Matrix Extension of the CompactDry™ "Nissui" YMR for Enumeration of Yeasts and Molds in Dried Cannabis Flower: AOAC *Performance Tested Method*℠ 092002

Authors and Affiliations

LAUREN HAMILTON[1], ANNA KLAVINS[1], RIANNA MALHERBE[1], JESSA YOUNGBLOOD[1], YUSUKE ITO[2], AND ANDRE HSIUNG[1]

[1]Hardy Diagnostics, 1430 West McCoy Lane, Santa Maria, CA 93455

[2]Nissui Pharmaceutical Co., Ltd., 3-24-6, Ueno, Taito-ku, Tokyo 110-0005, Japan

Corresponding author's email: hamiltonl@hardydiagnostics.com

ORCID iD number for all authors:

LAUREN HAMILTON (0000-0002-9891-0787), ANNA KLAVINS (0000-0002-5911-1501), RIANNA MALHERBE (0000-0002-5873-8142), JESSA YOUNGBLOOD (0000-0002-3392-7879), YUSUKE ITO (#), AND ANDRE HSIUNG (0000-0002-5655-2424)

Abstract

Background: CompactDry™ Yeast/Mold Rapid (YMR) is a ready to use dry media sheet using a chromogenic medium with selective agents for the enumeration of yeasts and molds in a variety of food products after incubation at 25 ± 1°C for 3 days. The method is certified as AOAC *Performance Tested Method*℠ 092002. *Objective*: The CompactDry YMR method was validated for a matrix extension to cannabis flower through the AOAC Emergency Response Validation process. *Methods*: The performance of the CompactDry YMR was compared to Dichloran Rose Bengal Chloramphenicol (DRBC) agar for the

enumeration of yeasts and molds in cannabis flower. Matrix data were normalized by $\log_{10}$ transformation and performance indicators included repeatability, difference of means (DOM), and inclusivity/exclusivity. *Results*: The results demonstrated that the CompactDry YMR method is equivalent to the DRBC agar method at 72 hours of incubation. In the independent laboratory validation study, there was no significant difference in detection, enumeration, or repeatability between the CompactDry YMR method and DRBC agar at 72 hours. Eighteen inclusivity and 16 exclusivity strains specific to cannabis plant materials that were not evaluated in the original CompactDry YMR method validation were tested in this study. All inclusivity organisms produced typical colonies on the CompactDry YMR. The two exclusivity bacterial strains that showed growth on CompactDry YMR at 72 hours were inoculated at a high concentration. *Conclusion*: CompactDry YMR is equivalent in performance to traditional culture media detection methods of yeasts and molds. *Highlight*: CompactDry YMR will streamline dried cannabis flower testing.

General Information

Yeasts and molds are important to the soil microbiome of the cannabis plant and can exhibit a symbiotic relationship as endophytes (1). However, some microorganisms can cause damage to the cannabis plant, buds, and/or roots leading to reduced quality or loss of the crop (2). Yeasts and molds can also affect product quality of cannabis inflorescences (2). Due to the heterotrophic nature and ubiquity in a wide range of environmental conditions, yeasts and molds can show amplified growth in greenhouse and high humidity conditions (3, 4).

Consumption and inhalation of certain yeasts, molds, and bacteria can cause a wide range of diseases including cryptococcal meningitis, invasive or lung aspergillosis, allergic reactions, fungal sinusitis, etc. (5). These types of infections are more frequently observed in immunocompromised

patient populations (5). While standard PCR-based testing can provide beneficial information on samples, the results provide limited information on the viability and physiological states of microorganisms which can cause products to be discarded that would otherwise be usable (6). Testing throughout cannabis farming and processing can help identify contamination before it leads to health issues or crop loss.

Principle of the Method

CompactDry Yeast/Mold Rapid (YMR) are ready-to-use dry media sheets comprising culture medium and a cold-soluble gelling agent. The film is rehydrated by inoculating 1 mL of diluted sample into the center of the self-diffusible medium. CompactDry YMR contains a special medium with nutrients, chloramphenicol to inhibit bacterial growth, and a chromogenic enzyme substrate, X-phos (5-bromo-6-chloro-3-indoxylphosphate), for the detection and enumeration of yeasts and molds after incubation at 25 ± 1°C for 72 hours. While most colonies are a shade of green/blue, any colored colony should be counted. In addition, mold colonies may have a diffuse or cottony appearance with blue/green or other color. CompactDry can be stored at room temperature, takes up less space due to the format of the product, and has a longer shelf life than most traditional media.

The CompactDry YMR was previously shown to be equivalent to ISO 21527-1:2008, *Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of yeasts and molds - Colony count technique in products with water activity greater than 0.95* (7) for enumeration of yeasts and molds in 10 matrixes, including cooked prawns, vegetable deli salad, tuna pâté, fermented strawberry yogurt drink, spinach and ricotta quiche, egg custard tarts, fruit and vegetable smoothie, cream cheese, egg salad sandwiches, and deli pasta salad (with chicken, bacon and corn) and awarded PTM certification number 092002 (8). The current matrix extension study compares the performance of the

CompactDry YMR to Dichloran Rose Bengal Chloramphenicol (DRBC) agar for the enumeration of yeasts and molds in dried cannabis flower [9-tetrahydrocannabinol (THC) >0.3%].

