



**Quality assurance (QA) protocols based on  
African capacities and international existing  
standards developed**

Milestone 3.3.1

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**N2Africa**

**Putting nitrogen fixation to work  
for smallholder farmers in Africa**





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## **QUALITY CONTROL TESTS FOR RHIZOBIAL INOCULANTS**

### **1 Introduction**

Inoculation of legumes with rhizobia has the overall objective of delivering to the plant's rhizosphere the maximum number of appropriate rhizobia at the time of nodule initiation. The delivery of high quality rhizobial inoculants to farmers is one of the cardinal objectives of the N2Africa project. To achieve this, it is imperative that an effective and efficient quality control (QC) framework is put in place in every country where the project is operating. The operation of QC will ensure that inoculant packages actually contain viable rhizobia in large enough numbers to provide for a reasonable number per seed at planting. This document is intended to provide project partners with streamlined procedures for carrying out requisite tests for inoculant quality in the various countries. Many of the procedures are adapted from NifTAL publications (see Bibliography).

### **2 Minimum criteria for inoculant quality**

The inoculant being supplied for N2Africa activities should meet the following criteria:

1.  $10^9$  cells/g of viable rhizobia.
2. Less than  $10^6$  contaminant organisms per gram inoculant.
3. Must include data on plant infection tests.
4. Specification on package should contain the above information as well as specify the crop for which it is meant, the manufacturer's details, storage and transport conditions, composition of the carrier, expiry date, recommended dose and instruction on how to apply the inoculant.

### **3 Quality tests**

Quality tests should include a plate count on yeast extract mannitol agar (YMA) containing Congo red (for viable counts of rhizobia and observation of contaminants) and a plant infection test. Enumeration of contaminants can be achieved through a plate count on peptone glucose agar.

#### **3.1 Viable cell counts (plate count methods)**

Viable rhizobial cell numbers in inoculants prepared with sterile carrier can be measured by plate count methods. Most inoculant prepared using non-sterile carrier contains so many fast-growing contaminants that plate count procedures are impractical. Plate count methods involve placement of a sample containing viable cells onto the nutrient media surface and



counting the resulting colonies after a period of incubation. Since rhizobial inoculants are expected to contain a very large number of viable cells, it is necessary to dilute the sample accurately prior to application to the media so that a countable number of discreet colonies will grow. The most widely used media for counting rhizobia is yeast-extract mannitol agar (YEMA or YMA), often containing Congo Red to assist in differentiating contaminants from rhizobia.

### 3.1.1 Serial dilutions

The first step in counting viable cells in rhizobial inoculant is to prepare a dilution series which will cover the range of expected viable cells. The most commonly used dilution ratio is 1:10 per dilution step. This is equivalent to 10 g (wet weight) of inoculant added to 90 mL diluent (or 11 g inoculant into 99 mL).

The dilution procedure must be a systematic and accurate sub-division of an inoculant. Consequently, transfer volumes must be removed prior to settling and the dilutions shaken prior to transfer onto plates. The bottle for the initial inoculant suspension should accommodate the addition of at least 11 g inoculant to 99 mL (a 1:10 dilution) diluent and still have adequate room for good mixing by shaking.

The dilution series is prepared as follows:

Prepare the diluent using sterile phosphate-peptone buffer of the following composition per 1 L distilled water: 1.0 g of Peptone; 0.34 g of  $\text{KH}_2\text{PO}_4$ ; and 1.21 g of  $\text{K}_2\text{HPO}_4$ . The pH of the final preparation should be adjusted to 7.0.

