Validation of a Quick PCR Method Suitable for Direct Sequencing: Identification of *Fusarium* Fungal Species and Chemotypes for Preventive Approaches in Food Safety

*Marine Pallez, Matias Pasquali*, Torsten Bohn, Lucien Hoffmann and Marco Beyer

Public Research Centre Gabriel Lippmann, Environment and Agro-Biotechnologies Department, 41, rue du Brill, LU-4422 Belvaux, Luxembourg

Received: April 26, 2013
Accepted: February 19, 2014

Summary

Species determination by sequencing and PCR genetic chemotyping, used to determine the toxigenic potential of *Fusarium* strains, are fundamental for developing preventive strategies in food safety. Here we propose and statistically validate a quick protocol for standardizing the procedure of species determination by sequencing of the elongation factor 1α and multiplex genetic chemotyping using the tri12 gene, based on fungal growth on Miracloth tissue coupled with microwave extraction. The test was validated on 75 *Fusarium culmorum* and *Fusarium graminearum* strains.

*Key words:* *Fusarium culmorum, Fusarium graminearum,* microwave DNA extraction, Ef1-α

Introduction

Polymerase chain reaction (PCR) is widely used for the identification of fungal species. Specific primers and sequencing procedures are needed for cross-checking morphological properties (1). More often, specific target sequences allow determining features of the strain that can be useful for its characterization. Genes coding for dangerous fungal toxins found in food items such as aflatoxins, trichothecenes and fumonisins have already been identified (2). For example, by using primers targeting genes involved in the biosynthetic pathway of trichothecenes, it is possible to determine the toxigenic
potential of *Fusarium* strains \((3,4)\) by predicting whether a specific strain will produce deoxynivalenol and its acetylated derivates, or nivalenol that is of significantly higher toxicity for human cell lines \((5)\). Moreover, fungal species determination can be used as a tool for identifying potential toxigenic risks linked to the presence of species able to produce toxic compounds. Therefore, PCR is essential for preventive approaches in the area of food safety.

For this purpose, simple, rapid and reproducible preparation of nucleic acids is required. However, as with plants, fungal nucleic acids are often complicated to extract due to the presence of cell walls and secondary metabolites that can inhibit enzymatic reactions, making the DNA extraction process a crucial step. Several protocols for fungal DNA extraction have been reported. Many methods rely on a mechanical disruption of the cell by grinding \((6,7)\), which can be preceded by a freezing step in liquid nitrogen \((8-11)\), by the use of a mortar/pestle \((12-15)\) or a bead milling method \((16-18)\). Other methods for breaking up the mycelium include sonication \((16)\) or thermolysis \((16,19,20)\) method to extract DNA. Chemical extraction such as the one using CTAB (cetyl trimethylammonium bromide), phenol, or chloroform is largely adopted \((1,15,16,20-24)\), as well as enzymatic extraction \((25-27)\) methods. These methods have gradually been incorporated or replaced by less cumbersome procedures such as the use of kits \((1,16,19,28-35)\) based on membrane filtration or magnetic separation. In all cited methods, sample preparation however is required and even with the use of automatic grinders the time and the possibility of upscaling the extraction procedures are limited and/or expensive.

Other methods were developed to simplify the extraction, such as techniques allowing a direct extraction into an Eppendorf tube using a pestle or a mortar \((36)\) from fresh or lyophilized mycelium. For example, Cenis \((9)\) cultivated fungi directly in an Eppendorf tube, which can reduce the risk of contamination. Based on the discoveries of Goodwin and Lee \((37)\) who showed that it is possible to extract DNA from eukaryotic cells with microwave radiation, Ferreira and Glass \((38)\) adapted this technique to fungal spores. Direct spore PCR is performed after irradiation of the spores and buffer solubilization, a stage of vortexing and a centrifugation. The availability of engineered taq has improved the efficiency of direct PCR approaches; therefore the implementation of direct PCR methods would facilitate large screening efforts on fungi. Recently, Fata et al. \((39)\) and Borman et al. \((40)\) developed a rapid method for the preparation of total genomic DNA using Whatman FTA filter papers (GE Healthcare, Fairfield, Connecticut, USA). The principle is based on the lysis and inactivation of microorganisms, provided by chelators and denaturants present in the Whatman paper, coupled with homogenous collection of mycelium on the surface of the paper. More recently, Ben Amar et al. \((41)\), developed a direct PCR-
based procedure for DNA amplification from crude samples or spores in *F. culmorum* by manual sampling of the mycelium.

