PathoSEEK® 5-Color and 2-Color Aspergillus Multiplex Detection Assays with SenSATIVAx® Purification and Grim Reefer® Free DNA Removal

Supplemental User Guide describing the addition of the Grim Reefer Free DNA Removal Steps

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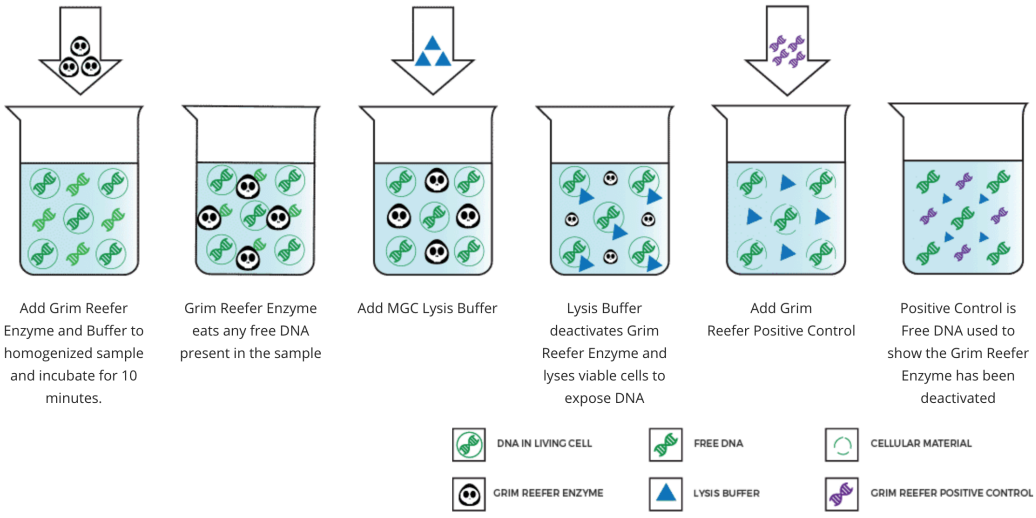
Introduction

This supplemental guide describes the process of using MGC's Grim Reefer free DNA removal reagents in conjunction with Medicinal Genomics PathoSEEK Aspergillus Detection Assays. Please note: the use of MGCs Grim Reefer Free DNA removal process was not included in the AOAC Validation process for the PathoSEEK Aspergillus 5 Color with SenSATIVAx or Aspergillus 2 Color with SenSATIVAx Assays. Also note that a **full 48** hour enrichment is required when implementing Grim Reefer.

Process Overview

SenSATIVAx Lysis with Grim Reefer Free DNA Removal



Kit Components

Item P/N	Item Name	Qty Provided
420145	Grim Reefer Free DNA Removal Enzyme and Buffer	100 Reactions
	Grim Reefer Enzyme	1 Bottle (2.5 mL)
	Grim Reefer Buffer	1 Bottle (12.75 mL)
420144	Grim Reefer Control	1 Tube (50 µL)
420143	Grim Reefer Assay	1 Tube (200 µL)

DNA Extraction

1. Prepare the Grim Reefer reagents:
    a. Allow a 1.5mL tube rack to come to temperature in a 37°C incubator
    b. Thaw the 10X GR Buffer
    c. Thaw Stock GR Positive Control and make a 1:10,000 dilution (fresh each day):
        i. Make a 1:100 dilution (1uL positive control + 99uL water mix well) - Spin down contents of tube after vortex
        ii. Make a second 1:100 dilution (1uL of 1st 1:100 dilution + 99uL water mix well) - Spin down contents of tube after vortex. This is your 1:10,000 dilution.
        iii. If running the 5 - Color Aspergillus assay, prepare an additional 1.5 mL tube that will get fresh TSB. This will be used to verify the deactivation of the Grim Reefer enzyme using the Grim Reefer Control spike.
2. 5- Color Aspergillus Assay sample preparation
    a. After a **full 48 hour enrichment**, homogenize the sample then aspirate **1 mL** from the side of the filter bag, free of plant debris, and dispense into a clean labeled 1.5mL tube.
    b. Add 1 mL fresh TSB to the extra clean 1.5 tube labeled GR deactivation.
    c. Add the following to all tubes (samples and TSB)
        i. Add 112 µL of 10X GR Buffer to all sample tubes and TSB tube and mix well by vortexing (briefly spin to remove liquid from cap).
        ii. Transfer 20 µL of GR Enzyme and mix well by vortexing (briefly spin to remove liquid from cap).
        iii. After addition of the GR Enzyme immediately, incubate the tubes at 37°C for 10 minutes. At the end of 10-minute incubation immediately proceed to the next step.
        iv. Add 50 µL of MGC Lysis buffer to the sample.
        v. Vortex for at least 10 seconds and incubate on the bench for 2 minutes.
        vi. Add **ONLY** to TSB only Extraction
            1. **Transfer** 10 µL of the diluted GR Positive control (1:10,000) to the TSB tube and mix by vortexing.

