CHAPTER 8

APPENDIX

All solutions were prepared in Milli Q water and filtered through 0.45 u membrane filters. Working solutions were prepared by diluting the stock solutions given below.

8.1 GROWTH MEDIUM AND SERUM

Earle's minimum essential medium (MEM-E) was purchased from HI-MEDIA-INDIA. Goat serum and Trypsin-Phosphate Buffered saline-Versene- Glucose (TPVG) was supplied by the Tissue Culture and Cell Biology Division of NIV, Pune.

Foetal-calf serum (FCS) and Dulbecco's modified minimum essential medium high glucose (DMEM) were purchased from GIBCO laboratories. The sera were heat inactivated at 56°C, for 30 min and stored in aliquots at -20°C.

DMEM was prepared as per instructions on the sachet by dissolving the contents of a 1 litre sachet in Milli-Q filtered water. To this 110 mg of sodium pyruvate (Sigma) and 3.7 g of sodium bicarbonate (Qualigens) was added. The pH of the medium was adjusted to 7.5 by bubbling carbon-di- oxide gas. The volume was made up to 1 litre with water and sterilized by filteration through a 0.22 u Millipore filter.

To all the tissue culture medium 30 mg/ml glutamine, 200 units/ml penicillin and 20 mg/ml streptomycin was added before use.

8.2 SOLUTIONS FOR PREPARATION OF RECOMBINANT PROTEIN

8.2.1 M 9 medium (For 1 liter.)

(A) Stock

Na_2HPO_4	(Qualigens)	6 g
KH_2PO_4	(Qualigens)	3 g
NaCl	(Qualigens)	0.5 g
NH_4Cl	(Qualigens)	1 g
Casamino acids (CA)	(DIFCO)	5 g

After autoclaving add.

(B) Stock

1 M $MgSO_4$	(Qualigens)	1 ml
*1 M $CaCl_2$	(Qualigens)	0.1 ml
*40% glucose	(Qualigens)	5 ml
*10 mg/ml Thiamine (Vit B1)	(Sigma)	1 ml

***Filter sterilize.**

Options :-

*4 mg/ml tryptophan(W)	(Sigma)	5 ml
*25 mg/ml Ampicillin	(Sigma)	4 ml
2 mg/ml indole-acrylic acid in 90% ethanol.	(Sigma)	0.5 ml

8.2.2 1 M Tris pH 7.5 (Sigma)

Dissolve 121.1 g Tris in around 500 ml water. Adjust the pH to 7.5 with concentrated HCl. Make up the volume to 1000 ml with water. Sterilize by autoclaving.

8.2.3 0.5 M EDTA (Ethylene diaminetetra acetic acid) (Sigma)

Dissolve 14.61 g EDTA in approximately 50 ml water. Add 1 N NaOH dropwise till pH stabilizes to pH 8.0. Make up volume to 100 ml. Sterilize by autoclaving.

8.2.4 1M Sodium chloride (Qualigens)

Dissolve 29.22 g NaCl in water. Make up volume to 500 ml. Sterilize by autoclaving.

8.2.5 8 M Urea (Sigma)

Dissolve 12.01 g urea in minimum amount of water. Make up volume to 25 ml. Prepare fresh before use.

8.2.6 5 M Guanidine hydrochloride (Sigma)

Dissolve 11.94 g guanidine hydrochloride in minimum amount of water. Make up volume to 25 ml. Prepare fresh before use.

8.2.7 Phosphate buffered saline

(10 mM phosphate pH 7.5 + 150 mM NaCl)

Soln.A : 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml).

Soln.B : 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ in 1000 ml).

Mix 62.5 ml of Soln.**(A)** with 37.5 ml of Soln.**(B)**. Add 30 ml of NaCl from a 1 M stock solution (Appendix 8.2.4). Make up the volume to 200 ml with distilled water.

8.3 BUFFERS FOR SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

8.3.1 Resolving Gel Buffer

Dissolve 18.10 gm Tris (Sigma) and 0.4 gm SDS (Sigma) in 50 ml D/W. Adjust pH to 8.9 with HCl. Make up volume to 100 ml.

8.3.2 Spacer gel buffer

Dissolve 5.9 gm Tris and 0.4 gm SDS. Adjust pH to 6.7 with HCl. Bring total volume upto 100 ml with DIW.

8.3.3 30% acrylamide (2.5 % Cross-linker)

29.25 gm acrylamide (Sigma) and 0.75 gm bisacrylamide (Sigma) were dissolved in minimum amount of DIW and volume was brought upto 100 ml. The acrylamide was filtered through Millipore membrane 0.45 u and stored at 4°C, in amber coloured bottle away from light.