Scope of method

(**a**) *Analyte(s).*–Yeasts and molds

(**b**) *Matrix.*– Cooked prawns, vegetable deli salad, tuna pâté, fermented strawberry yogurt drink, spinach and ricotta quiche, egg custard tarts, fruit and vegetable smoothie, cream cheese, egg salad sandwiches, deli pasta salad (with chicken, bacon and corn), and dried cannabis flower (THC >0.3%)

(**c**) *Summary of Validated Performance Claims*.–Performance of CompactDry™ Yeast/Mold Rapid (YMR) is equivalent to that of Dichloran Rose Bengal Chloramphenicol (DRBC) agar for cannabis flower.

Definitions

(**a**) *Repeatability (s_r).*—Standard deviation of replicates for each analyte at each concentration of each matrix for each method.

(**b**) *Difference of Means (DOM).*—Mean difference between candidate and reference method transformed results with 95% confidence interval for each analyte at each concentration of each matrix.

(**c**) *Confidence interval (CI)*.—A confidence interval displays the probability that a parameter will fall between a pair of values around the mean. Confidence intervals are calculated at the 90% and 95% levels.

(**d**) *Statistical equivalence*.—The acceptance criterion for statistical equivalence is that the 90% CI on the bias between the methods falls within -0.5, 0.5.

Materials and Methods

Test Kit Information

(**a**) *Kit Name*.—CompactDry™ "Nissui" YMR; distributed as CompactDry™ Yeast/Mold Rapid (YMR) by Hardy Diagnostics.

(**b**) *Catalog Number*.—Hardy Diagnostics #54084 (240 plates)

(**c**) *Ordering Information*.—US: Hardy Diagnostics, 1430 West McCoy Lane, Santa Maria, CA 93455; Phone: (800) 266-2222; FAX: (805) 346-2760; Email: OnlineOrders@HardyDiagnostics.com;

Online: https://catalog.hardydiagnostics.com/cp_prod/Hardy_Product_Catalog.aspx

Test Kit Component

(**a**) *CompactDry™ YMR plates.*

Additional supplies and reagents

(**a**) *Sterile Filtered sample bags*.—80-400 mL capacity

(**b**) *Maximum Recovery Diluent (MRD).*—Prepare according to ISO 21527-1:2008 or source commercially (Oxoid EB 0348D or equivalent).

(**c**) *Butterfield's Phosphate Buffered Diluent (BPBD).*—Hardy Diagnostics U190 or equivalent

(**d**) *Colony counter.*

Apparatus

(**a**) *Laboratory Paddle Blender.*—Stomacher® 400 (Seward, West Sussex, UK) or equivalent

(**b**) *Pipettes.*—Capable of delivering 1.0 mL

(**c**) *Incubator.*—Capable of maintaining 25 ± 1°C

Safety Precautions

(**a**) Immediately wash with water if medium or reagent comes into contact with eyes or mouth. Consult a physician.

(**b**) Manipulations with microorganisms involve certain risks of laboratory-acquired infections. Carry out manipulations under supervision of trained laboratory personnel with biohazard protection measures.

(**c**) Treat laboratory equipment or medium that comes in contact with the specimen as infectious and sterilize appropriately.

(**d**) Sterilize any medium, reagent or materials by autoclaving or boiling after use, and then dispose as industrial waste according to local laws and regulations.

General Precautions

(**a**) Read and follow the warnings and directions for use described in the package insert and/or label precisely.

(**b**) Do not use product after its expiration date. Quality of the product is not guaranteed after its shelf life.

(**c**) Do not use product that contains any foreign materials, is discolored or dehydrated, or has a damaged container.

(**d**) Use plates as soon as possible after opening. Any unused plates should be returned to the aluminum bag and sealed with tape to avoid light and moisture.

(**e**) Cap tightly after inoculation to avoid dehydration of gelled medium.

(**f**) Do not use CompactDry YMR for human or animal diagnosis.

(**g**) During inoculation, do not touch the surface of the medium.

(**h**) Use of filtered stomacher bags is recommended to eliminate risks of carryover of tiny pieces of foodstuffs onto the surface of the medium.

(**i**) If the nature of sample affects the reaction of the medium, inoculate the sample only after the factor has been eliminated by means such as dilution, pH adjustment, or others. This may include samples with high viscosity or deep color.

Sample Preparation

Viable count in solid foodstuffs:

(**a**) Weigh a 10 g test portion into a sterile filtered sample bag.

(**b**) Add 90 mL MRD and pummel for 1 min ± 10 s in paddle blender.

(**c**) Make 10-fold serial dilutions by diluting 10 mL homogenate in 90 mL MRD. Mix by shaking.

Viable count in liquid foodstuffs:

(**a**) Weigh a 10 g test portion into a sterile sample bag.

(**b**) Add 90 mL MRD and pummel for 1 min ± 10 s in paddle blender.

(**c**) Make 10-fold serial dilutions by diluting 10 mL homogenate in 90 mL MRD. Mix by shaking.

Viable count in cannabis flower:

(**a**) Weigh a 10 g test portion into a sterile filtered sample bag.

(**b**) Add 90 mL BPBD and pummel for 1 min ± 10 s in paddle blender.

