

# Instructions for Use

## BUFFERED CHARCOAL YEAST EXTRACT (BCYE) AGAR

<a href="#">Cat. no. G07</a>	BCYE Agar, 15x100mm Plate, 18ml	10 plates/bag
<a href="#">Cat. no. G08</a>	BCYE Selective Agar, 15x100mm Plate, 18ml	10 plates/bag
<a href="#">Cat. no. G108</a>	BCYE Agar without Cysteine, 15x100mm Plate, 18ml	10 plates/bag
<a href="#">Cat. no. G170</a>	BCYE Agar with CCVC, 15x100mm plate, 24ml	10 plates/bag
<a href="#">Cat. no. G209</a>	BCYE Agar with DGVP, 15x100mm Plate, 18ml	10 plates/bag
<a href="#">Cat. no. J61</a>	BCYE Agar / BCYE Agar without Cysteine, 15x100mm Biplate, 12ml/12ml	10 plates/bag
<a href="#">Cat. no. L24</a>	BCYE Agar Slant, 20x125mm tube, 10ml	20 tubes/box
<a href="#">Cat. no. W76</a>	BCYE Agar with GPVC without Cysteine, 15x100mm Plate, 28ml	10 plates/bag
<a href="#">Cat. no. W79</a>	BCYE Agar with VPC, 15x100mm Plate, 28ml	10 plates/bag
<a href="#">Cat. no. W169</a>	BCYE Agar with GPVC, 15x100mm Plate, 26ml	10 plates/bag

## INTENDED USE

Hardy Diagnostics Buffered Charcoal Yeast Extract (BCYE) Agar formulations are recommended for use in the cultivation and primary isolation of *Legionella* spp. in water and other samples suspected of harboring the bacteria. BCYE without Cysteine is for use in conjunction with BCYE Agar containing L-Cysteine for identification of *Legionella* spp. from clinical and environmental sources.

## SUMMARY

In 1977, MacDade et al. were the first to isolate the bacterium, *Legionella pneumophila*, responsible for causing Legionnaire's Disease. Following this discovery, there were numerous reports of *Legionella* being isolated from fresh water environments such as water distribution systems, evaporative condensers, air conditioning units, cooling towers, fountains, humidifiers, dentistry tools, shower heads, faucets and whirlpool spas or Jaccuzis®. To date, there are approximately 50 known species and 70 serogroups of *Legionella* that have been identified.

In 1978, Feeley et al. developed a medium to provide consistent isolation of *Legionella* species.<sup>(6)</sup> The medium demonstrated differential growth characteristics to aid in the identification of *Legionella* species. Feeley later modified the medium by substituting yeast extract for casein hydrolysate and beef extract, and replacing starch with activated charcoal. Feeley called this modified formula Charcoal Yeast Extract (CYE) Agar.<sup>(7)</sup> A further modification was made by Pasculle et al. in 1980.<sup>(8)</sup> This version employed the addition of ACES (N-2-acetamido-2-aminoethane sulfonic acid) buffer in order to maintain the proper pH for optimal growth. The new medium, designated BCYE for Buffered Charcoal Yeast Extract, could also be incubated aerobically. In 1981, Edelstein et al. increased the sensitivity of the medium by adding the potassium salt of alpha-ketoglutaric acid.<sup>(9)</sup> That same year, Wadowsky and Yee made the

medium more selective by suggesting that glycine, vancomycin and polymyxin be added, resulting in the formation of GVP medium.<sup>(10)</sup> Another modification in 1984 by Dennis et al. made the medium even more selective for *Legionella* by the addition of cycloheximide, resulting in GVPC medium.<sup>(11)</sup>

Hardy Diagnostics BCYE Agar (Cat. no. G07, G08, G209, W169, and G170) is based on Edelstein's formula and is formulated in accordance with ISO 11371:(1998)E.<sup>(15)</sup> This formulation employs the use of L-Cysteine, soluble ferric pyrophosphate, and alpha-ketoglutarate to enhance the growth of *Legionella* species. Activated charcoal removes toxic metabolic products. Protein and other growth nutrients are supplied by yeast extract.