To pour the resolving and spacer gels, the following cocktail was prepared for 2 gels. The Mini gel had dimensions 8.2x10x0.075 cm^3 while the dimensions of the preparative gel were 18x20x0.4 cm^3.

10% MINI GELS (FOR 2 GELS)

	Resolving Gel		Spacer Gel	
	Mini Gel	Big Gel	Mini Gel	Big Gel
DIW	5 ml	61.85 ml	3.5 ml	17.5 ml
30% acrylamide	4 ml	49.48 ml	1.0 ml	5 ml
Gel buffer	3 ml	37.11 ml	1.5 ml	7.5 ml
Temed (Sigma)	5 ul	62 ul	2.5 ul	12.5 ul
10% APS (Sigma) *(Ammonium Persulphate).*	75 ul	1000 ul	50 ul	250 ul

8.3.4 Reducing SDS sample buffer (3 X)

(Final SDS concentration 2% SDS)

Spacer gel buffer		1.0 ml
25% (w/v) SDS in DIW		0.8 ml
Beta-mercaptoethanol	(Sigma)	0.5 ml
Glycerol (BRL)		1.0 ml
1% Bromophenol blue	(Sigma)	0.2 ml

To prepare the sample, 2 parts of sample was mixed with 1 part of stock solution and held in a boiling water bath for 3-4 min.

8.3.5 Non-reducing SDS sample buffer (3X)

(Final SDS concentration 2%)

Spacer gel buffer		1.0 ml
25% (w/v) SDS in Distilled water	(Sigma)	0.8 ml
Distilled water		0.5 ml
Glycerol	(Qualigens)	1.0 ml
1% Bromophenol blue	(Sigma)	0.2 ml

To prepare the sample, 2 parts of sample were mixed with 1 part of stock solution and heed in a boiling water bath for 5 min.

8.3.6 Reducing SDS sample buffer (3X)

(Final SDS concentration 0.1%)

Spacer gel buffer		1.0 ml
25% (w/v) SDS in Distilled water	(Sigma)	40 ul
Beta-mercaptoethanol	(BioRad)	0.5 ml
Distilled water		0.5 ml
Glycerol		1.0 ml
1% Bromophenol blue	(Sigma)	0.2 ml

To prepare the sample, 2 parts of sample were mixed with 1 part of stock solution and held in a boiling water bath for 5 min.

8.3.7 Non reducing SDS sample buffer (3X)

(Final SDS concentration 0:1%)

Spacer gel buffer		1.0 ml
25% (w/v) SDS in Distilled water	(Sigma)	40 ul
Distilled water		0.5 ml
Glycerol		1.26 ml
1% Bromo-phenol blue	(Sigma)	0.2 ml

To prepare the sample, 2 parts of sample were mixed with 1 part of stock solution and held in a boiling water bath for 5 min.

8.3.8 Tank buffer

(0.52 mM Tris, 0.53 mM glycine, 0.1% w/v SDS.)

Tris	3.16gm
Glycine (Sigma)	2.0 gm
SDS	0.5 gm

The volume was brought upto 500 ml with DIW.

8.3.9 Coomassie brilliant blue stain

Coomassie brilliant blue R-250	(Sigma)	0.8 g
Methanol	(Qualigens)	200 ml
Acetic acid	(Qualigens)	28 ml
Distilled water		200 ml

Filter through Whatman paper No.3 and store.

8.3.10 Destaining solution

Methanol	(Qualigens)	100 ml
Acetic acid	(Qualigens)	140 ml
Distilled water		1760 ml

8.3.11 Preparation of molecular weight markers

The lyophilized MW markers (Sigma) were reconstituted in sterile distilled water to make up final concentration to 5 mg/ml. Depending on the MW of the sample, to be resolved, 4 markers in the range were selected.

BSA	66,000
Ovalbumin	45,000
Trypsinogen	24,000
Alpha Lactalbumin	14,200

0.1 ml of each was mixed together. To 0.4 ml of the resultant mixture, an equal volume of (2X) reducing SDS sample buffer (Appendix 8.3.4) was added. The entire mixture was placed in a boiling water bath for 5 min. The mixture of markers was aliquoted and stored at -70°C. The markers were boiled before use. 5 ul marker was loaded per track on an analytical gel.

8.3.12 Remazole prestained marker

(A) Remazole stock solution (RBB)

Prepare a 10 mg/ml solution of Remazole brilliant blue stain containing 10% (w/v) SDS.

