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Science SYMPOSIUM

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A Crisis of False Positive Results: Reality or a Mirage for the Cannabis Industry?

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bioMérieux

Objectives

- Understand the principle of the methods used and their limitations
- The complexity of *Aspergillus* as a target for detection
- Optimizing the accuracy of methods

Analysis of a Positive Sample

Retest Sample

New Assay

Cultural Confirmation

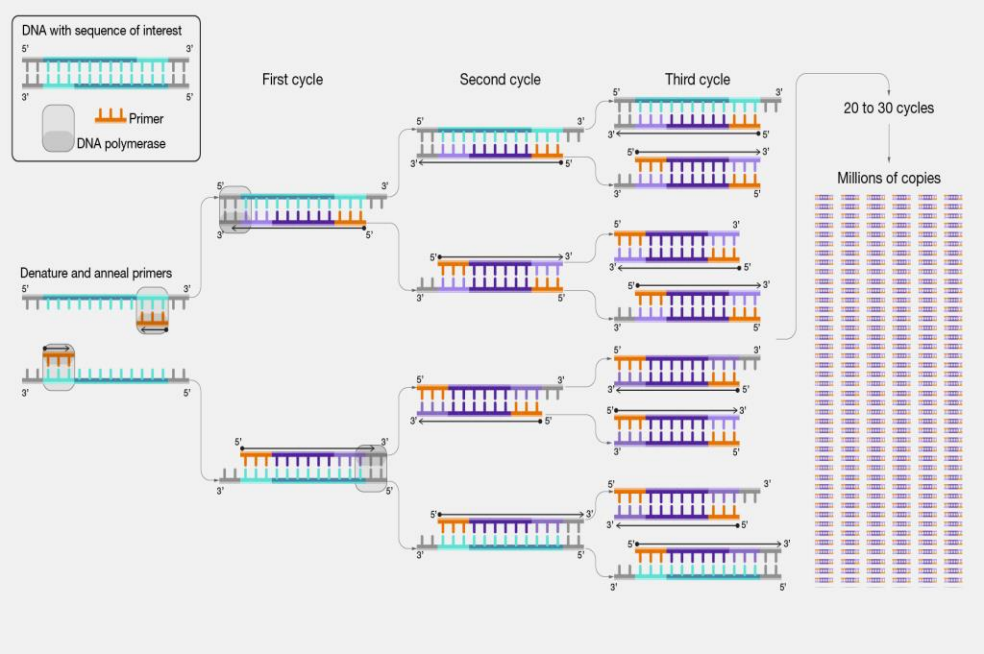
Reality or Mirage?

- Surveys of state data indicate a 10 – 20 % failure rate
- 90% of failures are due to microbial contaminants
- Fungal contamination (TYMC and *Aspergillus*) continue to be the leading cause of failure

What Causes (False) Positive Results?

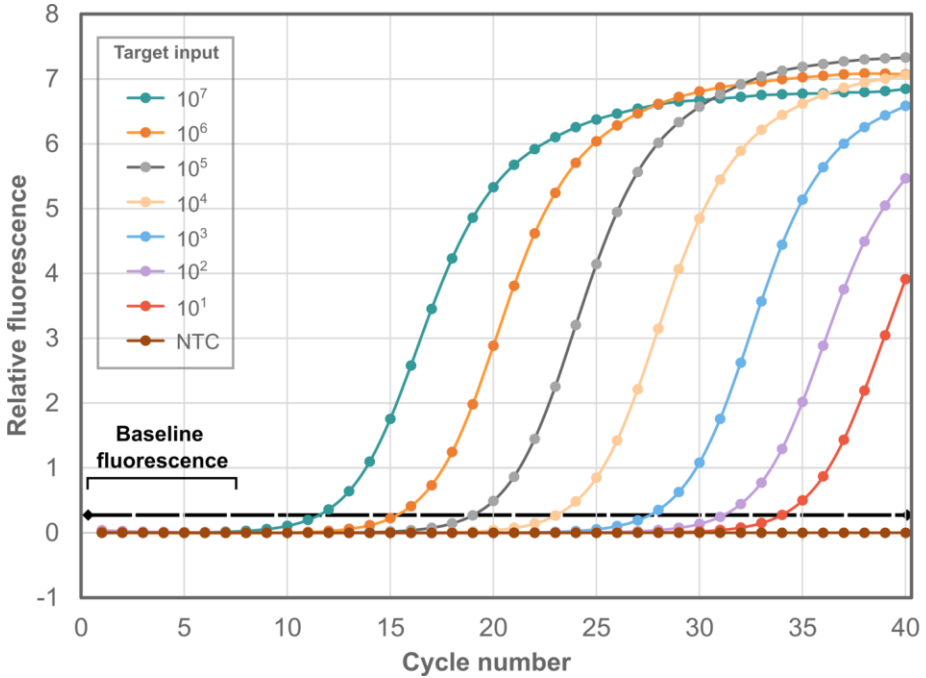
- Laboratory contamination
  - Sample workflow
  - Analyst technique
  - Reagents
- Non-specific amplification
- Cross-reactions
- Detectable but not culturable

