

# Technical Appendix

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
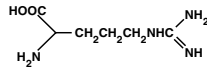
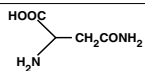
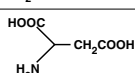
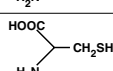
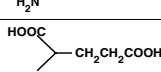
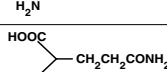

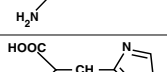
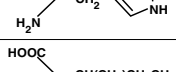
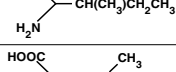
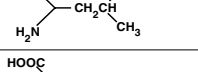
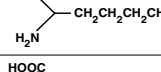
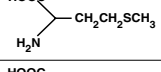
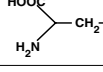
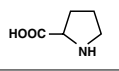
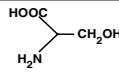
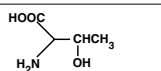
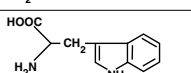
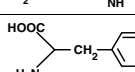
## Atomic weights of the elements

Element	Symbol	Atomic number	Atomic weight*	Element	Symbol	Atomic number	Atomic weight*
Actinium	Ac	89	227.02	Neodymium	Nd	60	144.24
Aluminum	Al	13	26.98	Neon	Ne	10	20.17
Americium	Am	95	(243.06)	Neptunium	Np	93	(237.04)
Antimony	Sb	51	121.75	Nickel	Ni	28	58.69
Argon	Ar	18	39.94	Niobium	Nb	41	92.90
Arsenic	As	33	74.92	Nitrogen	N	7	14.00
Astatine	At	85	(209.98)	Nobelium	No	102	(259.10)
Barium	Ba	56	137.32	Osmium	Os	76	190.2
Berkelium	Bk	97	(247.07)	Oxygen	O	8	15.99
Beryllium	Be	4	9.01	Palladium	Pd	46	106.42
Bismuth	Bi	83	208.98	Phosphorus	P	15	30.97
Boron	B	5	10.81	Platinum	Pt	78	195.08
Bromine	Br	35	79.90	Plutonium	Pu	94	(244.06)
Cadmium	Cd	48	112.41	Polonium	Po	84	(208.98)
Calcium	Ca	20	40.07	Potassium	K	19	39.09
Californium	Cf	98	(251.07)	Praseodymium	Pr	59	140.90
Carbon	C	6	12.01	Promethium	Pm	61	(144.91)
Cerium	Ce	58	140.11	Protactinium	Pa	91	(231.03)
Cesium	Cs	55	132.90	Radium	Ra	88	(226.02)
Chlorine	Cl	17	35.45	Radon	Rn	86	(222.01)
Chromium	Cr	24	51.99	Rhenium	Re	75	186.20
Cobalt	Co	27	58.93	Rhodium	Rh	45	102.90
Copper	Cu	29	63.54	Rubidium	Rb	37	85.46
Curium	Cm	96	(247.07)	Ruthenium	Ru	44	101.07
Dysprosium	Dy	66	162.50	Samarium	Sm	62	150.36
Einsteinium	Es	99	(252.08)	Scandium	Sc	21	44.95
Erbium	Er	68	167.26	Selenium	Se	34	78.96
Europium	Eu	63	151.96	Silicon	Si	14	28.08
Fermium	Fm	100	(257.09)	Silver	Ag	47	107.86
Fluorine	F	9	18.99	Sodium	Na	11	22.98
Francium	Fr	87	(223.01)	Strontium	Sr	38	87.62
Gadolinium	Gd	64	157.25	Sulfur	S	16	32.06
Gallium	Ga	31	69.72	Tantalum	Ta	73	180.94
Germanium	Ge	32	72.61	Technetium	Tc	43	(97.90)
Gold	Au	79	196.96	Tellurium	Te	52	127.60
Hafnium	Hf	72	178.49	Terbium	Tb	65	158.92
Helium	He	2	4.00	Thallium	Tl	81	204.38
Holmium	Ho	67	164.93	Thorium	Th	90	232.03
Hydrogen	H	1	1.00	Thulium	Tm	69	168.93
Indium	In	49	114.82	Tin	Sn	50	118.71
Iodine	I	53	126.90	Titanium	Ti	22	47.88
Iridium	Ir	77	192.22	Tungsten	W	74	183.85
Iron	Fe	26	55.84	Unnilhexium	Unh	106	(263.11)
Krypton	Kr	36	83.80	Unnilquadium	Unq	104	(261.11)
Lanthanum	La	57	138.90	Unnilpentium	Unp	105	(262.11)
Lawrencium	Lr	103	(262.11)	Unnilseptium	Uns	107	(262.12)
Lead	Pb	82	207.2	Uranium	U	92	238.02
Lithium	Li	3	6.94	Vanadium	V	23	50.94
Lutetium	Lu	71	174.96	Xenon	Xe	54	131.29
Magnesium	Mg	12	24.30	Ytterbium	Yb	70	173.04
Manganese	Mn	25	54.93	Yttrium	Y	39	88.90
Mendelevium	Md	101	(258.10)	Zinc	Zn	30	65.39
Mercury	Hg	80	200.59	Zirconium	Zr	40	91.22
Molybdenum	Mo	42	95.94				

