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i. Abstract

Measuring viability is an important and necessary assessment in studying microorganisms. Several methods can be applied to *Leptospira* spp, each with advantages and inconveniencies. Here, we describe the traditional colony-forming unit method, together with two other methods based respectively on the reducing capacity of live cells (Alamar Blue® Assay) and differential staining of live and dead cells (LIVE/DEAD *Bac*Light®). The Alamar Blue® Assay uses the blue reagent resazurin, which can be reduced into the pink reagent resorufin by live cell oxidoreductases. Production of resorufin can be quantified by absorbance or fluorescence reading. The LIVE/DEAD *Bac*Light® assay uses a mixture of two nucleic acid dyes (Syto9 and propidium iodide) that differentially penetrate and stain nucleic acid of cells with decreased membrane integrity. The colony-forming unit method is labor-intensive but the most sensitive and linear method. The two other methods are not laborious and well-adapted to high throughput studies but the range of detection and linearity is limited.

ii. Key words: Spirochetes, *Leptospira*, survival, plating, colony-forming unit, resazurin, resorufin, Syto9, propidium iodide, fluorescence, absorbance.

1. Introduction

Assessing and quantifying the viability of a microorganism is a basic but fundamental procedure in Microbiology. This is particularly important when the efficiency of an antibiotic or other antimicrobial compound needs to be determined, or to characterize bacterial species and their ability to survive under different environments.

Evaluating whether a bacterium is alive or dead is rather complex as finding the best criteria to define bacterial viability is still matter of debate. Several different qualitative and

quantitative techniques are available to measure bacterial survival, each having their advantages and limitations. Here, we will describe three methods that can be applied to *Leptospira* spp.

The gold standard is the colony-forming unit (CFU) method based on the ability of a bacterium to form visible colonies on agar plates ***(1)***. In this method, bacterial viability is defined by their cultivability and ability to divide on solid medium. This technique allows the determination of absolute number of bacteria and the range of detection is unlimited, although this method does not take into consideration and cannot be applied to viable but non-cultivable bacteria. After applying a treatment to a bacterial population, the bacterial suspension is diluted to obtain isolated, readily countable colonies on agar plate. This method is easy to implement and does not require sophisticated equipment, and is thus feasible with *Leptospira* spp. However, optimization to find the right bacterial suspension dilution for obtaining isolated colonies on agar plates might be time-consuming ***(2)***. Another limitation lies in the generation time of *Leptospira* spp. and, as a consequence, the results are obtained after one week or 3-4 weeks incubation for saprophytes and pathogenic species, respectively. An additional potential issue is that *Leptospira* spp. grow below the surface of the solid agar medium and appear as faint colonies, rendering their macroscopic visualization difficult. Nevertheless, this method was applied to *Leptospira* spp. and has allowed enumerating and quantifying their viability under different growth conditions ***(3–6)***.

Alternative methods using other viability criteria, such as metabolic activities, were developed and are now preferentially used due to their adaptability to high throughput screenings. One method is based on the fact that live and actively respiring cells maintain a reducing environment. This method uses resazurin (7-hydroxy-10-oxidophenoxasin-10-ium-3-one), a chromogen electron acceptor that is reduced into resorufin by cell oxidoreductases using NADH as cofactor (Figure 1) ***(7)***. Resazurin is permeable through cell membranes and

its reduction is therefore an indicator of the oxidoreduction state of the cells. Resazurin and resorufin have different spectrophotometric and fluorometric properties ***(8, 9)***. Resazurin is a blue non-fluorescent reagent and resorufin is a pink highly fluorescent reagent. When mixed with live cells, upon accepting electron, the blue-colored non-fluorescent resazurin is transformed into the pink fluorescent resorufin. Reduction of resazurin can be qualitatively determined by observing the visible change of the blue color into pink, indicative of the presence of live cells (Figure 2A). Resazurin reduction can be also quantified by absorbance (Figure 2B) or fluorescence measurement (Figure 2C). This method is easy to perform and not labor-intensive, as it does not require a high number of steps, and is therefore compatible with processing multiple samples. Reduction of resazurin can be visible after a few hours and the signal of reduced resazurin is stable for several days. In addition, it allows flexibility in the measurement method used (qualitative or quantitative). This method, commercially known as the Alamar Blue® Assay, has been extensively used with bacteria including the slow growing *Mycobacterium tuberculosis* ***(10)***. Since the EMJH medium used to cultivate *Leptospira* does not interfere with the reduction of resazurin, Alamar Blue® Assay was also successfully used to measure *Leptospira* viability ***(11−14)***.

