APPENDIX I

Preparation of Reagents

This appendix contains reagents that are used in several different protocols throughout the book. Specialized reagents used in only one protocol will generally be located within the Materials section of that protocol.

Note. Dilutions quoted as, for example, 1:10 or 1:100, are v/v and imply that the final volume is 10 or 100 parts, respectively.

Acetic/Methanol

Add 1 part glacial acetic acid to 3 parts methanol. Make up fresh each time used, and keep on ice.

Agar 2.5%

Agar..... 2.5 g
UPW..... 100 mL

(1) Boil to dissolve agar.
(2) Sterilize by autoclaving or boiling for 2 min.
(3) Store at room temperature.

Amido Black

See Naphthalene Black.

Amino Acids, Essential

See Section 9.4.1 and Table 9.3.
(Available as 50× concentrate in 0.1 M HCl from commercial suppliers such as Invitrogen, MP Biomedicals, and Sigma.)

(1) Make up tyrosine and tryptophan together at 50× in 0.1 M HCl and remaining amino acids at 100× in ultrapure water.
(2) Dilute for use as in Protocol 11.10.
(3) Sterilize by filtration.
(4) Store in the dark at 4°C.

Amino Acids—Nonessential

Ingredient	g/1 (100×)
L-Alanine	0.89
L-Asparagine H_2O	1.50
L-Aspartic acid	1.33
Glycine	0.75
L-Glutamic acid	1.47
L-Proline	1.15
L-Serine	1.05
Water	1000 mL

(1) Sterilize by filtration.
(2) Store at 4°C.
(3) Use at a concentration of 1:100.

Antibiotics

See under specific headings (e.g., penicillin, streptomycin sulfate, kanamycin sulfate, gentamycin, mycostatin).

Antifoam

e.g., RD emulsion 9964.40 (*see* Appendix II)

(1) Dispense into aliquots and autoclave to sterilize.
(2) Store at room temperature.
(3) Dilute 0.1 mL/liter (i.e., 1:10,000).

Bactopeptone, 5%

Difco Bactopeptone..... 5 g
Hanks' BSS..... 100 mL

(1) Stir to dissolve.
(2) Dispense in aliquots appropriate to use as a 1:50 dilution, and autoclave.
(3) Store at room temperature.
(4) Dilute 1/10 for use.

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Balanced Salt Solutions (BSS)

(*See* Table 9.2.)

(1) Dissolve each constituent separately, adding $CaCl_2$ last.
(2) Make up to 1 L.
(3) Adjust pH to 6.5.
(4) Sterilize the solution by autoclaving or filtration. With autoclaving, the pH must be kept below 6.5 to prevent calcium phosphate from precipitating; alternatively, calcium may be omitted and added later. If glucose is included, the solution should be filtered to avoid caramelization of the glucose, or the glucose may be autoclaved separately (*see* Glucose, 20%, in this appendix) at a higher concentration (e.g., 20%) and added later.
(5) With autoclaving, mark the level of the liquid before autoclaving. Store the solution at room temperature, and if evaporation has occurred, make up to mark with sterile ultrapure water before use. If borosilicate glass is used, the bottle may be sealed before autoclaving and no evaporation will occur.

Broths

See manufacturers' instructions for preparation.
See also Bactopeptone and tryptose phosphate broth.
Sterilize by autoclaving.

Buffered glycerol mountant

See Mycoplasma

Carboxymethylcellulose (CMC)

(1) Weigh out 4 g of CMC and place it in a beaker.
(2) Add 90 mL of Hanks' BSS, and bring the mixture to boil in order to wet the CMC.
(3) Allow the solution to stand overnight at 4°C to clarify.
(4) Make volume up to 100 mL with Hanks' BSS.
(5) Sterilize the solution by autoclaving. The CMC will solidify again, but will redissolve at 4°C.
(6) For use (e.g., to increase the viscosity of the medium in suspension cultures), use 3 mL per 100 mL of growth medium.