Based on 1987 IUPAC Table of Standard Atomic Weights of the Elements.

\* Values in brackets indicate the relative atomic mass of the isotope of that element with the longest known half-life.

## Amino acids table

Amino acid	Three-letter code	Single-letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic Acid	Asp	D	
Cysteine	Cys	C	
Glutamic Acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

## Amino acids table (continued)

Formula	FW	Middle unit residue (-H <sub>2</sub> O)		Charge at pH 6.0–7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
		Formula	FW				
C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub>	89.1	C <sub>3</sub> H <sub>5</sub> NO	71.1	Neutral	■		
C <sub>6</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub>	174.2	C <sub>6</sub> H <sub>12</sub> N <sub>4</sub> O	156.2	Basic (+ve)			■
C <sub>4</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>	132.1	C <sub>4</sub> H <sub>6</sub> N <sub>2</sub> O <sub>2</sub>	114.1	Neutral		■	
C <sub>4</sub> H <sub>7</sub> NO <sub>4</sub>	133.1	C <sub>4</sub> H <sub>5</sub> NO <sub>3</sub>	115.1	Acidic (-ve)			■
C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub> S	121.2	C <sub>3</sub> H <sub>5</sub> NOS	103.2	Neutral		■	
C <sub>5</sub> H <sub>9</sub> NO <sub>4</sub>	147.1	C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub>	129.1	Acidic (-ve)			■
C <sub>5</sub> H <sub>10</sub> N <sub>2</sub> O <sub>3</sub>	146.1	C <sub>5</sub> H <sub>8</sub> N <sub>2</sub> O <sub>2</sub>	128.1	Neutral		■	
C <sub>2</sub> H <sub>5</sub> NO <sub>2</sub>	75.1	C <sub>2</sub> H <sub>3</sub> NO	57.1	Neutral		■	
C <sub>6</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub>	155.2	C <sub>6</sub> H <sub>7</sub> N <sub>3</sub> O	137.2	Basic (+ve)			■
C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	131.2	C <sub>6</sub> H <sub>11</sub> NO	113.2	Neutral	■		
C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	131.2	C <sub>6</sub> H <sub>11</sub> NO	113.2	Neutral	■		
C <sub>6</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	146.2	C <sub>6</sub> H <sub>12</sub> N <sub>2</sub> O	128.2	Basic (+ve)			■
C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> S	149.2	C <sub>5</sub> H <sub>9</sub> NOS	131.2	Neutral	■		
C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	165.2	C <sub>9</sub> H <sub>9</sub> NO	147.2	Neutral	■		
C <sub>5</sub> H <sub>9</sub> NO <sub>2</sub>	115.1	C <sub>5</sub> H <sub>7</sub> NO	97.1	Neutral	■		
C <sub>3</sub> H <sub>7</sub> NO <sub>3</sub>	105.1	C <sub>3</sub> H <sub>5</sub> NO <sub>2</sub>	87.1	Neutral		■	
C <sub>4</sub> H <sub>9</sub> NO <sub>3</sub>	119.1	C <sub>4</sub> H <sub>7</sub> NO <sub>2</sub>	101.1	Neutral		■	
C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	204.2	C <sub>11</sub> H <sub>10</sub> N <sub>2</sub> O	186.2	Neutral	■		
C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>	181.2	C <sub>9</sub> H <sub>9</sub> NO <sub>2</sub>	163.2	Neutral		■	
C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	117.1	C <sub>5</sub> H <sub>9</sub> NO	99.1	Neutral	■		

