

**ab197008**

# **Beta Lactamase Activity Assay Kit (Colorimetric)**

Instructions for Use

For the rapid, sensitive and accurate measurement of Beta-Lactamase activity in various biological samples.

This product is for research use only and is not intended for diagnostic use.

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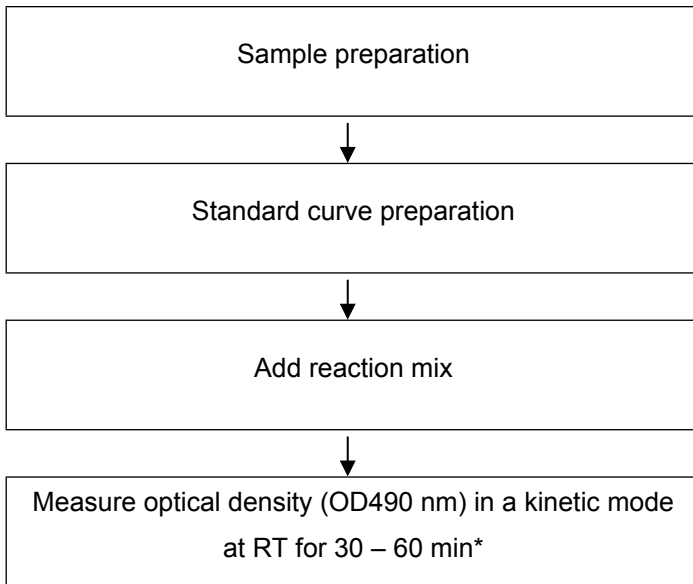
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## 1. OVERVIEW

Beta Lactamase Activity Assay Kit (Colorimetric) (ab197008) is a simple and sensitive assay that can detect and quantify the enzymatic activity of these hydrolases. The assay is based on the hydrolysis of the substrate Nitrocefin, a chromogenic cephalosporin, that results in the generation of a colored product (detectable at OD490 nm), which is directly proportional to the amount of beta-lactamase activity his product can detect as low as 0.06 mU of beta-lactamase activity from bacterial samples or from biological fluids infected with beta-lactamase expressing bacteria.

Beta-Lactamase ( $\beta$ L), (EC 3.5.2.6), is a bacterial enzyme that belongs to a large family of hydrolases comprising more than 850 identified members expressed in Gram-positive and Gram-negative bacteria.  $\beta$ Ls can be classified according to their substrate or inhibitor specificity. These enzymes are capable of hydrolyzing four atom rings known as  $\beta$ -lactams, the building block of  $\beta$ -Lactam antibiotics.  $\beta$ -Lactams, such as Penams, Carbapenems, Cephems, Clavams, Oxacephems and Monobactams, have been widely used for the treatment of bacterial infections since the discovery of penicillin, the first  $\beta$ -Lactam antibiotic, and its bactericidal effect more than eighty years ago. These drugs are highly susceptible to be hydrolyzed via enzymatic activity, which deactivates their antibiotic potency.  $\beta$ Ls have become a significant clinical threat due to the alarming number of cases of bacterial strains showing  $\beta$ -lactam antibiotic resistance.

## 2. ASSAY SUMMARY



*\*For kinetic mode detection, incubation time given in this summary is for guidance only.*

### **3. PRECAUTIONS**

**Please read these instructions carefully prior to beginning the assay.**

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

### **4. STORAGE AND STABILITY**

**Store kit at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 5.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

**5. MATERIALS SUPPLIED**

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Assay Buffer 42	25 mL	-20°C	-20°C
Nitrocefin	220 µL	-20°C	-20°C
β-Lactamase Positive Control	1 vial	-20°C	-20°C
βL Hydrolysis Buffer	100 µL	-20°C	-20°C

PLEASE NOTE: Assay Buffer 42 was previously labelled as Assay Buffer XLII and Beta Lactamase Assay Buffer, and βL Hydrolysis Buffer as Beta Lactamase Hydrolysis Buffer, and β-Lactamase Positive Control as Positive Control (Lyophilized), and Nitrocefin as Nitrocefin (in DMSO). The composition has not changed.

