OptiPrep™ Application Sheet V05

Analysis of gradients

♦ **To access other Application Sheets** referred to in the text: return to the **2020Virapp** file and select the appropriate V-number.

1. Density determination

Once gradients have been fractionated, it is often 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 20-50 µl of sample. The simple linear relationship between refractive index (η) and the density (ρ) is $\rho = A\eta - B$. The refractive index of gradient solutions is increased by the presence of other solutes (e.g. sucrose and NaCl), thus the values of the two constants A and B vary with the presence and concentration of the solute.

For extensive tables relating % (w/v) concentration of iodixanol, density and refractive index of solutions used for the fractionation of viruses see **Application Sheet V01**.

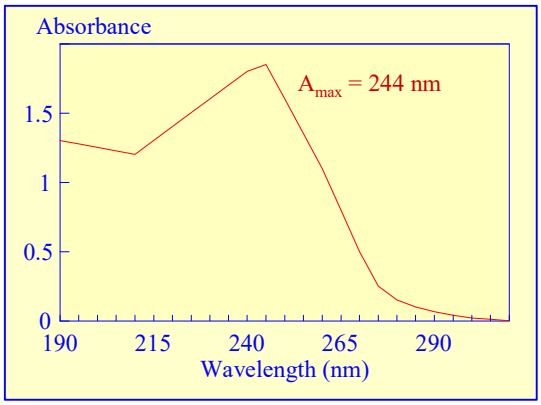

Figure 1 Absorbance spectrum of aqueous Nycodenz® solution (0.05 mg/ml in water)

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 (see Figure 1). If the absorbance is measured at approx 244 nm (the absorbance maximum for Nycodenz® and iodixanol) the gradient samples will need to be diluted 1:10,000 with water to get an absorbance value that can be measured accurately. Table 1 gives a few values for iodixanol solutions, measured in a standard 1 cm path length quartz cell in a single beam spectrophotometer. The need to dilute the solution also means that any other potentially interfering material will be diluted out at the same time.

Alternatively, if the absorbance is measured at a higher wavelength, dilution is not required. Table 2 gives a few absorbance values for Nycodenz® solutions at 350 nm 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.

Table 1 Absorbance at 244 nm of iodixanol solutions

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

Data from ref 1

Table 2 Absorbance at 350 and 360 nm of Nycodenz® solutions (undiluted)

Nycodenz %(w/v)	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

1a 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.

1. Transfer 100 µl of each of the fractions into 100 µl of water in the wells of a second plate.
2. Complete the transfer and mixing by three repeated aspirations into and expulsions from the pipette tips.
3. Measure the absorbance of the solutions in each well in a standard plate reader using a 340 nm filter, against a suitable blank.

- 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 produce by dilution of OptiPrep™ with either saline or 0.25 M sucrose are given in Tables 3 and 4 respectively. The absorbance measurements were made against saline and 0.25 M sucrose blanks, which accounts for the slight distortion of the measured values of samples diluted with sucrose.

Table 3 Absorbance (340 nm) and density of iodixanol solutions in 0.85% NaCl (solutions diluted 1:1 twice)

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

Table 4 Absorbance (340 nm) and density of iodixanol solutions in 0.25 M sucrose (solutions diluted 1:1 twice)

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

2. Particle detection

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 semi-quantified 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.

3. 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 (Table 5 and ref 2). Unlike metrizamide, neither Nycodenz® nor 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 [3]; polysaccharides and sugars can be determined using the phenol/H_2SO_4 assay [4]. Sensitive dye binding assays for protein [5,6] and DNA [7] 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 [8] 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 6 gives an example). In addition to these spectrophotometric methods, fluorimetric assays of nucleic acids [9,10] and proteins [11] can also be carried out in the presence of Nycodenz® or iodixanol. Many of these protocols are listed in ref 12.

Table 5 Compatibility of Nycodenz® and iodixanol with chemical assays

Assay for	Method (reagent)	Ref #	Interference from solute
DNA	Diphenylamine	3	No
	Methyl green	7	No
RNA	Orcinol	3	No
Protein	Folin-phenol	8	Yes, above 5% (w/v)
	Amido black	5	No
	Coomassie blue	6	No
Polysaccharides	Anthrone	9	Yes
Hexoses	Phenol/H_2SO_4	4	No

Table 6 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

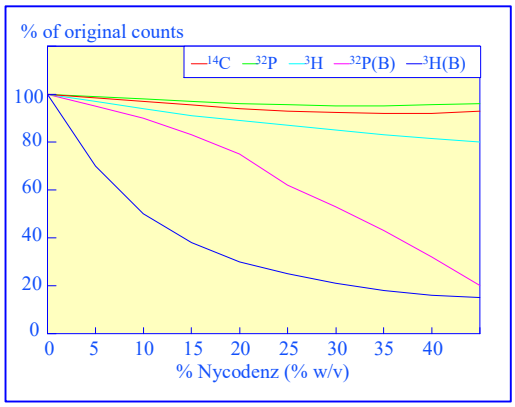

Figure 2: Quenching of radioactivity (scintillation counting) by Nycodenz®. In each case 100µl of the appropriate isotope was added to 4.5 ml of scintillant and counted for 5 min. Subsequently 0.4 ml of a solution containing 0-45% (w/v) Nycodenz® was added and recounted. The scintillant was either a standard PPO/POPOP/toluene/Triton mixture or Brays solution (B).

4. 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 that is dependent on the energy of the emission, although, as shown in Figure 2, the degree of quenching is also dependent upon the scintillant used. Toluene-based scintillant, containing 4.0 g 2,5-diphenyloxazole (PPO) and 0.05 g 1.4-bis - 2(5-phenyloxazolyl) benzene, (POPOP) per litre 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 may 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.

5. Electrophoresis

SDS-polyacrylamide and agarose gel electrophoresis can be carried out directly on gradient samples, as long as the concentration of protein or nucleic acid in the gradient fractions is sufficiently high for analysis. If the protein for example requires concentration, neither Nycodenz® nor iodixanol interfere with TCA precipitation.

6. 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. Viruses can be pelleted from fractions after dilution with 1-2 volumes of a low-density buffer such as a buffered salt or sucrose solution. 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 slightly 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 iodixanol and Nycodenz® from gradient samples containing virus is best achieved by filtration through microcentrifuge ultrafiltration cones such as those manufactured by Whatman

(Vectaspin®) or Millipore (Amicon® Ultra 4). Successful use of two other membrane devices has been reported in the literature - Vivaspin membranes from Sartorius and Centricon Plus 70 centrifugal filters from Millipore, or a PBHK Centrifugal Plus-20 filter unit with an Ultracel PL membrane (100 kDa cut off). An alternative is dialysis in large-pore size tubing or in a GeBAflex dialysis tube (Gene Bio Applications (GeBA) Ltd.). The latter are certainly more convenient than dialysis tubing for small volumes, the tubes are available with 0.25, 0.8 and 3.0 ml capacities and MWt cut-offs up to 14,000. Tangential flow filtration is also an effective alternative. Passage down a column of Sephadex G25 is another possibility.

7. References

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