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Abstract	The determination of aflatoxin production ability and differentiation of aflatoxigenic strains can be assessed by monitoring the expression of one or several key genes using reverse transcription polymerase chain reaction (RT-PCR). We herein describe the methods for RNA induction, extraction, and quality determination, and the RT-PCR conditions used to evaluate the ability of a given *Aspergillus* strain to produce aflatoxins.	

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Keywords (separated by " - ")	*Aspergillus flavus* - Mycotoxins - Aflatoxigenic fungi - RNA extraction - RT-PCR - Gel electrophoresis

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Chapter 15

Evaluating Aflatoxin Gene Expression in Aspergillus Section Flavi

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Abstract

The determination of aflatoxin production ability and differentiation of aflatoxigenic strains can be assessed by monitoring the expression of one or several key genes using reverse transcription polymerase chain reaction (RT-PCR). We herein describe the methods for RNA induction, extraction, and quality determination, and the RT-PCR conditions used to evaluate the ability of a given *Aspergillus* strain to produce aflatoxins.

Key words *Aspergillus flavus*, Mycotoxins, Aflatoxigenic fungi, RNA extraction, RT-PCR, Gel electrophoresis

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1 Introduction

Aflatoxins (AF) are the most widely studied of all mycotoxins produced by *Aspergillus* species. Although aflatoxin production ability has been detected in various species, *A. flavus* Link:Fr. and *A. parasiticus* Speare (belonging to *Aspergillus* section *Flavi*) remain the most important and representative aflatoxin producers occurring naturally in food commodities. Molecular techniques have been widely applied in order to discriminate between aflatoxigenic and non-aflatoxigenic strains, through the correlation of presence/absence of genes involved in the aflatoxin biosynthetic pathway with the ability/inability to produce aflatoxins. However, AF biosynthesis is based on a highly complex pathway which requires at least 25 structural and 2 regulatory genes [1], with possible alternative pathways. Additionally, there are reports on genes that are present but not expressed, even under highly aflatoxin-inductive conditions [2]. Furthermore, it is important to highlight that some genes are not exclusive of the aflatoxin biosynthetic pathway, which could create false positives in the case of sterigmatocystin-producing fungi [3] such as *Aspergillus nidulans*. More recently, aflatoxin

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production and aflatoxigenic strain differentiation are being assessed by monitoring the expression of one or several key genes using reverse transcription polymerase chain reaction (RT-PCR). Such systems have been applied to monitor AF production and biosynthetic gene expression based on various regulatory and structural AF pathway genes in *A. parasiticus* and/or *A. flavus* [2, 4–7]. Although with different levels of success, they were found to be rapid, sensitive, and reliable.

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). All materials and solutions involved in RNA-handling procedures must be RNase-free. Wear gloves during the whole process when working with RNA to protect samples from degradation by RNases.

2.1 Mycotoxin Safety Precautions

All the necessary safety precautions must be taken into account when handling mycotoxin solutions or other potentially contaminated materials since they are highly toxic and potent carcinogenic compounds. Handle contaminated material with protective gear; decontaminate all disposable materials by autoclaving before being disposed; decontaminate reusable materials by immersion in 10% commercial bleach overnight, followed by immersion in 5% acetone for 1 h and washing with distilled water several times.

2.2 Media Preparation

1. Malt extract agar (MEA): Malt extract 20 g/L, glucose 20 g/L, peptone 1 g/L, agar 20 g/L. Mix the components, autoclave (121 °C, 20 min), and plate in 90 cm Petri dishes.
2. Yeast extract sucrose (YES) broth: Yeast extract 20 g/L, sucrose 150 g/L. Mix the components, autoclave (121 °C, 20 min). Distribute 25 mL of YES in 50 mL Falcon tubes.
3. Yeast extract peptone (YEP) broth: Yeast extract 20 g/L, peptone 150 g/L. Mix the components, autoclave (121 °C, 20 min). Distribute 25 mL of YES in 50 mL Falcon tubes.

2.3 RNA Extraction

1. Paper towels: Cover a stack of paper towels by aluminum foil and sterilize in a sterilization oven at 160 °C, overnight.
2. Spatula, mortar, and pestle: Cover by aluminum foil, sterilize in a sterilization oven at 160 °C, overnight, and refrigerate (−20 °C) before use.
3. Eppendorf tubes, PCR tubes, pipette tips: Sterilize by autoclave (121 °C, 1 h). Whether possible, use RNase-free filter pipette tips.

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4. Liquid nitrogen.
5. RNeasy Plant Mini Kit (Qiagen) (*see* **Note 1**).