- i. An alternative diluent to phosphate-peptone solution is to use a one-quarter strength solution of the mineral salts present in yeast extract mannitol broth (the yeast extract and mannitol are omitted). The pH of diluents must be monitored and aseptically adjusted to near neutrality, if necessary.
- ii. Mix the inoculant thoroughly within its bag. Aseptically remove the inoculant (e.g., with a flamed and cooled spatula), and weigh out 11 g if using 99 mL dilution blanks (10 g if using 90 mL dilution blanks).
- iii. Suspend the inoculant in sterile diluent and place the bottle on a wrist action shaker for 10 minutes of vigorous mixing. Adjust the bottle horizontally on the shaker so that the suspension is hitting both ends of the bottle on each cycle. This represents the first dilution step ( $10^{-1}$ ).
- iv. Carry out the 10-fold dilution series to the required level. Serially dilute the final 10-



fold dilution by pipetting 1-5 mL (transfer volume) into sterile diluent to provide six serial 5-fold dilutions.

- v. The appropriate amount of diluent can be calculated as follows:

$$\text{Diluent volume} = [(\text{dilution ratio} - 1) \times \text{transfer volume}]$$

For example, for a dilution ratio of 5 and a transfer volume of 2 mL, the diluent volume is  $(5-1) \times 2 = 8$  mL.

### 3.1.2 Plating

Prepare serial 10-fold dilutions through the highest dilution required, and plate only those dilutions that are needed to enumerate cells within the range of interest. For the drop plate method, it is recommended to plate  $10^{-4} - 10^{-7}$ , while the  $10^{-5} - 10^{-7}$  dilutions are sufficient for the spread plate technique. Preparation of the inoculant dilution series is the same for drop or spread plate techniques.

Keep in mind that the accuracy of the plate count is dependent upon starting with a representative sample that is accurately diluted. Mix each dilution well and do not allow settling to take place before removing the chosen volume for the next dilution. Use a fresh pipette or pipette tip for each dilution level. Do not let sample dilutions stand for any longer than necessary before plating. Time the work so that it can be completed, from drawing of the sample to putting the plates in the incubator, in a single work session. Until considerable experience has been gained, work with one sample (start to finish) at a time.

#### 3.1.2.1 The drop plate technique

The drop plate technique involves dropping known volumes (usually 20–30  $\mu\text{L}$ ) of sample dilutions onto YEM agar plates. The drops are not spread, but are allowed to absorb into the agar surface. Resultant colonies are counted from the dilution which produces the largest number (usually about 20) of discreet non-confluent colonies. The plate is usually marked into eight sections to receive eight drops. Four replications of two different 10-fold dilutions can be accommodated on a single plate (see Fig. 1). The drop plate technique uses agar plates considerably more efficiently than the spread plate method. Experienced users report that more practice is needed to obtain consistent results with the drop plate technique than with the spread plate technique. If, however, a Pasteur pipette (using disposable tips and measuring accurately within the 20–30  $\mu\text{L}$  range) is used, the advantages in time and material saving can be considerable.

The drop plate procedure is as follows:



1. Prepare appropriate media and plates. It is important that the surfaces of the plates are not "wet." Plates prepared a few days in advance will have the chance to "dry." Mark the plates (on the bottom) into 8 equal pie shaped sections and label them appropriately.
2. From the most dilute of the chosen range of dilutions ( $10^{-7}$  dilution), drop 25  $\mu\text{L}$  of sample about 2.5 cm from the edge of the plates and from a height of about 2 cm. Replicate each dilution 4 times (4 drops individually delivered to 4 pie shaped sections).
3. Using a fresh sterile pipette tip and the next least dilute sample suspension ( $10^{-6}$ ), repeat step 2 above, filling the remaining 4 sections of the plate. The plate now has eight drops in total (4 replicate drops from each of two successive dilutions). Do not spread the drops. Leave the plate face up until the sample drops have been completely absorbed into the agar.
4. Repeat steps 2 and 3 above on a second plate, using the other two dilution levels selected for plating ( $10^{-5}$  and  $10^{-4}$  dilutions) . Two plates will accommodate 4 dilutions of four replicates each.
5. Allow all sample drops to be absorbed completely, then invert and incubate the plates.
6. Obtain counts from the dilution level drops which show the largest number of discreet colonies free of confluent growth. Count from the "underside" of the plate, marking the position of each colony with a marker as it is counted.
7. Average the counts of the four replicate drops and calculate the number of viable cells in the original sample by accounting for the sample dilution and the volume of the drops used. For example, if the average colony number is 20, the volume of the drops is 25  $\mu\text{L}$ , and the dilution level is  $10^{-5}$  then:  $20 \times 1000/25 \times 10^5 = 8 \times 10^5$  viable cells per gram.

