Technical Bulletin

β-Lactamase Activity Assay Kit

Catalog Number MAK221

Product Description

β-Lactamase (βL; EC 3.5.2.6) is an enzyme first identified in *Escherichia coli* and has been described as penicillinase. A number of βLs have since been identified from various bacteria. βLs specifically hydrolyze β-lactam rings present in antibiotics such as penicillin, cephalosporins, monobactam, and carbapenem, and confer resistance against these antibiotics.[1,2]

The β-Lactamase Activity Assay Kit provides a simple assay for measuring βL activity in βL-secreting bacteria in fermentation media and bacterial cultures. It can also be used to detect βL-secreting bacteria in saliva, urine, and serum of infected mammals and in food samples. βL activity is measured by hydrolyzing a chromogenic cephalosporin called nitrocefin, producing a colorimetric product with an absorbance maximum at 490 nm proportional to the enzymatic activity present. One unit of β-lactamase is the amount of enzyme required to hydrolyze 1.0 µmole of nitrocefin per minute at pH 7.0 at 25 °C.

Components

The kit is sufficient for 100 assays in 96-well plates.

β-Lactamase Assay Buffer Catalog Number MAK221A	27 mL
Nitrocefin, in DMSO Catalog Number MAK221B	220 µL
Positive Control Catalog Number MAK221C	1 vial
βL Hydrolysis Buffer Catalog Number MAK221D	100 µL

Reagents and Equipment Required but Not Provided

- Clear flat-bottom 96-well plates. Cell culture or tissue culture treated plates are **not** recommended.
- Spectrophotometric multiwell plate reader
- DMSO (dimethyl sulfoxide) (Catalog Number D2650 or equivalent)

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Briefly centrifuge vials before opening. Use ultrapure water for the preparation of reagents. To maintain reagent integrity, avoid repeated freeze/thaw cycles.

β-Lactamase Assay Buffer and βL Hydrolysis Buffer: Allow buffers to come to room temperature before use.

Nitrocefin (in DMSO): Store at –20 °C, protected from light. Warm to room temperature before use to melt DMSO. Use within 2 months.

Positive Control: Reconstitute with 20 µL of β-Lactamase Assay Buffer. Mix well by pipetting. Aliquot and store at –20 °C. Use within 2 months.

Storage/Stability

The kit is shipped on wet ice and storage at –20 °C, protected from light, is recommended.

Procedure

All samples and standards should be run in duplicate.

Nitrocefin Standards

Hydrolyze the Nitrocefin stock solution by mixing 4 µL of Nitrocefin, 8 µL of βL Hydrolysis Buffer, and 28 µL of DMSO to prepare a 2 mM (2 nmole/µL) Nitrocefin Standard Solution. Incubate at 60 °C for 30 minutes. Cool to room temperature and centrifuge. Prepare hydrolyzed Nitrocefin Standard Solution fresh, just prior to each use.

Add 0, 2, 4, 6, 8, and 10 µL of the hydrolyzed 2 mM (2 nmole/µL) Nitrocefin Standard Solution into wells of a 96-well plate generating 0 (blank), 4, 8, 12, 16, and 20 nmole/well standards. Add β-Lactamase Assay Buffer to each well to bring the total volume to 100 µL.

Sample Preparation

Clear liquid samples can be assayed directly.

For bacterial samples, centrifuge the samples at 10,000 × *g* for 10 minutes in a pre-weighed centrifuge tube. Discard the supernatant and determine the weight of the pellet. Resuspend the pellet with 5 µL of β-Lactamase Assay Buffer per mg of sample. Sonicate samples for 5 minutes and place them on ice for 5 minutes. Centrifuge the samples at 16,000 × *g* at 4 °C for 20 minutes. Transfer supernatant to a fresh tube.

Add 1–50 µL of the samples into duplicate wells. Bring samples to a final volume of 50 µL using β-Lactamase Assay Buffer.

Notes: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

For samples exhibiting significant background, include a Sample Blank for each sample by omitting the Nitrocefin. The Sample Blank readings can then be subtracted from the sample readings.

For a positive control (optional), dilute 2 µL of the Positive Control solution with 8 µL of β-Lactamase Assay Buffer and mix well. Add 1–10 µL of the diluted Positive Control solution to the desired wells. Adjust the final volume to 50 µL using β-Lactamase Assay Buffer.

Assay Reaction

1. Set up a Reaction Mix according to the scheme in Table 1. 50 µL of Reaction Mix is required for each reaction (well).

Table 1.

Reaction Mix

Reagent	Volume
β-Lactamase Assay Buffer	48 µL
Nitrocefin	2 µL

2. Add 50 µL of the Reaction Mix to wells containing samples and Positive Control. Mix well using a horizontal shaker or by pipetting.
3. Measure the absorbance at 490 nm (A_{490}) in a microplate reader in kinetic mode for 30-60 minutes at room temperature. Protect the plate from light during the incubation. It is recommended to take absorbance readings every minute.

Note: Incubation time depends on the activity of β-Lactamase in the samples.

4. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (20 nmole/well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve.

Note: The Nitrocefin Standards can be read at the end of the incubation time.

Results

Calculations

Plot the absorbance (A_{490}) for each well versus time.

Correct for the background by subtracting the measurement obtained for the 0 (blank) Nitrocefin standard from that of the standards, controls, and samples. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate Nitrocefin Standards to plot a standard curve.

Note: A new standard curve must be set up each time the assay is run.

Choose two time points (T1 and T2) in the linear range of the plot and determine the A_{490} at each time (ABS1 and ABS2).

Note: It is essential that ABS1 and ABS2 fall within the linear range of the standard curve.

Calculate the change in absorbance from T1 to T2 for the samples.

$$\Delta ABS = ABS2 - ABS1$$

Subtract the Sample Blank ΔABS value from the Sample ΔABS reading to obtain the corrected measurement. Using the corrected measurement, determine the amount of Nitrocefin (nmole/well) generated by the β-Lactamase assay between T1 and T2 (S_a).

β-Lactamase Activity

$$\text{β-Lactamase activity} = \frac{S_a}{(\text{Reaction Time}) \times S_v}$$

where:

S_a = Amount of Nitrocefin (nmole) hydrolyzed in unknown sample well between T1 and T2 from standard curve

Reaction Time = T2 – T1 (minutes)

S_v = Sample volume (mL) added to well

β-Lactamase activity is reported as nmole/min/mL = milliunit/mL.

One unit of β-lactamase is the amount of enzyme required to hydrolyze 1.0 µmole of nitrocefin per minute at pH 7.0 at 25 °C.

Sample Calculation:

Amount of Nitrocefin (S_a) = 5.84 nmole (from standard curve)

(T1) = 3 minutes

(T2) = 32 minutes

Sample volume (S_v) = 0.050 mL

β-Lactamase activity in sample well:

nmole/min/mL (milliunits/mL) =

$$\frac{5.84 \text{ nmole/well}}{(32 \text{ min} - 3 \text{ min}) \times 0.050 \text{ mL /well}} =$$

4.03 milliunits/mL

References

1. Abraham, E.P., and Chain, E., An enzyme from bacteria able to destroy penicillin. *Nature*, **146**, 837 (1940).
2. Drawz, S.M., and Bonomo, R.A., Three decades of beta-lactamase inhibitors. *Clin. Microbiol. Rev.*, **23**, 160–201 (2010).

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96-well plate used	For colorimetric assays, use clear plates
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of improperly stored reagents	Store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Reaction Mixes before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the Reaction Mixes
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare Reaction Mixes whenever possible

Problem	Possible Cause	Suggested Solution
Non-linear standard curve (continued)	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

Notice

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