International Seed Federation
Seed is Life

Best Practices for Dilution Plating Assays in Seed Health Tests

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DEVELOPED BY
International Seed Health Initiative (ISHI) of the International Seed Federation (ISF)

BEST PRACTICES AND THEIR PURPOSE

The following Best Practices have been developed to ensure accurate and reliable results when performing Dilution Plating Assays and to provide general guidance to laboratories developing and using seed health tests.
These guidelines are not intended to serve as a legal reference. They are not binding on ISHI nor ISF members.

DISCLAIMER

This document has been developed based on the current technical state of the art. ISF cannot be held liable for any possible claims associated with the application of these guidelines.

INTRODUCTION

This document describes best practices for the use of dilution plating assays in seed health tests to ensure accurate and reliable results. Best practices include process controls and assay conditions that should be applied to all experiments.

Controls and conditions of the assays are designated either as essential (necessary to perform for proper test execution), recommended (advised to perform for proper test execution) or optional (not necessary to perform for proper test execution).

1. DILUTION PLATING: A DIRECT TEST FOR ISOLATING PATHOGENIC BACTERIA

Dilution plating is an assay in which a seed extract is plated onto semi-selective media that allow for the cultivation of the target pathogen and its evaluation by visual examination/evaluation to assess the pathogen's growth and morphology. 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.

Treated seeds, seed that have 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 laboratory chooses to test seeds treated with protective chemicals or biological substances, it is the laboratory'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 performance.

2. 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 assays and proper test execution. Appropriate negative and positive controls should be included in every assay to ensure reliability of the test results. When handling the positive controls, it is important to ensure no cross-contamination occurs. As such, it is recommended to handle the positive controls last after setting up the negative controls and sample (e.g., use filter tips when using a dissolved reference isolate).

Table 1. Controls defined for dilution plating assays.

	Non Target Control (NTC)
Definition	Control that contains media and/or reagents used to prepare the sample that is tested and processed using the same assay at the same time as the samples. This control contains no extraction buffer, no target pathogen, and no spike.
Objective	To verify the reagents used are free from the target in the absence of extraction buffer and samples.
Expected Result	No detection of any pathogen and saprophyte, including the target.
Description	Agar plate free from any buffer, pathogen, or seed.
Dilution Plating	Recommended

	Negative Buffer Control (NBC)
Definition	Control that contains all buffers and reagents used to prepare the sample that is tested and processed using the same assay at the same time as the samples. This control contains no target pathogen and no spike.
Objective	To verify the sterility of the extraction buffer used in the absence of samples.
Expected Result	No detection of any pathogen, including the target.
Description	Extraction and dilution buffers.
Dilution Plating	Essential

	Negative Process Control (NPC)
Definition	Control that contains a well characterized healthy seed sample (with respect to the target pathogen) that is tested and processed using the same assay at the same time as the samples.
Objectives	To verify that no cross-contamination occurred under the assay conditions.
Expected Result	No detection of the target pathogen.
Description	Seed sample that contains no target pathogen.
Dilution Plating	Recommended

	Positive Control (PC)
Definition	Control that contains a known (isolated) target pathogen in the absence of seed that is tested and processed using the same assay at the same time as the samples.
Objective	To verify that the target pathogen can be detected under the assay conditions in the absence of a seed matrix.
Expected Result	Detection of distinct colonies of the target pathogen allowing for the observation of the typical morphology.
Description	Extraction buffer spiked with a known pathogenic reference isolate of the target pathogen.
Dilution Plating	Essential

Note: The reference isolate can be obtained from an external supplier.

	Positive Process Control (PPC)
Definition	Control that contains a well characterized positive seed sample, naturally infected or artificially contaminated with the target pathogen and is tested and processed using the same assay at the same time as the samples.
Objective	To verify that the target pathogen can be detected under the assay conditions in the presence of a seed matrix.
Expected Result	Detection of the target pathogen within expected range.
Description	A seed sample that contains the target pathogen **OR,** A seed extract spiked with living target pathogen or infected plant extract.
Dilution Plating	Recommended

	Inhibition Control
Definition	Control that contains an internal spiking of a predetermined quantity of an isolated target pathogen or related species, added directly to the seed samples that is tested and processed using the same assay at the same time as the samples.
Objective	To verify that no inhibition due to the sample seed matrix (e.g., chemical residues if treated and saprophytic load if not treated) occurs under the assay conditions.
Expected Result	Detection of the spiked pathogen within pre-determined expected range (i.e., either a minimum of 50% of the number of colony forming units (CFU) of the total bacterium spiked or a pre-determined specific threshold of the recovered target).
Description	Spiking of a predetermined quantity of target pathogen into a split subsample of the sample being tested ("early spike") **OR** Spiking of a predetermined quantity of a related species of the target pathogen into the sample being tested ("early spike") **OR** Spiking of a predetermined quantity of target pathogen into the seed extraction sample being tested after the seed extraction step ("late spike").
Dilution Plating	Recommended

Note: It is essential to use this control in case of testing disinfected and/or treated seeds.
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. When a Look-A-Like (LAL) is used as a non-target pathogen, the LAL resembles the target pathogen based on morphology on (semi-selective) growth media and genetic features. Use well-characterised strain(s) of the target that represent the population variations or related pathogen that give similar responses to the target pathogen (e.g., colour, shape, growth rates and requirements) and genetic features.

3. 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.	×	
Replicate plates: All dilution plating protocols should have two replicate plates for each dilution (or concentration) on all media types.	×	
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: • Inhibition (Spiked) Control plates: If recovery of spike in seed extract, in terms of number of colony forming unit	×	

Description	Essential	Recommended
(CFU), 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. • Non-spiked plates: If saprophytic bacteria are at a concentration that yields more than 250 CFU on medium (visually assessed). • Non-spiked plates: If saprophytes cover more than 50% of the plate surface.		
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., colour, size, shape, etc.). If any molecular assays are used to confirm suspect identity, then include all the appropriate controls defined in ISHI 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 as described in ISHI Best practices.	×	

4. OPTIMISATION 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.

4.1. 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 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., colour, 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.	×	
Colony growth and morphology: Generate single colonies by dilution streaking or dilution plating to check size, growth rate, and morphology.	×	
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 laboratories based on their own quality standards.		

4.2. 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% (w/v) 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.		×
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.		×
Dilution of the spiking solution: One part spiking solution to nine parts seed extract is the preferred dilution of the spiking solution.	×	
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.	×	
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.	×	

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