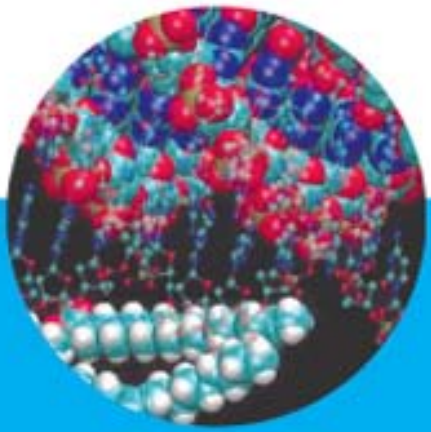
Markus R Wenk
Aaron Zefrin Fernandis



Manuals in Biomedical Research – Vol. 3

A Manual for Biochemistry Protocols

Jan-Thorsten Schantz *Series Editor*

A Manual for Biochemistry Protocols

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A Manual for Biochemistry Protocols

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Preface

The field of biochemistry is diverse and forms parts of diverse fields including cell biology, molecular biology and medical sciences. Biochemistry is the study of the molecules of life like proteins, lipids, carbohydrates and nucleic acids. Studying the structure, properties and reactions of these important molecules would help in better understanding life as a whole. The practical aspect along with the theoretical background would help in better understanding these mechanisms. This book tries to address and compile some of the routinely used protocols in biochemistry for easy access. The aim of this book is not only to bring together the protocols, but also to understand some of the basics behind following the methodologies. The target is to give students a view of biochemistry, especially those who have just ventured into the field of biochemistry and need a headstart.

The protocols are written as a handy guide that can be carried as a pocket guide for easy reference. The protocols are easy to follow with each step explained in layman terms. Even though the field of biochemistry is exhaustive, an effort has been made to list some of the protocols that could serve as a foundation for starting any biochemical investigation.

We would like to thank all the members of the lab, especially Dr Sravan Kumar Goparaju and Xue Li Guan, whose help in reviewing the manuscript is greatly appreciated. We would also like to thank all the people previously involved in designing these protocols.

Markus R. Wenk
Aaron Z. Fernandis

Contents

Preface v

List of Figures xi

List of Contributors xiii

A Protein Purification 1
A.1 Protein Precipitation 2
A.2 Column Chromatography 3
A.2.1 Ionic Exchange Chromatography 3
A.2.2 Size-Exclusion Chromatography 6
A.2.3 Affinity Chromatography 6
A.2.4 Purification of Recombinant Proteins 7
A.2.5 Commercially Prepacked Column Kits 9
Notes 12

B Protein Analysis 15
B.1 Protein Estimation 16
B.1.1 Absorbance Assays 16
B.1.2 Colourimetric Assays 17
B.1.3 Commercial Protein Estimation Kits 18
B.2 Spectrometric Analysis 19
B.3 SDS-PAGE 21
B.4 Gel Staining 26
B.4.1 Coomassie Blue Staining 26
B.4.2 Silver Staining 27
B.5 Western Blotting 28

B.6 Immunoprecipitations 32
Notes 35

C Lipid Analysis 39
C.1 Lipid Extraction 40
C.1.1 Modified Bligh and Dyers Method for Phospholipid Extraction 40
C.1.2 Folch Extraction 41
C.1.3 Grey Method for Phosphatidylinositol Phosphate Extraction 42
C.1.4 Modified Alex Brown Method for Phosphatidylinositol Phosphate Extraction 44
C.1.5 Hexane Extraction for Neutral Lipids 44
C.1.6 Glycolipid Extraction 45
C.2 Thin Layer Chromatography 46
Notes 49

D Mammalian Cell Culture 53
D.1 Primary Culture 54
D.1.1 Primary Cell Isolation and Culturing 54
D.1.2 Splenocyte Isolation 55
D.1.3 Isolation of Peripheral Blood Lymphocytes 57
D.2 Tissue Culture 58
D.2.1 Adherent Cells 58
D.2.2 Suspension Cells 60
D.2.3 Cell Maintenance 61
D.3 Cell Count 62
D.4 Mammalian Transfection 64
D.4.1 Calcium Phosphate Coprecipitation 64
D.4.2 Electroporation Protocol 65
D.4.3 Lipofectamine Transfection Protocol 66
Notes 68

E Microscopy 71
E.1 Poly-L-Lysine Immobilisation 73
E.2 Immunofluorescence and Confocal Microscopy 74
E.3 Immunohistochemistry 75
Notes 77

F Assays 81
F.1 Enzyme Assays 82
F.1.1 Enzyme Kinetics: Basics 82
F.1.2 Lipid Kinase Assay 84
F.1.3 Protein Kinase Assay 86
F.1.4 Protein Tyrosine Phosphatase Assay 88
F.1.5 Alkaline Phosphatase Assay 90
F.1.6 Caspase Assay 90
F.2 Functional Assays 92
F.2.1 Apoptosis Assay 92
F.2.2 XTT Cell Proliferation Assay [(2,3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)] 94
F.2.3 Chemotaxis Assay 95
F.2.4 Matrigel Invasion or Chemoinvasion Assay 97
Notes 100

G Autoradiography 103
Notes 105

Suggested Readings 109

Abbreviations 113

Appendices 115
Appendix 1 Safety Issues 116
Appendix 2 Buffer Chart 118
Appendix 3 Composition of Regular Buffers or Stock Solutions 120

Index 125

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List of Figures

Figure	Description	Page
A.1	Protein precipitation using ammonium sulfate	5
A.2	Ion exchange chromatography	5
A.3	Gel filtration chromatography	6
A.4	Affinity chromatography	7
A.5	Flow chart for column chromatography	8
B.1	Standard graph for protein estimation using known concentration of BSA as standard	20
B.2	Flow chart of spectrophotometer	20
B.3	SDS-PAGE. (A) Different parts of the electrophoretic apparatus. (B) Running the SDS-PAGE	23
B.4	Reaction occurring on the membrane	29
B.5	Cartoon depiction of the Western blot sandwich	30
C.1	Phase separation during lipid extraction	41
C.2	Thin layer chromatography	47

C.3	Thin layer chromatography chamber and TLC plate	47
D.1	Ficoll gradient for enrichment of peripheral blood lymphocytes	57
D.2	Tissue culture hood	59
D.3	CO_2 incubator	59
D.4	Cartoon depiction of a hemocytometer	63
E.1	A typical light microscope	72
E.2	Principle of fluorescence emission	73
F.1	Lineweaver–Burke plot and determination of K_m and $V_{\max}$	83
F.2	Cartoon depiction of the transwell plate used for chemotaxis assay	96
F.3	Cartoon depiction of the transwell plate used for Matrigel-based chemoinvasion assay	98

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A

Protein Purification

Summary

Protein expression is tightly regulated for normal functioning of a cell or organism. To understand protein structure and function in detail, they often need to be separated from other cellular components (lipids, nucleic acids, sugars, etc.) and isolated to homogeneity. After recovering a protein to near homogeneity, it should retain all its native biological characteristics of structure and activity. To achieve this objective, one needs to take into account the physical and chemical property of proteins (size, charge, solubility, hydrophobicity, precipitation, etc.). These common characteristics of the protein can be exploited to separate it from other components of the cell. With the introduction of recombinant DNA technology, protein purification technique has been enhanced and also simplified. Purification protocols vary, depending on the precise nature of the protein. General steps include (i) chromatography, (ii) precipitation and/or (iii) extraction.

A.1 Protein Precipitation

Many cytosolic proteins are water soluble and their solubility is a function of the ionic strength and pH of the solution. The commonly used salt for this purpose is Ammonium Sulphate, due to its high solubility even at lower temperatures. Proteins in aqueous solutions are heavily hydrated, and with the addition of salt, the water molecules become more attracted to the salt than to the protein due to the higher charge. This competition for hydration is usually more favorable towards the salt, which leads to interaction between the proteins, resulting in aggregation and finally precipitation. The precipitate can then be collected by centrifugation and the protein pellet is re-dissolved in a low salt buffer. Since different proteins have distinct characteristics, it is often the case that they precipitate (or 'salt out') at a particular concentration of salt.

Requirements:

(1) Ammonium sulphate
(2) Ice tray
(3) Magnetic bead and stirrer
(4) Swing-out rotor centrifuge

Protocol 1:

(1) Clarify the protein solution (in most cases the lysates) by centrifugation.
(2) Transfer the supernatant into an ice cold beaker with a magnetic bead.
(3) Note the exact amount of the supernatant (From Table A.1).
(4) Keep the beaker chilled by placing it in an ice tray.
(5) Transfer the beaker with the ice tray onto a magnetic stirrer (Fig. A.1).
(6) Weigh the amount of ammonium sulfate to be added. The amount depends on the volume of the solution and the percentage saturation of the salt needed. Refer to the precipitation chart. In case of protein purification, a step precipitation is carried out.
(7) Slowly add the ammonium sulphate with stirring. One needs to be careful as the addition of the salt should be very slow. Add a small amount at a time and then allow it to dissolve before further addition.
(8) Keep it on the stirrer for 1hr precipitation to occur in ice.
(9) Centrifuge at 10,000g for 15 min at 4^{o}C.
(10) The pellet contains the precipitated protein which could be dissolved in a suitable buffer for further analysis and purification.
(11) For a second round of precipitation of a different protein, the supernatant is again used and the above same steps are followed.

A.2 Column Chromatography

This method involves passing the protein through a column filled with resins of unique characteristics. Depending on the type of the resin or beads, purification can be achieved through (i) Ion Exchange, (ii) Size Exclusion or (iii) Affinity Chromatography.

A.2.1 Ionic Exchange Chromatography

This is one of the most useful methods of protein purification. Depending on the surface residues on the protein and the buffer conditions, the protein will have net a positive or negative charge

Table A.1 Amount of Ammonium sulfate required for protein precipitation.

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

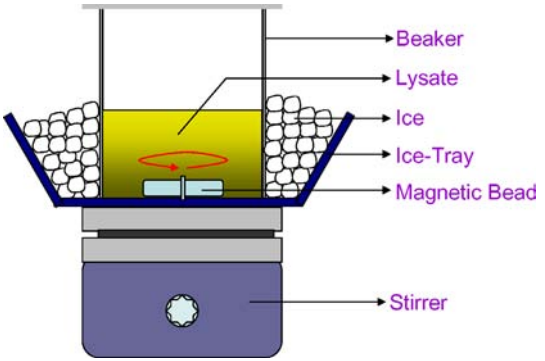

Fig. A.1 Protein Precipitation using ammonium sulfate.

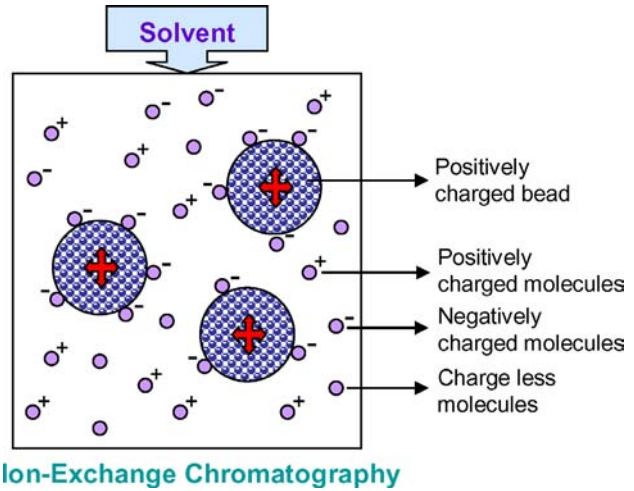

Fig. A.2 Ion Exchange Chromatography. The resins are charged and the protein molecules that bind are of opposite charge.

(Fig. A.2). An ideal buffer should be in the physiological pH range of 6 to 8. At this pH range, most of the proteins have been observed to be negatively charged. Hence, proteins would bind to positively charged molecules of the resin. Change in the buffer pH condition could make the protein relatively positive, thereby allowing it to bind to a negatively charged resin material. Among the most commonly used charged molecules are DEAE and CM. These charged molecules are coupled to an inactive material, often nanoparticle beads, loaded into a column. The protein is

loaded onto this packed column and is allowed to bind. The column is washed and the bound proteins are eluted depending on their tightness of binding, by subjecting them to either increasing concentrations of salt or changes in pH. Proteins with low charge will elute first.

A.2.2 Size-Exclusion Chromatography

In this approach, the size of the protein is taken into consideration. The size of the protein depends on the number of amino acids it contains. This property can be used in protein purification. The column material consists of a porous matrix for proteins to diffuse into (Fig. A.3). The smaller proteins get entangled inside the porous material and hence their mobility is restricted. In contrast, the larger proteins do not get entangled and could just pass through. Hence, in the elution profile, the larger molecules would be the first ones to elute, while the smallest ones will be last to elute.

A.2.3 Affinity Chromatography

As the name suggests, the principle is the use of a moiety or molecule which has high affinity for the protein of interest.

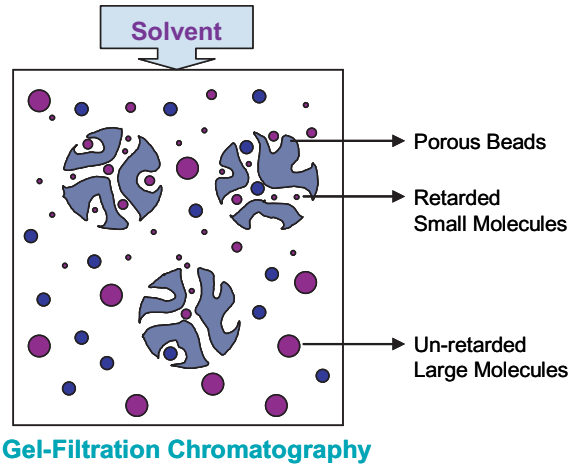

Fig. A.3 Gel filtration Chromatography. The resins are porous and the small molecules get trapped inside the pores whereas the bigger protein molecules exclude out.

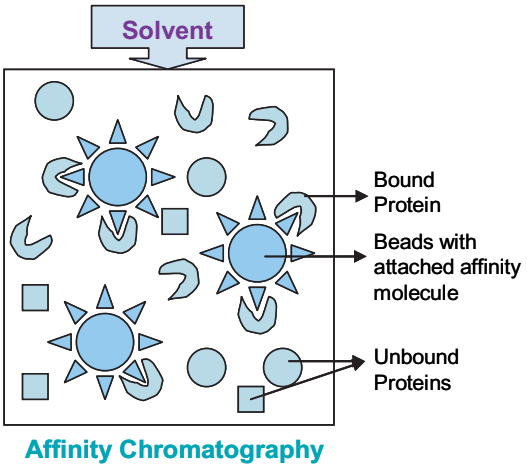

Fig. A.4 Affinity Chromatography. The resins have a head group which has a high binding affinity towards the protein of interest.

These molecules could either be co-factors, modified substrates, inhibitors or carbohydrates. This strategy of purification is used mostly in the later stages where the protein is relatively pure, and more specific approaches are required for additional purification. The affinity moiety or molecule is coupled to the matrix and used as a bait to fish the protein of interest (Fig. A.4). The protein could either be eluted with high salt in some cases or with increased amount of the affinity molecule itself.

