BBL CRYSTAL ANAEROBE IDENTIFICATION PROCEDURE

I. Principle

While BBL CRYSTAL ANR ID aids in the identification of anaerobes, it should be recognized that minor variations may exist in strains within species. The use of this system requires a competent microbiologist to take into consideration the source of the specimen, aerotolerance, specific cell morphology, colonial characteristics, and fluorescing qualities on various media. Further testing should be performed when warranted. Refer to Table 1 for the list of organisms included in the database.

The BBL Crystal Anaerobe ID panels contain 29 dried biochemical and enzymatic substrates. A bacterial suspension in the inoculum fluid is used for rehydration of the substrates. The tests used in the system are based on microbial utilization and degradation of specific substrates detected by various indicator systems. Enzymatic hydrolysis of fluorogenic substrates containing coumarin derivatives of 4-methylumbelliferone (4MU) or 7-amino-4-methylcoumarin (7-AMC), results in increased fluorescence that is easily detected visually with a UV light source. Chromogenic substrates produce color changes upon hydrolysis that can be detected visually. In addition, there are tests that detect the ability of an organism to hydrolyze, degrade, reduce, or otherwise utilize a substrate in the panel.

Refer to Table 2 for principles of tests employed in the BBL Crystal ANR ID system.

II. Materials

A. Kit contains 20 panels:

1. BBL CRYSTAL Anaerobe Panel Lids
2. BBL CRYSTAL Panel Bases
3. BBL CRYSTAL ANR Inoculum Fluid Tubes. *Each tube has approximately 2.3 mL of Inoculum Fluid containing: KCl 7.5 g, $CaCl_2$ 0.5 g, Tricine N-(2- Hydroxy-1, 1-bis hydroxymethyl) glycine 0.895 g, purified water to 1000 mL.*
4. 2 Incubation trays
5. BBL CRYSTAL ANR ID report pad

B. Other supplies include:

1. Sterile cotton swabs (do not use polyester swabs)
2. 35-37°C non-CO_2 incubator
3. McFarland No. 4 and No. 5 standards
4. BBL Panel Viewer
5. BBL CRYSTAL ANR electronic codebook *(Gram stain bench, Mycology and Blood Bench Computers)*
6. Indole reagent
7. Catalase *(Recommended: 15% H_2O_2 with 1.0% Tween 80 added)*

III. Procedure

A. Inoculation

1. Anaerobic organism must be a pure isolate, following 24-48 h of incubation (Actinomyces can be tested following 72-96 h, if necessary). *One whole plate of pure organism usually will be enough.*
2. Perform Gram stain, indole, and catalase tests. Record results on ANR ID Report form.
3. Remove lids with the wells from pouch, *these covered lids should be used within 1 h after removal from the pouch.*
4. Prepare a McFarland No. 4 standard (not to exceed a No.5*)* by suspending colonies in the provided inoculum tube.
5. Vortex for approximately 10-15 sec.
6. Pour entire contents of inoculum fluid into target area of the black base.
7. Distribute inoculum into each well by holding base in both hands and gently rolling inoculum along the tracks until all of the wells are filled.
8. Roll back any excess fluid to the target area. Make sure there is no excess fluid between the wells to prevent leaching of chemicals between wells.
9. Place base on bench top and align the lid so that the labeled end is on top of the target area of the base.
10. Snap lid shut by placing thumbs on both sides and pressing downward. Listen for two "clicks". Label with accession number and time.
11. Prepare a purity plate from inoculum tube. Incubate the plate anaerobically at 35-37°C for 24-48 h.

B. Incubation

1. Incubate panel **face down** *(larger window facing up; label facing down)* in a 35-37°C non-CO_2 incubator with 40-60% humidity for **4 h**. If there is a problem with making an acceptable identification at 4 h, continue incubating the panel overnight to intensify the fluorescence reactions. Note: the incubator door should not be opened repeatedly during the incubation period (preferably less than 3 times).
2. An incubation tray may be used if multiple panels are set up at the same time. Place panels face down in tray; 5 rows of 2 panels will fill one tray. One tray may be stacked upon the other, *but no more than two panels high during incubation.*

IV. Interpretation

A. Reading Panel

1. Place the panel face down on the "ledge-space "provided by the Panel Viewer. The 4A FCT well should be in the upper left-hand corner.
2. Turn the light on (side of viewer).
3. Refer to the color reaction chart (Table 3) or abbreviated color chart.
4. Read columns G through J first, using the white light source. Record results on the report pad. Note: H through J wells are negative if colorless

or very pale yellow. If more than 2 wells are borderline positive, consider resetting panel with fresh inoculum using a slightly heavier inoculum.

