

Standard Operating Procedure for the Determination of Tissue Fungal Burden Utilizing Quantitative Real Time Polymerase Chain Reaction (QPCR)

1. Purpose

This Standard Operating Procedure (SOP) will provide information necessary to conduct real time QPCR in assessing fungal tissue burden of secondary tissue homogenates from organs harvested from laboratory animals infected with experimental pulmonary aspergillosis at the molecular level. Additional information is provided to encompass additional processing such as purification of *Aspergillus* DNA from culture (for generation of standard curves) and a brief protocol on how to conduct real time Polymerase Chain Reactions utilizing Applied Biosystems TaqMan® reagents and equipment.

2. Scope

This SOP will provide information on how to assess fungal tissue burden of infected animals by use of a single copy (FKS) or multicopy gene (18s RNA) to assess the number of fungal cell nuclei present.

3. Definitions.

“conidial equivalents” means a unit of measurement that infers there is only one nuclei present per cell such as found in the conidiated form of *Aspergillus*.

4. Responsibilities

This SOP shall be utilized by employees of Research assistant status or higher without additional training. Research technicians may perform this work upon receipt of training.

5. Equipment

- Potato Dextrose Agar (PDA) Plates
- Potato Dextrose Broth (Difco) (40ml of broth in 125 ml flasks)
- High speed table top centrifuge (Eppendorf Centrifuge 5415 D)
- Whatman Paper #113, 11 cm diameter (Fisher Scientific)
- Sterile Plastic loops
- DNA bead bashing buffer [2% triton X-100,1%SDS, 100mMNaCl, 10mMTris, (pH 8.0), 1mM EDTA (pH 8.0)]
- phenol-chloroform-isoamyl alcohol (25:24:1)
- Tris-EDTA [10mM Tris·Cl, (pH 8.0) and 1mM EDTA (pH 8.0)]
- 100% ethanol
- 70% ethanol
- 100µg/ml RNase A stock (Sigma-Aldrich)
- 0.1M of Sodium Acetate
- phenol-chloroform isoamyl alcohol (Ambion, Austin, Tx)
- DNase/RNase free sterile water
- Epicentre MasterPure Yeast DNA Purification Kit (Epicentre, Madison, WI)
- Genomic *Aspergillus* DNA standards

- Barrier pipet tips
- DNA standards
- Primers and Probes used:

AF FKS gene¹	
Forward primer	5'GCCTGGTAGTGAAGCTGAGCGT-3'
Reverse primer	5'CGGTGAATGTAGGCATGTTGTCC-3'
Probe	6-FAM-TCACTCTCTACCCCCATGCCCGAGCC-MGB-3'
AF 18sRNA gene²	
Forward primer	5'-GGCCCTTAAATAGCCCGGT-3'
Reverse primer	5'TGAGCCGATAGTCCCCCTAA-3'
Probe	6-FAM-AGCCAGCGCCCGCAAATG-MGB-3'

- Forward and Reverse Primers 800nM final concentration of each/rxn
- **Ordered Exclusively from Applied Biosystems, Foster City CA**
 - Taq Man® Universal Master Mix (2x) (#4304437)
 - TaqMan® MGB probe 20,000pmoles (#4316033)
250nM final concentration/reaction.
5'-Fluorescent dye label: 6-FAM
 - ABI PRISM 96-well optical Reaction plates (#4306737)
Optical adhesive covers (#4311971)
 - Applied Biosystems 7900HT Fast Real Time PCR System or **7300/7500/7500** Fast Real Time PCR systems.

6. Procedure

- Preparation of *Aspergillus* Genomic DNA for QPCR Standard Curve
 - Phenol Chloroform extraction of genomic *Aspergillus fumigatus* DNA³
 - Streak for single colony isolation from an *Aspergillus* frozen glycerol stock culture four quadrant streak and on a PDA plate. Grow the initial culture at 30°C for 24-48 hours.
 - Select one isolated colony and streak it over an entire PDA plate. Incubate the plate at 30°C for 3-5 days in order to allow culture to conidiate.
 - Harvest conidia by adding approximately 5ml of PDA broth and adding it to the agar surface and gently rubbing so as to dislodge the conidia off the plate by aspiration with a 10ml pipet. Add conidial suspension to inoculate 35 ml PDA broth (in 125 ml flask) and incubate broth O/N at 30°C with constant agitation (225 rpm).
 - Harvest conidia from the overnight culture by filtering through Whatman paper. (Using an empty flask, place a plastic funnel lined with Whatman paper and pour the O/N broth culture through).
 - Wash the cells trapped on the Whatman filter with sterile water twice (2 x 100ml).
 - Add 1ml of 0.1M MgCl₂ to the filtered conidiae and allow it to air dry on paper towels.

