DROYCON BIOCONCEPTS INC
315 Dewdney Avenue, Regina, Saskatchewan, Canada S4N 0E7
(306) 585 1762
www.dbi.ca

Protocol

DBISOP06

IRB-BART

Testing Protocols for Iron Related bacteria

IRB-BART is made complex because of the numbers of reactions that can occur in what may be construed as a formless manner. This document attempts to provide a structured and logical breakdown of the various reaction sequences to enable better interpretation of the data and the establishment of a standard laboratory QM procedure. This document is therefore in two parts: (1) Interpretation of accepted and known reaction patterns; and (2) Establishment of a standard protocol for the quality management of the IRB-BART testers in a laboratory or field setting.

Definition of Iron Related Bacteria

Iron related bacteria is the name applied to that group of bacteria that, for various reasons, accumulate iron within the colloidal or colonial growths in a manner that significantly exceeds the immediate metabolic requirements for iron. Commonly this accumulation is in the form of crystallized forms of ferric iron in the form of oxides, hydroxides and sometimes carbonates. Generally this type of growth take on the color of the ferric accumulates and appears to be the color of rust. Hence the common name applied is iron bacteria to reflect the fact that these bacteria accumulate iron. The term “iron related bacteria” was developed to reflect in a more qualitative manner the various forms of bacteria that can be associated not just with the accumulation of ferric iron but also the dissolution of iron under more reductive conditions. The definition of iron related bacteria is therefore:

> Incorporates all bacteria that are able to accumulate iron in any form within the environmental matrix within which they function.

This brief definition encompasses a number of major bacterial groups that have been commonly recognized but not necessarily associated within a common grouping. These groups are described below separately and their role within the iron related bacterial group defined primarily in relation to the oxidation - reduction potential (ORP) of the environment within which they are active. Generally the different groups are active in specific parts of the ORP gradient and they are commonly attached to surfaces through the generation of biofilms, encrustations, nodules or some other from of growth.

Iron oxidising bacteria (ferrous oxidizing bacteria, FOB): is the name given to those bacteria that are able to accumulate ferric forms of oxide and hydroxide within the growing biomass. This accumulation appears commonly to be on-going until the Fe content reached between 40 and 95% of the dried weight. These events commonly occur at the redox front between oxidative and reductive conditions usually with ORP values of between +10 and -50 mV.

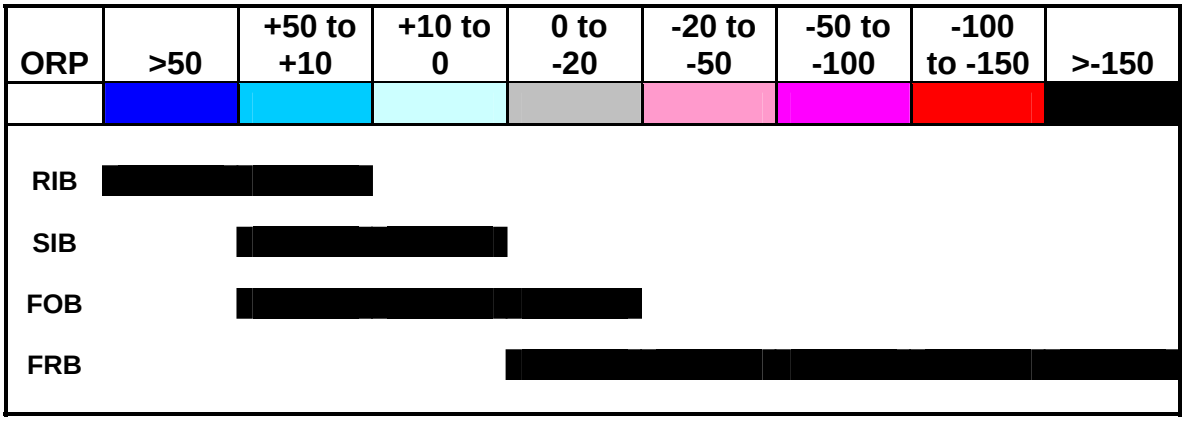
Iron reducing bacteria (ferric reducing bacteria, FRB): that group of bacteria which, over various reductive conditions, reduce ferric forms of iron to the more soluble ferrous that may then move by diffusive and biocolloidal process out of the immediate environment where they were created. Iron reducing bacteria are therefore most commonly found under more reductive where the ORP values are form 0 to -150mV.

Sheathed iron bacteria (SIB): these bacteria possess the ability to live for at least a part of their activity cycle with a slime-like sheath onto which ferric iron may, or may not, be accumulated. These sheaths usually are formed in the upper more oxidative part of the biomass and frequently the sheaths will extend out into the water flow. These bacteria are able to move into (for protection and growth) and out of (for colonization) the sheaths in accordance with the stage of the life cycle these bacteria are passing through. Generally the ORP values range from +5 to +150mV in order to get these sheathed bacteria to become a significant part of the biomass.

Ribbon iron bacteria (RIB): some bacteria have the ability to extrude slime-like ribbons out of the cell that are rich in ferric forms of iron. Usually only one ribbon is generated for each cell and it is thought that the energy release from the oxidation of ferrous to ferric forms of iron is a significant part of the total energy requirement. Generally these bacteria (dominated by the genus *Gallionella*) grow on the surface of the growing biomass with the ribbons extending outwards into the free water flow. It is thought that these ribbons act thought the Archimedean screw principles to move nutrients from the water phase down to the cell. When mature, these ribbons break off and move into the water flow where their presence is easy to observe microscopically. It should be remembered that the presence of these ribbons in a water sample does not mean that cells of *Gallionella* are present, it simply means that some *Gallionella* growths occurred upstream and that growth was shedding ribbons. Commonly species of *Gallionella* grow under oxidative conditions (>+50 mV) over very sharp redox fronts created within the biomass that allows ferrous iron to be come available to the cells. This ferrous iron could have originated in the water flowing over the biomass in the ferric form that was taken up

and reduced to the ferrous form or it could have been ferrous iron diffusing out from the deeper reductive parts of the biomass.