Another criterion that can be used to rapidly assess bacterial viability is the membrane integrity. In this method, bacterial viability is correlated with the level of membrane damage and is assessed using a mixture of two fluorescent nucleic acid stains, the Syto9 and the propidium iodide (PI). These two dyes differ in their spectroscopic properties and in their capacities to penetrate bacteria. The green-fluorescent Syto9 penetrates bacteria regardless their membrane integrity and stains DNA of all bacteria in a population. On the contrary, the red-fluorescent PI penetrates only permeable bacteria and stains DNA of only bacteria with damaged membranes. Importantly, PI has a higher affinity for nucleic acids than Syto9 ***(15)***. When a bacterial population containing intact and membrane-damaged bacteria is exposed to

a mixture of Syto9 and PI, intact bacteria will be stained by green fluorescence whereas membrane-damaged bacteria will be stained by red fluorescence. Because the two dyes have different fluorescent properties, respective nucleic acid staining can be quantified by measuring fluorescence of the bacterial suspension at different wavelengths. The green/red fluorescence ratio is proportional to the relative number of live bacteria and a standard curve will determine the proportionality factor. This method is known as the LIVE/DEAD *Bac*Light stain. Since Boulos *et al.* ***(16)*** evaluated this procedure in bacteria and demonstrated its ease twenty years ago, this method is widely used to assess bacterial viability. This method allows staining of viable and dead cells in a single step and the acquisition of results is rapid. In addition to quantify *Leptospira* viability by fluorescent measurement, staining of *Leptospira* by Syto9 and PI can be measured by flow cytometry ***(17)*** (see Chapter 4, Fontana et al.) and fluorescence microscopy ***(18)***. It should be pointed out however that several studies have raised some concerns about the Syto9/PI staining; in fact, under certain conditions, this method has led to under- or overestimation of the number of viable cells ***(19–21)***.

In conclusion, when *Leptospira* viability needs to be estimated, multiple approaches are available. The traditional gold standard colony-forming unit method is labor-intensive but the most sensitive and linear method. The Alamar Blue® and LIVE/DEAD *Bac*Light® assays are not laborious and well-adapted to high throughput studies but their sensitivity and linearity are limited to a short range of bacterial concentrations. We therefore would like to emphasize that accurate and meaningful bacterial viability assessment might require, under some circumstances, validation by two different approaches.

2. Materials

2.1 *Leptospira* cultivation.

1. Safety cabinet level 2.
2. EMJH albumin supplement: 10% (w/v) bovine serum albumin, 0.004% (w/v) zinc sulfate, 0.015% (w/v) magnesium chloride, 0.015% (w/v) calcium chloride, 0.1% (w/v) sodium pyruvate, 0.4% (w/v) glycerol, 1.25% (v/v) Tween 80, 0.0002% (w/v) Vitamin B12, 0.05% (w/v) ferrous sulfate (added at the last moment) in sterile water for injection (WFI) (*see* **Note 1**).
3. EMJH base: dissolve 2.3 g of Difco *Leptospira* medium base EMJH (Becton Dickenson) in 900 ml sterile WFI. Autoclave the solution.
4. EMJH medium: add 100 ml of EMJH albumin supplement to 900 ml of EMJH base. Adjust the pH to 7.5 and filter sterilize the solution.
5. Spectrophotometer.
6. Disposable serological plastic pipettes.
7. Culture flasks and/or tubes.
8. Incubator with orbital shaker at 30°C.
9. Dark-field microscope.
10. Petroff-Hauser counting chamber.

