Feb 19, 2025

Impranil Activity Assay for Plastic Degrading Enzymes

DOI
https://dx.doi.org/10.17504/protocols.io.14egn9bxml5d/v1

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Protocol Citation: Joao Vitor Molino, bsaucedo@ucsd.edu 2025. Impranil Activity Assay for Plastic Degrading Enzymes. **protocols.io** https://dx.doi.org/10.17504/protocols.io.14egn9bxml5d/v1

Protocol status: Working
We use this protocol and it's working

Created: October 23, 2024

Last Modified: February 19, 2025

Protocol Integer ID: 110627

Keywords: Plastic degrading, Plastic degradation, Enzymatic assay, Polyester, Polyurethane, impranil activity assay for plastic degrading enzyme, plastic degrading enzyme, impranil activity assay, enzyme activity on plastic substrate, assessing enzyme activity, degrading enzyme, enzyme sample, plate with the enzyme sample, impranil, assay, plastic substrate, impranil depletion, impranil depletion over time, fda

Funders Acknowledgements:
US Department of Energy
Grant ID: DE-EE0009671

Abstract

This protocol outlines an Impranil® DLN activity assay for plastic-degrading enzymes, offering an alternative to the FDA assay with improved resistance to esterase contamination. The assay involves checking cell density, preparing a 96-well plate assay with Impranil, incubating the plate with the enzyme sample, and analyzing the data. Key measurements include absorbance and chlorophyll fluorescence at specific wavelengths before and after incubation. Data analysis involves calculating delta variations, normalized activity, and Impranil depletion over time, providing a comprehensive approach for assessing enzyme activity on plastic substrates.

Guidelines

- **Cell Culture Handling:**
- Ensure that cell cultures are grown under optimal conditions before sampling to avoid variability in enzyme activity.
- Work under sterile conditions to prevent contamination that could affect the assay when handling cell cultures.
- **Centrifugation:**
- Separate cells from the supernatant using high-speed centrifugation (e.g., 20,000g). Incomplete separation could result in residual cells in the supernatant, impacting the results.
- After centrifugation, transfer the supernatant carefully to avoid disturbing the pellet and resuspending any cells.
- **Plate Preparation:**
- Work quickly with molten agarose and Impranil mixture, which will solidify upon cooling. Prepare it just before use to maintain a consistent state across wells.
- Ensure that the 96-well plates are clean and debris-free before adding solutions to avoid cross-contamination between wells.
- **Absorbance and Fluorescence Measurements:**
- Calibrate the absorbance and fluorescence plate readers before useto ensure that the gain and sensor distance settings are accurate.
- For consistency across time points and replicates, use the same plate reader settings (e.g., gain, sensor distance) throughout the experiment.
- **Incubation:**
- Ensure that the incubator is set to the correct temperature (37°C) and is stable throughout the incubation period.

- Use sealing film to prevent evaporation from the wells during prolonged incubation periods.
- **Data Analysis:**

- Carefully inspect raw data for outliers that may indicate technical issues (e.g., improper cell separation, contamination, or equipment malfunctions).

- Normalize all data points to initial measurements (e.g., absorbance at 750 nm or chlorophyll RFU) to account for any initial variations in enzyme concentrations.

Materials

- 1.7 mL Eppendorf tubes
- 96-well clear plates
- Impranil (0.25% v/v)
- Phosphate buffer (100 mM)
- Agarose (0.2%)
- Centrifuge
- An absorbance plate reader with top-reading configuration
- Fluorescence plate reader
- Clear sealing film (e.g., qPCR film)
- Excel or other data analysis software