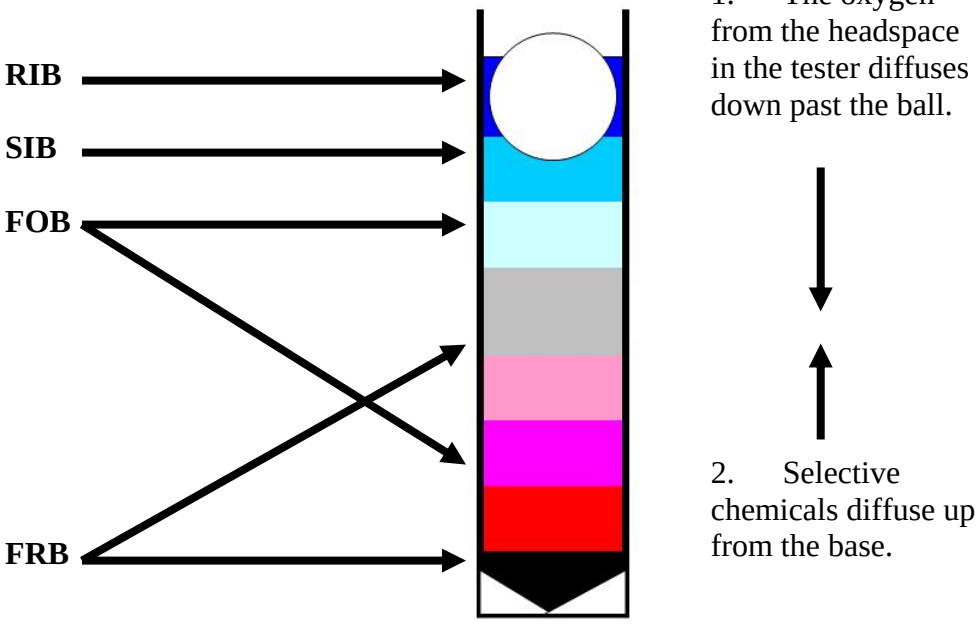
These observations can be summarized in the following ORP gradient chart: Here the likely occurrences of RIB, SIB, FOB and FRB are shown in relation to the ORP gradient from >+50 mV (blue) to < -150 (black). In the operation of an IRB-BART tester, it is normal for an ORP gradient to form if there is any microbial activity occurring within the tester since the intrinsic oxygen that would have been present in the sample or had entered the sample fluids during the dispensing of the test.



In the IRB-BART test, this generation of the ORP gradient causes a focussing of the bacterial community's activities in relationship to the types of bacteria present and active.

Note that the colors for the ORP are the same as rendered in the previous figure above. From this theoretical diagram, a number of statements can be drawn that are relevant to the success of the IRB-BART in encouraging the simultaneous growth of a range of iron related bacteria at different places within the culturing fluids within the tester. These are summarized as:

1. 1. As the oxidation gradient forms over a reduction gradient it creates a range of relatively stable ORP value laterally placed one above the other within the vertical column of culturing fluids.
2. 2. Selective chemicals including nutrients specifically for the enhanced activity of iron related bacteria diffuse slowly upwards from the crystallized pellet in the base of the tester.
3. 3. Microbes within the sample being tested will tend to gravitate towards the most favourable environment within the tester based upon the downward diffusion of oxygen (influencing the local ORP value) and upward diffusion of chemicals (significantly affecting the n local nutrient levels).
4. 4. Reactions observed as a result of iron related bacterial activity will give an indication of the relative aggressivity (activity) of these various sub-communities now active within the IRB-BART tester.



In the IRB-BART tester, there are two primary functions:

1. The oxygen from the headspace in the tester diffuses down past the ball.

2. Selective chemicals diffuse up from the base.

Reaction patterns in the IRB-BART tester are therefore chronologically sequenced reflecting the order within which the different sub-communities reach a sufficient dominance to be observable. Qualitative interpretation of the reaction patterns is summarized in section 1 below with the Quantitative interpretation in section 2 followed by the standard protocols that need to be applied in the setting up of an IRB-BART test as section 3.

1. Qualitative Interpretation of Reaction Patterns

IRB-BART reaction patterns follow in a sequence that indicates many of the characteristics of the iron related bacteria within the sample being tested. There are two major pathways for the sequence of reactions observable in the IRB-BART tester with a number of different combinations. There is a sequence in the occurrences of the reactions which is dependent mainly upon the microbes that are active within the sample now being tested. The term “microbes” is used here because fungi and possibly protozoa and algae may also influence the formation of a particular sequence of reaction. The common sequences for the reaction to occur are given in the Table below and divide them into four chronological sequences.

This sequence of possible reactions can become with the development of a WB reaction

which forms as a white deposit of carbonates in the base of the tester. Usually this occurs within 12 hours of the test starting and has traditionally not been recognized as a reaction in the interpretation of the iron related bacteria in the sample but it is important in the determination for the treatment of biofouling in wells, cooling towers and water treatment system because the presence of the WB does indicate that carbonates are capable of being accumulated and that any rehabilitation treatment should take into account the need to disrupt carbonate rich encrustations by such treatment techniques as include the use of acid.

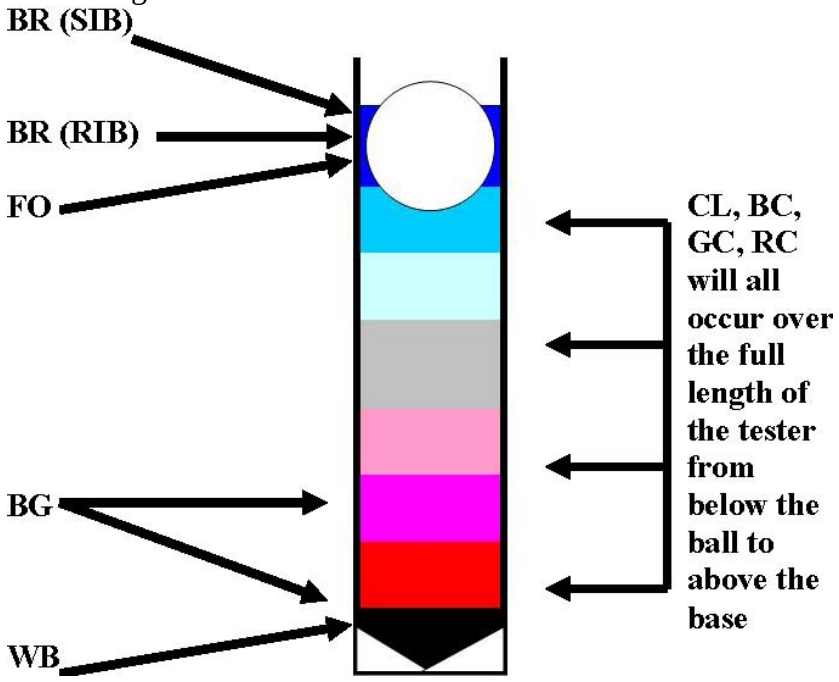
Sequence	Reaction pattern	Comments
1	**WB,** white base	Occurs more commonly in the slightly alkaline samples
2	**CL**, clouding **FO**, foam formation*	Indicates bacterial activity is occurring in the culturing fluids of the tester Reductive fermentative activity leads to the generation of gases including some of carbon dioxide, hydrogen, methane and nitrogen
3	**BC**, brown clouding** **BR**, brown ring **BG**, brown gel **RC**, red clouding **GC**, green clouding	Iron is being accumulated within the biocolloids created by some FOB. FOB, RIB and SIB are all capable of becoming significant when a ferric rich slime ring forms Some enteric bacteria (species of *Enterobacter* and *Klebsiella*) generate a dense ferric-rich gel that settles in the tester Some enteric bacteria (including species of *Serratia*) will cause a red color to be generated in the clouded growth This starts as a weak CL reaction that slowly turns a light shade of green that may turn darker and cloudier over time. This event occurs when *Pseudomonas* species are very active.
4	**BL**, black liquid	Walls and base of the tester get coated with black materials (possibly dominated by iron carbonates). This happens commonly when both enteric and pseudomonad bacteria are active

* It should be noted that foam can only be recognized when there is a ring of gas bubbles around the ball. Some water samples that are saturated with gases can cause gas bubbles to be generated on the walls of the tester. This is not a positive for an FO reaction.
** Colors may vary through orange, to various shades of brown.