    NOTE: The GR Positive Control is used to show the GR enzyme was completely deactivated during the lysis step.

3. 2 - Color Aspergillus Assay sample preparation
    a. After a **full 48 hour enrichment**, homogenize the sample then aspirate **1 mL** from the side of the filter bag, free of plant debris, and dispense into a clean labeled 1.5mL tube.
    b. Add the following to every Sample tube
        i. Add 112 µL of 10X GR Buffer and mix well by vortexing (briefly spin to remove liquid from cap).
        ii. Transfer 20 µL of GR Enzyme and mix well by vortexing (briefly spin to remove liquid from cap).
        iii. After addition of the GR Enzyme, immediately incubate the tubes at 37°C for 10 minutes. At the end of 10-minute incubation immediately proceed to the next step.
        iv. Add 50 µL of MGC Lysis buffer to the sample.
        v. Vortex for at least 10 seconds and incubate on the bench for 2 minutes.
        vi. Transfer 10 µL of the diluted GR Positive control (1:10,000) to each sample and mix by vortexing.

    NOTE: The GR Positive Control is used to show the GR enzyme was completely deactivated during the lysis step.

4. Spin all samples and TSB only for at least 1-3 minutes in a high speed or bench top mini centrifuge.

    Note: The supernatant should be translucent at this point. If the sample is still opaque (cloudy) spin for longer. This is important for removing cellular debris. See Figure 4.

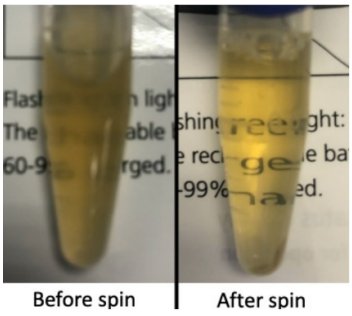


Figure 4: Example of translucent lysate after spinning.

5. Remove the 200µL of supernatant from the 1.5ml tube containing the centrifuged sample, being careful not to disturb the pellet at the bottom of the tube. Place the 200µL in a labeled 96 well extraction plate labeled with Extraction Plate Day1 [date]"

   Note: Pellet size will vary depending on trichome density.

6. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely re-suspended in the buffer for at least 30 seconds.

7. Add 200µL of MGC Binding Buffer to each sample, and pipette tip mix 15 times.

   Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.

8. Incubate the plate on the bench for at least 5 minutes.
9. Place the extraction plate onto the 96 well plate magnet plate for at least 5 minutes.
10. After the 5 min incubation, remove as much of the 400µL of the supernatant as possible. Be careful not to disturb or aspirate the beads. See Figure 5.
    a. Add 400µL of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.
    b. Wait at least 30 seconds and remove all the EtOH.

    Note: Place the pipet tip at the bottom center of the well to remove all liquid.

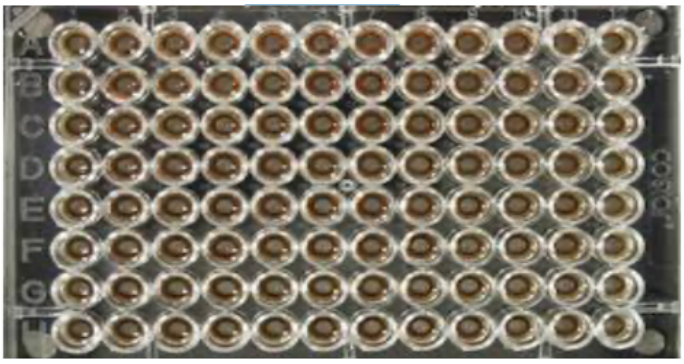


Figure 5: Extraction plate during wash step on magnetic plate.

