. graminearum* specific LAMP assay to analyze cultures of the fungus directly from SNAmod plates without testing other media.

4.2.1 Real time monitoring of the LAMP reaction using turbidity measurement and fluorescence

In many applications of LAMP, the reaction is used as endpoint analysis with readout of the fluorescence or turbidity signal after a defined period of time. However, using appropriate equipment, signals can also be measured continuously over the reaction time, which should allow for quantification of target concentrations. Using the LAMP assays developed during the current study, one of the objectives was to investigate whether or not quantification of target DNA was possible using two different technical platforms, turbidimetry and fluorometry. Real-time monitoring of DNA amplification during LAMP can be accomplished directly and indirectly. Indirect measurement relies on the detection of pyrophosphate produced as a by-product of enzymatic DNA biosynthesis (Saiki et al., 1992). Mori et al. (2001) observed that when nucleic acid is amplified during LAMP reactions, turbidity derived from precipitating magnesium pyrophosphate occurs. Its concentration was directly proportional to the concentration of DNA produced during the reaction. Mori et al. (2004) employed this effect by continuously measuring the turbidity of multiple samples simultaneously to monitor amplification of DNA in real-time. As an alternative to the measurement of magnesium

pyrophosphate turbidity, calcein can be used as a fluorescence indicator in indirect measurement of LAMP-based DNA amplification. Tomita et al. (2008) demonstrated that calcein fluorescence can be quenched by complexation to manganese ions. The complex is dissolved in favor of the formation of a pyrophosphate-manganese complex during DNA synthesis so that the free calcein displays its bright green fluorescence under UV-light. Direct measurement of the quantity of DNA produced during the LAMP reaction can be achieved by adding an intercalating fluorescent stain such like SYBR green 1 (Lucchi et al., 2010). The authors used SYBR green 1 in connection with the tube scanner device by direct addition to the LAMP reaction. However, it was observed that addition of SYBR green 1 at the beginning of the LAMP resulted in considerable inhibition of the reaction. Similar effects were described by Aoi et al. (2006) who, as a consequence, used YO-PRO stain as intercalating dye and did not observe inhibition of the LAMP reaction. The utilization of DNA-stain V13-01184 was described during the current study. The dye has absorption/emission maxima very similar to FAM, and is thus highly suitable for the set of filters available in the tube scanner instrument. Data obtained here show that the stain is highly suitable for real-time monitoring of LAMP reactions since fluorescent signals occurred as early as 6 min after the incubation temperature was reached and the measurement was completed within 15-20 min (figure 16). Results for the Hyd5 ID4 LAMP assay obtained show that the fluorescence-based real-time LAMP was considerably faster (>50 %) than the same reaction performed and monitored in a real-time turbidimeter. These findings confirm the data published by Aoi et al. (2006) who applied the same model of turbidimeter but a different type of real-time fluorescence reader and YP-PRO stain instead of V13-01184 for their study.

In contrast to PCR where DNA amplification is a function of the number of melting-amplification cycles, biosynthesis of DNA is a continuous process in LAMP and is therefore a function of incubation time only. Because of its discrete mode of dependence, the concentration of target molecules used in a PCR can be calculated rather precisely from the concentration of amplification product observed after a known number of amplification cycles. In reality, the equations used are only biased by factors, which result in suboptimal amplification efficiency. Because of the dependence of DNA amplification on reaction time in LAMP, a theoretical calculation of target concentrations from a given concentration of amplification product is impossible. Quantitative estimation of target concentrations in a given LAMP reaction therefore has to be done by comparison of the time needed for the signal to reach a certain threshold value T_t. The