Fusarium head blight (FHB), mainly caused by *Fusarium* species, is a major disease of small grain cereals. The disease can cause significant problems of yield and quality losses (42). Major concerns arise from the contamination of grains with mycotoxins and their impact on human health and animal development (5,43). The prediction of the presence or absence of *F. graminearum* and *F. culmorum*, the main producers of mycotoxins in wheat, as well as the determination of chemotypes may play an important role in preventive food safety strategies. For this purpose, a cheap, high throughput method is needed.

Here, by combining a homogeneous method of mycelium sampling (simplifying the sample preparation) and a PCR approach, we tested 4 protocols for their efficacy in multiplexing with 4 primers and sequencing of PCR products on a large set of *Fusarium* isolates.

**Materials and Methods**

**Samples**

*Fusarium* samples, deposited in the strain collection of the Centre de Recherche Public - Gabriel Lippmann (Luxembourg), were collected from different wheat fields across Luxembourg in 2011 (Table 1). After isolation as described previously (35), strains were stored at -80°C in 15 % of glycerol as spore suspensions.

Samples were prepared according to two protocols. First, for *Fusarium* spp. a potato dextrose agar medium (PDA, 39 g/L) was prepared, sterilized and poured into Petri dishes. To facilitate the sampling of mycelium, 5 mm diameter pieces of four types of materials were placed on the petri dish before mycelium inoculation: Miracloth (Merck Millipore, Darmstadt, Germany), Wypall L40 (Kimberly-Clark Professional), dialysis membrane (Spectra/Pol, cellulose, molecular porous membrane tubing, molecular weight cut-off: 12-14,000, Spectrum Laboratories Inc, Rancho Dominguez), and paper (Planet+, Laser, copier and inkjet, 80 g/m², Xerox). Afterwards, Petri dishes were inoculated with 10 μL of spore suspension (approximately 1000 spores) and kept at 22 °C in the dark for 5 days (or for 13 days to test the effect of aging of the mycelium on the extraction method). In the second procedure used for the Qiagen DNA extraction kit, potato dextrose broth medium (PDB, 24 g/L) was prepared and 50 mL distributed in an Erlenmeyer flask, then sterilized. It was inoculated with 10 μL of spore suspension and incubated on an orbital shaker at 150 rpm at 25°C in the dark. After
one week of growth, the mycelium was recovered by filtration and transferred into an 1.5 μL Eppendorf tube. The mycelium was freeze-dried for 24 hours (Christ Alpha 2-4 LSC, Marin Christ and Co., Osterode am Harz, Germany), immersed in liquid nitrogen and crushed with a mixer mill (Mixer mill MM200, Retsch GmbH and Co., Haan, Germany) at a frequency of 25 Hz (3 times, 20 seconds). The mycelium was stored at -20°C.

**DNA Extraction method**

DNA extraction methods used (Fig. 1) were:

1) a microwave DNA extraction,
2) an extraction by a heating step,
3) a direct extraction in PCR tubes,
4) a mycelium in-tube grinding extraction coupled with microwave extraction,
5) and, for comparison, the DNeasy plant Mini Kit extraction (Qiagen, Hilden, Germany).

**Microwave DNA extraction**

Discs of these materials covered with mycelium were removed from the Petri dishes with a sterilized toothpick and placed into an Eppendorf tube with 100 μL of AE buffer (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0) (8).

DNA was extracted using microwave irradiation, slightly modifying the protocol of Ferreira and Glass (16). Each tube containing the disc with mycelium on it and the AE buffer was irradiated in a domestic microwave (Easytronic, M571, Whirlpool) with full power (750 W) for 5 min.

**Extraction by a heating step**

The initial steps of the extraction were carried out according to the same protocol as in the microwave DNA extraction. Once the discs were removed from the Petri dishes, Eppendorf tubes were placed in a heating block at 95°C for 10 minutes.