11. Repeat 400 µL 70% EtOH wash with the extraction plate still on the magnet plate. Wait at least 30 seconds and remove all the EtoH.

    Note: If EtOH still remains in the wells, go back in with a smaller pipet tip to remove the excess. Leftover EtOH can inhibit qPCR efficiency.

12. After all the EtOH has been removed, let the beads dry at room temperature. Allow the beads to dry for up to 15 minutes. Necessary drying time will vary based on complete removal of the second ethanol wash, as well as lab environment. Visually inspect beads for residual ethanol before the elution step.

    Note: It is important to NOT allow the beads to dry for an extended period of time. Over-drying can cause a reduction in DNA yield.

13. Remove the extraction plate from the magnet plate and add 50 µL of MGC Elution Buffer.
    a. Tip mix approximately 15 times or until the beads are completely re-suspended.

    Note: The re-suspensions may appear varied in their appearance, but the result will be the same. See Figure 6.

    b. Incubate the plate for at least 1 minute on the bench, before returning the plate to the magnetic plate.
    c. Let the plate sit on the magnet for at least 1 minute before transferring the eluent to a new extraction plate labeled with "Final Extract [date]".

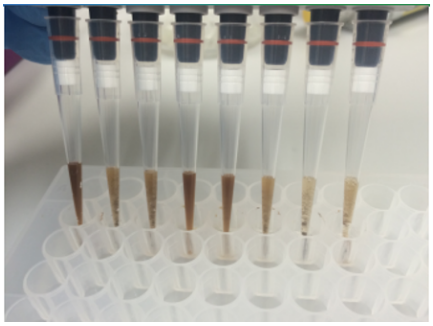


Figure 6: Multichannel pipette tips showing magnetic beads resuspended in elution buffer.

    d. Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal. Store at -20°C until ready to perform the qPCR protocol.

Real-Time Quantitative PCR (qPCR) Setup Protocol

1. Remove qPCR reagents including qPCR Master Mix, water, reaction buffer and assay probe mixes to be used from the -20ºC freezer. Place qPCR master mix on ice or leave at -20°C until ready to use. Allow remaining tubes to thaw at room temperature. Once thawed, immediately place tubes on ice.
2. Before preparing the reaction, invert or vortex and spin-down the reagents.
    a. Assay probe mix tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.
    b. qPCR Master Mix – Invert the tube 5 times (do not vortex), followed by a pulse spin-down in a microcentrifuge.
    c. Return all reagents to the ice.

    Note: Do not vortex the qPCR Master Mix at any point during the protocol.
3. Make a separate master mix in a 1.5mL tube for each assay type being run. All probe mixes contain the internal plant control, SCCG probe mix, and the probe for the microbial targets. Label each tube with [Assay Name] MM. Always prepare enough master mix for 1 or 2 additional reactions over the total number of tests to account for pipetting and dead volumes.

    Note: *It is best to add the largest volume reagent first, in this case water.*

Table 1: PCR Reagent Volumes

In order to detect the GR positive control that was spiked into the samples or the TSB only sample during the DNA extraction using Grim Reefer, 0.5µL of GR Assay probe mix needs to be added per reaction.

To keep the master mix identical, use GR assay probe mix for all samples including the TSB only sample. See the table below.

Reagents	1 Reaction	24 reactions (plus 1 excess rxn)	48 reactions (plus 2 excess rxn)
qPCR Master Mix	3.75µL	93.75µL	187.5µL
Assay Probe Mix (Assay Specific)	1µL	25µL	50µL
Grim Reefer Assay Probe Mix	0.5µL	12.5µL	25µL
Reaction Buffer	0.8µL	20µl	40µl
Water	7.7	192.5µL	385µL
Total	13.75µL	343.75µL	687.5µL