Chick Embryo Extract [Paul, 1975]

(1) Remove embryos from eggs as described in Protocol 12.2, and place the embryos in 9-cm Petri dishes.
(2) Take out the eyes, using two pairs of sterile forceps.
(3) Transfer the embryos to flat- or round-bottomed 50-mL containers, two embryos to each container.
(4) Add an equal volume of Hanks' BSS to each container.
(5) Using a sterile glass rod that has been previously heated and flattened at one end, mash the embryos in the BSS until they have broken up.
(6) Let the mixture stand for 30 min at room temperature.
(7) Centrifuge the mixture for 15 min at 2000 *g*.
(8) Remove the supernate, and, after keeping a sample to check its sterility (*see* Section 11.6.2), dispense the solution into aliquots and store at −20°C.

Extracts of chick and other tissues may also be prepared by homogenization in a Potter homogenizer or Waring blender [Coon and Cahn, 1966].

(1) Homogenize chopped embryos with an equal volume of Hanks' BSS.
(2) Transfer the homogenate to centrifuge tubes, and spin at 1000 *g* for 10 min.
(3) Transfer the supernate to fresh tubes, and centrifuge for a further 20 min at 10,000 *g*.
(4) Check the sample for sterility (*see* Section 11.6.2).
(5) Dispense into aliquots.
(6) Store at −20°C.

Citric Acid/Crystal Violet

See Crystal Violet.

CMC

See Carboxymethylcellulose.

Colcemid, 100× Concentrate

Colcemid..... 100 mg
Hanks' BSS..... 100 mL

(1) Stir to dissolve.
(2) Sterilize by filtration.
(3) Dispense into aliquots and store at −20°C

Δ ***Safety Note.*** Colcemid is toxic; handle it with care by weighing in a fume cupboard and wearing gloves.

Collagenase

2,000 U/mL in Hanks' BSS

Worthington CLS-grade collagenase or the equivalent (specific activity 1500–2000 U/mg).....100,000 U
Hanks' BSS..... 50 mL

(1) To dissolve the mixture, stir at 37°C for 2 h or at 4°C overnight.
(2) Sterilize the solution by filtration, as with serum (*see* Protocol 11.15).
(3) Divide into aliquots, each suitable for 1–2 weeks of use.
(4) Store at −20°.

Collagenase–Trypsin–Chicken Serum (CTC) [Coon and Cahn, 1966]

	Sterile	*Volume*	*Final Concentration*
Ca^{2+}- and Mg^{2+}-free saline [Moscona, 1952]		85 mL	
Trypsin stock, 2.5%, sterile	.....	4 mL	.....0.1%
Collagenase stock, 1%, sterile	.....	10 mL	.....0.1%
Chick serum	.....	1 mL	.....1.0%

Dispense into aliquots and store at −20°C.

Collection Medium (for Tissue Biopsies)

Growth medium..... 500 mL
Penicillin.....125,000 units
Streptomycin..... 125 mg
Kanamycin..... 50 mg
or
Gentamycin.....25 mg
Amphotericin..... 1.25 mg

Store at 4°C for up to 3 weeks or at −20°C for longer periods.

Crystal Violet, 0.1%, in 0.1 M Citric Acid

Citric acid..... 21.0 g
Crystal violet..... 1.0 g

(1) Make up to 1000 mL with deionized water.
(2) Stir to dissolve.
(3) To clarify, filter the solution through Whatman No. 1 filter paper.

Crystal Violet 0.1% in Water

Crystal violet..... 100 mg
Water..... 100 mL

Filter through Whatman No. 1 paper before use.
Available ready made from Merck.

Dexamethasone (Merck)

1 mg/mL (100×)
This reagent comes already sterile in glass vials.

(1) To dissolve, add 5 mL water by syringe to the vial.
(2) Remove when dissolved
(3) Dilute to 1 mg/mL, approximately 2.5 mM.
(4) Divide the solution into aliquots and store at −20°C.
(5) For use, dilute the solution to give 10–50 nM (physiological concentration range), 0.1–1.0 μM (pharmacological dose range), or 25–100 μM (high dose range).