**Direct PCR extraction**

Samples were also processed in a direct PCR approach using the Phusion® High-Fidelity PCR Master Mix (Thermo Scientific, Wilmington, State, USA). One of the discs covered with mycelium, was transferred from the petri dishes with a sterilized toothpick and placed into PCR tubes containing 50 μL
of the Master mix (Thermo Scientific, Wilmington, USA), 2.5 µL of each primer and 45 µL of DNase/RNase free water. The denaturation was extended to eight minutes at 98 °C.

Grinding of mycelium plus microwave extraction

Aerial mycelium was manually collected from a strain inoculated on PDA, mixed with 180 µL of AE buffer and grinded with a pestle for 1 min (4) in the Eppendorf tube. Five minutes of microwaving at 750 W were applied to the mixture. A centrifugation for 30 s at 12,000 g allowed the deposition of mycelia. Three µL of the upper phase were used for the single and multiplex PCRs carried out as described below.

DNeasy Plant Mini Kit extraction

Samples were also extracted according to Dubos et al. (35) using the Qiagen DNeasy Plant mini kit (Qiagen, Crawley, West Sussex).

After DNA extraction, an evaluation of the DNA quality and amount was done on precast 1% agarose gel (Biorad, Hercules, California, USA) using ethidium bromide coloration for staining. The gel was run at 110 Volt for 5 min, then at 80 V for 1h30min. Fluorescent bands, exposed to UV light, were compared relative to a 2-Log DNA Ladder (0.1–10.0 kb, New England BioLabs, Ipswich, Massachusetts, USA). Estimation of purity ratio (260/280) was calculated using the NanoDrop® ND-1000 (Thermo Scientific, Wilmington, Delaware, USA).

Single and multiplex PCR

Single and multiplex PCRs were carried out on the materials obtained from the different sampling and extraction procedures. Primers EF1 and EF2 (44) were used to identify the Fusarium species (1). The amplification was optimised using 2x Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific). This mix contains a Phusion DNA Polymerase, 2x Phusion HF Buffer and 400 µM of each dNTP and 1.5 mM MgCl₂. PCR was performed in 50 µL containing 5 µL of the supernatant liquid, 25 µL of the Master mix, 1.25 µL of each primer (10 µM) and 18 µL of DNase/RNase free were water. Gradient PCR was performed on a Biometra T- professional cycler (Goettingen, Germany) to define the optimum temperature for annealing, resulting in the final PCR program: 98°C during 2 min for DNA strand separation; then 40 cycles of denaturation at 98°C for 15 seconds, annealing at 55.2°C for 20 seconds, elongation at 72°C for 20 seconds; 5 minutes at 72 °C for the final extension followed by 4°C
until gel loading. A precast 3% agarose gel (1*32 wells, Bio-Rad Laboratories, Hercules, CA, USA) was used. The gel was exposed to 110 V for 40 min. Gel pictures were captured using an image analyzer (Ingenius Syngene Bio Imaging, Syngene, Cambridge, UK). Fluorescent bands were compared relative to a MassRuler™ Low Range DNA Ladder, ready-to-use (80-1031 bp, Thermo Scientific) and to the positive control strain PH1 from the USDA collection. A band between 700 bp and 800 bp was evidence for the presence of a *Fusarium* strain.

PCR products were then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Durham, North Carolina, USA) and quantified before PCR labelling. The concentration of each sample was adjusted to 25mg/µL. Labelling of PCR was carried out according to manufacturer procedures using the Big Dye Terminator v3.1 Cycle Sequencing Kit method (Applied Biosystems, 2002) on both forward and reverse strain using EF1 and EF2 primers. PCR conditions were the following: 96°C for 1 minute, 25 cycles of 10 seconds at 96°C and 4 minutes at 60°C and to finish a cooling at 4°C until purification. BigDye® Xterminator Purification kit was used for eliminating all the bases not incorporated and the primers.