(B) Marker preparation

Adjust the pH of the protein solution (1 mg/ml) to 8.8, with the addition of a few microlitres of 1% NaOH solution. Add RBB and protein solution in the ratio 1:5 (v/v). Heat for 30 min by placing in a boiling water bath. For preparing the sample for SDS-PAGE add 2 parts of sample and 1 part of (3X) reducing SDS sample buffer (Appendix 8.3.4). Heat for 5 min in a boiling water bath. Load 50 ul per track on a preparative SDS PAGE.

8.3.13 PMSF

(100 mM PMSF Mw 174.2).

Dissolve 174.2 mg PMSF in 10 ml isopropanol to prepare a 100 mM stock solution. Store at - 20°C in 1 ml aliquots.

8.3.14 Silver staining of proteins

8.3.14.1 Fixative

(50% methanol , 12% acetic acid, 0.01% formaldehyde)

Mix 500 ml of methanol, 120 ml acetic acid and 0.5 ml of 37% formaldehyde solution. Make up[the volume to 1000 ml with distilled water.

8.3.14.2 Silver nitrate

(2 gm/l silver nitrate containing 0.02% formaldehyde)

Dissolve 2 gm of silver nitrate in 500 ml of distilled water. Add 0.75 ml of 37 % formaldehyde and make up volume to 1000 ml. Prepare fresh.

8.3.14.3 Developer

(60 gm/l Na2CO3, 4 mg/l Na2S2O3 , 0.1% formaldehyde)

Dissolve 60 gm Na_2CO_3 in 500 ml distilled water. Add 4 mg $Na_2S_2O_3$ and 0.5 ml of 37% formaldehyde. Make up final volume to 1 litre.

8.3.14.4 Arresting solution

(50% methanol, 12% acetic acid)

Add 50 ml methanol, 12 ml acetic acid and 38 ml distilled water.

8.4 SOLUTIONS FOR WESTERN BLOT

8.4.1 Transfer buffer

(50 mM Tris, 39 mM glycine, 0.004% SDS and 20% methanol.)

Dissolve 6 gm Tris, 2.92 gm glycine and 0.4 gm SDS in 500 ml of Milli-Q water. Adjust pH to 9.2 with NaOH. Add 200 ml methanol. Make up volume to 1000 ml.

8.4.2 Ponceau S red stain (10X)

Ponceau S powder	(Sigma)	2 g
Trichloroacetic acid	(Sigma)	30 g
or		
Sulphosalicytic acid	(Sigma)	30 g

Dissolve in water and make up volume to 100 ml. Dilute one part of stock solution with nine parts of deionized water to make a working solution.

8.4.3 Amido black stain

Amido black powder	(Sigma)	5.0 g
Glacial acetic acid	(Qualigens)	300 ml
Sodium acetate trihydrate	(Qualigens)	68.0 g
Distilled water		4700 ml

Keep for 7 days at room temperature for maturation.

Filter through Whatman paper No.3 and store.

8.4.4 Western Blotting wash-buffer

(10 mM PB, 150 mM NaCl, 0.1% v/v Tween-20)

1 M Phosphate buffer pH 7.0	(Qualigens)	10 ml
NaCl		9 g
0.1% (V/V) Tween-20	(Sigma)	1 ml
Distilled water		1000ml

8.4.5 Substrate for alkaline phosphatase based conjugate

(A) NBT

30 mg NBT (Nitro Blue Tetrazolium,BioRad) was dissolved in 1ml of 70% solution of N-N Dimethyl formamide (DMF,Sigma). The solution was stored in an amber bottle at 4°C.

(B) BCIP

15 mg BCIP (5 bromo 4 chloro 3 indolyl phosphate p toludine salt, BioRad) was dissolved in 1ml DMF.

(C) Carbonate buffer

0.1 M $NaHCO_3$, 1.0 M $MgCl_2$, pH 9.8.

8.4 gm $NaHCO_3$ (BDH) and 0.029 gm $MgCl_2$ was dissolved in DIW. pH was adjusted to 9.8 and the volume was made upto 1000 ml .

Substrate Working Solution: 0.1% NBT,0.1% BCIP in carbonate buffer. To, 10 ml of carbonate buffer, 100 ul NBT and 100 ul BCIP was added.

8.4.6 Substrate for HRPO based conjugate (Western Blot)

Dissolve 6 mg Diaminobenzidine-tetrahydrochloride (DABT) (Sigma) and 3 mg urea peroxide in 10 ml PBS. Prepare in a vial covered with aluminium foil to prevent oxidation.

8.5 ELECTRO-ELUTION BUFFER

(80 mM Tris, 12.5 mM glycine, 20% Methanol)

Dissolve 9.6 g Tris and 0.9 g glycine in 500 ml water. Make up volume to 800 ml. Add 200 ml methanol.