A.2.4 Purification of Recombinant Proteins

This is the easiest method available for the purification of a protein, albeit it is a recombinantly expressed protein rather than an endogenous protein. The gene encoding a protein of interest is cloned into an expression vector (often with a tag such as GST or His) which is then introduced into the producer cell in order to express the protein as a fusion protein. The protein is then 'over-expressed' in higher than usual levels in a bacterial (e.g. BL21), yeast (e.g. S. cerevisiae), insect (e.g. sf9) or mammalian (e.g. CHO) cell system. The tag on the protein serves as a pull down, and thus separate and purify the protein from the cell lysate. The tag is usually a 6X His or Glutathione Transferase (GST). Thus, the column material is either Ni-NTA (Ni-nitrilotriacetic acid) which binds tightly to 6His, or Glutathione

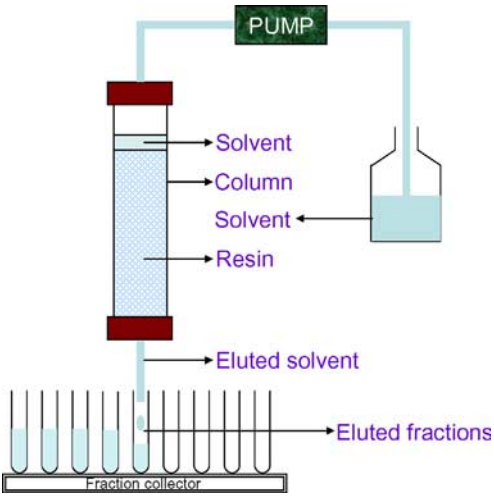

Fig. A.5 Flow Chart for column Chromatography. The Central part is the column from the sample and/or solvent is loaded at a controlled flow rate with a pump. The eluates from the column are collected in tubes of a fraction collector.

sepharose which binds to GST. Since these columns are very specific, the fusion protein is purified to near homogeneity. In order to attain complete purity, the protein then could be purified by other conventional chromatographic methods.

Protocol 2: (i) Column Preparation

(1) Make a slurry of the respective resin or beads in the equilibration buffer.
(2) Fill the glass column with the equilibration buffer with the nozzle of the column closed.
(3) Open the nozzle with a slow flow rate.
(4) Using a pipette, load the resin suspension onto the column.
(5) Allow the material to settle till the required level.
(6) Wash the column thoroughly with 2 to 3 column volumes of equilibration buffer before loading the sample onto the column.

(ii) Column Run: (Fig. A.5)

(1) The sample is loaded at a slow rate onto the column from the top. The eluate from the column is collected as a flow through. In the case of size exclusion, the concentrated sample is layered on the top of the column bed.
(2) The equilibration buffer or wash buffer is applied on the column at a monitored flow rate. The eluate is collected as the wash. For size exclusion, the eluates are collected in fractions.
(3) The protein level can be monitored by scanning the eluates at O.D. 280 nm.
(4) The bound proteins are eluted with increasing concentrations of salt or other elution buffers, depending on the column and enzyme. The elution can be carried out as step elution or gradient.
(5) The eluates are collected as fractions.
(6) The fractions can then be analysed for enzyme activity and run on SDS-PAGE for purity.

A.2.5 Commercially Pre-packed Column Kits

The columns are here designed specifically for a defined purpose. These columns are easier to use, faster and they require much less resources. Some of the columns include the NAP-25 or PD 10 desalting columns (from Amersham Biosciences), His Tag columns such as Ni-NTA spin column from Qiagen, His Bind from Novagen or His GraviTrap from GE Healthcare.

Desalting columns

The NAP-25 /PD-10 column contains Sephadex G-25 and is used for a rapid desalting or buffer exchange of nucleic acids, proteins and oligonucleotides.

Protocol 3:

(1) Remove the top cap and pour off the excess liquid.
(2) Cut the end of the column tip.

(Continued)

(Continued)

(3) Support the column over a suitable receptacle and equilibrate the gel with approximately 25 ml of the required buffer.
(4) Allow the equilibration buffer to completely enter the gel bed.
(5) Add the sample to the column in a maximum volume of 2.5 ml. If the sample volume is less than 2.5 ml, do not adjust it at this time. Allow the sample to enter the gel bed completely.
(6) For sample volumes less than 2.5 ml, add equilibration buffer so that the combined volume of sample added in Step 5 and buffer added in Step 6 equals 2.5 ml. Allow the equilibration buffer to enter the gel bed completely.
(7) Place a test tube for sample collection under the column.
(8) Elute the purified sample with 3.5 ml buffer.

Purification of His-Tag Proteins

These columns are used for purification of recombinant fusion proteins tagged to 6XHis. The commercial columns contain the precharged Ni coupled to a tetradentate chelating absorbent such as the NTA (nitrilotriacetic acid), bound to a matrix which could be Sepharose or Cellulose.

Protocol 4:

(1) Lyse the cells in the presence of protease inhibitors either by enzymatic lysis (0.2 mg/ml lysozyme, 20μg/ml DNAse, 1 mM $mgCl_2$) or by mechanical lysis (Sonication, homogenization, repeated freeze/thaw) in 20 mM sodium phosphate, 500 mM NaCl. Adjust the pH of the lysate to pH 7.4 using a dilute acid or base.
(2) Centrifuge the lysate at 10000 rpm for 30 min at 4°C.
(3) Collect the supernatant for purification step.
(4) Cut off the bottom tip, remove the top cap, pour off excess liquid and place the column in the Workmate column stand.

(Continued)

(Continued)

(5) Equilibrate the column with 10 ml of 20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.4
(6) Load the sample.
(7) Wash with 10 ml 20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4.
(8) Apply 3 ml elution buffer (20 mM sodium phosphate, 500 mM NaCl, 100 mM imidazole, pH 7.4) and collect the eluate containing the purified protein.

Notes

Notes

Notes

B

Protein Analysis

B.1 Protein Estimation

Protein determination is necessary to estimate the amount of protein in the sample, to normalise against the protein concentration or during purification procedures. Depending on the amount of sample, accuracy and presence of interfering agents, one needs to decide on the method to be used. For accurate quantification, the sample protein is compared with a known amount of a standard protein which could either be the commonly used bovine serum albumin (BSA) or it could sometimes be immunoglobulin G (IgG). The various methods and their specifications are outlined below:

B.1.1 Absorbance Assays

The aromatic rings in the protein absorb ultraviolet light at an absorbance maximum of 280 nm, whereas the peptide bonds absorb at around 205 nm. The unique absorbance property of proteins could be used to estimate the level of proteins. These methods are fairly accurate with the ranges from 20 μg to 3 mg for absorbance at 280 nm, as compared with 1 to 100 μg for 205 nm. The assay is non-destructive as the protein in most cases is not consumed and can be recovered. Secondary, tertiary and quaternary structures all affect absorbance; therefore, factors such as pH, ionic strength, etc can alter the absorbance spectrum. This assay depends on the presence of a mino acids which absorb UV light (mainly tryptophan, but to a lesser extent also tyrosine). Small peptides that do not contain such a mino acids cannot be measured easily by UV.

Requirements

(1) Quartz Cuvettes
(2) UV-Spectrophotometer

Protocol 1:

(1) Start the UV-spectrometer at least 15 min before taking the reading, so that the instrument is stabilised.

(Continued)

(Continued)

(2) Load the buffers solution into the cuvette and measure the absorbance at 280 nm and 260 nm. The 260 nm absorbance is done in order to avoid the interference by nucleic acids.
(3) Now put in the sample for which the protein needs to be quantified and measure the absorbance at 280 and 260 nm.
(4) Calculate the concentration of the protein in the sample using the following formula:

Concentration (mg/ml) = (1.55 × A280) − 0.76 × A260) or
Concentration = $A_{280\,nm}$ divided by absorbance coefficient

Absorbance coefficients of BSA is 63 OD/M/cm and Bovine, human or rabbit IgG is 138 OD/M/cm.

B.1.2 Colorimetric Assays

Bradford protein assay

This is the assay of choice in most cases due to its simplicity, scalability and sensitivity. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm upon protein binding. Both hydrophobic and ionic interactions stabilise the anionic form of the dye, causing a visible color change. Range: 1 to 20 micrograms (micro assay); 20 to 200 micrograms (macro assay).

Requirements

(1) Bradford reagent: Dissolve 100 mg Coomassie Brilliant Blue G-250 in 50 ml of 95% ethanol and add 100 ml of 85% (w/v) phosphoric acid. Dilute to 1 litre when the dye has completely dissolved, and filter through Whatman #1 paper just before use
(2) 1M NaOH
(3) Colorimeter
(4) Glass or polystyrene cuvettes

Protocol 2:

(1) Dilute unknowns between 5 and 100 μg protein in a 100 μl sample.
(2) Prepare standards containing a range of 5 to 100 μg BSA in 100 μl volume.
(3) Add an equal volume of 1M NaOH and vortex.
(4) Add 5 ml dye reagent and incubate 5 min.
(5) Measure the absorbance at 595 nm in a glass or polystyrene cuvette.

Analysis

Prepare a standard curve of absorbance versus μg of protein. Use this curve to determine the concentrations of original samples.

B.2 Commercial Protein Estimation Kits

The commercial kits are easy to use. One of the most used kits is the DC protein estimation kits from Bio-Rad. The assay is sensitive and is also reliable in the presence of reducing agents and detergents. The assay is a modified Lowry method. This method is rapid and the reading can be done in 15 min with the color change not varying with time, making the reading stable and reliable. The reaction involves protein with the alkaline copper tartrate and the Folin reagent. The protein residues, especially, tyrosine and tryptophan, and to a lesser extent, cysteine cysteine and histidine, reduces the Folins reagent producing a blue color.

Requirements

(1) Reagent S
(2) Reagent A
(3) Reagent B
(4) 96 well flat bottom clear microtitre plate
(5) Microtitre plate spectrophotometer

Protocol 3:

(1) Prepare the working reagent by adding 20 µl of reagent S to each ml of reagent A.
(2) Prepare dilutions of protein standards like BSA in the range of 0.2 mg/ml to 2 mg/ml.
(3) Make the necessary dilution of the sample whose protein needs to be measured.
(4) Pipette 5 µl of standards and samples into a clean, dry 96 well microtitre plate.
(5) Add 25 µl of working reagent to each well.
(6) Add 200 µl of reagent B to each well.
(7) Mix the contents in the plate by gently shaking on a shaker at RT (RT). If bubbles form, pop them with a clean, dry pipette tip.
(8) After 15 min, measure the absorbance at 750 nm.
(9) Plot a standard graph as depicted in Fig. B.1.
(10) Estimate the concentration of the protein in the sample using the standard graph.

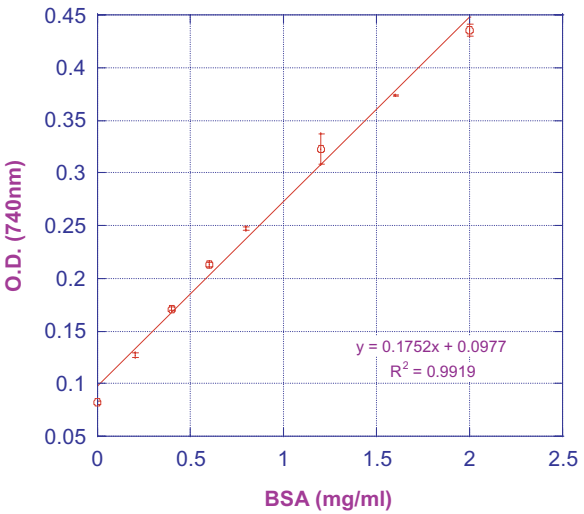

Fig. B.1 Standard graph for protein estimation using known concentration of BSA as standard.

B.3 Spectrometric Analysis

A spectrometer is one of the most widely used instruments in any biochemistry lab. Its application ranges from protein or DNA determination to enzyme assays. This instrument has a capability of measuring the absorbance of light by the sample as a function of the wave length. The instrument consists of a spectrometer which produces a light of desired wavelength and a photometer for measuring the intensity of the light after it has passed through the sample. The sample is placed inside a cuvette so that the light beam passes from the spectrometer through the sample to the photometer (Fig. B.2). The extent of absorption of light depends on the concentration of the sample. Hence, the intensity of the light transmitted to the photometer is proportional to the concentration of the sample. This relationship is defined by the Beer's law. Optical density or O.D. is the scale which is used by the spectrophotometers. The O.D. is directly proportional to the concentration of the sample and O.D. $= \varepsilon cd$, where ε is the extinction coefficient of the sample (protein), c its concentration, and d the length of the path which the light takes in the solution.

Requirements

(1) Cuvettes
(2) Spectrophotometer

Protocol 4:

(1) Switch on the instrument at least 15 min prior using.

(Continued)

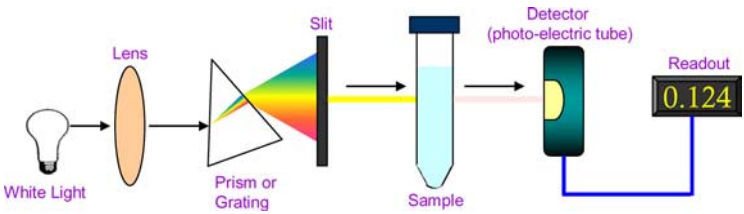

Fig. B.2 Flow chart of spectrophotometer.

(Continued)

(2) Set the wavelength of the instrument to the desired wavelength.
(3) Transfer the buffer in which the sample is prepared into two cuvettes.
(4) Wipe the cuvettes and insert them into the slots.
(5) Close the sample cover and auto Zero the instrument.
(6) Remove the buffer and load the sample into the cuvette.
(7) Wipe and insert the cuvette in the slot.
(8) Read the absorbance.
(9) Remove the sample, clean the cuvette and fill with another sample for readings.
(10) Calculate the concentration of the sample based on the standards (or e and d, in case they are known).

B.4 SDS-PAGE

SDS-PAGE (Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis) is a powerful technique which is used for the separation of proteins and nucleic acids. Electrophoresis is the migration of charged molecules in a media upon application of an electric field. The rate of migration depends on the charge on the molecule, its molecular mass, size and the strength of the electric field. Usually, this technique is routinely used for the analysis of proteins. The most commonly used matrix is agarose or polyacrylamide. These matrix forms a porous support and the size of the pores can be varied by changing the concentration of the matrix. Agarose is used mostly for separation of larger macromolecules, including nucleic acids, proteins and their complexes. On the other hand, polyacrylamide is used for the separation of proteins and small oligonucleotides. The charge on a protein is determined by the pH of the medium and the a mino acid composition of the protein. Each protein has an isoelectric point which is the pH at which the protein has no net charge. Thus, at a pH below the isoelectric point, the protein will be net positive charge and migrate towards cathode, but at higher pH, it will be negatively charged and move towards anode. Thus, the movement of protein will not only depend on the mass, but also on the charge. Nucleic acids, however, remain negative at any pH

due to the presence of the phosphate group of each nucleotide. Electrophoretic separation of nucleic acids is therefore strictly according to size.

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by binding to the polypeptide backbone. This makes the protein molecule negatively charged. This negative charge is proportionately distributed throughout the molecule, yielding the same charge density per unit length. In order to remove the disulphide bridges in proteins before they adopt the random-coil configuration necessary for separation by size, the proteins are reduced either by 2-mercaptoethanol or dithiothreitol. Thus, in denaturing SDS-PAGE separations, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight.

