DENSITY GRADIENT MEDIA

Analyzing density gradients

Density

Once gradients have been fractionated, it is extremely important that the density of each fraction is measured accurately. The most direct method is to weigh accurately known volumes of liquid using a pycnometer; however, this is very time consuming and it is more convenient to determine the density of a fraction by measuring the refractive index which has the added advantage of requiring as little as 20 µl of sample. There is a simple linear relationship between refractive index and the density. The refractive index of gradient solutions is increased by the presence of other solutes (e.g. sucrose and NaCl).

If a refractometer is not available then an alternative method of determining the density of gradient fractions is to measure the absorbance (optical density) of the fractions. All iodinated density gradient media absorb strongly in the UV. If the absorbance is measured at approx 244 nm (the absorbance maximum for Nycodenz® and iodixanol) the gradient samples will need to be diluted with water (table below gives a few values for iodixanol solutions) to get an absorbance value that can be measured accurately. The need to dilute the solution also means that any interfering material will be diluted out at the same time. Alternatively, if the absorbance is measured at a higher wavelength, dilution is not required. The table below gives a few absorbance values for Nycodenz® solutions at 350 nm

Absorbance at 244 nm of iodixanol solutions in water

Density of undiluted solution	A_{244} after dilution 1:10,000
1.050	0.152
1.075	0.375
1.100	0.569
1.125	0.777
1.150	0.964

Determination of density of solutions of Nycodenz® from absorbance measurements.

Nycodenz %	Density (g/ml)	$A_{350\ nm}$	$A_{360\ nm}$
1	1.004	0.06	0.03
2	1.009	0.12	0..07
4	1.020	0.25	0.15
6	1.030	0.38	0.23
8	1.040	0.51	0.31
10	1.052	0.64	0.39
15	1.078	0.97	0.58
20	1.105	1.29	0.79
25	1.131		0.99

and 360 nm. Care must be taken to use the correct blank to ensure that other components in the gradient fractions that absorb at, or near these wavelengths do not interfere with the measurement of the gradient medium.

Absorbance measurements using a Multi-well Plate Reader

The wide availability of Multi-well Plate Readers which routinely have the facility for measurement of absorbance at 340 nm considerably simplify the measurement of absorbance on gradient fractions, particularly if the gradient has already been collected in a multi-well plate. Multiple-channel automatic pipettes also facilitate the transfer of liquid aliquots between plates.

• Transfer 100 µl of each of the gradient solutions into 100 µl of water in the wells of a second plate. Complete transfer and mixing is achieved by three repeated aspirations into and expulsions from the pipette tips. The absorbance of the solutions in each well is measured in a standard

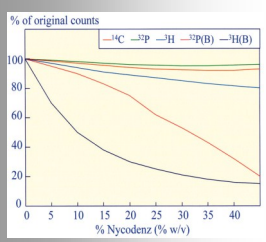


plate reader using a 340 nm filter, against

• For iodixanol concentration above 35% (w/v), it may be necessary to make a second dilution of the solutions (again 100 µl into 100 µl of water) to avoid absorbance values above 1.2.

• Six different types of multi-well plate have been tested for their suitability. A flat-bottomed 96-well polystyrene plate which has the lowest background absorbance of any plate tested (approx 0.130 at 340 nm) is available from Greiner BioOne Inc (Cat. # 655101). The inter-well variability of the absorbance was also one of the lowest of all those tested (± 0.007).

• Absorbance values of a range of iodixanol solutions produced by dilution of OptiPrep™ with either saline or 0.25 M sucrose are given in the tables below respectively. The absorbance measurements were made against saline and 0.25M sucrose blanks, which accounts for the slight distortion of the measured values of samples diluted with sucrose.

Particle concentration

Although the quantitative distribution of cells through a gradient, can be determined by using a haemocytometer or an electronic particle counter, turbidometric analysis is a more general method used for all types of light-scattering particles. Particulate matter can be detected and semiquantified by light-scattering measurements at 500-600 nm, while particles containing macromolecules bearing porphyrin prosthetic groups (e.g. haem groups) can be monitored by Soret band absorbance at 400-420 nm.

