Enhancing *Aspergillus* Enrichment in a qPCR Workflow

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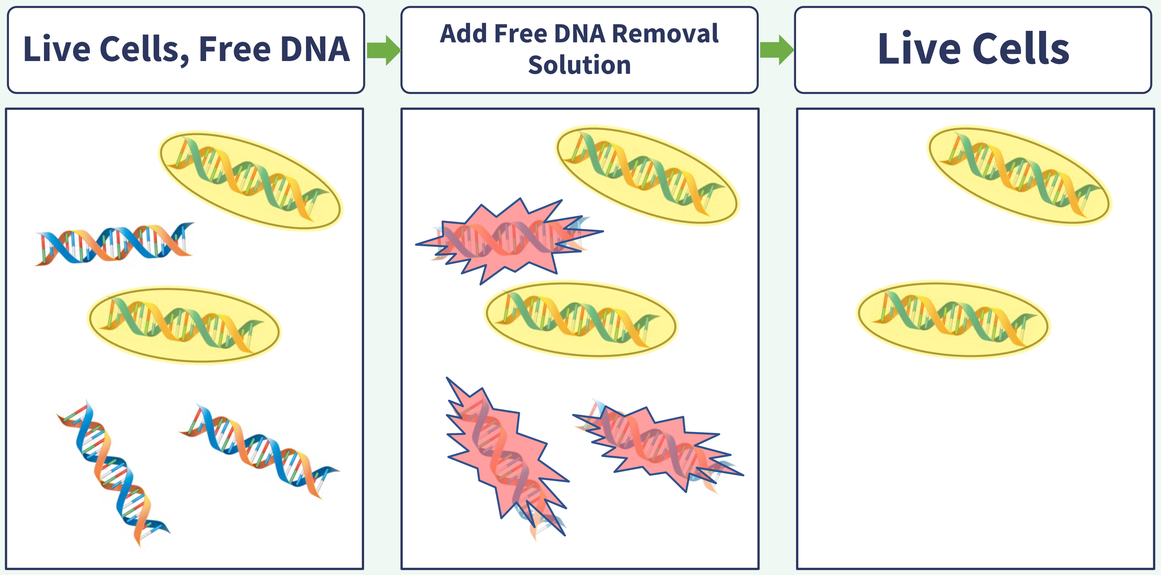


Simple, 30-minute procedure to remove free DNA

Free DNA Removal Solution (FDRS) is added to an aliquot of enriched sample and incubated at 37 °C for 30 minutes.

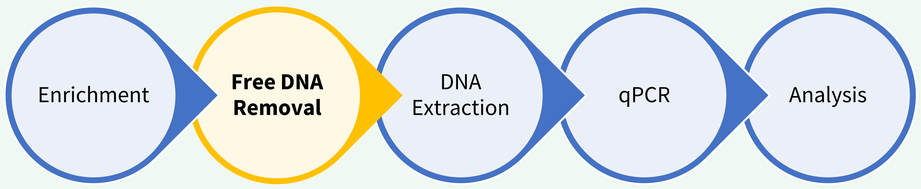


Figure 1. The Free DNA Removal step is incorporated into the post-enrichment step of a standard qPCR workflow.