To increase the resolution of protein separation, a discontinuous buffer system is often used. The stacking gel contains a low pH, range of 6.8. At this pH, the major ion species, glycine, from the buffer is less ionized and hence moves very slowly. This leads to a trapping effect of the protein molecules, thereby concentrating them in the form of a band. As the protein enters the smaller pore sized separating gel and a higher pH, glycine is ionized, the voltage gradient is dissipated and the protein is separated based on size.

Requirements

(1) 30% Acrylamide Solution: Can be bought in solution form directly from suppliers such as Biorad, or it can be prepared by dissolving 30 g of Acrylamide and 0.8 g of Bis-Acrylamide in 50 ml of water. Bring the volume to 100 ml with water
(2) 1.5 M Tris buffer, pH 8.9 (200 ml): Dissolve 36.34 g of Tris in 150 ml of water. Adjust the pH to 8.9 with HCl, and then bring the volume to 200 ml
(3) 1M Tris pH 6.8 (100 ml): Dissolve 12.11g of Tris in 60 ml of water and pH adjusted to 6.8 with HCl. Bring the volume to 100 ml
(4) 10% SDS (50 ml): Dissolve 5 g of SDS in 50 ml water

(Continued)

(Continued)

(5) 10% Ammonium persulphate (APS) (Freshly prepared): 0.1 g of Ammonium persulphate in 1 ml of water
(6) TEMED
(7) Running Buffer (1litre): Dissolve 3.03 g Tris, 14.04 g Glycine and 1 g of SDS in water and bring the volume to 1 litre
(8) 4X sample buffer (10 ml): To 4 mg of Bromophenol Blue, add 0.6 ml of 1M Tris pH 6.8, 2.5 ml of 100% Glycerol, 3 ml of 10% SDS and 1.4 ml of water. Aliquot 375 µl in fresh eppendorf tube. Store at −20°C. Add 125 µl of 2-Mercaptoethanol before using
(9) Gel apparatus including power pack

Protocol 5: Gel Casting: (Fig. B.3A): System 3 gel apparatus from Biorad is popular and is widely used.

(1) The separating or the resolving gel mix is first prepared by adding water, Acrylamide, Tris buffer and SDS according to the table mentioned below.
(2) Set up the gel casting apparatus with the plates.
(3) Insert the combs and mark the length of the comb.
(4) Add APS and TEMED to initiate gel polymerisation.
(5) Immediately pour into the space between the plates about 0.5mm below the comb marks.

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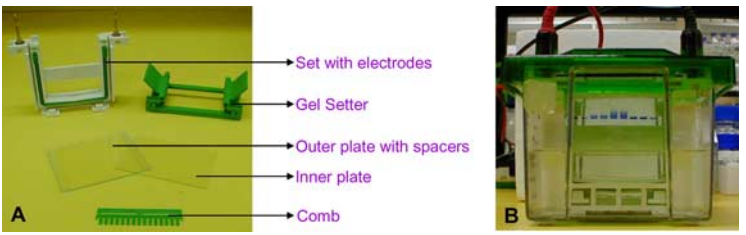

Fig. B.3 SDS-PAGE. (A) Different parts of the electrophoretic apparatus. (B) Running the SDS-PAGE.

(Continued)

(6) Layer the gel with water or ethanol or water saturated butanol.
(7) Allow the gel to polymerise.
(8) Till that time, make the stacking gel mix excluding APS and TEMED.
(9) Once the gel is polymerised, discard the upper layering media.
(10) Pour in the stacking gel mix after addition of TEMED and APS.
(11) Immediately put in the comb and allow the gel to polymerise.
(12) Once polymerised, carefully remove the combs and wash the wells with water.

Sample preparations

- Take a defined amount of Sample in an eppendorf tube.
- Add 4X sample buffer to make it 1X.
- Boil the samples for 5 min.
- Cool down the samples to RT before loading.

Running gel (Fig. B.3)

- Set up the gel apparatus.
- Transfer the gel plates from the gel casting apparatus to the running unit.
- Add the running buffer in the reservoirs.
- Load carefully the samples.
- Connect the power cords and run the gel at 200 mA.

Processing

- After the gel run is over with the dye front reaching the end of the gel, stop the power.
- Lift the plates and transfer the gel for further analysis like staining or for Western blot.

Table B.1 Composition of SDS-PAGE.

RESOLVING GEL							
Components	5 ml	10 ml	15 ml	20 ml	25 ml	30 ml	50 ml
6% Gel							
Water	2.60	5.30	7.90	10.6	13.2	15.9	26.5
30% Acrylamide	1.00	2.0	3.00	4.0	5.0	6.0	10.0
1.5 M Tris (pH 8.8)	1.30	2.50	3.80	5.0	6.30	7.50	12.50
10% SDS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
10% APS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
TEMED	0.004	0.008	0.012	0.016	0.02	0.024	0.04
8% Gel							
Water	2.30	4.6	6.90	9.3	11.5	13.9	23.2
30% Acrylamide	1.30	2.7	4.0	5.3	6.70	8.0	13.3
1.5 M Tris (pH 8.8)	1.30	2.5	3.80	5.0	6.30	7.50	12.5
10% SDS	0.05	0.1	0.15	0.2	0.25	0.30	0.50
10% APS	0.05	0.1	0.15	0.2	0.25	0.30	0.50
TEMED	0.003	0.006	0.009	0.012	0.015	0.018	0.03
10% Gel							
Water	1.90	4.0	5.90	7.90	9.90	11.9	19.80
30% Acrylamide	1.70	3.30	5.00	6.70	8.30	10.0	16.70
1.5 M Tris (pH 8.8)	1.30	2.50	3.80	5.0	6.30	7.50	12.50
10% SDS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
10% APS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.02
12% Gel							
Water	1.60	3.30	4.90	6.6	8.20	9.90	16.5
30% Acrylamide	2.00	4.0	6.00	8.0	10.0	12.0	20.0
1.5 M Tris (pH 8.8)	1.30	2.50	3.80	5.0	6.30	7.50	12.5
10% SDS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
10% APS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.02
15% Gel							
Water	1.10	2.3	3.40	4.6	5.70	6.90	11.5
30% Acrylamide	2.50	5.0	7.50	10.0	12.5	15.0	25.0
1.5 M Tris (pH 8.8)	1.30	2.50	3.80	5.0	6.30	7.50	12.5
10% SDS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
10% APS	0.05	0.10	0.15	0.20	0.25	0.30	0.50
TEMED	0.002	0.004	0.006	0.008	0.01	0.012	0.02

Table B.1 Contd.

STACKING GEL							
Components	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	10 ml
Water	0.68	1.40	2.10	2.70	3.40	4.10	6.80
30% Acrylamide	0.17	0.33	0.50	0.67	0.83	1.00	1.70
1.0 M Tris (pH 6.8)	0.13	0.25	0.38	0.50	0.63	0.75	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.10
10% APS	0.01	0.02	0.03	0.04	0.05	0.06	0.10
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.01

B.5 Gel Staining

For visualisation of proteins after separation on gel, one could use different stains such as Coomassie blue stain or more sensitive silver staining. The Coomassie blue staining is relatively less sensitive than silver staining, but is highly convenient to use.

B.5.1 Coomassie Blue Staining

The dye Coomassie Brilliant Blue R250 nonspecifically binds to all the protein. The gel is soaked in the dye for it to seep in and bind to the proteins. The gel is then destained to remove the unbound dye. The dye binds to the protein and not the gel, and hence the protein bands can be visually seen. The binding of the dye to the protein is approximately in stoichiometry, so the relative amounts of protein can be determined by densitometry. For most SDS and native gels, separated proteins can be simultaneously fixed and stained in the same solution.

Requirements

(1) Staining Solution: Dissolve 0.25 g of Coomassie Brilliant blue R250 in 125 ml methanol, before adding 25 ml of acetic acid and 100 ml of water
(2) Destaining Solution: Mix 400 ml of Methanol and 70 ml of acetic acid, finally bringing the volume to 1 litre with water

Protocol 6:

(1) Transfer the gel gently into a tray containing staining solution.
(2) Shake the gel for 1 hr on a rocking shaker. Have a rapid staining microwave on high for 1 min and shake it for 10–15 min.
(3) Remove the staining solution slowly and wash the gel with water.
(4) Add the destaining solution and shake it slowly. For rapid destaining, microwave on high for 1 min and add some Kim wipes or folded paper towel or cotton.
(5) Shake it till it destains. One could also replace if the staining solution is too coloured.

B.5.2 Silver Staining

In silver staining, the gel is Impregnated with soluble silver ions and developed by treatment with formaldehyde, which reduces silver ions to form an insoluble brown precipitate of metallic silver. This reduction is promoted by protein.

Requirements

(1) Fixative (200 ml): Mix 100 ml of methanol with 100 ml of water and 100 µl of formaldehyde. Formaldehyde is added freshly
(2) Dithiothreitol (DTT) (1 mg/ml): Dissolve 10 mg of DTT in 10 ml of water. Store as 1 ml aliquots at −20°C
(3) 20% Silver Nitrate (25 ml): Dissolve 5 g of silver nitrate in 25 ml of water. Store in an amber-colored bottle at 4°C
(4) 4% Sodium Carbonate, freshly prepared (500 ml): 20 g of sodium carbonate dissolved in 500 ml of water. Add 400 µl of formaldehyde to it
(5) 2.5 M Citric acid (25 ml): Dissolve 13.13 g of citric acid in 25 ml of water
(6) Gel tray
(7) Gel shaker

Protocol 7:

(1) Transfer the gel to a tray containing fixative solution.
(2) Keep it covered in a shaker for 2 hr under cover.
(3) The size of the gel shrinks, indicating proper fixation.
(4) Rinse the gel once with distilled water.
(5) Add 200 ml of water and two aliquots of DTT to get a final concentration of 10 μg/ml DTT.
(6) Incubate the gel by shaking 30–45 min. This swells the gel back to its original size.
(7) Rinse once with 200 ml of water.
(8) Add 200 ml of water and 1.5 ml of 20% silver nitrate.
(9) Incubate by shaking for 30–45 min.
(10) Wash thrice with 200 ml of water to completely remove the silver nitrate.
(11) Pre-rinse with 4% sodium carbonate containing formaldehyde.
(12) Allow the gel to develop with a fresh solution of sodium carbonate.
(13) Stop the reaction with 2.5M citric acid after the development of bands.
(14) Wash the gel finally in water.

Precautions

- Water used should be deionized water only
- Do not touch the gel with bare hands. Use gloves.
- All solutions should be at RT before use.

B.6 Western Blotting

Western Blotting or immunoblotting allows determining the presence of a specific protein in a sample after separation on SDS-PAGE. The term Western blotting is used after a similar term 'Southern blotting', which was invented by and named after E. M. Southern. That method allows for detection of nucleic acids in a blot technique. The proteins are separated on SDS-PAGE and then transferred to a membrane (generally nitrocellulose or PVDF). The membrane is incubated with a source of non-specific

protein (such as milk proteins) to bind to any remaining sticky places on the membrane. A primary antibody is then added to the solution which is able to bind to its specific target protein followed by washes and incubation in a solution of secondary antibody.

The secondary antibody recognizes the primary antibody and binds at locations on the blot where the primary antibody is bound as well. The secondary antibody is furthermore conjugated with an enzyme or marked with a nucleotide, thus allowing detection (Fig. B.4).

Requirements

(1) Transfer buffer (1 litre): Dissolve 5.82 g of Tris base, 2.93 g of Glycine, 3.75 ml of 10% SDS in 800 ml water. Make it to 1 litre with 200 ml of Methanol
(2) TBST (1 litre): Dissolve 8.8 g of Tris base, 1.2 g of Sodium Chloride and 500 µl of Tween 20 in 750 ml of water. Adjust the pH of the solution to 7.5 and finally bring the volume to 1 litre
(3) Blocking solution: 5 g of fat free milk in 100 ml of TBST. (Carnation milk works well)
(4) Stripping buffer (1 litre): To 125 ml of 0.5M Tris (pH 6.8) buffer add 100 ml of 10% SDS and 8 ml of 2-Mercaptoethanol. Bring the volume to 1 litre with water

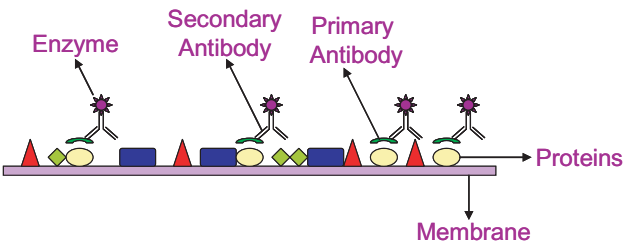

Fig. B.4 Reaction occurring on the membrane. The membrane has the proteins after transfer which is tightly adhered to the membrane. The primary antibody bins to the speci c protein followed by the secondary which has an enzyme linked whose reaction can be visualized.

Protocol 8:

(1) After the gel run, transfer into the transfer buffer.
(2) Soak the filter paper in the transfer buffer and lay flat.
(3) Soak the nitrocellulose membrane and lay it on top of the filter paper.
(4) Place the gel on top of the nitrocellulose membrane.
(5) Layer the gel again with a filer paper soaked with transfer buffer (Fig. B.5).
(6) Roll a pipette so as to remove trapped air bubbles.
(7) Transfer the sandwich directly to a transfer apparatus in case of semi-dry apparatus, or to a cast in case of wet transfer.
(8) Place the gels with the membrane on the anode or positive electrode (usually Red) and the gel on the cathode or negative electrode (usually Black).
(9) Cover the apparatus and transfer as per the specification of the instrument as stated in the manufacturer's instructions.
(10) After the transfer, mark the blot and then wash with TBST buffer.
(11) Block the membrane in blocking solution for at least 1 hr at RT or overnight at 4°C.
(12) After blocking, incubate the blot with primary antibody at an appropriate dilution in 10 ml of blocking solution.

(Continued)

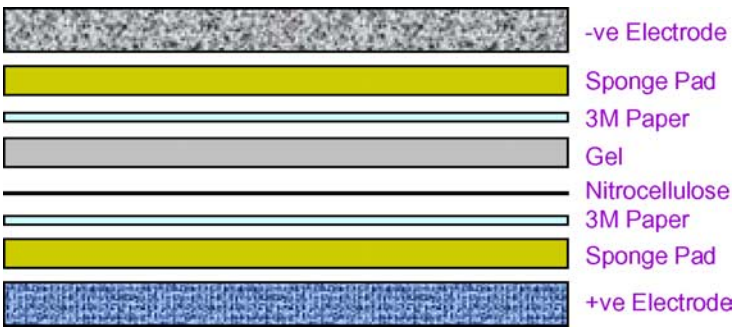

Fig. B.5 Cartoon depiction of the Western Blot sandwich. The gel is placed with the membrane between a set of sponge pad and 3M papers on either side and the transfer is done with the membrane on the negative electrode.

(Continued)

(13) Incubate on rocking, shake for at least 2 hr at RT or overnight at 4°C.
(14) Wash the blot 3 times for 5 min each with TBST.
(15) Add 15 ml of blocking solution with appropriate amount of secondary antibody (Either Alkaline phosphatase or HRP conjugated antibody).
(16) Incubate the blot on the rocker for 2 hr at RT.
(17) Wash the blot 3 times for 5 min each with TBST.
(18) Develop the blot with the respective developer depending on the secondary antibody.