(**c**) Make 10-fold serial dilutions by diluting 10 mL homogenate in 90 mL BPBD. Mix by shaking.

Analysis

(**a**) Open aluminum pouch and remove the set of 4 plates.

(**b**) Detach the necessary number of plates from a set of four by bending up and down while pressing the lid. Use a connected set of four plates when serial dilution measuring is intended.

(**c**) Remove the cap from the plate, pipette 1 mL of sample (to be diluted further if necessary) in the middle of the dry sheet, and replace cap. The sample diffuses automatically and evenly over the entire sheet (total medium of 20 cm^2) to transform it into gel within seconds.

(**d**) Write the appropriate information on the memorandum section. Invert the capped plate and place in incubator at 25 ± 1°C for 72 ± 3 h.

(**e**) From the backside of the plate, count the number of any colored colonies (usually green/blue) and "cottony" colonies in the medium. White paper placed under the plate can make colony counting easier. For large numbers of colonies, use the grids carved on the backside consisting of 1 cm x 1 cm, or 0.5 cm x 0.5 cm, at the four corners.

(**f**) The enumeration range of the CompactDry YMR is 1-150 CFU/plate. Dilute samples further in the appropriate diluent as necessary to achieve a concentration level in the countable range.

(**g**) The full plate size is 20 cm^2. The backside contains carved grids of 1 cm x 1 cm and 0,5 cm x 0.5 cm to make colony counting easier. If large numbers of colonies are present on the medium, the total viable count can be obtained by averaging the number of colonies per large grid (1 cm x 1 cm), counted from several grids, and multiplying by 20. Alternatively, when large numbers of colonies are present, the total viable count can be obtained by averaging the number of colonies per small grid (0.5 cm x 0.5 cm) and multiplying by 80.

(**h**) If more than 10^4 CFU are inoculated onto a CompactDry YMR plate, no distinguishable colored colonies will form and the entire plate may become colored.

Validation Study

This validation study was conducted as an Emergency Response Validation (ERV) process within the AOAC Research Institute *Performance Tested Method*[SM] (PTM) program. The validation followed the *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* (9) and the draft *Standard Method Performance Requirements for Viable Total Yeast and Mold Count Enumeration* (10). Hardy Diagnostics (Santa Maria, CA), a distributor of CompactDry products, provided CompactDry YMR plates for the matrix study, performed part of the inclusivity/exclusivity study, and provided CompactDry YMR plates for the remainder of the inclusivity/exclusivity study. Steadfast Analytical Laboratory (Hazel Park, MI) performed the matrix study under the direction of the AOAC Research Institute as the Coordinating Laboratory preparing the blind coded test portions and the Independent Laboratory performing the analysis of test portions for the CompactDry YMR method. Q Laboratories (Cincinnati, OH), a Contract Laboratory, performed a partial inclusivity/exclusivity study.

Method Developer Study

Inclusivity/Exclusivity Study

An inclusivity/exclusivity study of the CompactDry YMR method was previously performed using 51 strains of yeasts and molds and 32 strains of non-target organisms as part of the original method validation (8). The ERV protocol required data for specific inclusivity and exclusivity organisms commonly found in cannabis matrixes that had not already been tested in the original validation were included in this study. Hardy Diagnostics examined 4 inclusivity and 11 exclusivity organisms in a randomized blind-coded study. Q Laboratories examined 14 inclusivity and 5 exclusivity organisms in a randomized blind-coded study.

Methodology (Q Laboratories).—All yeast inclusivity organisms were propagated from a stock culture stored at -70°C to Potato Dextrose Broth and incubated at temperatures optimal for growth. Following incubation, all yeast inclusivity organisms were diluted to 100x the limit of detection (LOD) of the CompactDry YMR. All mold inclusivity organisms were propagated from a stock culture stored at -70°C to Sabouraud Dextrose Agar (SDA, 4% dextrose) and incubated for 5-7 days at 30 ± 1 °C. Following incubation, all mold spores were harvested for mold inclusivity testing by washing cultures with Butterfield's Phosphate Buffered Dilution Water (BPBD). The mold wash was then diluted to 100x the LOD of the CompactDry YMR.

All exclusivity organisms were propagated from a stock culture stored at -70°C to trypticase soy agar with 5% sheep blood (SBA) and incubated at conditions optimal for growth. Following incubation, exclusivity organisms were transferred to non-selective Brain Heart Infusion (BHI) broth and incubated at conditions optimal for growth. All exclusivity cultures were analyzed undiluted.

Methodology (Hardy Diagnostics).— All strains tested at Hardy Diagnostics were maintained in Brucella Broth with 10% glycerol at -80°C. Prior to use, each inclusivity strain was subcultured onto an SDA and incubated for 5-10 days at 25±1°C. After incubation, an isolated colony from each strain was then subcultured onto a SDA plate and incubated for 5-10 days at 25°C ± 1°C. Suspensions of approximately $1.5x10^6$ CFU/mL were prepared for each inclusivity strain in tryptic soy broth (TSB) and were compared to a McFarland Turbidity standard. Serial dilutions were performed in TSB to achieve a $1.5x10^2$ CFU/mL suspension. Using aseptic technique, 100 µL of the $1.5x10^2$ CFU/mL suspension was spread-plated onto a SDA plate to confirm the suspension concentration.