2.2 Determination of colony-forming unit

1. Safety cabinet level 2.
2. P1000, P200, P20 micropipettes and sterile tips.
3. Sterile 1.5 ml polypropylene tubes.
4. EMJH medium (see section 2.1).
5. Concentrated EMJH medium: dissolve 2.3 g of Difco *Leptospira* medium base EMJH (Becton Dickenson) in 560 ml sterile WFI. Add 100 ml of albumin supplement. Adjust the pH to 7.5 and filter sterilize the solution.

6. Agar solution: Dissolve 12 g of agar noble in 340 ml of sterile WFI. Autoclave the solution.
7. Solid EMJH agar medium: melt 340 ml of agar noble solution and keep it at 50-55°C in a water bath. Pre-warm 660 ml of concentrated EMJH medium at 50-55°C. Mix the agar noble solution with the concentrated EMJH medium. Pour 25-30 ml of the solution into petri plates (*see* **Note 2**).
8. 90 mm × 14.2 mm petri dishes.
9. Sterile inoculation loop or glass spreader or glass beads.
10. Microbiological incubator at 30°C.
11. Aluminum foil.
12. Parafilm.

2.3 Measurement of resazurin reduction by the Alamar Blue® Assay

1. Safety cabinet level 2.
2. P1000, P200, P20 micropipettes and sterile tips.
3. 10× Resazurin solution (Alamar Blue® Assay).
4. EMJH medium (see section 2.1).
5. Sterile 96-well flat bottom microplates for absorbance or fluorescence reading.
6. Microplate reader for absorbance or fluorescence reading.
7. Plastic storage box (to accommodate 96-well microplates).
8. Microbiological incubator at 30°C.

2.4 DNA staining with Syto9 and PI

1. Safety cabinet level 2.
1. P1000, P200, P20 micropipettes and sterile tips.

2. Sterile 15 ml conical polypropylene tubes.
3. Sterile 1.5 ml polypropylene tubes.
4. 10 ml disposable serological sterile plastic pipettes.
5. Sterile ultrapure H_2O.
6. 0.5% NaCl solution: 0.5% NaCl in ultrapure sterile H_2O.
7. Water bath at 55°C.
8. Centrifuge with rotor for 2600×g.
9. Syto9 and PI nucleic acid dye mix: Prepare a mixture of 10 μM of Syto9 and 60 μM of PI in ultrapure sterile H_2O (*see* **Note 3**). Keep the stock and the mixed solutions at -20°C and protected them from light.
10. 96-well flat bottom microplates for fluorescence reading.
11. Microplate reader for fluorescence reading.

3. Methods

3.1 Preparation and treatment of a bacterial suspension

1. Inoculate EMJH medium-containing flasks or tubes with *Leptospira* cultures to achieve a final concentration of $\approx 10^7$ bacteria/ml as assessed by absorbance measurement at 420 nm ($OD_{420} \approx 0.01$-0.02) or enumerating the bacteria under a dark-field microscope with a Petroff-Hauser chamber.
2. Cultivate *Leptospira* at 30°C with shaking at 100 rpm until they reach the suitable and desirable growth phase (*see* **Note 4**).
3. If comparing different *Leptospira* strains, adjust all samples to the same bacterial concentration with EMJH medium (*see* **Note 5**).
4. Divide the bacterial suspension in different samples according to the number of treatments you want to apply to the bacterial suspension.

5. Apply the treatment to the samples, e.g. temperature shift, addition of chemicals or antibiotics, for a given time. You should have the proper control of the treatment, i.e. a sample that is not subjected to the treatment (*see* **Note 6**).

3.2 Assessing survival by determination of colony-forming unit (cfu)

The colony-forming unit method is considered as the most quantitative and linear method if the appropriate dilution is applied to the bacterial suspension.