Blot development

When using Alkaline Phosphatase based secondary antibody:

- Add the commercially available substrate solution containing 5-Bromo-4-chloro-3-indolyl Phosphate (BCIP)/Nitroblue Tetrazolium (NBT) (SIGMA) to the blot till it is just submerged.
- Incubate the blot in the substrate solution for 10–30 minutes until the development of bluish purple color.
- Stop the reaction by washing the strips in several changes of water.
- Air-dry the strip and photograph or scan it for records.

When using the HRP conjugated secondary antibody:

- Just before developing, prepare the substrate solution by mixing equal parts of reagents 1 and 2 (Commercially available like Pierce ECL Western Blotting Substrate).
- Incubate the blot with gentle shaking in the substrate for 1 min at RT.
- Using a forceps, lift the blot from the substrate and drain off the excess solution by placing the tip of the blot on a filter paper.
- Wrap the blot in a saran wrap.
- Place the blot in a film cassette.

- Take the cassette to the developing room without lights except for the safelight, place an X-ray film on the top of the blot.
- Expose the film to the blot for various time points.
- Develop the film.

Reprobing blot

- Wash the blot once with TBST.
- Add 10 ml of stripping buffer to the blot in an air tight container.
- Strip the blot at 50°C for 30 min.
- Wash the blot till no smell of 2-Mercaptoethanol can be detected.
- Block the blot and use for reprobing.

B.7 Immunoprecipitations

Immunoprecipitation involves pulling down or separation of a protein of interest using a specific antibody against the protein. The immuno complex is subsequently pulled down from solution by using Protein G or Protein A which has a high affinity for antibodies. The pull down products are subsequently washed and subjected to SDS-PAGE analysis or other assays. Using this technique, one can pull down a protein (or protein complex) from whole cell lysates, culture supernatants or tissues lysates. This technique can be applied to check for biochemical characteristics, such as post-translational modifications (phosphorylation or glycosylation), expression levels or interaction with other proteins (which may or may not be pulled down in a complex). In some cases, instead of analyzing the proteins on SDS-PAGE, they could be used for the enzymatic assays. Depending on the source of the primary antibody used, the choice of Protein A or Protein G is according to the species in which the primary antibody was raised.

Requirements

(1) Protein A-Sepharose CL-4B or GammaBind™ sepharose beads

(Continued)

Table B.2 List of Immunoglobulin with their binding ability to Protein A or Protein G

Immunoglobulin		Protein A	Protein G
Mouse	IgG1	+	+ +
	IgG2a	+ + +	+ + +
	IgG2b	+ +	+ +
	IgG3	+	+ + +
Rat	IgG1	+	+
	IgG2a	–	+ + +
	IgG2b	–	+ +
	IgG2c	+	+ +
Human	IgG1	+ + +	+ + +
	IgG2	+ + +	+ + +
	IgG3	–	+ + +
	IgG4	+ + +	+ + +

(Continued)

(2) RIPA buffer (see Appendix for composition)
(3) Phosphate buffered saline (see Appendix for composition)
(4) Rotary Shaker
(5) Refrigerated Microfuge

Protocol 9:

(1) Transfer equal amounts of proteins to be immunoprecipitated into a fresh eppendorf tube.
(2) Add protein A-Sepharose CL-4B or GammaBind™ sepharose beads for 1 hr at 4°C for clarification.
(3) The sepharose beads were removed by brief centrifugation.
(4) Transfer the supernatant into a different tube.
(5) Incubate with the primary antibody for 2 hr at 4°C.
(6) Immunoprecipitation of the antibody-antigen complexes is performed by incubation at 4°C overnight with proteinA-Sepharose or Gammabind™ sepharose.

(Continued)

(Continued)

(7) Nonspecific interacting proteins are removed by washing the beads thrice with modifiedradioimmune precipitation assay buffer (RIPA) and once with phosphate-buffered saline.
(8) Immune complexes are solubilised in 50 μl of 2X Laem mli buffer, boiled and subjected to SDS-polyacrylamide gel electrophoresis or washed once with the enzyme assay buffer and used for enzymatic assays.

Notes

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C

Lipid Analysis

C.1 Lipid Extraction

Lipids play an important role in various cellular and physiological functions. Biochemical analysis of lipids requires their isolation. Depending on the type of the sample, extraction protocols vary considering the tissue structure, texture and lipid contents. The high sensitivity of some analytical methods employed to measure lipids requires the use of very pure solvents and clean glassware. Furthermore, all lipids must be protected against degradation through oxidation by solvent, oxygen, and enzymes in combination with temperature and light. Lipids can be broadly classified as polar and non polar lipids based on the head group present.

Method	Lipid Class Extracted
Bligh Dyers	Acidic Phospholipids
Folch Method	Phospholipids
Grey Method	Phosphoinositides
Alex Brown	Phosphoinositides
Hexane Extraction	Neutral Lipids like MAG, DAG and TAG
Glycolipid extraction	Sphingolipids

C.1.1 Modified Bligh and Dyers Method for Phospholipid Extraction

This method is particularly suitable for lipid extraction of incubation medium, tissue homogenates or cell suspensions.

Requirements

(1) Chloroform : Methanol (1:2, v/v)
(2) Chloroform
(3) 1M HCl
(4) Chloroform : Methanol (1:1, v/v)

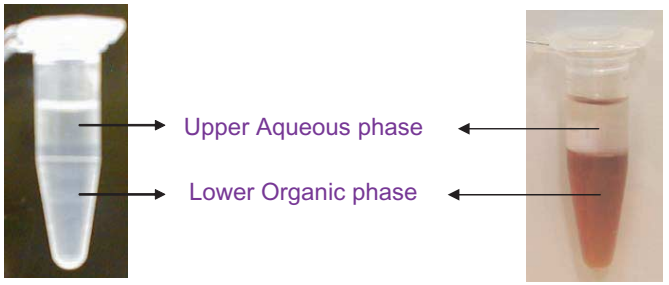

Fig. C.1 Phase separation during lipid extraction.

Protocol 1:

(1) Transfer 50 µl of sample into a 1.5 ml eppendorf tube.
(2) Add 600 µl of Chloroform : Methanol (1:2, v/v).
(3) Vortex the suspension for 1 min.
(4) Incubate on ice for 10 min.
(5) Add 300 µl of Chloroform.
(6) Add 200 µl of 1M HCl.
(7) Vortex hard for 2 min.
(8) Spin down at max speed for 2 min.
(9) Transfer the bottom organic layer into a fresh tube (Fig. C.1).
(10) Re-extract the aqueous phase with 0.6 ml of Chloroform.
(11) Dry the sample in a speed vac (takes about 45 min).
(12) Store the lipids in the freezer at −80°C.

C.1.2 Folch Extraction

Requirements

(1) Chloroform : Methanol (2:1, v/v)
(2) 0.9% NaCl in water
(3) Homogeniser
(4) Lyophiliser

Protocol 2:

(1) The tissue is homogenised with Chloroform : Methanol (2:1, v/v) to a final volume 20 times the volume of the tissue sample (1 g in 20 ml of solvent mixture).
(2) Agitate the mixture for 15 min in an orbital shaker at RT.
(3) Centrifuge at 14,000 rpm for 10 min to get the clarified supernatant.
(4) Measure the volume of the supernatant and transfer to a fresh tube.
(5) Break phase by adding 0.2 volumes of 0.9% NaCl.
(6) Vortex the tube hard for 1 min.
(7) Centrifuge at a low speed of 2000 rpm to separate the two phases.
(8) Transfer the upper aqueous phase for analysis of gangliosides and polar molecules.
(9) Collect the lower organic phase containing lipids in a fresh tube.
(10) Evaporate in a lyophilizer or under a nitrogen stream and store below −80°.

C.1.3 Greys Method for Phosphatidylinositol Phosphate Extraction

Requirements

Prechilled

(1) PBS
(2) 0.5M Trichloroacetic acid (TCA)
(3) 5% TCA/1mM EDTA
(4) Chloroform
(5) 0.1M HCl

Room temperature

(1) Chloroform : Methanol (1:2, v/v)
(2) Chloroform : Methanol: HCl (20:40:1, v/v)

Protocol 3:

(1) Grow tissue cultures in appropriate media in 10 cm cell culture dishes until ~ 60–80% confluent for adherent cells or suspension cells.
(2) For adherent cells, keep the culture dish on a bed of ice during cell harvest and aspirate media from dish. For suspension cells, spin the cells at 1700 rpm for 5 min.
(3) Wash the cells with 10 ml cold PBS.
(4) Repeat the wash again with 10 ml cold PBS.
(5) To the adherent cells, add 0.6 ml cold 0.5M TCA and immediately scrape cells loose from dish. For suspension cells, suspend them in 0.6 ml cold 0.5M TCA.
(6) Transfer the suspension to a microfuge tube.
(7) Vortex tubes for 10s and then store in ice till other samples are processed.
(8) Incubate all samples for a minimum of 5 min in ice bath.
(9) Microfuge for 2 min at 14,000 rpm at 4°C. Decant supernatant to waste.
(10) Resuspend pellet in 1.0 ml cold 5% TCA/1mM EDTA.
(11) Microfuge for 2 min at 14,000 rpm at 4°C. Decant supernatant to waste.
(12) Repeat wash one more time.
(13) Resuspend pellet in 1.0 ml RT Chloroform : Methanol 1:2, v/v.
(14) Incubate 10 min at RT, vortexing every 3 min.
(15) Microfuge for 2 min at 14,000 rpm at RT. Decant supernatant to waste.
(16) Repeat neutral extraction one more time.
(17) Resuspend pellet in 0.75 ml Chloroform : Methanol : HCl 40:80:1, v/v.
(18) Incubate 15 min at RT, vortexing every 5 min.
(19) Transfer sample tube back to ice bath.
(20) Add 0.25 ml cold Chloroform and 0.45 ml cold 0.1M HCl.
(21) Vortex for 1 min, then return to ice bath.
(22) Microfuge for 2 min at 9000 rpm.
(23) Transfer organic layer to a clean microfuge tube.
(24) Dry organic layer under a stream of N_2 gas or in a lyophiliser.
(25) Store at −80°C.

C.1.4 Modified Alex Browns Method for Phosphatidylinositol Phosphate Extraction

Requirements

(1) Chloroform : Methanol (1:1, v/v). (prechilled in ice)
(2) Chloroform : Methanol (2:1, v/v) 0.25% 12N HCl. To 1 ml of Chloroform : Methanol (2:1, v/v) add 2.5 µl of conc. HCl
(3) 1N HCl
(4) Chloroform : Methanol: Water (1:1:0.3, v/v)

Protocol 4:

(1) To 50 µl of sample, add 400 µl of ice cold Chloroform : Methanol (1:1, v/v).
(2) Vortex the mixture for about 1 min.
(3) Centrifuge at 9000 rpm for 5 min at 4°C.
(4) Decant the supernatant and discard it.
(5) Add 200 µl of Chloroform to the pellet : Methanol (2:1, v/v) containing 0.25% 12N HCl.
(6) Vortex the sample for 5 min.
(7) Pulse spin the samples.
(8) Transfer the supernatant to a fresh tube.
(9) Add 40 µl of 1N HCl and vortex for 15s.
(10) Centrifuge at 14000 rpm for 2 min.
(11) Collect the lower organic layer in a fresh tube and dry.
(12) Store at −80°C.

C.1.5 Hexane Extraction for Neutral Lipids

Requirements

(1) 0.3% NaCl
(2) Methanol
(3) Hexane

Protocol 5:

(1) Add 5 ml of Methanol/ 0.3%NaCl (10:1) to the cell pellet.
(2) Vortex to make a cell suspension.
(3) Form an emulsion by shaking at 250 rpm for 1 hr at RT.
(4) Add 2.5 ml of Hexane.
(5) Emulsify again by shaking for 1 hr at 250 rpm RT.
(6) Centrifuge at 4000 rpm for 10 min to separate the phases.
(7) Transfer the upper phase into a fresh glass vial.
(8) Repeat steps 4 to 6 to re-extract the lipids.
(9) Combine both upper phases in glass vial.
(10) Dry and store the lipids at −80°C.

C.1.6 Glycolipid Extraction

Requirements

(1) Methanol
(2) Chloroform
(3) 1M KOH in methanol
(4) Glacial acetic acid

Protocol 6:

(1) Place 50 µl of sample or cells in a 2 ml eppendorf or 13×100 mm screw-capped, borosilicate glass test tubes with Teflon caps.
(2) Add 0.5 ml of methanol and then 0.25 ml of chloroform and the internal standards.
(3) Sonicate the test tubes in a bath-type sonicator until they appear evenly dispersed; incubate overnight at 48°C in a heating block. This heating step can often be shortened, but it is conventional in the extraction of sphingolipids because they have high phase transition temperatures.

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(4) Cool the tubes and add 75 μl of 1M KOH in methanol, sonicate and incubate for 2 hr at 37°C. This step removes most of the interfering glycerolipids, in particular phosphatidylcholines, that can mask sphingomyelins in a simple Mass spectrometry scan.
(5) Cool the samples to RT and transfer the content to 15 ml falcon tubes.
(6) Add 6 μl of glacial acetic acid to bring the pH near neutral.
(7) Add 2 ml of chloroform and 4 ml of water, mix (vortex) and centrifuge to separate the phases.
(8) Carefully transfer the organic layer to an eppendorf and evaporate the solvent using a Speed Vac-type concentrator or under a stream of N_2 gas.
(9) The extracts are kept refrigerated and should be analysed as soon as possible to minimise possible changes in composition.

C.2 Thin Layer Chromatography

Thin layer chromatography (TLC) consists of a thin layer adsorbent such as silica gel, alumina or cellulose on a flat carrier like a glass plate, a thick aluminum foil, or a plastic sheet. This layer of adsorbent acts as a stationary phase. This method is widely used in lipid analysis or is the standard method in organic chemistry for qualitative analysis of organic reactions. The adsorbent-like silica has hydroxyl groups which act as interacting groups. The sample partitions between the mobile and the stationary phase. Individual components in the sample will interact with the stationary phase based on charge, solubility and adsorption. The components could then be visualised by iodine vapors or fluorescent dyes. The retention factor or the Rf value is a characteristic of the substance. This is a constant for a particular substance for that specific solvent and plate system. The Rf value is the ratio of the distance moved by the compound to that moved by the solvent.

Requirements

(1) TLC plate
(2) Mobile phase solvent
(3) Glass jar

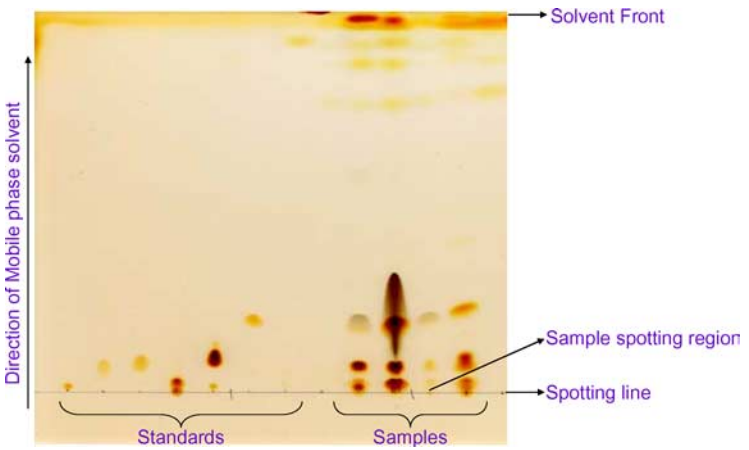

Fig. C.2 Thin Layer Chromatography. An example of a lipid containing sample developed and stained with Iodine vapors.