# General Information / Nucleic Acids

## Nucleic Acid Conversion Data

### Common abbreviations

bp	-	base pair(s)	Mb	-	megabase; 10 <sup>6</sup> bases (or base pairs)
dNTP	-	2'-deoxynucleoside 5'-triphosphate	m	-	molecular mass (g/mol), 1/12 the mass of <sup>12</sup> C
ddNTP	-	2', 3'-dideoxynucleoside 5'-triphosphate	M <sub>r</sub>	-	relative molecular mass (molecular weight), ratio between the mass of 1 mole of a substance and 1 mole of <sup>12</sup> C. M <sub>r</sub> is dimensionless.
ds	-	double-stranded (i.e., double-stranded DNA)	mol	-	mole(s)
kb	-	kilobase; 1000 bases (or base pairs)	NTP	-	ribonucleoside 5'-triphosphate
kDa	-	kilodalton	ss	-	single-stranded (i.e. single-stranded DNA)
M	-	molarity; number of moles per liter of solution			

### Codon dictionary

5'-OH Terminal base	Middle base				3'-OH Terminal base
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G

5'-OH Terminal base	Middle base				3'-OH Terminal base
	U	C	A	G	
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val*	Ala	Glu	Gly	G

\* Codes for Met if in the initiator position.

### Metric conversions

1 kg (kilogram)	= 10 <sup>3</sup> g
1 g (gram)	= 1 g
1 mg (milligram)	= 10 <sup>-3</sup> g
1 µg (microgram)	= 10 <sup>-6</sup> g
1 ng (nanogram)	= 10 <sup>-9</sup> g
1 pg (picogram)	= 10 <sup>-12</sup> g
1 fg (femtogram)	= 10 <sup>-15</sup> g

### Spectrophotometric quantitation of DNA or RNA

Spectrophotometric measurements of nucleic acid solutions are typically taken at wavelengths of 260 and 280 nm. The A<sub>260</sub> reading is used to determine the concentration of nucleic acid in solution. For a solution with an A<sub>260</sub> = 1.0, the following approximations hold:

- 1 A<sub>260</sub> unit of dsDNA = 50 µg/ml
- 1 A<sub>260</sub> unit of ssDNA = 37 µg/ml\*
- 1 A<sub>260</sub> unit of ssRNA = 40 µg/ml

\* For oligonucleotides, an A<sub>260</sub> of 1.0 represents anywhere from 20 to 33 µg/ml with the actual conversion factor dependent on the length and base sequence of the oligonucleotide (1).

The ratio between measurements at 260 and 280 nm provides an indication of the purity of a nucleic acid solution. In solution, pure DNA and RNA typically have A<sub>260</sub>/A<sub>280</sub> ratios of 1.8 and 2.0, respectively. If the absorbance ratio is significantly less than the values above, the nucleic acid solution is probably contaminated with protein or phenol. Accurate quantitation of a contaminated nucleic acid solution is not feasible without prior purification, and the efficacy of this can be established by the A<sub>260</sub>/A<sub>280</sub> ratio.

Most of the lyophilized polynucleotides are sold as A<sub>260</sub> units (2). For an approximation of quantity, use the conversion factors provided above to convert the A<sub>260</sub> units into micrograms—which must be known if a certain concentration is desired.