1. Treated and non-treated *Leptospira* samples are ten-fold diluted serially. For instance, 15 µl of bacterial suspension are added to 135 µl of EMJH medium in 1.5 ml polypropylene sterile tubes. A wide range of 10-fold dilutions is necessary to determine the appropriate dilution allowing obtaining readily countable isolated colonies on agar plate (*see* **Note 7**).
2. Place 5-6 cleaned and sterile glass beads at the surface of the EMJH agar plates. Add 100 µl of the diluted treated or non-treated leptospires samples onto EMJH agar plates in duplicate and spread the cells by agitating the plates (*see* **Note 8**).
3. Allow some time for the plated bacterial suspension to penetrate the agar and remove the glass beads (*see* **Note 9)**. Wrap the plates in Parafilm to avoid drying off the medium and in aluminum foil to protect them from light and lower risk of contamination.
4. Incubate the plates at 30°C for 3-4 weeks (*see* **Note 10**).
5. Count the number of colonies on each plate and calculate the mean of the replicates (*see* **Note 11**). Multiplicate this number by the dilution factor and by 10 to obtain the number of cfu/ml. Calculate the ratio of cfu of the treated sample to the cfu of the non-treated sample. The percentage of survival after treatment is determined by multiplying this ratio by 100.

3.3 Assessing survival by measuring cellular reducing environment.

We describe the protocol to measure *Leptospira* spp. viability with the Alamar Blue® Assay (*see* **Note 12**). You can perform this assay with the bacterial suspension as prepared in section 3.1 or directly perform the cell treatment in 96-well plates, depending on the nature of the treatment or whether cell viability will be simultaneously assessed by different techniques.

1. Transfer 100 µl of treated cells or non-treated control cells into each well of a 96-well plate (*see* **Note 13**) and add 80 µl of EMJH medium to each well. Proceed directly to step 3.
2. If the treatment is being performed directly in a 96-well plate, pipette 100 µl of *Leptospira* suspension obtained at step 3 of section 3.1 into each well of the plate. Add the desired amount of the chemical used in the survival test and complete with EMJH medium to a final volume of 180 µl. Mix by pipetting and incubate the plate at 30°C for a given time.
3. Add 20 µl of the 10× resazurin solution beforehand warmed at room temperature. Mix by pipetting (*see* **Note 14**).
4. Prepare a negative control sample that does not contain *Leptospira*: pipette 180 µl of EMJH medium and add 20 µl of the 10× resazurin solution. Mix by pipetting (*see* **Note 15**).
5. Place the 96-well plate into a plastic box and incubate the plate at 30°C (*see* **Note 16**) until the reaction is completed, as seen by the appearance of a pink solution (*see* **Note 17**).
6. You can record the results simply by taking a picture (Figure 2A).

7. The quantification can be done by absorbance measurement at 570 (absorbance maximum of resorufin) and 600 (absorbance maximum of resazurin) nm (Figure 2B). The optical zero is the made using the negative control well that does not contain any cell. Percentage of resazurin reduction is calculated using the equation indicated in Figure 3 (as recommended by the manufacturer). The quantification can be also done by fluorescence measurement with an excitation and emission wavelengths of 560 nm and 590 nm, respectively (Figure 2C) (*see* **Note 18**).

3.4 Quantitative survival test based on membrane integrity

We describe the LIVE/DEAD *Bac*Light staining method to assess *Leptospira* spp. viability. In this method, the viability will be quantified by measurement of Syto9 and PI fluorescences. The ratio of green (Syto9) to red (PI) fluorescence is proportional to the relative number of live bacteria. A standard curve is necessary to verify the linearity of the assay and determine the proportionality factor between the fluorescence ratio and percentage of live bacteria.