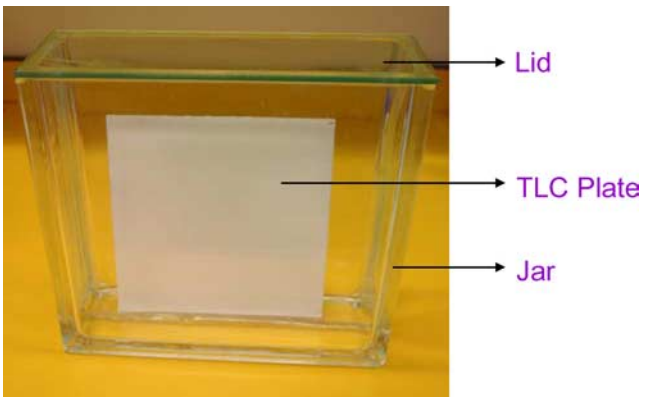

Fig. C.3 Thin Layer chromatography chamber and TLC plate.

Protocol 7:

(1) Bake the TLC plate in an oven set at 100°C for 1 hr before use.
(2) Pour the appropriate developing solvent into a glass jar at least one hr before use. This is to saturate the jar with the running solvent vapors.
(3) Mark the TLC plate with pencil as shown in the Fig. C.2.
(4) Spot the sample and the respective standards onto the plate.
(5) Dip the plate in the running solvent just below the sample load.
(6) Allow the solvent to run due to capillary action till it reaches nearly the end of the plate (Fig. C.3).
(7) Remove the plate from the jar and let it dry.
(8) Stain the plate for visualisation of the compound.
(9) Measure the distance of the solvent and the compound travelled to obtain the Rf values.

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D

Mammalian Cell Culture

D.1 Primary Culture

D.1.1 Primary Cell Isolation and Culturing

Requirements

(1) Sterile Petridish
(2) Sterile balanced salt solution
(3) 0.25% Trypsin in PBS
(4) Growth media

Protocol 1:

(1) Transfer the organ of interest into a sterile petridish.
(2) mince or cut the tissue into small pieces of about 2–5 millimeter size using a sterile scissors or scalpels.
(3) Transfer the tissue to a sterile balanced salt solution on ice.
(4) Add appropriate amount of 0.25% Trypsin in PBS and incubate at 4°C for 12–16 hr for complete penetration of the trypsin.
(5) Decant the trypsin slowly without disturbing the tissue. Leave a small amount of trypsin behind.
(6) Incubate with the residual trypsin for 30 min at 37°C.
(7) Add media prewarmed to 37°C.
(8) Gently disperse the tissue so as to dislodge the cells.
(9) filter the cell suspension through a sterile stainless steel mesh.
(10) Wash the cells 3 times with the same buffer.
(11) Resuspend the cells in medium or buffer.
(12) Count the number of cells.
(13) Quantitate cell yield and viability.
(14) Seed the culture cell if required.

Note: Instead of trypsin, one could also use other enzyme or combination of enzymes such as Collagenase (100 units/ml) or Dispase (2 units/ml).

D.1.2 Splenocyte isolation

The spleen serves as a rich source of lymphocytes. The spleen is the source used most often as it is easily obtainable. Lymph nodes and peripheral blood are also occasionally used as lymphocyte sources. In humans, peripheral blood is often the only source used, unless the spleen or some lymph nodes were being surgically removed (and thus available).

Requirements

(1) 0.17M Ammonium Chloride: Dissolve 0.45 g of Ammonium Chloride in 50 ml of water
(2) Phosphate Buffered Saline pH 7.4 (See Appendix for composition)
(3) Surgical instruments
(4) 70% Alcohol
(5) Syringe plunger

Protocol 2A. Spleen removal:

(1) Set up a petri dish containing about 5–10 ml of cold, sterile PBS before removing the spleen from the mouse.
(2) Place the dead or the anaesthetised mouse on its back or right side on a clean surface. Wipe the abdomen and left side thoroughly with 70% alcohol to clean it off and to wet the fur — this is not going to sterilise the animal's surface, but as long as the spleen does not touch the surface, it will not result in contamination.
(3) Using the set of sterile forceps and scissors, cut a slit into the abdomen. Enlarge the slit with the scissors.
(4) Using the second set of sterile forceps, locate the spleen by gently pushing the stomach to the animal's right side. The spleen will appear as a dark red, thin, flat organ about one inch in length by one-quarter inch wide.

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(5) Hold the spleen with the sterile forceps and cut it free using the sterile scissors.
(6) Place the spleen in a sterile, glass petri dish containing a few ml of cold, sterile PBS.
(7) Trim away and dispose of excess fatty or connective tissue.

2B. Spleen cell dissociation

(1) Cut the spleen in to small pieces using a sterile scissors.
(2) Using sterile forceps, place the spleen onto the fine sterile wire mesh.
(3) Add a few drops of cold, sterile PBS to the spleen on the wire mesh to keep it moist.
(4) Open a sterile 5cc syringe. Remove the plunger and press firmly on the spleen with the end of the plunger portion of the syringe to force the spleen to break apart and the cells to pass through the wire mesh.
(5) Gentle ''grinding'' of the spleen across the mesh with the plunger will help to hasten the breaking apart of the tissue.
(6) Collect the mesh flow through harboring the cells and transfer it into a fresh sterile falcon tube.
(7) Centrifuge the cell suspension (200 × g) for 5 min.
(8) Discard the supernatant.
(9) Suspend the cells in 5 ml of 0.17M ammonium chloride solution.
(10) Keep the tubes at RT for 5 min, allowing the RBC's to lyse.
(11) Spin the tubes at 1700 rpm for 5 min.
(12) Wash the cells with 10 ml of PBS buffer.
(13) Re-suspend in 1ml of PBS.
(14) Count the number of cells and use it for culturing or for other assays.

D.1.3 Isolation of Peripheral Blood Lymphocytes

Requirements

(1) RPMI 1640 media
(2) Ficoll Hypaque ($d = 1.070$)
(3) Fetal Bovine Serum (FBS)
(4) Penicillin/Streptomycin (PS)

Protocol 3:

(1) Collect blood in sterile heparin coated vacutainers.
(2) Keep the tubes on a rocker for about 15 min.
(3) Dilute 100 ml blood with 100ml of RPMI or PBS.
(4) Take 20 ml of ficol (Pharmacia) in a 50 ml Falcon tube (5 tubes).
(5) Lay 20 ml of the diluted blood over Ficol.
(6) Spin at 3000 rpm for 25 min. at RT.
(7) Collect the lymphocyte layer carefully, which is a white layer as shown in Fig. D.1.
(8) Add 3 volumes of RPMI w/o serum to this cell suspension.
(9) Spin at 1700 rpm for 7 min to remove RBC.

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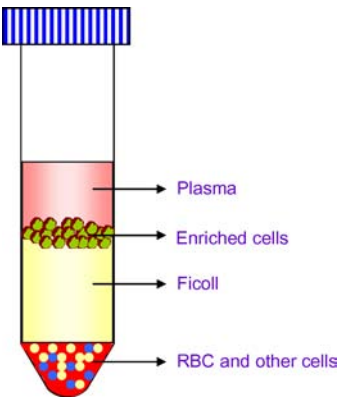

Fig. D.1 Ficol gradient for enrichment of peripheral blood lymphocytes.

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(10) Repeat the wash twice again with RPMI.
(11) Suspend in 50 ml of RPMI +10% FBS +1% PS.
(12) Count the number of cells and adjust the concentration of cell to 1×10^6 cells/ml.
(13) Transfer the cells to a tissue culture flask.
(14) Incubate the flask at 37°C overnight.
(15) Change another flask the next day.
(16) Analyse by flow cytometry and use.

D.2 Tissue Culture

Many lines of investigations in the life sciences are incomplete without the use of cell lines. Maintenance of cells is a very delicate process and requires careful monitoring and handling. This is done in a sterile environment such as the hood (Fig. D.2). Sterile growth media containing pH sensitive dyes are supplemented with serum. Serum is a source of essential ingredients such as hormones, growth factors etc. The growth medium also contains antibiotics in order to avoid contamination due to unwanted microbial activity. Cells are typically cultured in the presence of a controlled amount of carbon dioxide and oxygen, usually 5% and 95%, respectively in a specialised incubator (Fig. D.3). This is also a means of buffering the medium at physiological pH values. The base media composition depends on the type of cells used. Based on their growth characteristics, cells can be classified as 'adherent' or 'suspension cells'. Adherent cells attach in monolayers to the culture vessel which acts as a substratum. Adherent cells need to be uplifted using a usual trypsinisation protocol for passaging.

D.2.1 Adherent Cells

Cell release procedure

The cells which form a monolayer on the dish or ask need to be released from the substratum into suspension in order to be

Fig. D.2 Tissue culture hood.

Fig. D.3 CO_2 incubator.

diluted and transferred to another culture vessel. This is accomplished by using purified trypsin.

Requirements

(1) Sterile PBS
(2) Trypsin Solution
(3) Culture media with the components

Protocol 4:

(1) Aspirate the media from the culture dish.
(2) Add sterile PBS and pre-warmed to 37°C till the cells are covered up.
(3) Swirl the PBS gently so as to rinse the cells.
(4) Add minimal amount of Trypsin solution.
(5) Incubate the plate at 37°C or at RT for several min.
(6) Tap the plates to dislodge the cells completely.
(7) Observe the plate under microscope to see if all the cells are dislodged. If not, incubate for a longer time.
(8) Add media and swirl to mix. The media has trypsin inhibitors which will stop the action of trypsin.
(9) Count the number of cells.
(10) Transfer a required amount of cells to a fresh culture flask of dish.
(11) Replenish the cells with a fresh media.
(12) Transfer the vessels to the CO_2 incubator.

D.2.2 Suspension Cells

Requirements

(1) Media with serum

Protocol 5:

(1) The cell density of suspension cells in culture should be maintained between 1×10^5 cells/ml to 2×10^6 cells/ml.
(2) To subculture cells, resuspend the cells by pipetting the cells up and down to break down cell clusters. Count the cells using a hemocytometer.
(3) Start a new culture with fresh media and seed cells such that the seeding density is not lower than 1×10^5 cells/ml. Cells can be centrifuged and subsequently resuspended at 1×10^5 cells/ml.

D.2.3 Cell Maintenance

Cells are stored in liquid nitrogen for longer shelf life. The freezing and thawing protocols are as follows:

Making Frozen Stocks

Requirements

(1) Freezing media: Fetal Bovine Serum containing 10% DMSO
(2) Cryovial
(3) Cryo-cooling chamber
(4) Liquid Nitrogen storage tank

Protocol 6:

(1) For making frozen stocks, the cells should be counted using a hemocytometer.
(2) The cells are resuspended in freezing media, 3×10^6 cells in 1 ml of freezing media
(3) Transfer the cells immediately into a labelled cryovial and place into the cryo-cooling chamber.

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(4) Place the cryo-cooling chamber overnight at −80°C.
(5) The vials are then transferred into freezer boxes and stored in the Liquid Nitrogen storage tank the next day.

For thawing cells from a frozen vial

Requirements

(1) 37°C water bath
(2) Pre-warmed growth media
(3) Tissue culture flask

Protocol 7:

(1) Thaw the cells rapidly (1 to 2 minutes) by incubating the cryovial in a 37°C water bath.
(2) The cells are immediately resuspended into pre-warmed growth media such that the cell density is around 2×10^6 cells/ml.

D.3 Cell Count

A hemocytometer can be used to estimate exactly the number of cells in a suspension. The hemocytometer consists of two chambers, each of which is divided into nine 1.0 mm squares (Fig. D.4). A cover glass is supported 0.1 mm over these squares such that the total volume over each square is 1.0 mm × 0.1 mm or 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is approximately equivalent to 1 ml, the cell concentration per ml will be the average count per square $\times 10^4$.

The Trypan blue stain can be used to stain the dead cell, but is excluded from the live cells. This gives a good estimate of the viable cells. The reactivity of trypan blue is based on the fact that

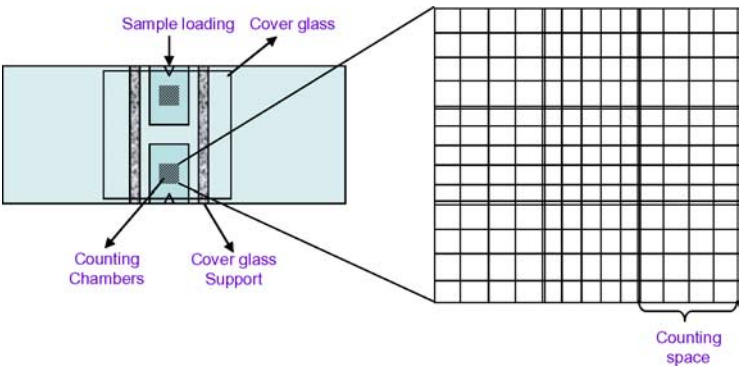

Fig. D.4 Cartoon depiction of a Hemocytometer.

the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged.

Requirements

(1) PBS
(2) 0.4% Trypan Blue
(3) Hemocytometer
(4) Microscope

Protocol 8:

(1) Dilute 0.2 ml of Trypan blue stain with 0.8 ml of PBS. This diluted stain is stable.
(2) Make a proper cell suspension by agitation of the cells.
(3) Transfer 20 µl of the cell suspension into an eppendorf tube.
(4) Add 20 µl of diluted trypan blue stain. Mix by vortexing.
(5) Place a cover glass over the hemocytometer chamber.
(6) Load 20 µl of the stained cells through the sample loading area by capillary action (Fig. D.4).

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(7) Using a light microscope, count the number of cells which have excluded the dye.
(8) Calculate the number of viable lymphocytes/ml using the following equation:

$$\#\ \text{lymphocytes/ml} = \frac{\text{total cells counted} \times 2 \times 10^4}{\#\ \text{of squares counted}}$$

D.4 Mammalian Transfection

Transfection is the process of introducing DNA or RNA into eukaryotic cells. The use of transfection is to study the role and regulation of proteins or to understand the mechanisms of a pathway. Transfection can be 'transient' for rapid analysis or 'stable', mostly for induction of expression. There are various methods of transfection which include electroporation, viral vectors, DEAE-Dextran, calcium phosphate or Lipofectamine. The choice of transfection depends on the cell type used. The most desirable technique is the one which gives high efficiency of nucleic acid transfection with less interference to the cells physiology and high reproducibility.

