Product Manual

abbexa®

Trimethoprim ELISA Kit

Catalog No.: abx364830

Revision date: 4-Sep-19

Size: 96T

Storage: Store at 4°C for up to 12 months.

Application: For quantitative detection of trimethoprim in serum, plasma, tissues, urine, and feed.

Sensitivity: 0.01 ppb (ng/g)

Detection Limit: Serum – 0.4 ppb, Plasma – 0.4 ppb, Tissues – 0.4 ppb, Urine – 0.4 ppb, Feed – 1.6 ppb.

Cross-reactivity: Trimethoprim – 100%, Sulfonamide antibiotics – < 0.1%

Introduction: Trimethoprim (TMP) is an antibiotic from the dihydrofolate reductase inhibitor class. The molecule consists of 2,4-diaminopyrimidine group conjugated to 1,2,3-trimethoxybenzene via a linking methyl group. TMP prevents the reduction of dihydrofolic acid to tetrahydrofolic acid, and has a higher affinity for bacterial dihydrofolate reductase than for the human equivalent. It is used to treat urinary tract infections and various aerobic bacteria. Adverse effects of TMP use include nausea, diarrhoea, rash, pruritus, and photosensitivity.

Principle of the Assay

This kit is based on indirect-competitive enzyme-linked immuno-sorbent assay technology. Trimethoprim is pre-coated onto a 96-well plate. The standards and samples and a biotin-conjugated antibody specific to trimethoprim are added to the wells and incubated. After washing away the unbound conjugates, avidin conjugated to Horseradish Peroxidase is added to each microplate well. After TMB substrate solution is added only wells that contain trimethoprim will produce a blue colour product that changes into yellow after adding the stop solution. The intensity of the yellow colour is inversely proportional to the amount of trimethoprim bound on the plate. The O.D. absorbance is measured spectrophotometrically at 450 nm in a microplate reader, and then the concentration of trimethoprim can be calculated.

Kit components

1. One pre-coated 96 well plate
2. Standard vials – 0 ppb, 0.03 ppb, 0.09 ppb, 0.27 ppb, 0.81 ppb, 2.43 ppb: 1 ml
3. 100 ppb standard: 1 ml
4. HRP conjugate reagent: 5.5 ml
5. Substrate reagent A: 6 ml
6. Substrate reagent B: 6 ml
7. Stop solution: 6 ml
8. Wash buffer (20X): 40 ml
9. Reconstitution buffer (2X): 50 ml
10. Plate sealers: 3
11. Hermetic bag: 1

Materials Required But Not Provided

1. 37°C incubator
2. Microplate reader (450 nm)
3. High-precision pipette and sterile pipette tips
4. Automated plate washer (optional)
5. ELISA shaker (optional)
6. Centrifuge and microfuge tubes
7. Absorbent filter papers
8. Nitrogen evaporator or water bath
9. Homogenizer
10. Vortex mixer

Reagents Required But Not Provided

1. Deionized water
2. Methanol (pure)
3. 0.1 mol/L hydrochloric acid
4. n-Hexane (unbranched hexane)
5. Sodium hydroxide (NaOH)

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Abbexa Ltd., Cambridge Science Park, Cambridge, CB4 0EY, UK
www.abbexa.com · Email: info@abbexa.com · Phone: +44 (0) 1223 755950 · Fax: +44 (0) 1223 755951
Registered in England: 8475531 · VAT Reg. No: GB 167 9240 79

Protocol

A. Preparation of sample and reagents

1. Preparation of reagents

- **Wash buffer**
  Dilute the 20× wash buffer 20-fold with deionized water (i.e. dilute 40 ml 20× wash buffer in 760 ml deionized water) to make the 1× wash buffer solution.

- **1 mol/L sodium hydroxide solution**
  Dissolve 4 g of sodium hydroxide in 100 ml of deionized water.

- **Reconstitution buffer**
  Dilute the 2× reconstitution buffer 2-fold with deionized water (i.e. dilute 50 ml 20× wash buffer in 50 ml deionized water) to make the 1× reconstitution buffer solution.

- **Standard solutions**
  Add 22.4 µl of the 100 ppb standard solution and 900 µl of 1X reconstitution buffer to the 2.43 ppb vial. Mix well, then add 300 µl of this dilution to 600 µl of reconstitution buffer in the 0.81 ppb vial. Mix well, then add 300 µl of this dilution to 600 µl of reconstitution buffer in the 0.27 ppb vial. Repeat these steps for the 0.09 ppb and 0.03 ppb vials. To the 0.00 ppb vial add 900 µl of reconstitution buffer directly.

2. Sample pretreatment

Store samples to be assayed within 24 hours at 2-8°C. Alternatively, aliquot and store at -20°C or -80°C for long term. Avoid repeated freeze-thaw cycles.

- **Feed:** Weigh 2 g of crushed sample and add to a 50 ml centrifuge tube. Add 20 ml of hydrochloric acid and vortex for 15 minutes. Centrifuge at 3000 rpm for 10 min. Aspirate 1 ml of supernatant into a microfuge tube and adjust pH to 6-8 using 1 mol/L sodium hydroxide. Centrifuge at 3000 rpm for 10 min. Aliquot 0.5 ml of supernatant into a microfuge tube and add 0.5 ml of reconstitution buffer. Mix well and analyze immediately.
  Note: Sample dilution factor: 20. Minimum detection dose: 1.6 ppb.