β-Methasone (Glaxo) and methylprednisolone (Sigma) may be prepared in the same way.

Dissection BSS (DBSS)

(1) To Hanks' BSS without bicarbonate, previously sterilized by autoclaving, add the following (all sterile):

Penicillin..... 250 U/mL
Streptomycin..... 250 μg/mL
Kanamycin..... 100 μg/mL
or
Gentamycin..... 50 μg/mL
Amphotericin B..... 2.5 μg/mL

(2) Store at −20°C.

Dulbecco's PBS without Ca^{2+} and Mg^{2+} (D-PBSA)

(*See* Protocol 11.6)

EDTA (Versene)

(1) Prepare as a 10 mM concentrate, 0.374 g/L in D-PBSA.
(2) Sterilize by autoclaving or filtration.
(3) Dilute 1:10, or 1:5 for use at 1.0–2.0 mM, or, exceptionally, 1:2 for use at 5 mM, diluted in D-PBSA or trypsin in D-PBSA.

EGTA

As for EDTA, but EGTA may be used at higher concentrations because of its lower toxicity.

Ficoll, 20%

(1) Sprinkle 20 g of Ficoll (GE Heathcare) on the surface of 80 mL UPW
(2) Leave overnight for the Ficoll to settle and dissolve.
(3) Make up to 100 mL in UPW.
(4) Sterilize by autoclaving.
(5) Store at room temperature.

Fixative for Tissue Culture

See Acetic/methanol.
Alternatively, use pure anhydrous ethanol or methanol (*see* Protocol 16.2), 10% formalin, 1% glutaraldehyde, or 5% paraformaldehyde.

Gentamycin

Dilute to 50 μg/mL for use.

Gey's Balanced Salt Solution

	g
NaCl	7.00
KCl	0.37
$CaCl_2$	0.17
$MgCl_2 \cdot 6H_2O$	0.21
$MgSO_4 \cdot 7H_2O$	0.07
$Na_2HPO_4 \cdot 12H_2O$	0.30
KH_2PO_4	0.03
$NaHCO_3$	2.27
Glucose	1.00
Water, up to	1000 mL
CO_2	5%

Giemsa Stain

Giemsa stain can be applied undiluted and then diluted with buffer or water (*see* Protocol 16.2), or diluted in buffer before use. The author has found the first method more successful for cultured cells.

(1) Prepare buffer:

$NaH_2HPO_4 \cdot 2H_2O$..... 0.01 M..... 1.38 g/L
$Na_2HPO_4 \cdot 7H_2O$..... 0.01 M..... 2.68 g/L

Combine to give pH 6.5.

(2) Dilute prepared Giemsa concentrate 1:10 in 100 mL of buffer.
(3) Filter the solution through Whatman No. 1 filter paper to clarify.
(4) Make up a fresh solution each time, because the concentrate precipitates on storage.

Glucose, 20%

Glucose..... 20 g
Hanks' BSS to..... 100 mL

(1) Sterilize by autoclaving.
(2) Store at room temperature.

Glutamine, 200 mM

L-glutamine..... 29.2 g
Hanks' BSS..... 1000 mL

(1) Dissolve the glutamine in BSS and sterilize by filtration (*see* Protocols 11.12, 11.13).
(2) Dispense the solution into aliquots and store at −20°C.

Glutathione

(1) Make 100× stock (i.e., 0.1 M in HBSS or D-PBSA), and dilute to 1 mM for use.
(2) Sterilize by filtration.
(3) Dispense into aliquots and store at −20°C.

Ham's F12

See Table 9.3.

Hanks' BSS

See Section 9.3 and Balanced Salt Solutions, in this appendix.

Hanks' BSS without phenol red:

Follow the preceding instructions, but omit phenol red.