D.5 Calcium Phosphate Co-Precipitation

Requirements (all sterile)

(1) 2M NaCl
(2) 0.5M HEPES, pH 7.10
(3) 2M $CaCl_2$
(4) 1M $NaPO_4$, pH 7.0

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(5) 2X HEPES buffered saline:
7.75 ml H_2O
1.25 ml 2M NaCl
1.0 ml 0.5M HEPES
15 µl 1M $NaPO_4$
Adjust the pH to 7.07–7.12 with NaOH, filter sterilise
(6) Cell culture medium

Protocol 9:

(1) Plate the cells 24 hr prior to transfection in a 35 mm dish.
(2) Dilute 4 to 5 µg of DNA into 0.1 ml of 250 mM $CaCl_2$. Carrier DNA such as salmon sperm or empty plasmid can be used to make up the amount if the DNA is less than 4 to 5 µg.
(3) Add the DNA dropwise to 0.1 ml of 2X HEPES, while bubbling air into the solution with a pipette tip.
(4) The solution should turn cloudy indicating that a precipitate is forming. Let the mixture stand for 20–30 minutes, and then add drop-wise to cells.
(5) After 12–20 hr, remove the DNA and medium and add fresh medium.
(6) Harvest 24–36 hr after changing the medium.

D.6 Electroporation Protocol

Requirements

(1) Serum free media
(2) Electroporator

Protocol 10:

(1) Trypsinise cells for adherent cells or directly use suspension cells and transfer to 15 ml conical tube.
(2) Determine the total number of cells.
(3) Pellet cells in by centrifugation at 1200 pm for 5–7 min.
(4) Resuspend the cells in medium with no serum at a concentration of 2×10^6 cells/ml. Chill on ice.
(5) Set the electroporator to 500 V/capacitance and resistance low voltage, set the capacitance to 950 μF, set the resistance to R10, and the recommended voltage is 250 V.
(6) Into a chilled electroporator cuvette, pipette approximately 10 μg of DNA and 0.450 ml of chilled cells.
(7) Insert into chamber and pulse.
(8) Remove and plate cells into warm (37°C) medium with the required composition for growth including serum.
(9) Wait 48–72 hr before assaying.

D.7 Lipofectamine Transfection Protocol

Requirements

(1) Serum free medium
(2) Lipofectamine
(3) Plasmid to be transfected

Protocol 11:

(1) Plate the cells as for transfection ($2–4 \times 10^4$ cells/cm^2) the day before.
(2) For each transfection, dilute 1 to 2 ug of DNA in 100 μl of serum-free medium. In a separate tube, dilute 8 μl of lipofectamine in 100 μl of serum-free medium.

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(3) Combine the two solutions with gentle mixing and incubate at RT for 15–45 minutes.
(4) Rinse the cells once with 2 ml of serum-free medium.
(5) Add 0.8 ml of serum-free medium to the tube of DNA and lipofectamine. Mix and overlay the mixture onto the rinsed cells. Do not use antibacterial agents to the media during transfection.
(6) Incubate for 6–10 hr, then add 1 ml of complete medium.
(7) Change medium again 18–24 hr after the start of transfection.

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E

Microscopy

The magnification of small things is a necessary facet of biological research. The most basic microscope is the light microscope which employs visible light to amplify and detect small objects (Fig. E.1). Other specialised microscopes which are used are fluorescence and confocal and electron microscopes. In cell biology and biochemistry, one uses a microscope especially to check cells from tissue culture, tissue sections, as well as subcellular fractions.

During fluorescence, the emitted light is different from the incident light. A fluorescent molecule absorbs light of high energy while emitting light at a higher wavelength, thus reducing energy (Fig. E.2). Most of these molecules absorb light in the UV or the

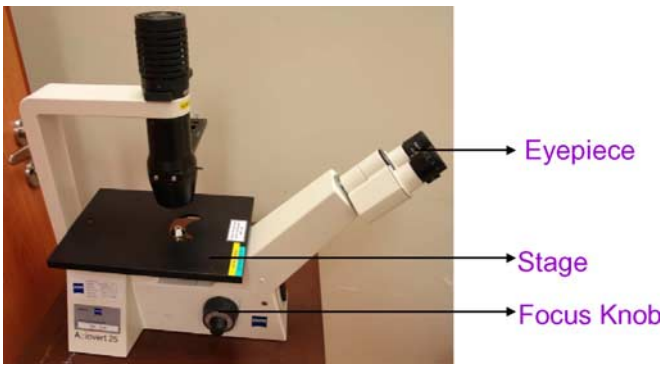

Fig. E.1 A typical light microscope.

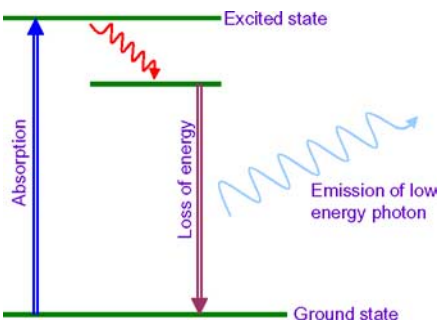

Fig. E.2 Principle of fluorescence emission. The molecules absorb the light and reach an excited state. It losses some amount of energy to go a lower state of excitation. In order to reach the ground state again it releases photons. This photon is of lower energy level and there is a shift in wavelength. In most cases from UV to visible.

blue region of the light spectrum and emit in the visible spectrum. Fluorescein is a commonly used dye which emits green light when hit with blue excitation light. Some of the other known molecules include Texas Red, Malachite green, Cy2, Cy3 and Cy5. These dye molecules can also be coupled to other molecules (e.g. proteins or lipids) of interest for localisation studies.

A confocal microscope is a high end fluorescent microscope. It is able to give a pin point image. Thus, it is like getting a sliced picture of the object. By scanning different sections, one could then build up the three-dimensional image of the specimen. In contrast to the regular fluorescent microscope which captures all the light emitted, the confocal picture images are generally sharper. The microscope is efficient in rejecting the out-of-focus fluorescent light.

E.1 Poly-L-Lysine Immobilisation

This method is suitable for growing the adherent cells on a coverslip for microscopy purposes. The simplest step is poly-L-Lysine mobilisation for growing the adherent cells on the slides.

Requirements (Sterile)

(1) Coverslip
(2) Poly-L-lysine solution
(3) Water
(4) Glass Petri dish

Protocol 1:

(1) Clean cover slips.
(2) Dilute poly-L-lysine 1:10 with sterile deionised or double deionised water.
(3) Immerse cover slips in poly-L-lysine for 5 min.
(4) Transfer the cover slips to a Petri-dish.

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(5) Keep the dish at 60°C for one hr or at RT overnight in hood to dry.
(6) Drop culture on clean microscope slide.
(7) Cover with coated cover slip.
(8) Transfer the slide to CO_2 incubator for cells to attach and grow.

E.2 Immunofluorescence and Confocal Microscopy

Requirements

(1) Poly-L-lysine coated cover slips or the chamber slides
(2) PBS
(3) 0.2% Triton X-100 in PBS
(4) Primary antibody
(5) Rhodamine and/or FITC labeled secondary antibody
(6) No. 1 coverslip
(7) Fluorescence or confocal microscope

Protocol 2:

(1) Grow cells on the slide either on poly-L-lysine coated cover slips or the chamber slides.
(2) Serum starve the cells followed by the required stimulation.
(3) Stop the stimulation by fixing the cells in 1 ml of 4 % paraformaldehyde at RT for 20 min.
(4) Wash the cells twice with PBS.
(5) Permeabilise the cells with 0.2% Triton X-100 for 10 min.
(6) Wash the cells twice with PBS.
(7) Incubate with primary antibody for 1 hr. at RT.
(8) Wash the cells thrice with PBS.

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(9) Incubate with Rhodamine and/or FITC labeled secondary antibody for 1 hr at RT.
(10) Wash the cells thrice with PBS.
(11) Remove the excess fluid.
(12) Mark the slide on the opposite end for the slots.
(13) Air-dry the slide.
(14) Add few drops of the mounting solution.
(15) Overlay with a No. 1 coverslip.
(16) Analyse the images using fluorescence or confocal microscope.

Note: Avoid exposing the cells to light as much as possible from step 15 onwards. For suspension cells carry out the staining protocol in an eppendorf tube till step 10. To adhere the cell to the slide, cytofuge the cells.

E.3 Immunohistochemistry

Immunohistochemistry is used for detecting the constituents of tissue by using specific antibody tagged to a visible label which will bind to the antigen inside the tissue. The tissues are paraffinised, frozen or embedded in resin.

Requirements

(1) 2% Paraformaldehyde
(2) Xylene
(3) Ethanol
(4) BSA
(5) 0.25M Tris, pH 7.5
(6) 2% Fetal bovine serum in 0.25M Tris-HCl, pH 7.5

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(7) PBS
(8) DAB solution: 10 mg DAB (3, 3-diaminobenzidine) +20 µl of 38% H_2O_2 in 20 ml 0.1M Tris pH 7.2

Protocol 3:

(1) Dissect the tissue out and transfer into 2% paraformaldehyde solution.
(2) Incubate in fixative for 30 min or overnight depending on the size of the tissue.
(3) Section the tissue with the thickness of less than 10 µmeters and fix on slide.
(4) Incubate the tissues in Xylene for 3 times with each incubation for 10 min.
(5) Transfer the tissue into 100% Ethanol and incubate for 2 min. Repeat once more.
(6) Hydrate the tissue by placing in 95%, 70%, 50%, 30% ethanol for 2 min each.
(7) Give 3 washed with PBS or running water for 20 min.
(8) Incubate at 0.25M Tris-HCl, pH 7.5 for 5 min.
(9) Block the tissue with 2% Fetal bovine serum.
(10) Incubate the slide in a humidifier chamber overnight with primary antibody.
(11) Rinse the slides with a stream of buffer.
(12) Wash the slides for 5 min with the buffer.
(13) Incubate with the secondary antibody in a humidifier chamber for minimum of 1 hr.
(14) Wash the slide with the buffer three times.
(15) In case of fluorescent labelled secondary antibody mount the slide and visualise under fluorescent or Confocal microscope. For peroxide visualisation incubate with a DAB solution till brown color is seen followed by stopping the reaction under running tap water.

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F

Assays

F.1 Enzyme Assays

F.1.1 Enzyme Kinetics: Basics

Enzymes are biological catalysts that carry out tightly controlled biological reactions with high specificity. Like a chemical catalyst, an enzyme acts by lowering the activation energy of a reaction, thereby inducing the formation of the products from the substrates. Enzymes are regulated by a multitude of mechanisms (e.g. phosphorylation, product inhibition) or by localisation. The rate of an enzyme-catalysed reaction depends on multiple factors. These include:

(i) Temperature of the reaction: This increase in heat increases the collision frequency between the reactants. However, beyond a particular temperature, the protein enzyme also tends to denature, thereby making it inactive.
(ii) Substrate concentration: Increase in substrate concentration favours the reaction, as this increases the collision between the enzyme molecule and the substrates.
(iii) pH of the reaction: The protein's active site needs a particular charge inducing a favourable microenvironment for the reaction to occur. This microenvironment varies as the charge of the amino acids also changes with the change in pH.

In order to understand the mechanisms of the enzymatic process and also predict the reaction characteristics, one needs to understand the kinetics of the reaction. The important factor that effects the enzyme reaction is the availability and concentration of the substrates. An important model that gives a mathematical relationship is the Michaelis–Menten and Hill equation. The equation is denoted as

$$v_i = \frac{V_{\max}[S]}{K_m + [S]}$$

where v_i is the initial velocity, S is the substrate concentration, $V_{\max}$ is the maximal velocity, and K_m is the Michaelis constant which is the substrate concentration at which the velocity is half of $V_{\max}$. K_m denotes the affinity of the substrate to the enzyme; a low K_m indicates that the enzyme binds to the substrate tightly.

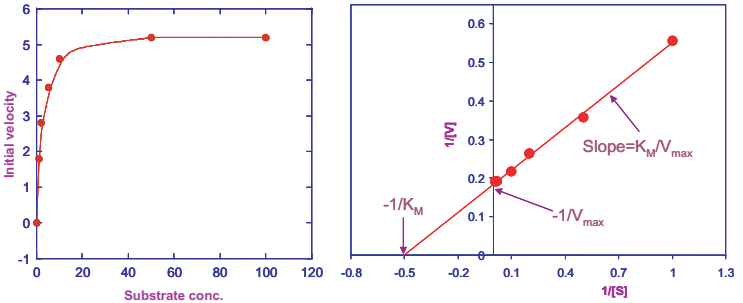

Fig. F.1 Lineweaver–Burke plot and determination of K_m and $V_{\max}$.

The linear form of this equation is denoted by the Lineweaver–Burk or double reciprocal plot, which is derived from the Michaelis–Menten and Hill equation and is denoted as:

$$\frac{1}{v_i} = \frac{K_m}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}}.$$

A plot of $1/v_i$ vs. $[S]$ will give a straight line with a slope of $K_m/V_{\max}$ and a y-intercept as $1/V_{\max}$ (Fig. F.1).

Protocol 1:

(1) Set up an assay tube with varying concentrations of the substrate.
(2) Initiate the reaction with a constant amount of the enzyme.
(3) At a defined time point, stop the reaction and measure the amount of product formed. Calculate the rate of reaction.
(4) Plot the rate of reaction or the amount of product formed as a function of concentration of substrate.
(5) At low amount of substrate, the rate of the reaction would be linear; but at very high concentration, this rate remains nearly constant. This rate is the $V_{\max}$ of the reaction.
(6) The K_m can be calculated from the graph as the concentration of the reaction when the velocity is half the maximal velocity or $V_{\max}/2$.

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(7) Plot the reciprocal of the same values. This gives a straight line.
(8) V_{max} is determined by the inverse of the *y*-intercept, i.e. $1/V_i$.
(9) K_m can be found either by the slope or the *x*-intercept (Fig. F. 1). The slope of the line is the ratio of K_m to V_{max}, and the *x*-intercept is the negative reciprocal of K_m.

F.1.2 Lipid Kinase Assay

Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase phosphorylates the 3 position of the inositol ring of phosphatidylinositol, phosphatidylinositol-4 phosphate (PtdIns4P) and phosphatidylinositol-4,5 bisphosphate (PtdIns4,5P_2). It forms important signalling molecules that are involved in the regulation of cell adhesion and proliferation. Thus, PtdIns 3-kinase has been a potential target for the design of drugs to control cancer. Fluorescent-based or radiometric PtdIns 3-kinase assays have been described. The radiometric assays are very sensitive assays, but involve the use of radioisotopes. The fluorescent-based assays are easy to handle, but have other limitations such as interference from other coloured molecules in the reaction mixture. In radiometric assays, the activity is measured as the amount of radiolabelled ATP which is incorporated into the lipid substrate and is next separated chromatographically.

Requirements

(1) *Assay Buffer*: pH 7.6

25 mM HEPES
5 mM $MgCl_2$
2mM EDTA

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(2) 50 μM working ATP in assay buffer with 5 μCi [γ-^{32} P]-ATP
(3) 1 mg/mL PtdIns/PtdIns4P or PtdIns4,5P_2. The lipids are suspended in 0.02% Triton X 100.

    Sonicate for 5 min before use.