2.4 RNA Analysis by Gel Electrophoresis

1. RNase-free water: Treat ultrapure water with 0.1% diethyl pyrocarbonate (DEPC) (v/v), mix thoroughly, and store overnight. Autoclave at 121 °C for 1 h to eliminate DEPC. Prepare all solutions with DEPC-treated water (*see* **Notes 2** and **3**).
2. EDTA 0.5 M: pH 8.0: Weigh 93.05 g EDTA-Na_2 (FW = 372.2). Dissolve in 400 mL RNase-free water and adjust to pH 8.0 with NaOH. Make up to a final volume of 500 mL with water (*see* **Note 4**).
3. Tris-acetate-EDTA (TAE 50×): 2 M Tris-acetate, 0.05 M EDTA. Weigh 242 g Tris base (FW = 121.14) and dissolve in approximately 750 mL of RNase-free water (*see* **Note 5**). Carefully add 57.1 mL glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) previously prepared. Adjust the solution up to a final volume of 1 L. The pH of this buffer does not need to be adjusted and should be about 8.5. Store in the dark at room temperature.
4. Tris-acetate-EDTA (TAE 1×): Dilute the stock solution TAE 50× in RNase-free water. For example, to prepare 1 L of TAE 1×, dilute 20 mL of TAE 50× in 980 mL of water. Final solution contains Tris–HCl (40 mM), glacial acetic acid (40 mM), and EDTA (1 mM).
5. SDS washing solution (SDS 10%): Weight 50 g of sodium dodecyl sulfate (SDS) and dissolve in RNase-free water to a final volume of 500 mL (*see* **Note 6**).
6. DNA/RNA dye (*see* **Notes 7** and **8**).
7. Non-denaturing agarose gel (*see* **Note 9**): Prepare a 1.2% agarose gel in TAE 1× and add the recommended amount of DNA/RNA dye.
8. RNA loading buffer (6×): 30% (v/v) glycerol; 0.25% (w/v) bromophenol blue. Store at 4 °C.
9. RNA molecular weight marker.
10. Horizontal electrophoresis apparatus (*see* **Note 10**).
11. Ultraviolet (UV) transilluminator (preferentially coupled to a gel image analysis software).

2.5 RNA Analysis by Spectrophotometry

1. Tris-EDTA (TE) buffer: 10 mM Tris–HCl, 1 mM EDTA (pH 8.0). Add 1 mL of a 1 M Tris–HCl (pH 8.0) stock solution and 0.2 mL of 0.5 M EDTA (pH 8.0) stock solution to 98.8 mL of RNase-free water. Store at room temperature.
2. Spectrophotometer.
3. Quartz cuvette.

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2.6 Analysis of Gene Expression

1. One-Step RT-PCR Pre-Mix (*see* **Note 11**).
2. Primers for *β-tubulin* and *aflQ* genes (Table 1).
3. RNase-free water.
4. RNase-free filter tips.
5. Agarose gel and electrophoresis apparatus (as described for RNA analysis).
6. DNA molecular weight marker (100 pb ladder or similar).

3 Methods

3.1 Biological Material Preparation

1. Grow the isolates under both AF inductive and noninductive conditions (*see* **Note 12**). For that, inoculate a loop full of spores from a 7-day-old culture in MEA into 25 mL of YES (AF inductive) and YEP (noninductive) broths (in 50 mL Falcon tubes).
2. Incubate the cultures horizontally for 4 days at 28 °C, in the dark, with slight agitation (100 rpm).
3. Collect the mycelium with a sterilized spatula, dry the mycelium in sterilized absorbent paper, and rapidly divide it into aliquots of 100 mg.
4. Preserve the mycelium at −80 °C until use or proceed with the RNA extraction protocol immediately (*see* **Note 13**).

3.2 RNA Extraction

1. The Qiagen RNeasy Plant Mini Kit is used for RNA isolation according to the manufacturer's protocol. Grind 100 mg of mycelium to a fine powder with liquid nitrogen (N_2) in a cold mortar and pestle (*see* **Note 14**).

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Table 1
Details of the target genes, primer sequences and expected product length in base pairs (bp) for PCR and RT-PCR

Primer pair	Gene	Primer sequence (5′ → 3′)	PCR product size (bp)	RT-PCR product size (bp)	Reference
Tub1-F Tub1-R	*tub1*	GCT TTC TGG CAA ACC ATC TC GGT CGT TCA TGT TGC TCT CA	1406	1198	[5]
Ord1-gF Ord1-gR	*aflQ*	TTA AGG CAG CGG AAT ACA AG GAC GCC CAA AGC CGA ACA CAA A	719	599	[4]

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2. Transfer the powder with a residual amount of N_2 into a 2.0 mL Eppendorf tube previously refrigerated by immersion in N_2.
3. Leave the N_2 to evaporate completely and immediately follow the extraction protocol as described by the manufacturer (*see* **Notes 15–17**).
4. Store RNA at −70 °C in 5 µL aliquots, to avoid repeated freeze and thaw that would damage RNA.