5. Lift ledge up in order to use fluorescent light. Read column A through F next. Well 4A is the fluorescent negative control (FCT). It will fluoresce a very slight blue color. Positive wells **must exhibit greater fluorescence than the 4A FCT well** to be called positive. (4A will always be recorded as negative)
6. Record results on Crystal report pad.
7. Calculate the 10-digit profile number by scoring each Row (A-J) by adding the numbers vertically. Example: *If all wells were positive in row C (4+2+1), the profile number for that row is 7.*

B. Computerized Code Book (located on Gram stain, Fungus and Blood Culture terminals)

1. Select **BBL CRYSTAL ICON** from computer desktop.
2. User Name: **BBL Crystal**
3. Password: **BBL**
4. Select **Data Entry**.
5. Type in Accession # *(Patient ID and Name are optional)*. Enter X 3
6. Select panel type **(ANRCDC)** with mouse arrow. Left click mouse.
7. Enter biochemical reactions. (Click Keyboard Entry with mouse and enter profile number)
8. Enter gram stain, indole, catalase by selecting and clicking the appropriate box at the bottom of the screen..
9. Click "**ADD**" Click "ID"
10. Click "**Review**". Scroll to the correct accession number.
11. **Your answer will be in the middle of the screen**. You may click on the answer to see additional information about this organism. (Realize that not all organisms will "key out". This is called a NO CODE.)
12. If you wish to modify your result, click "**Modify**" and then change results. Click "**Modify**" again. Click "Review" and scroll to accession number and click appropriate entry. To close, Click "Close".
13. If you need to check on results from any accession number, click "Review".
14. If you would like to see the percentage chart, click "**Percentage**".
15. To close, click "Close".

V. Quality Control

A. Inoculate a panel with *Bacteroides fragilis* ATCC 25285 per procedure.
B. Prior to incubation, let the panel remain at room temperature for 1 min (not more than 2 min).
C. Read and record reactions with the aid of the viewer and color reaction chart.
D. If any of the wells, except 1F, are positive per the color reaction chart after 1-2 min, DO NOT USE PANELS from this lot. Contact Becton Dickinson Microbiology Systems Technical Services. (Note: Well 1F (Escosyl) should be positive upon rehydration.)

E. If all wells are negative, incubate the panel for 4 h at 35-37° C. Read panel with the panel viewer and record reactions on the report pad. Refer to Table 4 “Quality Control Chart” for acceptable well reactions for *Bacteroides fragilis* ATCC 25285.
F. Additional quality control strains for BBL CRYSTAL ANR ID System include the following organisms.
    1. *Bacteroides distasonis* ATCC 8503
    2. *Peptostreptococcus asaccharolyticus* ATCC 29743
    3. *Lactobacillus acidophilus* ATCC 314
    4. *Fusobacterium varium* ATCC 27725

