CHAPTER 19. COMPETITIVE ENZYME-LINKED IMMUNOASSAY (CELIA) FOR THE DETECTION AND QUANTITATION OF CHLORAMPHENICOL

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19.1 Introduction and Principles

Enzyme Immunoassays (EIA) have become increasingly popular to detect and quantitate a wide range of biological molecules of interest. The excellent specificity and sensitivity afforded by EIA are two major factors contributing to the development and use of this technique for quantitative detection of low molecular weight haptenic molecules such as antibiotics. The Immunology Section of the Microbiology Division developed and published an original EIA procedure to detect and quantitate the antibiotic chloramphenicol (CA).

The specific type of EIA developed was an indirect Competitive Enzyme-linked Immunoassay (CELIA) system. The principles of this assay are as follows. The binding of the limiting number of specific rabbit CA antibody molecules in liquid phase to solid phase bound CA antigen is competitively inhibited by free liquid phase CA in the sample under assay. Bound antibody (not displaced) is indicated by using an enzyme linked anti-rabbit antibody preparation which is subsequently reacted with an appropriate substrate. Enzyme activity, measured spectrophotometrically, is inversely proportional to the concentration of CA in the sample.

The CELIA procedure for CA when performed on bovine muscle tissue extracts or phosphate buffered saline CA standards has the following characteristics: sensitivity of 1 ng/ml ($P<0.05$), linear quantitative displacement over the range of 1-100 ng/ml, a mean 50% displacement end point of 15 ng/ml and excellent specificity with respect to other antibiotics and related chemicals.

The specific procedure subsequently described provides the complete information necessary to perform the CELIA for CA. This procedure represents a modified version of the originally developed and published manual method. This modified version is automated and employs 96 well microtiter plates and Flow (ICN) automatic plate washing and optical density reading equipment. This automated version affords the potential opportunity for high volume sample analysis and effective cost savings by the reduced use of extremely expensive developmental biochemical reagents.

19.2 Equipment and Supplies

a. Flow (ICN) Laboratories Titertek Multiskan MC plate reader; #78-530-00.
b. Flow (ICN) Laboratories Titertek Microplate Washer; #78-431-00.
c. Flow (ICN) Vacuum pump for above washer; #78-426-00.
d. Flow (ICN) Titertek Multichannel pipette; 8 channel, adjustable 50-200 ul volume; #77-859-00.
e. Eppendorf Repeater Pipette (Daigger Scientific Co.#G20551) with accessory of 2.5 ml capacity Combitips (Daigger #G20552C) and 5.0 ml capacity Combitips (Daigger #G20552D).
f. Dynatech Laboratories Microelisa plates, Immulon I, flat bottom, 96 wells, #11-010-3350 and covers.
g. Incubator, 37°C (any properly operating brand).
h. Stomacher®, Model 80 (Tekmar Co., Cincinnati, OH).
i. Whirl-pak® bags; 75 x 180 cm size.
j. Centrifuge, capable of operation at 15,600 x g (Eppendorf, Model 5412; Brinkman Instruments, Inc.), and appropriate centrifuge tubes.
k. Refrigerator (4°C).
l. Microtest Manifold, Wheaton, straight, 8 place with Luer Lock connection (Daigger #G20560A).

19.21 Chemicals and Reagents

a. Na_2HPO_4 (Fisher, S-374).
b. NaH_2PO_4 (Fisher, S-369).
c. NaCl (Fisher, S-271).
d. Citric acid, anhydrous (Fisher, A-940).
e. Hydrogen peroxide, 30% reagent grade (Fisher, H-323).
f. Tween 80 (Fisher, T-164).
g. Sodium azide†; NaN_3, purified (Fisher, S-227).
h. Bovine Serum Albumin, powder, fraction V (Sigma, A-4503), store in refrigerator.
i. Chloramphenicol, crystalline (Sigma, C-0378), store in refrigerator.
j. ABTS substrate indicator; 2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid), (Sigma, A-1888).

19.22 Biochemical Reagents and Supplies

a. Anti-chloramphenicol serum (undilute).
b. Chloramphenicol-BSA conjugated antigen (50 µg/ml stock).

c. Goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate; Miles-Yeda, Israel, (undilute).
d. Chloramphenicol negative beef tissue (initial supply only; used to set up tissue-CA standards).
e. Normal Rabbit Serum (undilute).

NOTE: The above 5 items must be stored in the frozen state at all times to maintain stability.

19.23 Preparation of Stock Reagent Solutions

a. 0.15 M Phosphate Buffered Saline at pH 7.2 (PBS)

Add 10.35 grams of NaH_2PO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "acid" solution. Add 10.65 grams of Na_2HPO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "base" solution. While mixing with a magnetic stirrer and monitoring the pH on a pH meter, add a sufficient quantity of the "acid" solution to the "base" solution to achieve a final, stabilized pH of 7.2. Dispense into glass containers, autoclave at 121°C for 15 minutes and store at room temperature. It is most convenient to make up this buffer in 5 liter quantities at a time.

b. Phosphate Buffered Saline Containing 0.05% Tween 80 (PBS-Tween)

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2 add 0.5 ml of Tween-80 and mix (not on magnetic stirrer) for several hours at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

c. Phosphate Buffered Saline Containing 0.5% Bovine Serum Albumin (PBS-BSA)

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2, add 5 grams of powdered bovine serum albumin and 1 gram of sodium azide (NaN_3) and mix (not on magnetic stirrer) at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

d. ABTS - H_2O_2 Substrate Buffered Solution

Prepare a 0.1 M citric acid solution by dissolving 1.92 grams of anhydrous citric acid in 100 ml of distilled water. Prepare a 0.1 M dibasic sodium phosphate stock solution by dissolving 1.42 grams of Na_2HPO_4 in 100 ml distilled water. Add sufficient quantities of these two stock solutions together while mixing with a magnetic stirrer and monitoring the pH on a pH meter to prepare 100 ml of a 0.1 M citrate-phosphate buffer at a final stabilized pH of 4.0.

To 100 ml of the above prepared 0.1 M citrate-phosphate buffer add 22 mg of ABTS [2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid)] and 15 µl of stock 30% hydrogen peroxide, mix gently by hand (no magnetic stirrer) until completely dissolved. Pass this substrate solution through a 0.45 µm Millex® filter, place in a sterile glass container, and store in the dark at room temperature until needed. This substrate solution should be prepared 24 h in advance of need and may be used as long as it retains its original light green color. A solution which has deteriorated to the point where it cannot be used is evidenced by a dark azure-green color formation.

e. PBS Chloramphenicol (CA) Standards

Prepare a stock 1 mg/ml chloramphenicol (CA) solution by weighing out 10 mg powdered, pure CA on an analytical balance and placing in 10 ml PBS. Allow the CA to dissolve thoroughly into solution by occasional mixing over a period of 24-48 h, or longer if necessary, due to limited solubility of the CA. From this stock 1 mg/ml CA solution make serial ten-fold dilutions in PBS (10 ml quantities) to produce CA standards at concentrations of 10,000, 1,000, 100, 10, and 1 ng/ml respectively. Store these standards in the refrigerator (4°C) until used in the assay.

f. Tissue Extract CA Standards

Prepare tissue extract from known CA free, raw, bovine muscle tissue by the following manner:

i. Place 5 grams of diced tissue in a 75 x 180 cm Whirl-pak® bag.

ii. Add 10 ml PBS.

iii. Place bag in Model 80 Stomacher® and stomach for 30 seconds.

iv. Remove bag from Stomacher® and leave undisturbed for 1 h at room temperature.

v. Pour off the liquid contents from the extraction bag into a centrifuge tube.

vi. Centrifuge at 15,600 x g for 15 minutes.

vii. Collect the clear supernatant tissue extract. If necessary, filter to remove all debris and lipid particulates, and place in a sterile glass container.

Using the PBS-CA standards prepared in (e) above, make ten-fold dilutions of each needed 10X higher concentration standard into the freshly prepared beef tissue extract to produce CA standards at concentrations of 1,000, 100, 10, and 1 ng/ml respectively. These tissue extract CA standards should be made fresh each time a standard curve is to be run in the CELIA. The tissue extract originally prepared, without CA, should be stored in the refrigerator and may be used for subsequent CA standards preparation as long as the extract shows no evidence of microbial contamination or protein precipitation. Tissue extracts should always be prepared from tissues similar to those being analyzed for the presence of CA with respect to species and organ or tissue type.

19.3 Performance of CELIA for CA

a. Obtain a flat bottom, 96 well Dynatech Immulon I microelisa plate and cover from stock supplies.

b. Prepare a sufficient quantity of the Chloramphenicol-Bovine Serum Albumin (CA-BSA) conjugated antigen for plate well sensitization. Make a small volume dilution

of the stock 50 µg/ml CA-BSA antigen solution in PBS such that a final concentration of 50 ng/ml CA is obtained.

c. By using the 8 channel pipette, place 200 µl of the 50 ng/ml CA-BSA (in PBS) sensitizing antigen solution into all wells except those of column 2. Leave these wells empty for the present time.

d. Place a cover on the plate and allow the CA-BSA antigen to passively absorb to the wells by incubating the plate for 3 h at 37°C.

e. Test sample extractions should now be concurrently started at this stage in the following manner:

   i. Place 5 grams of diced tissue in a 75 x 180 cm Whirl-pak® bag.

   ii. Add 10 ml of PBS.

   iii. Place bag in Model 80 Stomacher® and stomach for 30 seconds.

   iv. Remove bag from Stomacher® and leave undisturbed for 1 h at room temperature.

   v. Pour off liquid contents from the extraction bag into a centrifuge tube.

   vi. Centrifuge at 15,600 x g for 15 minutes. (Eppendorf Model #5412 centrifuge using 1.5 ml volume centrifuge tubes is very convenient for this).

   vii. Place the clear, test sample supernatant extracts in the refrigerator (4°C) until called for in step (p) of this assay procedure.

f. Remove the plate from the incubator [continued from step (d)], remove the cover and mount on the carrier of a Titertek Microplate Washer which has been primed with PBS-Tween and set to deliver 300 µl fluid to each well.

g. Remove the CA-BSA sensitizing antigen solution from the wells by aspiration with the washer and wash the wells <u>once</u> with 300 µl of PBS-Tween per well.

h. Remove the plate from the washer, invert over a sink, hold the plate tightly in one hand and flick several times to remove any remaining excess liquid from the wells.

i. Tap the plate in an inverted position several times on a soft paper towel (Sorg Laboratory Towels) placed on the surface of the lab bench and allow the plate to remain inverted for 1-2 minutes to complete the draining process. Place the plate right-side up and cover until next reagent addition.

j. Block unwanted reactive sites on the plastic wells by filling all wells (including those in column 2) with 250 µl of PBS-BSA per well, dispensed by using a 8 place microtest manifold attached to a Cornwall syringe.

k. Replace the cover on the plate and incubate for 2 h at 37°C.

l. Remove the plate from the incubator, place on the carrier of the washer, aspirate the PBS-BSA blocking solution out of each well and wash the wells twice with 300 µl of PBS-Tween per well.

m. Repeat steps (h) and (i).

n. With an appropriate pipetting device place 150 µl of PBS in the wells of column 1, 2, 3, and 4 of row A and B. Place 150 µl of CA free tissue extract in the wells of column 1, 2, 3, and 4 of row C and D and the wells of column 1 and 2 of row E, F, G, and H. These wells all serve as negative reagent controls (column 1 and 2) or 0 level controls (column 3 and 4).

o. Place 150 µl of PBS CA standards at concentrations of 1, 10, 100, and 1000 ng/ml in wells of column 5 and 6, 7 and 8, 9 and 10, 11 and 12 respectively of rows A and B. Place 150 µl of tissue extract CA standards at concentrations of 1, 10, 100, and 1000 ng/ml in wells of column 5 and 6, 7 and 8, 9 and 10, 11 and 12 respectively of rows C and D. These wells serve to produce the standard CA inhibition curves in PBS (rows A and B) and tissue extract (rows C and D).

p. Place 150 µl of each test sample extract [from step (e)] in 2 adjacent wells (duplicates) of an individual row. All wells of column 3-12 of row E-H are available for use for duplicate analysis of individual test sample extracts (20 test sample capacity/plate). Record in some appropriate fashion the location of each test sample extract within the available wells for sample analysis for future reference.

q. With the use of an Eppendorf Repeater pipette and a 2.5 ml Eppendorf combitip attached, add 50 µl of normal rabbit serum diluted 1:700 in PBS to all wells of column 1. The wells of this column serve as zero blank normal rabbit serum controls, producing no visible reactions and are used to blank in the reader making spectrophotometric measurements of the reactions in all subsequent wells in each row.

r. With the use of the repeater pipette and a new 2.5 ml combitip attached, add 50 µl of anti-chloramphenicol serum diluted 1:700 in PBS to all remaining wells.

s. Carefully mix and distribute the contents in each well by gently rocking the plate and tapping the ends against your fingers. DO NOT allow the contents of any well to spill out as this will invalidate this result.

t. Place the cover on the plate and incubate overnight (16-18 h) in the refrigerator at 4°C.

u. Remove the plate from the refrigerator, allow equilibration to room temperature, place on the carrier of the washer, aspirate the contents out of each well and wash the wells twice with 300 µl PBS-Tween per well.

v. Repeat steps (h) and (i).

w. By using the 8 channel pipette, add 200 µl of goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate diluted 1:5000 in PBS-Tween to all wells.

x. Place the cover on the plate and incubate for 2 h at 37°C.

y. Remove the plate from the incubator, place on the carrier of the washer, aspirate the contents out of each well and wash the wells three times with 300 µl of PBS-Tween per well.

z. Repeat steps (h) and (i).

aa. With the use of the 8 channel pipette, add 200 µl of ABTS-H_20_2 substrate buffered solution to all wells.

bb. Place the cover on the plate and incubate for 90 minutes at 37°C.

cc. Twenty minutes prior to the end of the above incubation period, turn on the power to the Titertek Multiskan MC plate reader and allow it to warm up.

dd. After the 90 minute incubation period of step (bb) is complete, remove the plate from the incubator, remove the cover and place the plate on the carrier of the Multiskan MC plate reader.

ee. Program the reader for the current date, Mode 1 (single wavelength absorbance), Wavelength filter #2 (414 nm), push the carrier and plate into the measuring head and blank the instrument (zero O.D. point set) on column 1.

ff. Press the START button and obtain a printed paper strip of the Optical Density (O.D.) values for all of the reaction wells on the plate.

gg. Remove the plate from the reader and visually examine the plate to see that the obvious colored reaction intensities generally correspond to the numerical values on the printed data sheet to assure that the instrument properly read the plate.

hh. Turn off the power to the Multiskan MC plate reader and discard the plate (save the cover for reuse) after completion of the Data Analysis, Plotting, and Sample Interpretation Section described below.

19.4 Data Analysis, Plotting, and Sample Interpretation

a. All wells in column 1, which serve as the zero-blank normal rabbit serum control, should have no color

reaction. This indicates a proper lack of non-specific attachment of rabbit serum or GARP conjugate to the bound CA antigen in the wells. Under these conditions these wells are excellent controls to blank in (zero point set) the O.D. reading instrument.

b. All wells in column 2 serve as BSA negative controls to assess non-specific attachment of anti-CA antibody (and also GARP). Since these wells were never sensitized with CA antigen and only blocked with BSA, no positive reactions (high O.D. values) should be observed. These controls may also be considered as a check on the other half of the primary antigen-antibody component of the assay system initiated in column 1.

c. Wells in columns 3 and 4 of rows A, B, C, and D should demonstrate maximum binding of anti-CA antibody (zero inhibition) and have the highest O.D. values. These represent the zero controls for the standard inhibition curves produced by subsequently increasing concentrations of CA.

d. The remaining wells of rows A and B represent the standard inhibition curve for PBS CA standards and those of rows C and D represent the standard inhibition curve for tissue extract CA standards. The O.D. values in both of these series of wells should decrease with increasing concentrations of CA due to inhibition of binding of anti-CA antibody.

e. The remaining wells of the plate (columns 3-12 of rows E-H) represent reaction values for test sample extracts relative to the presence or absence of CA in the original samples.

f. For each pair or set of wells containing exactly the same test materials, calculate the average O.D. value.

g. Obtain a piece of 5 cycle semi-logarithmic graph paper containing 100 numerical scale divisions. Label the ordinate (100 numerical scale divisions) with O.D. values from 0 to 2.0 in increments of 0.2 units. Label the abscissa (5 cycle logs) with CA concentrations of 0, 1, 10, 100, and 1000 ng/ml.

h. Plot the average O.D. values generated for the PBS CA standards and tissue extract CA standards from 0 to 1000 ng/ml respectively on the graph paper. Draw straight lines from point to point. You will now have two inhibition (displacement) curves for increasing concentrations of CA in PBS or tissue extracts.

i. Examine the two inhibition curves and compare the slopes and overall O.D. values. The PBS CA standard displacement curve represents the basic reaction level of the primary antigen-antibody system influenced only by pure CA. The tissue extract CA standard displacement curve represents the influence of CA and interaction of various proteinaceous materials extracted from the test sample. If the tissue extract CA inhibition curve is significantly different from the PBS CA inhibition curve (which it usually is) use the former for determining positive CA concentration levels in test samples.

j. Calculate the 50% displacement end point for both standard inhibition curves (50% of the 0 standard O.D.). Values in the range of 5 to 20 ng/ml with a mean value of around 15 ng/ml should be obtained as an indication of properly operating displacement systems.

k. To determine if a test sample contains CA and to quantitate the amount, if it is present, proceed as follows:

   i. Obtain the O.D. value for the test sample and determine the relationship to the tissue extract CA standard curve.

   ii. If this value is between 0 and 1 ng/ml (i.e. O.D. greater than the 1 ng/ml standard), the sample is considered to be free of CA.

   iii. If the value falls within the linear portion of the standard curve, from 1-100 ng/ml, the sample is considered to contain CA. To determine the amount of CA present per gram of tissue, interpolate from the curve the ng/ml CA value on the abscissa relative to the particular O.D. obtained for that sample and multiply it by 2. This assumes that all of the CA from the original 5 gram of tissue is extracted into the 10 ml PBS volume and the

resulting dilution therefore is 1:2 rather than the usual 1:3.

iv. If the O.D. value falls beyond the linear portion of the standard curve (ie. O.D. less than the 100 ng/ml standard), the sample is also considered to contain CA but accurate quantitation is not possible from this particular analytical run. More accurate quantitation in this case would be achieved by taking this sample extract, making serial ten-fold dilutions of it in PBS (10^1 -10^6), repeating the CELIA analysis a second time on these dilutions and determining which dilution produced an O.D. value within the linear portion (1-100 ng/ml) of the PBS CA standard curve.

Calculations for this sample would then be reduced to: interpolated CA value of ng/ml from the PBS CA standard curve abscissa x ten-fold dilution factor x 2 = ng CA/gram of tissue.

19.5 Quality Control Procedures

a. The assay reagents have been evaluated for use only with Dynatech Immulon I microtiter plates. No other plates should be used.

b. All stock reagent solutions must be properly prepared and maintained free of contamination or chemical breakdown.

c. All stock immunochemical reagents must be stored in the frozen state at all times to maintain stability.

d. The stock ABTS-H_2O_2 substrate buffered solution should not be used if it has turned to a significantly darker shade of green from that of the original preparation.

e. Be sure the stock, commercial preparation of Goat anti-rabbit immunoglobulin G horseradish peroxidase (GARP) conjugate reagent has not deteriorated to the point of producing improper final O.D. readings. Use only an unexpired lot of this reagent.

f. To insure validity of the quantitative aspects of this assay, extreme care must be exercised to accurately

prepare the standard CA concentrations in PBS and CA free tissue extracts from stock sources of the pure CA drug.

g. The CA free tissue used to prepare extracts for subsequent preparation of the CA tissue extract standards should be initially validated as being free of CA by a reliable procedure.

h. Standard curves for CA in PBS and CA in tissue extracts must always be run in an analytical determination for the presence of CA in test samples.

i. The tissue source used to prepare the CA tissue extract standard curve must be of the same species and organ type as that of the test sample to be quantified.

j. The standard CA inhibition curves should always be quite similar from run to run and the 50% displacement end point should always be in the same general range. Drastic deviations in the above indicates an improperly operating displacement system due to critical reagent deterioration or technical error in the assay set-up and must therefore be corrected.

k. A valid test run can only be assured by the demonstration of proper CA standard inhibition curves for each particular analytical determination.

† Safety Caution: Do not dispose of spent sodium azide PBS-BSA solution by pouring down sink drains.

Collect in separate liquid waste container and dispose of as hazardous waste according to standard waste management procedures for your laboratory.

Accumulation of sodium azide in lead sink drains may result in an explosion.

19.6 Selected References

Campbell, G. S., R. P. Mageau, B. Schwab, and R. W. Johnston. 1984. Detection and quantitation of chloramphenicol by competitive enzyme-linked immunoassay. Antimicrob. Agents Chemother. 25:205-211.

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