Microbiology Focus

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Fluka® Analytical

Spoilage Microbes in Non-Alcoholic Beverages

Lactobacilli are some of the major spoilage organisms found in beverages, causing an awful flavor in even the smallest concentrations.

SIGMA-ALDRICH®

Spoilage Organisms in Non-Alcoholic Drinks

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Microbiological quality of non-alcoholic drinks.

Non-alcoholic drinks are the target of spoilage organisms such as yeast (e.g., *Saccharomyces, Zygosacchromyces, Brettanomyces, Torulaspora, Pichia, Candida* and bacteria of the genera *Lactobacillus, Acetobacteraceae* and *Alicyclobacillus*. As a result of contamination with such organisms, drinks begin to taste awful within hours or days of incubation. The beverage producers are often in a difficult situations since on the one hand they would like their fruit juice as natural as possible with vitamins and healthy metabolites, while on the other hand, the product should be stable and free of pathogens and spoilage organisms. Typically, beverages like apple and orange juice are the most susceptible products. They are either fresh or pasteurized and often kept at ambient temperatures. Their naturally low pH tends to prevent the growth of most organisms, but some acid tolerant organisms can grow and produce metabolites (e.g, phenolic compounds) with a bad odor. Also, fruit juice concentrates can be the source of contamination, which is usually present in the form of endospores from bacteria such as *Alicyclobacillus acidoterrestris*.

For the microbiologists, the product analysis is often time consuming and the matrix can be problematic for modern tests. The ability to rapidly send the juice to market is important in order to keep the quantity of stock small since the time period until expiration can be very short. The ability to produce a product of consistently high quality is important for the manufacturer's reputation, since a product recall can be both expensive and quite damaging.

Did you know…

Beverage-spoiling microbes are very thermotolerant?

Compared to pathogens or protozoans, vegetative cells from beverage-spoiling organisms are very heat resistant. Some lactic acid bacteria survive at 55 °C and some alicyclobacilli survive even up to 60 °C and are able to build extremely heat resistant endospores (Tribst et al. 2009, Lawlor et al. 2009).

Figure 1: Drinks in the focus

Because non-alcoholic drinks such as juice require very specialized quality control, highly skilled laboratory staff are needed to perform microbiological analysis in breweries and laboratories. One disadvantage is that most of the laboratories still use conventional, standard-based cultivation methods, which are very time consuming and require a delay of three to seven days before non-alcoholic drinks can be available for sale.

The microbiological testing should be performed on the final packages of the run to catch any possible contamination. It is important that the samples tested are representative, so a sampling scheme is important.

Figure 2: Beverage filling.

HybriScan™*D* Drink kit is a rapid test system which detects organisms based on ribosomal RNA and leads to a faster product release by detecting the relevant spoiling contaminants within two hours and in a single assay. After two hours (pre-enrichment for 24 hours, if necessary), the beverage test could have its first reliable results.

In contrast to other rapid test systems, the robustness of the HybriScan assay enables detection of bacterial and yeast contamination and leads to efficient use of this valuable resource. Not only can non-alcoholic beverage spoiling organisms be detected with this innovative rapid test system, but also all Eubacteria (HybriScan Total Count) can be quantified. Therefore, the HybriScan test system is the perfect tool for microbiological control of dispensing equipment and bioburden of water and other material. Often the detection of microorganisms is possible without performing a pre-enrichment-procedure and is available within two hours for analysis.

Comparison of HybriScan and Other Rapid Test Systems

Performing quality control using the standard cultivation-based method will take a long time and will delay the release of products. In recent years, many companies have developed rapid test systems to hasten this procedure. For quality control of beer and beverages, three technologies are currently on the market:

- HybriScan (sandwich hybridization)
- PCR (Polymerase Chain Reaction)
- VIT (Vermicon Identification Technology)

Comparison of these different technologies is given in **Table 2**. By comparing HybriScan to PCR or VIT, the benefits of this rapid test system become obvious:

- Fast and cost efficient analysis
- Inexpensive readout technology
- High sensitivity and specificity

By using two different probes for detection of microbial RNA, false-positive results are almost impossible. In **Figure 3**, results of quantification of *Lactobacillus buchneri* within a starter culture (silage) of three different samples are presented. Comparison of the HybriScan test with a cultivation-based analytical method (MRS agar) displays very slight differences in the determination of the cell count.