The first major diagnostic sequence that occurs usually in one to five days is the generation of either a cloudy (CL) or foam reaction (FO). CL occurs more commonly

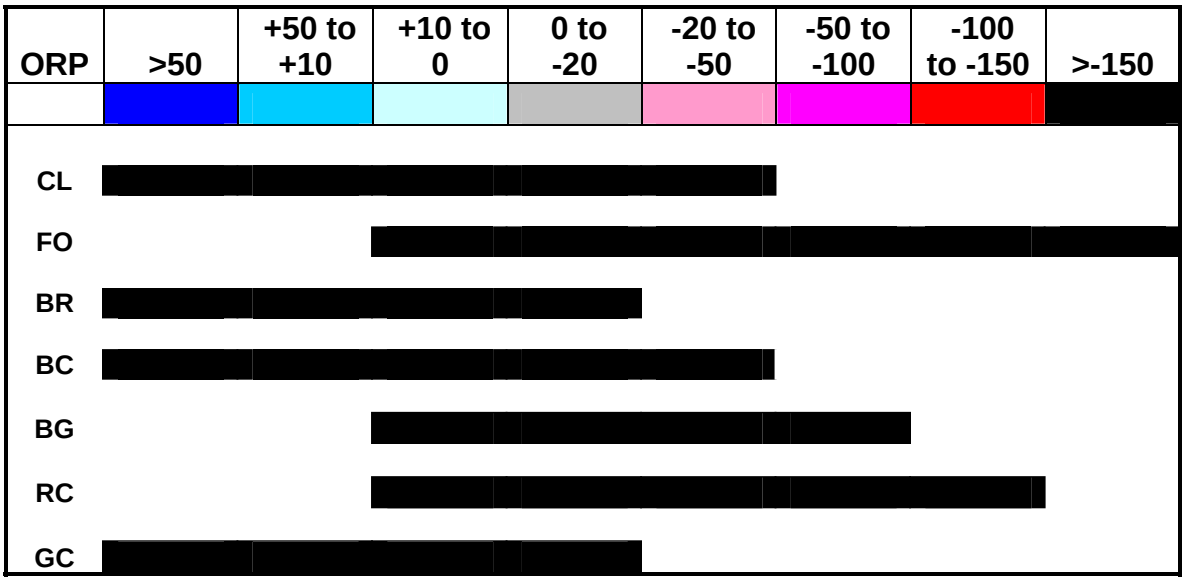
when the sample comes from an oxidative region or at the redox front (where there is an interface between oxidative and reductive conditions). Both the FO and CL reactions can occur together meaning that the microbes in the sample are mixtures of cells capable of being active both oxidatively (through respiration) and reductively (through fermentation). If the FO precedes the CL then the sample is more dominated by fermentors whereas if the CL precedes the FO then it is dominated by "respirers." Reactions found in sequence three tend to occur in an order reflecting the dominance of those particular bacteria. Sequence four is a terminal reaction which occurs only when there are significant populations of enteric and pseudomonad bacteria present.

The location of any given reaction type within the IRB-BART tester gives information about the active bacterial community present in the sample. It also can be used to determine the ORP state of the state where the sample was taken. This is summarized in the next figure showing the sites which each of the reaction start and move to.



For five of the reactions, therefore, the location is restricted to one region of the tester while for the other four reactions, the reaction occurs throughout the column of the culturing fluids from beneath the ball to the base of the tester. These sites for growth in the IRB-BART tester do not necessarily relate to the ORP conditions from which the sample was taken. This is shown in the next figure: From this chart, a relationship can be generated between the reaction patterns observed (as a reaction pattern signature, RPS) and the probable ORP conditions in the sample. It should be noted that for water that is flowing over a surface, the types of iron related bacteria present in the sample, will only reflect those species that were either already in suspension or sheered from the surfaces. It therefore only reflects the ORP in the water phase and not that of the attached biological growths over which the water sample was passing. In that event that a semi-

solid (e.g. sludge) or a solid (e.g. soil) sample is to be tested then there may well be significant ORP gradients within the sample that is used in the IRB-BART tester. In these cases it can be expected that the sample will go through some dispersion releasing a broader spectrum of bacteria to cause the RPS to reflect this. Diagnosis of the RPS for the IRB-BART to the major active communities and genera can be achieved using BART-SOFT (version 4.0).



The term “iron related bacteria” implies strongly that all of the microbes cultured using the IRB-BART tester are most likely to be involved in some form of excessive manipulation of iron beyond the basic metabolic needs. For microorganisms to be cultured and recognized within the IRB-BART tester there are a number of constraints that reduce the probability of non-iron related bacteria growing within these culturing fluids. Within the nutrient pellet dispensed in the tester are chemicals that will restrict the ability of many bacteria to grow. Restrictive ingredients are listed within the table below: Through the application of a crystallized pellet for the delivery of these selective nutrients, there is a gradual concentration increase in these selective chemicals as they diffuse up through the culturing fluids. This then forms a vertically moving diffusion front that further limits the types of bacterial activity to the iron related bacteria.

2. Quantitative Interpretation of Reaction Patterns

While the RPS which is formed as a product of section 1 to qualitatively determine the types of iron related bacteria in the sample, this section addresses the projection of a population count to the sample. Such population counts have been traditionally set by such determinations as the number of countable colonies on a known surface that have grown from a know mass of sample. This is commonly referred to as the number of “colony forming units” (c.f.u.) and is in common use in microbiology laboratories for reporting populations. For the BART methodologies it is not possible to count colonies since a fluid is employed for the cultivation of the microorganisms. Colonies therefore

cannot be seen and certainly cannot be counted and so an alternative approach has been employed. As a basic premise it is assumed that the bacterial population in the sample is formed by two primary parts: (1) active cells that have the potential to be cultured in the BART tester; and (2) active and inactive cells that do not have such abilities to be cultured under the conditions established in the tester.

Ingredient	Restrictive function
Ferric iron	Ferric iron is provided in sufficiently high concentrations to be inhibitory to bacteria that do not have a high tolerance. It has been found that commonly the ferric form will be reduced to the ferrous form by many of the iron related bacteria to allow the iron cycle to occur.
Citrate	By experiment it has been shown that most of the iron related bacteria are able to most commonly utilize citrate as carbon source. Citrate therefore acts as a functional restrictor for the many heterotrophic bacteria that cannot use this carbon source
Nitrate	This chemical provides an alternate electron acceptor to oxygen and many of the iron related bacteria are able to "respire" using this nitrate. This therefore provides an improved selectivity for the growth of iron related bacteria.
Dipotassium hydrogen phosphate	Iron related bacteria commonly have the ability to store phosphate primarily as polyphosphates. The use of a high concentration of phosphate restricts the range of bacteria able to grow to those that are phosphate tolerant.