Prior to use, each exclusivity strain was subcultured onto a SBA or Nutrient Agar (NA) plate and incubated for 24-48 hours at 35±1°C. After incubation, an isolated colony from each culture was subcultured onto a SBA or NA plate and incubated for 24-48 hours at 35±1°C. Using fresh cultures, each

exclusivity strain was suspended into TSB to achieve a turbidity of approximately $1.5x10^8$ CFU/mL compared to a McFarland Turbidity Standard. Exclusivity strains were analyzed undiluted at $1.5x10^8$ CFU/mL.

Results.—The inclusivity results are presented in Table 1. All of the 18 inclusivity strains tested showed growth on the CompactDry YMR medium with typical morphology. Yeasts and molds typically form green/blue colonies. While most colonies are a shade of green/blue, any colored colony should be counted. In addition, mold colonies may have a diffuse or cottony appearance.

Table 2 shows the exclusivity results. Of the 16 exclusivity strains tested, two exclusivity microorganisms showed growth (*Klebsiella pneumoniae* and *Pseudomonas fluorescens*) on the CompactDry YMR medium after 72 h at 25±1°C. *Klebsiella pneumoniae* grew weakly and had less than 10 small blue colonies after 72 hours of incubation. This organism is inhibited by the media since only a few colonies were observed at the concentration tested ($1.5x10^8$ CFU/mL). *Pseudomonas fluorescens* had no distinct colonies present, but the background of the plate turned green and was likely due to a lawn of bacterial growth since the plate was inoculated with $1.5x10^8$ CFU.

Table 1. Inclusivity testing of yeast and mold species on CompactDry YMR for Cannabis Emergency Response Validation

No.	Organism	Fungus Type	Source[c]	Origin	Result[d]
1	*Arthrinium aureum*[a]	Mold	ATCC 56042	Not available	+
2	*Aspergillus aculeatus*[a]	Mold	ATCC 56925	Grape	+
3	*Aspergillus caesiellus*[a]	Mold	ATCC 42693	Dried chilies	+
4	*Botrytis cinerea*[a]	Mold	ATCC 11542	Azalea flowers	+
5	*Candida tropicalis*[b]	Yeast	ATCC 750	Patient with bronchomycosis	+
6	*Cryptococcus laurentii*[b]	Yeast	ATCC 18803	Palm wine	+
7	*Cryptococcus neoformans*[b]	Yeast	ATCC 32045	Fermenting fruit juice	+
8	*Fusarium proliferatum*[a]	Mold	QL 0567112-1C	Environmental	+
9	*Mucor circinelloides*[a]	Mold	ATCC 24905	Rice fermentations	+
10	*Mucor hiemalis*[a]	Mold	ATCC 34334	Cow dung	+
11	*Penicillium rubens*[b]	Mold	ATCC 9179	Culture contaminant	+
12	*Penicillium venetum*[a]	Mold	ATCC 16025	*Hyacinthus* sp. bulb	+
13	*Purpureocillium lilacinum*[a]	Mold	ATCC 10114	Soil	+
14	*Rhizopus stolonifera*[a]	Mold	QL 14181-2A	Not available	+
15	*Scopulariopsis acremonium*[a]	Mold	ATCC 58636	Chicken house soil	+
16	*Stemphylium* sp.[a]	Mold	QL 15229-1	Potable water	+
17	*Talaromyces pinophilus*[a] (*Penicillium pinophilum)*	Mold	NRRL 11797	Corn	+
18	*Yarrowia lipolytica*[a]	Yeast	ATCC 9773	Not available	+

[a]Tested by Q Laboratories

[b]Tested by Hardy Diagnostics

[c]ATCC = American Type Culture Collection (Mannassas, VA, USA); QL = Q Laboratories culture collection (Cincinnati, OH, USA); NRRL = Agriculture Research Service (Northern Regional Research Laboratory) Culture Collection (Peoria, IL, USA)

[d]"+" indicates that growth typical for these species occurred.

Table 2. Inclusivity testing of yeast and mold species as presented in original validation study (8)