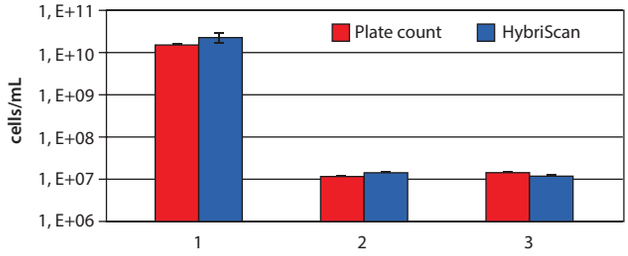


Figure 3: Comparison of cell numbers of HybriScan*D* kit versus Plate Count Agar in three different samples.

Genus Lactobacillus:	*Lactobacillus acidophilus*	Yeasts:	*Saccharomyces*
	Lactobacillus brevis		*Zygosacchromyces*
	Lactobacillus brevisimilis		*Brettanomyces*
	Lactobacillus buchneri		*Torulaspora*
	Lactobacillus casei		*Pichia*
	Lactobacillus collinoides		*Candida*
	Lactobacillus coryniformis	Acetobacteriaceae:	*Acetobacter pasteurianus*
	Lactobacillus curvatus		*Gluconacetobacter liquefans*
	Lactobacillus fermentum	Alicyclobacillus:	*Alicyclobacillus acidiphilus*
	Lactobacillus lindneri		*Alicyclobacillus acidoterrestris*

Table 1: Some examples of species of beverage spoiling microorganisms which can be detected with the HybriScan*D* Drink Kit (**Fluka Cat. No. 68301**, 96 assays). Additional species of the different genera are detected as well.

	Cultivation-based method	PCR	VIT	HybriScan
Method	Cultivation-based method with optical or microscopic readout	PCR/real-time PCR	Fluorescence microscopy	Sandwich hybridization and photometrical signal read out
Detection Spectrum	Detection and identification of all beverage spoiling microorganisms	All relevant beer spoiling microorganisms, identification of all relevant beverage spoiling microorganisms possible	*Lactobacillus sp.* and *Alicyclobacillus*	All relevant beverage spoiling microorganisms, identification of all relevant beverage spoiling microorganisms possible
Sample Preparation	Selective pre-enrichment	Enrichment and lysis of bacteria, if necessary pre-enrichment	Selective pre-enrichment	Enrichment and lysis of bacteria, if necessary pre-enrichment
Time	3 to 7 days	3 hours to 2 days	2 days	2 hours to 2 days
Est. Cost per Test	1€	10€	15€	3€
Detection Limit (cfu)	1	$1\text{-}5 \times 10^3$	1×10^3	$1\text{-}5 \times 10^3$
Devices	None	PCR cycler	Fluorescence microscope	Microplate reader
Advantage	High sensitivity, relatively cheap	High sensitivity, quantitative analysis	Simple detection technology set up, detects only living cells (RNA)	Rapid and sensitive, qualitative and quantitative detection of living cells, cost efficient analysis
Disadvantage	Time consuming, no detection of unculturable microbes, labor expensive	Expensive devices needed, no discrimination between live and dead cells, not officially accepted	Time consuming, low sample throughput, expensive, not automatable, difficult data analysis, not officially accepted	No differentiation of serotypes or subspecies, limited probe design (rRNA target), not officially accepted

Table 2: Comparison of different technologies for detection of beverage spoiling bacteria.

Note: $1 = 0.86€

Test Format

The test method is designed on a simple 96-well microplate with 12 strips of 8 wells and uses sandwich hybridization. The test is not sensitive to sample matrix and detects only living cells. No special expensive equipment is needed and the test is done in approximately 2 to 2.5 hours. A positive result is visible to the naked eye, but can also be read by a standard microplate reader to quantify the number of cells at 450 nm.