8.6 SOLUTIONS FOR HYBRIDOMA

8.6.1 Normal saline

8.5 gm NaCl dissolved in 1000 ml DIW. The solution was autoclaved at 15 lbs, 15 min and stored at 4°C.

8.6.2 0.34 M Sucrose

11 gm Sucrose in 100ml DIW. The solution was sterlized by filtration and stored at 4°C.

8.6.3 50% PEG solution

5 gm polyethylene glycol (PEG 1450, Sigma) was autoclaved at 15 lbs, 15 min. While the PEG solution was still liquid, 5 ml of DMEM without serum was added. The solution was stored at 4°C.

8.6.4 8-Azaguanine medium

Stock solution: 2mg of 8-azaguanine powder was dissolved per ml of DMSO(Sigma) This stock was diluted 1:100 in DMEM with 10% FCS.

8.6.5 Freezing mixture

20% DMSO + 20% FCS in medium (either DMEM, MEM(E) or L-15).

8.6.6 HAT medium

50 X HAT (Sigma) was diluted in DMEM containing 20% FCS to give 1 X concentration. The final concentrations of HAT in the medium are :

Hypoxanthine	100 uM
Aminopterin	0.4 uM
Thymidine	16 uM

8.6.7 HT medium

50 X HT (Sigma) was diluted in DMEM containing 20% FCS to give 1 X concentration. The final concentration of H and T in the medium are:

Hypoxanthine	100 uM
Thymidine	16 uM

8.6.8 RBC lysis buffer

Soln.A - Preparation of 100 ml.

NH_4Cl	3.5 g
KCl	0.18 g
$Na_2HPO_4.12H_2O$	0.15 g
KH_2PO_4	0.01 g
Glucose	0.5 g

Dissolve the following in 100 ml of distilled water. Sterilize by filtration through a 0.2 u filter.

Soln.B : For the preparation of 100 ml.

$MgCl_2.6H_2O$	0.42 g
$MgSO_4.7H_2O$	0.14 g
Or	
$MgSO_4$	0.07 g
$CaCl_2$	0.34 g

Sterilized by autoclaving.

Soln.C : For the preparation of 100 ml.

$NaHCO_3$	2.25 g

Sterilized by autoclaving.

For the preparation of working solution mix 4 ml of Soln.(A) + 1 ml of Soln.(B) + 1 ml of Soln.(C) and 14 ml of distilled water. To 1 ml of cell suspension add 19 ml of lysis buffer and incubate on ice for 1 min. Immediately neutralize by adding 20 ml of medium containing 10% serum.

8.7 SOLUTIONS FOR PURIFICATION OF IgG (Protein G - Pharmacia)

8.7.1 Binding buffer

For preparing 20 mM sodium phosphate buffer, 2 ml of 1M PB was added to 98 ml D.W.and autoclaved at 15 lbs, 15 min. and stored at room temperature.

8.7.2 Elution buffer

0.1 M Glycine-HCl pH 2.7, 3.75 gm glycine was dissolved in DIW, pH adjusted to 2.7 with concentrated HCl and volume made upto 50 ml with DIW. This is 1M stock solution. 10 ml of stock was diluted to 100ml to give 0.1M glycine.

8.8 SOLUTIONS FOR ELISA

8.8.1 Carbonate buffer

Solution 1: 4.29 gm. $NaHCO_3$ (BDH) in 250 ml DIW.

Solution 2: 2.129 gm Na_2CO_3 (BDH) in 100 ml DIW.

204 ml solution 1 was mixed with 96 ml solution 2 and pH was adjusted to 9.6.

Working Solution:- 0.2 M Carbonate buffer was diluted to 50 mM (1:4 dilution)

8.8.2 1% BSA

1 gm BSA in 100 ml of 0.01 M PBS pH 7.2. Filter through 0.22 u Millipore filter and store.

8.8.3 Wash buffer

(10 mM PB pH 7.0, 150 mM NaCl, 1% gelatin and 0.1% Tween-20)

For the preparation of 1 litre buffer. 10 g gelatin (LOBA) was dissolved in 500 ml water by heating in a Microwave oven. To the warm solution, 10 ml PB (10X) stock solution and 9 g NaCl was added. The volume was made up to 1 litre. The solution was cooled to room temperature and used for saturating the ELISA wells. For using as a wash buffer 1 ml Tween-20 was added.

8.8.4 Substrate solution (for HRPO based conjugate) (ELISA)

(A) Citrate buffer:

7 gm citric acid (Thomas Baker and Co.) in 1000 ml of DIW.