3.4.1 Preparing a standard curve.

1. Cultivate *Leptospira* in 20 ml of EMJH medium at 30°C to reach the exponential phase and a concentration of at least 10^8 bacteria/ml.
2. Transfer 10 ml of the culture in two different sterile conical polypropylene tubes.
3. Obtain killed *Leptospira* by incubating one of the samples 2h at 55°C in a water bath while the other sample (live bacteria) is maintained at 30°C (*see* **Note 19**).
4. Harvest the bacteria by centrifugation for 15 min at 2600×g at room temperature (*see* **Note 20**).
5. Wash the bacteria twice with 10 ml of 0.5% NaCl (*see* **Note 21**).

6. Resuspend the bacteria in 10 ml of 0.5% NaCl.
7. Adjust the two samples (live and killed bacteria) to the same concentration (as assessed by absorbance measurement at 420 nm or enumeration with a Petroff-Hauser chamber under a dark-field microscope). We generally work with a bacterial suspension at about 10^8 *Leptospira*/ml.
8. Mix different proportions of killed and live *Leptospira* suspensions in sterile 1.5 ml polypropylene tubes in order to have, for instance, the following live:killed bacteria ratio: 0:100, 10:90, 25:75, 50:50, 75:25, 90:10, and 100:0.
9. In a 96-well flat bottom plate suitable for fluorescence measurement (*see* **Note 22**), mix 100 µl of bacterial suspension containing different proportions of killed and live *Leptospira* with 100 µl of Syto9/PI mixture. Mix thoroughly by pipetting up and down.
10. Incubate for 15 min at room temperature in the dark by enveloping the plate in aluminum foil.
11. Measure the fluorescence of each well with a plate reader (excitation at 485 nm and emission at 530 nm for the green fluorescent Syto9 (F530); excitation at 485 nm and emission at 630 nm for the red fluorescent PI (F630)).
12. Calculate the ratio of Syto9 fluorescence (F530) to that of the PI (F630) for each sample.
13. The standard curve is obtained by plotting the F530/F630 value for each well corresponding to the different live:killed bacteria ratio versus the percentage of live bacteria (Figure 4). A fitted linear regression method will provide with an equation to deduce the percentage of live bacteria from the F530/F630 value of any tested samples (*see* **Note 23**).

3.4.2 Survival test of a bacterial suspension

1. Harvest the bacteria (treated and non-treated control samples obtained in section 3.1) by centrifugation for 15 min at 2600×g at room temperature in conical polypropylene tubes (*see* **Note 20**).
2. Wash the cells twice with 0.5% NaCl and resuspend them in 0.5% NaCl (*see* **Note 24**).
3. Adjust all the samples to the same concentration (as assessed by absorbance measurement at 420 nm or enumeration with a Petroff-Hauser chamber under a dark-field microscope) (*see* **Note 25**).
4. In a 96-well flat bottom plate suitable for fluorescence measurement (*see* **Note 22**), mix 100 µl of bacterial suspension washed in 0.5% NaCl with 100 µl of Syto9/PI mixture. Mix thoroughly by pipetting up and down.
5. Incubate for 15 min at room temperature in the dark by enveloping the plate in aluminum foil.
6. Measure the fluorescence of each well with a plate reader as described in step 11 in section 3.4.1.
7. Calculate the ratio of Syto9 fluorescence (F530) to that of the PI (F630) for each sample.
8. You can deduce the percentage of live bacteria from the F530/F630 value using the standard curve and its proportionality factor (Figure 4, *see* **Note 23**). Alternatively, you can normalize the fluorescence ratio of the treated sample with that of the non-treated control one and calculate a percentage of live cells.