**6. MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- MilliQ water or other type of double distilled water (ddH<sub>2</sub>O)
- Cold PBS
- DMSO
- Microcentrifuge
- Pipettes and pipette tips
- Colorimetric microplate reader – equipped with filter for OD 490 nm
- 96 well plate: clear plate for colorimetric assay
- Sonicator
- Heat block or water bath
- Vortex

### 7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

### **8. TECHNICAL HINTS**

- **This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Keep enzymes and heat labile components and samples on ice during the assay.
- Make sure all buffers and developing solutions are at room temperature before starting the experiment.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure complete removal of all solutions and buffers from tubes or plates during wash steps.
- Make sure you have the appropriate type of plate for the detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

## 9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

### 9.1 **Assay Buffer 42:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

### 9.2 **$\beta$ L Hydrolysis Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

### 9.3 **Nitrocefin:**

Ready to use as supplied. Warm by placing in a 37°C bath for 1 – 5 minutes to thaw the DMSO solution before use.

**NOTE: DMSO tends to be solid when stored at -20°C, even when left at room temperature, so it needs to melt for few minutes at 37°C.** Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light and moisture. Use within 2 months.

### 9.4 **$\beta$ -Lactamase Positive Control:**

Reconstitute with 20  $\mu$ L Assay Buffer 42 and mix gently by pipetting. Aliquot  $\beta$ -Lactamase Positive Control so that you have enough volume to perform the desired number of assays. Store aliquots at -20°C. Avoid repeated freeze/thaw. Use within 2 months.

## 10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Hydrolyzed nitrocefin solution should be prepared immediately prior the assay. Do not store and discard unused standard.

10.1 Hydrolyze Nitrocefin stock solution (see Section 9.3) by adding adding 8  $\mu\text{L}$  of Nitrocefin to 16  $\mu\text{L}$  of  $\beta\text{L}$  Hydrolysis Buffer and 56  $\mu\text{L}$  of DMSO (ratio 1:2:7) in an eppendorf tube.

10.2 Incubate the reaction at 60°C for 30 min. Cool the reaction to room temperature and briefly centrifuge the tube.

10.3 Using the hydrolyzed Nitrocefin solution (2 mM stock solution) prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of standard ( $\mu\text{L}$ )	Assay Buffer ( $\mu\text{L}$ )	Final volume standard in well ( $\mu\text{L}$ )	End Conc. hydrolyzed Nitrocefin in well
1	0	250	100 $\mu\text{L}$	0 nmol/well
2	5	245	100 $\mu\text{L}$	4 nmol/well
3	10	240	100 $\mu\text{L}$	8 nmol/well
4	15	235	100 $\mu\text{L}$	12 nmol/well
5	20	230	100 $\mu\text{L}$	16 nmol/well
6	25	225	100 $\mu\text{L}$	20 nmol/well

Each dilution has enough amount of standard to set up duplicate reading (2 x 100  $\mu\text{L}$ ).

## 11. SAMPLE PREPARATION

### **General Sample information:**

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze bacteria cells in liquid nitrogen upon extraction and store the samples immediately at  $-80^{\circ}\text{C}$ . When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

### **11.1 Bacterial samples:**

- 11.1 Pre-weight collection sample tube and label
- 11.2 Collect bacterial samples by centrifugation at  $10000 \times g$  during 10 minutes.
- 11.3 Remove supernatant and determine wet weight of the pellet.
- 11.4 Resuspend pellet in of  $5 \mu\text{L}$  of Assay Buffer 42 per mg of sample. More BL buffer can be added if required to resuspend the pellet, but keep in mind that final sample might be more diluted.
- 11.5 Sonicate samples for 5 minutes. Keep samples on ice for 5 minutes. Remove insoluble material by centrifugation at  $16,000 \times g$  at  $4^{\circ}\text{C}$  for 20 minutes. Collect supernatant.
- 11.6 Keep sample on ice.