threshold can either be linked to the dynamics of the reaction ($1^{st.}$ derivative of signal over time, slope validation) or to absolute signal intensity. In the current study, the slope validation method was used to determine T_t values. With the slope validation method used, a standard curve was created for the Hyd5 ID4 LAMP assay using a serial dilution of a known concentration of the target DNA. Because some authors have constructed the standard curve without loop primers (Aoi et al., 2006; Fall et al., 2010) and some with loop primer (Han and Ge, 2010; Suzuki et al., 2006), tests were performed with and without loop primers on the real-time turbidimeter (figure 13). The standard curves with loop primers display a less steep slope as compared to a LAMP assay involving loop primers. Results also indicate that the standard deviation of data points from the reaction without loop primers is much higher than the standard deviation of data points generated with loop primers. However, data presented here suggest that quantitative evaluation of DNA concentrations is more accurate using loop primers as compared to a standard curve generated with LAMP not involving loop primers. With the experience of the real-time turbidimeter a standard curve was also generated on the tube scanner device using the Hyd4 ID4 primer set with loop primers and V13-01184 as intercalating fluorescent dye. Although the measuring process of the tube scanner was very rapid, the results showed particularly high standard deviations when compared to the turbidimetric system. In contrast to the turbidimeter, the tube scanner uses a data acquisition interval of at least 30 s, while the turbidimeter measures turbidity every 6 s. This means that only $1/5^{th}$ of the amount of data are available per time using the fluorimeter. Since LAMP reactions proceed very quickly, there is mostly a time slot of only 2-3 minutes between the onset of the fluorescence signal and its maximum readout (see figure 15). Given a data acquisition interval of 30 s in the tubescanner device, this means that the calculation of the time to threshold value for a given target DNA concentration is based on 4 to 6 data points only. Under these conditions it becomes highly improbable that an even vaguely exact quantification will be possible on this system at present. As a consequence of the results described, further experiments using real-time monitoring of LAMP reactions were exclusively performed on the turbidimeter device.

4.2.2 Analysis of sample materials

In addition to fungal pure cultures, the LAMP assay was applied to analyze samples of cereal grains for the presence of gushing inducing fungi. Due to the assay's high sensitivity a very simple protocol could be applied in which the grains were just shaken in water and heated so that the supernatant was directly added as template to the LAMP master mix. Addition of single visibly infected grains of wheat malt to 200 g portions of uninfected brewing barley revealed that the assay fulfills the quality requirements adopted by many European breweries in their raw material contracts according to which the number of visibly infected ("relevant") grains should be five or less in 200 g of brewing barley (MEBAK, 2006). The detection limit of the current assay was three grains in 200 g of non-infected barley grains (see chapter 0.). The results show that the LAMP-based assay developed during the current study may provide breweries and malting companies but also agricultural producers and suppliers of brewing cereals with a powerful new tool for quality control. In another experiment, field infected barley (*F. culmorum*) was mixed with uninfected barley in order to model different infection levels (0-40 %) (figure 17). Samples with different infection levels were analyzed with the turbidimeter-based real-time LAMP assay to investigate whether the resulting T_t values were correlated to infection levels of samples. Data were well correlated in samples containing between 10 % and 40 % of infected barley. However, at levels below 10 % a significant correlation between parameters was observed but standard deviations were too high to allow determination of infection levels with the desired accuracy. The lowest percentage level of infection, which could be reliably detected was 1 %. Performing the experiment on the tube scanner device showed that also here 1 % of infection could be reliably detected in samples (data not shown). Samples with infection levels below 1 % showed too much variation of results to be detected with sufficient accuracy. Using the threshold value determined for a 1 % infection level on the turbidimeter, MCT gushing tested barley malt samples were analyzed in order to correlate LAMP data with gushing potential of samples. The analysis shows that the results of the LAMP assays are in agreement with MCT results in 50 % of samples. At first sight, these results would suggest a low level of correlation between the results obtained with the MCT and with the *hyd5* gene based real-time LAMP assay. However, the use of *in vitro* tests for prediction of the gushing potential of malted and unmalted cereals has been a matter of controversial debate over the last 30 years. Two different systems have been established as standard methods, i.e. the MCT and the "Donhauser Test" (Donhauser et al., 1990).

The differences and particulars of both tests and of novel methods for gushing analysis have been recently reviewed by Christian et al. (2011). Rath (2009) studied the reproducibility of the MCT in particular between different labs and came to the conclusion that results are widely irreproducible and that the test tends to over-estimate the number of gushing positive samples when compared to the actual occurrence of gushing in beers produced from tested malts. A similar tendency may be visible when comparing the results obtained during the presented work by estimating gushing inducing fungi using the *hyd5* gene as a potential marker in the LAMP assay (see table 10). However, it is generally difficult to compare the results of two different independent assay methods such as the MTC and LAMP since both provide an indirect measure for the potential of a malt to induce gushing in the beer actually produced from it. In order to ultimately judge the usefulness of the *hyd5* gene as a marker for gushing and the LAMP method as a tool to measure its presence in samples, future studies should focus on the correlation between LAMP results and beer gushing rather than with the results of any of the predictive gushing tests available. Attempts to set up such a study were made during the current study. However, attempts to find German breweries, which were ready to participate in such an investigation failed!