4. Notes

1. We use autoclaved glassware dedicated only to EMJH medium preparation. To avoid contaminating the glassware with components that could prevent growth of *Leptospira*, we rinse beforehand the glassware with sterile water for injection (WFI) and all the chemical stock solutions are prepared with sterile WFI.
2. We recommend pouring rather thick EMJH agar plates to avoid drying during the long storage at 30°C.
3. This Syto9/PI combination has given satisfactory results; however it might be necessary to adapt dye concentrations to the condition the assay will be performed. We have used the LIVE/DEAD™ BacLight™ Bacterial Viability Kit from ThermoFisher Scientific. This kit provides with 3.34 mM Syto9 and 20 mM PI solutions. Separated Syto9 and PI solutions allow finding the optimal dye combination for your specific assay condition.
4. We usually perform survival tests with exponentially growing *L. interrogans* serovar Manilae, which are at a concentration of about 10^8 bacteria/ml ($OD_{420} \approx 0.1$-0.3). These values might vary depending of laboratory conditions including the oxygenation level, composition of EMJH medium, and the size of leptospiral strain used. We advise the experimenter to perform a growth curve with the strain used in survival test beforehand in order to have a knowledge of its growth feature in EMJH medium in his/her laboratory condition.
5. Rigorous viability comparison implies that all bacterial samples are taken at the same growth phase.
6. If a killing kinetic is performed, the non-treated control sample can be a bacterial suspension incubated in the same conditions and for the same duration without the killing agent. A sample of the bacterial suspension before the addition of the killing agent can also serve as non-treated control sample.

7. Finding the suitable dilution for plating is crucial for the accuracy of bacterial enumeration on agar plates. Indeed, to accurately count *Leptospira* on plates, it is important to obtain isolated colonies. Also, discrepancies in counting might occur when having too few (less than 10) or too many (more than 100) colonies on plate. Generally, sample dilution will also dilute the killing chemical agent used for the survival test to sublethal concentration. However, in some cases, it might be necessary to wash the bacteria with EMJH before plating them on solid media.
8. We generally use cleaned and autoclaved 4 mm glass beads from Sigma. You can also use a glass spreader or inoculation loops to spread bacteria, but this will make handling multiple plates difficult.
9. Glass beads are reusable. Wash them thoroughly with a decontaminating solution, rinse them extensively with distilled water and autoclave them for further use.
10. It is important to ensure that the colonies will remain isolated. It might be necessary to check regularly the plates during their incubation at 30°C as *Leptospira* spp. are motile and can produce spread large and confluent colonies when overgrown on agar plate.
11. The use of a black paper sheet facilitates the visualization of *Leptospira* colonies.
12. We generally use the Alamar Blue® Assay provided by ThermoFisher Scientific Invitrogen but a resazurin solution from another manufacturer might give satisfactory results as well.
13. You have to anticipate which method will be used to quantify resazurin reduction. If fluorescence will be used, the reaction has to be performed in plates that are compatible with fluorescence measurement. We recommend using clear-bottom black-walled 96-well plates. We have obtained satisfactory results with the µClear

Black 96-well plate provided by Greiner. But, these plates are not suitable to take a picture for recording the change in color upon resazurin reduction.

14. The optimum pH for resazurin reduction is around 7.0 ***(9)***. In fact, resazurin and resorufin will be respectively pink and colorless under acidic pH. You have to ensure that the reaction mix for resazurin reduction is buffered.
15. It is crucial to verify that the chemical used as killing agent in the survival test does not interfere with resazurin reduction. This can be done by pipetting the chemical at the desired concentration into a well of the 96-well plate and completing with EMJH medium to have a final volume of 180 µl. Add 20 µl of the 10× resazurin solution and mix by pipetting.
16. The optimal temperature for resazurin reduction is 37°C but we prefer to perform the resazurin reduction reaction at 30°C to reduce evaporation. 200 µl of sterile deionized water can also be added to all outer-perimeter wells of the 96-well plate. You can also seal the 96-well plate with Parafilm for a longer incubation or storage.
17. The time for the completion of the resazurin reduction reaction depends on the number of viable cells used in the assay. The change of blue to pink color can be seen after few hours but it can also require longer incubation time (up to three days). Extended incubation times are not recommended to record the results. Indeed, the resazurin reduction into resorufin is irreversible but resorufin can be further reduced into hydroresorufin, a colorless and non-fluorescent chemical ***(8)***.
18. Resazurin reduction into resorufin is detectable only if *Leptospira* concentration is above 10^6/ml (Figure 2). A good linearity is observed between resazurin reduction and *Leptospira* concentration only between 10^6 and 10^8 bacteria/ml, as assessed by absorbance (Figure 2B) or by fluorescence (Figure 2C).