- Scrape conidial cells off the Whatman paper with a sterile plastic loop into 1.7 ml microcentrifuge tubes (approx 0.2mg of washed mycelia/ tube)
- Add 400 µl of DNA bead bashing buffer, 400 µl of phenol-chloroform-isoamyl alcohol and glass beads (measurement equal to 700µl volume of an eppendorf tube) and is bead beaten on the homogenization setting (3200 rpm) for 1-2 minutes on a Bead beater homogenizer (Biospec). Place directly on ice.
- The supernatant is recovered by centrifuging the tubes immediately after bead-beating at 8,000rpm for 10min. and pipetted into a new tube.
- Add 400 µl phenol-chloroform-isoamyl alcohol, vortex for 30 seconds
- Take the upper phase layer and carefully transfer it to a new tube. Add 2 volumes of 100% ethanol invert gently for 30s, then pellet 5 minutes at high speed.
- Centrifuge at 14,000rpm for 10 min. Discard supernatant and wash the pellet with chilled 70% ethanol. Air dry pellet only!
NOTE: do not use the speed vacuum or the pellet will become difficult to resuspend.
- Dissolve pellet in 0.5ml of TE, pH 8.0 (this is done in order to inhibit nucleases that can degrade DNA.) Add 1 µl RNase A (100mg/ml) /100 µl of sample. Incubate for 1-2hr at 65°C.
- Add 500 µl of chloroform-isoamyl extraction to the sample and centrifuge at 14,000 rpm for 10 min. Collect the supernatant.
- Add 2 volumes of 100% ethanol and 1/10th volume of 0.1M of Sodium Acetate and place at -70°C for 30 min.
- Precipitate by spinning at 14,000 rpm for 10 min discard supernatant and wash pellet a final time with 70% ethanol. Let air dry.
- Dissolve pellet in TE, pH 8.0 ,and quantify genomic DNA spectrophotometrically and by gel electrophoresis to also check for quality of the genomic extraction.
- 500 µl aliquots of 200ng/µl stock solutions of genomic *Aspergillus* DNA are stored in eppendorf tubes at -20°C until use.
- B. Alternative method: Epi-centre Master Pure Yeast DNA Purification Kit.
 - DNA extraction was followed according to the manufacturer's instructions with a minor modification which consisted of the addition of a phenol-chloroform isoamyl alcohol (PCIA) extraction.
 - Following the Protein Precipitation step as directed in the kit, supernatants were added to new micro-centrifuge tubes and precipitated with the addition of 2 volumes of ice-cold 100% ethanol.
 - Tubes are inverted several times and incubated at -20°C for at least 30 minutes if not longer.
 - Tubes were centrifuged at 14,000 rpm for 10 min and DNA was recovered as described in the manufacturer's instructions.
- Preparation of Samples for RT-PCR

- TaqMan® probes must be kept in the dark at all times at -20°C. As a precaution, aliquots of fluorescent probe should be wrapped in aluminum foil.
- The sequences for Forward and Reverse primers used are preferentially designed utilizing the use of the Primer Express® software provided by Applied Biosystems. This program will offer the most efficient sequences to be used in the assay.
- There will be three types of templates prepared for each run:
 - Non-template controls, reaction mixes containing no DNA template (negative control).
 - *Aspergillus sp.* genomic DNA to be used for construction of the standard curve. (genomic DNA extraction procedures are included in this SOP).
 - Experimental samples extracted from infected tissues.
- DNA Standards are prepared in the following manner:
 - Aliquot 120µl of *Aspergillus* genomic stock solution [200ng/µl] into a clean microcentrifuge tube. Add 1080µl of DNase/RNase-free water to the tube and vortex. Concentration = 20ng/ µl .
 - Take 600 µl of 20ng/µl solution and add to a fresh tube containing 600 µl of DNase/RNase-free water. Vortex again. Concentration = 10ng/µl
 - Repeat 4 times to obtain standard DNA samples of [20.0, 10.0, 5.0, 2.5, 1.25, 0.625] ng/µl.
 - When entering standard quantity in the program, multiply the standard concentration by 5 to reflect the true concentration in the well based on volume added to the reaction mix.
 - Each sample is prepared in a 50 µl reaction volume as shown in the following table:

Reagents	Concentration	Volume
TaqMan® Universal PCR Master Mix (2x)	1x	25µl
Forward Primer	800nM	5µl
Reverse Primer	800nM	5µl
TaqMan Probe	250nM	5µl
DNA Template/Standards	10-100ng	5µl
DNase/RNase-free water	-	5µl
Total		50 µl

- Each standard, non-template control and sample is run in triplicate (3 wells/sample). Therefore the sample reaction mix volumes in the table should be tripled.
- Samples are placed in 96 well-plates and assay run in the ABI system machine and assay run as directed by manufacturer’s instructions.
- Parameters for Run are as follows:
 - 2min. @ 50°C – ramp time

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Version 1.1

- 10 min @ 95°C - Hold
- 15 sec at 95°- Melt
- 1 min @ 65°C –Anneal/ Extension
- Steps c & d run for 40 cycles.

The associated software will automatically calculate the cycle threshold, standard deviation of the samples etc. as well as the number of gene copy present in the 5 µl sample as arrived at by utilization of the standard curve constructed/run. Subsequent calculations to arrive at the amount of cell nuclei present/gram of tissue are performed based on the gene copy number and dilution factors that were done with the initial tissue sample to arrive at the 5 µl volume tested in this assay..

7. Attachments

N/A

8. References:

1. Costa C, Vidaud D, Olivi M, Bart-Delabesse E, Vidaud M, Bretagne S. Development of two real-time quantitative TaqMan PCR assays to detect circulating *Aspergillus fumigatus* DNA in serum. *J Microbiol Methods*. 2001 Apr;44(3):263-9.
2. Bowman JC, Abruzzo GK, Anderson JW, Flattery AM, Gill CJ, Pikounis VB, Schmatz DM, Liberator PA, Douglas CM. Quantitative PCR assay to measure *Aspergillus fumigatus* burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. *Antimicrob Agents Chemother*. 2001 Dec;45(12):3474-8.
3. Jin J., Lee, Y.K. Wickes, B.L. (2004) Simple Chemical Extraction Method for DNA Isolation from *Aspergillus fumigatus* and Other *Aspergillus* Species. *Journal of Clinical Microbiology*, 42 (9): 4293-96.

Online help: http://www.appliedbiosystems.com/support/apptech/#rt_pcr

9. History:

Changed to version 1.1 due to the addition of primer and probe sequences for the FKS gene (single copy) and 18sRNA gene (multi-copy).

10. Version 1.1.

11. Examples of Deliverables

12. N/A