HAT Medium

Drug	*Concentration*	*Dissolve in*	*Molarity (100× final)*
Hypoxanthine (H)….	136 mg/100 mL….	0.05 N HCl….	1×10^{-2} M
Aminopterin (A)……	1.76 mg/100 mL….	0.1 N NaOH…	4×10^{-5} M
Thymidine (T)……	38.7 mg/100 mL….	….HBSS…..	1.6×10^{-3} M

(1) For use in the HAT selective medium, mix equal volumes of each, sterilize by filtration, and add the mixture to medium at 3% v/v.
(2) Store H and T at 4°C, A at −20°C.

HB Medium

Add the following to CMRL 1066 medium:

Insulin…… 5 μg/mL
Hydrocortisone…… 0.36 μg/mL
β-Retinyl acetate…… 0.1 μg/mL
Glutamine……1.17 mM
Penicillin…… 50 U/mL
Streptomycin……50 μg/mL
Gentamycin……50 μg/mL
Fungizone……1.0 μg/mL
Fetal bovine serum…… 1%

HBSS

See Section 9.3 and Balanced Salt Solutions, in this appendix.

Hoechst 33258 [Chen, 1977]

2-[2-(4-Hydroxyphenol)-6-benzimidazoyl]-6-(1-methyl-4-piperazyl)benzimidazole trihydrochloride

(1) Make up 1 mg/mL stock in D-PBSA or HBSS without phenol red.
(2) Store the solution at −20°C. For use, dilute 1:20,000 (1.0 μL in 20 mL) in D-PBSA or HBSS without phenol red at pH 7.0.

Δ ***Safety Note.*** Because this substance may be carcinogenic, handle it with extreme care. Weigh in a fume cupboard and wear gloves.

Kanamycin Sulfate (Kannasyn), 10 mg/mL

Kanamycin…… 4, 1-g vials
Hanks' BSS…… 400 mL

(1) Add 5 mL of HBSS from a 400-mL bottle of HBSS to each vial.
(2) Leave for a few minutes to dissolve.
(3) Remove the HBSS and kanamycin from the vials, and add them back to the HBSS bottle.
(4) Add another 5 mL of HBSS to each vial to rinse and return to the BSS bottle. Mix well.
(5) Dispense 20-mL aliquots of the solution into sterile containers and store at −20°C.
(6) Test for sterility: Add 2 mL of reagent to 10 mL of sterile medium, free of all other antibiotics, and incubate the solution at 37°C for 72 h.
(7) Use at 100 μg/mL.

Lactalbumin Hydrolysate 5% (10×)

Lactalbumin hydrolysate…… 5 g
HBSS……100 mL

(1) Heat to dissolve.
(2) Sterilize by autoclaving.
(3) Use at 0.5%.

McIlvaines Buffer, pH 5.5

	To make 20 mL	*To make 100 mL*
0.2 M Na_2HPO_4 (28.4 g/L)……	11.37 mL……	56.85 mL
0.1 M citric acid (21.0 g/L)……	8.63 mL……	43.15 mL

Media

The constituents of some media in common use are listed in Chapters 9 and 10 (*see* Tables 9.3, 10.1, and 10.2), together with the recommended procedure for their preparation. For those media not described, *see* Morton [1970], or suppliers' catalogs (*see* Appendix II: Media).

MEM

See Section 9.4 and Table 9.3.

2-Mercaptoethanol (M.W. 78)

Stock solution, 5 mM…… 4 μL
HBSS…… 10 mL

(1) Sterilize by filtration in fume cupboard.
(2) Store at −20°C or make up a fresh solution each time.

Methocel

See Methylcellulose.

Methylcellulose (1.8%)

(1) Weigh out 7.2 g of Methocel, and add it to a 500-mL bottle containing a large magnetic stirrer bar.
(2) Sterilize by autoclaving with the cap loose for penetration of steam.
(3) Add 400 mL of sterile UPW heated to 90°C to wet the Methocel.
(4) Stir at 4°C overnight to dissolve. (The Methocel will form a solid gel if the magnet does not keep stirring.)
The resulting solution is now Methocel 2×, and for use, it should be diluted with an equal volume of 2× medium of your choice. It is more accurate to use a syringe (without a needle) than a pipette to dispense Methocel.