Safety warnings

- **Risk of Contamination:**
- **Warning:** Residual cells or lysates may interfere with assay results, particularly in chlorophyll fluorescence readings. Ensure proper cell separation and clean transfer of supernatant.
- Cross-contamination between wells can occur if pipette tips are not changed between samples or if improper pipetting techniques are used.
- **Temperature Sensitivity:**
- **Warning:** Temperature fluctuations in the incubator can affect enzyme activity, leading to inconsistent results. Ensure the incubator remains stable throughout the assay period.
- **Volatile Reagents:**
- **Warning:** Impranil has volatile component and can degrade over time. Always prepare fresh Impranil-agarose solutions and avoid reheating previously prepared batches.
- **Safety:**
- **Warning:** When working with Impranil or other chemicals, wear appropriate personal protective equipment (PPE) such as gloves, lab coats, and eye protection to avoid exposure.
- Handle hot molten agarose with care to prevent burns. Use appropriate heat-resistant gloves and pipette carefully when transferring molten agarose to the plate.
- **Interference in Fluorescence Readings:**
- **Warning:** Elevated chlorophyll fluorescence (>50 RFU) may indicate the presence of cells or lysate, which can affect the accuracy of the assay. High fluorescence values should be flagged and carefully evaluated during data analysis.
- **Time Sensitivity:**
- **Warning:** Take readings at consistent time intervals (every 5 minutes) during the incubation period to ensure accurate time-dependent data. Delayed readings may distort time-course analysis.

Cell Density Check

1 From your culture, take a **1 mL** aliquot and place it in a **1.7 mL** Eppendorf tube.

2 From the aliquot, take a sample of the culture and place it in a **96-well clear plate**.

3 Centrifuge the aliquot at maximum speed (e.g., 20,000g) for at least **3 minutes**.

4 Collect the supernatant in a new Eppendorf tube and set aside.
Note: Take care not to transfer cells, as they will continue to express enzymes.

Assay Plate Preparation

5 In a clean flask, add the desired volume of buffer (e.g., **10 mL of 100 mM phosphate buffer**), and then add **agarose** to final concentration **0.2%**. Melt the agarose by boiling the solution, and add **Impranil** to **0.25% (v/v)**. Swirl the flask to ensure the Impranil is evenly mixed to obtain a homogenous solution. Use immediately.

6 Using a reagent reservoir to displace the hot molten solution, transfer **150 µL** to each well in the plate.
Note: Warm the plastic tip by leaving it inside the molten solution and pipetting up and down a couple of times.

7 Add **50 µL** of the supernatant sample containing the plastic-degrading enzyme (e.g., the supernatant of a culture producing the enzyme).

8 Seal the plate with clear sealing film (e.g., used for qPCR).
Note: Make sure the plate is tightly sealed to avoid evaporation of samples.

Absorbance and Chlorophyll Fluorescence Measurements

9 Using a top-reading configuration, measure the absorbance at **350 nm** and **750 nm**, and chlorophyll fluorescence at **440/680 nm** with a gain of 100 and a sensor distance of **18,000 µm**.

10 Readings are taken every **5 minutes** while the plate is incubated at **37°C** for **72 hours to 168 hours**.

Data Analysis:

11 **Delta variation**

11.1 Calculate the change in absorbance at 350 nm (initial time point minus the final time point).

12 **Normalization**

12.1 Normalize the results using wells containing only gel and buffer (no enzyme) to account for variations due to interactions between the buffer, gel, and Impranil® DLN.

Note: Normalization is necessary because absorbance readings can fluctuate during the first two days, often increasing initially and then decreasing over time if PHL7 enzymes are present.

These fluctuations may result from changes in the gel suspension, such as partial precipitation or degradation, altering absorbance independently of enzyme activity. Wild-type controls are used in conjunction with the blanks for normalization to ensure the accuracy of enzyme activity measurements.

If an initial increase in absorbance is observed, then calculate the change in absorbance at 350 nm only in the linear parts with a negative slope, as this is evidence of true enzymatic activity and indicates that any underlying molecular interactions among the buffer, gel, and Impranil components have stabilized.

13 **Calculate Normalized Activity:**

13.1 Calculate the normalized activity by dividing the delta value (change in absorbance at 350 nm) by the cell density performed prior to setting up the assay (absorbance at **750 nm** or RFU chlorophyll).

14 **Impranil Variation Calculation**

14.1 Calculate the Impranil variation per day by dividing the delta absorbance at **350 nm** by the number of days elapsed from the initial reading.