OptiPrep™ Application Sheet C51

Analysis of gradients

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1. Density determination

Once samples have been collected from a gradient, it may be important to determine their density by collecting fractions from a blank gradient run in parallel. The most direct method is to weigh accurately known volumes of liquid using a pycnometer; however, this is very time consuming. It is more convenient to determine the density of a fraction by measuring the refractive index (RI), which has the added advantage of requiring as little as 20-50 µl of sample. For extensive tables relating % (w/v) concentration of iodixanol, density and RI of iodixanol solutions produced by the dilution of OptiPrep™ with routine buffered saline solutions **see Application Sheet C01**. Because the RI of gradient solutions is increased by the presence of other solutes (e.g. salts, buffer etc), the precise value of the RI will vary with the presence and concentration of these solutes. Thus it might be wise to construct a simple graph of RI against iodixanol concentration, from measurements made on solutions of iodixanol prepared by mixing OptiPrep™ with the specific cell medium used in the study

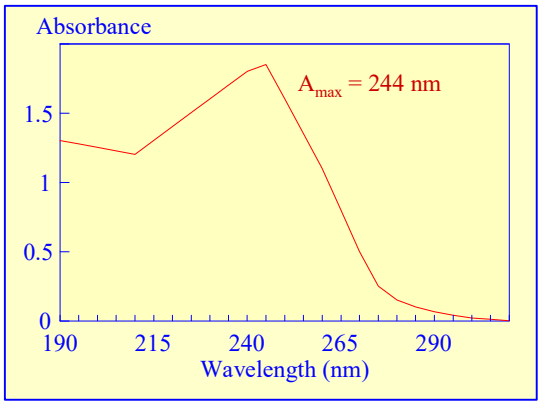

Figure 1 Absorbance spectrum of aqueous Nycodenz® solution (0.05 mg/ml in water)

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

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 blank gradient fractions. 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 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.

- 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 saline are given in Table 3. The absorbance measurements were made against saline blanks.

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

2. Nucleic acids, proteins and polysaccharides

Nucleic acids, proteins and polysaccharides in isolated gradient fractions are often assayed spectrophotometrically by chemical methods (Table 4 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 5 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 4 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 5 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

3. 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. This contrasts with Percoll® whose colloidal silica particles interfere with the smooth migration of macromolecules into the gel; this gradient medium must therefore be removed prior to analysis. If the protein for example requires concentration, neither Nycodenz® nor iodixanol interfere with TCA precipitation.

4. 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. Cells may be pelleted from fractions after dilution with 1-2 volumes of a low-density buffer such as a buffered salt solution.

5. References

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