Sequencing was carried out using an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems Carlsbad, United States). Use of the sequence scanner V1.0® (Applied Biosystems) and CLC main workbench 6® (CLC bio Germany, Muehltal, Germany) software allowed to complete the sequence translation from the fluorescence signal intensity to nucleotide sequence. Sequencing was performed two times in order to evaluate the technical variability. The sequencing parameters QV20+ and sequence length were investigated.

The chemotyping PCR was performed using the primers 12 CON, 12 NF, 12-15F and 12-3F (Table 2) (4,45). The amplification was optimised using the Phusion master mix (Thermo Scientific, Wilmington, USA). PCR was performed in 50 µL containing 4 µL of the supernatant liquid, 25 µL of the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific, Wilmington, USA), 1 µL of each primer (10 µM) and 17 µL of DNAse/RNAse free water. In all comparative tests among methods, three strains (*F. culmorum* 4000, 4016, 4033) were used.

To test long term storage features of DNA extracted from Miracloth growing mycelium, samples were stored for 18 months at -20 °C and then used for a EF1-α amplification as described above.
Data analysis
Statistical analyses were performed using SPSS version 19 software (SPSS INC. Chicago, IL, USA) and Sigmasstat v 2.03 (Systat Software Inc, Chicago, Illinois, USA). In order to evaluate the normality of the distribution, the Kolmogorov-Smirnov test was applied. Because comparisons were carried out between methods, different strains processed with the same method were considered as replicates to also take into account the biological variability.

To assess the purity of the samples, statistical analysis were done on the 260/280 ratio using one way analysis of variance in Sigmasstat.

To compare the quality of the sequencing results between each modality, two parameters were chosen: the contiguous read length of the sequence and the QV20+ which is a score of accuracy of sequencing of 99% or above. QV 20+ and contiguous read length are assessed automatically by the Sequencing Analysis software (Life Technologies, USA).

First, the effect of the two primers was compared using independent samples Mann-Whitney U test. As no significant effect was evident, data of both primers were combined for each treatment. Contiguous length and QV20+ parameters were analysed by the Kruskal-Wallis test for comparison of the five independent distributions. A corrected P-value <0.05 (2-sided) was considered as statistically significant.

Results and Discussion
The quick standardized sampling of the microwave extraction procedure was analysed and compared to the other methods. Five different procedures for species identification by PCR were compared. Three isolates (F. culmorum 4000, 4016, 4033) were used to compare the methods with each other.

Only three of the methods extracted DNA and allowed to consistently amplify the EF gene from Fusarium species as confirmed by agarose gel electrophoresis (number 1, 4, 5 from Fig. 1): DNeasy Plant Mini Kit, microwave, and mycelia grinding with pestle plus microwave (Fig. 2). Indeed, extraction by a heating step and the use of direct PCR using Phusion master mix without further processing were not able to amplify the EF factor consistently (data not shown). This contrasts with the result of Ben Amar et al. (41), who successfully applied direct PCR to characterise a number of F. culmorum strains. This may be due to two factors: the taq-polymerase and master mix combination, different from the present conditions (41), and the mycelium sampling which could play a role in the efficiency of PCR reaction. We observed that the most difficult aspect of direct PCR is that reproducibility varies
according to the amount of mycelium collected and the strain used. For this reason we developed a quick and cheap method of growing colonies over a standardized defined area of material that can be easily transferred to PCR tubes, and we validated the assay on a large number of samples. We also observed that microwave processing increased the reproducibility of the assay and for this reason we focused on the optimal method able to combine the standardized extraction and the speed in the process.

We therefore compared the different materials used for collecting the mycelium from the plate with a standard kit procedure (Qiagen kit) and with a previously developed method based on manual grinding in Eppendorf tubes (4). No statistically significant difference ($P = 0.333$) between Miracloth, paper, Wypall, dialysis membrane and Qiagen was found with respect to the purity of the sample as tested by the 260/280 ratio (Table 3).

When the DNA was then used for EF1/EF2 amplification it was evident that the paper modality was working less efficiently (Fig. 2), suggesting a potential increase of inhibitory effects that could be linked to the more intense colouring of the mycelium on the substrate. The grinding method performed well in PCR amplification with a lower efficiency of amplification of one isolate (Fig. 2). This difference may be explained by the collection method that did not allow a sampling with a homogeneous amount of mycelium, further suggesting the advantage of a defined area of mycelium to obtain consistent results.