No.	Species	Source[a]	Origin	Growth at 72 h[b]	Morphology at 72 h
1	*Alternaria alternata*	IFO 31188	living leaf, *Stevia rebaudiana*	+	blue green - dark green, unclear edge
2	*Aspergillus brasiliensis*	NBRC 9455	Blueberry, *Vaccinium* sp.	+	white - blue green
3	*Aspergillus flavus*	NBRC 6343	Shoe sole	+	unclear edge, black spots in pale blue green
4	*Aspergillus fumigatus*	NBRC 33022	unknown	+	atypical, black spots in hypha pale blue green
5	*Aspergillus niger*	NBRC 105649	Leather	+	rather unclear colony deep blue green; black spots in center
6	*Aspergillus oryzae*	NBRC 5375	unknown	+	blue green; pale brown center
7	*Aspergillus terreus*	NBRC 6346	Haversack	+	White; not clearly formed edge
8	*Aspergillus versicolor*	NBRC 4098	tobacco	+	pale blue green; not clearly formed edge
9	*Aureobasidium pullulans*	NBRC 6353	unknown	+	blue - dark green
10	*Candida albicans*	NBRC 1594	Clinical bronchomycosis	+	white - pale green
11	*Candida apicola*	NBRC 10261	intestine of bee	+	blue green
12	*Candida lactis-condensi*	NBRC 1286	fermenting condensed milk	+	blue green; blue green edge
13	*Chaetomium globosum*	NBRC 6347	Stored cotton	+	blue green
14	*Cladosporium cladosporioides*	NBRC 6348	unknown	+	pale blue green - blue green; not clearly formed colony
15	*Cladosporium halotolerans*	NBRC 4460	Air	+	white - pale blue green; not clearly formed colony
16	*Debaryomyces hansenii*	IFO 0026	beef-and-pork sausage	+	white
17	*Debaryomyces maramus*	NBRC 0668	Air	+	white
18	*Fusarium oxysporum*	NBRC 7155	unknown	+	pale blue green; deep blue green center
19	*Fusarium solani*	NBRC 5232	unknown	+	blue green; cottony center
20	*Geotrichum candidum*	NBRC 4598	unknown	+	pale greenish white; cottony
21	*Hormoconis resinae*	NBRC 100535	unknown	+	very pale blue green; atypical growth, not clearly formed
22	*Monascus purpureus*	NBRC 32316	red rice	+	blue green; pale brown center
23	*Moniliella acetoabutans*	NBRC 9482	sweet fruit sauce	+	blue green
24	*Myrothecium verrucaria*	NBRC 6113	Deteriorated baled cotton	+	blue green; pale brown center
25	*Neosartorya fischeri*	IFO 8789	rubber tire scrap	+	pale blue green - blue green; not clearly formed edge
26	*Paecilomyces variotii*	NBRC 33284	unknown	+	pale blue green - blue green
27	*Penicillium aurantiogriseum*	NBRC 7733	Rotting grain of *Zea mays*	+	blue green

No.	Species	Source[a]	Origin	Growth at 72 h[b]	Morphology at 72 h
28	*Penicillium brevicompactum*	NBRC 5727	soil	+	blue green; white - pale brown center
29	*Penicillium chrysogenum*	IFO 32030	cheese	+	blue green; white cottony center
30	*Penicillium citrinum*	NBRC 6352	unknown	+	yellow green; white cottony center
31	*Penicillium funiculosum*	NBRC 100958	Mercury-treated fabric	+	pale white; not clearly formed colony
32	*Penicillium martensii*	NBRC 8142	unknown	+	Green; not clearly formed edge
33	*Penicillium ochrochloron*	NBRC 4612	unknown	+	blue green; not clearly formed edge
34	*Penicillium pinophilum*	NBRC 33285	unknown	+	white - very pale blue green; not clearly formed colony
35	*Phialophora fastigiata*	IFO 6850	unknown	+	blue green; not clearly formed edge
36	*Phoma herbarum*	NBRC 107643	Polyester straw on drinking pot	+	white - very pale blue green; not clearly formed edge
37	*Pichia anomala*	IFO 10213	unknown	+	blue green (nearly green)
38	*Pseudocochliobolus lunatus*	NBRC 30883	leaf of sudangrass 'Greenleaf'	+	pale blue green; not clearly formed edge
39	*Rhizopus oryzae*	NBRC 31005	Radio set	+	partly pale yellow brown; atypical growth
40	*Rhodotorula acuta*	IFO 1912	grape must	+	blue green; pale blue green edge
41	*Rhodotorula glutinis*	NBRC 1125	Air	+	blue green; pale blue green edge
42	*Rhodotorula mucilaginosa*	NBRC 0889	unknown	+	pale blue green; very pale blue green edge
43	*Saccharomyces cerevisiae*	NBRC 101557	Fermenting sake mash	+	white - pale green
44	*Scopulariopsis brevicaulis*	NBRC 100536	Chrysalis of silkworm	+	pale blue green - blue green; not clearly formed colony
45	*Torulaspora delbrueckii*	IFO 1180	grape must	+	blue green (nearly green); pale blue green edge
46	*Trichoderma citrinoviride*	IFO 31137	Soil in organic layer	+	pale blue green; not clearly formed edge, deep blue green
47	*Trichoderma virens*	NBRC 6355	Soil	+	blue green - yellow green; atypical growth
48	*Trichophyton mentagrophytes*	IFO 6202	unknown	+	pale blue green
49	*Trichosporon asahii*	NBRC 103889	case of trichosporia cutis	+	blue green
50	*Zygosaccharomyces bailii*	NBRC 1098	unknown	+	pale blue green - blue green; very pale blue green edge
51	*Zygosaccharomyces rouxii*	NBRC 1960	cane sugar	+	white - pale blue green; very pale blue green edge

[a]IFO = Campden Culture Collection (Campden BRI, Chipping Campden, UK); NBRC = National Institute of Technology and Evaluation Biological Resource Center (Tokyo, Japan)

[b]"+" indicates that growth typical for these species occurred.