(4) Methanol:1N HCl::1:1, v/v
(5) Chloroform
(6) Pretreatment solvent:

    60 mM EDTA
    2% Na-K tartrate
    50% ethanol

(7) Silica gel 60 plates dipped and shaken gently in pretreatment solvent, and then dried and baked
(8) Running solvent: chloroform:methanol:NH_4OH:H_2O ::90 :90:7:20, v/v

Protocol 2:

(1) The assay protocol is suitable for purified enzyme or the immunoprecipitates. For inhibitor studies, the protein needs to be preincubated with the inhibitor for at least 30 min.
(2) Immunoprecipitate the PtdIns3-kinase activity by using p85/4G10/PY20 antibodies.
(3) Add 35 μL of assay buffer.
(4) Add 5 μL of sonicated phosphatidylinositol.
(5) Initiate the reaction with 10 μL of 50 μM ATP (containing hot ATP).
(6) Incubate at RT for 15 min.
(7) Stop the reaction with 300 μL of methanol:1N HCl, v/v.
(8) Vortex the tube.
(9) Add 250 μL of chloroform.
(10) Vortex the tube carefully, but vigorously.
(11) Microfuge the tube so as to separate the phases.

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(12) Discard the upper aqueous phase (the aqueous phase is highly radioactive).
(13) Dry the lipids using a speed vac (do not use high temp.). The lipids can be stored at −80°C if needed.
(14) Resuspend the lipids in 25–30 μL of chloroform.
(15) Spot on the pretreated TLC plates.
(16) Develop the plate in the developing solvent.
(17) Air-dry the plate after the run.
(18) Expose the plate to X-ray film to visualise the product formation.
(19) Mark the spots corresponding to the product, cut and count.

For PtdIns 4 kinase and PtdIns (4) P5 kinase activities, the assay can be modified with: assay buffer (50 mM Tris-HCl, pH 7.2; 10 mM $MgCl_2$; 1 mM DTT; 0.4% Triton X-100), 0.5 mM of substrate PtdIns or PtdIns4P, and 1 μCi [γ-^{32}P]-ATP. Terminate the reaction with 0.6 mL chloroform:methanol (1:1, v/v). After addition of 0.5 mL 12N HCl, phosphoinositides are extracted into the lower chloroform phase, which are washed with 1 mL methanol:1M HCl (1:1, v/v) followed by 1 mL methanol:0.1 mol/L HCl (1:1, v/v). The radioactive reaction product can be isolated by TLC and quantified by liquid scintillation counting.

F.1.3 Protein Kinase Assay

Requirements

(1) Assay buffers (5X):

Buffer A:

40 mM MOPS, pH 7.0
1 mM EDTA
50 mM magnesium acetate

(Continued)

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Buffer B:

125 mM Tris, pH 7.5
0.1 mM EGTA
50 mM magnesium acetate

Buffer C:

250 mM Tris, pH 7.5
0.5 mM EGTA
50 mM magnesium acetate
0.5% β-Mercaptoethanol (add fresh)

Buffer D:

250 mM Tris, pH 7.5
0.5 mM EGTA
50 mM magnesium acetate
0.5 mM sodium orthovanadate (activated)

(2) Mg/ATP cocktail (10X):

500 μM ATP and 75 mM $MgCl_2$ in assay buffer with 5 μCi [γ-^{32}P]-ATP

(3) Substrate: 0.4 mM stock

Protocol 3:

(1) Take 1–5 μg of purified protein per assay in a final volume of 25 μL.
(2) Add 10 μL of the respective assay buffer (refer Table F.1).
(3) Add 5 μL of the substrate.

(Continued)

Table F.1 Buffer to be used for various kinases for assay.

Assay buffer	Kinase
Buffer A	Abl, Akt, Aurora, CDKs, CHK, cKit, cSrc, EGFR, GSK, JAK, PAK, PDGFR, PKA, Pyk2, SGK
Buffer B	cRaf, MAPK, RockII, SAPK
Buffer C	JNK
Buffer D	Fyn, Lck, Lyn, ZAP-70

(Continued)

(4) Initiate the reaction with 10 μL of diluted [γ^{32}P] ATP working solution.
(5) Vortex the mixture gently.
(6) Incubate for 30 min at 30°C with shaking (can be vortexed gently and intermittently).
(7) Pulse-spin the samples.
(8) Add 20 μL of 40% TCA to terminate the reaction.
(9) Incubate for at least 5 min.
(10) Transfer 50 μL of reaction mixture to pencil-labelled p81 phosphocellulose paper.
(11) Allow the radio-labelled substrate to bind the paper for 10 s.
(12) Immerse the paper into a tray containing 0.75% phosphoric acid.
(13) Gently shake the squares for 5 min on a rotator.
(14) Discard the wash into a liquid radioisotope waste container.
(15) Repeat the wash step twice.
(16) Rinse the papers in acetone for 5 min.
(17) Drain and allow the papers to dry.
(18) Count the radioactivity on the papers.

F.1.4 Protein Tyrosine Phosphatase Assay

Requirements

(1) RIPA lysis buffer (without vanadate and sodium fluoride inhibitor as they inhibit phosphatase activity)
(2) 10X phosphatase assay buffer:

500 mM Tris, pH 8.0
5 mg/mL BSA
5 mM DTT
10 mM $MgCl_2$

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(3) Substrate peptide: END(pY)INASL or DADE(pY)LIPQQG
(4) Malachite green:

Solution 1: 0.034% Malachite green in 10 mM ammonium molybdate, 1N HCl and 3.4% ethanol
Solution 2: 0.5 mL of 1% Tween 20 to 1 mL of Solution 1. Add 10 mL of Solution 2 and use it at RT.

(5) Phosphate standard: 1mM KH_2PO_4

Protocol 4:

(1) Lyse the cells in RIPA buffer without vanadate or NaF (lysis buffer).
(2) Immunoprecipitate the phosphatase enzyme using the respective antibody.
(3) Wash the precipitates twice with the RIPA buffer, followed by one wash with the 1X phosphatase assay buffer.
(4) Add 50 µL of 1X phosphatase assay buffer with the phosphotyrosine-labelled peptide.
(5) Incubate overnight at 30°C for 30 min or overnight at 37°C in a shaking incubator.
(6) At the end of the incubation pulse, spin the tube to pellet the immunocomplex.
(7) Transfer 25 µL of the supernatant into a 96-well microtitre plate.
(8) Add 100 µL of the Malachite green mix. Avoid formation of air bubbles.
(9) Allow the colour to develop by incubating for 15 min.
(10) Read the absorbance from 620–660 nm.
(11) Prepare a standard graph by using the standard solution in the dilution range from 0.2 to 2 µmol.
(12) Using the standard curve, estimate the amount of phosphate released in the sample assay.

F.1.5 Alkaline Phosphatase Assay

Requirements

(1) RIPA lysis buffer (without vanadate and sodium fluoride inhibitor as they inhibit phosphatase activity)
(2) 10X phosphatase assay buffer:

500 mM Tris, pH 8.0
5 mg/mL BSA
5 mM DTT
10 mM $MgCl_2$
50 mM *p*NPP

(3) Stop buffer: 13% K_2HPO_4

Protocol 5:

(1) Lyse the cells in RIPA buffer without vanadate or NaF (lysis buffer).
(2) Take 100 μg of the cell lysates in 100 μL of lysis buffer in a 91-well microtitre plate.
(3) Add 100 μL of 2X phosphatase assay buffer.
(4) Incubate for 1 h or until colour develops.
(5) Stop the reaction with 13% K_2HPO_4.
(6) Read the absorbance at 410 nm.
(7) Calculate the concentration of *p*-nitrophenolate ion produced using molar absorbtivity of 1.78×10^4/M/cm.

F.1.6 Caspase Assay

The assay involves a chromogenic tag such as pNA linked to a specific peptide corresponding to the cleavage site of the respective caspase. Proteolysis of this peptide releases the chromogenic tag, thereby increasing the chromophore intensity.

Requirements

(1) 10X assay buffer:

250 mM HEPES pH 7.4
1M NaCl
1% CHAPS
100 mM DTT
1mM EDTA

(2) Respective caspase substrate

Caspase 1: Ac-YVAD-pNA
Caspase 2: Ac-VDQQD-pNA
Caspase 3: Ac-DEVD-pNA
Caspase 4: Ac-LEVD-pNA
Caspase 5: Ac-WEHD-pNA
Caspase 6: Ac-VEID-pNA
Caspase 8: Ac-IETD-pNA
Caspase 9: Ac-LEHD-pNA

(3) Spectrophotometer
(4) 96-well plates

Protocol 6:

(1) In the 96-well plate, add 10 µL of 10X assay buffer.
(2) Add the sample in either cell lysate or purified enzyme.
(3) Bring the volume to 90 µL.
(4) Cover the plate with a plate seal.
(5) Mix the contents by keeping the plate on a plate shaker for 1 min at 300 rpm.
(6) Prewarm the plates at 30°C for 10 min.
(7) Add 10 µL of the colourimetric substrate.
(8) Incubate the plates at 37°C for 1 h.
(9) Read the colour of the reaction at 405 nm.
(10) The specific activity of caspase can be calculated from the standard graph generated using increasing concentration of free pNA.

F.2 Functional Assays

F.2.1 Apoptosis Assay

Apoptosis or programmed cell death is one of the regulatory mechanisms for the removal of unwanted cells. Apoptosis is induced by the stimulation of several different cell surface receptors in association with caspase activation. Apoptosis of a cell is thus a complicated process and can be assayed by various methods. Among widely used methods, the TUNEL assay is described here.

TUNEL Assay (Terminal Transferase dUTP Nick End Labelling)

During apoptosis, there is a fragmentation of genomic DNA. This fragmentation yields double- or single-stranded breaks. This process releases free 3'-OH group, which can be modified with labelled nucleotide in an enzymatic process. This methodology detects apoptosis at a single-cell level based on such DNA labelling. This method is also more appropriate to distinguish between apoptotic cells and necrotic cells.

Requirements

(1) 1% paraformaldehyde in PBS
(2) Reaction buffer:
    1 M potassium cacodylate
    125 mM Tris-HCl pH 6.6
    1.25 mg/mL BSA
(3) 25 mM cobalt chloride
(4) Ethanol
(5) PBS
(6) Triton X-100
(7) BSA
(8) Terminal transferase enzyme (TdT)
(9) FITC-dUTP
(10) RNAse A

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(11) TdT staining solution:

5 mL of $CoCl_2$ (25 mM)
12.5 units of TdT in its storage buffer (usually 0.5 mL)
0.25 nmol of FITC-dUTP (or BODIPY-dUTP) in its storage buffer
Add distilled water to 50 mL.

(12) Rinsing buffer: PBS containing 0.1% Triton X-100 and 5 mg/mL BSA
(13) PI staining solution: 5 μg/mL propidium iodide, RNase A 200 μg/mL in PBS
(14) Centrifuge
(15) Incubator
(16) Fluorescence microscope or flow cytometer

Protocol 7:

(1) Wash cells (1×10^6) in PBS and centrifuge at 200 g for 5 min. For parafinised tissue sections, deparaffinise sections in two changes of xylene for 5 min each, and hydrate with two changes each of 100%, 95%, 80%, 75% and 50% ethanol for 2 min. Rinse the slide thoroughly with water.
(2) Prepare a positive control by treating the tissue or cells with DNase I for 10 min at 25°C. For negative control, incubate with staining solution only without the enzyme.
(3) Fix cells in 1 mL of 1% paraformaldehyde for 15 min on ice. Avoid this step in case of dewaxed tissue sections and proceed directly to step 7.
(4) Centrifuge the cells at 200 g for 5 min and resuspend the cell pellet in 5 mL of PBS.
(5) Centrifuge and resuspend cells in 0.5 mL of PBS.
(6) Post-fix the cell suspension in 5 mL of ice-cold 70% (vol/vol) ethanol. Leave cells in ethanol for at least 1 h (the cells can be stored in ethanol at –20°C for several days).

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(7) Centrifuge, remove ethanol, resuspend cells in 5 mL of PBS and centrifuge. For tissues, wash the slide with PBS thrice.
(8) Resuspend the cell pellet in 0.5 mL of TdT staining solution or layer the slide with TdT staining solution.
(9) Incubate for 60 min at 37°C in a humidifier chamber.
(10) Wash the suspension cells thrice with 1.5 mL of rinsing buffer or wash the slide with the rinsing buffer thrice.
(11) Resuspend cell pellet in 1 mL of PI staining solution or overlay the tissue with PI staining solution.
(12) Incubate for at least 30 min at RT in the dark and analyse cells by flow cytometry or by fluorescence microscope.
(13) For flow cytometer analysis: construct a dot plot of PI red fluorescence on *x*-axis vs. green fluorescence of incorporated nucleotides on *y*-axis.

F.2.2 XTT Cell Proliferation Assay [(2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)]

XTT assay is suitable for measuring cell proliferation, cell viability or cytotoxicity. The tetrazolium salts are converted into a coloured formazan product by cellular enzymes present in the mitochondria of a metabolically active cell. These enzymes are rapidly inactivated when a cell dies, and hence the activity of these enzymes can be used to monitor the viability of a cell.

Requirements

(1) XTT stock solution (fresh): Dissolve XTT in serum-free media as a 1 mg/mL solution. XTT solution is warmed at 37°C for 5 min to dissolve the XTT completely (cloudiness will disappear).

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(2) PMS stock solution: Dissolve PMS (phenazine methosulfate) in 1X PBS as a 0.383-mg/mL solution. Make 100-μL aliquots and freeze at −20°C.
(3) XTT mix: Add 100 μL of PMS solution to 5 mL of XTT solution immediately prior to use. Mix well before using.

Protocol 8:

(1) Plate the cells in a volume of 100 μL of growth media per well.
(2) Allow the cells to incubate overnight at 37°C in the CO_2 incubator.
(3) Observe the cells under the microscope to ensure cell viability and also to verify that an equal number of cells have been plated in each well.
(4) At the end of the incubation time point, perform the XTT assay.
(5) For the XTT assay, add 50 μL of the XTT mix solution to each well containing cells in 100 μL of growth media.
(6) Incubate the cells at 37°C in the CO_2 incubator and take absorbance measurements at 465 nm after the coloured product starts developing. For HeLa, A549, A2058, MCF-7 and Caco2 cells, 2 or 3 h of incubation is sufficient. For Jurkat cells, 18 h (overnight) incubation is required before absorbance measurements should be made.

F.2.3 Chemotaxis Assay

Migration of cells in response to stimuli is responsible for several physiological functions mostly in the case of inflammatory responses and immune functions. The transwell chemotaxis assay is useful to study mechanisms of migration during chemotaxis. The main purpose of this assay is to determine if a molecule of interest exhibits chemotactic activity. Molecules that

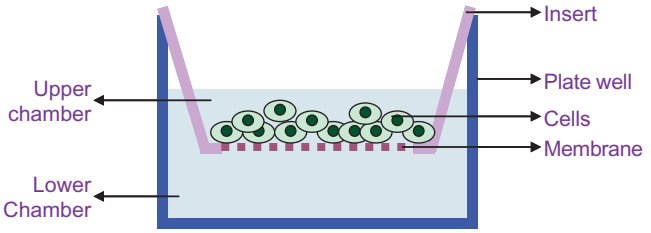

Fig. F.2 Cartoon depiction of the transwell plate used for chemotaxis assay.

attract specific cells are called chemoattractants. One of the major classes of chemoattractants is the cytokine family members named chemokines. The setup involves a transwell plate that has two chambers separated from each other by a membrane of defined pore size (Fig. F.2). The chemotactic agent is placed in the bottom well and the cells are placed in the upper chamber. This creates a gradient of the molecules across both of the chambers. If the molecule of interest is a chemoattractant, then the cells would squeeze through the porous membrane into the bottom well. The cells in the bottom well can then be counted to get the efficiency of chemotaxis. The role of inhibitors can be analysed by adding the inhibitor in the media of both chambers.