Principle

The HybriScan method is based on the detection of rRNA via hybridization events and specific capture and detection probes. Sandwich hybridization is very sensitive, detecting attomoles of the respective target rRNA molecules. The ideal hybridization target for bacteria and yeast is rRNA. These cells contain a large number of rRNA-containing ribosomes; a single cell therefore contains several thousand copies of rRNA but only one DNA. Sandwich hybridization also provides sensitivity in crude biological samples because it is not susceptible to matrix interference. Specificity is achieved by targeting conserved or unique rRNA sequences. A biotin-labeled capture probe is used to immobilize the target sequence on a solid support plate (streptavidin-coated microtiter plate). A digoxigenin-labeled detection probe provides an enzyme-linked optical signal readout. Detection results from application of anti-DIG-horseradish peroxidase Fab fragments. The bound complex is visualized by horseradish peroxidase substrate TMB (3,3',5,5'-tetramethylbenzidine). Photometric data are measured at 450 nm and compared with standard solutions.

Cultural Method

Another possibility is classical media. The enrichment of the organisms can take a long time as some of them grow very slowly because of the low pH, and some anaerobes can have an especially slow growth.Typically, samples are incubated at 30-35 °C in a closed container. If thermophilic spoilage is expected, duplicates may also be incubated at 55 °C. The incubation time depends to some extent on the nature of the product, but is likely to be within a range of 4-15 days.

After the enrichment step, the samples are plated out on common agars like potato dextrose agar, orange serum agar and MRS agar. Duplicate plates should be incubated under aerobic and anaerobic conditions for 48-72 hours and then examined for visible colonies. A microbiologically stable product should normally contain less than 100 CFU/ml after pre-incubation.

Further examination is done under a microscope and further identification media and tests may be done.

Medium	Cat. No.
Malt Extract Agar	70145
Malt Extract Broth	70146
MRS Agar	69964
MRS Agar, original acc. DeMan-Rogosa-Sharpe	30912
MRS Agar, Vegitone	41782
MRS Broth	69966
MRS Broth modified, Vegitone	38944
Orange-serum Agar (OSA)	75405
Potato Glucose Agar (PDA)	70139
Potato Glucose Agar (PDA) ready prepared in flasks	51684
Sabouraud 2% Glucose Agar (SDA)	84086
Sabouraud 4% Glucose Agar (SDA)	84088
Sabouraud 4% Glucose Agar (SDA) ready prepared in flasks	55277
Sabouraud Glucose Broth (SDB)	S3306
Tomato Juice Broth (TJB)	17218
Yeast Malt Agar (YMA)	Y3127

Table 3: Common media for microbiological testing of beverages.

References:

1. Tribst, A.A., Sant'Ana Ade, S. and de Massaguer, P.R., Review: Microbiological Quality and Safety of Fruit Juices–Past, Present and Future Perspectives., Critical Reviews in Microbiology, Vol. 35, p. 310-339 (2009).
2. Lawlor, K., Schuman, J., Simpson, P. and Taormina, J., Compendium of the Microbiological Spoilage of Foods and Beverages, Food Microbiology and Safety, Sperber, W.H. and Doyle, M.P. (eds.) p. 245–283, Springer, New York (2009).

Winner of the Microbiology Photo Competition 2014

For the 5th microbiology photo competition, we received more images than ever before!

A diverse group of microbiologists gave their best to show us the world of microbiology through impressive and funny images. As you look through the photos and read through the descriptions, it quickly becomes obvious how broad the world of microbiology really is. It is clear that microorgansims are present everywhere – in food and beverage production and control, in the hospital and mainly in the environment. It is also clear that although a lot of research has been done in this area, we still have a lot of questions as to their nature.

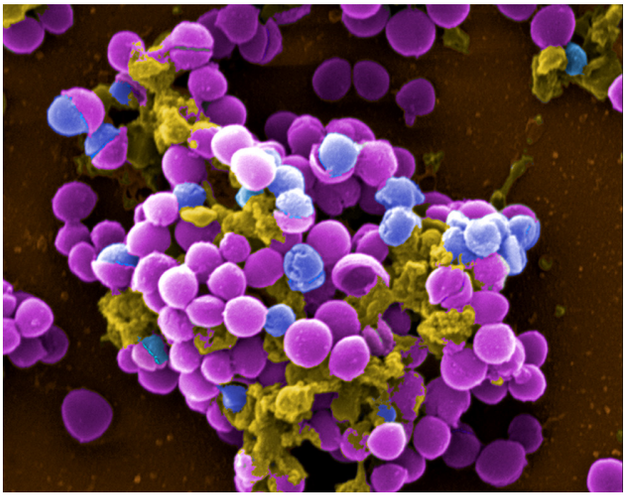
Although the winning photos are presented in this issue of Microbiology Focus, for the complete display of images, we recommend that you visit our website (**sigma-aldrich.com/microbiology**) where you'll find many more fascinating and creative images and their descriptions.