### **3.1.2.2 The spread plate technique**

The spread plate is the most reliable and commonly used method of plate count enumeration of rhizobia. The method involves spreading a known volume (usually 100  $\mu\text{L}$ ) of a known dilution of sample over the agar solidified surface of a nutrient media. Following incubation for growth, the resulting colonies are counted at a dilution yielding 30–300 colonies per plate. Since plating at each dilution level is normally replicated 3–5 times, the amount of material and time invested plating and counting can become considerable.



The spread plate procedure is as follows:

1. Prepare the required number of YEMA plates and sterile spreaders ("hockey sticks"). The plates should not be "wet" on the surface. Label all plates with a standard nomenclature
2. Prepare 10-fold dilutions of the sample  $10^{-1}$ – $10^{-7}$ .
3. Transfer 100  $\mu$ L of the highest dilution to be plated ( $10^{-7}$ ) to each of three replicate plates. Have sterile, cool "hockey stick" spreaders ready. A 100  $\mu$ L Pasteur pipette using disposable tips is much better for sample delivery than attempting to hand deliver 0.1 mL volumes from a 1 mL pipette. Use a fresh, sterile pipette tip for each dilution level.
4. Minimize the time that the samples are on the plate before spreading. Spread the sample over the agar surface with a sterile spreader. Move the spreader back and forth in a straight line while rotating the plate on a turntable for approximately 10 seconds. Do not allow the tip of the spreader to deliver sample liquid to the extreme edge of the media where it touches the Petri dish. Do not let the spreader dig into the media.
5. Repeat steps 3 and 4 for the selected number of dilutions to be plated working from the most dilute sample to the least dilute. Use a sterile spreader for each dilution level.
6. Allow the plates to stand right side up until the sample liquid is completely absorbed, then invert the plates for incubation.
7. After an appropriate incubation period, count the colonies on the replicate plates of the dilution showing discreet colonies in the countable range of 30–300. Do not count the plates too early. **If the colonies seem to have grown up too fast, they are probably not rhizobia.**
8. Calculate the number of viable cells in the original sample by accounting for all dilutions and for the volume plated. For example, if the average number of colonies from plating 100  $\mu$ L per plate from the  $10^{-5}$  dilution is 62, then  $62 \times 10 \times 10^5 = 6.2 \times 10^7$  viable cells per gram. Use the mean from the three replicate plates.



### 3.1.3 Reagents for yeast-extract mannitol agar (YMA) media

**Table 1.** Concentrated stock solutions for preparation of YMA

|                         |               |                             |             |
|-------------------------|---------------|-----------------------------|-------------|
| 1. $K_2HPO_4$           | 25.0 g/500 mL | 6. $H_3BO_3$                | 50mg/500mL  |
| 2. NaCl                 | 10.0 g/500 mL | 7. $ZnSO_4$                 | 50mg/500 mL |
| 3. $CaSO_4 \cdot 2H_2O$ | 10.0 g/500 mL | 8. $CuSO_4 \cdot 5H_2O$     | 25mg/500 mL |
| 4. $MgSO_4 \cdot 7H_2O$ | 10.0 g/500 mL | 9. $MnCl_2 \cdot 4H_2O$     | 25mg/500 mL |
| 5. Fe-EDTA              | 1.25 g/25 mL  | 10. $Na_2MoO_4 \cdot 2H_2O$ | 5mg/500 mL  |
| (Sequestrene)           |               |                             |             |

To prepare YMA, add to 950 mL of purified water 10 mL of stock concentrates 1, 2, 3, 4, 6, and 0.2 mL of stock concentrate 5.