- 1 For a more accurate approximation, refer to Borer in the *Handbook of Biochemistry and Molecular Biology*, 3rd edition (G.D. Fasman, ed.) CRC Press, Cleveland, OH, page 589 (1975).
- 2 Unit definition: One unit is that quantity of oligonucleotide or polynucleotide which has an absorbance of 1.0 at a given wavelength when dissolved in 1 ml of buffer and measured in a 1 cm cuvette at 20°C. The wavelength at which the absorbance is measured is printed on the Certificate of Analysis which accompanies the product. For nucleic acids, typically an absorbance is taken at 260 nm in 20 mM sodium phosphate (pH 7.0), 0.1 M NaCl.

### Molar masses of nucleic acids

Average molar mass of a deoxynucleotide base	= 324.5 g/mol
Average molar mass of a deoxynucleotide base pair	= 649 g/mol
Average molar mass of a ribonucleotide base	= 340.5 g/mol
1 kb of dsDNA (sodium salt)	= $6.5 \times 10^5$ g/mol
1 kb of ssDNA (sodium salt)	= $3.3 \times 10^5$ g/mol
1 kb of ssRNA (sodium salt)	= $3.4 \times 10^5$ g/mol

$1 \times 10^6$ g/mol of dsDNA (sodium salt)	= 1.54 kb
$\lambda$ DNA	= $3.1 \times 10^7$ g/mol (1)
pBR322 DNA	= $2.8 \times 10^6$ g/mol (1)
$\phi$ X-174 DNA	= $3.6 \times 10^6$ g/mol (1)
<i>E. coli</i> DNA	= $3.1 \times 10^9$ g/mol (1)

1. Ausubel, F. M. et al. (eds.), in *Current Protocols in Molecular Biology*, Wiley and Sons, Inc., NY (1989).

### Mass-to-mole conversions

1 $\mu$ g/ml of DNA	= 3.08 $\mu$ M phosphate
1 $\mu$ g/ml of a 1 kb DNA fragment	= 3.08 nM 5'-ends
1 $\mu$ g of a 1 kb DNA fragment	= 1.5 pmol = $9.1 \times 10^{11}$ molecules
1 $\mu$ g of a 1 kb DNA fragment	= 3.0 pmol 5'-ends
1 pmol of a 1 kb DNA fragment	= 0.65 $\mu$ g
1 $\mu$ g of pUC18/19 DNA (2686 bp)	= 0.57 pmol = $3.4 \times 10^{11}$ molecules
1 pmol of pUC 18/19 DNA	= 1.77 $\mu$ g
1 $\mu$ g of pBR322 DNA (4361 bp)	= 0.35 pmol = $2.1 \times 10^{11}$ molecules

1 $\mu$ g of linear pBR322 DNA	= 0.70 pmol of 5'-ends
1 pmol of pBR322 DNA	= 2.83 $\mu$ g
1 pmol of 5'-ends of linear pBR322	= 1.4 $\mu$ g
1 $\mu$ g of M13mp18/19 DNA (7249 bp)	= 0.21 pmol = $1.3 \times 10^{11}$ molecules
1 pmol of M13mp18/19 DNA	= 4.78 $\mu$ g
1 $\mu$ g of $\lambda$ DNA (48 502 bp)	= 0.033 pmol = $1.8 \times 10^{10}$ molecules
1 pmol of $\lambda$ DNA	= 32.01 $\mu$ g

### Determining the molar mass and moles of ends of a double-stranded DNA fragment

Molar mass of a dsDNA fragment	= (# of bp) $\times$ (649 g/mol/bp)
Moles of ends of linear DNA	= $2 \times$ (g of DNA)/(# of bp) $\times$ (649 g/mol/bp)
Moles of ends generated by a restriction digest:	
for linear DNA	= (# of cuts) $\times$ (moles of DNA) $\times$ 2 (ends per cut) + 2 (ends of linear DNA) $\times$ (moles of DNA)
for circular DNA	= (# of cuts) $\times$ (moles of DNA) $\times$ 2 (ends per cut)

# Nucleic Acids

## Nucleic Acid Conversion Data

### Determining molar masses using $S_{20,W}$ values

An  $S_{20,W}$  value for many of our polynucleotides is printed on the Certificate of Analysis that accompanies the product. This value may be used to determine the average molecular mass and average length of the polymer. Our polynucleotides consist of molecules with a distribution of varying lengths; therefore, only an average length can be calculated from an  $S_{20,W}$  value.

