International Seed Federation
Seed is Life

Best Practices for Dilution Plating Assays in Seed Health Tests
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This document describes best practices for the use of dilution plating assays in seed health testing to ensure accurate and reliable results. Best practices include process controls and assay conditions that should be applied to all trials.

Controls and conditions are either designated as essential (must/shall be included) or recommended (can be included).

I. Dilution Plating: A direct test for isolating pathogenic bacteria

Dilution plating is a direct test for isolating pathogenic bacteria from a seed sample. Different non-selective and semi-selective media are used to recover suspect pathogen colonies. Suspect colonies are identified by confirmation assays such as PCR and/or confirmed as pathogenic via pathogenicity assays. In some cases, dilution plating is used to confirm pathogen viability for indirect molecular pre-screen assays.

Process controls and assay conditions in this document are described for routine dilution plating assays used for isolating suspect bacterial pathogens.

II. Controls and their Purpose

The types of controls for dilution plating assays are defined in Table 1. Their purpose is to verify both the quality of the material used in the assay and proper test execution. Treated seeds, seed that has been treated using physical or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection can be tested if an inhibition (spike) control is included to rule out assay failure due to chemical or biological inhibition. If a lab chooses to test seeds treated with protective chemicals or biological substances, it is the lab's responsibility to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on the method results. Proper negative and positive controls should be included in every assay to ensure reliable test results.

Unless exceptions are stated, the following controls should be applied to all bacterial plating methods.

Table 1: Controls to be included in routine dilution plating assays

Control type	**Negative control (NC) – Recommended**
Definition	The extraction buffer used for seed samples. This control contains no targets, related, or saprophytic organisms.
Expected Result	No recovery of the target pathogen(s) or saprophytes on semi-selective and/or non-selective media, or both.
Description	The NC checks for contamination of the extraction buffer.

Note: The scope of this document has been revised to include best practices for the routine use of dilution plating assays in seed health testing and pertains to bacterial pathogens alone. It has been reformatted and the presentation modified to improve readability. Version 2.1 includes some editorial changes.

Control type	Negative process control (NPC) – Essential
Definition	This is either extraction buffer or a negative seed sample that is processed in the same way and at the same time as the test samples but contains no target organism or spike. The extraction buffer used is the same as the one used for seed samples.
Expected Result	No recovery of the target pathogen(s) on both semi-selective and non-selective media.
Description	The NPC checks for cross-contamination of extraction buffer, plating materials, and lab equipment.

Control type	Inhibition (Spike) Control – Recommended
Definition	Target or related pathogen added to seed sample extracts and recovered on medium at a predetermined concentration defined by test method or characterized laboratory glycerol stock.
Expected Result	A minimum of 50% of the number of CFU of the bacterium spiked into the aliquot drawn from seed subsamples should be recovered on the spiked plates unless protocol specifies threshold of the recovered target.
Description	It is recommended that all dilution plating protocols include a spiking control to assess potential inhibition of the target organism by seed background (e.g. chemical residues if treated and saprophytic load if not treated). Three options are available for a spiking control: 1. Spiking of the target pathogen into a separate aliquot of each subsample of homogenized seed extract and plated separately and in parallel to the seed subsamples 2. Spiking of a related organism into subsamples of homogenized seed extracts for plating 3. Early spike into the seed subsample with a related organism. **Note**: The use of isolates for spike, either target or related pathogen, carries some risk in that the isolate may respond differently than the target pathogen. The use of a target or related pathogen requires that the isolate be well characterized prior to use and have shown responses similar to the target pathogen.

Control type	Positive Process Control (PPC) - Positive Seed – Recommended
Definition	A well characterized seed lot naturally or artificially infected with the target pathogen that is processed in parallel with seed samples.
Expected Result	Detection of the target pathogen within expected range.
Description	The performance of the positive process control serves to evaluate the entire assay.

Control type	Positive Control (PC) - Pure Culture – Essential
Definition	Plate a pure culture dilution in extraction buffer of a reference isolate, target pathogen, on all media used in the assay at the same time as the samples.
Expected Result	The concentration used should yield distinct colonies where morphology of the target pathogen is visible.
Description	The pure culture control ensures that the reference isolate, target pathogen grows on the media and in the conditions used in the test. It also serves as reference for target colony morphology when evaluating assay plates.