4. Once combined, gently tip mix or invert the tube 5 times to combine the assay master mix.
    a. Pulse spin-down tube in microcentrifuge.
    b. Place qPCR Master Mix tubes on ice until used.
    c. For the positive control, make a 1:10 dilution
        i. Add 1 µL of Positive Control to 9 µL nuclease free water (found in the kit), vortex to mix well and spin down the tube.
        ii. For the negative control, use water (found in the kit)

    Note: It is best to add the largest volume reagent first, in this case the 9 µL water then the 1 µL of positive control, pipette mix or vortex control dilution to ensure control DNA is in solution.
5. Place the Extraction Plate on the magnet. This is to ensure no magnetic beads are transferred into the qPCR reactions if there are some left over from the extraction elution process.
6. Use a 96-well optical qPCR plate and label the plate “qPCR Plate_ [date]”.
7. Carefully remove the seal from the Extraction Plate. If frozen, let the DNA thaw completely, spin the plate to avoid cross contamination between samples, and pipette tip mix the DNA. Transfer 5

µL of each sample into the corresponding well on the qPCR plate. Keep the extraction plate on the magnet when aspirating the 5 µL.

a. Add 5 µL of the diluted Positive Control to the corresponding positive control well. Then add 5 µL of water to the corresponding negative control well.

Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate

8. Add 13.75 µL of specific Assay Probe MM to each corresponding sample well, TSB only well if applicable, positive control well, and negative control well in the qPCR plate. Gently tip mix a few times after each addition of qPCR master mix. Be careful not to introduce bubbles during this mix.
9. Seal the plate with strip caps or an adhesive seal.
10. Spin-down for at least 1 minute in plate microcentrifuge to bring well contents to the bottom of wells and help to get rid of reaction bubbles.

    Note: Check for bubbles at the bottom of the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin-down for another minute.
11. For the Agilent Aria: If using an adhesive seal; place the reusable compression pad (gray side down) on the plate directly lining up the holes in the pad with the holes in the plate.
12. Place the sealed plate onto the PCR instrument, positioning the A1 well in the top left corner.
13. Follow the software specific instructions to initiate the run.

PathoSEEK® Aspergillus 2-color Data Analysis Quick Reference Table

PathoSEEK Target	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus flavus, fumigatus, niger and terreus	≤ 40	FAM	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA ** Grim Reefer Pos control verifies successful inactivation of Grim Reefer Enzyme	
Grim Reefer Pos Control**	≤35	Cy5		
Assay Positive Control	≤35	FAM		

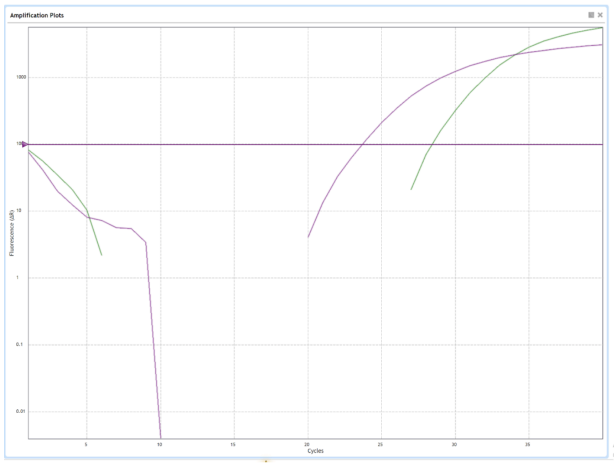
PathoSEEK® Aspergillus 5-color Data Analysis Quick Reference Table

PathoSEEK Target	Cq Value	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus flavus	≤ 40	Cy5	No Cq	Presence/Absence
Aspergillus fumigatus	≤ 40	FAM	No Cq	Presence/Absence
Aspergillus niger	≤ 40	ROX	No Cq	Presence/Absence
Aspergillus terreus	≤ 40	Cy5.5/Atto425	No Cq	Presence/Absence
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA ** Grim Reefer Pos control verifies successful inactivation of Grim Reefer Enzyme (this should only be observed in TSB only reaction)	
Grim Reefer Pos Control**	≤35	Cy5		
Assay Positive Control	≤35	FAM/HEX/ROX/Cy5 /Cy5.5 or Atto425		