### **11.2 Liquid samples (biological fluids, fermentation media):**

Samples can be tested directly by adding sample to the sample to the microplate wells.

**NOTE:** *We suggest using different volumes of sample to ensure readings are within the Standard Curve range.*

## 12. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

### 12.1 Set up Reaction wells:

- Standard wells = 100  $\mu$ L Standard dilutions.
- Sample wells = 1 – 50  $\mu$ L samples (adjust volume to 50  $\mu$ L/well with Assay Buffer 42).
- $\beta$ -Lactamase Positive Control (from Section 9.4): dilute 100-fold in Assay Buffer 42 (2  $\mu$ l of  $\beta$ -Lactamase Positive Control with 198  $\mu$ l Assay Buffer 42) just before detection. Use 1 – 10  $\mu$ L diluted  $\beta$ -Lactamase Positive Control enzyme (adjust volume to 50  $\mu$ L/well with Assay Buffer 42).

### 12.2 Reaction Mix:

Prepare of 50  $\mu$ L of Reaction Mix for each reaction:

Component	Reaction Mix ( $\mu$ L)
Assay Buffer 42	48
Nitrocefin	2

Mix enough reagents for the number of assays (samples and positive control) to be performed. Prepare a Master Mix of the Reaction Mix to ensure consistency. We recommend the following calculation:

X  $\mu$ L component x (Number samples + positive controls +1).

- 12.3 Add 50  $\mu$ L of Reaction Mix into each sample and positive control wells. DO not add reaction mix to the standards. Mix well.
- 12.4 Measure output immediately ( $T_1$ ) on a colorimetric microplate reader at OD = 490 nm to obtain corresponding absorbance ( $A_1$ ).

- 12.5 Measure absorbance on a microplate reader at OD=490 nm in a kinetic mode, every 2 – 3 minutes, for at least 30 – 60 minutes at RT protected from light.

**NOTE:** *Incubation time depends on the Beta Lactamase activity in samples. Longer incubation times may be required if sample's Beta Lactamase activity is low.*

*We recommend measuring the OD in kinetic mode, and choosing two time points ( $T_1$  &  $T_2$ ) in the linear range to calculate the beta lactamase activity of the samples.*

*The Nitrocefin Standard Curve can be read in Endpoint mode (i.e., at the end of the incubation time [60 min.]) The Standard Curve will not change as incubation time increases.*

## 13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
  - 13.1 Average the duplicate reading for each standard and sample.
  - 13.2 Subtract the mean absorbance value of the blank (Standard #1) from all standard and sample readings. This is the corrected absorbance.
  - 13.3 Plot the corrected absorbance values for each standard as a function of the final concentration of hydrolyzed Nitrocefin.
  - 13.4 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).
  - 13.5 Extrapolate sample readings from the standard curve plotted using the following equation:

$$\text{Time point value} = \left( \frac{\text{Corrected absorbance} - (y - \text{intercept})}{\text{Slope}} \right)$$

$$\text{Time point} = A_1 \text{ or } A_2$$

- 13.6 Activity of Beta Lactamase is:

$$\Delta\text{OD}_{490\text{nm}} = A_2 - A_1$$

- 13.7 Use the  $\Delta\text{OD}_{490\text{nm}}$  to obtain B nmoles of hydrolyzed Nitrocefin:

$$\text{BL Activity} = \left( \frac{B}{(T_2 - T_1) \times V} \right) * D$$

$$= \text{nmol/min/mL} = \text{mU/mL}$$

Where:

B = Amount of hydrolyzed Nitrocefin from Standard Curve (nmol)

T1 = Time of the first reading ( $A_1$ ) in minutes.

T2 = Time of the second reading ( $A_2$ ) in minutes.

V = volume added into the reaction well (mL).

D = sample dilution factor.

