Version 09-01/25

User's Manual

Cyclic di-AMP ELISA Kit

REF **DEIA-CA04**

Σ **96T**

RUO

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

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Cyclic di-AMP ELISA Kit is a competitive assay that can be used for quantification of cyclic di-AMP in bacterial cell lysates. The assay has a range of 15.6-2,000 pg/ml (23.7-3,038 pM) with a midpoint (50% B/Bo) of approximately 180 pg/ml (273 pM) and an average sensitivity (80% B/Bo) of approximately 66 pg/ml (100 pM)

Principles of Testing

This assay utilizes the competitive interaction between native cyclic di-AMP and a cyclic di-AMP-horseradish peroxidase conjugate (Cyclic di-AMP-HRP Tracer) for a limited quantity of Cyclic di-AMP Monoclonal Antibody. The concentration of the Cyclic di-AMP-HRP Tracer is kept constant, while that of native cyclic di-AMP varies; the binding of Cyclic di-AMP-HRP Tracer to the antibody inversely correlates with the concentration of native cyclic di-AMP present.

The formed antibody-cyclic di-AMP complex binds to anti-mouse IgG coated on the well. Following a wash to eliminate unbound reagents, 3,3',5,5'-tetramethylbenzidine (TMB) Substrate Solution is added, followed by HRP Stop Solution. The enzymatic reaction produces a yellow color measurable at 450 nm, with the color intensity, assessed spectrophotometrically, directly proportional to the quantity of bound Cyclic di-AMP-HRP Tracer, and inversely proportional to the amount of free cyclic di-AMP during incubation.

Reagents And Materials Provided

1. Cyclic di-AMP-HRP Tracer: 2-8°C
2. ELISA Tracer Dye: 2-8°C
3. Cyclic di-AMP Antibody: 2-8°C
4. Standard: 2-8°C
5. ELISA Antiserum Dye: RT
6. Wash Buffer Concentrate (400X): RT
7. Buffer A Concentrate (10X): RT
8. Polysorbate 20: RT
9. Pre-coated Microplate: 2-8°C
10. TMB Substrate Solution: 2-8°C
11. Stop Solution: RT

Materials Required But Not Supplied

1. Distilled/demineralized water
2. Graduated mono- or multichannel pipettes (2-20 µL, 20-200 µL et 100-1000 µL range) and single-use tips
3. Microplate reader (450nm filter)
4. Microplate washer

5. Incubator at 37±2°C
6. Standard laboratory equipment: graduated cylinder, tube rack, lid, ...

Storage

2-8°C

Specimen Collection And Preparation

This assay has been demonstrated to work with bacterial cell lysates prepared in Bacterial Protein Extraction Reagent without causing interference in the assay. Other lysis buffers or concentrated lysates may cause interference and require sample purification or a minimum dilution determined by the end user outlined below. Please read this section thoroughly before beginning the assay.

NOTE:

1. All samples must be free of organic solvents prior to assay.
2. Samples should be assayed immediately after collection; samples that cannot be asayed immediately should be stored at -80°C.

Testing for Interference

To test for interference, dilute one or two test samples to obtain at least two different dilutions of each sample between approximately 90 pg/ml and 400 pg/ml (i.e., between 25-70% B/Bo, which is the linear portion of the standard curve). If two different dilutions of the same sample show good correlation (differ by 20% or less) in the final calculated cyclic di-AMP concentration, purification is not required. If you do not see good correlation of the different dilutions, purification is advised. Purification methods will need to be determined by the end user and tested for compatability in the assay.

Plate Preparation

The 96-well plate(s) that come with this kit must be washed five times with Wash Buffer (1X) (~300 µl per well) before they are used in the ELISA. NOTE: If you do not need to use all the strips at the same time, please place the unused strips back in the plate packet and store them at 4°C, ensuring the packet is sealed with the desiccant inside. Each plate or strip set must have at least two Blank wells, two NSB wells, and two B0 wells, as well as an eight-point standard curve run in duplicate. NOTE: This minimum configuration is necessary for obtaining accurate and reproducible results in each assay. Each sample should be tested at no fewer than two dilutions, with each dilution performed in duplicate. For optimal statistical accuracy, it is advisable to test samples in triplicate.