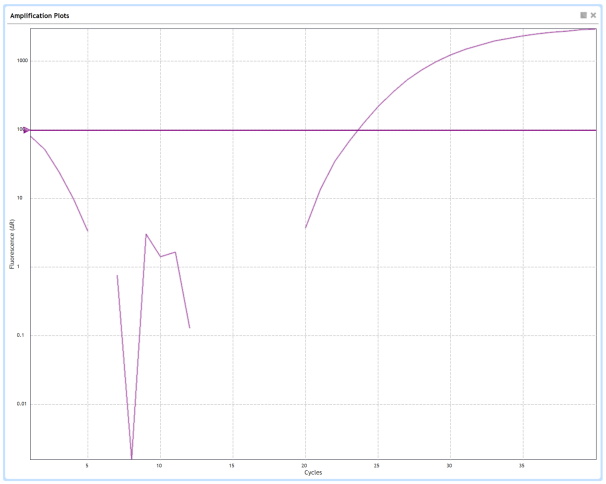
Results

For detailed data analysis refer to the appropriate User Guide. When using Grim Reefer in conjunction with the Aspergillus detection assays, the following guidance should also be observed:

Aspergillus 2 - Color Assay: Amplification of Cy5 should be observed in all sample reactions in which the Grim Reefer control was spiked in after lysis buffer addition.



Aspergillus 5 - Color Assay: Amplification of Cy5 should be observed in the reaction prepared from the TSB only extraction in which the Grim Reefer control was spiked in after lysis buffer addition. If Cy5 amplification is observed in a sample reaction, this indicates the presence of *Aspergillus flavus* in the sample.



Troubleshooting Guide for Grim Reefer

Symptom	Reason	Solution
No Amplification of Grim Reefer Control	Grim Reefer Control dilutions not freshly prepared	Make a fresh GR control dilution. Repeat SenSATIVAx and PathoSEEK by following the protocol.
	Lysis Buffer was not added/insufficient vortexing after lysis buffer addition	GR Enzyme was not deactivated and GR Control DNA Was degraded
	Mix up in qPCR Reaction Setup	Repeat the qPCR by following the protocol.
	Missing Cy5 Fluorophore on plate set up	Check plate setup in qPCR platform data file and ensure the Cy5 fluorophore was chosen for the wells that include the GR Control spike
	qPCR inhibition	Dilute Elution 1:10 with nuclease free water and re-perform qPCR
All samples positive for A. flavus on 5-color Detection Assay	Grim Reefer control spiked into all samples when using 5-Color Assay. Grim Reefer Control and A. flavus are both detected on Cy5.	Re-extract samples spiking Grim Reefer positive control into a TSB blank only as described in this guide.
Total run failure	Excessive vortex of the qPCR Master Mix	Repeat the qPCR by following the protocol.

REVISION HISTORY

Version	Date	Description
v1	October 2024	Creation of supplemental guide for use of Grim Reefer Free DNA Removal with PathoSEEK Aspergillus Detection Assays with SenSATIVAx Purification. This supplemental guide is not included in Medicinal Genomics' Aspergillus AOAC PTM Method.

DISCLAIMER

This test was developed, and its performance characteristics determined by Medicinal Genomics Company, for laboratory use. Any deviations from this protocol are not supported by MGC.

The use of MGCs Grim Reefer Free DNA Removal Kit was not included in the validation process for the PathoSEEK 5 - Color Aspergillus Assay with SenSATIVAx or PathoSEEK 2 - Color Aspergillus Assay with SenSATIVAx. A 48 hour enrichment is recommended when implementing Grim Reefer with the PathoSEEK 5 - Color Aspergillus Assay with SenSATIVAx or 2 - Color Aspergillus Assay with SenSATIVAx.

This test has not been validated on remediated (irradiated, ozone treated, acid treated, hydrogen peroxide treated, etc.) samples. Samples that have undergone remediation may cause discordant results between plating methods and PathoSEEK methods. When remediated samples produce a result above the action limit on qPCR, we recommend confirming viability with an approved plating method.

The results may vary based on laboratory conditions. Altitude and humidity are among factors known to affect the growth of bacterial and fungal species. All thresholds were determined based on the results using the BIO-RAD CFX96 Touch® Real-Time PCR Detection System. It is recommended that thresholds be calibrated for each specific laboratory setting.

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