Add mannitol, yeast extract, and if desired, agar,  $CaCO_3$ , and Congo red.

**Table 2.** YMA rhizobial plate-count media.

| Nutrients            | g per litre | Micronutrients          | mg per litre |
|----------------------|-------------|-------------------------|--------------|
| $K_2HPO_4$           | 0.5         | $H_3BO_3$               | 1.0          |
| NaCl                 | 0.2         | $ZnSO_4$                | 1.0          |
| $CaSO_4 \cdot 2H_2O$ | 0.1         | $CuSO_4 \cdot 5H_2O$    | 0.5          |
| $MgSO_4 \cdot 7H_2O$ | 0.2         | $MnCl_2 \cdot 4H_2O$    | 0.5          |
| Mannitol             | 10.0        | $Na_2MoO_4 \cdot 2H_2O$ | 0.1          |
| Yeast extract        | 2.0         | Fe-EDTA (Sequestrene)   | 10.0         |
| Agar                 | 18.0        |                         |              |

Adjust pH to 7.0 with 1N HCl. To add Congo red, add 10 mL of a 1:400 (0.1 per 40 mL  $H_2O$ ) aqueous solution of dye to the media before autoclaving.



## 3.2 Peptone-glucose test for contamination on plates

Almost all rhizobia grow poorly, if at all, on glucose-peptone media, whereas many potential contaminants grow readily and produce pH changes. Bromthymol blue (BTB) and bromcresol purple (BCP) are pH indicators. If either dye is incorporated in peptone-glucose media it will indicate major pH shifts which are often associated with growth of contaminating organisms, but not with rhizobia (because the rhizobia do not grow). Bromthymol blue turns yellow at pH 6.0, blue at pH 7.6, and is green between pH 6.0 and 7.6. Bromcresol purple turns yellow at pH 5.2 and purple at pH 6.8. Rapid growth (24 -48 hours) of streaked culture on this media, particularly if associated with colour change (pH reaction), strongly indicates contamination.

### 3.2.1 Composition of Peptone-glucose media

- 1000 mL purified water
- 5 g glucose
- 10 g peptone
- 15 g agar
- 10 mL of 1% bromcresol purple in ethanol (0.10 g BCP in 10 mL EtOH) or
- 5.0 mL of 0.5% BTB

### 3.2.2 Plating on peptone glucose agar (PGA)

Enumeration of contaminants on peptone glucose agar is done using the spread plate method. Prepare the required number of PGA plates and prepare 10-fold dilutions of the sample  $10^{-1}$ – $10^{-7}$ . Proceed with plating as explained for the spread plate method. Contaminants should not exceed  $10^6$  per gram of inoculant.

## 3.3 Enumeration of rhizobia in inoculant using the most probable number (MPN) plant infection assay

The most-probable-number (MPN) technique is a means to estimate microbial population sizes. The technique is widely used to enumerate rhizobia based upon the ability of rhizobia to nodulate appropriate host legume plants. The method relies upon the pattern of positive and negative nodulation responses of host plants inoculated with a consecutive series of dilutions of rhizobia containing sample suspension. The results are used to derive a population estimate based upon the mathematics of Halvorson and Zeigler (1933). MPN enumeration of rhizobia is dependent upon the ability of the researcher to grow the legume hosts in a healthy and replicated fashion, and to keep the plants free from rhizobial contamination for a period of up to 4 weeks.