In the evaluation of population size for the BART testers, the primary method is to take the link between the speed with which the first reaction happens, the activity level of the cells in the sample and then project a population. In the use of speed to determine the bacterial activity, it is the time lag (commonly measured in seconds, hours or days) that is used to predict the population size. Here the preferred unit for quantifying the population is predicted active cells (p.a.c.). This means that the unit of expression (i.e. p.a.c.) relates to an evaluation of active cells in the sample and not the total number since a fraction of the cells would be either dormant or unable to react to the conditions established in the IRB-BART and generate a distinguishable reaction. The population predictions (based commonly based on either the CL or FO time lag is given in the Table below:

Note that when readers are being used to automatically project time lags, the normal upper limit in seconds is 345,600 seconds. Estimations of populations using daily readings usually starts at daily intervals. If more frequent visual observations are desired then record can be measured in hours. The program BART QuickPop is available on the web site (www.dbi.ca) where more precision (to the nearest second of time lag is desired. For the purposes of uniformity the time lag to the first recognized reaction (WB is not considered a recognized reaction for this purpose) forms the base data for the projection

of the p.a.c regardless of which recognized reaction has occurred. Commonly this would be the time lag created by either the CL or FO reactions. No other populations are computed from subsequent reactions and these reactions are used solely for development of the RPS and qualitative evaluation of the iron related bacterial content of the sample. In the calculation of the p.a.c. / ml the data is automatically modified to correct from the 15ml sample size to predict the p.a.c. / ml.

TL (secs)	TL (hrs)	TL (days)	p.a.c./ml
21,600	6		1,500,000
43,200	12		1,070,000
64,800	18		760,000
86,400	24	1**	540,000
172,800	48	2	137,000
259,200	72	3	36,000
345,600*	96	4	9,000
	120	5	2,300
	136	6	580
	168	7	150
	192	8	38
	216	9	9

3. Establishment of Standard Protocols.

A standard protocol is limited to specific conditions in the manner and from with which the sample will be received for testing, the nature of the set up of the test procedure relevant to the sample, conditions for the ongoing observation of the IRB-BART during the testing phase, and the manner in which the time lag and reaction patterns will be observed. There will be two protocols listed below: (3a) water and waste water samples; and (3b) semi-solid and solid samples such as sludges and soils.

Recommended Protocol for Water and Waste Water Samples

There are standard procedures for the collection of water and waste water samples but these are not so well defined for well water. Essentially iron related bacteria tend to be able survive several days in water samples with no significant loss in activity (being reflected in longer time lags). However there remain a number of basic considerations that should be considered to ensure precision and validity in the final interpreted data. These can be summarized as needing to include the following conditions:

(i) If the sample is going to be tested within four hours of collection then the sample may be allowed to acclimatize to room temperature at a site away from direct sunlight in a place where there are no vibrations or disturbances of any type.

(ii) If a water sample is taken from a continuous stream flow then the sample should be taken at midpoint as far as possible to avoid contamination from any growth occurring on the walls of the conduit.

(iii) If it is desired to examine the types of iron related bacteria growing attached within the conduit with which the water is in contact, then changing the environmental conditions through stopping or radically changing the flow rate will cause stress amongst the attached bacteria and there is likely to be more releases into the water with subsequently shorter time lags and changes in the RPS.

(iv) If a water sample has to be collected longer than four hours prior to testing then the sample should be cooled down over ice to approximately 4^{o}C. Water samples for IRB determinations can be kept for 48 hours in this state with no significant changes in activity. When the sample has been cooled down then it should be left at room temperature for one hour to acclimatize before starting test. Water samples should always be kept in sterile containers such as those provided by Public Health authorities for coliform testing.

(v) In the event that sterile containers are not available in the field for on-site testing then the outer vial of the IRB-BART tester may be used to collect the sample.

(vi) It is not necessary for the sample container to have a chemical tablet to neutralize residual chlorine since: (a) the iron related bacteria do have a resistance to the normal levels of residual chlorine in water; and (b) the IRB-BART incorporates a chlorine neutralizing chemical to eliminate any potential inhibition.

(vii) It is not necessary to completely fill the sterile sample container with the sample water since this would cause some restriction in the available oxygen since it would be used up through indigenous respiration leading the a more reductive ORP to be generated possibly stressing some of the strictly aerobic bacteria.

(viii) Cleanliness should be imposed in all stages of the collection and handling of the sample and rigorous efforts need to be applied to assure no casual contamination of the sample in any manner.

(ix) ③ It is recommended that latex gloves be worn by the operator when working with the IRB-BART testers.

Charging the IRB-BART with the sample

Filling the IRB-BART tester involves a sequenced approach that ensures that there is no casual contamination of the tester and that a suitable volume of sample has been added. There are two different types of IRB-BART tester are the: (1) field tester; and (2) laboratory tester. The field tester is designed for use away from the laboratory and can be

charged with the sample close to the sampling site and monitored in the field at a suitable temperature. This type of tester includes an outer vial which surround and protects the inner vial in which the test is actually performed. The other type of IRB-BART tester is meant to be used in the laboratory under normal conditions for the handling of microbiological materials. This tester includes the essential inner vial necessary for undertaking the IRB-BART test but without the use of the outer vial. The field tester therefore provides additional protection for the user from any potential leakages of odorous gases or the cultured fluids that may occur from the inner vial. Instructions for the charging of the field and laboratory versions of the IRB-BART tester are described below:

(1) Charging the field IRB-BART tester

The field tester comes in packs of three each separately sealed within an aluminum foil pouch. To remove a field tester from the pouch, locate the “tear down” tab on one end of the pouch, grip it tightly and pull it down over the pouch. This will exposed the field tester that can then be removed. Make sure that there is a clean flat surface to place the tester on in a place where it is not likely to be knocked over.

The container containing the sample to be tested should be placed next two the field tester on a clean surface and it is important to make sure that there is not likely to be any strong winds or air currents that could bring dust into the area where the test is going to be set up. Follow the sequence of activities as described below:

(i) Label the top cap of the field tester with the essential information to recognize the sample (e.g. name, date, time) using a black fine point permanent marker. Do not write on the walls of the field tester because this may seriously impair the ability of the operator to recognize reactions. Make sure that the ink from the marker is dry before attempting to charge the field tester. If this is not done then the samples information may become smudged and unreadable.

(ii) When ready to charge the field tester with the sample, the first step is to unscrew and remove the outer cap of the tester. It should be noted that lifting up the outer cap causes the inner tester vial to be lifted up as well.

(iii) As the outer cap is lifted up clear of the outer vial then gently remove the inner test vial from the cap and place the cap down on a clean surface without turning it over and place the inner tester next to the sample. It is strongly recommended that charging of the tester be done one at a time to reduce the potential for inadvertent mixing of the samples and testers.