Requirements

(1) Cell media
(2) Chemoattractant
(3) Transwell plates
(4) Microscope

Protocol 9:

(1) Wash the cells twice with fresh media and suspend at 1×10^7 cells/mL.

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(2) In the case of inhibitor studies, preincubate the cells with the inhibitor/s or the appropriate control solvent (DMSO).
(3) Add 0.6 mL of medium containing the required amount of chemokines with or without inhibitor or DMSO to the bottom well.
(4) Load 100 μL (1×10^6 cells) of cell suspension from each sample to the upper well or inserts of the 24-well transwell plates.
(5) Place the inserts or the upper well on the bottom well carefully, avoiding formation of air bubbles.
(6) Incubate the plates for 3–6 h at 37°C in 5% CO_2.
(7) After incubation, remove the porous inserts carefully and count the cells in the bottom chamber using standard procedures.
(8) The results can be expressed as the percent of migrated cells compared with the control (untreated cells).

F.2.4 Matrigel Invasion or Chemoinvasion Assay

Tumour cells are characterised by metastatic behaviour during which the cells develop an ability to invade through extracellular matrix (ECM). The tumour cells induce secretion of several proteolytic enzymes including metalloproteinases such as MMPs. This induces dissolution of the basement membrane surrounding the blood, thereby leading to gaps in the basement. The chemotactic ability of the tumour cells then allows the cells to loosen up and invade blood vessels, and migrates to the other parts of the body, where they settle and grow, forming secondary tumours. Boyden chambers are commonly used for chemoinvasion assays. These chambers consist of inserts with a porous membrane coated with a Matrigel. The Matrigel contains basement membrane components such as growth factors, collagen, laminin and proteoglycans. The inserts are commercially available (Fig. F.3).

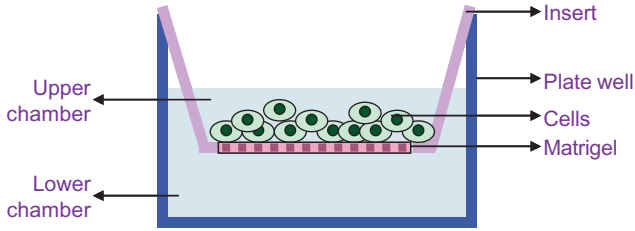

Fig. F.3 Cartoon depiction of the transwell plate used for Matrigel-based chemoinvasion assay.

Requirements

(1) DMEM
(2) 10 mM EDTA in PBS
(3) DMEM with 0.1% BSA
(4) Chemokine
(5) Matrigel
(6) Transwell plates
(7) Diff-Quick staining solution

Protocol 10:

(1) Dislodge the adherent cells from the plates with 10 mM EDTA in PBS.
(2) Wash the cells twice with DMEM and suspend as 2.5×10^6 cells/mL in medium containing DMEM with 0.1% BSA.
(3) Before performing the migration assay, pretreat the cells with inhibitors or the appropriate control solvent (DMSO).
(4) Load 150 µL of cells from each sample onto the upper well or inserts containing the Matrigel, and 0.6 mL medium containing chemokines in the presence or absence of inhibitors or DMSO in the bottom well.
(5) Place the inserts or the upper well on the bottom well carefully, avoiding formation of air bubbles.

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(6) Incubate the plates for 24 h at 37°C in 5% CO_2.
(7) After incubation, remove the inserts carefully and then fix and stain the cells using Diff-Quick staining solution.
(8) Scrape off noninvaded cells on the top of the transwell with a cotton swab.
(9) The results are expressed as the percentage of migrated cells compared with the control (untreated cells).

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Autoradiography

Summary

Autoradiography is an image formed on photographic film by radiation from a radioactive substance. The radioactive substance could be from a radiometric assay or from metabolic labelling of tissue. In the case of biochemical assays, the labelled products are separated from the radioactive substrates either by extraction or separation using chromatographic techniques, commonly thin layer chromatography (TLC; see also Sec. C.2). The TLC plates can then be directly exposed to the photographic plates. In the case of labelled tissues, the compound of interest that could be a metabolite or nucleic acid is radiolabelled and incubated with the tissue. The tissue is washed and then exposed to the photographic plates. The localisation of the radioactive spot indicates the localisation of the molecules of interest. In specialised cases such as Western blots or Southern blots, the samples are probed with a radioactive probe or enzyme that induces the formation of photons. This will again darken photographic films. ^{35}S, ^{14}C, ^{32}P and ^{125}I can be visualised by autoradiography, while ^{3}H requires enhancer materials due to its low energy of emission. In addition, exposure towards the film can be enhanced by the use of screens that reflect the radiation or photons.

Protocol 1:

(1) Dry the tissue slide/gel/TLC plates.
(2) Label them with the isotope or the photomarkers to know the orientation.
(3) Transfer the slide/gel/TLC plates to a transparent plastic bag.
(4) Align them on the cassette containing the screens.
(5) In the dark room under red light, open the film pack and keep it carefully on the top of the plate.
(6) After a specified time point, remove the film and develop it.
(7) If the signals are too low, keep the plate for a longer time.

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Abbreviations

APS	ammonium persulfate
ATP	adenosine trisphosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CHO	Chinese hamster ovary
CM	carboxymethylcellulose
DAB	3,3-diaminobenzidine
DEAE	diethylaminoethyl
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo) tetraacetic acid
FBS	fetal bovine serum
GST	glutathione transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	horseradish peroxidase
IgG	immunoglobulin G
NBT	nitroblue tetrazolium
NTA	nitrilotriacetic acid
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PI	propidium iodide
PMS	phenazine methosulfate
pNA	p-nitrophenolate
pNPP	p-nitro phenyl phosphate
PS	penicillin/streptomycin
PtdIns	phosphatidylinositol
RIPA	radio-immunoprecipitation assay buffer
RPMI	Roswell Park Memorial Institute
RPMI	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
TBST	Tris buffered saline Tween 20
TCA	trichloroacetic acid
TdT	terminal transferase
TEMED	tetramethylethylenediamine
TLC	thin layer chromatography
Tris	Tris (hydroxymethyl)-aminomethane
TUNEL	terminal transferase dUTP nick end labelling
XTT	(2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)

Appendices

Appendix 1 Safety Issues

1. Before beginning any work in the lab, check out all the safety regulations of the lab.
2. Beverages and eatables are to be avoided. They are not to be consumed or permitted in the laboratory.
3. Every lab has its own dress code. The more common ones are as follows:

   (1) Always wear a laboratory coat or apron.
   (2) Closed-toe shoes, not sandals, are to be always worn.
   (3) Short shorts or short skirts are not allowed unless covered completely.

4. Always wear gloves while working or handling any potentially hazardous chemicals.
5. Before the start of work, make a note of the location of shower, eye wash and other emergency equipment.
6. Check the location of the emergency fire exits and fire extinguishers.
7. When handling chemicals with low boiling temperature, i.e. volatile compounds, work in the fume hood.
8. It is potentially safe to wear safety glasses when working in the lab.
9. Before beginning a new experiment, check the safety-related issues of the protocol as well as the chemicals involved. Take note of precautionary measures in case of accidents. Presumably all chemicals are harmful.
10. Make a special note when handling strong acids or bases, phenol, ethidium bromide and acrylamide — the main chemicals.
11. Before leaving the lab, wash hands thoroughly with soap.
12. In case of accidents, immediately take the necessary precautions to reduce the harm. This is usually by washing the affected area thoroughly with soap water or running tap water.
13. All accidents and injuries should be reported to the lab safety officer in charge.
14. When spills occur, contain the spills with a paper towel or sponge, or according to the spillage containment protocol, and then report the incident.

15. Keep the work area as clean as possible. Always store personal belongings away from the working area.
16. After completing the experiments, clean the work bench and do not leave any hazardous chemicals unattended.
17. Label the contents of the reagents using a proper marker pen and stickers.
18. Before using any equipment, read the instruction manual for operating conditions and the limitations of the instruments.
19. Each institution has its regulations for waste disposal. Adhere strictly to the institutional policies. Do not put hazardous waste down the drain including organic solvents, strong acids and bases. Dispose the hazardous waste in their respective containers. This is also applicable for solid wastes.
20. All sharp disposables should be handled carefully and disposed in containers specifically assigned for sharps. DO NOT dispose sharps in regular bins.
21. To a maximum extent, avoid working alone in the lab. In extreme cases, if need arises, keep the contact numbers of the responsible people handy for emergency use.
22. For biological hazards that include bacterial, viral or mammalian cultures, follow stringent precautionary measure as per the lab safety standards.

Appendix 2 Buffer Chart

Choice of Buffers

Effective pH range	pKa 25°C	Buffer
1.2–2.6	1.97	Maleate (pK1)
1.7–2.9	2.15	Phosphate (pK1)
2.2–3.6	2.35	Glycine (pK1)
2.2–6.5	3.13	Citrate (pK1)
3.0–4.5	3.75	Formate
3.0–6.2	4.76	Citrate (pK2)
3.2–5.2	4.21	Succinate (pK1)
3.6–5.6	4.76	Acetate
5.0–7.4	6.27	Cacodylate
5.5–6.5	5.64	Succinate (pK2)
5.5–6.7	6.1	MES
5.5–7.2	6.4	Citrate (pK3)
5.5–7.2	6.24	Maleate (pK2)
5.8–7.2	6.46	Bis-Tris
5.8–8.0	7.2	Phosphate (pK2)
6.0–12.0	9.5	Ethanolamine
6.0–8.0	6.35	Carbonate (pK1)
6.1–7.5	6.78	ACES
6.1–7.5	6.76	PIPES
6.5–7.9	7.14	MOPS
6.8–8.2	7.48	HEPES
7.4–8.8	8.05	Tricine
7.5–9.0	8.06	Tris
8.5–10.2	9.23	Borate
9.5–11.1	10.33	Carbonate (pK2)
9.7–11.1	10.4	CAPS

Phosphate (Sodium) Buffer Chart

Stock solution A: 2M monobasic sodium phosphate, monohydrate (276 g/L).
Stock solution B: 2M dibasic sodium phosphate (284 g/L).

Mixing an appropriate volume (mL) of A and B as shown in the table below and diluting to a total volume of 200 mL, a 1M phosphate buffer of the required pH at RT.

A	B	pH
90.0	10.0	5.9
87.7	12.3	6.0
85.5	15.0	6.1
81.5	19.5	6.2
77.5	22.5	6.3
73.5	26.5	6.4
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5
13.0	87.0	7.6
10.5	89.5	7.7
8.5	91.5	7.8

Appendix 3 Composition of Regular Buffers or Stock Solutions

RIPA

50 mM Tris-HCl, pH 7.4
1% NP-40
150 mM NaCl
1 mM EDTA
Protease inhibitors added fresh.

PBS (5X in 500 mls)

20.45 g NaCl
0.465 g KCl
10.142 g Na_2HPO_4, $7H_2O$
0.545 g KH_2PO_4, pH 7.2

1M $MgSO_4$ Stock

24.074 g $MgSO_4$ distilled water to 200 mL, store at RT.

1 M HEPES, pH = 7.0 Stock

119.15 g HEPES (free acid) distilled water to 400 mL. Add solid NaOH a few pellets at a time while mixing until the pH is ~6.8; add concentrated NaOH dropwise to achieve pH = 7.0 distilled water to 500-mL sterile filter and store at 4°C.

0.5 M EDTA Stock

16.81 g EDTA (sodium salt) distilled water to 90 mL. Adjust pH to 7.0. Bring the volume to 100 mL with distilled water. Store at RT.

0.5 M EGTA Stock

19.02 g EGTA (sodium salt) distilled water to 90 mL. Adjust pH to 7.0. Bring the volume to 100 mL with distilled water. Store at RT.

1M Dithiothreitol (DTT)

1.542 g dithiothreitol distilled water to 10 mL. Disburse into 500-mL aliquots and store at −20°C.

100 mM MgATP Stock

Check formula weight of the lot of ATP you have and determine the amount required for 10 mL of a 100 mM solution. Add 8.5 mL distilled water to the determined amount and add 1 mL of 1M $MgSO_4$ stock. Adjust pH to 7.0 distilled water to 10 mL. Disburse into 200-mL aliquots, and store at −20°C.

100 mM Sodium Orthovanadate

1.839 g sodium orthovanadate distilled water to 8 mL in a screw cap tube. Adjust pH to 10 if solution is yellow. Place in boiling water until clear and recheck pH; repeat as necessary and adjust to final concentration by checking A265 nm, ext. coeff. = 2925/M/cm and adding distilled water as needed. Store at −20°C.

Notes

Notes

Notes

Index

K_m, 82
V_{max}, 82

Abl, 87
absorbance, 16
absorbance coefficients, 17
adherent cells, 58
affinity chromatography, 3
Akt, 87
alkaline phosphatase, 31, 90
ammonium sulphate, 2
apoptosis, 92
Aurora, 87

BCIP, 31
Beer's law, 19
Bradford, 17

caspase, 90
CDKs, 87
chemoinvasion, 97
chemokines, 96
chemotaxis, 95
CHK, 87
chromatography, 2
cKit, 87
column preparation, 8
confocal, 74
Coomassie, 17
Coomassie blue, 26
cRaf, 87
cSrc, 87

DAG, 40
DC protein, 18
desalting, 9

EGFR, 87
extraction, 2

Ficoll–Hypaque, 57
FITC, 74
Fyn, 87

glycolipid, 40
GSK, 87

hemocytometer, 61
Hill, 82
His-Tag, 10
HRP, 31

immunohistochemistry, 75
immunoprecipitation, 32
immunofluorescence, 74
ion exchange, 3

JAK, 87
JNK, 87

Lck, 87
Lineweaver–Burk, 83
Lowry, 18
Lyn, 87

MAG, 40
MAPK, 87
Matrigel, 97
Michaelis–Menten, 82

NBT, 31
nitrocellulose, 28

PAK, 87
PDGFR, 87
peripheral blood lymphocytes, 57
phosphatidylinositol, 42
phosphatidylinositol 3-kinase, 84
phosphoinositides, 40
phospholipids, 40
PKA, 87
polyacrylamide gel electrophoresis, 21
precipitation, 2
primary antibody, 29
propidium iodide, 93
Protein A, 32
Protein G, 32
protein kinase, 86
protein tyrosine phosphatase, 88
PtdIns (4) P5 kinase, 86
PtdIns 4 kinase, 86

PVDF, 28
Pyk2, 87

radioactive, 104
recombinant proteins, 7
Rf value, 46
RockII, 87

SAPK, 87
secondary antibody, 29
SGK, 87
silica gel, 46
silver staining, 26
size exclusion, 3
sodium dodecyl sulphate, 22
spectrometer, 19
spectrophotometer, 16
sphingolipids, 40
stationary phase, 46
suspension cells, 60

TAG, 40
terminal transferase, 92
transfection, 64
transwell chemotaxis assay, 95
Trypan blue, 62
TUNEL assay, 92

ultraviolet light, 16

XTT, 94

ZAP-70, 87