III. Assay Set-up

The essential and recommended conditions for the setup of a routine dilution plating assay are described in table 2.

Table 2: Set-up and determination of results of a dilution plating assay

Description	Essential	Recommended
Plating volumes: All dilution plating protocols shall plate a minimum of 0.1 ml of seed extract and serial diluted extract per plate.	x	
Replicate plates: All dilution plating protocols should have 2 replicate plates for each dilution (or concentration) on all media types.	x	
Unreadable Plates: Any plate that is considered 'unreadable' reduces the confidence in finding the target organism if present. Plates are considered "unreadable" when the target organism is not detected **and** one or more of the circumstances described below occur: 1. Inhibition (Spiked) Control plates: If recovery (CFU) of spike in seed extract is < 50% of the expected number of colonies of reference isolate added to the buffer control on the same media, or if recovery of reference isolate is less than that defined in protocol. 2. Non-spiked plates: If saprophytic bacteria are at a concentration that yields more than 250 CFU on medium (visually assessed). 3. Non-spiked plates: If saprophytes cover more than 50% of the plate surface.	x	
Suspect colony confirmation: Select suspect colonies by comparing colony morphology of target colonies from the positive control and the inhibition control keeping in mind that colonies from pure culture and seed extract may present differently (e.g. color, size, shape, etc.). If any molecular assays are used to confirm suspect identity then include all the appropriate controls defined in ISHI-Veg's Best Practices for PCR Assays in Seed Health Tests. If pathogenicity assays are used to confirm suspect identity, use appropriate controls, hosts and environmental conditions and refer to ISHI-Veg's Best Practices for Biological Assays in Seed Health Tests (https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/).	x	

IV. Optimization of media and spike controls

Certain components of dilution plating can greatly influence the performance and outcome of the test. The components described in Tables 3a and 3b must be controlled for quality and validated prior to use in routine seed health testing.

i. Evaluation of media

The performance of a fresh media batch (i.e. in-house or pre-made commercial) must be checked with well-characterized target pathogens prior to or during its use in the assay.

Table 3a: Components of dilution plating testing – Evaluation of media

Description	Essential	Recommended
Detection of isolates: Plate well-characterized strains of the target pathogen that represent the population variation. Familiarity with different morphologies (e.g. color, shape, growth rates and requirements) is important for detection of pathogens when variation has been reported. If there is no morphological variation one strain is sufficient to evaluate overall growth and morphology.	x	
Colony growth and morphology: Generate single colonies by dilution streaking or dilution plating to check size, growth rate, and morphology.	x	
Percent suppression relative to a non-selective media: Dilutions of a pure culture are plated on both the semi-selective medium and a non-selective medium (such as King's B or Nutrient Agar) and suppression of colony growth relative to the non-selective media should be determined. Acceptance of the suppression level in the target media is established in individual labs based on their own quality standards.		x

ii. Preparation and performance of spike control

The following conditions are to be considered for the performance of the spike control.

Table 3b: Components of dilution plating testing – Preparation and performance of spike control

Description	Essential	Recommended
Preparation of the spiking solution: The spike suspension is preferably made from fresh cultures from agar plate or liquid media. It can also be prepared from a 20% glycerol stock stored at -20°C or -80°C (preferred). Check the concentration of glycerol stocks and recovery of glycerol stocks in seed extract or buffer before use; recovery may be lower in buffer.		x
Concentration of the spiking control: The desired concentration of the target for the spiking control is 20-100 CFU per 0.1 ml. of seed extract on the semi-selective media unless the protocol specifies the threshold of spike recovery.		x
Dilution of the spiking solution: One (1) part spiking solution to nine (9) parts seed extract is the preferred dilution of the spiking solution.	x	

Description	Essential	Recommended
Recovery rate of spiking solution: Recovery (CFU) of spike in seed extract is at least 50% of the expected number of colonies of reference isolate added.	x	
Cross-contamination: In order to minimize the chances of cross contamination, it is essential to separate, in space and/or time, the preparation of the spiking solution and further processing of the spiked samples from the processing of the regular samples. An antibiotic resistant strain of the target pathogen could be used to rapidly identify possible false-positives due to cross-contamination.	x	