Sigma-Aldrich® would like to say thank you to all microbiologists who took part in our competition and also to our independent jury members – Professor, Dr. Lars Fieseler (ZHAW) and Professor, Dr. Mohammad Manafi (Medical University of Vienna). The aim was to encourage microbiologists to promote their work, and the entries were supposed to illustrate any microorganisms (living or dead) or a microbiologist in action at work.

The winning image will also have the honor of being on the front cover of a future Microbiology Focus in 2015.

1st Place: Jose Ramos Vivas (Hospital Universitario Marqués de Valdecilla & Instituto de Formación e Investigación Marqués de Valdecilla)

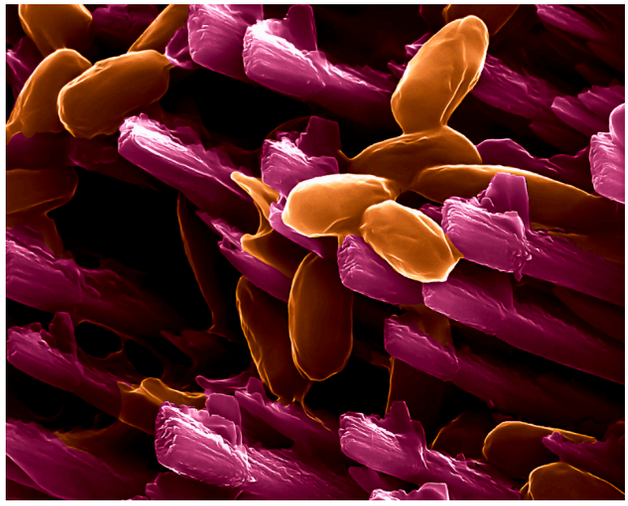
Title: Killing S. *aureus* biofilms



Description: Biofilms are 3D communities of microbial cells held in association and firmly attached to surfaces via an extracellular polymeric matrix. Growth in biofilm enables bacterial populations to survive better in hostile environments and during host infections (i.e., in the presence of antibiotics) increasing the probability of causing infections. The pseudocolored scanning electron micrograph shows a *Staphylococcus aureus* biofilm disrupted by an antibiotic, showing cell-shape deformation and cell wall breakdown. Bacterial cytoplasm is shown in blue, bacterial cell wall in purple and extracellular matrix in yellow. Magnification × 30.000.

2nd Place: Stefan Andrei (Babes-Bolyai University)

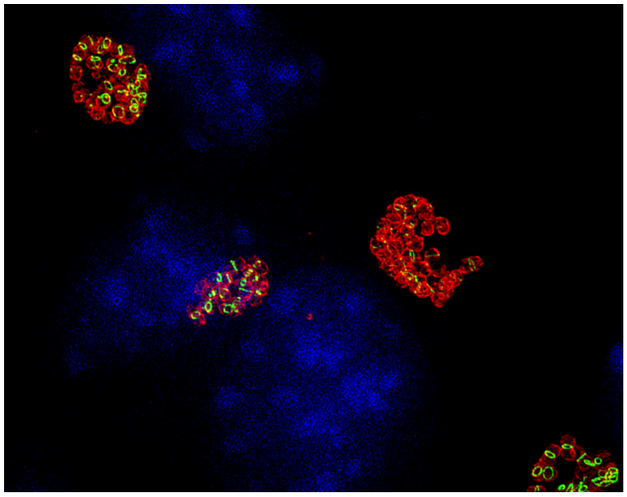
Title: Aragonite microcrystals formation induced by ureolytic bacteria



Description: Artificially colored SEM image of ureolytically-driven aragonite microcrystals formation (purple) in the presence of *B. pumiluls cells* (orange)

3rd Place: Kuru Erkin (Indiana University)