Molecular Detection Using PCR



National Human Genome Research Institute, *Polymerase Chain Reaction*. NIH, 2022

Molecular Detection Using PCR



qPCR: How SYBR Green and TaqMan assays work | INTEGRA (integra-biosciences.com)

What makes Detecting Aspergillus Difficult?

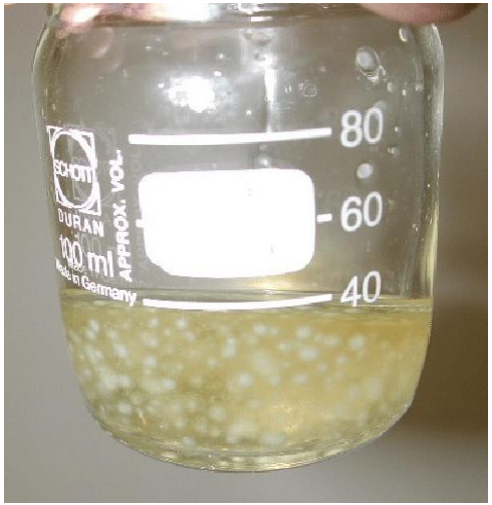
- From the family *Aspergillaceae*
- >1000 known species (mostly in *Aspergillus* and *Penicillium* genera)
- Many species are used in food production (soy sauce, vinegar), biotechnology (amylase, cellulase) and drug industries (cholesterol drugs)

What makes Detecting Aspergillus Difficult?

- Some strains are pathogenic
- Inhalation of spores can lead to multiple diseases
- Produce mycotoxins, known human carcinogens, cause other disease and lead to death.

What makes Detecting Aspergillus Difficult?

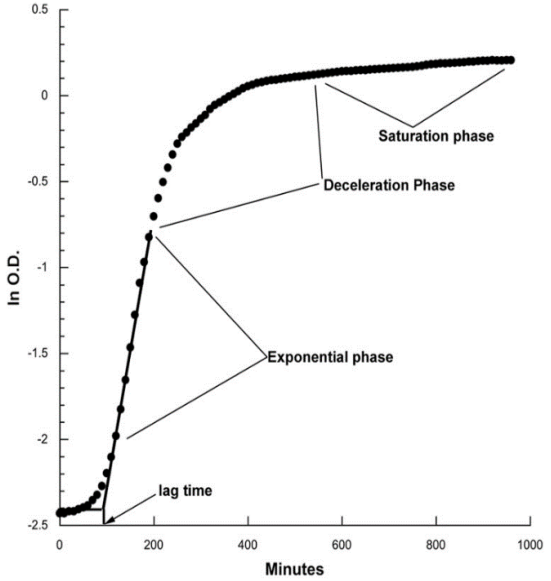
- Traditional process for detection is enrichment followed by PCR.
- Unlike bacteria, mold often grow in the form of **aggregated mycelium clumps** or **pellets**



Bioenergy Research Group (hawaii.edu)

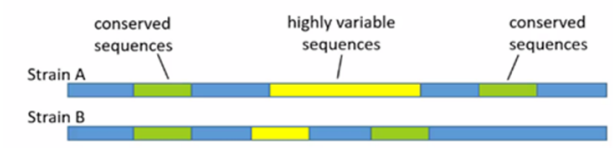
What makes Detecting Aspergillus Difficult?

- When molds grow in this aggregated form, the growth kinetics become more complex leading to higher variation in growth rates
- Studies indicate that **doubling time** for fungal pathogens, such *Aspergillus*, is around 2 – 4 h



What makes Detecting Aspergillus Difficult?