(B) Phosphate buffer:

11.9 gm. Na_2HPO_4. $2H_2O$ in 1000 ml of DIW.

25 ml citric acid + 25ml phosphate buffer were mixed together in an amber bottle as the substrate is photosensitive. The pH of the solution is adjusted to 5.0. To 50 ml substrate buffer, 20 mg Orthophenyl diamine (OPD, Sigma) and 10 mg of Urea peroxide (UP,Sigma) were added. The substrate was weighed in dark.

8.9 SOLUTIONS FOR IMMUNOFLORESCENSE

(A) EVAN'S BLUE:

10 mg Evan's blue powder (Loba Chemicals).

Dissolved in 300 ml DIW. This was diluted 1:30,000 in DIW and used.

(B) DABCO:

250 mg DABCO (1,4 Diazobicyclo (2,2,2) Octane, Aldrich Chemicals and Co.) powder in 10 ml Glycerol. Heat gently in water bath, store at room temperature.

8.10 BOVINE ALBUMIN IN PHOSPHATE SALINE (BAPS)

For the preparation of 0.75% and 1.25% BAPS, following stock solutions were prepared.

(A) Phosphate Buffer (10 x)

NaCl	(Qualigens)	35.0 gm
Na_2HPO_4	(BDH)	8.0 gm
NaH_2PO_4	(BDH)	4.2 gm

These were dissolved in Deionized water (DIW) and the volume was made upto 500 ml. The solution was autoclaved at 15 lbs, 15 min.

(B) 7.5% solution of Bovine Albumin:

37.5 gm. Bovine albumin powder (Sigma) was dissolved in distilled water at 4°C. The solution was sterlized by filtration.

8.10.1 0.75% BAPS

100 ml solution A was mixed with 100 ml solution B. To it, 800 ml autoclaved DIW was added and pH of the solution was adjusted to 7.2 by 1N NaOH.

8.10.2 1.25% BAPS

100 ml solution A was mixed with 166 ml of solution B and 734 ml autoclaved DIW was added. pH was adjusted to 7.2 by 1N NaOH.

Both solutions were distributed in 100 ml aliquots and stored at 4°C.

8.10.3 Reagents for plaque assay

8.10.3.1 Agarose overlay medium (AOM)

AOM is prepared by dissolving one sachet of MEM (E) (used for preparing 1 litre medium) in 500 ml distilled water so as to obtain two fold concentrated medium. The solution was sterilized by autoclaving.

8.10.3.2 1.8% Agarose

1.8 gms of agarose (Sigma lot No. A6013), priorly tested for non-toxicity to cells, was dissolved in 100 ml of distilled water and sterilized by autoclaving.

8.10.3.3 Preparation of AOM-Agarose overlay

The 1.8% Agarose was melted and placed in a water bath maintained at 45_oC along with AOM. The agarose and AOM were mixed in equal proportions containing 2% (v/v) goat serum. The mixture was placed in a 45_oC water bath for at least 1 hr prior to use.

8.10.3.4 0.1% Glutaraldehyde

Stock solution of glutaraldehyde (Sigma) was 25% aqueous solution. To 0.4 ml stock solution, distilled water was added to make final volume to 100 ml.

8.10.3.5 0.2% Crystal violet in 10% formaldehyde.

Formaldehyde stock solution is 37% aqueous. Add 27 ml from stock solution to 73 ml distilled water to prepare 100 ml of 10% formaldehyde. Dissolve 0.2 gms of crystal violet powder (Sigma) to 100 ml of 10% formaldehyde.

8.11 BUFFERS FOR VIRUS PURIFICATION BY SUCROSE DENSITY GRADIENT

8.11.1 NTE buffer

(20 mM Tris, 150 mM NaCl and 1 mM EDTA.)

Dissolve 2.42 g Tris (Sigma) and 8.766 g NaCl in 800 ml D/W. Add 2 ml EDTA from a 500 mM stock solution (Appendix 8.2.3). Adjust the pH to 8.0 with concentrated HCl. Make up volume to 1000 ml. Autoclave and store at 4°C.

8.11.2 10% Sucrose for gradient

Dissolve 10.3 g sucrose in NTE buffer pH 8.0. Make up volume to 100 ml. Adjust the refractive index to 1.3479.

8.11.3 40% Sucrose for gradient:

Dissolve 47.06 g sucrose in 100 ml NTE buffer pH 8.0. Make up volume to 100 ml. Adjust the refractive index to 1.3997.

8.12 REAGENTS FOR HAEMAGGLUTINATION

Stock Solutions

(a) 1.5 M sodium chloride. : 87.67 g NaCl in 1000 ml DIW.