Colonies of the various species of *Legionella*. may or may not exhibit fluorescence on BCYE Agar when exposed to UV light (wavelength of 365nm). See Interpretation section below.

BCYE Selective Agar (Cat. no. G08) contains anisomycin, colistin, and vancomycin. Anisomycin inhibits the growth of fungi. Gram-negative organisms are inhibited by colistin. Gram-positive organisms are inhibited by vancomycin.

It has been shown that *Legionella* spp. require the addition of L-cysteine for growth. BCYE Agar without Cysteine (Cat. nos. G108 and W76) can be used in conjunction with traditional BCYE medium. Organisms that fail to grow on BCYE without Cysteine but grow on traditional BCYE Agar can be presumptively identified as *Legionella* spp.

BCYE Agar with DGVP (Cat. no. G209) contains the dyes bromcresol purple and bromothymol blue, along with glycine, vancomycin and polymyxin. Antibiotics in the medium improve the recovery of *Legionella* species by inhibiting the growth of contaminating microorganisms, while dyes in the medium facilitate the differentiation and identification of *Legionella*.

BCYE Agar with GPVC (Cat. no. W169) contains glycine, vancomycin, polymyxin and cycloheximide for the greater inhibition of secondary microflora and further selective isolation of *Legionella* spp.

BCYE Agar with CCVC (Cat. no. G170) contains cephalothin, colistin, vancomycin, and cycloheximide. This is based on the formulation of Bopp et al.<sup>(14)</sup>, where they obtained the improved recovery of *L. pneumophila* with this selective media with an acid wash treatment to reduce the contaminating microbial flora present in environmental water samples.

## FORMULA

Ingredients per liter of deionized water:\*

BCYE Agar (Cat. no. G07, and L24):	
Yeast Extract	10.0gm
ACES Buffer	10.0gm
Activated Charcoal	2.0gm
Potassium Hydroxide	2.8gm
Alpha-Ketoglutarate	1.0gm
L-Cysteine	0.4gm
Ferric Pyrophosphate	0.25gm
Agar	12.0gm

BCYE without Cysteine (Cat. no. G108, J61 section II, W76) is made using the above formulation, without the addition of L-cysteine.

In addition, BCYE Selective Agar (Cat. no. G08) contains:

Anisomycin	80.0mg
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Colistin	10.0mg
Vancomycin	0.5mg

In addition, BCYE Agar with DGPV (Cat. no. G209) contains:

Bromcresol Purple	5.0ml
Bromothymol Blue	10.0ml
Glycine	3.0gm
Polymyxin	3.0ml
Vancomycin	1.0mg

In addition, BCYE Agar with GPVC (Cat. no. W169, W76\*, W79\*\*) contains:

Glycine	3.0gm
Polymyxin	80,000iu
Cycloheximide	80.0mg
Vancomycin	1.0mg

\* BCYE Agar with GPVC (Cat. no. W76) does not contain L-cystiene

\*\*BCYE with VPC (Cat. no. W79) does not contain Glycine

In addition, BCYE Agar with CCVC (Cat. no. G170) contains:

Cycloheximide	40.0mg
Colistin Solution	3.0ml
Cephalothin	4.0mg
Vancomycin	1.0mg

Final pH 6.8 +/- 0.2 at 25°C.

\* Adjusted and/or supplemented as required to meet performance criteria.

## STORAGE AND SHELF LIFE

Storage: Upon receipt store at 2-8°C. away from direct light. Media should not be used if there are any signs of deterioration (shrinking, cracking, or discoloration), contamination, or if the expiration date has passed. Product is light and temperature sensitive; protect from light, excessive heat, moisture, and freezing.

The expiration date on the product label applies to the product in its intact packaging when stored as directed. The product may be used and tested up to the expiration date on the product label and incubated for the recommended incubation times as stated below.

Refer to the document "[Storage](#)" for more information.

## PRECAUTIONS

This product may contain components of animal origin. Certified knowledge of the origin and/or sanitary state of the animals does not guarantee the absence of transmissible pathogenic agents. Therefore, it is recommended that these products be treated as potentially infectious, and handle observing the usual Universal Precautions for blood. Do not ingest, inhale, or allow to come into contact with skin.