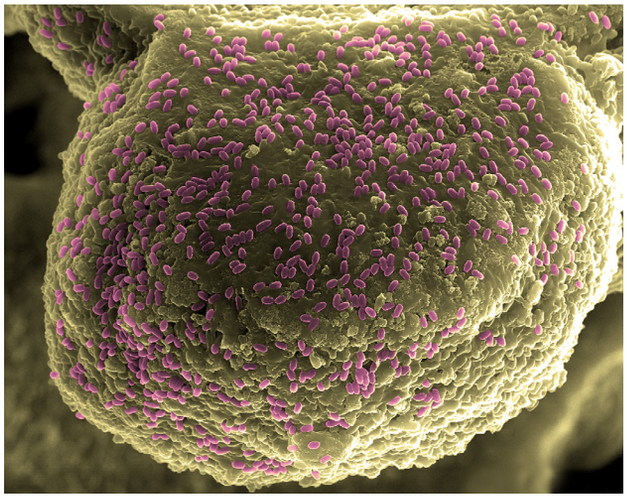
Title: The Chlamydial Anomaly Resolved



Description: New metabolic labels for bacterial peptidoglycan (PG) cell walls for the first time revealed that the sexually transmitted pathogen *Chlamydia trachomatis* makes a limited, but essential peptidoglycan cell wall. Unlike other bacteria, the chlamydial PG appears to be localized exclusively to the mid-cell of actively growing chlamydial cells suggesting a novel role of PG for supporting the growth and division of these bacteria. The Structured Illumination Microscopy (OMX, Delta Vision) image shows the L2 cells infected for 18 h with *C. trachomatis i*n the presence of the new bio-orthogonal PG probe and stained for PG (green), chlamydial outer membranes (red) and eukaryotic nucleus (blue).

4th Place: Pausan Manuela-Raluca (Babes-Bolyai University)

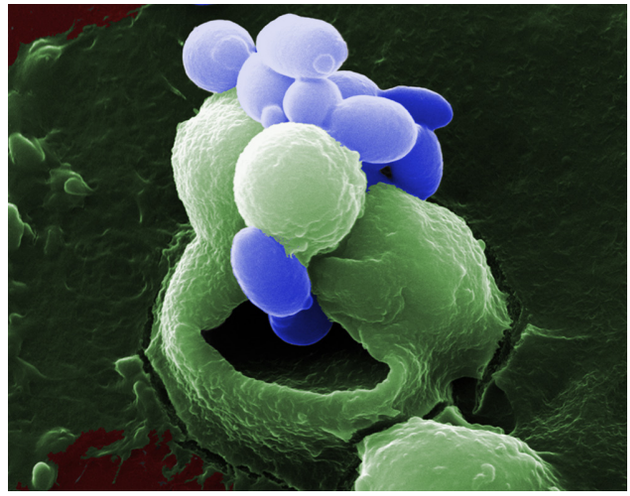
Title: Biological synthesis of calcium carbonate



Description: The picture represents a false colored SEM image of *Bacillus pumilus* attached to the surface of biogenic calcite. *Bacillus pumilus* is one of the many bacteria capable of producing calcite (calcium carbonate).

5th Place: Jose Ramos Vivas (Hospital Universitario Marqués de Valdecilla & Instituto de Formación e Investigación Marqués de Valdecilla)

Title: Eating those yeasts



Description: Fungal adhesion to the epithelium is a prerequisite for colonization, and some fungal infections are characterized by the invasion of epithelial cells. The pseudocolored micrograph shows a human epithelial cell (green) being invaded by some yeast (blue). Some yeast have already entered the host cell through a great mouth or hole formed in the cell. Scanning electron microscopy, Magnification × 10.000.

Biologics Safety Testing Services

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Genetic identification of microbial organisms using sequence analysis of the ribosomal RNA gene (rDNA)

BioReliance, a Sigma-Aldrich® company, offers a genotypic identification method for eubacteria, yeasts and filamentous fungi which involves sequencing the highly conserved and universally functional gene that encodes a portion of the ribosome (rDNA). This genotypic method offers improved accuracy, precision and faster turnaround to result compared to classic methods that rely on morphology, biochemistry, phenotype and culture requirements.

Bacterial identification targets the 16S rDNA, which is comprised of nine hypervariable regions interspersed among nine conserved regions. Sequence analysis targets approximately 500 base pairs of the 16S rDNA gene which incorporates three of the hypervariable regions, providing a high level of discrimination that is sufficient to make a successful identification for most bacterial organisms. Fungal and yeast identification target the D2 region of the nuclear large-subunit rDNA that offers ample variation to identify most yeast and fungi to the species level.