In order to compare the efficacy of the method on old colonies, a PCR was performed comparing five and thirteen day old colonies. Strains of F. culmorum (4043, 4044, 4056, 4057) were used and DNA 260/280 values were obtained by using the NanoDrop® ND-1000 (Thermo Scientific, Wilmington, USA). The purity of the extract was slightly lower for the older colonies (1.73 ± 0.15 at 5 days and 1.44 ± 0.07 at 13 days) suggesting that the amount of pigments may increase with the age of the colony ($P = 0.004$).

Nonetheless, no evident difference on the PCR efficiency of the elongation factor 1 alpha could be observed comparing the extraction of colonies having an age of 5 days and 13 days (Fig. 3A). Storing, similarly, did not influence the ability to efficiently amplify EF1 alpha as 1.5 years storage of samples allowed good quality amplification (Fig. 3B).

As the method using the Miracloth filter paper was performing efficiently and allowed to identify all the 75 strains as listed in Table 1, the same extraction was used for determining the chemotype, by using primers developed on TRI12. The procedure included multiplexing 4 primers, requiring therefore a good DNA quality to be performed. Indeed, chemotypes could be distinguished by using DNA amplification (Fig. 4).
To evaluate the possibility to perform sequencing reactions on the quickly extracted mycelium, sequencing was performed on the different EF products using EF1 and EF2 primers obtained from the strains 4000, 4016, 4033.

Few differences could be detected among the methods such as that Qiagen had a better QV20+ (Table 4a) and was significantly different from the membrane ($P = 0.0001$), the Miracloth ($P = 0.025$) and the paper ($P = 0.0001$) (Table 4b) extraction method. Concerning the length of the sequence, Wypall was significantly different from Qiagen ($P = 0.012$) and paper ($P = 0.024$), but did not differ from the two other methods (Table 4b).

Despite the higher intensity of the sequencing signal obtained with the Qiagen kit, sequences obtained using Miracloth, Wypall and dialysis membrane were readable and allowed the identification of the sequences without mistakes by a blast search in the Fusarium database (19), (http://isolate.fusariumdb.org/blast.php) and the National Center for Biotechnology Information Blast tool (http://blast.ncbi.nlm.nih.gov/blast.cgi?CMD=web&page_type=blasthome). Aligned sequences showed identical results. All sequences are deposited at NCBI database (GenBank IDs: KJ170153-KJ170227).

The efficacy of the paper method for subsequent DNA extraction and sequencing was lower compared to the other methods due to the variability of results obtained with the paper. As paper may induce different colorations in the strains it is probably activating secondary metabolites pathways that may produce inhibitory molecules of PCR. PCR amplification had an effect on the sequence quality afterwards. For this reason the use of the paper is not ideal for our purposes.

In order to study the application of the method to other fungi, the method was also tested on 69 strains of *Z. tritici* (*data not shown*) confirming the effectiveness and speed of this method.

**Conclusions**

In conclusion, we demonstrated that coupling growth of mycelium on Miracloth with microwave processing is a valuable, cheap and reliable method for large screenings of *Fusarium* isolates in order to determine the toxigenic potential of the strains. We verified that direct sequencing of the products and a multiplex with 4 primers could be performed with 100% efficiency on 75 *Fusarium* strains. We could not reproduce recent results reported by Ben Amar et al. (2012) (41) of direct PCR with our master mix. It seems as though the method developed by the latter authors relies on operator ability to collect similar amounts of mycelium in all assays. For this reason, the use of a surface with defined area for collecting
the mycelium guaranteed better reproducibility for large screenings. Given the easy handling, rapid preparation of samples and the high reproducibility of the results for the identification and characterization of strains, the method proposed here will be further used in currently running chemotyping studies aiming at the characterisation of Fusarium chemotypes over Europe.

Acknowledgements
We would like to thank Sylvain Legay and Laurent Solinhac for running the sequencer and Servane Contal and Boris Untereiner for the technical assistance. This study was realized in the framework of the ANTREPP project.