- Assays are designed for conserved sequences in target species

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A.fumigatus   GGGTTCCGATTGAGGCCTCTGGGTCCACCTCCCACCCGTGTCTATCGTACCTTGTTGCTT  61
              ||||| ||| ||||||||||||| ||||||||||||||||||||||||||||||||||||
LR7           GGGTTTCGAGTGAGGCCTCTGGGT-CACCTCCCACCCGTGTCTATCGTACCTTGTTGCTT  59

A.fumigatus   cggcgggcccgccgtttcgacggccgccggggaggccttgcgcccccgggcccgcgcccg  121
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LR7           CGGCGGGCCCGCCGTTTCGACGGCCGCCGGGGAGGCCTTGCGCCCCCGGGCCCGCGCCCG  119

A.fumigatus   ccgAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCGTAAT  181
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LR7           CCGAAGACCCCAACATGAACGCTGTTCTGAAAGTATGCAGTCTGAGTTGATTATCGTAAT  179

A.fumigatus   CAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA  241
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LR7           CAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA  239

A.fumigatus   TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTG  301
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LR7           TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATTG  299

A.fumigatus   CGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGG  361
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LR7           CGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCACGG  359

A.fumigatus   CTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGC  421
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LR7           CTTGTGTGTTGGGCCCCCGTCCCCCTCTCCCGGGGGACGGGCCCGAAAGGCAGCGGCGGC  419

A.fumigatus   ACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGCCGGCG  481
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LR7           ACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCTGCTCTGTAGGCCCGGCCGGCG  479

A.fumigatus   CCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGC  541
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
LR7           CCAGCCGACACCCAACTTTATTTTTCTAAGGTTGACCTCGGATCAGGTAGGGATACCCGC  539

A.fumigatus   TGAACTTAAGCATATCAATAAGGCGGAGGAA  572
              |||||||||||||||||||| ||||||||
LR7           TGAACTTAAGCATATCAATAAG-CGGAGGAA  569
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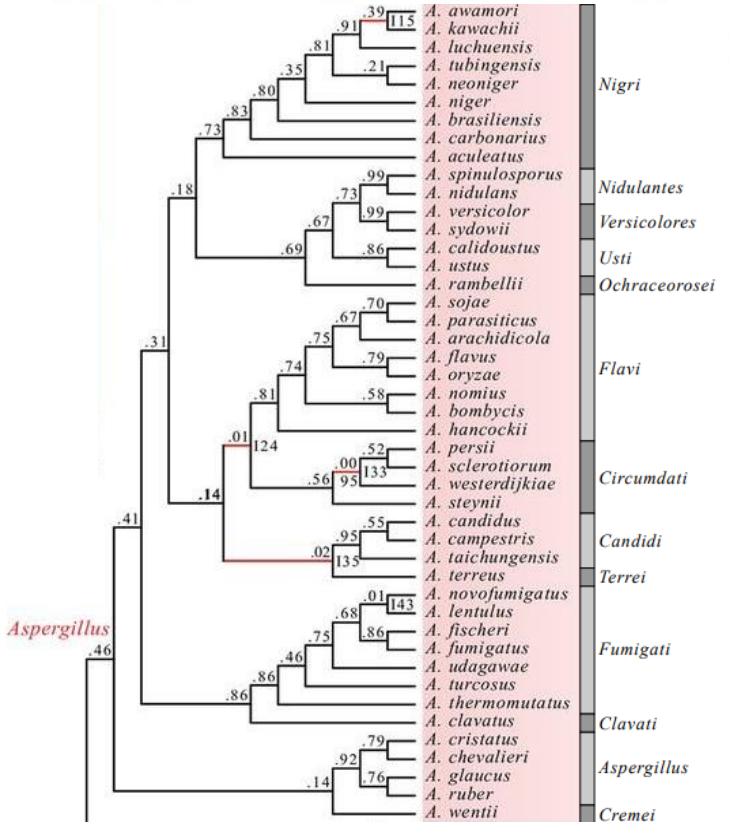
Ali, F. et al. (2021). Isolation and identification of Aspergilli causing Banana fruit rot. Open Journal of Chemistry. 4. 8-18. 10.30538/psrp-ojc2021.0019.