#### For DNA

The relationship between the sedimentation velocity ( $S_{20,W}$ ) of sodium DNAs and molar masses (m) for solutions in 1 M sodium salt is expressed in the formulae (1):

#### Native, dsDNA

$$S_{20,W} = 0.0882 m^{0.346}$$

$$\log m = 2.89 (\log S_{20,W} + 1.055)$$

#### Neutral, denatured ssDNA

$$S_{20,W} = 0.0105 m^{0.549}$$

$$\log m = 1.82 (\log S_{20,W} + 1.979)$$

The chart below is derived from the above equations and may be used for quick estimates of the average molar masses and average lengths of our polynucleotides:

$S_{20,W}$ Value	Native ds DNA		Neutral ss DNA	
	m (g/mol)	bp	m (g/mol)	bases
1.1	1474	2	4754	14
2.0	8297	12	14 114	43
3.0	26 781	41	29 521	90
4.0	61 504	94	49 834	152
5.0	117 212	179	74 800	229
6.0	198 521	304	104 235	319
7.0	309 944	475	137 993	423
8.0	455 910	699	175 955	539
9.0	640 782	982	218 022	668
10.0	868 860	1332	264 107	810
11.0	1 144 392	1755	314 133	963
12.0	1 471 579	2257	368 035	1128
13.0	1 854 583	2844	425 752	1305
14.0	2 297 523	3523	487 228	1494
15.0	2 804 488	4301	552 415	1694

#### For RNA

The relationship between the sedimentation velocity ( $S_{20,W}$ ) of sodium RNAs and molar masses (m) for solutions in 1 M sodium salt is expressed in the formula:

#### Native, single-stranded RNA

$$S_{20,W} = 0.030 m^{0.459} \text{ [Average of two equations (2, 3)]}$$

$$\log m = 2.179 (\log S_{20,W} + 1.523)$$

The chart below is derived from the above equations:

$S_{20,W}$	m (g/mol)	Approx. base length (Avg. FW = 361)*
4	42 658	118
5	69 502	193
6	103 276	286
7	144 544	400
8	195 147	535
9	249 459	691
10	314 775	872
11	386 367	1070
12	467 735	1296
13	557 186	1543
14	654 636	1813

\* Average Formula Weight (Avg. FW) is obtained by averaging the following formula weights of the sodium salts of A, C, G and U:

$$A = 371$$

$$C = 344$$

$$G = 384$$

$$U = 345$$

#### References

- Studier, F. W., *J. Mol. Biol.* **11**, 373 (1967).
- Eisenberg, H. and Felsenfeld, G., *J. Mol. Biol.* **30**, 17 (1967).
- Inners, L. D. and Felsenfeld, G., *J. Mol. Biol.* **50**, 373 (1970).

### Concentration of DNA in solution\*

Double-stranded DNA (50 $\mu$ g/ml)	Molecules/ml	Moles/ml	Molar concentration	Molar concentration of termini
Bacteriophage $\lambda$	$9.78 \times 10^{11}$	$1.62 \times 10^{-12}$	1.62 nM	3.24 nM
pBR322	$1.09 \times 10^{13}$	$1.81 \times 10^{-11}$	18.1 nM	36.2 nM
pUC18/pUC19	$1.77 \times 10^{13}$	$2.94 \times 10^{-11}$	29.4 nM	58.8 nM
Segment of DNA (1 kb)	$4.74 \times 10^{13}$	$7.87 \times 10^{-11}$	78.7 nM	157.4 nM
Octameric double-stranded linker	$5.92 \times 10^{15}$	$9.83 \times 10^{-9}$	9.83 $\mu$ M	19.7 $\mu$ M