References


**Table 1.** Strain designation, sampling site, geographic coordinates expressed according to the Luxembourg Reference Frame (LUREF) datum, sampling date and species identified according to the quick PCR method.

<table>
<thead>
<tr>
<th>Strain designation</th>
<th>Sampling site</th>
<th>Geographical coordinates</th>
<th>Sampling year</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>4000, 4004, 4005, 4006, 4007, 4008, 4009, 4010, 4011, 4012, 4013, 4014, 4015</td>
<td>Bettel</td>
<td>82476, 109697</td>
<td>2011</td>
<td><em>F. culmorum</em></td>
</tr>
<tr>
<td>4016, 4020, 4021, 4022, 4023, 4024, 4025, 4026, 4027, 4028, 4029, 4030, 4031, 4032</td>
<td>Fingig</td>
<td>62534, 75512</td>
<td>2011</td>
<td><em>F. culmorum</em></td>
</tr>
<tr>
<td>4033, 4037, 4038, 4039, 4040, 4041, 4042, 4043, 4044, 4045, 4046, 4047</td>
<td>Hamiville</td>
<td>60812, 122138</td>
<td>2011</td>
<td><em>F. culmorum</em></td>
</tr>
<tr>
<td>4048, 4052, 4053, 4054, 4055, 4056, 4057, 4058, 4059, 4060, 4061, 4062, 4063</td>
<td>Hivange</td>
<td>63997, 76842</td>
<td>2011</td>
<td><em>F. culmorum</em></td>
</tr>
<tr>
<td>4064, 4068, 4069, 4070, 4071, 4072, 4073, 4074, 4075, 4076, 4077, 4079</td>
<td>Strassen</td>
<td>73998, 75159</td>
<td>2011</td>
<td><em>F. culmorum</em></td>
</tr>
<tr>
<td>4083, 4084, 4085, 4086, 4087, 4088, 4089, 4090, 4091, 4092, 4093</td>
<td>Erpeldange</td>
<td>76367, 102557</td>
<td>2011</td>
<td><em>F. graminearum</em></td>
</tr>
</tbody>
</table>
Table 2. Gene target, primer sequence, expected product size, reference of the study and chemotype identified (when available).

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Product size</th>
<th>References</th>
<th>Chemotype identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tri 12</td>
<td>12CON</td>
<td>5’-CATGAGCATGGTGATGTC-3’ 5’-TCTCCTCGTTGTATCTGG-3’</td>
<td>840 bp</td>
<td>(46)</td>
<td>NIV 15-ADON 3-ADON</td>
</tr>
<tr>
<td></td>
<td>12NF</td>
<td>5’-TACAGCGGTGCAACTTC-3’ 5’-CTTGGCAAGCCGTGCA-3’</td>
<td>670 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-15F</td>
<td></td>
<td>410 bp</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12-3F</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elongation factor 1</td>
<td>EF1</td>
<td>5’-ATGGGTAAGGAGGACAAGAC-3’</td>
<td>704 bp</td>
<td>(44)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>EF2</td>
<td>5’-GGAAGTACCAGTGATCATGTT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NIV=Nivalenol; 15-ADON=15-acetylated deoxynivalenol; 3-ADON=3-acetylated deoxynivalenol
Table 3. Ratio of the 260/280 absorbance obtained using the NanoDrop® ND-1000 (Thermo Scientific, Wilmington, USA) from 2 replicates of 3 biological samples. No significant differences were detected among the methods (ANOVA P-value = 0.333).

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>260/280 Average</th>
<th>STD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miracloth</td>
<td>1.73</td>
<td>0.023</td>
</tr>
<tr>
<td>Wypall</td>
<td>1.69</td>
<td>0.006</td>
</tr>
<tr>
<td>Dialysis Membrane</td>
<td>1.70</td>
<td>0.050</td>
</tr>
<tr>
<td>Paper</td>
<td>1.68</td>
<td>0.053</td>
</tr>
<tr>
<td>Qiagen</td>
<td>1.73</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Table 4. A. Sequence read length and QV20+ values obtained with different extraction methods. B. Corrected P-values of each pairwise comparison among all methods tested for read length and QV20+ values.