What makes Detecting Aspergillus Difficult?

- Multiple clades have evolved from a single ancestor
- Genetic similarity between species is conserved
- Reclassification occurs frequently.



Steenwyk et al. *A Robust Phylogenomic Time Tree for Biotechnologically and Medically Important Fungi in the Genera Aspergillus* and Penicillium. mBio (10)2019
https://doi.org/10.1128/mBio.00925-19

Determining Cross Reactivity

AOAC SMPR® 2019.001

***Standard Method Performance Requirements* (SMPRs®) for Detection of *Aspergillus* in Cannabis and Cannabis Products**

Intended Use: Consensus-Based Reference Method

1 Purpose

AOAC SMPRs describe the minimum recommended performance characteristics to be used during the evaluation of a method. The evaluation may be an on-site verification, a single-laboratory validation, or a multi-site collaborative study. SMPRs are written and adopted by AOAC composed of representatives from industry, regulatory organizations, contract laboratories, test kit manufacturers, and academic institutions. AOAC SMPRs are used by AOAC expert review panels in their evaluation of validation study data for methods being considered for *Performance Tested Methods*℠ or AOAC *Official Methods of Analysis*℠ and can be used as acceptance criteria for verification at user laboratories. [Refer to Appendix F: *Guidelines for Standard Method Performance Requirements*, *Official Methods of Analysis of AOAC INTERNATIONAL* (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, USA.]

2 Applicability

Candidate methods used to detect *Aspergillus* (*Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Aspergillus terreus*) in cannabis (plants/flowers) and/or cannabis products (concentrates, infused edibles, and infused nonedibles). Candidate methods may be validated for specific matrices, categories or broader claims. *See* Table 1 for matrix/category claim acceptance criteria.

3 Analytical Technique

Any analytical technique that meets the method performance requirements is acceptable.

4 Definitions

Aspergillus.—Filamentous, cosmopolitan, and ubiquitous fungus found in nature producing colonies typically of 1–9 cm in size (select species produce 0.5–1 cm colonies). Colonies are powdery in texture and color varies based on species. Reverse color is typically uncolored to pale yellow. Growth is typical at 20–30°C. *Aspergillus fumigatus* is thermotolerant and can grow at a temperature range of 20 to 50°C. For all species, hyphae are septate and hyaline. The conidiophores originate from the basal foot cell located on the supporting hyphae and terminate in a vesicle at the apex. Vesicle is the typical formation for the genus *Aspergillus*. The morphology and color of the conidiophore vary from one species to another. Covering the surface of the vesicle entirely ("radiate" head) or partially only at the upper surface ("columnar" head) are the flask-shaped phialides, which are either uniseriate and attached to the vesicle directly or are biseriate and attached to the vesicle via a supporting cell, metula. Over the phialides are the round conidia (2–5 µm in diameter) forming radial chains. Other microscopic structures include sclerotia, cleistothecia, aleuriconidia, and Hulle cells are of key importance in identification of some *Aspergillus* species. Cleistothecium is a round, closed structure enclosing the asci which carry the ascospores. The asci are spread to the surrounding when the cleistothecium bursts. Cleistothecium is produced during the sexual reproduction stage of some *Aspergillus* species. Aleuriconidium is a type of conidium produced by lysis of the cell that supports it. The base is usually truncate and carries remnants of the lysed supporting cell. These remnants form annular frills at its base. Hulle cell is a large sterile cell bearing a small lumen. Similar to cleistothecium, it is associated with the sexual stage of some *Aspergillus species*. *See* Tables 2 and 3 for more macroscopic and microscopic information on *Aspergillus* species.

Chen, S.C.A., Meyer, W., Sorrell, T.C., & Halliday, C.L. (2019) *Manual of Clinical Microbiology*, 12th Ed., Landry, M.L., McAdam, A.J., Patel, R., & Richter, S.S. (Eds) ASM Press, Washington, DC, USA, pp 2103–2131

Anaissie, E.J., McGinnis, M.R., & Pfaller, M.A. (2009) *Clinical Mycology*, 2nd Ed., Churchill Livingstone, New York, NY, USA, 687 pp

Walsh, T.J., Hayden, R.T., & Larone, D.H. (2018) *Larones Medically Important Fungi: A Guide to Identification*, 6th Ed, ASM Press, Washington, DC, USA, 500 pp

Candidate method.—Method submitted for validation [Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, Official Methods of Analysis of AOAC INTERNATIONAL* (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, USA]

Candidate method confirmed result.—Final result obtained for a test portion after cultural confirmation of a candidate method.