This product is for *in vitro* diagnostic use only. It is to be used only by adequately trained and qualified laboratory personnel. Observe approved biohazard precautions and aseptic techniques. All laboratory specimens should be considered infectious and handled according to "standard precautions." Refer to the document "[Guidelines for Isolation Precautions](#)" from the Centers for Disease Control and Prevention.

For additional information regarding specific precautions for the prevention of the transmission of all infectious agents from laboratory instruments and materials, and for recommendations for the management of exposure to infectious disease, refer to CLSI document M29: *Protection of Laboratory Workers from Occupationally Acquired Infections*.

Sterilize all biohazard waste before disposal.

Refer to the document "[Precautions When Using Media](#)" for more information.

## PROCEDURE

**Specimen Collection:** Infectious material should be submitted directly to the laboratory without delay and protected from excessive heat and cold. If there is to be a delay in processing, the specimen should be inoculated onto an appropriate transport media and refrigerated until inoculation. Consult listed references for information on specimen collection.<sup>(1-5)</sup>

The media should be brought to room temperature prior to use and inoculated to produce isolated colonies. Incubate aerobically in a humidified atmosphere at 35°C. Incubate for a minimum of four days and examine daily for growth. Media may be reincubated for up to seven days.

### **Method of Use (Environmental Samples):**

1. Obtain a water sample under aseptic conditions and prepare the sample according to the protocol of the standard being applied. Analysis of the sample can be accomplished either by direct seeding or filtration, usually followed by concentration, heat and/or acid treatment, then plating.
2. In direct seeding, spread 0.1 to 0.5ml of the water sample or dilution, using a sterile spreading device, across the agar surface. If using a membrane filter, pour the water sample through a 0.22µm polycarbonate membrane filter, place the membrane in a sterile flask containing 5ml of the initial water sample and centrifuge or sonicate the sample to concentrate. Proceed with the appropriate protocol, as required. If plating the filter directly, place the membrane, filtered side up, on the agar surface.
3. Incubate plates at 35 +/- 2°C. for a minimum of 3 days. Growth is typically visible after 3-4 days, but may take up to 2 weeks.
4. Note the number of each colony type and select at least three distinct colonies of *Legionella* from each plate.
5. Re-streak each colony onto a separate plate of BCYE without Cysteine (Cat. no. G108) and incubate using the same parameters as above.
6. Consider as positive for *Legionella* all colonies that develop on BCYE Agar, but present no growth on BCYE without Cysteine.
7. Proceed with immunofluorescence or serological tests for further identification.

## INTERPRETATION OF RESULTS

In general, colonies of *Legionella* spp. present a white to gray coloration. They may also have blue, pink, purple, maroon, greenish-yellow or dark red pigmentation that fades, becoming whiter and more filamentous with age. The colony surface is generally smooth with precise edges, but some strains may give a ground glass or "fried egg" appearance when viewed microscopically. Some species fluoresce under UV light.

On BCYE and BCYE Selective Agars, colonies of *Legionella pneumophila* appear white-gray to blue-gray and fluoresce yellow-green under long-wave UV light. Colonies of *Legionella bozemanii* appear white-gray to blue-gray and fluoresce blue-white under long-wave UV light. Colonies of *Legionella micdadei* do not fluoresce under long-wave UV light.

On BCYE with DGVP, all strains of *L. pneumophila* produce round, shiny and white colored colonies with a barely discernable green hue at 3 days incubation. Colonies will be larger by 5 to 7 days incubation, becoming more distinctly green, flat, dull, and opaque in appearance. *Tatlockia micdadei* (syn. *Legionella micdadei*) yields shiny, round, blue-gray colonies. Colonies become larger and more intensely blue in color during prolonged incubation. Colonies of *Fluoribacter* spp. appear round, shiny and distinctly green in appearance after 3 days incubation; the green coloration will deepen with continued incubation and colonies will remain shiny. Colonies of *L. bozemanii* will exhibit a bright pastel green coloration that will intensify over prolonged incubation; colonies will fluoresce blue-white under long-wave UV light.