### 3.3.1 Designing an MPN assay

The process of designing an MPN assay for legume inoculants involves selecting the degree of initial dilution of the sample, base dilution ratio of the serial dilutions applied to plants, number of serial dilution steps applied to plants, number of replicate plants to be inoculated at each dilution level, and volume of inoculant to be applied to each plant unit. Care must be taken to design MPN assays for which population estimates can be obtained through MPN tables. Standard MPN tables do not provide estimates for all dilution ratio-replicate combinations.

The base dilution ratio and the number of replicate plants per dilution level are used in the calculation of a confidence factor which describes the reliability of the MPN result. The population estimate is multiplied or divided by the confidence factor to establish the upper and lower limits, respectively, of the confidence interval for the population estimate. Decreasing the base dilution ratio or increasing the number of replicate plants per dilution level results in a narrowing of the range of the confidence interval and a greater resolution of the MPN estimate. Conversely, increasing the base dilution ratio or decreasing replication results in broader confidence intervals. Confidence factors associated with different dilution ratios and replicate combinations are presented in Table 3. For two population estimates to be significantly different from one another, the lower limit of the greater population must be higher than the upper limit of the lesser population.

Altering the volume of inoculant applied to the root system of the host legume is another option in the design of plant-infection assays. Most published MPN tables assume a 1.0 mL inoculant volume. An MPN population estimate can be obtained from those tables after applying greater or lesser inoculant volumes by using the relationship:

$$\text{Population estimate} = (1 / \text{inoculant volume}) \times \text{tabular estimate}$$

For example, if an inoculant volume of 2 mL is applied to the root system, the population estimate is half that of the tabular MPN estimate. Similarly, if 0.5 mL is applied, the population estimate is twice that of the tabular value. Increased inoculant volumes (greater than 1 ml) are useful when larger plant growth containers are used, or when the researcher wants to lower the range of population detection. Decreased inoculant volumes (less than 1 ml) are useful when host plants are grown in small culture tubes or when the researcher wants to extend the upper range of population detection. Adjustments in the inoculant volume do not affect the confidence factor.



**Table 3.** Factors for calculating the confidence intervals of Most-Probable-Number estimates

| Replicates per dilution | Factor for 95% confidence interval at various dilution ratios <sup>1</sup> |      |      |      |       |
|-------------------------|--|------|------|------|-------|
|                         | 2  | 3    | 4    | 5    | 10    |
| 1                       | 4.01   | 5.75 | 7.14 | 8.31 | 14.45 |
| 2                       | 2.67   | 3.45 | 4.01 | 4.47 | 6.61  |
| 3                       | 2.23   | 2.75 | 3.11 | 3.40 | 4.67  |
| 4                       | 2.00   | 2.40 | 2.67 | 2.88 | 3.80  |
| 5                       | 1.86   | 2.19 | 2.41 | 2.58 | 3.30  |
| 6                       | 1.76   | 2.04 | 2.23 | 2.37 | 2.98  |
| 7                       | 1.69   | 1.94 | 2.10 | 2.23 | 2.74  |
| 8                       | 1.63   | 1.86 | 2.00 | 2.11 | 2.57  |
| 9                       | 1.59   | 1.79 | 1.93 | 2.03 | 2.44  |
| 10                      | 1.55   | 1.74 | 1.86 | 1.95 | 2.33  |

<sup>1</sup> Population estimates are multiplied and divided by the confidence factors to establish the upper and lower confidence intervals at ( $P = 0.05$ ), respectively. Confidence factors were calculated using MPNES software (Woomer *et al.*, 1990) after Cochran (1950).

### 3.3.2 MPN procedure for inoculant evaluation

The following is a commonly used MPN design for the evaluation of legume inoculant products. Using growth pouches and a suitable supporting rack, prepare 30 pouches (also called growth units) with a suitable number of healthy host plants per pouch and sterile N-free nutrient solution (30 mL for whole pouches, 15 mL per side for split pouches). This makes six sets of 5 growth units.