4.3 Real-time LAMP detection of trichothecene producing *Fusarium* spp.

The Duplex LAMP assay was developed as an application mainly for the inspection of incoming goods (cereals) in the brewing and malting industry. Presently, at least the bigger malt producers obtain their raw materials from a worldwide supply. Accordingly, also malt coming to the brewery can be supposed to go back to that same worldwide supply. For malt producers as well as for the brewing industry it is therefore important to have a test available which can be used to analyze the presence of producers of any trichothecene in order to be able to have an estimate of potential risks for mycotoxin contamination associated to their incoming goods. As the presence of certain *Fusarium* spp. in cereals may vary regionally, it is important to have an assay available which detects a maximal wide spectrum of species. The Duplex LAMP assay developed during the current study was demonstrated to detect at least the most important species of trichothecene producers in one rapid analysis and may therefore provide a hint to the potential presence of trichothecene mycotoxins which can then be analyzed further with chemical methods if needed. No quantification was done with the Duplex LAMP assay for

reasons of focusing the detection on as many trichothecene producing *Fusarium* spp. as possible. Experiments revealed that with two LAMP assays combined in one reaction it is not possible to create a standard curve, because two different target genes were chosen having different amplification characteristics and which cannot be determined independently due to the nature of LAMP reactions.

4.3.1 Analysis sample material

DON is the most frequently occurring trichothecene mycotoxin in German wheat. In order to verify whether there is a correlation between DON content and T_t value for the Duplex LAMP signal, 100 wheat samples from the 2012 harvest were obtained from different areas of the German federal state of Bavaria and analyzed for both parameters. LAMP reactions were carried out using total DNA extract from wheat grains as template for the Duplex LAMP. In parallel experiments, water was used as template in which wheat grains had been vigorously shaken for 1 min by hand as a rapid method for sample preparation. For reasons discussed previously, the advantage of the Duplex LAMP assay is its broader spectrum of species detected as compared to either of the two *tri*-gene specific assays which makes it a more powerful tool for sample analysis. Results obtained with both target preparation methods revealed a weak correlation between DON-HPLC data and T_t values in corresponding samples. One reason for this poor correlation could be that not only DON producing *Fusarium* spp. are prevalent in the samples but also *F. sporotrichioides, F. langsethiae, F. cerealis* or *F. poae,* which all gave a signal in the Duplex LAMP assay but do not produce DON but other toxins for which no data were available. Another explanation might be that DON contents tend to vary in samples due to the genetic predisposition of the prevailing strains or the environmental conditions during growth in the field or during storage. In previous publications (Homdork et al., 2000a; Snijders and Perkowski, 1990) it was shown to also vary considerably between years, regions, cultivars and agricultural technologies. Under field conditions there are several factors, which have been found to influence the level of mycotoxin production by *Fusarium* spp. on the physiological level. Water availability, temperature and interactions with fungicides were demonstrated to impact the production of DON as the major trichothecene mycotoxin found in cereals in temperate regions (Magan et al., 2002). However, *Fusarium* spp. may not only produce mycotoxins under field conditions, but also during storage (Birzele et al., 2000). Homdork et al. (2000b) reported that DON concentrations in severely infected grain samples remained

unchanged under any storage conditions, but increased during storage in samples with only slight or moderate infections with DON producing *Fusarium* spp. Moreover, Mesterházy (2002) demonstrated that even in the field, toxin production of a given DON producing *Fusarium* isolate showed considerable variation depending on the host plant cultivar analyzed. Several methods for identification and quantification of *Fusarium* spp. from cereal grain using real-time PCR have been described previously (Edwards et al., 2001; Sarlin et al., 2006; Schnerr et al., 2001; Waalwijk et al., 2004).