Table 3. Exclusivity testing of non-yeast and non-mold species on CompactDry YMR for Cannabis Emergency Response Validation

No.	Species	Source[c]	Origin	Growth[d]
1	*Aeromonas hydrophila*[a]	ATCC 7966	Tin of milk with fishy odor	-
2	*Citrobacter braakii*[a]	ATCC 43162	Clinical isolate	-
3	*Edwardsiella tarda*[a]	ATCC 15947	Human feces	-
4	*Erwinia amylovora*[b]	ATCC 51852	Plant	-
5	*Escherichia coli* O157:H7[a]	ATCC 43888	Human feces	-
6	*Escherichia hermanii*[b]	ATCC 33650	Mouse brain	-
7	*Escherichia vulneris*[b]	ATCC 29943	Human wound	-
8	*Hafnia alvei*[a]	ATCC 29926	Clinical isolate	-
9	*Klebsiella oxytoca*[a]	ATCC 43165	Clinical isolate	-
10	*Klebsiella pneumoniae*[a]	ATCC 13883	Not available	+
11	*Pantoea agglomerans*[b]	ATCC 19552	Sewage	-
12	*Pseudomonas fluorescens*[a]	ATCC 13525	Pre-filter tanks	+
13	*Pseudomonas putida*[a]	HDX 9061	Clinical isolate	-
14	*Ralstonia pickettii*[b]	ATCC 27511	Clinical isolate	-
15	*Rahnella aquatilis*[a]	ATCC 33071	Drinking water	-
16	*Stenotrophomonas maltophilia*[a]	ATCC 13636	Spinal fluid	-

[a]Tested by Hardy Diagnostics

[b]Tested by Q Laboratories

[c]ATCC = American Type Culture Collection (Mannassas, VA, USA); HDX = Hardy Diagnostics Culture Collection (Santa Maria, CA, USA)

[d]"-" indicates growth did not occur. "+" indicates growth occurred.

Table 4. Exclusivity testing of non-yeast and non-mold species as presented in original validation study (8)

No.	Species	Source[a]	Origin	Growth[b]
1	*Acinetobactor baumannii*	JCM 6841	Urine	-
2	*Acinetobacter calcoaceticus*	ATCC 19606	unknown	-
3	*Alcaligenes faecalis*	IFO 13111	unknown	-
4	*Bacillus cereus*	IFO 13494	unknown	-
5	*Bacillus licheniformis*	NBRC 12200	unknown	-
6	*Bacillus subtilis*	NBRC 3134	unknown	-
7	*Burkholderia cepacia*	NBRC 15124	10% benzalkonium chloride solution	-
8	*Citrobacter freundii*	IFO 12681	unknown	-
9	*Enterobacter aerogens*	ATCC 13048	Sputum, South Carolina Dept. of Health and Environmental Control	-
10	*Enterobacter cloacae* subsp. *cloacae*	NBRC 13535	Spinal fluid	-
11	*Enterococcus faecalis*	NBRC 12965	Citrus juice	-
12	*Enterococcus faecium*	ATCC 19434	unknown	-
13	*Escherichia coli*	NBRC 3301	Human feces	-
14	*Escherichia coli*	NBRC 3972	Feces	-
15	*Flavobacterium odoratum*	ATCC 4651	unknown	-
16	*Kocuria rhizophila*	ATCC 9341	Soil	-
17	*Lactobacillus casei*	NBRC 15883	Cheese	-
18	*Lactococcus lactis*	NS 6938	human	-
19	*Listeria monocytogenes*	VTU 206	unknown	-
20	*Micrococcus luteus*	NBRC 3333	unknown	-
21	*Morganella morganiii*	ATCC 25830	Patient with summer diarrhea	-
22	*Paenibacillus polymyxa*	NBRC 15309	unknown	-
23	*Proteus mirabilis*	IFO 3849	unknown	-
24	*Pseudomonas aeruginosa*	NBRC 13275	Outer ear infection	-
25	*Pseudomonas stutzeri*	ATCC 17587	Bile	-
26	*Rhodococcus equi*	IFO 14956	Lung abscess of foal	-
27	*Salmonella* Typhimurium	ATCC 14028	Tissue, animal - pools of heart and liver from 4-week-old chickens	-
28	*Serratia marcescens* subsp. *marcescens*	NBRC 102204	Pond water	-
29	*Shigella flexneri*	ATCC 12022	unknown	-
30	*Staphylococcus aureus* subsp. *aureus*	NBRC 12732	unknown	-

No.	Species	Source[a]	Origin	Growth[b]
31	*Staphylococcus epidermidis*	ATCC 35984	Catheter sepsis, Tennessee	-
32	*Streptococcus pyogenes*	JCM 5674	Scarlet fever	-

[a]JCM = Japan Collection of Microorganisms (RIKEN BioResource Center, Ibaraki, Japan); ATCC = American Type Culture Collection (Manassas, VA, USA); IFO = Campden Culture Collection (Campden BRI, Chipping Campden, UK); NBRC = National Institute of Technology and Evaluation Biological Resource Center (Tokyo, Japan); VTU = Visvesvaraya Technological University (Bangalore, India)
[b]"-" indicates growth did not occur.

Independent Laboratory Study

Matrix Study

Methodology.—Cannabis test materials were prepared in the Coordinating Laboratory from Steadfast Analytical's inventory of retained samples from its Michigan licensed grower, patient, and caregiver customers. Individual samples within a specified contamination level were combined to produce batch materials of at least 1000 g at a low level (<1000 CFU/g), a medium level (1000–10,000 CFU/g), and a high level (10,000–100,000 CFU/g). Batches were manually mixed in an aseptic manner until homogeneous. For each contamination level, five replicate test portions (10 g) were quantified by spread plating aliquots of diluted test portions onto DRBC agar plates. Table 5 summarizes the average CFU/g of yeast and mold for each contamination level that was provided to laboratories for analysis in the study.