Candidate method presumptive result.—Preliminary result for a test portion produced by following a candidate method's instructions for use.

Cannabis.—Genus of flowering plants within the Cannabinaceae family that commonly contain 9-tetrahydrocannabinol (THC), cannabidiol (CBD), and other cannabinoids and terpenes. Cannabis includes, but is not limited to, high-THC and high-CBD cultivars.

Cannabis concentrates.—Extracts (primarily composed of cannabinoids and/or terpenes) manufactured through the extraction and concentration of compounds derived from the cannabis plant or flower. Final products can be many forms, including oils, wax, or hash (Category II).

Cannabis infused edibles.—Food and drinks containing extracts of cannabis and/or cannabis materials (Category III).

Cannabis infused nonedibles.—Products containing extracts of cannabis and/or cannabis materials intended to be applied to the human body or any part thereof. Final products can be many forms, including creams, ointments, cosmetics, and therapeutic pads (Category IV).

Cannabis plant and flower.—General terms for the structural and flowering unadulterated parts of the cannabis plant (Category I).

Cannabis products.—Products (edible, and nonedible) extracted or infused with compounds derived from the cannabis plant, including, but not limited to, CBD and THC.

Exclusivity.—Study involving pure nontarget strains, which are potentially cross-reactive, that shall be not detected or enumerated by the candidate method. *See* Table 4 for a list of recommended nontarget strains. [Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces, Official Methods of Analysis of AOAC INTERNATIONAL* (2019) 21st Ed., AOAC INTERNATIONAL, Rockville, MD, USA]

Table 4. *Aspergillus* exclusivity panel[a]

Organism	Reference ID (where applicable)
Acinetobacter baumanii	
Alternia alternata	
Aspergillus aculeatus	
Aspergillus alabamensis	
Aspergillus brasiliensis Varga et al.	ATCC 9642[b]
Aspergillus caesiellus	
Aspergillus carbonarius	
Aspergillus carneus	
Aspergillus clavatus	
Aspergillus deflectus	
Aspergillus fijiensis Varga et al.	ATCC 20611[b]
Aspergillus fischeri	
Aspergillus glaucus	
Aspergillus janponicus	
Aspergillus nidulans	
Aspergillus oryzae (Ahlburg) Cohn	ATCC 10124[b]
Aspergillus parasiticus Speare	ATCC 15517[b]
Aspergillus pseudoterreus Peterson et al.	ATCC 10020[b]
Aspergillus steynii	
Aspergillus tamarii	
Aspergillus tubingensis (Schober) Mosseray	ATCC 1004[b]
Aspergillus tubingensis (Schober) Mosseray	ATCC 10550[b]
Aspergillus ustus	
Aspergillus versicolor	

Determining Cross Reactivity

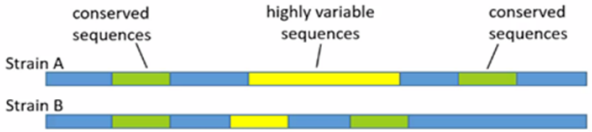


Table 6. Exclusivity List (1)