A solution containing 50  $\mu$ g/ml of dsDNA has an absorbance of 1.0 at 260 nm, i.e.,  $A_{260} = 1.0 = 50 \mu\text{g/ml}$  of dsDNA. A solution containing 37  $\mu$ g/ml of ssDNA has an absorbance of 1.0 at 260 nm, i.e.,  $A_{260} = 1.0 = 37 \mu\text{g/ml}$  of ssDNA. These values are calculated assuming that the mass of a nucleotide pair in DNA is 660 g/mol. (Note: At GE Healthcare, we employ the approximation that 1.0  $A_{260}$  unit of ssDNA = 37  $\mu$ g/ml.)

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## MicroSpin column selection guide

DNA	Purpose	First choice	Second choice*	Third choice*	Others OK?
PCR products	buffer exchange	G-25	G-50 or AutoSeq (lower yield)	S-200 (lower yield)	All except ProbeQuant
	primer removal prior to sequencing	S-300	S-400 (lower yield)	S-200 (lower purity)	None
	primer removal prior to cloning	S-400	S-300 (lower purity)	None	None
labeled dsDNA	removal of unincorporated nucleotides	ProbeQuant	G-50 or AutoSeq (lower yield)	S-200 (lower yield)	All OK
Dye-terminator labeled DNA	removal of unincorporated fluorescent dye-terminators	AutoSeq	G-50 (poss. salt effects)	S-200 (poss. salt effects/lower yield)	None
labeled oligonucleotides	removal of unincorporated nucleotides from end-labeled oligonucleotides	G-25	ProbeQuant (lower yield)	G-50 (lower yield)	None
plasmid DNA	removal of NaOH from denatured plasmid DNA, RNase treated	G-25	G-50 or AutoSeq (lower yield)	S-200 (lower yield)	All except ProbeQuant
	removal of NaOH from denatured plasmid DNA, RNA present	S-300	S-400 (lower yield)	S-200 (lower purity)	None
oligonucleotides	buffer exchange/desalting of oligonucleotides after synthesis	G-25	None	None	None
unlabeled dsDNA	buffer exchange of unlabeled dsDNA	G-25	G-50 or AutoSeq (lower yield)	S-200 (lower yield)	All except ProbeQuant

\* Reasons why columns are second or third choice are given in parentheses.

## Centrifuges, rotors, and carriers for use with MicroPlex 24

Centrifuge model	Manufacturer's number	Manufacturer	Rotor	Separate carriers needed?	Number of places	Maximum g-force and rpm
GS-15	#360908	Beckman	S2096	no	2	1100 × g, 3000 rpm
GS-15R	#360902		(#361111)			
Sorvall RT-6000B	#07983	DuPont	H1000B (#11706)	yes #11093	2	1900 × g, 3000 rpm
6K	#83794	Fisher	#04-976-420	yes #04-975-410 MT	2	1506 × g, 3500 rpm
Megafuge 1.0	#C1725-3 (Baxter #)	Heraeus	#C1725-10	yes #C1725-35	4	1280 × g, 2800 rpm
Z-320	#C-0320	Hermle	#C-0320-50	no	2	1506 × g, 3500 rpm
GP8	#3121	IEC	#216 or	yes	2 or 4	2340 × g, 3500 rpm
GP8R	#3122		#228	#5785		
HN-SII	#2355	IEC	#244	no	2	1450 × g, 3575 rpm
MP4	#2437	IEC	#244	no	2	1450 × g, 3575 rpm
MP4R	#2438					
C312	#11175087	Jouan	E4	yes	4	2290 × g, 3300 rpm
CR312	#11175090		(#11174153)	#11174168		
1130	Not required	Kubota	S11222	no	2	1107 × g, 3000 rpm
1140	Not required	Kubota	S11222	no	2	1107 × g, 3000 rpm

## Rotor speed (rpm) calculation for illustra AutoSeq 96 Dye Terminator Clean-up Kit

AutoSeq96 Dye Terminator Clean-up Kit requires a relative centrifugal force (RCF) of 910 × g for optimal performance.