(5) For use, add a cell suspension in a small volume of growth medium (*see* Protocol 14.5)

Mitomycin C
Stock solution, 10 μg/mL (50×)

Mitomycin..... 2-mg vial

(1) Measure 20 mL of HBSS into a sterile container.
(2) Remove 2 mL of HBSS by syringe and add it to a vial of mitomycin.
(3) Allow the mixture to dissolve, withdraw the resulting solution, and add it back to the container.
(4) Store for 1 week only at 4°C in the dark. (Cover the container with aluminum foil.)
(5) For longer periods, store at −20°C.
(6) Dilute to 0.25 μg/mL for 18-h exposure or 20 μg/mL for 10-h exposure (*see* Protocols 14.3, 23.4)

Δ ***Safety Note.*** Because mitomycin is toxic, reconstitute it in the vial. Work in a fume hood when handling the substance in powder form.

MTT
(1) Dissolve 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) 50 mg/mL in D-PBSA
(2) Sterilize by filtration.

Δ ***Safety Note.*** MTT is toxic; weigh it in a fume cupboard and wear gloves.

Mycoplasma Reagents
Stain (*see* Hoechst 33258)
Mountant: Glycerol in McIlvaines Buffer pH 5.5

	To make 40 mL
0.4 M Na_2HPO_4 (56.8 g/L)	11.37 mL
0.2 M Citric acid (42.0 g/L)	8.63 mL
Glycerol	20.00 mL

(1) Add Vectashield (Vector) to reduce fluorescence fade (*see* manufacturer's instructions)
(2) Check the pH and adjust to 5.5.

Mycostatin (Nystatin)
(1) Prepare at 2 mg/mL (100×):

Mycostatin..... 200 mg
Hanks' BSS..... 100 mL

(2) Make up by same method as kanamycin.
(3) Final concentration, 20 μg/mL.

Naphthalene Black
(1) Prepare at 1% in Hanks' BSS

Naphthalene Black..... 1 g
Hanks' BSS..... 100 mL

(2) Dissolve as much as possible of the stain in the HBSS.
(3) Filter the resulting saturated solution through Whatman No. 1 filter paper.

PBS
See Phosphate-Buffered Saline.

D-PBSA
See Phosphate-Buffered Saline.

PE
See Phosphate-Buffered Saline/EDTA.

Penicillin
(e.g., Crystapen benzylpenicillin [sodium]) 1,000,000 units per vial

(1) Make up as for kanamycin, stock concentration 10,000 U/mL.

Crystapen..... 4 vials (4×10^6 U)
HBSS..... 400 mL

(2) Store frozen at −20°C in aliquots of 5–10 mL.
(3) Use at 50–100 U/mL.

Percoll
(1) Ready made and sterile as purchased, Percoll should be diluted with medium or HBSS until the correct density is achieved.
(2) Check the osmolality. Adjusting it to 290 mOsm/Kg will require the diluent to be hypo- or hypertonic, so it is better to dilute a small sample first and check its osmolality, and then scale up.

Phosphate-Buffered Saline (Dulbecco Solution A; D-PBSA)
See Table 9.2 and Protocol 11.6.

Phosphate-Buffered Saline/EDTA, 10 mM (PE)
(1) Make up D-PBSA.
(2) Add EDTA disodium salt, 3.72 g/L, and stir.
(3) Dispense, autoclave, and store at room temperature.
(4) Dilute D-PBSA/EDTA 1:10 to give 1 mM for most applications or 1:2 (5 mM) for high chelating conditions (e.g., trypsinization of CaCo-2 cells).

Phytohemagglutinin (PHA)
(1) Prepare stock 500 μg/mL (100×) from lyophilized PHA by adding HBSS by syringe to an ampoule.
(2) Dispense into aliquots and store at −20°C.
(3) Dilute 1:100 for use.