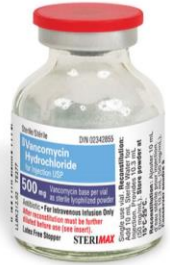
No.	Organism	Source[a]	Origin	Result SenSATIVax Flower AriaMx[b]	Result SenSATIVax Flower CFX-96[b]
1	*Acinetobacter baumanii*	ATCC 19606	Urine	-	-
2	*Alternia alternata*	ATCC 6663	Not Available	-	-
3	*Aspergillus aculeatus*	ATCC 24147	Not Available	-	-
4	*Aspergillus brasiliensis*	ATCC 9642	Wireless radio equipment, New South Wales, Australia	-	-
5	*Aspergillus casiellus*	ATCC 42693	Dried chilies, New Guinea	-	-
6	*Aspergillus carbonarius*	ATCC MYA-4641	Grape berry, Brindis, Apulia, Italy	-	-
7	*Aspergillus clavatus*	ATCC 1007	Not Available	-	-
8	*Aspergillus deflectus*	ATCC 62502	Wheat, China	-	-
9	*Aspergillus fijiensis*	ATCC 20611	Not Available	-	-
10	*Aspergillus niveo-glaucus*	ATCC 10075	Not Available	-	-
11	*Aspergillus japonicus*	ATCC 16873	Soil, Panama	-	-
12	*Aspergillus nidulans*	ATCC 38163	Not Available	-	-
13	*Aspergillus oryzae*	ATCC 10124	Not Available	+[c]	+
14	*Aspergillus parasiticus* Speare	ATCC 15517	Not Available	+[c]	+
15	*Aspergillus pseudoterreus*	ATCC 10020	Not Available	+[d]	+
16	*Aspergillus tamarii*	ATCC 1005	tomato	-	-
17	*Aspergillus tubingensis*	ATCC 1004	Not Available	-	-
18	*Aspergillus tubingensis*	ATCC 10550	Not Available	-	-
19	*Aspergillus ustus*	ATCC 1041	Culture containment, USA	-	-
20	*Aspergillus versicolor*	ATCC 11730	Cellophane gas mask, India	-	-
21	*Botrytis cinerea* Persoon	ATCC 11542	Azalea flowers, Washington, D.C.	-	-
22	*Candida albicans*	ATCC 10231	Man with bronchomycosis	-	-
23	*Cryptococcus laurentii*	ATCC 18803	Palm wine, malaffou, Congo	-	-
24	*Cryptococcus neoformans*	ATCC 208821	Patient with Hodgkin's disease, New York	-	-
25	*Fusarium proliferatum*	ATCC 76097	Raw cotton, North Carolina	-	-
26	*Fusarium oxysporum*	ATCC 62506	Celery, *Apium graceolens* var. *dulce*, California, USA	-	-
27	*Fusarium solani*	ATCC 52628	Cardamom fruit pod, *Elettaria* cardamomum, Guatemala	-	-
28	*Mucor circinelloides*	ATCC 38592	N/A	-	-
29	*Mucor hiemalis*	ATCC 28935	Soil in spruce forest, Germany	-	-
30	*Penicillium chrysogenum*	ATCC 18476	Cheese?, USSR	-	-
31	*Penicillium rubens*	ATCC 11709	Selected from Wis. 48-701, after N-mustard exposure	-	-
32	*Penicillium venetum*	ATCC 16025	*Hyacinthus* sp. Bulb, England	-	-
33	*Pseudomonas aeruginosa*	ATCC 35554	Not Available	-	-
34	*Rhizopus stolonifer*	ATCC 14037	Not Available	-	-
35	*Yarrowia lipolytica*	ATCC 20390	Nonsporulating diploid	-	-

[a]ATCC – American Type Culture Collection, Manassas, VA; [b](+) = positive, (-) = negative; [c] Cross reacts with *A. flavus*; [d] Cross reacts with *A. terreus*

AOAC PTM Validated Method Database [September 25, 2000 (aoac.org)]

Enrichment Media and Supplements

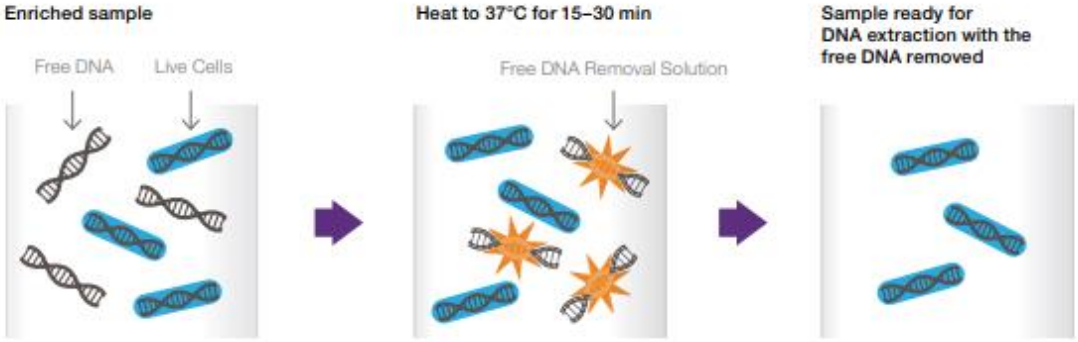
- Specialized enrichment media
- Selective supplements
- Neutralization of product inhibitors