Reagent Preparation

Store all diluted buffers at 4°C; they will be stable for about two months.

1. **Buffer A (1X) Preparation**

Dilute the contents of one vial of Buffer A (10X) (with 90 ml of pure water. Be certain to rinse the vial to remove any salts that may have precipitated. NOTE: It is normal for the concentrated buffer to contain crystalline salts. These will completely dissolve upon dilution with pure water.

2. **Wash Buffer Preparation**

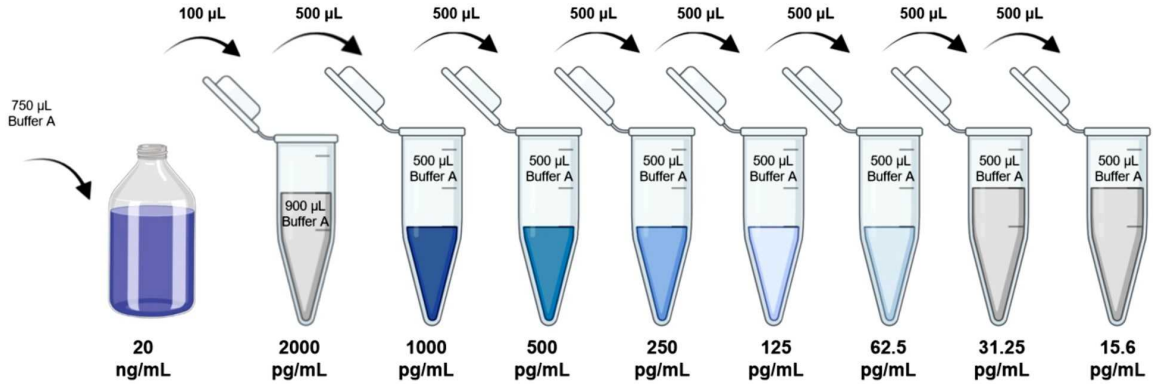
Dilute the contents of one vial of Wash Buffer Concentrate (400X) with pure water to a total volume of 2 L and add 1 ml of Polysorbate 20. Smaller volumes of Wash Buffer (1X) can be prepared by diluting the Wash Buffer Concentrate (400X) 1:400 and adding Polysorbate 20 to an end concentration of 0.5 ml/L. NOTE: It is normal for the concentrated buffer to contain crystalline salts. These will completely dissolve upon dilution with pure water. Polysorbate 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

3. **Cyclic di-AMP ELISA Standard**

Resconstitute the lyophilized Cyclic di-AMP ELISA Standard in 0.75 ml of Buffer A (1X). The concentration of this solution (the bulk standard) is 20 ng/ml. It will be stable for at least four weeks when stored at 4°C.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and label them #1-8. Aliquot 900 µl Buffer A (1X) to tube #1 and 500 µl Buffer A (1X) to tubes #2-8. Transfer 100 µl of the bulk standard (20 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing

500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than two hours.



4. **Cyclic di-AMP-HRP Tracer**

Dilute the Cyclic di-AMP-HRP Tracer with 5 ml of Buffer A (1X). Store the diluted Cyclic di-AMP-HRP Tracer at 4°C (do not freeze!)

and use within four weeks. A 20% surplus of tracer has been included to account for any incidental losses.

Dye Note (optional)

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the diluted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer). Do not store tracer with dye for more than four weeks at 4°C.

5. **Cyclic di-AMP Monoclonal Antibody**

The Cyclic di-AMP Monoclonal Antibody is ready to use as supplied. Store the Cyclic di-AMP Monoclonal Antibody at 4°C (do not freeze!). A 20% surplus of antibody has been included to account for any incidental losses.

Dye Note (optional)

This dye may be added to the antibody, if desired, to aid in visualization of antibody-containing wells. Add the dye to the supplied antibody at a final dilution of 1:100 (add 60 µl of dye to 6 ml antibody). Do not store

antibody with dye for more than four weeks at 4°C.

Assay Procedure

· Use different tips to pipette each reagent.

· Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).

· Do not expose the pipette tip to the reagent(s) already in the well.

1. **Immunoassay Buffer C**

Add 100 µl Buffer A (1X) to NSB wells. Add 50 µl Buffer A (1X) to B0 wells.