(b) 0.5 M Boric Acid. : 30.92 gm. H_3BO_3 (BDH) was dissolved in 700 ml of hot DIW. The solution was allowed to cool to room temperature and the volume was made upto 1000 ml with DIW.

For the preparetion of borate saline:

160 ml solution (a), 200 ml solution (b) and 47 ml 1 M NaOH were mixed. The pH was adjusted to 9.0 either by addition of 0.5 M boric acid or 1 M NaOH. The volume was made upto 2000 ml with DIW. The solution was stored at 4°C.

8.12.1 Bovine albumin in borate saline (BABS)

400 mg Bovine serum albumin in 100 ml of borate saline pH 9.0.

8.12.2 VIRUS ADJUSTING DILUENT (VAD):

(A) VAD 6.6

100 ml solution (a), 160ml solution (c) and 120 ml solution (d) were mixed together and volume was made upto 1000 ml.

(B) VAD 6.2

100 ml solution (a); 62 ml solution (c); 169 ml solution (d) were mixed together and volume was made upto 1000 ml.

For DEN-2, DEN-3, DEN-4, JE and WN viruses, VAD 6.6 was used, whereas, for DEN-1 VAD 6.2 was used.

8.13 SOLUTIONS FOR COMPLEMENT FIXATION

The solutions were supplied by Central Serology, NIV, Pune.

8.13.1 5 X Veronal Saline (V.S.)

5.57 gm Diethyl barbituric acid (Veronal, Fluka); 3.750 gms sodium barbitone (BDH); 85.0 gm NaCl; 1.68 gm Magnesium chloride ($MgCl_2$. $6H_2O$, BDH); 0.37 gm Calcium Chloride ($CaCl_2$. $2H_2O$, Sarabhai Chemicals)in 500 ml DIW. The solution was autoclaved and kept for maturation for two months. Just before use, 5 X V.S. was diluted 1:5 with DIW and used within 48 hours.

8.13.2 Antigen preparation

JE virus antigen was prepared by acetone extraction of infected mouse brain suspensions. They were lyophilized and stored at -20°C. The antigens were titrated by checker board CF test and the antigen dilution showing 75-100% fixation with the specific antibody and complement corresponds to one unit.

8.13.3 Complement

Guinea pig serum was used as a source of complement. Richardson Sol. A and B was used as a preservative. Eight parts of complement was mixed with 1 part each of solution A and 3.

Sol. A : 0.930 gm boric acid; 2.29 borax (BDH); 11.47 gm Sorbitol (BDH) was dissolved in a saturated solution of NaCl. Volume was made upto 100 ml with the same.

Sol. B. 0.570 gm Borax, 0.810 sodium azide (Loba Chemicals) was dissolved in a saturated solution of NaCl. The volume was made upto 100 ml with the same.

The preserved complement was lyophilized and stored at - 20°C. To 1 part of reconstituted complement, 7 parts of chilled DIW was added to give 1:10 dilution. Serial dilution of complement were tested. The highest dilution giving complete haemolysis is taken as one unit of complement 2.5 units of complement were used in the test.

8.13.4 Haemolytic System

Rabbits were immunized with 5-6 doses of sheep R.B.Cs. The serum was collected, lyophilized and stored at -20°C. This is "haemolysin". Before use, the haemolysin ampoule was reconstituted in DIW, diluted 1:5 in glycerin (used as a preservative). The haemolysin was titrated. Highest dilution of haemolysin giving haemolysis is considered as one unit of haemolysin. Four units of haemolysin were used in the test.

For haemolytic system - equal volumes of O.D. (0.6) adjusted sheep RBCs were mixed with equal volume of diluted haemolysin (4 units). This was mixed thoroughly and kept at room temperature for 10-15 min.

8.14 SOLUTIONS FOR LOWRY

8.14.1 Standard BSA

10 mg of BSA was dissolved in 10 ml of DIW. 1 ml aliquots were stored at -20°C.

8.14.2 Lowry C

Stock solutions for Lowry C:

(1) Lowry A: 2% Na_2CO_3 in 0.1 N NaOH.

10 gm Na_2CO_3 (BDH) in 500 ml 0.1N NaOH.

(2) Lowry B: 0.1% $CuSO_4$ in Sodium citrate.

Working solution: 50 ml A + 1ml B.

8.14.3 LOWRY D

3 ml Folin Ciocalteau (SISCO Laboratories) reagent + 3 ml DIW.