Absorbance (340 nm) concentration and density of iodixanol solutions in 0.85% NaCl (solutions diluted 1:1 twice)

Absorbance	% iodixanol	Density (g/ml)
0.045	2	1.016
0.085	4	1.027
0.125	6	1.037
0.165	8	1.048
0.205	10	1.058
0.245	12	1.069
0.285	14	1.079
0.325	16	1.090
0.365	18	1.100
0.405	20	1.111
0.445	22	1.121
0.485	24	1.132
0.525	26	1.142
0.565	28	1.153
0.605	30	1.163
0.645	32	1.174
0.685	34	1.184
0.725	36	1.195
0.765	38	1.205
0.805	40	1.215

Absorbance (340 nm) concentration and density of iodixanol in 0.25 M sucrose (solutions diluted 1:1 twice)

Absorbance	% iodixanol	Density (g/ml)
0.112	6	1.059
0.148	8	1.069
0.184	10	1.079
0.220	12	1.088
0.256	14	1.098
0.294	16	1.107
0.330	18	1.117
0.366	20	1.127
0.403	22	1.136
0.440	24	1.146
0.477	26	1.156
0.514	28	1.165
0.550	30	1.175
0.537	32	1.185
0.625	34	1.194
0.662	36	1.204
0.698	38	1.214
0.735	40	1.223
0.772	42	1.233
0.808	44	1.243
0.845	46	1.252
0.882	48	1.262
0.918	50	1.272

Nucleic acids, proteins and polysaccharides

Although solutions of iodinated media absorb strongly in the ultraviolet region of the spectrum, as their absorbance maximum is different to that of proteins and nucleic acids, it may be possible in some cases, through use of the correct blank (i.e. from a blank gradient unloaded in exactly the same manner as the test gradient) to determine their distribution spectrophotometrically. Normally however, nucleic acids, proteins and polysaccharides are assayed spectrophotometrically by chemical methods (see table below). Unlike metrizamide, neither Nycodenz® or iodixanol contain a sugar residue therefore they do not interfere with the orcinol or diphenylamine reactions for the estimation of the ribose and deoxyribose of RNA and DNA respectively and polysaccharides and sugars can be determined using the phenol/H_2SO_4 assay. Sensitive dye binding assays for protein and DNA are also unaffected by the presence of the gradient media. Protein assays based on

Coomassie blue give the most reliable data. The Folin Ciocalteu reagent however cannot be carried out unless the concentration of Nycodenz® or iodixanol is less than 5%(w/v): this situation however can often be attained if the final assay volume is 1-2 ml and the volume of gradient fraction used is 50 µl. Even at higher concentrations of gradient medium, an appropriate correction can be made to produce a linear relationship between protein concentration and absorbance (table below gives an example). In addition to these spectrophotometric methods, fluorimetric assays of nucleic acids and proteins can also be carried out in the presence of Nycodenz® or iodixanol.

Enzymes

So long as the concentration of a subcellular membrane in a gradient fraction is sufficiently high, then many standard marker enzyme assays can be performed in the presence of either Nycodenz® or iodixanol. Little or no inhibition is observed with these media (see table above). This is in contrast to Percoll®, which because of its light scattering properties must be removed prior to spectrophotometric enzyme analysis. If the membrane does require concentration, then this can be done efficiently either by sedimentation or one of the other methods described below.

Compatibility of Nycodenz with chemical assays

Assay for	Method (reagent)	Interference from Nycodenz®
DNA	Diphenylamine	No
	Methyl green	No
RNA	Orcinol	No
Protein	Folin-phenol	Yes, above 5% (w/v)
	Amido black	No
	Coomassie blue	No
Polysaccharides	Anthrone	Yes
Hexoses	Phenol/H_2SO_4	No

Effect of iodixanol on protein assay using the Folin reagent

Protein (µg)	$A_{660\ nm}$		
	Water	30% iodixanol	30% iodixanol (-0.226)
0	0	0.226	0
20	0.146	0.289	0.063
50	0.364	0.383	0.157
100	0.680	0.529	0.303