- **Urine, serum, plasma:** Aliquot 0.5 ml of sample and centrifuge at 4000 rpm for 5 min. Aspirate 50 µl of supernatant and add 200 µl of 1X reconstitution buffer. Mix well and analyze immediately.
  Note: Sample dilution factor: 5, minimum detection dose: 0.4 ppb.

- **Tissues:** Homogenise samples. Weigh 2 g of sample and add to a 50 ml centrifuge tube. Add 6 ml of methanol and 2 ml of hexane. Vortex for 5 minutes. Centrifuge at 4000 rpm for 10 min. Aspirate and discard the upper hexane layer. Aspirate 0.5 ml of the middle layer into a glass test tube (do not disturb the fat layer). Dry at 50-60°C in a water bath or nitrogen evaporator. Add 0.4 ml of 1X reconstitution buffer and 0.5 ml of hexane. Vortex for 1 minute. Centrifuge at 4000 rpm for 5 min. Aspirate and discard the upper hexane layer. Aliquot the lower liquid layer and analyze immediately.
  Note: Sample dilution factor: 5, minimum detection dose: 0.4 ppb.

Note:

» Fresh samples or recently obtained samples are recommended to prevent degradation and denaturalization that may lead to erroneous results. It is recommended to store samples to be used within 5 days at 4°C, within 1 month at -20°C and within 2 months at -80°C.

» Samples should be clear and transparent. Samples must be diluted so that the expected concentration falls within the kit's range.

» Please bring sample slowly to room temperature. Sample hemolysis will influence the result. Hemolyzed specimen should not be used. Samples that contain NaN_3 cannot be detected as it interferes with HRP.

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B. Assay Procedure

Bring all reagents to room temperature prior to use.

1. Set standard, and sample wells on the pre-coated plate and record their positions. It is recommended to measure each standard and sample in duplicate.
2. Add 50 µl of the standard solutions to the standard wells. Add the solution at the bottom of each well without touching the side wall.
3. Add 50 µl of prepared sample to the sample wells.
4. Add 50 µl of HRP conjugate to each well.
5. Cover the plate with the plate sealer. Tap the plate gently to ensure thorough mixing. Incubate at 37°C for 45 minutes in dark conditions.
6. Remove the cover, and discard the solution without touching the side walls. Blot the plate on an absorbent material. Add 350 µl of wash buffer to each well and soak for at least 30 seconds. Discard the contents and blot the plate on absorbent material. Repeat the wash procedure for a total of five times.

Note: For automated washing, discard the solution in all wells and wash five times overfilling the wells with Wash buffer. After the final wash invert the plate and tap on absorbent filter papers or other absorbent material. It is recommended that the washer be set for a soaking time of 1 minute.

7. Add 50 µl of substrate solution A and 50 µl of substrate solution B into each well. Tap the plate gently to ensure thorough mixing. Cover the plate and incubate at 37°C in dark conditions for 15-20 minutes (incubation time is for reference only, do not exceed 30 minutes). When an apparent gradient appears in the standard wells the reaction can be terminated.
8. Add 50 µl of stop solution into each well. There should be a colour change to yellow. Gently tap the plate to ensure thorough mixing.
9. Ensure that there are no fingerprints or water on the bottom of the plate, and that the fluid in the wells is free of bubbles. Measure the absorbance at 450 nm immediately.
10. OPTIONAL: measure the absorbance at 630 nm and subtract these values from the 450 nm values for analysis.
11. OPTIONAL: Calculate the absorbance percentage by dividing each OD value by the OD value of the 0 ppb standard, and multiplying by 100 to give a percentage.

This assay is competitive, therefore there is an inverse correlation between trimethoprim concentration in the sample and the absorbance measured. Create a graph with the log of the standard concentration (x-axis) and absorbance measured (y-axis). (Alternatively, divide each average OD value by the average OD of the 0 ppb standard and plot this against the standard concentration.) Apply a best fit trendline through the standard points. Use this graph calculate sample concentrations based on their OD values. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Note: If the samples measured were diluted, multiply the dilution factor by the concentrations from interpolation to obtain the concentration before dilution.

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C. Precautions

1. Equilibrate all reagents to room temperature (18-25°C) prior to use and centrifuge the tubes briefly in case any contents are trapped in the lid.
2. For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 10 minutes.
3. Wash buffer may crystallize and separate. If this happens, please warm the tube and mix gently to dissolve.
4. Avoid foaming or bubbles when mixing or reconstituting components.
5. It is recommended to assay all standards, controls and samples in duplicate or triplicate.
6. Do NOT let the plate dry out completely during the assay as this will inactivate the biological material on the plate.
7. Ensure plates are properly sealed or covered during incubation steps.
8. Complete removal of all solutions and buffers during wash steps is necessary for accurate measurement readings.
9. To avoid cross contamination do not reuse pipette tips and tubes.
10. Do not use components from a different kit or expired ones.
11. Substrate solution A and substrate solution B are easily contaminated; work under sterile conditions when handling these solutions. The substrate solutions should also be protected from light. Unreacted substrate should be colorless or very light yellow in appearance. Aspirate the dosage needed with sterilized tips and do not replace the residual solution back into the vial.

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