3.3 RNA Analysis

Determine general quality and yield of extracted RNA, as well as contamination with genomic DNA, by native agarose gel electrophoresis.

3.3.1 Native (Non-denaturing) AgaroseGel Electrophoresis (*See* **Note 9**)

1. Thaw a 5 µL aliquot of each RNA sample (at all times kept on ice) and add 1 µL of 6× loading buffer. Gently mix by reflux and load into the gel (on native gels, the samples are loaded directly without heating).
2. Make sure to include an RNA marker and/or a positive control RNA (commercial RNA or one of your samples known to be intact) in the gel to rule out unusual results due to gel artifacts and to aid in yield determination.
3. Run the gel in TAE buffer, at constant voltage of 5 V/cm (measured between the electrodes) for approximately 1 h.
4. Observe the gel under UV light. Compare fluorescence intensities between samples and standards, and estimate RNA concentration. Even though you might want a more accurate RNA quantitation (*see* below), the gel is still essential to determine RNA quality in terms of degradation and contamination (with protein or genomic DNA) (Fig. 1).

3.3.2 Spectrophotometry (*See* **Note 18**)

1. Place the sample in a quartz cuvette. Zero the spectrophotometer with the solvent. For accurate readings, dilute the sample with TE (*see* **Note 19**) to obtain absorbance (optical density, OD) values between 0.1 and 1.0.
2. The OD at 260 nm (OD260) equals 1.0 for a 40 µg/mL solution of RNA. For RNA concentration apply the following calculation: RNA concentration = 40 µg/mL × OD260 × dilution factor.
3. For an indication of RNA purity, calculate the OD260/OD280 and OD A260/A230 ratios. For pure RNA, both ratios should be very close to 2.0 in TE (*see* **Note 20**) [8]. Lower or higher ratios could be caused by protein, salts, or ethanol contamination.
4. Dilute some aliquots of RNA in water to obtain a working solution of approximately 1 µg/mL and retain others at the original concentration (stock solution).
5. Store RNA at −70 °C in 5 µL aliquots.

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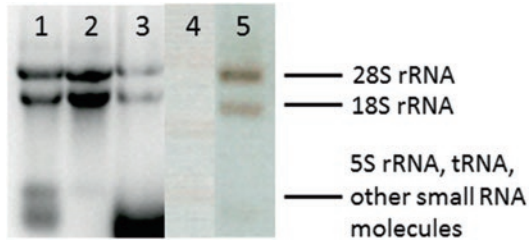

Fig. 1 Native (non-denaturing) agarose gel electrophoresis of RNA samples obtained by different maceration and extraction methods, showing various types of RNA molecules: 1—N_2, RNeasy Plant Mini Kit (RLT buffer); 2—N_2, RNeasy Plant Mini Kit (RLC buffer); 3—N2, Trizol method (Invitrogen); 4—Glass Beads, RNeasy Plant Mini Kit (RLC buffer); 5—TissueRuptor (Qiagen), RNeasy Plant Mini Kit (RLC buffer)

3.4 Analysis of Key Aflatoxin Gene Expression

1. Perform a Multiplex RT-PCR with the obtained RNA (1 μg/mL) using a One-Step RT-PCR Premix (e.g., iNtRON Biotechnology) (*see* **Note 11**).
2. Prepare the mix as described in Table 2, or adjust to the manufacturer's instructions.
3. Prepare a multiplex reaction by using both primer pairs Ord1-gF/gR and Tub1-F/R (Table 1) in the same tube. Primer pair Ord1-gF/gR will amplify the aflatoxin-related gene *aflQ* (formerly *ord1*) gene (*see* **Note 21**). The pair Tub1-F/R will amplify a part of the housekeeping β-tubulin gene *tub1*, which will be used as internal control of amplification (*see* **Note 22**).
4. Set the amplification program in the thermal cycler as described in Table 2.
5. Check for contamination with genomic DNA. Carry out a PCR as described for the amplification step of RT-PCR (Table 2), using the same primers and 1 μg of total RNA as template (*see* **Note 16**). Use the following PCR mix: Taq buffer 1×, $MgCl_2$ 1.5 mM, dNTPs 0.2 mM, each primer 0.2 μM, Taq 1 U (e.g., GoTaq® Flexi DNA Polymerase, Promega), 1 μg of RNA, make up to 20 μL with ultra pure water.
6. Prepare a 1.2% agarose gel in TAE 1× (not necessary to be cautious such as in the case of gels to run RNA). Confirm that you have amplification for the internal control (Fig. 2). The absence of a band at the internal control position (Fig. 2, lane 4) reflects a failed reaction, potentially due to bad RNA quality or amplification inhibitors (false negative). The presence of a product with the expected RT-PCR size confirms aflatoxin gene expression (Fig. 2, lanes 2 and 3) whereas its absence implies no expression (Fig. 2, lanes 1 and 5). The presence of a band with the PCR expected size confirms genomic DNA contamination, but that will not interfere with your analysis,