- i. Taking a representative sample of the inoculant, dilute the sample by successive 10-fold dilutions (in sterile diluent) to a suitable starting point based on the anticipated number of rhizobia in the product. Given an anticipated rhizobial cell number of greater than  $10^9$  per g, a suitable starting point for further dilutions would be a dilution of  $10^{-6}$ .
- ii. From the  $10^{-6}$  dilution make an additional six serial 5-fold dilutions in sterile diluent (the initial dilution is  $10^{-6}$  the base dilution ratio is 5).
- iii. Apply 1.0 mL to each of four replicate growth units from each of the six 5-fold dilutions. Leave the 5<sup>th</sup> growth unit in each series uninoculated. Whether one works



from the most dilute of the 5-fold dilutions to the least dilute, or vice versa, depends upon the technique used to apply the inoculant suspension, but in any case work in order, and be consistent.

- iv. Place the growth-unit rack containing the MPN assay in an appropriate growth environment and maintain suitable plant condition by watering with sterile water.
- v. After 3-4 weeks (depending upon the legume host used) examine the plants and record each growth pouch unit as "+" or "-" for nodulation. Even one nodule per growth unit means a "+" score for that unit. Do not count "maybe" nodules. All six negative control units must be free of nodules (if not, discard the test, repeat, and work on technique).
- vi. For each of the six successive 5-fold dilutions a number of pouches between 0 and 4 will have been scored as "+" for nodulation. The number of "+" scores taken in order of increasing dilution will yield a six digit number. A typical result could be: 4, 4, 4, 1, 1, 0.
- vii. Using either the MPNES computer program or an MPN probability table based on six 5-fold dilutions, 4 replicates, and 1.0 mL volume applied per growth unit, determine the population estimate and 95% confidence limits. This estimate refers to the number of rhizobia present in 1 mL of the suspension from which the first 5-fold dilution applied to the plants was made. Multiply the MPN estimate by the reciprocal of the dilution level of the inoculant suspension prior to beginning the 5-fold serial dilutions (in this case  $10^6$ ) to get an estimate for the number of viable rhizobia in the original inoculant. For the example given above the estimate is  $379 \times 10^6 = 3.79 \times 10^8$  rhizobia per g.

## References

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- Vincent, J. M. 1970. A manual for the practical study of the root-nodule bacteria. International Biological Programme Handbook No.15. Blackwell Scientific Publications, Ltd., Oxford.
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which this compilation has been made.



## List of project reports

1. N2Africa Steering Committee Terms of Reference
2. Policy on advanced training grants
3. Rhizobia Strain Isolation and Characterisation Protocol
4. Detailed country-by-country access plan for P and other agro-minerals
5. Workshop Report: Training of Master Trainers on Legume and Inoculant Technologies (Kisumu Hotel, Kisumu, Kenya-24-28 May 2010)
6. Plans for interaction with the Tropical Legumes II project (TLII) and for seed increase on a country-by-country basis
7. Implementation Plan for collaboration between N2Africa and the Soil Health and Market Access Programs of the Alliance for a Green Revolution in Africa (AGRA) plan
8. General approaches and country specific dissemination plans
9. Selected soybeans, common beans, cowpeas and groundnuts varieties with proven high BNF potential and sufficient seed availability in target impact zones of N2Africa Project
10. Project launch and workshop report
11. Advancing technical skills in rhizobiology: training report
12. Characterisation of the impact zones and mandate areas in the N2Africa project
13. Production and use of Rhizobial inoculants in Africa
18. Adaptive research in N2Africa impact zones: Principles, guidelines and implemented research campaigns
19. Quality assurance (QA) protocols based on African capacities and international existing standards developed



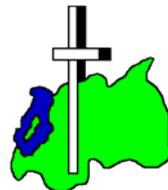
## Partners involved in the N2Africa project



Caritas Rwanda



Diobass



Eglise Presbiterienne Rwanda



Resource Projects-Kenya



Université Catholique de Bukavu



University of Zimbabwe