(iv) Unscrew the inner cap from the inner and place the cap down on a clean surface without turning it over. Note this cap is not labelled with the sample information and so it is important to do one test at a time to prevent mixing up of samples. Note also that the contents of the inner IRB-BART tester are now exposed to possible contamination from the outside environment and so the next steps should be done quickly. Unscrew the cap from the sample container and slowly pour sufficient water into the inner tester vial to bring

the water level up to the fill line indicating 15ml of sample has been added. The ball will float up on the rising water column and the water level is observed against the ball. When pouring water into the inner tester vial, every effort should be made to direct a stream of the sample water over the center of the ball rather than allow it to trickle down the side. Care should be taken to ensure that the final water sample level is to within 2mm of the etched fill line on the BART tester. For the effective use of the field tester, maximum tolerance for error for filling the IRB-BART tester is 5% so that the amount of water sample being tested falls within the range of 14.25 and 15.75ml. It should be noted that the sample container retains a headspace of air over the sample and so some oxygen will diffuse down into the liquid sample. To assure that there is oxygen in the sample along with a dispersion of any settled or particulate materials within the sample it is recommended that the sample container be inverted five times before being used to charge the field tester. This assures a better level of precision since the oxygen concentration at the start of the test would be at a saturated level and the possible bacteria of interest are more likely to be evenly dispersed throughout the sample.

(v) Once the inner tester vial has been charged, immediately screw back the inner cap down on the vial. This cap does not need to be screwed down hard. Do not shake or disturb the contents of the inner tester vial in any manner. Lift the outer cap without turning it over and clip the inner tester vial back into the underside of the outer cap. Now grip the outer cap (inner charged inner test vial should now be tightly held inside this cap) and lower it back down into the outer tester vial. Screw the outer cap down firmly but without excessive force. The charged field tester may now be moved to a safe place for periodic observation. In moving the charged tester remember that the test has started and that complex diffusion fronts for oxygen (moving downwards) and nutrients (moving upwards) are occurring and that severe movement may affect the precision of the tester. It is therefore recommended that the field testers be charged close to the site where the testers are going to be observed to minimise motion-induced disruption.

(vi) Testing begins as soon as the sample is added. If the sample has a much lower temperature than the temperature at which the tester will be maintained (e.g. room temperature) then there is a probability that excess oxygen might be released into the culturing fluids causing gas bubbles to form on the walls. This is not a positive reaction. To minimise this risk, the sample should be given time acclimatise to the temperature at which the testers will be monitored (e.g. room temperature). This acclimatization would be dependent upon the sample bulk volume but should, under normal conditions, take no longer than one hour.

(vii) Incubation temperature commonly recommended for the field IRB-BART tester is room temperature which is considered ideally to be between 21 and 25°C away from direct sunlight that could cause internal heating of the tester. If the temperature goes above 25°C then there is most likely to be an increase in the rate of iron related bacterial activity and the time lags for given population sizes can be expected to drop. This would cause higher

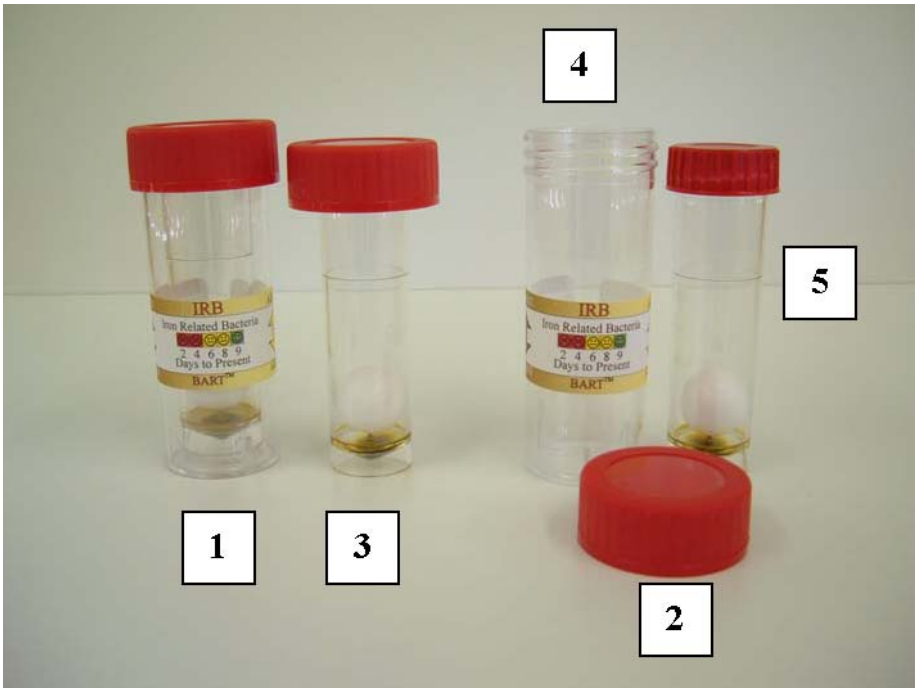
populations of IRB to be predicted. If the temperature drops below 21°C then this may commonly slow down the level of bacterial activity causing losses in precision (where duplicate tests are performed) and much lower predicted populations.

(viii) Observation for the occurrence of activities and reactions in the tester should ideally be performed daily until day 6 and then again on day 8 and day 10. Critical days for the observation of activities and reactions are days 1, 2 and 3 along with such other days up to day 10 if the objective is to obtain a qualitative and/or quantitative assessment from the sample in the tester. If the IRB-BART is just to be used to determine whether iron related bacteria present or not then a single observation on either day 7 or 8 will be sufficient to indicate whether these bacteria are present or absent.

.(ix) To observe a tester for any significant reactions or activities then the recommended technique is to follow the same procedure each day (and at the same time of day as the test was started). This procedure would consist of lifting the tester up towards a light such as a fluorescent light fixture or the northern sky during daylight hours. The ideal position to view the tester is to place it directly in front of the light source at arms length. In moving the tester it is important not to use any sudden movements since this could disturb the activities and reactions occurring within the IRB-BART tester. To detect all possible events the tester needs to be moved slowly viewing from the bottom first, followed by the side and ending inspecting down around the ball. The sequence for detecting reaction during his bottom up inspection would be to look for WB (in the base), CL (looking through the tester at a light), BG (close to the base as a dark brown gel), BL (the whole tester below the ball appears to have a black film on the inside of the tube with the culturing fluids clear inside); BC / RC /GC may be recognized throughout the culturing fluids; FO will be seen as gas bubbles under the underside of the ball covering at least 10% of the surface and/or a foam ring of bubbles all around the balls; and BR is most easily seen looking slightly downwards as the born slime ring around the ball. It should be noted that no color chart is used to determine whether a reaction has, or has not, occurred since each operator has slightly different color perception. In stead it is recommended that only a clearly recognized color should be considered to be positive. In the event of uncertainty it is better to err on the side of caution and only accept reactions that are easily recognized. IRB-BART testers that are negative will show some changes in color but will not go cloudy. These changes in color are usually to a pale yellow that is crystal clear, or depending upon the chemistry of the sample a dark green that is usually occupies the lower 5 to 15mm of the culturing fluids. Control test can be set up to confirm negative (absent) reactions. Such control tests should employ sterile distilled water or phosphate buffer and be set up using aseptic procedures to minimize the risk of casual contamination of the control testers.