Because of the weak correlation between parameters, the set of data generated during the current study was further analyzed for the presence of a threshold T_t value, which could be used as a quality indicator for sample testing. Analysis of data obtained from testing of samples with the total DNA extraction method revealed that setting a threshold at $T_t = 47$ min produced a minimum number of false results relative to the presence or absence of DON content at a minimum level of ≥ 163 ppb. Analysis of false negative and false positive samples with reference methods revealed that the majority of such samples obviously contained trichothecene producers, which were not picked up by the LAMP assay applied. However, all in all the percentage of false results was low and confirmed the usefulness of the assay for quality control applications. The limit DON concentration of ≥ 163 ppb corresponding to the T_t threshold of the LAMP assay (47 min) can be considered far below the maximum level of 1250 ppb set by Commission Regulation (EC) No 1881/2006 (2006) for DON in unprocessed cereals. Apart from its high sensitivity and specificity, the possibility of using quite impure DNA as template is one of the major advantages of the LAMP method. Advantage was taken of these facts during the current study using a more rapid alternative to total DNA extraction by shaking seeds in water and to test the supernatant directly in the Duplex LAMP assay. Setting a threshold at $T_t = 39$ min the alternative sample preparation method produced a minimum of false positive/false negative results relative to the presence or absence of DON content at a threshold level of ≥ 1000 ppb. Samples showing false positive LAMP results were found to be also positive in the Duplex LAMP when total DNA was used as template. Only 1 % of the false positive samples were not positive when analyzed with the total DNA extraction method of template preparation. The differences in results can be explained by the different limits of DON concentrations chosen with the two methods of sample preparation. False negative results (3 % of samples) obtained with the shaking method were found to give positive results when total DNA from the same samples was used as template. This can be explained by the

fact that some grains internally carry fungal material rather than on their outside. As a conclusion from the results obtained the percentage of false results was low and also confirmed the usefulness of the faster method for quality control applications. However, this method is much faster as the method using total extracted DNA and the limit DON concentration of ≥ 1000 ppb is closer to the legal maximum limit of 1250 ppb set by Commission Regulation (EC) No 1881/2006 (2006). The T_t thresholds given above were set using a dedicated type of real-time turbidimeter. Utilization of this assay with other equipment means that thresholds will need to be verified and possibly adjusted accordingly. With the Duplex LAMP assay for detection of important trichothecene producing *Fusarium* spp. and sample preparation protocols described, the user can choose between two alternatives: 1.) Extraction of total DNA from samples is more time-consuming but almost 10 times more sensitive especially concerning the infestation inside the grain or 2.) Shaking seeds in water and taking the supernatant for testing with the Duplex LAMP assay. The latter method provides a 4-times higher rapidity of sample preparation and the threshold of T_t of 34 min relates to a threshold in DON concentrations which is closer to the legal limits of the toxin. However, since the assay described here is intended as a rapid test for quality inspection and because of the well-established high level of variation of DON levels it would be wise to use a test which indicates levels of DON contamination well below the official maximum level in order to separate sound samples from samples which have to be checked by further HPLC analysis at an early stage during the processing of cereals.

4.4 Optimization of LAMP assays for speed and user-friendliness

One of the objectives of the current work was to optimize the developed assays for rapidness and user-friendliness. Several attempts were made to this end by optimizing primer and enzyme concentrations as well as by using alternative DNA polymerases and additives to the master mix. Moreover, experiments were performed aiming at the production of an assay in a ready to use format for maximum convenience.

As a first step to reach this objective, loop primers were used routinely in all LAMP assays during the current study. As shown in fig. 12, addition of loop primers to the master mix resulted in a reduction of the time needed to reach the endpoint of the reaction from 60 to 20 min when turbidity measurement was used. According to the author’s experience, reductions from 90 to 45 min can also be achieved in assays using calcein fluorescence to detect the LAMP signal (data not shown). A further step to

achieve higher user–friendliness of LAMP assays, the use of PEG in the LAMP master mix was tested. Addition of this water soluble polymer resulted in a further enormous acceleration of the assay's performance with an increase of assay reaction time of 16 % and 35 % for the turbidimeter-based assay and the tube scanner-based assays, respectively. Minton (2006) speculates that this effect may be due to a phenomenon termed macromolecular crowding. *In vivo* biological reactions take place in an environment in which around 20 % to 40 % of total volume in a cell consists of macromolecules. This leads, among other effects, to an increase in association rates and equilibrium constants of enzymes and the reactions they catalyze. Macromolecular crowding agents such as ficoll, dextran or polyethylene glycol have been shown to increase reaction speed of several enzymes. Tong et al. (2011) demonstrated that among these substances, PEG had a strong accelerating effect to the amplification process in a helicase-dependent amplification assay.