RCF can be calculated using the formula:

$$RCF = 1.12r(RPM/1000)^2$$

where r = the radius of the centrifuge rotor in mm.

For illustra AutoSeq 96, this formula can be simplified by using 910 × g for RCF and solving for RPM:

$$RPM = (2.85 \times 10^4) / r^{1/2}$$

Check the user manual of the rotor to be used to verify the rotor radius. If this information is not available, the rotor radius can be determined by measuring the distance, in mm, from the center of the rotor shaft to the surface of the adapter where the illustra AutoSeq 96 plate will be resting when the rotor arm is in its fully extended position.

# Nucleic Acids

## Nucleotides

### Nucleotide quick reference chart

Nucleotide	Code Number	FW (1)	Chemical Formula (1)	$\lambda$ max (pH 7.0)	$A_m$ (pH 7.0) (2)
<b>2'-Deoxynucleoside 5'-Triphosphates, Solutions</b>					
2'-dATP, 100 mM Solution	28-4065-01, -02, -03	579.2	$C_{10}H_{12}N_5O_{12}P_3Na_4$	259 nm	$15.2 \times 10^3$ d
2'-dCTP, 100 mM Solution	28-4065-11, -12, -13	555.1	$C_9H_{12}N_3O_{13}P_3Na_4$	280 nm <sup>a</sup>	$13.1 \times 10^3$ a,e
2'-dGTP, 100 mM Solution	28-4065-21, -22, -23	595.1	$C_{10}H_{12}N_5O_{13}P_3Na_4$	253 nm	$13.7 \times 10^3$ f
2'-dTTP, 100 mM Solution	28-4065-31, -32, -33	570.1	$C_{10}H_{13}N_2O_{14}P_3Na_4$	267 nm	$9.6 \times 10^3$ g
2'-dUTP, 100 mM Solution	28-4065-41	556.1	$C_9H_{11}N_2O_{14}P_3Na_4$	262 nm	$10.2 \times 10^3$ i
c7'-2'-dGTP, 5 mM Solution	27-2090-02	595.1	$C_{11}H_{13}N_4O_{13}P_3Na_4$	257 nm	$10.5 \times 10^3$ c
<b>2'-Deoxynucleoside 5'-Triphosphates</b>					
2'-dATP, Disodium, Crystalline	27-1850-04	589.2	$C_{10}H_{14}N_5O_{12}P_3Na_2 \bullet 3 H_2O$	259 nm	$15.2 \times 10^3$ d
2'-dCTP, Sodium, Lyophilized	27-1860-04	569.1	$C_9H_{12}N_3O_{13}P_3Na_3 \bullet 2 H_2O$	280 nm <sup>a</sup>	$13.1 \times 10^3$ a,e
2'-dGTP, Sodium, Lyophilized	27-1870-04	609.2	$C_{10}H_{13}N_5O_{13}P_3Na_3 \bullet 2 H_2O$	253 nm	$13.7 \times 10^3$ f
2'-d2'-dTTP, Sodium, Lyophilized	27-1880-04	584.1	$C_{10}H_{14}N_2O_{14}P_3Na_3 \bullet 2 H_2O$	267 nm	$9.6 \times 10^3$ g
<b>2',3'-Dideoxynucleoside 5'-Triphosphates, Solutions</b>					
2',3'-ddATP, 100 mM Solution	27-2051-01	563.1	$C_{10}H_{12}N_5O_{11}P_3Na_4$	259 nm	$15.2 \times 10^3$ d
2',3'-ddCTP, 100 mM Solution	27-2061-01	539.1	$C_9H_{12}N_3O_{12}P_3Na_4$	280 nm <sup>a</sup>	$13.1 \times 10^3$ a,e
2',3'-ddGTP, 100 mM Solution	27-2071-01	579.1	$C_{10}H_{12}N_5O_{12}P_3Na_4$	253 nm	$13.7 \times 10^3$ f
2',3'-ddTTP, 100 mM Solution	27-2081-01	554.1	$C_{10}H_{13}N_2O_{13}P_3Na_4$	267 nm	$9.6 \times 10^3$ g
<b>Ribonucleoside 5'-Triphosphates, Solutions</b>					
ATP, 100 mM Solution	27-2056-01	595.1	$C_{10}H_{12}N_5O_{13}P_3Na_4$	259 nm	$15.4 \times 10^3$
CTP, 100 mM Solution	27-2066-01	571.1	$C_9H_{12}N_3O_{14}P_3Na_4$	280 nm <sup>a</sup>	$13.0 \times 10^3$ a
GTP, 100 mM Solution	27-2076-01	611.1	$C_{10}H_{12}N_5O_{14}P_3Na_4$	252 nm	$13.7 \times 10^3$
UTP, 100 mM Solution	27-2086-01	572.1	$C_9H_{11}N_2O_{15}P_3Na_4$	262 nm	$10.2 \times 10^3$
<b>Ribonucleoside 5'-Triphosphates</b>					
ATP, Disodium, Crystalline	27-1006-01, -03	605.2	$C_{10}H_{14}N_5O_{13}P_3Na_2 \bullet 3 H_2O$	259 nm	$15.4 \times 10^3$
CTP, Sodium, Lyophilized	27-1200-04	585.1	$C_9H_{13}N_3O_{14}P_3Na_3 \bullet 2 H_2O$	280 nm <sup>a</sup>	$13.0 \times 10^3$ a
GTP, Sodium, Lyophilized	27-2000-04	607.2	$C_{10}H_{13}N_5O_{14}P_3Na_3 \bullet 1 H_2O$	252 nm	$13.7 \times 10^3$