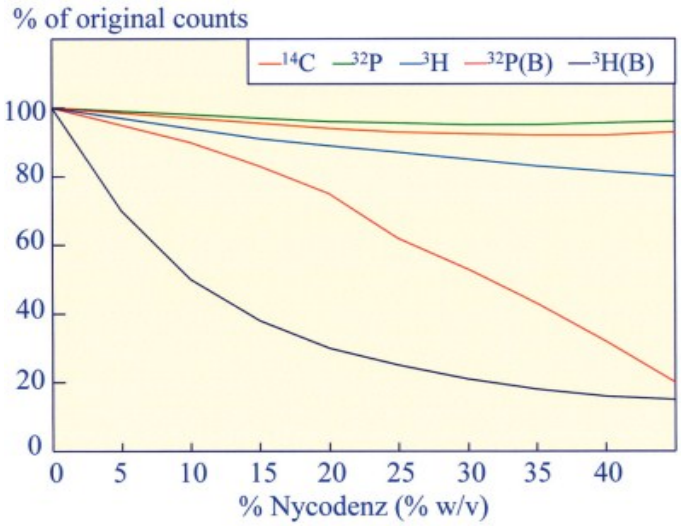
Effect of gradient media on marker enzymes

Enzyme	Iodixanol	Nycodenz®	Sucrose
Succinate deHase	96; 103; 104	74; 87; 88	67; 90; 86
β-Galactosidase	101; 96; 102	93; 100; 100	95; 85; 77
Acid phosphatase	95; 101; 102	94; 102; 101	87; 93; 92
Alkaline phosphatase	143; 131; 135	152; 152; 152	115; 117; 112
Catalase	143; 140; 137	100; 91; 104	112; 111; 121
Mg^{2+}-ATPase	71; 74; 79	76; 77; 80	105; 84; 98
5'-Nucleotidase	109; 95; 99	96; 100; 98	91; 103; 97
NADPH-cyt c reductase	105; 112; 122	100; 108; 115	100; 113; 119
Leucine aminopeptidase	102; 88; 96	108; 100; 97	104; 93; 73

All figures are given as a percentage of the activity in the control medium (0.25 M sucrose, 1 mM EDTA, 20 mM Tricine-NaOH, pH 7.4). The three figures are the % activity after 0.5, 1.0 and 3.0 h incubation in 30% (w/v) medium.

Radioactivity assays

Analysis of gradients material may either involve the radiolabeling of the material prior to fractionation or the use of radiolabeled reagents in functional assays. Nycodenz® and iodixanol quench ^{3}H, ^{32}P and ^{14}C to an extent which is dependent on the energy of the emission, although, as shown in the figure below, the degree of quenching is also dependent upon the scintillator used. Toluene scintillator, containing 4.0 g 2,5 diphenyloxazole (PPO) and 0.05 g 1.4- bis - 2(5-phenyloxazolyl) benzene, (POPOP) per liter and mixed with one half its volume of Triton X-100 is quite resistant to quenching, while Brays scintillant is much less suitable. The extent of quenching can be minimized by diluting the samples prior to counting, or it can be eliminated completely by acid-precipitating the material in the gradient fractions and counting each precipitate after collection on filters and drying.



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Removal of gradient medium and concentration of particles

It may be necessary to remove either Nycodenz® or iodixanol from the gradient fractions either to concentrate the particles or if the medium does interfere with some add-on process. Larger particles can be pelleted from fractions after dilution with 1-2 volumes of a low-density buffer such as a buffered salt solution (cells and viruses) or the homogenisation medium (organelles and membrane vesicles). Particles should be sedimented at either a slightly higher RCF and/or longer centrifugation time than that used to pellet the particles from the low-density solution itself, to take account of the raised density and viscosity caused by the presence of the gradient medium. RCFs in excess of 150,000g should be avoided for iodixanol-containing solutions; otherwise there may be some sedimentation of the solute molecule itself. Removal of Percoll® particles from organelle suspensions provides a major problem since the organelles and the silica particles sediment under the same conditions and there is evidence for considerable loss of peroxisomes, for example, during this process. If the protein requires concentration, neither iodixanol or Nycodenz® interfere with TCA precipitation.

Removal of iodixanol and Nycodenz® from gradient samples containing macromolecules and macromolecular complexes is best achieved by filtration through microcentrifuge ultrafiltration cones such as those manufactured by Whatman (Vectaspin®). Alternatives are dialysis in large pore size tubing and passage down a column of Sephadex G25.

Electrophoresis

SDS-polyacrylamide and agarose gel electrophoresis can be carried out directly on gradient samples, as long as the concentration of particles in the gradient fractions is sufficiently high for analysis. Colloidal particles of Percoll® interfere with the smooth migration of macromolecules into the gel, therefore this gradient medium must be removed prior to analysis.

For detailed protocols and references see the OptiPrep™ Application CD or online at: www.axis-shield-density-gradient-media.com

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