In addition to maximum speed of the LAMP assays developed here, experiments were performed to simplify the handling and set up of LAMP reactions to provide a maximum of convenience to users of the assays. The advantages of LAMP assays over other molecular diagnostic formats such as PCR is its higher user friendliness in terms of equipment and effort necessary for sample preparation. However, the preparation of LAMP master mixes still is a problem in the diagnostic process since it has to be performed under proper hygienic conditions in order to prevent cross contaminations. It would therefore be desirable to have LAMP master mixes available in a ready to use contamination free format to which only the sample has to be added with no further manipulations necessary before assay incubation. A major prerequisite, which must be met to set up such a system is that the master mix must be provided to the user in a stable condition in order to guarantee its activity after a period of storage and/or during shipping. During the current study, experiments were performed to optimize the stability of Hyd5 ID4 LAMP master mixes in order to demonstrate the feasibility and usefulness of vacuum drying to reach this goal. Preliminary experiments showed that storage of a LAMP master mix with *Bst* polymerase and primers included was impossible for a period longer than a few hours before complete loss of activity (data not shown). This observation would mean that shipment of ready to use LAMP assays can only be done under refrigeration and only over short distances providing that the recipient of a shipment will use the assay immediately after reception. In order to maintain assays active over a longer period of time and allow for their shipment even to

farer destinations, vacuum drying of complete LAMP master mixes was performed and tested during the current study. Vacuum drying instead of freeze drying was chosen to avoid the frozen state which had a negative impact on the activity of the *Bst* polymerase. For the vacuum drying optimization of the master mix and testing of different protective additives and conditions it revealed that with the addition of 15 % (v/v) sucrose, complete LAMP master mixes could be vacuum dried maintaining the activity after reconstitution with a template containing aqueous solution. Preliminary experiments showed that even after storage at 37 °C activity remained unchanged for at least 3 days. Storage at 4 °C kept the activity for at least 37 days (see figure 23). However, the reaction time for the vacuum dried assay was almost twice as long as for the reaction without vacuum drying. Despite the different temperatures applied during storage and although the vacuum drying was performed in several different batches, the reaction time of the *Bst* WarmStart™ DNA polymerase showed a standard deviation of only 5 % between the different time points of testing. Long term shelf life experiments are currently under way. However, data were still unavailable at the time of finishing the current thesis. In case the results of the experiments should indicate a shelf life of 6 month or longer at 4 °C the Hyd5 ID4 LAMP assay could be produced, vacuum dried, and sent out to the malt producing companies or breweries for testing their raw materials as regards gushing inducing *Fusarium* spp. Even when the test cannot be run immediately, it can be stored at 4 °C under standard storage conditions.

It is well established that some sugars and sugar alcohols have the property to protect proteins by preferential hydration. In the work of Higl (2008), the influence of sugar on proteins during the drying process was analyzed. Under normal conditions a hydration layer will protects proteins, in this case the polymerase, from dehydration (Higl, 2008). In order to prevent structural changes of the polymerase after water removement during drying, protective substances such as sugars or sugar alcohols must be added to maintain the enzyme's activity. During the process of drying under these conditions, t sugar molecules replace the water molecules by replacing hydrogen bonds and thus stabilize the tertiary structure of the polymerase molecule by forming an amorphic glass-like layer around the enzyme (Higl, 2008). Tests during the current study were performed with three different types of thermophilic polymerases fit for LAMP: *Bst* DNA polymerase, *Bst* 2.0 DNA polymerase, and *Bst* WarmStart™ DNA polymerase. It revealed that all polymerases with the exception of *Bst* WarmStart™ DNA polymerase were unsuited for vacuum drying since their activity loss even under normal handling

conditions was too high. New England Biolabs GmbH (NEB) guarantees thermostability for the enzyme for up to 4 h at 20 °C-65 °C (https://www.neb.com/products/m0538-bst-20-warmstart-dna-polymerase#pd-properties-usage). Vacuum drying experiments were therefore done using the *Bst* WarmStart™ DNA polymerase since the enzyme had a high thermostability which is necessary for the vacuum drying..

4.5 Conclusion

The objective of this work was to investigate the contamination of raw materials used in the malting and brewing industry with quality relevant *Fusarium* species using newly developed gene specific loop-mediated isothermal amplification based assays as specific and sensitive yet rapid and easy to use analytical tools, to evaluate and to optimize these assays for maximum user-friendliness, and to apply them to the analysis of naturally infected sample materials.

Results obtained demonstrate that the assays developed are well fit for analysis of typical materials used in the malting and brewing industries. Assay results were correlated to important quality parameters associated with the contamination of materials with typical *Fusarium* species, e.g. induction of gushing and trichothecene mycotoxin contamination. According to the investigation of sample materials, the assays developed can be a useful tool in the analysis of incoming goods in the malting and brewing industry. The current work contains several features, which have not been published by other authors before. The Hyd5 ID4 assay is the first description of the application of LAMP for the detection of several fungal species in a group specific or gene specific manner. Also the combination of two independent primer sets in one LAMP assay for the detection of a non-overlapping set of species has not hitherto been demonstrated. To the author's knowledge, also the application of vacuum drying of a complete and active LAMP master mix and its storage over a prolonged period of time was as yet undescribed in the literature and also the addition of PEG as an additive to significantly accelerate LAMP reactions is a feature newly described in the current work.