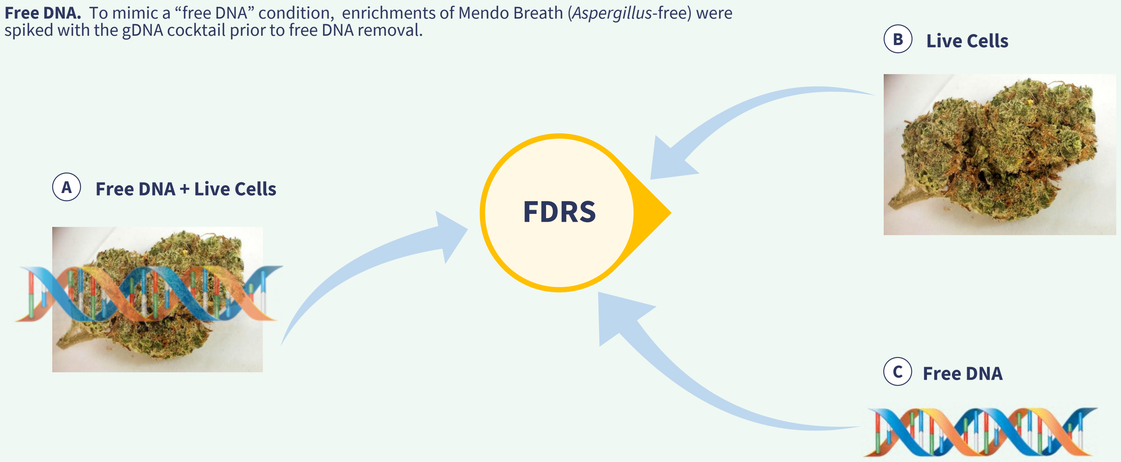
Experimental design

Experiments were designed not only to challenge the Free DNA Removal Solution, but also to simulate processes that may result in the presence of free DNA from *nonviable* or lysed organisms.

Ⓐ **Free DNA + Live Cells.** To evaluate performance of FDRS on free DNA mixed with live cells, a cocktail of *A. flavus, A. fumigatus, A. niger,* and *A. terreus* gDNA was spiked into the naturally contaminated Godbud, followed by the free DNA removal procedure.

Ⓑ **Live Cells.** To assess the effects of FDRS on living cells, Godbud was enriched and assayed with or without FDRS.

Ⓒ **Free DNA.** To mimic a "free DNA" condition, enrichments of Mendo Breath (*Aspergillus*-free) were spiked with the gDNA cocktail prior to free DNA removal.



Principle of the method

This protocol uses Bio-Rad's iQ-Check Free DNA Removal Solution to remove free DNA from cannabis enrichments prior to qPCR, resulting in **fewer false positives**.

- Degradation of free DNA occurs via enzymatic reaction and requires a unique buffer. Following treatment, enriched samples will only contain DNA from intact and living cells. The enzyme is deactivated during the subsequent cell lysis and has no activity prior to DNA extraction.
- Free DNA Removal Solution can be integrated into any qPCR workflow with a thermal lysis step. **Canalysis Laboratories** recommends Bio-Rad's iQ-Check *Aspergillus* Kit and CFX96 Real-Time PCR Detection System, which leverages a user-friendly protocol and automated results interpretation.

Why *Aspergillus*?

An increasing number of jurisdictions requires compliance testing to include detection of four species of *Aspergillus* (*A. flavus, A. fumigatus, A. niger,* and *A. terreus*) that can cause disease in individuals with underlying health issues. Pulmonary aspergillosis, an invasive lung disease, has been reported in immunocompromised medical cannabis patients, with multiple cases resulting in death despite antifungal interventions. In some patients, a few inhaled *Aspergillus* spores can lead to irreversible organ damage. Consequently, it is crucial that the detection system for *Aspergillus* must be robust, specific, and highly sensitive.

Why qPCR?

With qPCR, **target DNA** is detected in a relatively short period of time. In contrast to traditional plating, where spores may be **unculturable**, samples are enriched in media selective for *Aspergillus* prior to qPCR. Dead DNA is diluted in the enrichment, and only living / reproducing cells will result in a **strong** signal. However, there is theoretically free DNA that persists due to the decontamination process. To further enhance *Aspergillus* detection and ensure analysis of viable cells, a free DNA removal step can be implemented. The Free DNA Removal Solution described here is a cost-effective alternative to propidium monoazide (PMA) assays and an efficient alternative to lengthy culture-based tests, which risks missing *Aspergillus* living *within* cannabis plant cells. (Not to mention, a **poorly timed sneeze** over a culture plate can contaminate an entire lab).

Results: dead DNA removal

The Free DNA Removal procedure successfully reduced signal arising from gDNA-spiked Godbud. Samples treated with FDRS are indicated with **blue** circles (*A. niger, A. flavus,* and *A. fumigatus*) or **red** circles (*A. terreus*) in the figure below.

