Product No. C-2545
Lot 025H8868

Chloramphenicol-Horseradish Peroxidase Conjugate
For Immunoassay

Chloramphenicol derivative (Chloromycetin®, D-threo-2,2-dichloro-N-[β-hydroxy-α-(hydroxymethyl)-4-nitrophenyl] acetamide) conjugated to horseradish peroxidase for use in immunoassay is supplied ready to use for 100 double antibody enzyme immunoassay (EIA) tests when used with Rabbit Anti-Chloramphenicol antiserum (Sigma Product No. C-1168).

The Chloramphenicol-HRP EIA is a competitive binding immunoassay in which Chloramphenicol-HRP and unlabeled chloramphenicol (standard or unknown sample) compete for a limited number of combining sites present in the rabbit anti-chloramphenicol antiserum. Separation of the bound and free Chloramphenicol-HRP is accomplished using a specific immunoprecipitation reagent containing goat anti-rabbit IgG antiserum. The ratio of Chloramphenicol-HRP conjugate bound in the presence of chloramphenicol to that bound without chloramphenicol is inversely proportional to the concentration of unlabeled chloramphenicol (See sample data).

The Chloramphenicol-HRP EIA procedure described in this product insert will allow the determination of as little as 0.03 ng of chloramphenicol per assay tube. The cross reactivity with chloramphenicol-glucuronide, the major metabolite of chloramphenicol, is 0.617%.

Lot Specific Data

Chloramphenicol-HRP	12.5 ng/ml
Volume per bottle	10.0 ml

Diluent: 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA, 150 mM NaCl, 200 µg/ml rabbit IgG, 10% bovine calf serum and 0.01% thimerosal as a preservative.

Sample Data

Below is an example of an antigen addition curve generated using the reagents and methods described in this product insert.

Chloramphenicol-HRP (Sigma Product No. C-2545)
Rabbit Anti-Chloramphenicol antiserum (Sigma Product No. C-1168)
Chloramphenicol standards prepared in 1% bovine calf serum
Sample size: 100 µl per assay tube
Microtiter plate reader: Dual wavelength, 490/540 nm 300 µl/well

ng/ml	Absorbance	%B/B_0	Intercepts	
0.0	0.913	100.0		
0.3	0.839	91.9	90%	0.3 ng/ml
0.7	0.672	73.6	50%	1.1 ng/ml
1.3	0.382	41.9	20%	2.7 ng/ml
2.0	0.249	27.3		
2.9	0.165	18.1		

Cross Reactivity

The specificity of the Chloramphenicol-HRP EIA was determined by calculating the ratio of the moles of chloramphenicol to moles of chloramphenicol analog at the 50% intercept of the dose response curve and multiplying the result by 100%.

Analog	**%**
Chloramphenicol Glucuronide	0.617
Thiamphenicol	0.009

Recommended EIA Procedure

Reagents Required	Sigma Product No.
Chloramphenicol-HRP	C-2545
Rabbit Anti-Chloramphenicol antiserum	C-1168
Rabbit IgG Immunoprecipitation Reagent	R-8633
Chloramphenicol	C-0378
Phosphate-Citrate Buffer Capsules with Sodium Perborate	P-4922
o-Phenylenediamine Dihydrochloride Tablets (10mg)	P-8287

Preparation of Reagents

Chloramphenicol-HRP (C-2545)

Store the Chloramphenicol-HRP at –20°C until use. One hour prior to assay, remove the Chloramphenicol-HRP conjugate from the freezer and thaw at room temperature. Carefully remove and discard the inner seal in the Chloramphenicol-HRP bottle and firmly replace bottle cap. If the Chloramphenicol-HRP conjugate is to be used within 1 week, store at 4°C, otherwise, return Chloramphenicol-HRP to the freezer. The conjugate is sensitive to temperature and prolonged exposure to temperatures greater than 4°C should be avoided.

EIA Assay Buffer

The EIA assay buffer is not supplied by Sigma. The recommended buffer composition is: 0.1 M phosphate, pH 7.4 containing 1% bovine calf serum and 0.01% thimerosal as a preservative.

Prepare EIA assay buffer by dissolving the contents of 1 bottle Gal-Pac® (Sigma Product No. 936-4GP) in approximately 3 liters deionized water. Add 0.38 g thimerosal (Sigma Product No. T-5125) and 38 ml bovine calf serum (Sigma Product No. C-3284). Stir until all components are completely dissolved and bring the volume to 3.8 liters with deionized water. Store EIA assay buffer at 4°C.

Chloramphenicol Standards

Standards should be prepared in a matrix equivalent to the unknown samples. The recommended range of the standards is 0.3 - 3 ng/ml for the procedure described below. Chloramphenicol (Sigma Product No. C-0378) is readily soluble in EIA assay buffer. Chloramphenicol standards are stable at 4°C with a preservative such as 0.01% thimerosal.
NOTE: Sodium azide interferes with horseradish peroxidase and should not be used.

Rabbit Anti-Chloramphenicol Antiserum (C-1168)

Reconstitute the anti-chloramphenicol antiserum with 10 ml EIA assay buffer to obtain a 10x stock antiserum solution. The stock antiserum can be aliquoted into convenient volumes and stored frozen. To prepare assay strength chloramphenicol antiserum, further dilute 1 part of 10x stock antiserum with 1.5 parts EIA assay buffer. Store assay strength antiserum at 4°C.

Rabbit IgG Immunoprecipitation Reagent (R-8633)

Rabbit IgG Immunoprecipitation Reagent is supplied as a ready to use mixture of goat anti-rabbit IgG antiserum in 0.1 M phosphate buffer, pH 7.4, containing 5 mM EDTA, 3.9% polyethylene glycol (M.W. = 8,000) and 0.01% thimerosal as a preservative. Before use, gently mix by inversion. Store the immunoprecipitation reagent at 4°C.

Horseradish Peroxidase Substrate

The Chloramphenicol-HRP EIA described in this product insert uses hydrogen peroxide (generated by sodium perborate) as the substrate and *o*-phenylenediamine dihydrochloride (OPD) as the hydrogen donor. The OPD forms a colored oxidation product that is measured at 490 nm after acidification. The absorbance of the reaction mixture is proportional to the Chloramphenicol-HRP concentration in the assay tube.

Substrate composition

Phosphate-Citrate Buffer	0.05 M
OPD	0.4 mg/ml
Sodium Perborate	0.03%
pH 5.0	

Phosphate-Citrate Buffer with Sodium Perborate

Dissolve the contents of one phosphate-citrate buffer capsule with sodium perborate (Sigma Product No. P-4922) in 100 ml of deionized water. Use within 30 minutes.

Substrate Solution (sufficient for 80 tubes)

Prior to use, measure 25 ml of phosphate-citrate buffer with sodium perborate into a convenient container and then add one 10 mg OPD tablet (Sigma Product No. P-8287). After the tablet has dissolved, mix well and use immediately. If the substrate solution has a yellow tint it should be discarded and a fresh solution prepared.

1 M Sulfuric Acid

The Chloramphenicol-HRP reaction with the substrate is stopped by acidification with 1 M sulfuric acid.
Prepare 1 M sulfuric acid by carefully adding 56 ml of concentrated sulfuric acid (Aldrich Product No. 25,810-5) to 900 ml deionized water. Bring volume to 1 liter with deionized water.

Equipment

Micropipets: 100 µl (Sigma Product No. MP-100)
300 µl (Sigma Product No. MP-300)
750 µl (Sigma Product No. MAP-750)
1000 µl (Sigma Product No. MAP-1000)
Refrigerated centrifuge capable of 2,000x g
Microtiter plate reader or spectrophotometer
Vacuum aspirator or equivalent
Test tube rack for 12 x 75 mm test tubes

Miscellaneous Supplies

12 x 75 mm glass test tubes
Pipet tips: 100 µl (Sigma Product No. MPT-2)
300-1000 µl (Sigma Product No. MPT-4)
Microtiter plates

Procedure

1. Label 12 x 75 mm tubes with the appropriate standard or test sample identification.
2. Pipet 100 µl of Chloramphenicol-HRP to all tubes.
   Optional: Add 100 µl Chloramphenicol-HRP to non-specific binding tubes (NSB).
3. Pipet 100 µl standard or test sample to the appropriate tube.
   Optional: Add 100 µl assay buffer to the NSB tubes.
4. Pipet 100 µl antibody to all tubes (except optional NSB tube).
   Optional: Add 100 µl assay buffer to NSB tubes.

5. Vortex gently to ensure complete mixing and incubate at ambient temperature for 2 hours, protected from light.
6. Pipet 1 ml Rabbit IgG Immunoprecipitation Reagent to all tubes.
7. Centrifuge at 2,000x g for 15 minutes at 4 °C.
8. Aspirate the supernatant from each tube.
9. Disrupt the precipitate with brief, vigorous vortexing with the tube held vertically to reduce the amount of immune precipitate on the sides of the tubes above the 300 µl level.
10. Add 300 µl of freshly prepared substrate solution to each tube. Note the order and timing of the substrate addition.
11. Incubate with occasional agitation at ambient temperature for 30 minutes. Protect from light.
12. In the same order and with similar timing of substrate addition, add 750 µl 1 M sulfuric acid to all tubes.
13. Measure the absorbance at 490 mn for each tube in an appropriate spectrophotometer, reduce the data and calculate the results as appropriate.

References

Sadee, W., and Beelen, G., In: "Drug Level Monitoring, Analytical Techniques, Metabolismk and Pharmacokinetics", Wiley & Sons, New York, P. 167 (1980)