Decontamination of Mold

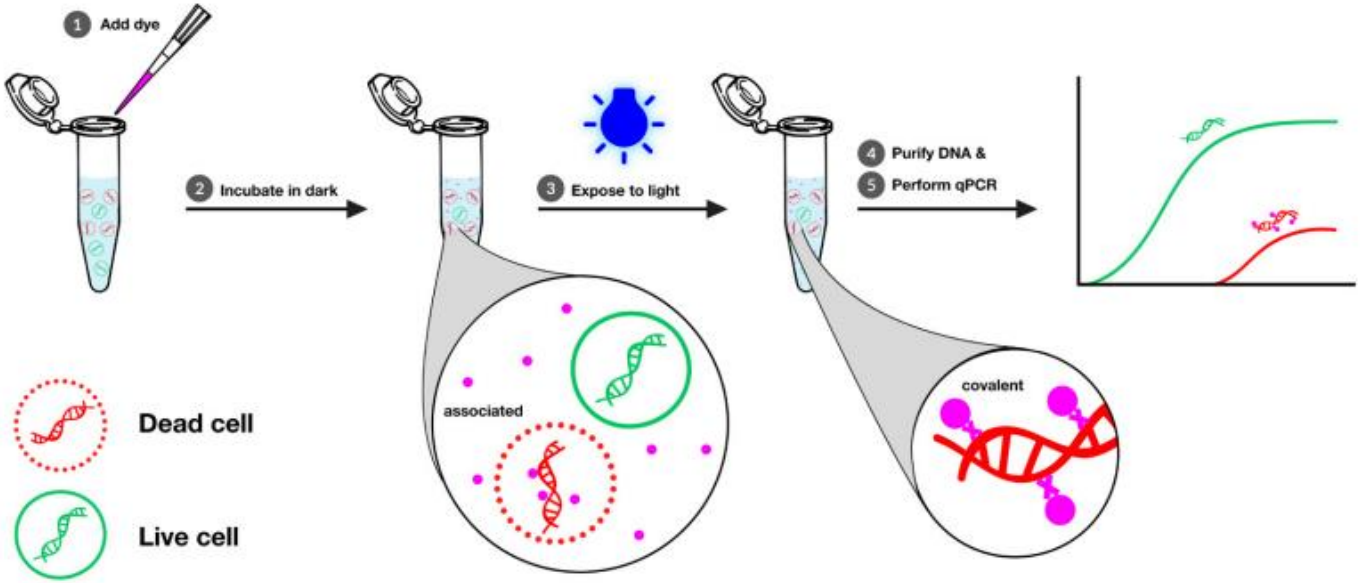
- Chemical
- X-Ray
- Gaseous
- Gamma
- Radio/Photonic
- Microwave
- UV Light

Extracellular DNA Removal



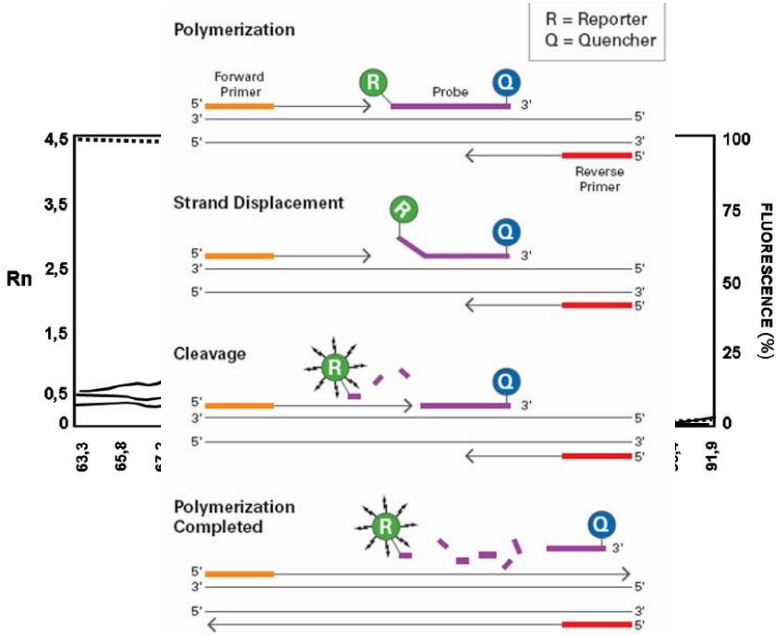
[iQ-Check Free DNA Removal Solution for Food, Water, and Environmental Samples | Bio-Rad]