Table 5. Average contamination level of yeast and mold in test batches

Batch	n	DRBC[a] (CFU/g)
Low	5	350
Medium	5	5600
High	5	48,000

[a]DRBC = Dichloran Rose Bengal Chloramphenicol agar

Individual 10 g test portions from each contamination level were placed in sterile filter Whirl-Pak bags. Five bagged test portions from each of the 3 contamination levels were selected for each candidate method participating in the ERV project. Test portions were assigned an identification tag in Michigan's Marijuana Regulatory Agency (MRA) seed-to-sale system for distribution and tracking. This served to blind code the contamination level of the test portions. The test portions were also assigned random sample numbers for reporting results to AOAC.

Personnel from each of the participating independent laboratories were responsible for picking up and transporting the test portions to their laboratories on Monday, December 7, 2020. Participating laboratories were instructed to analyze samples on Tuesday, December 8, 2020 following the user guides provided with the candidate methods. In addition to the candidate methods, all test portions were enumerated using DRBC agar. For the CompactDry YMR method, Steadfast Analytical Laboratories was the independent laboratory.

Candidate Method

All testing was performed using paired test portions. Test portions were prepared for analysis as described in the CompactDry YMR method. Plates containing counts between 10-150 from multiple dilutions were used to determine the final result using the equation provided in the FDA Bacteriological Analytical Manual (BAM) Chapter 3 *Aerobic Plate Count* (11):

$$N = \frac{\sum c}{[(1 \times n_1) + (0.1 \times n_2)] \times d} \qquad \text{Eq. 1}$$

where c = total colony forming units (CFU) from plates in the countable range; n_1 = number of plates in the first dilution that produced results in the countable range; n_2 = number of plates in the second dilution that produced results in the countable range; d = dilution factor corresponding to the first dilution.

Dichloran Rose Bengal Chloramphenicol (DRBC) Confirmation

Paired test portions, prepared following the candidate method dilution protocol, were confirmed by spread plating aliquots of each dilution onto DRBC agar plates. From the initial dilution of sample prepared in BPBD (10^{-1} dilution, 10 g test portion into 90 mL BPBD), 2 additional tenfold serial dilutions

were prepared by transferring 10 mL of the previous dilution into 90 mL of BPBD. Dilution bottles were shaken 25 times in a one-foot arc within 7 secs to ensure homogeneity.

To plate dilutions, 1.0 mL from the 10^{-1} dilution was spread plated across 2 DRBC agar plates (0.5 mL per plate) in triplicate (6 total DRBC agar plates). Additionally, 0.1 mL of the 10^{-1}, 10^{-2}, and 10^{-3} dilutions was plated in triplicate on DRBC. The agar plates were allowed to dry and were subsequently incubated at 25 ± 1°C for 5-7 days before enumeration. Plates containing counts between 10-150 colonies were enumerated. Plates containing counts in the countable range from multiple dilutions were used to determine the final result using Equation 1 above.

Results.— The matrix study data summary is presented in Table 6. Statistical analysis was conducted for each contamination level comparing the candidate method result to CFU/g obtained on the DRBC agar plates (12). For each test portion, results were logarithmically ($\log_{10}$) transformed using the equation (CFU/g +0.1f, where f is the reported CFU/g corresponding to the smallest reportable result) and subjected to the Grubbs test for outliers. No outliers were found. After transformation, replicate test portion results for each contamination level for each method were averaged, and the difference of means between methods with 95% and 90% confidence intervals were determined. Repeatability (s_r) and relative standard deviation of repeatability (RSD_r) were also calculated. A 90% confidence interval for the difference of means that falls between -0.5 $\log_{10}$ and 0.5 $\log_{10}$ indicates that the candidate method and DRBC agar plate results are equivalent. Data obtained during the validation indicated that each level evaluated produced equivalent results between the CompactDry YMR method and DRBC result.

Table 6. Method comparison data summary and statistics for dried cannabis flower

		CompactDry™ YMR			DRBC[d]				95 % CI[f]		90 % CI	
Contamination level	n[a]	Mean Log_{10} CFU[b]/g	s_r	RSD_r[c], %	Mean Log_{10} CFU/g	s_r	RSD_r, %	DOM[e]	LCL[g]	UCL[h]	LCL	UCL
Low	5	2.828	0.048	1.70	2.819	0.046	1.63	0.009	-0.076	0.094	0.056	0.074
Medium	5	4.851	0.029	0.60	4.818	0.022	0.46	0.033	-0.007	0.073	0.003	0.064
High	5	5.769	0.078	1.35	5.776	0.095	1.64	-0.008	-0.135	0.120	-0.106	0.090

[a]n = number of replicate test portions
[b]CFU = Colony-forming units
[c]RSD_r = Relative standard deviation of repeatability
[d]DRBC = Dichloran Rose Bengal Chloramphenicol agar
[e]DOM = Difference of Means
[f]CI = Confidence Interval for DOM
[g]CL = Lower confidence limit for DOM
[h]UCL = Upper confidence limit for DOM

Discussion

CompactDry YMR was previously shown to be equivalent to ISO 21527-1:2008 and awarded PTM certification number 092002 (7, 8). The current matrix extension study compared the performance of the CompactDry YMR to DRBC agar at 72 hours for the enumeration of yeasts and molds in cannabis flower [THC >0.3%]. Naturally contaminated low, medium and high-level cannabis test materials were evaluated. The plate counts from the CompactDry YMR plate method were compared with colony counts from the reference agar.