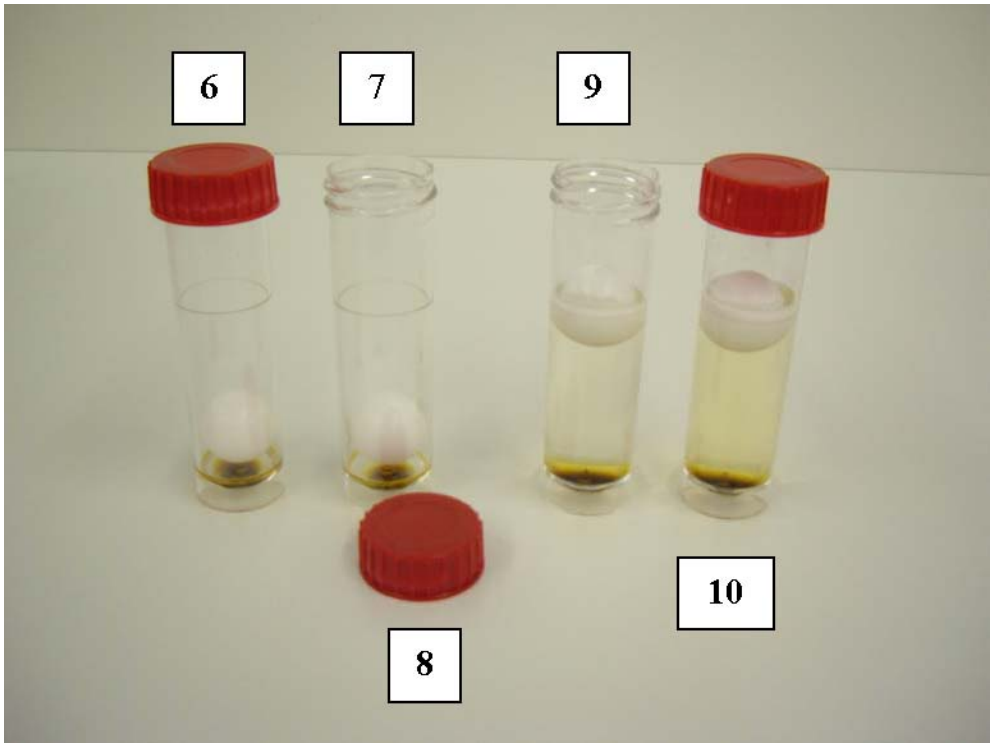
.(x) Once the tests are finished after a maximum of 10 days of incubation and observation, confirmatory tests are possible in a certified microbiology laboratory able to undertake bacterial identification. Data should be stored

using a chain of custody that confidently links the data sets to the interpretation and sample origin. Finished tests should be disposed of using one of the techniques recommended in (4) below.



Components in the IRB-BART field tester

The sequence for preparing the IRB-BART field tester for testing is summarised as: (1) take a field IRB-BART tester; (2) unscrew the cap and place on clean dry surface; (3) remove the inner tester vial from the cap; (4) put the outer vial aside until required at the end of charging the tester with the sample; and (5) use the inner tester vial for filling with the sample. Note these activities are undertaken in rapid succession leaving the operator with items 2, 4 and 5. The next plate shows the method for charging the field tester with sample.



Sequence for Charging the Inner Vial of the tester with a Sample

In the above plate the sequence for charging an inner IRB-BART tester (6) as requiring the following steps: (7) unscrew the cap; (8) place the cap down on a clean surface without turning the cap over; (9) carefully fill the tester vial with the sample until the water level is at the same level as the fill line, note that the ball floats up as the water level rises; and (10) screw the inner cap back onto the vial without using force. Once this has been done then the outer cap can be positioned over, and pressed down onto, the inner test vial that will now become seated into the outer cap. Once this has been done then the outer cap (with the inner tester attached can be returned to the outer vial and screwed down. The test for the presence of iron related bacteria has now started.

Charging and incubating the laboratory IRB-BART tester

The IRB-BART laboratory tester is a minimal form of the field tester including only necessary minimal steps to assure that suitable testing can be conducted effectively in the laboratory setting. When used in this environment there can be a higher level of precision due to the skills of the laboratory technologists but also opportunities to select different incubation temperatures. Procedures are similar to the field test but involve fewer steps

and the differences are summarized below. Samples are dispensed into the laboratory tester using sterile pipettes under aseptic procedures. Technician error in filling the vial with water sample is reduced through the water sample being pipetted into the vial at the midpoint over the ball using a 25ml pipette. This restricts the filling error to ±0.2ml. In the event that 25ml sterile pipettes were not available then 10ml sterile pipettes cold be used with two aliquots (e.g. 10 and 5ml) being employed to charge the laboratory tester.

For the sample coding on the individual tester it is recommended that this be done only on the top of the cap using a black permanent fine tip marker. Once dispensed, the testers should be placed in a test tube rack having a hole diameter of 26 to 28mm that would allow the testers to be suspended at least 90mmabove the floor of the rack giving a 10mm clearance beneath the tester. A rack should contain only a single row of testers so the activities and reactions can be more easily recognized when the rack is lifted up for observation purposes.

Incubation can be at room temperature (21 to 25°C) but other temperatures can be selected since different incubators are likely to be available. Faster reactions and activities can be achieved sometimes by incubating the testers at 27 to 29°C but this will cause the time lags for a given population to shorten. BART QuickPop software has interpretation methods to project the population when this higher temperature is used but a summary table below gives some of the time lag to p.a.c. / ml population relationships. Temperatures above 29°C are not recommended since there is a high probability of radical changes in the reaction pattern with FO reactions becoming almost universal at blood heat.

When a water or waste water sample is taken from a cold environment when the temperature is commonly below 8°C for several months at a time then it is probable that the iron related bacteria may have adapted to these temperatures and will not grow even at room temperatures. In this event it is quite likely that false negatives will occur when tests are performed at room temperature. If this is a serious concern then the tests should be incubated in a refrigerated incubator at 8 to 10°C. At these temperatures the activities and reactions are similar to those seen at room temperature but the time lag will be affected by the populations of iron related bacteria that are only able to grow at these low temperatures. For laboratory testing using the IRB-BART laboratory tester there are therefore three selectable temperature ranges for incubation (depending upon the clients needs): low temperature range of 8 to 10°C, room temperature range of 21 to 25°C, and the elevated temperature range of 27 to 29°C. Temperatures above 29oC are not recommended but in the event that a client requires information on the iron related bacteria population in a water sample taken from higher temperatures than the recommended range then the 27 to 29°C range should be selected Conversion of time lags to p.a.c. / ml would involve different computations and these are incorporated into BART QuickPop and are summarized in the table below:

TL secs	TL hours	TL days	8 to 12oC	21 to 25oC	27 to 29oC
21,600	6		1.9M	1.5M	1.7M
43,200	12		1.3M	1.1M	1.3M
64,800	18		806T	760T	1.1M
86,400	24	1	516T	540T	850T
172,800	48	2	76T	137T	343T
259,200	72	3	14T	36T	137T
345,600	96	4	2.4T	9T	55T
	120	5	405	2.3T	22T
	136	6	67	580	9.0T
	168	7	11	150	3.6T
	192	8	1	38	1.5T
	216	9	1	9	585
	240	10	1	1	235
	360	15	1	1	1

Note: M – millions; T – thousands; units are iron related bacterial cells as p.a.c. / ml; Additionally note that it is not recommended that the IRB-BART testers be incubated at above 30°C since FO and CL reactions become unstable.