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Table 2
RT-PCR conditions used for the multiplex amplification of genes *tub1* and *aflQ*

Reaction mix (20 μL)		
One-step RT-PCR pre-mix	8 μL	
Each primer forward	0.2 μM	
Each primer reverse	0.2 μM	
Total RNA	1 μg	
Amplification program		
Reverse transcription	45 °C, 30 min	
Initial denaturation	94 °C, 4 min	
Denaturation	94 °C, 1 min	5×
Annealing	60 °C, 1 min	
Extension	72 °C, 1 min	
Denaturation	94 °C, 1 min	30×
Annealing	55 °C, 1 min	
Extension	72 °C, 1 min	
Final extension	72 °C, 6 min	

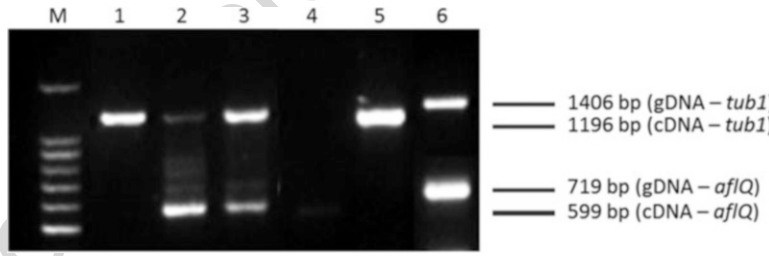

Fig. 2 Electrophoretic pattern of RT-PCR products for *Aspergillus flavus* and *Aspergillus parasiticus* isolates. Lanes: *M*—100 bp DNA ladder (Promega); *1* and *5*—*A. flavus* AF non-producing strain; *2* and *3*—*A. parasiticus* AF producing-strain; *4*—false negative result for *A. parasiticus*; *6*—*A. parasiticus* DNA-PCR control

because primers have been constructed in such a way that genomic DNA and cDNA amplification products will have different sizes (*see* **Note 16**).

4 Notes

1. The *RNeasy Plant Mini Kit* (Qiagen) is one of the most cited methods for fungal RNA extraction and it seems to show the best results for RNA extraction from *Aspergillus* mycelium and conidia. It is, though, more expensive than other routine

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protocols. An alternative protocol using the *TRIzol* reagent (Invitrogen) is also available (Fig. 1) [9].

2. Diethylpyrocarbonate (DEPC) treatment is the most commonly used method for eliminating RNase contamination from water, buffers, and other solutions, as it destroys enzymatic activity by modifying –NH, –SH, and –OH groups in RNases. Solutions containing Tris and EDTA cannot be DEPC-treated. Solutions that cannot withstand autoclaving also cannot be DEPC-treated since autoclaving is essential for inactivating DEPC. It is thus preferable to prepare all solutions with DEPC-treated water instead of treating the solutions themselves. DEPC will dissolve some plastics; glass should be used whether possible.
3. DEPC is highly toxic (oral, dermal, and inhalation), so take special care while handling the reagent. DEPC must be always handled at the fume hood wearing high-protection gloves. When mixing DEPC with water, take special care to avoid spilling; make sure that the flask is tightly closed and even cover the flask with absorbent paper. After the treatment, autoclave the gloves and any other material that has been in contact with DEPC. After being autoclaved, DEPC-treated water is safe to be handled without special care.
4. EDTA solutions must be prepared ahead of time since EDTA dissolution only takes place when the pH is about 8.
5. Tris will dissolve better if you already have *ca*. 100 mL of water and a magnetic stirrer in the cylinder before you add the salt.
6. Wear face mask or use the fume hood when preparing SDS solutions to avoid inhalation of SDS dust. SDS is synonymous to sodium lauryl sulfate. 10% SDS solution will precipitate at room temperature and this solution has to be kept at 37 °C.
7. There are numerous new generation fluorescent DNA and RNA dyes designed to replace the highly toxic ethidium bromide (EtBr) such as SYBR Green I, Gel Red, or Green Safe. The amount of dye recommended by the manufacturer is usually excessive, and you can try to reduce it by one-half or one-third. However, depending on the sample a loss of sensibility might occur using these dyes.
8. If you are not able to avoid EtBr, it is preferable to add it directly to the gel (0.5 μg/mL) to avoid the additional step of gel staining (potentially RNase-prone). EtBr is highly toxic and potentially carcinogenic; make sure that you wear protective gear (highly protective gloves and goggles) when handling it and that you use it in a confined and appropriately identified area.
9. A denaturing gel system (which involves the use of acrylamide, TEMED and formamide) is sometimes suggested because