Growth of most other flora should be inhibited or greatly reduced on BCYE Selective Agar, BCYE Agar with DGVP, BCYE Selective Agar with GPVC, and BCYE Selective Agar with CCVC.

Use a 365nm wavelength handheld UV Lamp ([Cat. no. UVL56](#) or [LSS3](#)) to detect colony fluorescence. These handheld lamps require that the room lights be turned off, since ambient light will interfere with fluorescence detection. Alternatively, a dark viewing box ([Cat. no. CM10A](#)) with its companion UV lamp ([Cat. no. EA160](#)) may be used so that the room lights will not need to be turned off.

**CAUTION:** Not all UV wavelengths are capable of producing sufficient fluorescence effects. It is important to use a UV light with a wavelength at or near 365nm, one with higher power (in watts, not lumens), and one that is high efficiency. Use of UV lights not meeting these criteria will fail to produce sufficient fluorescence. Most inexpensive battery operated LED UV lights produce light at multiple wavelengths, use less watts, and/or low power, and are thus **not acceptable** and will produce erroneous results. [Cat. no. LSS3](#) is an exception and has been verified to work well. Please do not use cheaper versions.

### Tips for using fluorescence

1. Use a 366nm or 365nm handheld UV lamp ([Cat. no. UVL56](#)) or ([Cat. no. LSS3](#)) to detect colony fluorescence. See 'CAUTION' above regarding inexpensive UV lights. Alternatively, a dark viewing box with its compatible UV lamp may be used as described above. Viewing must be done in the dark.
2. Hold the lamp directly over isolated colonies on the plate, approximately 3 to 4 inches (7 to 10cm) away.
3. Some species of *Legionella* isolated colonies will fluoresce yellow-green or blue-white glow. See Interpretation section above.
4. Only well isolated colonies will fluoresce. Colonies in areas of confluent growth will not.
5. Fluorescence will fade over time.

## LIMITATIONS

It is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on colonies from pure culture for complete identification of bacteria and/or fungi.

It is also recommended that more than one type of medium be used for isolating *Legionella* spp. and that non-selective and selective BCYE Agar plates be inoculated in parallel.

The Centers for Disease Control and Prevention (CDC) recommend incubation of environmental samples with 2.5%

CO<sub>2</sub>; however, *L. gormanii* is the only known species with enhanced growth under this condition.<sup>(12,13)</sup>

This medium is to be used for the isolation and presumptive identification of *Legionella*.

Colonies of *Legionella* that develop on white membrane filters may have a different appearance to those that develop against a black or dark background filter.

Fluorescence must be read in a darkened environment with a 365nm wavelength UV lamp of adequate power (see “Tips for Using Fluorescence” above).

When handling *Legionella* spp., it is important to avoid aerosol formation. Thoroughly clean and disinfect all work areas.

Refer to the document "[Limitations of Procedures and Warranty](#)" for more information.

## MATERIALS REQUIRED BUT NOT PROVIDED

Standard microbiological supplies and equipment such as loops, other culture media, swabs, UV lamps, applicator sticks, incinerators, handheld UV lamp ([Cat. no. UVL56](#) or [LSS3](#)) or dark viewing box ([Cat. no. CM10A](#)) with compatible UV lamp ([Cat. no. EA160](#)), and incubators, etc., as well as serological and biochemical reagents, are not provided.

## QUALITY CONTROL

Hardy Diagnostics tests each lot of commercially manufactured media using appropriate quality control microorganisms and quality specifications as outlined on the Certificate of Analysis (CofA) and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media*. The following microorganisms are routinely used for testing at Hardy Diagnostics:

Test Organisms	Inoculation Method*	Incubation			Results
		Time	Temperature	Atmosphere	
<b>BCYE Agar (Cat. no. G07, J61 (Section I), and L24):</b>					
<i>Legionella pneumophila</i> ATCC® 33152**	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces yellow-green under long-wave UV light
<i>Legionella pneumophila</i> clinical strain	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces yellow-green under long-wave UV light
<i>Legionella bozemanii</i> ATCC® 33217	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies; fluoresces blue-white under long-wave UV light
<i>Legionella micdadei</i> ATCC® 33204	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray, no fluorescence under long-wave UV light
<b>BCYE Selective Agar (Cat. no. G08):</b>					
<i>Legionella pneumophila</i> ATCC® 33152**	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces yellow-green under long-

					wave UV light
<i>Legionella pneumophila</i> clinical strain	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces yellow-green under long-wave UV light
<i>Legionella bozemanii</i> ATCC® 33217	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies; fluoresces blue-white under long-wave UV light
<i>Legionella micdadei</i> ATCC® 33204	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, no fluorescence under long-wave UV light
<i>Escherichia coli</i> ATCC® 25922**	B	24hr	35°C	Aerobic	Inhibited
<i>Staphylococcus aureus</i> ATCC® 25923	B	24hr	35°C	Aerobic	Inhibited
<i>Candida albicans</i> ATCC® 10231	B	72hr	35°C	Aerobic	Inhibited
<b>BCYE with VPC (Cat. no. W79):</b>					
<i>Legionella pneumophila</i> ATCC® 33152**	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces yellow-green under long-wave UV light
<i>Legionella pneumophila</i> clinical strain	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces yellow-green under long-wave UV light
<i>Legionella bozemanii</i> ATCC® 33217	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies; fluoresces blue-white under long-wave UV light
<i>Legionella micdadei</i> ATCC® 33204	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, no fluorescence under long-wave UV light
<i>Escherichia coli</i> ATCC® 25922**	B	24hr	35°C	Aerobic	Inhibited
<i>Staphylococcus aureus</i> ATCC® 25923	B	24hr	35°C	Aerobic	Inhibited
<b>BCYE without Cysteine (Cat. no. J61 (Section II) and G108) and BCYE with GPVC without Cysteine (Cat. no. W76):</b>					
<i>Campylobacter jejuni</i> ATCC® 33291**	A	24-72hr	35°C	Microaerophilic	Growth
<i>Legionella pneumophila</i> ATCC® 33152**	A	48-72hr	35°C	Aerobic	Inhibited
<b>BCYE Agar with DGVP (Cat. no. G209):</b>					



<i>Legionella pneumophila</i> ATCC® 33152**	A	48-120hr	35°C	Aerobic	Growth; pale green colonies, fluoresces yellow-green under long-wave UV light
<i>Legionella pneumophila</i> clinical strain	A	48-120hr	35°C	Aerobic	Growth; pale green colonies, fluoresces yellow-green under long-wave UV light
<i>Legionella bozemanii</i> ATCC® 33217	A	48-120hr	35°C	Aerobic	Growth; bright green colonies; fluoresces blue-white under long-wave UV light
<i>Legionella micdadei</i> ATCC® 33204	A	48-120hr	35°C	Aerobic	Growth; blue-gray colonies, no fluorescence under long-wave UV light
<i>Escherichia coli</i> ATCC® 25922**	B	24hr	35°C	Aerobic	Partial to complete inhibition
<i>Staphylococcus aureus</i> ATCC® 25923	B	24hr	35°C	Aerobic	Partial to complete inhibition
<b>BCYE Agar with GPVC (Cat. no. W169):</b>					
<i>Legionella pneumophila</i> ATCC® 33152**	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces pale green under long-wave UV light
<i>Legionella pneumophila</i> clinical strain	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces pale green under long-wave UV light
<i>Legionella bozemanii</i> ATCC® 33217	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces pale green under long-wave UV light
<i>Legionella micdadei</i> ATCC® 33204	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, no fluorescence under long-wave UV light
<i>Escherichia coli</i> ATCC® 25922**	B	24hr	35°C	Aerobic	Partial to complete inhibition
<i>Staphylococcus aureus</i> ATCC® 25923	B	24hr	35°C	Aerobic	Partial to complete inhibition
<i>Aspergillus brasiliensis</i> ATCC® 16404	G	24-48hr	35°C	Aerobic	Partial to complete inhibition
<b>BCYE Agar with CCVC (Cat. no. G170):</b>					
<i>Legionella pneumophila</i> ATCC® 33152**	A	48-96hr	35°C	Aerobic	Growth; white-gray to blue-gray colonies, fluoresces pale green under long-wave UV light
					Growth; white-gray to blue-