The difference in the predicted populations using these three incubation temperatures relates to the manner in which the bacteria react to the temperatures. At 8 to 12°C, the IRB that are active in those water samples are likely to be more aggressive than at room temperature and indicate higher populations. However these bacteria do not have a great deal of ability to adapt to these lower temperatures if they had been growing in warmer waters (e.g. 20°C). At the higher temperatures of 27 to 29°C there is a greater ability for the indigenous iron related bacteria to grow after a period of adaptation. Hence the higher temperature is more suitable for the late detection of significant iron related bacterial populations.

Samples waters, such as those form cooling towers, hot water systems and other warm water sources cannot be easily tested using the IRB-BART tester at incubation temperatures above 30°C due to the generation of reactions that do not relate to iron related bacteria but rather other bacteria that now dominate the culturing fluids causing excessive gassing (with foam formation) and heavy iron-rich clouding. To compensate for this, the IRB-BART testers can be used at 27 to 29°C and will be able to detect the IRB able to grow within waters having a natural temperature of between 30 and 45°C.

Protocol DBIFWO6

Using the IRB-BART (Field version) tester to test for IRB in water

Filling the IRB-BART tester involves a sequenced approach that ensures that there is no casual contamination of the tester and that a suitable volume of sample has been added.

Instructions for the charging the IRB-BART field tester is described below:

The field tester comes in packs of three each separately sealed within an aluminum foil pouch. To remove a field tester from the pouch, locate the “tear down” tab on one end of the pouch, grip it tightly and pull it down over the pouch. This will exposed the field tester that can then be removed. Make sure that there is a clean flat surface to place the tester in a place where it is not likely to be knocked over.

The container containing the sample to be tested should be placed next to the field tester on a clean surface and it is important to make sure that there is not likely to be any strong winds or air currents that could bring dust into the area where the test is going to be set up. It is recommended that latex gloves be worn during the setting up of the IRB-BART testers to reduce the risk of contamination. Follow the sequence of activities as described below:

(i) Label the outer red cap of the field tester with the essential information to recognize the sample (e.g. name, date, time) using a black fine point permanent marker. Do not write on the walls since this might seriously impair the ability of the operator to read reactions. Make sure that the ink from the marker is dry before attempting to charge the field tester. If this is not done then sample information may become smudged and unreadable.

(ii) When ready to charge the field tester with the sample, the first step is to unscrew and remove the outer cap of the tester. It should be noted that lifting up the outer cap causes the inner tester vial to be lifted up as well. This is displayed in the figures shown below.

(iii) As the outer cap is lifted up clear of the outer vial then gently remove the inner test vial from the cap and place the cap down on a clean surface without turning it over and place the inner tester next to the sample. It is strongly recommended that charging of the tester be done one at a time to reduce the potential for inadvertent mixing of the samples and testers.

(iv) Unscrew the inner cap from the inner vial and place the cap down on a clean surface without turning it over. Note this cap is not labelled with the sample information and so it is important to remember to do one test at a time to prevent mixing up of samples. Note also that the contents of the inner IRB-BART tester are now exposed to possible contamination from the outside environment and so the next steps should be done quickly. Unscrew the cap from the sample container and slowly pour sufficient water into the inner tester vial to bring the water level up to the fill line indicating 15ml of sample has been added. The ball will float up on the rising water column and the water level is observed against the ball. When pouring water into the inner tester vial, every effort should be made to direct a stream of the sample water over the center of the ball rather than allow it to trickle down the side. Care should be taken to ensure that the final water sample level is to within 2mm of the etched fill line on the inner vial of the BART tester. For the effective use of the field tester, maximum tolerance for error for filling the IRBBART tester is 5% so that the amount of water sample being tested falls within the range of 14.25 and 15.75ml. It should be noted that the sample container retains a headspace of air over the sample and so

some oxygen will diffuse down into the liquid sample. To assure that there is oxygen in the sample along with a dispersion of any settled or particulate materials within the sample it is recommended that the sample container be inverted five times before being used to charge the field tester. This assures a better level of precision since the oxygen concentration at the start of the test would be at a saturated level and the possible bacteria of interest are more likely to be evenly dispersed throughout the sample.

(v) Once the inner tester vial has been charged, immediately screw back the inner cap down on the vial. This cap does not need to be screwed down hard. Do not shake or disturb the contents of the inner tester vial in any manner. Lift the outer cap without turning it over and clip the inner tester vial back into the underside of the outer cap. Now grip the outer cap (inner charged inner test vial should now be tightly held inside this cap) and lower it back down into the outer tester vial. Screw the outer cap down firmly but without excessive force. The charged field tester may now be moved to a safe place for periodic observation. In moving the charged tester remember that the test has started and that complex diffusion fronts for oxygen (moving downwards) and nutrients (moving upwards) are occurring and that severe movement may affect the precision of the tester.

(vi) Testing begins as soon as the sample has been added. If the sample has a much lower temperature than the temperature at which the tester will be maintained (e.g. room temperature) then there is a probability that excess oxygen might be released into the culturing fluids causing gas bubbles to form on the walls. This is not a positive reaction and should not be confused with the FO (foaming) reaction that will occur around the ball. To minimise this risk, the sample should be given time acclimatise to the temperature at which the testers will be monitored (e.g. room temperature). This acclimatization would be dependent upon the sample bulk volume but should, under normal conditions, take no longer than one hour.

Protocol DBILWO6

Using the IRB-BART (laboratory) tester to test for IRB in water

The IRB-BART laboratory tester is a minimal form of the IRB-BART tester including only necessary minimal steps to assure that suitable testing can be conducted effectively in the laboratory setting. When used in this environment there can be a higher level of

precision due to the skills of the laboratory technologists but also opportunities to select different incubation temperatures. Procedures are similar to the field test but involve fewer steps and the differences are summarized below. Samples are dispensed into the laboratory tester using sterile pipettes under aseptic procedures. Technician error in filling the vial with water sample is reduced through the water sample being pipetted into the vial at the midpoint over the ball using a 25ml pipette. This restricts the filling error to ±0.2ml. In the event that 25ml sterile pipettes were not available then 10ml sterile pipettes cold be used with two aliquots (e.g. 10 and 5ml) being employed to charge the laboratory tester. Other procedures are addressed in DBIFWO6

For the sample coding on the individual tester it is recommended that this be done only on the top of the cap using a black permanent fine tip marker. Once dispensed, the testers should be placed in a test tube rack having a hole diameter of 26 to 28mm that would allow the testers cap to be suspended at least 90mmabove the floor of the rack giving a 10mm clearance beneath the tester. A rack should contain only a single row of testers so the activities and reactions can be more easily recognized when the rack is lifted up for observation purposes.

Protocol DBILSO6

Using the IRB-BART (laboratory) tester to test for IRB in soil

Soil samples require a different approach since it is not possible to simply dispense the soil into the BART tester. If this were to be done then the soil particles would perch around the ball and disturb the effective generation of the redox gradient essential to the functioning of the tester. It is therefore important to remove the ball from the tester before adding the soil sample.