2. **Cyclic di-AMP ELISA Standard**

Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

Add 50 µl sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Cyclic di-AMP-HRP Tracer**

Add 50 µl to each well except the Total Activity and Blank wells.

5. **Cyclic di-AMP ELISA Monoclonal Antibody**

Add 50 µl to each well except the TA, NSB, and Blank wells within 15 minutes of addition of the tracer.

Incubation of the Plate

6. Cover each plate with a 96-Well Plate Cover Sheet and incubate 2 hours at room temperature on an orbital shaker.

Development of the Plate

7. Empty the wells and rinse five times with ~300 µl Wash Buffer (1X).
8. Add 175 µl of TMB Substrate Solution to each well.
9. Add 5 µl of the diluted tracer to the Total Activity wells.
10. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker at room temperature for 30 minutes.
11. Remove the plate cover being careful to keep TMB Substrate Solution from splashing on the cover. NOTE: Any loss of TMB Substrate Solution will affect the absorbance readings.
12. DO NOT WASH THE PLATE. Add 75 µl of HRP Stop Solution to each well of the plate. Blue wells should turn yellow and colorless wells should remain colorless. NOTE: The stop solution in this kit contains an acid. Wear appropiate protection and use caution when handling this solution.

Reading the Plate

13. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
14. Read the plate at a wavelength of 450 nm.

Calculation

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells (Non-Specific Binding).
2. Average the absorbance readings from the B0 wells (Maximum Binding).
3. Subtract the NSB average from the B0 average. This is the corrected B0 or corrected maximum binding.
4. Calculate the B/B0 (Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B0 (from Step 3). Repeat for S2-S8 and all sample wells. (To obtain %B/Bo for a logistic four-parameter fit, multiply these values by 100.)

NOTE: The Total Activity values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool. Erratic absorbance values could indicate the presence of organic solvents in the buffer or other technical problems. Low or no absorbance from a Total Activity well could indicate a dysfunction in the enzyme-substrate system.

Plot the Standard Curve

Plot %B/B0 for standards S1-S8 versus cyclic di-AMP concentration using linear (y) and log (x) axes and perform a four-parameter logistic fit.

Alternative Plot - The data can also be linearized using a logit transformation.

The equation for this conversion is shown below. NOTE: Do not use %B/B0 in this calculation.

logit (B/B0) = ln [B/B0/(1 - B/B0)]

Plot the data as logit (B/B0) versus log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the B/B0 (or %B/B0) value for each sample. Determine the concentration of each sample by identifying the %B/B0 on the standard curve and reading the corresponding values on the x-axis. NOTE: Remember to account for any dilution of the original sample concentration prior to its addition to the well. Samples with %B/B0 values greater than 70% or less than 25% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample indicates interference, which could be eliminated by purification.

NOTE: If there is an error in the B0 wells it is possible to calculate sample concentrations by plotting the absorbance values and back calculating sample absorbance off the standard curve.

Performance Characteristics

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve.

Detection Range

15.6-2000 pg/ml (23.7-3038 pM)

Detection Limit

20.7 pg/ml (31.4 pM)

Sensitivity

65.5 pg/ml (99.5 pM)

Specificity

Cyclic di-AMP: 100%

pApA: 0.011%

c[A(3',5')pA(3',5')pG(3',5')p]: 0.014%

pG(2',5')pA: <0.01%

c-tetra-AMP: <0.01%

Cyclic di-GMP: <0.01%

Linearity

To assess dilutional linearity, E. coli was lysed in Bacterial Protein Extraction Reagent, spiked with cyclic di-AMP, serially diluted with Buffer A (1X), and evaluated for linearity using the Cyclic di-AMP ELISA Kit.

Dilution factor 60, Dilution Linearity 101%

Dilution factor 240, Dilution Linearity 106%

Recovery

E. coli was lysed in Bacterial Protein Extraction Reagent, spiked with different amounts of cyclic di-AMP, serially diluted in Buffer A (1X), and analyzed using the Cyclic di-AMP ELISA Kit. The error bars represent standard deviations obtained from multiple dilutions of each sample.

Spike Concentration (150 pg/mL), Recovery 109%

Spike Concentration (2000 pg/mL), Recovery 96%

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