8.14.4 Table

Sample (ul)	DIW (ul)
Std. BSA	
0	200
20	180
40	160
60	140
80	120
100	100
120	80
Test Sample	
20	180
40	160

To each tube, 2.5 ml of Lowry C (Appendix 8.14.2) was added and incubated at RT for 10 min. This was followed by addition of 0.25 ml of Lowery D (Appendix 8.14.3). Each tube was shaken and incubated in the dark for 20 min. The optical density (O.D.)was measured at 500 nm. A graph of concentration of standard BSA (ug/ml) verses O.D. was plotted and concentration of sample determined from std. graph

8.15 BUFFERS FOR STUDIES ON pH SENSITIVITY

8.15.1 100 mM glycine - HCl pH 2.6

Stock solution :

(A) 0.2 M glycine (15.01 g glycine in 1000 ml)

(B) 0.2 M HCl

50 ml of solution A + 24.2 ml of solution B was added to obtain pH 2.6. The solution was diluted to 100 ml with distilled water.

8.15.2 100 mM Acetate buffer pH 4.6

Stock solution :

(A) 0.2 M solution of acetic acid (11.55 ml in 1000 ml)

(B) 0.2 M sodium acetate (16.4 g of $C_2H_3O_2Na$ in 1000 ml)

25.5 ml of solution A was mixed with 24.5 ml of solution B. The final volume was made upto 100 ml with distilled water.

8.15.3 100 mM Acetate buffer pH 5.6

4.8 ml of solution (A) was mixed with 45.2 ml of solution B. The final volume was made upto 100 ml with distilled water.

8.15.4 100 mM Phosphate buffer pH 6.6

(A) 0.2 M solution of monobasic sodium phosphate (27.8 g in 1000 ml)

(B) 0.2 M solution of dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ in 1000 ml)

62.5 ml of solution A was mixed with 37.5 ml of solution B. The volume was made upto 200 ml with distilled water.

8.15.5 100 mM Phosphate buffer pH 7.6

13.0 ml of solution A was mixed with 87 ml of solution B and volume made upto 200 ml with distilled water.

8.15.6 100 mM Tris buffer pH 8.6

2.42 g Tris was dissolved in 50 ml water. HCl was added dropwise to titrate the pH to 8.6. The volume was made upto 8.6.

8.15.7 100 mM Carbonate-bicarbonate buffer pH 9.6

(A) 0.2 M solution of anhydrous sodium carbonate (21.2 g in 1000 ml)

(B) 0.2 M solution of sodium bicarbonate (16.8 g in 1000 ml).

16 ml of solution A was mixed with 34 ml of solution B and volume made upto 100 ml.

8.15.8 100 mM Carbonate-bicarbonate buffer pH 10.6

42.5 ml of solution A was mixed with 7.5 ml of solution B and volume made upto 100 ml.

Buffer for endoglycosidase digestion.

8.15.9 100 mM Phosphate buffer pH 5.7

(A) 0.2 M Monobasic sodium phosphage (27.8 g in 1000 ml).

(B) 0.2 M dibasic sodium phosphate (53.65 g of $Na_2HPO_4.7H_2O$ in 1000 ml).

93.5 ml of solution A + 6.5 ml of solution B and volume made to 200 ml with distilled water.

8.16 TRITON X-114 PHASE PARTITIONING BUFFER

(50 mM Tris-Cl pH 7.5 + 1 mM EDTA + 150 mM NaCl containing 2% v/v Triton X-114 and 1 mM PMSF).

For the preparation of 100 ml buffer add 5 ml, 1 M Tris Cl buffer pH 7.5 (Appendix 8.2.2) + 0.2 ml EDTA from 0.5 M stock (Appendix 8.2.3) + 15 ml NaCl from a 1M stock solution (Appendix 8.2.4) + 2 ml Triton X-114 and 1 ml PMSF from 100 mM stock solution (Appendix 8.3.13). Make up the volume to 100 ml with distilled water. Store at 4°C.

8.17 REAGENTS FOR RADIO IMMUNO PRECIPITATION

8.17.1 RIPA buffer

(50 mM Tris-Cl pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% v/v Triton X-100, 1% w/v sodium deoxycholate, 0.1% w/v SDS and 1 mM PMSF).

For the preparation of 100 ml RIPA lysis buffer to 50 ml distilled water, add 5 ml Tris-Cl pH 7.5 from a 1 M stock (Appendix 8.2.2) + 0.2 ml EDTA from a 500 mM stock (Appendix 8.2.3) + 1 ml Triton X-100 + 1 g sodium deoxycholate 0.1 g SDS + 1 ml 100 mM PMSF was added. The contents were aliquoted and stored at -20°C.