Non-Viable DNA Removal

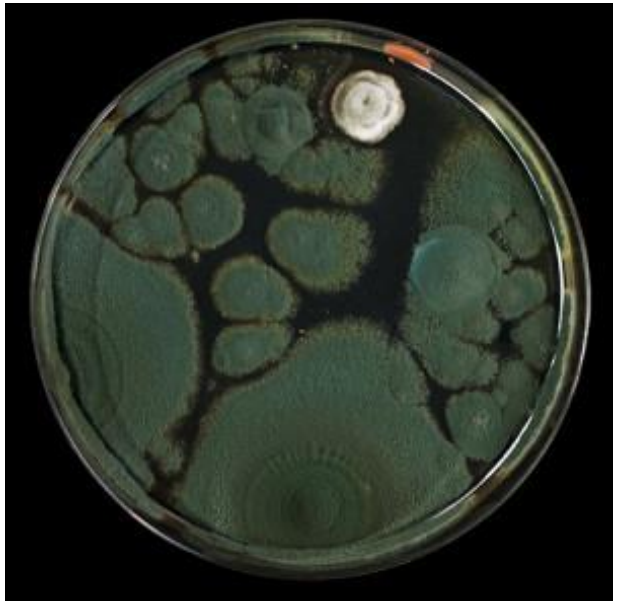


Enhanced PCR Design

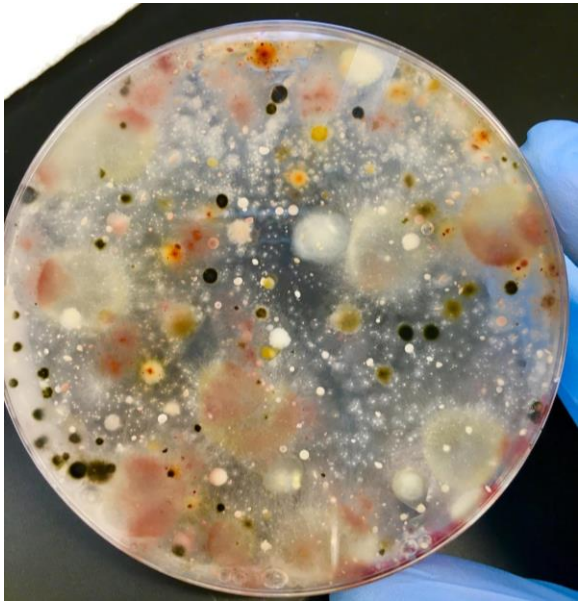
- Streamlined extraction
- Melt curve analysis
- NTC
- Hydrolysis probes



Verifying Positive Results

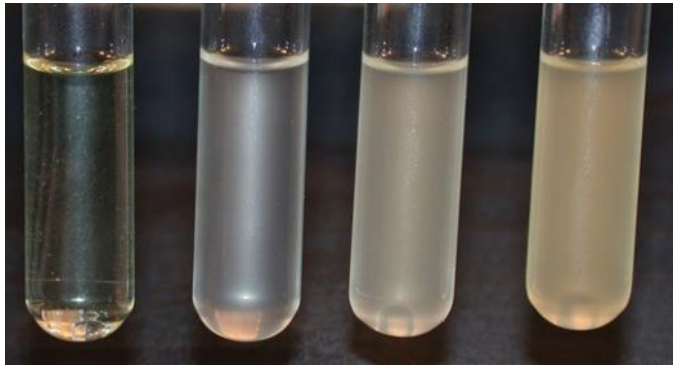


Microbiologics Blog, 2016.
https://blog.microbiologics.com/methods-for-growth-success-yeasts-and-molds/



https://www.reddit.com/r/labrats/comments/iqgaic/one_of_our_yeast_and_mold_plates_on_pda_i_just/

Verifying Positive Results



9780321776082.pdf (unr.edu.ar)

- Reanalysis on a different assay isn't accurate
- Retain tests are not directly comparable
- Enhanced solutions (NGS) are available

Thank you!!!

Patrick Bird

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