SF12
Ham's F12 (*see* Table 9.3) with additional 2× Eagle's MEM essential amino acids and 1× nonessential amino acids but lacking thymidine, and with 10× folic acid concentration.

Sodium Citrate/Sodium Chloride See SSC
SSC 20× (Sodium Citrate/Sodium Chloride)
(1) Prepare concentrated SSC:

Trisodium citrate (dihydrate)	0.3 M	88.2 g
NaCl	3.0 M	175.3 g
Water		1000 mL

(2) Dilute to 1× or 2× as appropriate

Streptomycin Sulfate

(1) Take 2 mL from a bottle containing 100 mL of sterile HBSS, and add to a 1-g vial of streptomycin.
(2) When streptomycin has dissolved, return the 2 mL to the 98 mL of HBSS.
(3) Dilute 1:200 for use. The final concentration should be 50 μg/mL.

Trypsin

2.5% w/v in 0.85% (0.14 M) NaCl
Trypsin solutions can be bought commercially. Alternatively:

(1) Prepare a 2.5% solution:

Trypsin (e.g., Difco 1:250)..... 25 g
NaCl, 0.85%..... 1 L

(2) Stir trypsin for 1 h at room temperature or 10 h at 4°C. If the trypsin does not dissolve completely, clarify it by filtration through Whatman No. 1 filter paper.
(3) Sterilize by filtration.
(4) Dispense into 10- to 20-mL aliquots and store at −20°C.
(5) Thaw and dilute 1:10 in D-PBSA or PE for use.
(6) Store diluted trypsin at 4°C for a maximum of 3 weeks.

Note. Trypsin is available as a crude (e.g., Difco 1:250) or purified (e.g., Worthington or Sigma 3× recrystallized) preparation. Crude preparations contain several other proteases that may be important in cell dissociation, but may also be harmful to more sensitive cells. The usual practice is to use crude trypsin, unless the viability of the cells is diminished or reduced growth is observed, in which case purified trypsin may be used. Pure trypsin has a higher specific activity and should therefore be used at a proportionally lower concentration (e.g., 0.01 or 0.05%). Check for mycoplasma when preparing from raw trypsin.

Trypsin/EDTA

See Trypsin, Step (5)

Trypsin, Versene, Phosphate (TVP)

Trypsin (Difco 1:250)..... 25 mg (or 1 mL 2.5%)
Phosphate-buffered saline, D-PBSA..... 98 mL
Disodium EDTA ($2H_2O$)..... 37 mg
Chick serum (MP Biomedicals)..... 1 mL

(1) Mix D-PBSA and EDTA, autoclave the mixture, and store it at room temperature.
(2) Add chick serum and trypsin before use. If powdered trypsin is used, sterilize it by filtration before adding the serum.
(3) Dispense the solution into aliquots and store at −20°C.

Tryptose Phosphate Broth

(1) Prepare at 10% in HBSS

Tryptose phosphate (Difco)..... 100 g
Hanks' BSS..... 1000 mL

(2) Stir until dissolved.
(3) Dispense into aliquots of 100 mL and sterilize in the autoclave.
(4) Store at room temperature
(5) Dilute 1:100 (final concentration, 0.1%) for use.

Tyrode's Solution

	g
NaCl	8.00
KCl	0.20
$CaCl_2$	0.20
$Mg_2Cl_2 \cdot 6H_2O$	0.10
$NaH_2PO_4 \cdot H_2O$	0.05
Glucose	1.00
UPW, up to	1 L
Gas phase	Air

Versene

See EDTA.

Viability Stain

See Naphthalene Black.
Trypan Blue is available from most tissue culture media suppliers (*see* Appendix II).

Vitamins

Detailed in media recipes (*see* Tables 9.3, 10.1, 10.2) and available commercially as 100× concentrates.

(1) Make up individually as 1000–10,000× stocks and combine as required to make up a 100× concentrate.
(2) Sterilize by filtration.
(3) Store at −20°C in the dark.