5 Summary

The quality of cereals used as the basis for beer production is a highly important factor to the brewing and cereal processing industry. Infestation of raw commodities and malt by members of the fungal genus *Fusarium* may result in gushing and in contamination with mycotoxins. Apart from technical problems related with handling of the contaminated materials, processing is also connected with economical and legal risks for producers of beer as well as a health risk for consumers. Economic risks refer to increase in production costs, costs for recall of affected batches and losses in sales, all of which may lead to a decrease in revenues. Moreover, the legal risk refers to the risk of exceeding the legal maximum limits for mycotoxins in which case also the consumer is at risk of being chronically intoxicated. Proper quality of incoming goods is one possible way of reducing risk for the industry as well as for consumers.

Aimed at improving and facilitating quality control in the brewing and malting industry, the objective of this work was to investigate the contamination of raw materials used in the malting and brewing industry with quality relevant *Fusarium* spp. using newly developed gene specific assays as specific and sensitive yet rapid and easy to use analytical tools, to evaluate and to optimize these assays for maximum user-friendliness, and to apply them to the analysis of naturally infected sample materials.

To reach these objectives, new methods were developed during the current study, which was based on the loop-mediated isothermal amplification (LAMP) technology. The new assays enable identification of pure cultures of quality relevant *Fusarium* spp. as well as their detection in contaminated cereals and malts in less time and with less effort in terms of equipment and personnel as compared to classical and PCR-based methods. Applying the novel assays, naturally infected samples of brewing cereals and malts were tested and results compared with mycotoxin contents and gushing potentials of respective samples in order to correlate LAMP results to quality parameters.

The Hyd5 ID4 LAMP assay targets the *hyd5* gene in typical gushing-inducing *Fusarium* spp. in a group specific manner. Results showed that *F. graminearum, F. culmorum* and *F. cerealis* are detected with high specificity with no cross reactions found with DNA isolated from 133 other fungal species. The assay was applied to the analysis of malt samples which had been tested for their gushing potential using the industry standard method (MCT). Comparison between the Hyd5 ID4 LAMP assay and the results of the MCT in corresponding samples showed that consistency of results between the two

methods occurred only in 50 % of samples. However, the fact that the new assay indicated 44 % false-negative results relative to the MCT would support observations made by quality control officials according to which the standard test over-exaggerates positive results which may speak in favor of the LAMP assay.

Apart from the assay for testing gushing inducing fungi, another LAMP assay was developed for the group specific detection of *Fusarium* spp. well known as producers of trichothecene mycotoxins. The assay was based on genes within the trichothecene gene cluster. Two genes were used as the basis for primer design: the *tri5* gene coding for trichodiensynthase and the *tri6* gene coding for a regulatory protein. The two genes are potentially present in all fungi which have been identified as trichothecene producers. Separate LAMP assays were developed and optimized for both genes. Analysis of both assays showed that the *tri5* specific primer set was specific for the detection of three major species known for the production of B trichothecenes such like DON and NIV. With the *tri6* specific primer set typical producers of A trichothecenes such like T2-toxin, HT-2 toxin or diacetoxyscirpenol were specifically detected. In order to detect all relevant trichothecene producers in one assay, both primer sets were combined to result in a Duplex LAMP assay. The range of detected *Fusarium* spp. consisted of *F. graminearum, F. culmorum, F. sporotrichioides, F. langsethiae, F. cerealis* and *F. poae*. The capability of the newly developed Duplex LAMP assay for quality control was tested by correlation of results to DON contamination data in 100 wheat samples collected randomly from the 2012 harvest in Bavaria (Germany). As a result, only a weak correlation between the DON content and Duplex LAMP results in corresponding samples was statistically established. However, further evaluating of results indicated that based on the data, a threshold useful for quality analysis could be established. For the more sensitive method of sample preparation (total DNA extraction), threshold T_t value for the Duplex LAMP assay was 47 min with 95 % of samples being correctly identified as having DON concentrations of ≥163 ppb of DON. Using a more rapid method of sample preparation, a threshold T_t of 39 min was found to apply with 91 % of correct results for samples with ≥1000 ppb of DON.