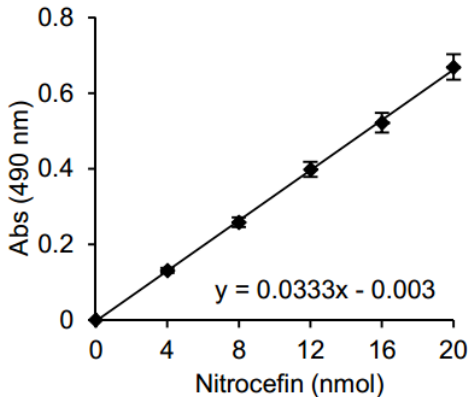
Sample Beta Lactamase Activity can also be expressed as mU/mg (nmoles/min hydrolyzed Nitrocefin generated per mg) of protein.

### **Unit Definition:**

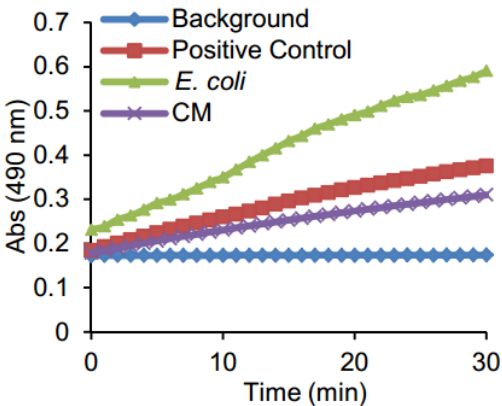
**1 Unit Beta Lactamase activity** = amount of enzyme that generates 1.0  $\mu\text{mol}$  of nitrocefin per minute at pH7.0 at 25°C.

## 14. TYPICAL DATA

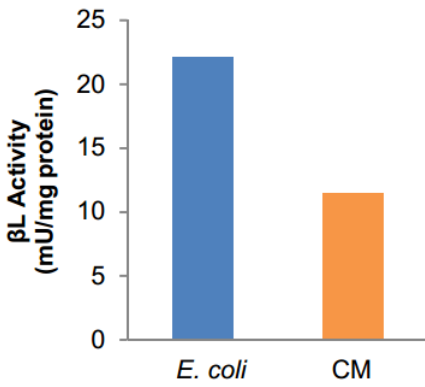
**TYPICAL STANDARD CURVE** – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



**Figure 1:** Typical Nitrocefin Standard Curve.



**Figure 2:** Beta Lactamase activity in *E. coli* culture (green; 5 µL), contaminated media (CM) (purple; 30 µL) and β-Lactamase Positive Control (red; 4 µL). Background reading is shown in blue.



**Figure 3:** Beta Lactamase Activity of *E.coli* and contaminated media expressed per milligram of protein. Assay was performed following the kit protocol.

## 15. QUICK ASSAY PROCEDURE

**NOTE:** This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

- Thaw nitrocefin, solubilize  $\beta$ -Lactamase Positive Control (aliquot if necessary); get equipment ready.
- Prepare standard curve for colorimetric detection.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings, make up to 50  $\mu$ L with Assay Buffer 42).
- Set up a plate for standard (100  $\mu$ L); samples (50  $\mu$ L) and positive control (50  $\mu$ L).
- Prepare Reaction Mix (50  $\mu$ L/well) (Number samples + positive control + 1).

Component	Reaction Mix ( $\mu$ L)
Assay Buffer 42	48
Nitrocefin	2

- Add 50  $\mu$ L Reaction mix to samples and positive control wells.
- Measure output plate ( $A_1$ ) at time  $T_1$  at OD = 490 nm.
- Measure plate ( $A_2$ ) at time  $T_2$  at OD = 490 nm, while incubating plate at RT during 30 – 60 minutes, protected from light.

## 16. TROUBLESHOOTING

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

## RESOURCES

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 $\mu\text{L}$ ) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. FAQ

## **18. INTERFERENCES**

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure:

- RIPA buffer – contains SDS which can denature proteins and affect enzyme activity.

19. NOTES

## **Technical Support**

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