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RNA might form secondary structures. Denaturing conditions prevent RNA from migrating strictly according to its size. Native agarose gel electrophoresis is sufficient to judge the integrity and overall quality of a total RNA preparation by inspection of the 28S and 18S rRNA bands (Fig. 1). Bands are generally not as sharp as in denaturing gels, but native gels are safer and easier to prepare.

10. Use electrophoresis equipment (tank, trays, and combs) exclusively for RNA analysis, and wash it regularly with 10% SDS and RNase-free water.

11. You may choose between one-step RT-PCR or two-step RT-PCR procedures. Both have pros and cons. Using sequence-specific primers, it might be better the former since it allows easier processing of large numbers of samples and helps minimize carryover contamination (all steps happen in the same tube). However, in some situations two-step procedures are the best option. Independent PCR reactions need to be performed if: (i) you want to test in the same cDNA sample the expression of several genes which require different amplification conditions; (ii) the amplification products are similar in size or; (iii) some interference or cross reaction might be suspected.

12. In order to confirm that AF genes are only expressed under inductive conditions, some isolates should also be tested on YEP (non-AF inductive) broth. It is important to perform this analysis in a wide range of isolates. While testing your method, the presence or absence of AF must be checked by HPLC in both YEP and YES broths used for fungal growth to confirm the correlation between expression of the test gene and AF production. Because AF production is extremely dependent on growth conditions, it is important to determine aflatoxigenic ability under the same test conditions as gene expression. The description of the HPLC method for AF analysis is not within the scope of this text.

13. RNA extraction should be performed on freshly produced material immediately after harvest to avoid RNA degradation. If you are not able to carry out RNA isolation immediately, you must store the harvested mycelium either at –70 °C or at 4 °C after immersion in an RNA-stabilizing solution (e.g., *RNAlatter*, Ambion) until use.

14. The maceration of biological material for RNA extraction is probably the most important and critical step of the procedure. Maceration with liquid nitrogen will result in higher RNA yield and quality (Fig. 1), but requires extra care and skills to avoid RNA contamination and degradation.

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15. Using the Qiagen protocol, we found RLC extraction buffer to work slightly better than RLT buffer for *Aspergillus* mycelium and conidia (Fig. 1).
16. A DNase treatment is recommended to avoid contamination with genomic DNA but it is not mandatory if you choose primers that differentially amplify genomic DNA (gDNA) and complementary DNA (cDNA). Make sure to select primers that span a part of the gene containing at least one intron. That way, you can easily differentiate gDNA from cDNA on the basis of the amplification product size (Fig. 2; Table 1).
17. At the final step of the procedure, elute the RNA in water instead of Elution Buffer to avoid buffer interferences in subsequent reactions.
18. RNA analysis can be performed using a NanoDrop spectrophotometer (ThermoScientific), which is simpler to use and requires smaller amounts of sample than classic spectrophotometers.
19. OD ratios will vary depending on the solvent. While RNA concentration is independent of the solvent you use, OD260/230 and OD260/280 ratios are more reliable if TE is used as solvent (turning pH-dependent variations in the OD230 and OD280 readings null). RNA samples are eluted with water for optimal subsequent reactions, but for spectrophotometer analysis it is better to dilute the samples in TE.
20. In water, OD260/230 and OD260/280 ratios are expected to be 1.8–2 [8].
21. Besides *aflQ*, other key genes in the aflatoxin pathway have been used, e.g., *aflD*, *aflO*, *aflP*, and *aflR* [2, 4–7]. We recommend *aflQ*, since it is the last known gene in the pathway necessary for aflatoxin production and the only one specific for aflatoxin producers.
22. When testing isolates for presence/absence of specific genes, you must include an internal control, which consists of a housekeeping gene, universally expressed in all isolates tested regardless of aflatoxin production ability and culture conditions. Make sure that you choose control primers that work exactly under the same conditions as your test primers. The detection of expression of this internal control is mandatory to rule out false negatives.

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