<i>Legionella pneumophila</i> clinical strain	A	48-96hr	35°C	Aerobic	gray colonies, fluoresces pale green under long-wave UV light
<i>Escherichia coli</i> ATCC® 25922**	B	24hr	35°C	Aerobic	Partial to complete inhibition
<i>Staphylococcus aureus</i> ATCC® 25923	B	24hr	35°C	Aerobic	Partial to complete inhibition
<i>Aspergillus brasiliensis</i> ATCC® 16404	G	24-48hr	35°C	Aerobic	Partial to complete inhibition

\* Refer to the document "[Inoculation Procedures for Media QC](#)" for more information.

\*\* Recommended QC strains for User Quality Control according to the CLSI document M22 when applicable.

## USER QUALITY CONTROL

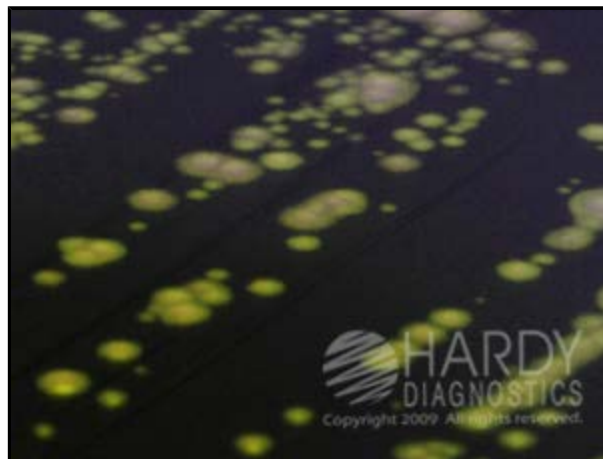
End users of commercially prepared culture media should perform QC testing in accordance with applicable government regulatory agencies, and in compliance with accreditation requirements. Hardy Diagnostics recommends end users check for signs of contamination and deterioration and, if dictated by laboratory quality control procedures or regulation, perform quality control testing to demonstrate growth or a positive reaction and to demonstrate inhibition or a negative reaction, if applicable. Hardy Diagnostics quality control testing is documented on the certificate of analysis (CoFA) available from Hardy Diagnostics [Certificate of Analysis](#) website. Also refer to the document "[Finished Product Quality Control Procedures](#)," and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media* for more information on the appropriate QC procedures. See the references below.

## PHYSICAL APPEARANCE

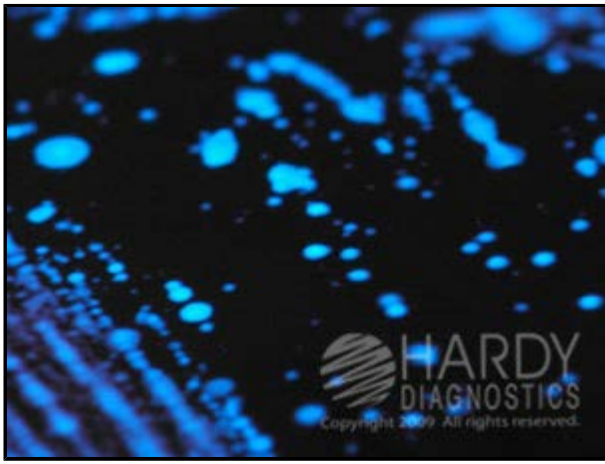
All formulations of BCYE Agar listed should appear opaque, and black in color.



*Legionella pneumophila* (ATCC® 33152) colonies growing on BCYE Selective Agar (Cat no. G08). Incubated aerobically for 48 hours at 35°C.



*Legionella pneumophila* (ATCC® 33152) colonies growing on BCYE Selective Agar (Cat. no. G08) under UV light. Incubated aerobically for 72 hours at 35°C.



*Legionella bozemanii* (ATCC® 33217) colonies growing on BCYE Selective Agar (Cat. no. G08) under UV light. Incubated aerobically for 48 hours at 35°C.

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