The method comparison data demonstrated statistically equivalent enumeration between the CompactDry YMR method and the reference DRBC agar at all levels at 72 hours for cannabis material. For this matrix, DRBC and CompactDry repeatability values were comparable at all inoculum levels. No negative feedback was reported to the study directors from the independent laboratory.

All inclusivity organisms evaluated were recovered on CompactDry YMR. Two exclusivity organisms, *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas fluorescens* (ATCC 13525), showed growth at 72 hours. *Klebsiella pneumoniae* grew weakly and had less than 10 small blue colonies after 72 hours of incubation. This organism is inhibited by the media since only a few colonies were observed at the concentration tested ($1.5x10^8$ CFU/mL). *Pseudomonas fluorescens* had no distinct colonies present, but the background of the plate was green, likely due to a lawn of bacteria as the plate was inoculated with about $1.5x10^8$ CFU.

Conclusion

These studies demonstrate that the CompactDry YMR method is a robust method that detects and enumerates yeasts and molds at 72 hours from dried cannabis flower, yielding equivalent values to the reference agar, DRBC.

Acknowledgements

Submitting Company: Hardy Diagnostics, 1430 West McCoy Lane, Santa Maria, CA 93455

Financial Support: Nissui Pharmaceutical CO., Ltd., 3-23-9, Ueno, Taito-ku, Tokyo 110-8736, Japan

Independent Laboratory: Steadfast Analytical Laboratory, 21928 John R Road, Hazel Park, MI 48030

Contract Laboratory: Q Laboratories, 1930 Radcliff Drive, Cincinnati, OH 45204

Reviewers:

Yvonne Salfinger, Consultant, Tallahassee, FL, USA

Farahath Shenaz Dave, Maryland Medical Cannabis Commission

Michael Brodsky, Brodsky Consultants, Toronto, ON, Canada

References

(1) Scott, M.; Rani, M.; Samsatly, J.; Charron, J.B.; Jabaji, S. Endophytes of industrial hemp (*Cannabis sativa* L.) cultivars: Identification of culturable bacteria and fungi in leaves, petioles, and seeds. *Can. J. Microbiol. 2018, 64*, 664–680

(2) Punja, Zamir K et al. "Pathogens and Molds Affecting Production and Quality of *Cannabis sativa* L." *Frontiers in Plant Science* vol. 10 1120. 17 Oct. 2019, doi:10.3389/fpls.2019.01120

(3) APHA (2015) *Compendium of Methods for the Microbiological Examination of Foods, Fifth Edition,* APHA, Washington, D.C.

(4) McPartland, J.M.; McKernan, K.J. Contaminants of concern in *Cannabis*: Microbes, heavy metals and pesticides. *Canabis Sativa L. Bot. Biotechnol.* 2017, 22, 457–474.

(5) Vujanovic, Vladimir et al. “Scientific Prospects for Cannabis-Microbiome Research to Ensure Quality and Safety of Products.” *Microorganisms* 8.2 (2020): 290. *Crossref*. https://www.mdpi.com/2076-2607/8/2/290/html.

(6) Cangelosi, Gerard A, and John S Meschke. “Dead or alive: molecular assessment of microbial viability.” *Applied and environmental microbiology* vol. 80,19 (2014): 5884-91. doi:10.1128/AEM.01763-14 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4178667/

(7) Anonymous (2008) International Organization for Standardization (ISO) Method 21527-1:2008, Geneva, Switzerland, https://www.iso.org/standard/38275.html, accessed 2 April 2021

(8) AOAC Research Institute (2020) *Performance Tested Method*SM 092002, https://members.aoac.org/AOAC_Docs/RI/20PTM/21C_092002_NissuiYMR.v3.pdf , accessed January 25, 2021.

(9) *Official Methods of Analysis* (2019), 21st Ed., Appendix J, AOAC INTERNATIONAL, Gaithersburg, MD, http://www.eoma.aoac.org/app_j accessed 2 April 2021.

(10) *Standard Method Performance Requirements for Viable Total Yeast and Mold Count Enumeration* (Draft Version 7, March 5, 2021), https://www.aoac.org/wp-content/uploads/2021/03/CASP_Yeast-Mold_SMPRv71.pdf, accessed 2 April 2021.

(11) US Food and Drug Administration (2001) *Bacteriological Analytical Manual*, Chapter 3, *Aerobic Plate Count*, https://www.fda.gov/food/laboratory-methods-food/bam-chapter-3-aerobic-plate-count, accessed 2 April 2021.

(12) Paired Method Analysis for Micro Testing (2010), Version 1.2, Least Cost Formulations, Ltd., Virginia Beach, VA.