A

<table>
<thead>
<tr>
<th>Extraction method</th>
<th>Contiguous length</th>
<th>QV20+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miracloth</td>
<td>639 ± 4</td>
<td>632 ± 5</td>
</tr>
<tr>
<td>Wypall</td>
<td>640 ± 5</td>
<td>634 ± 3</td>
</tr>
<tr>
<td>Membrane (dialysis)</td>
<td>634 ± 7</td>
<td>608 ± 42</td>
</tr>
<tr>
<td>Paper</td>
<td>577 ± 88</td>
<td>574 ± 75</td>
</tr>
<tr>
<td>Qiagen</td>
<td>630 ± 8</td>
<td>662 ± 15</td>
</tr>
</tbody>
</table>

Values are expressed as length of sequence reads ± S.D. and average of QV20 score ± S.D. Standard deviation is based on n=3 independent biological replicates.
### B

<table>
<thead>
<tr>
<th>Pairwise comparison</th>
<th>Length p-values</th>
<th>QV20+ p-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane – Paper</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Paper – Miracloth</td>
<td>0.200</td>
<td>0.499</td>
</tr>
<tr>
<td>Paper - Wypall</td>
<td>0.024</td>
<td>0.120</td>
</tr>
<tr>
<td>Paper - Qiagen</td>
<td>1.000</td>
<td>0.0001</td>
</tr>
<tr>
<td>Membrane - Miracloth</td>
<td>1.000</td>
<td>0.564</td>
</tr>
<tr>
<td>Membrane – Wypall</td>
<td>0.284</td>
<td>0.140</td>
</tr>
<tr>
<td>Membrane – Qiagen</td>
<td>1.000</td>
<td>0.0001</td>
</tr>
<tr>
<td>Miracloth - Wypall</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>Miracloth - Qiagen</td>
<td>0.116</td>
<td>0.025</td>
</tr>
<tr>
<td>Qiagen - Wypall</td>
<td>0.012</td>
<td>0.133</td>
</tr>
</tbody>
</table>

Each row tests the null hypothesis that the two sample distributions are the same. Significant differences are marked in grey.
Figure 1. The scheme represents the different extraction procedures tested in this work. The procedure that is proposed and validated in this work is highlighted in bold letters. The numbers associated with the different extraction methods refer to the description in the text.
Figure 2. Agarose gel (3%) showing PCR products of elongation factor of 4 *Fusarium culmorum* strains: (1, 5, 9, 13) Miracloth, (2, 6, 10, 14) Wypall L40, (3, 7, 11, 15) dialysis membrane, (4, 8, 12, 16) paper, (17, 18, 19, 20) mycelia in tube grinding, (21) positive control PH1 obtained using Qiagen DNA extraction kit, (22) negative control, (M) marker : Mass Ruler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Scientific).
Figure 3. A. Agarose gel (3%) showing five PCR products obtained by quick extraction method of DNA from *F. culmorum* isolates (4043, 4044, 4056, 4057) using miracloth filter paper. From 1 to 4: sampling five days after inoculation. From 5 to 8: thirteen days after inoculation. Lane + is a positive control and lane – is a negative control. M is the marker: MassRuler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Scientific). B. Agarose gel (3%) showing four PCR products obtained by quick extraction method of DNA from *F. culmorum* isolates (4021, 4022, 4045, 4046) using miracloth filter paper. From 1 to 4: the direct PCR was realized the 18.12.2013 directly after quick DNA extraction. From 5 to 8: the PCR after storage was realized the 22.05.2012 after one year and a half of storage at -20°C. Lane + is a positive control and lane – is a negative control. M is the marker: MassRuler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Scientific).
Fig. 4. Agarose gel (3%) showing PCR amplification products obtained using primers developed in (45). M is the marker: MassRuler Low Range DNA Ladder, Ready-to-Use, 80-1031 bp (Thermo Scientific). From 1 to 6: Multiplex PCR from *F. culmorum* (2, 3, 5, 6) and *F. graminearum* (1, 4). From 7 to 9: positive control of chemotype 15-ADON (7), NIV (8), 3-ADON (9) as from Pasquali et al., 2011 (46). Lane 10 is a negative control.