19. We have verified that two hours incubation at 55°C results in more than 99.99% of *Leptospira* killing as measured by colony-forming unit on EMJH agar plate.
20. Do not centrifuge *Leptospira* at a speed above 2600×g as it is generally believed that this might result in damage to *Leptospira*.
21. Complete removal of EMJH medium is important as we have observed that this medium interferes with fluorescence measurement of Syto9 and PI. We have also verified that incubating *Leptospira* with 0.5% NaCl did not affect their viability. Noteworthily, *Leptospira* resuspended in 0.5% NaCl cannot be used in the Alamar Blue® Assay. Therefore, if different methods are simultaneously used to assess *Leptospira* viability, bacteria will have to be treated accordingly to each assay.
22. We generally use clear-bottom black-walled 96-well plates and we have obtained satisfactory results with the µClear Black 96-well plate provided by Greiner.
23. The F530/F630 value exhibits a linear relationship with the percentage of live bacteria. The linear regression by the least-squares method gave a R square coefficient of 0.9907, indicative of a very good linearity.
24. You should expect to lose some bacteria upon washing and a decreased bacteria concentration at the end of the washing steps.
25. We have observed that a bacterial concentration below 10^7 *Leptospira*/ml is not detected by this method. We generally perform this assay with a bacterial suspension at 10^8 *Leptospira*/ml.

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Figure captions

Figure 1: Reaction of resasurin reduction into resorufin.

Resazurin (a blue reagent) is an electron receptor that is reduced into resorufin (a pink reagent) in the presence of cellular oxidoreductases and NADH.

Figure 2: Linear relationship between the number of *Leptospira* and resazurin reduction.

Exponentially growing *Leptospira* were serially diluted to obtain the indicated concentration and 100 µl of *Leptospira* were transferred in a flat bottom 96-well plates (TPP® Tissue Culture Test Plate in (A) and (B); µClear Black 96-well plate in (C)). 80 µl of EMJH medium and 20 µl of 10× resazurin solution (provided by the Alamar Blue® Assay) were added. The plates were incubated 24 h at 30°C.

(A) The change of the blue resazurin solution into a pink resorufin solution upon reduction is shown.

(B) The percentage of resazurin reduction was calculated as described in Figure 3 and plotted in function of the bacterial concentration (step 7 in section 3.3).

(C) The fluorescence was measured (λ_{exc}=560 nm, λ_{em}=590 nm) and plotted in function of the bacterial concentration (step 7 in section 3.3).

Figure 3: Calculation of percentage of resazurin reduction.

This formula is used to calculate the percentage of resazurin reduction with $E_{oxi}600$, the molar extinction coefficient of oxidized Alamar Blue at 600 nm (117216); $E_{red}570$, the molar extinction coefficient of reduced Alamar Blue at 570 nm (155677); A_{570}, absorbance of the tested well at 570 nm; A_{600}, absorbance of the tested well at 600 nm; C_{570}, absorbance of the negative control well at 570 nm; and C_{600}, absorbance of the negative control well at 600 nm.

Figure 4: Linear relationship between the percentage of live *Leptospira* and Syto9/PI fluorescence.

Live and heat-killed *Leptospira* were obtained and treated with the Syto9/PI mixture in a flat bottom 96-well plates (µClear Black 96-well plate) as described in section 3.4.1. Green Syto9 fluorescence (F530; λ_{exc}=485 nm, λ_{em}=530 nm) and red PI fluorescence (F630; λ_{exc}=485 nm, λ_{em}=630 nm) were measured. The F530/F630 values (green Syto9 to red PI fluorescence ratio) were plotted versus the percentage of live bacteria.

Acknowledgments

We would like to thank Jan Bayram for his help with Figure 1.