8.17.2 RIPA wash buffer

(50 mM Tris-Cl pH 7.5, 1 mM EDTA, 150 mM NaCl and 0.05% NP- 40).

For the preparation of 100 ml RIPA wash buffer, to 50 ml distilled water add 5 ml Tris-Cl pH 7.5 from a 1 M stock (Appendix 8.2.2) + 0.2 ml EDTA from 500 mM stock (Appendix 8.2.3) + 15 ml NaCl from a 1 M stock (Appendix 8.2.4) + 50 ul NP-40.

8.17.3 50% Protein A coated Sepharose CL-4B beads

Protein A coated Sepharose CL-4B beads from Pharmacia were swollen in excess of 0.01 M PBS pH 7.2 (Appendix 8.2.7) on 10 ml viral for 30 min at room temperature. Beads were pelleted by centrifugation at 1000 rpm 5 min. and excess PBS decanted off. The free protein binding sites were blocked by the addition of 1% gelatin in PBS for 10 min at room temperature. The beads were washed with three changes of PBS and allowed to settle by gravity. The excess PBS was decanted off and to the bed volume of beads settled by gravity an equal volume of PBS was added to make 50% bead suspension.

8.17.4 Reagents for autoradiography

(i) X-ray film developer was prepared according to the instructions given on the pack (May and Baker India Ltd.).

(ii) Fixer for X-ray film - Sodium thiosulfate - 1 kg
Potassium metabisulfate - 150 g
Acetic acid - 40 ml
DDW to make volume upto 4 lit.

8.17.5 Scintillation fluid

(2,5 diphenyl oxaxole) PPO - 10 g

[1,4-Di(2-(5-phenyloxazole))] POPOP - 125 mg

Toluene - 2.5 lit.

Stirred overnight at room temperature and stored in amber coloured bottle.

8.18 BUFFERS FOR IgG-HRP CONJUGATION

8.18.1 100 mM carbonate buffer pH 9.6

Soln.A : 0.2 M solution of anhydrous sodium carbonate (21.2 g in 1000 ml).

Soln.B : 0.2 M solution of sodium bicarbonate (16.8 g in 1000 ml).

16 ml of Soln.(A) was mixed with 34 ml of Soln.(B) and volume made upto 100 ml.

8.18.2 100 mM sodium metaperiodite

Dissolve 213.9 mg sodium metaperiodite (Sigma) in 10 ml of distilled water.

8.18.3 1 mM sodium acetate pH 4.4

Stock solution :

(A) 0.2 M solution of acetic acid (11.55 ml in 1000 ml with distilled water).

(B) 0.2 M solution of sodium acetate (16.4 g $C_2H_3O_2Na$ or 27.2 g of $C_2H_3O_2Na.3H_2O$ in 1000 ml with distilled water).

Add 3.05 ml of Soln.(A) + 1.95 ml of Soln.(B) and dilute to 1000 ml with distilled water.

8.18.4 Sodium borohydride

Dissolve 4 mg of sodium borohydride in 1 ml distilled water to prepare a 4 mg/ml solution.

8.19 REAGENTS FOR COUPLING IgG TO CNBR ACTIVATED SEPHAROSE 4B

8.19.1 Coupling buffer

(0.1 M $NaHCO_3$ buffer containing 0.5M NaCl)

8.4 gms of $NaHCO_3$ was dissolved in 500 ml D/W and pH adjusted to 8.3 with 1N NaOH. 29.22 gm of NaCl was dissolved and volume made upto 1000 ml.

8.19.2 0.2 M glycine pH 8.0

1.50 gm of glycine was dissolved in 50 ml D/W. pH was adjusted to 8.0 with 1N NaOH and volume made upto 100 ml with D/W.

8.19.3 0.1 M Acetate buffer containing 0.5 M NaCl

Stock solutions :

A) 0.2 M solution of acetic acid (11.55. ml in 1000ml)

B) 0.2 M sodium acetate (16.4 gm of sodium acetate in 1000 ml).

41 ml of solution A was mixed with 9 ml of solution B. The final volume was made to 100 ml after the addition of 2.92 gm of NaCl.

8.20 REAGENTS FOR DISSOCIATING ANTIGEN-ANTIBODY COMPLEXES

8.20.1 3M Potassium thiocynate (KCNS)

Dissolve 2.9 gms KCNS in 8 ml D/W. Make up total volume to 10 ml.

8.20.2 0.1 M diethylamine (DEA) pH 11.6

Stock solution is 9.6 M. To prepare 0.1 M DEA, add 10.4 ml to 89.6 ml D/W so as to make 100 ml of 0.1 M DEA.