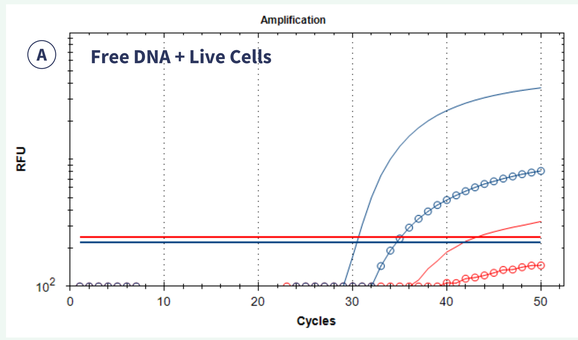
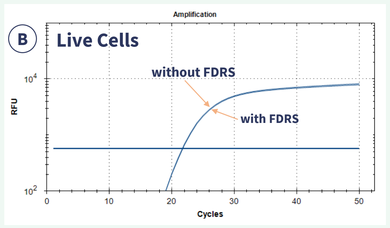


Figure 2. Signal from spiked gDNA is reduced in a naturally infected sample. Note that the sample was *not* naturally infected with *A. terreus.*

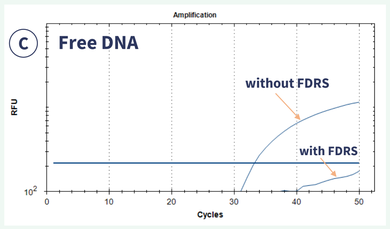
Results: live…

The naturally contaminated Godbud flower was enriched and assayed with or without Free DNA Removal Solution. qPCR confirmed the removal solution had no effect on living cells.



…and let die

qPCR on Mendo Breath (spiked with a genomic DNA cocktail) demonstrated reduction of signal arising from free DNA. A 10× inoculation (not shown) resulted in a DNA reduction of 8.7 cycles, or 3.1 logs.

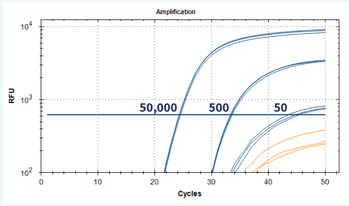


Performance

To assess enzyme efficiency, the Free DNA Removal Solution was tested on a serially-diluted *Aspergillus* gDNA cocktail.

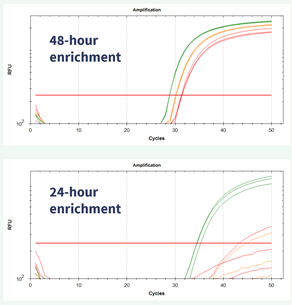
Copies of genome	Ct (no removal)	Ct (with removal)
50,000	24.3	Not Detected
500	33.4	Not Detected
50	44.5	Not Detected

Multiplex qPCR was run in triplicate, and an average Ct was calculated. The lowest dilution (~10 copies / µL) represents an equivalent amount of free DNA in a 1 gram enrichment, or roughly 100,000 dead cells. Note that 100,000 dead cells in an enrichment would barely generate a signal in qPCR *without* Free DNA Removal. In the figure below, the three signals that do not reach the threshold (**orange**) represent 50,000 copies treated with FDRS; treatments on 500 and 50 copies resulted in no signal at all. (Signal arising from *A. terreus* is not shown and was not included in the Ct calculation).



48 > 24

The Bio-Rad iQ-Check *Aspergillus* workflow utilizes a 48-hour incubation for the enrichment step; this enrichment is required for detection of low-level contamination, particularly for slow growing species like *A. terreus.* To demonstrate susceptibility to **false negatives** when using a 24-hour incubation, 1-10 CFU (**red**), 10-100 CFU (**orange**), and 100-1000 CFU (**green**) of *A. terreus* was inoculated into an enrichment, incubated for 24 or 48 hours, and assayed using the iQ-Check *Aspergillus* Kit at both timepoints.



Conclusions

The Free DNA Removal Solution offers an efficient and easy qPCR implementation for the removal of signal arising from dead cells and free DNA. Combined with an assay like Bio-Rad's iQ-Check *Aspergillus* Kit, this method provides high quality, easy-to-interpret data, without the need for cultural confirmation. Armed with *accurate* data, independent testing labs can report results with higher confidence.

For more information, visit **www.canalysislaboratories.com/FDRS**

Acknowledgments

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