Results obtained during the current study demonstrate that the assays developed are well fit for the analysis of typical materials used in the malting and brewing industries. According to the investigation of sample materials, the assays developed can be an useful tools in the analysis of incoming goods in the malting and brewing industry. The

current work contains several features, which have not been published by other authors before:

1. The Hyd5 ID4 assay is the first description of the application of LAMP for the detection of several fungal species in a group specific or gene specific manner.
2. The combination of two independent primer sets in one LAMP assay for the detection of a non-overlapping set of species has not hitherto been demonstrated.
3. To the author's knowledge, the application of vacuum drying to a complete and active LAMP master mix and its storage over a prolonged period of time is as yet undescribed in the literature.
4. The addition of PEG as an additive to significantly accelerate LAMP reactions is a feature newly described in the current work.

6 Zusammenfassung

Die Rohstoffqualität von Getreide als Basis für die Bierproduktion spielt bei der Brauwirtschaft und getreideverarbeitende Industrie eine wichtige Rolle. Durch den Befall von Rohstoffen und Malz mit Schimmelpilzen der Gattung *Fusarium* kann es zum sogenannten Gushing kommen und zur Kontamination mit Mykotoxinen. Neben wissenschaftlich-technischen Problemen, die sich durch eine *Fusarium*-Kontamination von Braugetreide und Malz ergeben, birgt die Verarbeitung solcher Rohstoffe für die Brauereien auch ein hohes wirtschaftliches und rechtliches Risiko sowie ein gesundheitliches Risiko für die Konsumenten. Das wirtschaftliche Risiko ergibt sich aus vergeblich aufgewendeten Produktionskosten, Kosten durch den Rückruf betroffener Chargen und Umsatzverlusten, die alle zu einem Rückgang der Umsatzerlöse führen. Darüber hinaus resultiert das rechtliche Risiko aus der Gefahr der Überschreitung der gesetzlichen Grenzwerte für Mykotoxine, so dass in diesem Fall auch der Verbraucher in Gefahr ist sich chronisch zu vergiften. Die richtige Wareneingangskontrolle ist eine Möglichkeit der Risikoreduzierung für die Industrie als auch für die Verbraucher.

Um eine Verbesserung und Erleichterung der Qualitätskontrolle in der Brauerei und Mälzerei Industrie zu erreichen, war es das Ziel dieser Arbeit, die Kontamination mit qualitätsrelevanten *Fusarium*-Arten in der Mälzerei-und Brauindustrie mit einem neu entwickelten Gen-spezifischen Test ein Analyse Werkzeug zu schaffen, das schnell und einfach, spezifisch und empfindlich Rohstoffe untersucht und dieses für maximale Benutzerfreundlichkeit zu optimieren, um sie für die Analyse von natürlich infizierten Probenmaterialien anzuwenden.

Um dieses Ziel zu erreichen, ist in der vorliegenden Arbeit eine neue Methode entwickelt worden, das auf dem Prinzip der loop-mediated isothermal amplification of DNA (LAMP) beruht. Die neuen Tests ermöglichen Identifizierung von Reinkulturen von qualitätsrelevanten *Fusarium* spp. sowie deren Nachweis in belastetem Getreide und Malz in weniger Zeit und mit weniger Aufwand an Material und Personal im Vergleich zu klassischen und PCR-basierten Methoden. Für die Anwendung der neuen Tests wurden natürlich infizierte Proben von Getreide und Braumalz getestet und die Ergebnisse verglichen mit Mykotoxingehalt und Gushing Potential der jeweiligen Proben, um die LAMP Ergebnisse mit den Qualitätsparametern zu korrelieren.

Der Hyd5 ID4 LAMP-Test ist ein gruppenspezifischer Test auf der Basis des *hyd5* Gens das typischerweise in Gushing induzierenden *Fusarium* spp. vorkommt. Die Ergebnisse

zeigen, dass *F. graminearum, F. culmorum* und *F. cerealis* sehr spezifisch detektiert werden ohne Kreuzreaktionen mit DNA isoliert von 133 anderen Pilzen. Zur Analyse wurden Malzproben aus der Praxis, die bereits auf ihr Gushing-Potential (MCT) untersucht wurden, getestet. Vergleich zwischen dem Hyd5 ID4 LAMP-Test und der Ergebnisse des MCT in entsprechenden Proben haben gezeigt, dass die Übereinstimmung der Ergebnisse der beiden Verfahren in nur 50% der Proben eintrat. Allerdings unterstützt die Tatsache, dass der neue Test 44% falsch-negative Ergebnisse in Bezug auf die MCT-Beobachtungen angibt, die Beobachtung, dass der Standard-Test in der Qualitätskontrolle mit zu vielen positiven Ergebnissen übertreibt und dies damit zu Gunsten des LAMP-Tests spricht.