To prepare to add the soil sample for testing, the screw cap has to be removed from the laboratory tester and place with the inside facing upwards. This inside surface is sterile and so should not be contaminated in any way. The ball is now rolled out of the tube into the cap and the tube placed upright again. It is now ready for the soil sample to be added and the test started.

0.1g of soil is added using a clean spatula to drop the soil onto the floor of the tester. It should be noted that a sandy soil may require 0.5g of soil to be used in order to get effective testing. If 0.1 g of soil is used then the population calculated using the IRB-BART system would need to be multiplied by x150, if 0.5 g of soil is used then the population should be multiplied by x30 to get an accurate population count. Once the soil has been added then 15 mL of sterile saline (0.1% NaCl) should be added followed by the ball being rolled back into the tube. Once the tube is now capped again then the test can start at the appropriate temperature. Incubation can be at room temperature (21 to 25^{o}C) but other temperatures can be selected since different incubators are likely to be available. Faster reactions and activities can be achieved sometimes by incubating the

testers at 27 to 29^{O}C but this will cause the time lags for a given population to shorten. BART QuickPop software has interpretation methods to project the population when this higher temperature is used but a summary table below gives some of the time lag to p.a.c. / ml population relationships. Remember that the IRB-BART only functions giving the full range of reactions at incubation temperatures up to 30^{o}C.

It should be noted that soil with a high fraction of oil is likely to create odd effects in the tester during incubation. When there is significant hydrocarbons (i.e., >1% by weight) the soil particles may become mobilized and float up into fluids. This may also be accompanied by heavy gassing. Such activity may disrupt the observation of the reactions and activities in the tester and so the testing of high oil content soils is not recommended.

4. Data sheet specifications

It has been found that the most efficient manner to collect the data for an IRB-BART test is to use ten cells in a row to record the data generated and observed on each of the first ten days of the test. Each cell in the row would be recognized by the column as to which day in the test procedure is being evaluated:

Sample	**date**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**

Information added to the day cells is strictly limited to the two character reaction codes only. Where more than one reaction code is recognized as having occurred on that day into the test then both are entered separated by a dash. The population of iron related bacteria are calculated (using BART QuickPop) or determined using a conversion table using the day number (or hour number) from the start of the test to the first observation of a reaction. This would commonly be either a CL or FO reaction. It has to be remembered that the WB reaction is not recognized for the purposes of calculating the size of the iron related bacterial population. An example of the entry of data for tow IRB-BART testers (A & B) is shown below:

Sample	**Date/temp**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
A	mn/da/tm RT	**WB**		**FO**	**CL BR**	**BC**		**BL**			
B	mn/da/tm RT		**CL**	**BG**			**BCBR**				

These may now be summarized to a time lag (days) of 3 (A) and 2 (B) with the reaction pattern signatures being: for A, WB, FO, CL-BR, BC, and BL; while for B, CL, BG, and BC-BR. Populations using the conversion table from TL to population would give an iron related bacteria population (p.a.c. / ml) of 36,000 (A) and 137,000 (B). Both samples showed that iron related bacteria were positive through the generation of ferric oxides

and hydroxides (i.e. for A it was the BR and BC reactions and for B it was the BG, BC and BR reactions. Further interpretations of the make up the community of iron related bacteria inhabiting and active within the samples can be obtained by using BART-SOFT available at www.dbi.ca.

5. Disposal methods

IRB-BART testers, when charged with a sample and incubated, are likely to contain active bacterial populations whether the tester has gone positive for iron related bacteria or not. Such testers may be used for confirmatory tests in a certified microbiology laboratory but most would then be disposed off since they are single use disposable test methods. Disposal may vary with the location of the completed testers. In the laboratory setting, the testers should be placed in a biohazard bag which would then be sealed prior to steam or gas sterilization. Once sterilized then the testers do not present a health risk issue and should be disposed of with the regular laboratory solid wastes.

In the event that the testers have been used at too great a distance from a suitable certified microbiology laboratory or do not any arrangement to get the samples to such a laboratory then the testers do have the potential to contain active bacterial cultures. To eliminate the hygiene risks from these bacteria to the general society through disposal as domestic garbage, the testers need to be disinfected or pasteurized prior to final disposal. Recommended methods for this are listed below:

Disinfection of Used Testers. Take an 10.5” x 11.25”(27 x 28.5 cms) plastic freezer bag that has a zip lock that can securely open and close the bag. Open the bag and place six 8.5” x 11” (22.5 x 29 cms) sheets of household paper towel which have been folded along the longer side of the sheet to make a “v” shape fold. These sheets are placed in fold side down and opened so that there are six sheets on each side of the bag. Up to 9 field testers or 15 laboratory testers can be placed in the center of the bag lying on their side (make sure the outer caps have been screwed down tightly onto the outer vials). Once the testers have been added then 125ml of household bleach is poured into the bag. This bleach is soaked up by the paper towel. The bag is now sealed and can be disposed of with domestic garbage. Note that the normal function in trash collection includes compressing the garbage which would cause the plastic vials to fracture and leak. There is sufficient active disinfectant in the bleach to assure the disinfection of the contents so that the risks are no greater than for the rest of the domestic garbage.

Pasteurization of Used Testers. Heat can be employed to kill the bacteria that have grown in the tester. The recommended method involves the use of dedicated 800 to 1,000 watt microwave that would only be used for this purpose. To perform this treatment the initial steps are the same as given above for disinfection using a plastic freezer bag and placing the finished testers inside the bag but here there are two differences: (1) the testers are set up right; and (2) the caps are not screwed down tightly. The microwave should be activated for 50 seconds for up to 9 field testers or 65 seconds for up to 15

laboratory testers. This amount of heat would be sufficient to pasteurize the contents and cause sufficient distortion in the plastic vials to allow the contents to leak out and be absorbed by the paper towel. After the heat treatment then the sealed bags can be disposed with domestic garbage.

August 13, 2006

Appendix One

Primary Claims

The IRB-BART generates, when charged with a water sample, a sufficient diversity of environments that will encourage the determination of observable activities of the IRB within the water sample being tested. From experiences to date the IRB-BART tester appears to be superior to any other field-applicable testing system due to the broad scope of IRB that can be recovered using this tester. It is proposed that the methodologies and technical information relating to the IRB-BART tester were sufficient for the verification of the Biodetector as a suitable system for the detection of IRB in water-based samples.

These claims would be subject to the following limitations:

1. 1. The limits of detection for the IRB in a given water sample would be 67cells/l.
2. 2. Any water sample taken for testing using the IRB-BART tester would have to be collected following the protocols established for the collection of a water sample for microbiological analysis. Transportation and storage of the sample should similarly follow the standard guidelines practiced for sample handling prior to the initiation of microbiological examination. These should include hygienic aseptic handling, the use of sterile sample containers and minimizing the sample storage time to less than four hours at room temperature or twenty four hours when cooled to refrigeration temperatures.
3. 3. The IRB-BART can be used for both field and laboratories based investigations and generate similar data with respect to time lag and reaction patterns where a sample is split and incubated under similar conditions in field and laboratory settings.
4. 4. While the IRB-BART technology commonly operates at ambient room temperatures there is the ability for the testers to be used at incubation temperatures ranging from +1 to +30°C under exceptional circumstances.