Comparison of the test sequence against the fully validated MicroSEQ® microbial libraries, containing in excess of 2,000 microbial sequences, ensures a reliable identification from a range of key genera present within the library, some of which include *Bacillus, Brevibacillus, Chryseobacterium, Coryneforms, Paenibacillus, Pseudomonas* and *Staphylococcus*.

Regulatory bodies including the Food and Drug Administration (FDA) recommend the use of DNA based identification methods for bacteria, such as sequence analysis of the 16S rDNA gene[1]. BioReliance offers this service as a GMP compliant and validated identification system (**Figure 1**).

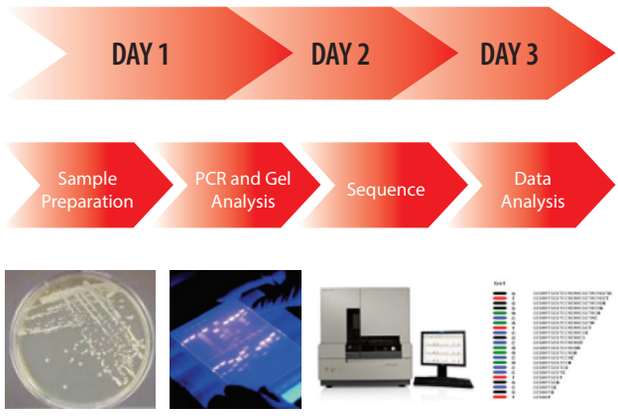

Figure 1: Workflow Process

Assay Method

For a successful identification, pure cultures are required. The assay initiates DNA isolation from the microbial cells by a crude lysis step. DNA is then collected and prepared for PCR. Amplification is performed using a standard primer set which targets the appropriate rDNA.

The PCR amplified product is analyzed by agarose gel electrophoresis. The successfully amplified products are sequenced using standard sequencing primers which target the amplified PCR product in the forward and reverse orientation.

The resulting DNA sequence is analyzed using MicroSEQ® ID Analysis Software and compared to the appropriate rDNA sequence library. Data quality is assessed in the Analysis QC report and the closest matches to the test sample are detailed in the Library Search Report. Raw data is also available in the form of an electropherogram (**Figure 2**).

Identification	Sample Specification
Microbial Cell Bank	**Pure microbial culture required.**
Microbial Isolate from Environmental Monitoring Microbial Isolate from Investigation	For organisms that produce hyphal structures and spores, it is preferred that samples are submitted with minimal spore formation where possible, e.g., hyphal mass cell pellet or freshly prepared sub-culture on solid media such as a nutrient agar plate. For organisms that produce homogenous colonies, it is preferred that samples are submitted on solid based media where possible, e.g., nutrient agar plate.

Table 1: Assay Appropriateness and Use: BioReliance has a dedicated Program Management Team which can further assist you if you require additional information or wish to discuss your requirements in more detail.

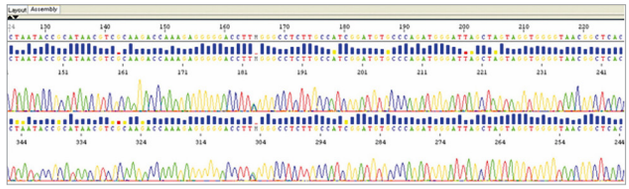

Figure 2: Electropherogram data for *E. coli* bacterial positive control.

Ordering Information

Assay No.	Assay Description	Regulatory Compliance	Sample Requirements
106701GMP.BUK	Genetic identification of bacterial microorganisms using the Fast MicroSEQ® 500 Protocol (3 day turn-around time)	GMP	Provide a pure bacterial culture in one of the following formats: 1 × agar plate/slant or 1 × cell pellet containing 5 × 107 or 1 × 1 mL broth containing 5 × 107 cells.
106708GMP.BUK	Genotypic identification of yeast and fungi using the Fast MicroSEQ® D2 LSU rDNA system (3 day turn-around time)	GMP	Provide a pure yeast or fungal culture in one of the following formats: 1 × agar plate/slant or 1 × cell pellet containing 5 × 107 or 1x1mL broth containing 5 × 107 cells or 1 × 1 cm^3 hyphal mass pellet.

For ordering please contact **info@bioreliance.com**

Reference:

1. FDA Guidance for Industry—Sterile Drug Products Produced by Aseptic Processing. US FDA, September 2004, Pharmaceutical cGMPs.

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