Neben dem Test zur Prüfung Gushing induzierender Pilze, wurde ein weiterer LAMP-Test für den gruppenspezifischen Nachweis von Trichothecene produzierenden *Fusarium* spp. entwickelt. Der Test basiert auf Genen innerhalb des Trichothecene-Gen-Clusters. Als Grundlage dazu wurden zwei Gene verwendet: das für die Trichodiensynthase kodierende *tri5* Gen und das für ein regulatorisches Protein bei der Trichothecen Biosynthese kodierende *tri6* Gen. Diese beiden Gene kommen prinzipiell in allen Pilzen vor, die Trichothecene bilden können. Für beide Gene wurde je ein LAMP Test entwickelt und optimiert. Untersuchungen zu den von beiden Tests zeigten, dass das *tri5*-spezifische Primerset spezifisch für die Detektion von drei Arten waren, die für die Produktion von B-Trichothecenen wie DON und NIV bekannt sind. Das tri6-spezifische Primerset erfasst dagegen Arten, die A-Trichothecene wie T2-Toxin, HT-2 Toxin oder Diacetoxyscirpenol bilden. Um jedoch einen Test zur Verfügung zu haben, mit dem alle relevanten Trichothecenbildner zusammen erfasst werden können, wurden die beiden Primersets in einer Duplex-LAMP zusammengefasst. Das Spektrum der nachgewiesenen *Fusarien* besteht aus *F. graminearum, F. culmorum, F. sporotrichioides, F. langsethiae, F. cerealis* und *F. poae*. Um die Tauglichkeit des entwickelten Verfahrens zu zeigen und gleichzeitig zu untersuchen, ob es eine Korrelation zwischen dem Nachweis von Trichothecenbildnern und dem Vorliegen einer Mykotoxinbelastung gibt, wurden 100 Proben von Weizen aus ganz Bayern (Deutschland) des Jahres 2012 mit dem so optimierten Duplex-LAMP Test getestet. Als Ergebnis wurde nur eine schwache Korrelation zwischen den DON-Gehalt und Duplex LAMP Ergebnisse in entsprechenden Proben statistisch festgestellt. Aber bei weiterer Auswertung der Ergebnisse zeigte sich, dass auf der Grundlage der Daten, ein Grenzwert nützlich für die qualitativen Analysen festgestellt werden konnte. Für die empfindlichere Methode der Probenvorbereitung

(Gesamt-DNA-Extraktion) war der T_t Schwellenwert für den Duplex-LAMP-Test bei 47 min mit dem 95 % der Proben korrekt mit einer DON-Konzentration von ≥ 163 ppb DON identifiziert werden konnte. Unter Verwendung eines schnelleren Verfahrens zur Probenvorbereitung, wurde ein Schwellwert T_t von 39 min festgestellt, bei dem 91% der Proben korrekt mit ≥ 1000 ppb DON identifiziert wurden.

Ergebnisse aus der aktuellen Studie demonstrieren, dass die entwickelten Tests für die Analyse von typischen Materialien in den Mälzerei-und Brauindustrie gut anzuwenden sind. Nach der Untersuchung von Probenmaterialien können die entwickelten Tests ein nützliches Werkzeug in der Analyse bei der eingehenden Waren in der Mälzerei-und Brauindustrie sein. Die aktuelle Arbeit enthält mehrere Ergebnisse, die nicht von anderen Autoren zuvor veröffentlicht worden sind:

1. Der Hyd5 ID4 Test ist die erste Beschreibung einer Anwendung der LAMP für den Nachweis von verschiedenen Pilzarten in einer bestimmten Gruppe oder genspezifischen Weise.
2. Die Kombination der zwei unabhängigen Primersets in einem LAMP Test zum Nachweis von einer nicht-überlappenden Gruppe von Spezies wurde bisher nicht gezeigt.
3. Nach Kenntnis der Autorin ist die Anwendung der Vakuumtrocknung zu einem vollständigen und aktiven LAMP Master-Mix und ihre Lagerung über einen längeren Zeitraum in der Literatur bisher unbeschrieben.
4. Die Zugabe von PEG als Additiv beschleunigt deutlich die LAMP Reaktionen was eine Funktion ist, die neu in dieser Arbeit beschrieben wird.

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