VI. Limitations

A. The BBL CRYSTAL ANR ID System is designed for the taxa provided. Taxa other than those listed in Table 1 are not intended for use in this system. It is important to note that some organisms will “NO CODE”.
B. Since *Actinomyces spp.* often are slow growing, it is acceptable to incubate the culture 72-96 h before inoculating the BBL CRYSTAL. In the mean time, it is helpful to determine key characteristics of the organism. NOTE: If a gram-positive rod **is both branching and catalase negative**, the presumptive ID is: *Actinomyces israelii*, *Actinomyces spp.* not *viscosus*, or *Propionicum propionicus* (70% are catalase negative). If the system gives “no identification”, *A. israelii* can be differentiated from *Propionicum propionicus* by performing the following API20A sugars: Mannitol 99/50, Salicin 99/0, Xylose 99/0, Arabinose 97/5, Cel 90/0, Trehalose 90/30. *Actinomyces israelii* can be differentiated from *Actinomyces spp.* (not *viscosus*) by performing the following API20A sugars: Mannitol 99/26, Xylose 99/62, Arabinose 97/37, Trehalose 90/46, and molar tooth colony (+)/molar tooth colony (-).
C. Not all *Actinomyces* spp. branch or are catalase negative; for example, *A. pyogenes* is non-branching and catalase negative but beta-hemolytic. *Actinomyces viscosus* is branching, catalase positive, and can have a rough colony.
D. Only cotton tipped applicator swabs, wooden applicator sticks, or disposable plastic loops should be used to prepare the inoculum suspension since some polyester swabs may cause the inoculum fluid to become viscous. This may result in insufficient inoculum fluid to fill the wells.
E. Once the lids containing the biochemical wells are removed from the sealed pouches, they must be used within 1 h to ensure adequate performance. The plastic cover should remain on the lid until used.
F. If the test profile yields a “No identification” result and the culture purity has been confirmed, it is possible that the test isolate is producing atypical results or that the isolate is not included in the BBL CRYSTAL database. Resetting the panel may be necessary to avoid borderline results or reading errors. The inoculum must be pure, fresh, and between a 4 and 5 McFarland standard. Too light of an inoculum may result in borderline results.

VII. Validation of Method

A total of 14 ATCC strains, including anaerobic gram-negative rods, gram-negative cocci, and gram-positive rods, were tested with the BBL CRYSTAL ANR System. A total of 13 of the 14 (93%) gave the correct genus and species with a confidence level over 94%. One of the 14 (7%) gave the correct genus/species with a confidence level of 62% (*Clostridium sporogenes*).

A total of 48 anaerobic organisms, including the 14 ATCC strains noted above and 34 fresh clinical isolates, were tested using the Crystal system. A total of 39 of the 48 (81%) organisms were identified correctly to the genus level, with greater than 80% confidence. Overall, there were 7 (15%) no codes, and 2 (4%) isolates were incorrectly identified. For the anaerobic gram-negative rods, 10 of 12 (83%) isolates were correctly identified to the genus level, with 1 (8%) no code and 1 (8%) incorrectly identified. For the gram-positive rods, 26 of 32 (81%) were correctly identified to the genus level, with 4 (16%) no codes and 1 (3%) incorrectly identified. Some of the no codes would be expected to resolve following 24 hours of incubation, which the procedure now includes.

The Crystal anaerobe system was compared to the API 20A system. A total of 32 of 63 (51%) organisms were correctly identified to the genus level in the API 20A, with greater than 80% confidence. Overall, there were 18 (29%) no codes, and 13 (21%) isolates were incorrectly identified. For the anaerobic gram-negative rods, 12 of 18 (67%) isolates were correctly identified to the genus level, with 4 (22%) no code and 2 (11%) incorrectly identified. For the gram-positive rods, 16 of 40 (40%) were correctly identified to the genus level, with 13 (33%) no codes and 11 (28%) incorrectly identified.

VIII. References

1. API 20A Identification insert. July 1998.
2. BBL Crystal Identifications Systems insert. Revised: June 2001.
3. Engelkirk, P., J. D. Engelkirk, and V. R. Dowell. 1992. Clinical Anaerobic Microbiology. Star Publishing, Belmont, CA.
4. Santala, A. M., N. Sarkonen, V. Hall, P. Carlson, H.J. Somer, and E. Kononen. 2004. Evaluation of four commercial test systems for identification of *Actinomyces* and some closely related species. J. Clin. Microbiol. 42: 418-420.

Effective 05/07/2004
Reviewed by Microbiology Director, Dr. Ann Robinson: 05/07/2004
Reviewed by Medical Director, Dr. Joseph Schappert: 03/10/2010
Reviewed by Microbiology Supervisor, Jerry Claridge: 11/2005, 11/2006, 10/2007, 05/2008, 05/2009, 04/01/2011, 03/2013, Jason Ammons 05/2015
Updates and Revisions: