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ULTRASENSITIVE DETECTION OF CHEMICAL SUBSTANCES

REPORTING PERIOD
JULY 1, 1982 - FEBRUARY 29, 1984

EFFECTIVE DATE OF THE CONTRACT
JULY 1, 1982

EXPIRATION DATE OF THE CONTRACT
FEBRUARY 29, 1984

FINAL REPORT

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FINAL REPORT

PERIOD OF DECEMBER 31, 1983 TO JANUARY 31, 1984

We report in this final period of our extension the satisfactory completion of all tasks as stated in our contract. Indeed in many task areas we have significantly exceeded task requirements. Our extra effort in selected task areas has been valuable in our determination of the feasibility of our approach. We are now quite satisfied that our approach to ultrasensitive detection is reasonable and we look forward to renewing our contract to demonstrate its functionality. In the following report, we discuss under each task assignment our progress and accomplishments during the course of our contract together with data that is pertinent to the discussion. In the Appendix we summarize procedures that have been developed during our contract period and refer to these procedures in the appropriate sections of the text.

Task 1. Preparation/purification hapten carrier/trapping mojety ;

As explained in our early quarterly reports, we have found acetylcholinesterase (EC 3.1.1.7) purchased from Sigma Chemical Company to be adequate for our needs without further purification. This includes use in immunizing mice or rabbits and for binding to solid supports to select monoclonal antibodies or evaluate ultrasensitive assays. The enzymatic assay procedure for the colorametric determination of AChE is given in the Appendix, Procedure #1. In addition, we have been in the process for some months of developing a radiological assay for AChE activity that would considerably improve our assay sensitivity. This would be useful as an independent and sensitive assay for quantitating AChE bound to solid support surfaces. We have made a number of improvements in this assay procedure. However, we are not yet satisified with the reproducibility of this assay. We, therefore, continue to rely largely on our colorametric assay described in the Appendix.

While the Sigma Chemical Company's AChE is quite adequate for our purposes, it is expensive and our requirements for large amounts of this enzyme have proven costly. As an alternate source of inexpensive AChE, we have isolated this enzyme from human red blood cells which we have been able to obtain at a very modest cost. We now have several milligrams of crude enzyme which can be pu.ified by affinity column chromatography if needed. If this source of AChE proves to be of less cost than the enzyme purchased from Sigma, we would consider it as an alternative source in our experiments.

Task 2. Formation of hapten carrier adducts -

We have successfully formed AChE-DFP conjugates and reported our completion of this task in our second quarterly report. The current procedures used to form AChE-DFP conjugates are given in the Appendix, Procedure 2. In addition, the protocol used for immunizing mice with this antigen is given (Procedure 3).

Task 3. Production of hybridoma cell lines

Hybridization of NS1 myeloma cells with immunized mouse splenocytes is now routinely done in our laboratory. Most difficulties with both the in vitro and in vivo immunization and hybridization protocols have been overcome. As with any delicate procedure, difficulties can and do arise. However, we have been able to overcome these difficulties and produce successful fusions and hybridoma cell lines. At present we have five lines, as well as ten clones from each line, frozen in liquid nitrogen for storage. These lines were selected for their production of monoclonal antibody to DFP-AChE conjugates. Procedures for fusion are given in the Appendix, Procedure 4.

Task 4. Selection, cloning, preservation and characterization of AChE-DFP producing hybridomas

A. Selection

1. Antibody production - Hybridomas are identified visually. Supernatants from culture wells containing hybridomas are tested for the presence of mouse immunoglobulin (MIg) utilizing the streptovidin HyBRL-Screen assay produced by Bethesda Research Laboratories. (See Appendix, Procedure 5.)

2. Specific Ab production - MIg producing culture supernatants are tested for the production of AChE/AChE-DFP specific Ab using an enzyme linked immunosorbant assay (ELISA) developed in this lab. (See Appendix, Procedure 6.)

B. Cloning

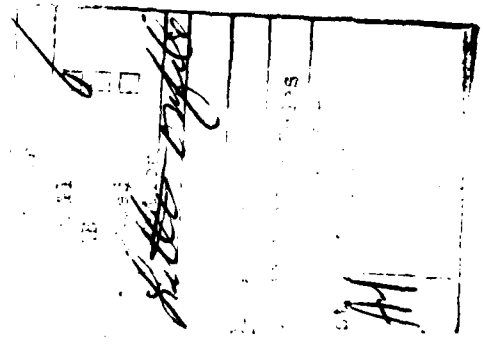
Primary AChE/AChE-DFP specific Ab producing hybridoma cultures are expanded from microfilter plate wells to flasks. The cultures are then cloned by limiting dilution. At least 10 AChE-DFP Ab producing clones from each primary culture are expanded in culture flasks. (See Appendix, Procedure 7.)

C. Preservation

AChE/AChE-DFP specific Ab producing original hybridomas and their clones are preserved in liquid nitrogen. (See Appendix, Procedure 8.)

D. Characterization

AChE/AChE-DFP specific MIg subclass is determined by immunoelectrophoresis. (See Appendix, Procedure 9.)



Task 5. Fabrication of solid supports with bound trapping moiety

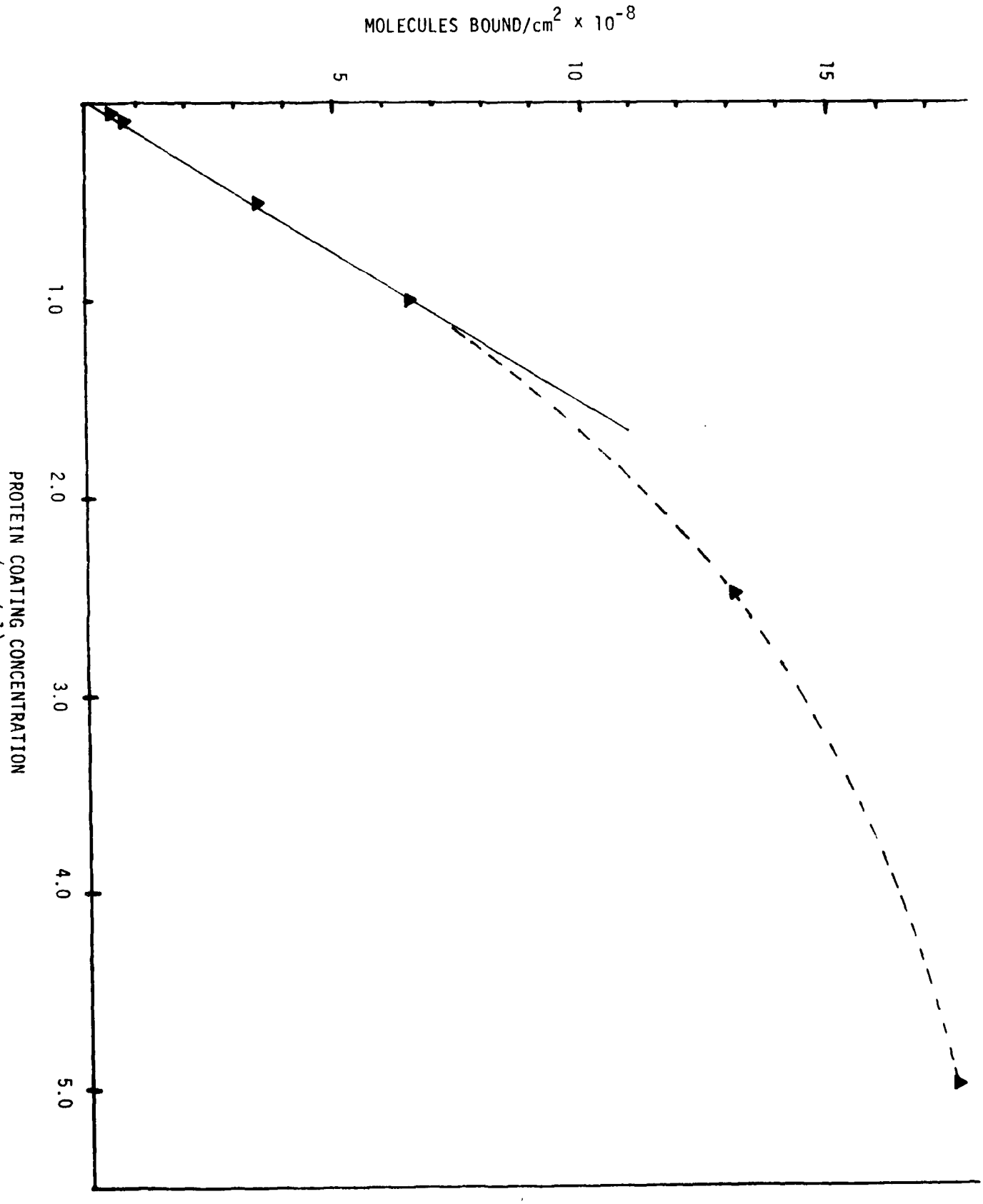
We have successfully completed our objective of binding trapping agent (AChE) to solid supports. However, we have continued to make improvements in binding and noise characteristics of solid support surfaces during the course of our contract in an effort to examine certain variables associated with the ultrasensitive detection system we propose.

The need to develop solid supports with bound trapping moiety is twofold. First, we require a solid phase system adapted to ELISA procedures so that monoclonal antibodies with the proper specificity may be selected. Second, the ultrasensitive detection system we propose requires a solid phase with appropriately bound trapping moiety.

Very early in our work we were successful in binding AChE to polystyrene and polyvinyl wells in microtiter plates. This has been reported in our first, second, and third quarterly reports. We found that polystyrene bound greater amounts of AChE than polyvinyl and that polystyrene plate wells coated with AChE retained good AChE activity up to 24 hours after coating. This binding and retained activity is quite adequate for evaluating monoclonal antibody specificity to select monoclonal antibodies as described in Task 4. We continue to update and improve our methods in this area, but basically we have met or exceeded our objectives. The procedure for binding AChE to polystyrene is given in Appendix, Procedure 6.

The requirements for ultrasensitive detection using solid supports complexed with trapping moiety are more stringent than requirements necessary for isolating monoclonal antibodies. The trapping moiety must bind to the surface with reasonable stability as we have demonstrated with polystyrene binding. In addition, however, the surface with the bound trapping moiety must be essentially noiseless and yet provide uninhibited access for detector structures (antibody bound to fluorescent spheres) to bind to the trapping target molecule complex. A great deal of our contract effor has been devoted to exploring the variables involved with the above basic requirements. Three areas have been investigated relating to these questions. First, we quantitated protein bound to solid surfaces independently of fluorescent bead analysis. Second, we studies factors and procedures which lowered noise, yet retained highest possible degree of specific binding. Third, we briefly surveyed materials other than polystyrene as potential solid supports. Since we had not generated the appropriate monoclonal antibodies when we initiated these studies, model systems utilizing mouse IgG and their antibodies have been developed. Findings obtained from these model systems should transfer well to the AChE system when employed due to common proteinaceous and antibody binding characteristics of both systems.

Independent quantitization of protein bound to solid substrates is necessary if we are to correlate fluorescent sphere binding to surface bound protein trapping moieties. Figure 5-1 shows the binding of goat anti-rabbit IgG (^{125}I) Fab' fragments to polystyrene surface at various concentrations for 2 hours at 37°C. The procedure is given in Appendix, Procedure 10. The measurements are not sensitive at the low levels of molecules we propose to


MOLECULES BOUND/cm^2 x 10^{-8}
5
10
15
PROTEIN COATING CONCENTRATION
(ngm/ml)
1.0
2.0
3.0
4.0
5.0

detect with ultrasensitive detection. However, the last 3 to 4 points of the curve shown appear to be linear. Using this linear slope we can extrapolate smaller treatment concentrations to their corresponding lower numbers of bound protein molecules. With this extrapolated data, we can make estimates as to the number of fluorescent beads that should bind to surface associated proteins at lower treatment concentrations. This method of estimation depends heavily on extrapolation over 2 to 3 orders of magnitude and, in final cases, as much as 8 orders of magnitude. To improve the accuracy of extrapolation, we hope in the future to use (^{125}I) labelled avidin or other proteins with as much as 1000 fold greater specific activity which will enable us to compare projected levels of fluorescent bead binding three orders of magnitude lower than done at this time. Detailed procedures have been instituded to provide testing of variables that affect nonspecific and specific binding of fluorescent beads to treated polystyrene surfaces. The procedure is given in Appendix, Procedure 11.

Results have been quite variable as we have stated in our most recent quarterly report. Our best results indicate detection of about one out of twenty rabbit IgG molecules bound to polystyrene. In some cases, binding does not develop at all or control levels (non-specific binding) are unacceptably high. To date we have determined several factors to be responsible in part for the variability observed. As a result, we prepare all BSA blocking and washing solutions fresh and beads are coated using distilled water rather than PBS. We are encouraged that good results are attainable because we have observed low non-specific binding with high specific binding. In addition, during the course of our studies with binding, we have innovated procedures that will give information about both specific and non-specific binding. One of these procedures is reverse centrifugation of plates with fluorescent beads bound to the surface. By centrifuging the beads off from the surface, we can determine quantitatively the force with which they are bound.

Our survey (reported in our third quarterly report) of materials that could be used to replace polystyrene in a solid phase detection system has been most encouraging. After testing several plastic and glass surfaces, we have found two surfaces (agarose coated mylar and matt mylar) whose noise levels are very near zero. We hope in a renewal of this contract to be able to investigate these surfaces more completely.

Task 6. Production of fluorescent loaded antibody labeled signal packets

We have reported on the use of covaspheres as fluorescent signal packets many times in our quarterly reports. Covaspheres continue to be our choice as signal packets due to their availability, range of surface binding characteristics and range of sizes, down to 0.3 microns, that are supplied. We have recently revised our procedure for binding antibody to covasphere beads (See Appendix, Procedure 12) and we have developed a simple ELISA assay to determine the amount of antibody bound to each bead (Appendix, Procedure 13).

Task 7. Initial evaluation of concept feasibility

To evaluate our approach to ultrasensitive detection, several elements must be considered. The most important element in our scheme of detection is the ability to produce monoclonal antibodies, particularly to the determinate specific for the complex between the hapten and trapping agent. We have been quite sucessful in producing monoclonal antibodies. We have now five monoclonal antibodies against AChE-DFP conjugates.

Another important element in developing an ultrasensitive assay is to produce signal packets with appropriate fluorescent signal characteristics and the ability to bind antibody. In addition, characteristics of low non-specific binding to solid substrates and the ability to link to a solid substrate with specific binding that is stable to various washing procedures are important considerations in selecting signal packets.

We were very fortunate that Covaspheres, a new product of the Covalent Technology Corporation, were being developed shortly before we began work. These beads have a very strong fluorescent signal and very little fading which gives excellent counting characteristics with a microscope at only 100 magnification. In addition, these spheres can be obtained with a variety of activated surfaces that bind antibody covalently. Using these beads, we have been able to demonstrate their feasibility as signal packets. The availability of these beads has enabled us to do some preliminary investigation of low noise factors and specific binding to solid substrates which is scheduled to be done in the second year of our contract. All indications are that Covaspheres will work well in an ultrasensitive assay. While many factors contribute to the success of our project, the ability to make the appropriate antibody and utilize fluorescent signal packets are the most crucial. Our success with the covaspheres, together with our ability to produce monoclonal antibodies against the AChE-DFP antigen certainly indicate that our approach to ultrasensitive detection is feasible.

APPENDIX

Procedure 1 - Colorimetric Method for Measuring AChE Activity

Procedure 2 - DFP-AChE Binding Procedure

Procedure 3 - Immunization Protocol

Procedure 4 - Fusion Protocol

Procedure 5 - Screening for Ig Production

Procedure 6 - ELISA for Determination of the Presence of Antibody Specific for Acetylcholinesterase (AChE)

Procedure 7 - Cloning by Limiting Dilution

Procedure 8 - Freezing Procedure

Procedure 9 - Immunoelectrophoresis

Procedure 10 - Binding Radioactive Goat IgG

Procedure 11 - Fluorescent Bead Binding to Treated Plates

Procedure 12 - Coating Bead with Protein

Procedure 13 - Measurement of Antigen Bound to Fluorescent Beads

APPENDIX

Procedure 1

COLORIMETRIC METHOD FOR MEASURING AChE ACTIVITY

The procedure used is the published Ellman Procedure where acetylcholine iodide is used as substrate and dithiobisnitro benzoic acid as color reagent in phosphate buffer at pH 8.0. The reaction is done at room temperature and followed with a Beckman Du-6 Spectrophotometer at 450 nm for color appearance. Units of activity are calculated using the change in absorbance with a given time.

APPENDIX

Procedure 2

DFP-AChE BINDING PROCEDURE

Dissolve acetylcholinesterase from electric eel in 0.01 M MOPS buffer at pH 7.6. To it add 1/10 volume of DFP diluted 1:500 in MOPS buffer. Incubate overnight at room temperature. Use directly for aluminum precipitated immunization. For soluble injections, the above material is dialyzed against three changes of PBS in the cold. Aliquots are taken before and after DFP reaction and at the end of dialysis.

Activity measurement - Enzyme activity of initial and final material is measured as described in Procedure #1.

Protein Concentration Measurement - Protein concentrations are measured to determine recovery efficiency as described by the Bio-Rad kit manual. A standard curve is prepared using bovine y-globulin supplied by the manufacturer. Amounts vary from 1.44 to 17.88 mg in 0.8 ml PBS buffer. Samples are appropriately also diluted to 0.8 ml. To samples and standard is added 0.2 ml of Bio-Rad dye reagent concentrate. Vortex and leave at room temperature for 5 minutes to one hour. Read absorbance at 595 nm. Construct a standard curve and read unknowns from it.

APPENDIX

Procedure 3

IMMUNIZATION PROTOCOL

Mice used for immunization are Balb/C at 6 weeks of age. The first three injections were of aluminum precipitated enzyme at 3 week intervals given ip. Acetylcholinesterase-DFP is precipitated with aluminum potassium sulfate after addition of sodium biocarbonate. Let it sit 15 minutes. Centrifuge and wash three times with PBS buffer. Suspend the precipitate in PBS at 1 mg/ml and add pertussis at a concentration of 2×10^9 organisms per mouse.

One week before fusion, 50 nm of enzyme is given in soluble form followed by 200 and 50 nm starting from day 4 to day before fusion.

APPENDIX

Procedure 4

FUSION PROTOCOL

1. NS1 and splenocytes at 1:1 (viable) ratio.
2. Fuse 1.0 to 5.0 x 10^7 total (NS1 + splenocytes) per tube (16 x 12.5 mm round bottomed tube).

   NOTE: All media is at room temperature.
3. Centrifuge combined cells at 300 xg for 8 minutes. Remove all media by pipette.
4. Disrupt the cell pellet in the remaining media by tapping the tube.
5. Add 0.2 ml 50% PEG in Standard Iscoves/0 slowly while shaking.
6. Centrifuge for 6 minutes at 200 xg.
7. Add 5 ml medium slowly by setting the pipette against the side of the tube and allowing the medium to drain down the side of the tube (Standard/0). Let stand 2 minutes.
8. Add 5 ml medium (Standard/0). Resuspend cells by tapping the tube.
9. Centrifuge at 300 xg for 5 minutes. Decant SN.
10. Add Complete/20 containing 10^7 thymocytes per ml to produce an end concentration of NS1 + splenocytes of 10^7/ml. Let stand for 30 minutes. Resuspend the cell pellet by tapping the tube.
11. Place 1 drop/well in 96 well plate with 5 ml pipette. Add 1 drop per well of Complete/20 or alternatively, Complete/20 + HAT, in which case step 13 is omitted.
12. Incubate at 37°C/7% CO_2 in humid air overnight.
13. After incubation overnight, add 2 drops Complete/20 + HAT via 5 ml pipette.
14. Feed each culture on days 3, 6, 9, and 12 by removing 1/2 the medium and replacing it with Complete/20 + HAT.
15. Growing hybrids should be visible by day 10.
16. Discontinue addition of aminopterin on the first feeding day after day 12. Continue using Complete/20 + H T until transfer to 1 ml cultures.

Modification of the procedure of:
R.P. Siragania, P.C. Fox, E.H. Berenstein. Methods of enhancing the frequency of antigen-specific hybridoma. Methods in Enzymology 92:17-26 (1983).

MATERIALS - GENERAL

Insulin Sigma I-5500
100X = 0.2 u/ml DH_2O

Aminopterin Sigma A-2255
100X = .189 g/ml DH_2O (4.3×10^{-5} M)

Hypoxanthine Sigma H9377 (1×10^{-5} M)
100X = 1.36 mg/ml DH_2O

Thymidine Sigma T9250
100X = 0.387 mg/ml DH_2O (1.6×10^{-3}M)

Glutamine Sigma G3126
100X = .0292 g/ml DH_2O (200 mM)

Pyruvate MA Bioproducts #13-1154
(100 mM)

Oxaloacetate Sigma O-4126
100X = 13.20 mg/ml

2-Mercaptoethanol
2500X = .1 ml in 14.1 ml medium (0.1 M)
(40 ul/100 ml medium)

NCTC-135 Gibco 320-1350

Polyethylene glycol (PEG) 1500
(MW 500-600)
MA Bioproducts #17-780Z

Medium

Iscoves Modified Dulbecco's Medium
(Iscoves) Irvine Scientific 9422

Formulations

1. Basal - No additives

2. Standard/X*

*[X = % Fetal Bovine Serum (FBS)]

pen strep
glutamine/pyruvate
FBS

3. Complete/X*

pen/strep	FBS
glutamine pyruvate	NCTC (10%)
oxalacetate	2-Mercaptoethanol
insulin	

4. Complete/X* + HAT

   As #3 except that it also contains hypoxanthine, thymidine, and aminopterin.

5. Complete/X* + HT

   As #4 except that aminopterin is omitted.

APPENDIX

Procedure 5

SCREENING FOR Ig PRODUCTION

Materials:

Streptavidin - HyBRL Screen Kit; Bethesda Research Laboratories
BRL #9505SA

Buffer A - Washing Buffer

0.01 M PO_4 buffer pH 7.2

0.385g KH_2 PO_4

1.248g K_2HPO_4

8.76g NaCl

.5g BSA (.05% BSA)

qs DH_2C to 1000 ml

Buffer B - Dilution Buffer

0.01 M PO_4 buffer pH 7.2

0.385g $1KH_2$ PO_4

1.248g K_2HPO_4

8.76g NaCI

1.0g BSA (1.0% BSA)

qs DH_2O to 1000 ml

Store buffers A & B at 4° C - Warm to room temperature prior to use.

Buffer C - Substrate buffer

0.1 M citrate buffer pH 4.5

0.588g citric acid

0.647g Na citrate .$2H_2O$

qs DH_2O to 100 ml

Make fresh for each assay.

Just prior to use:

Add: 0.04 ml 30% H_2O_2/100ml Buffer C and 1 mg o-phenylenediamine per ml Buffer C.

Buffer D - Antigen Adhesion Buffer

.05 M carbonate buffer

0.159g Na_2CO_3
0.293g $NaHCO_3$
0.2g NaN_3
DHO_2 qs 100 ml

Method: 3 days post last medium replenishment

A. Plate preparation

1. Coat each well of microtiter plate by placing 100 ul of hybridoma SN diluted 1:10 with 0.05 M carbonate buffer, pH 9.6, in the well and incubate overnight at 4°C on rotating platform.

2. Well 1-A will be a blank well. Add 0.1 ml of 0.05 M carbonate buffer containing 1% BSA. Well 2-A will be a positive control. Dilute mouse serum 1:100 with 0.15 M PBS, pH 7.2. Dilute 50 ul of the above dilution to 100 ul with 50 ul of 0.05 M carbonate buffer.

3. At the end of incubation, wash wells 4 times with buffer A at room temperature. (Washing - place 0.1 to 0.2 ml in each well. Invert plate over sink and hit edge of plate twice. Repeat as directed.)

B. Reaction of hybridoma SN with Biotinylated Second Ab

1. Dilute biotinylated Ab 1:1000 with Buffer B (stable 2-3 days at 4°C).

2. Add 100 ul of dilute Ab to each well.

3. Incubate at room temperature for 1 hour with constant shaking.

4. Wash plate 4 times with Buffer A as directed in Step A.3.

C. Incubation with Bridging Reagent

1. Dilute bridging reagent 1:1000 in Buffer B at room temperature.

2. Add 100 ul of diluted bridging reagent to each well.

3. Incubate at room temperature for 30 minutes with constant shaking.

4. Wash plate 6 times with Buffer A.

5. Color development. (Light will cause color development in the O-phenylenediamine solution. KEEP IN DARK.)

   a. Add 100 ul of 1 mg/ml O-phenylenediamine in Buffer C to each well.

   b. Incubate for 15-30 minutes at room temperature with constant shaking in the dark.

   c. Determine OD_{450} of each well.

APPENDIX

Procedure 6

ELISA

For Determination of the Presence of
Antibody Specific for Acetylcholinesterase (AChE)

Materials:

1. Acetylcholinesterase/Acetylcholinesterase-DFP

   .250 U/0.1 ml* in carbonate buffer, pH 9.68
   (*units are determined based upon original unit activity prior to DFP inactivation)

2. Carbonate buffer, pH 9.68 (1X)
   1.05g Na_2CO_3
   1.74g $NaHCO_3$
   qs 500ml DH_2O

3. PBS, pH 7.2 (10X)
   2.0g KCl
   2.0g KH_2PO_4
   80.0g NaCl
   21.6g Na_2HPO_4 $7H_2O$
   qs 1000ml PH_2O

   a. for 5% BSA - add 5g BSA/100ml buffer

   b. for 0.5% BSA - add .5g BSA/100ml buffer

   c. for 1% BSA - add 1.0g BSA/100ml buffer

4. Peroxidase labeled anti-mouse IgG diluted 1:1000 with PBS/1% BSA

5. ABTS solution
   200mg ABTS per 10ml DH_2O

6. Citric Acid
   2.5g citric acid/100ml DH_2O
   adjust to pH 4.0

7. Substrate:
   9.8ml citric acid, pH 4
   0.1ml ABTS solution
   0.1ml 0.5% H_2O_2 (0.100ml 30% H_2O_2 in 6ml DH_2O)

8. Nunc Immunoplate
   Plate I-96F
   #4-39454

Procedure:

1. Place 0.1 ml of 0.25 U AChE in carbonate buffer in wells of Nunc immunoplate.
2. Incubate plate at 4°C for 16 hours on rotating table.
3. Wash wells 3X with PBS/0.5% BSA.
4. Place 0.1 ml of PBS/5% BSA in each well (to block open sites in well)
5. Incubate at room temperature for one hour on rotating table.
6. Wash wells 3X with PBS/0.5% BSA.
7. Place 0.1 ml of undilute hybridoma SN in each well (except in well A1 in which PBS/0.5% BSA should be placed).
8. Incubate at room temperature for one hour on rotating table.
9. Wash wells 3X with PBS/0.5% BSA.
10. Place 0.1 ml of peroxidase labeled anti-mouse IgG diluted 1:1000 in PBS/1% BSA.
11. Incubate at room temperature for 1 hour with constant shaking.
12. Wash 3X with PBS/no BSA.
13. Add 0.1 ml substrate (ABTS/H_2O_2) and incubate at room temperature with constant shaking for 30 minutes.
14. Determine optical density of wells at 405 nm. (Well A1 will be blank...See Step 7.)

APPENDIX

Procedure 7

CLONING BY LIMITING DILUTION

1. Determine number of cells/ml of culture to be cloned.

2. Place 1 x 10^5 cells in a conical 15 ml centrifuge tube.

3. Suspend the cells in 10 mls Std/10 yielding 1 x 10^4 cells/ml.

4. Remove 1 ml from this tube, place in a second tube, increase volume to 10 mls, yielding 10^3 cells/ml.

5. In a 50 ml centrifuge tube, place:

   0.45 ml of 1 x 10^3 cells/ml, and
   44.55 ml of either:

   a. medium consisting of: 50% Complete/20 + HT
      50% NS1 treated Standard/10 supplemented with 10% PBS + hypoxantine, thymidine, glutamine and pyruvate

   or

   b. Complete/20 + HAT if 68 x 10^6 thymocytes

6. Place 0.2 mls of the cell suspension in the wells of each of two 96 well tissue culture plates. This volume will result in approximately two hybridoma cells/well.

7. Place the microtiter plates in a 37°C incubator at 7% CO_2 in humid air. Examine for growing clones at 14 days. Note and record the number of clones in each well.

8. During the fourth week, transfer clones to 24 well tissue culture plates in 0.5 ml of the medium used for cloning.

9. Feed the 24 well plates with 0.5 ml on days 5 and 10. Assay the supernatant for specific antibody activity on day 14.

10. Expand positive wells to 5 ml cultures and then to 75 ml flasks prior to freezing in liquid nitrogen.

<Methods in Enzymology 92:17-26 (1983)>

APPENDIX

Procedure 8

FREEZING PROCEDURE

1. Cells should be in logarithmic growth phase. Ensure that this is so by reculturing the cells to be frozen at 1.0×10^5 cells/ml 18 hours prior to harvest.

2. Pellet $1\text{-}2 \times 10^7$ cells by centrifugation at 400 xg for 10 minutes.

3. Remove the supernatant and resuspend the cells in 1 ml of their usual growth medium and place in a freezing vial.

4. Add 75 ul of Spectral Grade DMSO. Mix.

5. Immediately place the vial in the temperature gradient apparatus with the ring in the "D" notch and place in the liquid nitrogen container.

6. After 4-24 hours, place the vial in a cane and submerge into the liquid nitrogen.

APPENDIX

Procedure 9

IMMUNOELECTROPHORESIS

Buffer:

Barbitol-Na	$C_8H_{11}O_3N_2Na$	MW = 206	20.6 g/liter
Barbitol	$C_8H_{12}O_3N_3$	MW = 184	4 g/liter

Dissolve the barbitol in boiling distilled water. After the barbital is dissolved, remove from heat. Add barbital-Na.

Bring volume to desired level with DH_2O.

A pH range of 8.5 to 8.7 is acceptable.

Store at 4° C.

For use, dilute as follows:

1 volume buffer + 4 volumes DH_2O. Ed ionic strength = 0.02.

Immunoprecipitate are more distinct in this dilution- and heat production during electrophoresis is minimized. Duration of electrophoresis is limited, however, to maximum of 24 hours at 2 V/cm or 6 hours at 10 V/cm, due to pH changes at the poles. Use new buffer with each electrophoresis to avoid artifacts.

Gel Preparation:

1% Agarose in barbital buffer

Thickness 1.5 mm.

Multiply area of plate (in mm^2) by .0015 to determine the volume of agar (in mls).

Bring the buffer containing agarose to a boil - continue at high temperature until agarose is dissolved.

Pour solution slowly and steadily into center of level plate. If solution does not reach edges, coax there with a pipette.

For glass: The plate must be clean and dry.

Wash in detergent followed by repeated rinses in DH_2O, and a final rinse in 70% ethanol, then allow to dray prior to pouring gel.

For gel bond: The gel is applied to the hydrophilic side (the side protected by an interleaf). To confirm that the hydrophilic side is facing up, apply a drop of water and not that it will "spread" on the hydrophilic surface and will "bead" on the hydrophobic surface.

Allow agar to harden for 1 hour at 4° C.

Electrophoresis:

1. Punch sample holes in gel with .4 mm or 2.5 mm punch. Offset the holes so that they are nearer the cathode (negative electrode) than the anode. Use the template to ensure continuity in spacing for different sample wells.

2. Fill the wick reservoirs with 200 ml barbital buffer.

3. Attach the water lines to the cooling plate and start the water flowing.

4. Place the agar plate on the cooling plate, position squarely to avoid protein electrophoretic paths out of square with the gel.

5. Wet the wicks (Val-u-sorb underpants) with buffer. Place one end on gel, the other in the buffer reservoir. The wicks should be the same width as the gel.

6. Ensure the orientation of the gel is correct with respect to the current flow.

7. Apply samples to the wells. A positive sample with bromphenyl blue should be included to allow determination of the extent of electrophoresis.

8. Place the cover on the electrophoresis bed.

9. Apply power: 250V, 20 mA.

DETERMINATION OF HYBRIDOMA SUBCLASS

Diffusion in second dimension

1. Remove plate from electrophoresis apparatus.

2. Cut troughs between wells.

3. Add Ab specific for M IgG subclasses to the wells.

4. Incubate in a moisture box at 4° C for 16 hours.

5. Stain gel with Coomassie Brilliant Blue.

See Staining Procedure which follows.

STAINING OF AGAROSE GELS

Staining solution (for 1 liter)

Coomassie Brilliant Blue R 250	1 g
Ethanol (96%)	450 ml
Glacial acetic acid	100 ml
DH_2O	450 ml
	1000 ml

Destaining solution (for 1 liter)

Ethanol	450 ml
Glacial acetic acid	100 ml
DH_2O	450 ml

Preparation of gel for staining

1. Dehydration of gel

   A. Fill wells and troughs with DH_2O.
   B. Place wet filter paper over gel. Insure that no air bubbles are present.
   C. Place sheets of blotting paper over filter paper. The blotting paper layers must be homogeneous to avoid irregularities in the gel.
   D. Place weight over filter paper ($\leq$ 1 kg.).
   E. Let stand for 10-15 minutes.
   F. Remove blotting paper, filter paper. Repeat C - F.

2. Washing of gel

   A. Place gel in 0.1 M NaCl.
   B. Incubate at RT for 15 minutes.
   C. Change 0.1 M NaCl 2X, with 15 minute waiting period between changes.
   D. Remove 0.1 M NaCl, add DH_2O. Allow to stand for 15 minutes.

If heavy background staining of gels is noted, wash gel in DH_2O overnight. If this is required, precoad the gel (glass). Support with a thin layer of agarose (0.01% agarose).

   E. Dehydrate gel as in #1 above, but for 1 cycle.

3. Drying of gel

   A. Leave the filter paper covering the gel.
   B. Dry the gel at a temperature $\leq$ 50 C for 10-15 minutes.

4. Staining of gel

   A. Place gel in stain for 10 minutes.
   B. Destain at least 3X for periods of 10 minutes each. If faint precipitates disappear using this procedure, destain for a shorter p iod of time.

APPENDIX

Procedure 10

BINDING RADIOACTIVE GOAT IgG

Varying amounts of I^{125} Goat Anti-Rabbit antibody in 2 ml of carbonate suffer were used to coat the surface of polystyrene plates. The plates with the antibody were incubated at room temperature for 1 hour, then washed six times with 0.1% BSA in PBS. Several circles of 1 cm diameter were cut from the surface of the plate and counted in a gamma counter. The total amount of antibody bound to the plate was calculated from these counts.

APPENDIX

Procedure 11

FLUORESCENT BEAD BINDING TO TREATED PLATES

Rabbit IgG at various concentrations is bound to the surface of polystyrene petri dishes with carbonate buffer at pH 9.68. Incubation time is 2 hours at 37°C. The surface is then washed with 0.1% BSA in carbonate buffer three times and then blocked with 1% BSA in carbonate buffer for one hour at room temperature. The plates are futher acclimatized with 0.5% BSA in PBS for 30 minutes before incubation with beads for 2 hours. Excess beads are then washed off and bound beads are counted using a Zeiss fluorescent microscope with calibrated eyepiece.

APPENDIX

Procedure 12

COATING BEAD WITH PROTEIN

100 ul of beads 0.5 u in diameter suspension received from Covalent Technology Corporation are incubated with 10 ug of protein in 1 ml of distilled water at room temperature for 75 minutes. After centrifugation, supernatant is discarded and beads are incubated in 1 ml of 1% BSA in PBS at room temperature for 1 hour.

Beads are again centrifuged. Wash with PBS three times and suspended in PBS with 1% BSA to a concentration of 1×10^{11} per ml.

APPENDIX

Procedure 13

MEASUREMENT OF ANTIGEN BOUND TO FLUORESCENT BEADS

0.1 ml of beads suspension coated with mouse IgG (See Procedure #8) was incubated with peroxidase labeled anti-mouse at room temperature for 1 hour. Beads were then centrifuged and washed with PBS buffer. Beads were suspended in 0.1 ml of a mixture made with citric acid, ABTS (2,2"-azino-di-[3-athylbenzthiazolin-sulfonate(6)] and peroxide, incubated at room temperature with shaking for 30 minutes and centrifuged. Supernatants were counted in a ELISA counter at 410 nm.

Beads' washes	OD_{410}
2	1.072
3	1.082
4	0.671
5	0.852
6	0.708

After 2 washes, we find 1.2% of total antibody added to the beads.