1 Salt and water composition may vary slightly from lot to lot.

2 The spectral terms and definitions used are those recommended by the National Bureau of Standards Circular LC 857, May 19, 1947 and are as follows:

$$\text{Molar Absorbancy } (A_m) = \frac{\alpha}{\text{Moles per liter}} = \frac{(\alpha) \text{ (g/mol)}}{\text{grams per liter}}$$

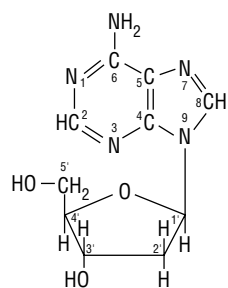
Where:  $\alpha$  = absorbancy =  $\log_{10} T = 2 - \log_{10} (100T)$

T = transmittance of a solution in a 1 cm cell with reference to a solvent blank set at T = 1.

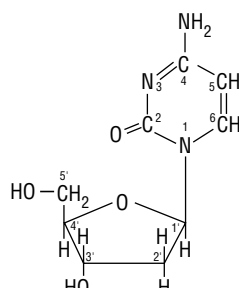
- a Spectral analysis done at pH 2.0.
- b Spectral analysis done at pH 6.0.
- c Value determined at GE Healthcare.
- d 2'-dAMP N.R.C. reference spectral constants employed.
- e 2'-dCMP N.R.C. reference spectral constants employed.
- f 2'-dGMP N.R.C. reference spectral constants employed.
- g 2'-dTMP N.R.C. reference spectral constants employed.
- h 2'-dIMP N.R.C. reference spectral constants employed.
- i 2'-dU N.R.C. reference spectral constants employed.

## Structures of representative nucleosides

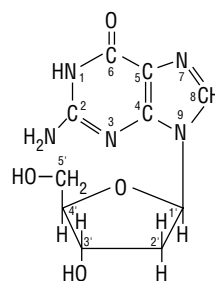
\* Purine  
 † Pyrimidine  
 R = Purine or pyrimidine base



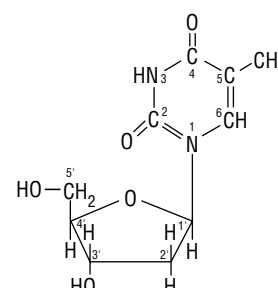
2'-deoxyadenosine\*



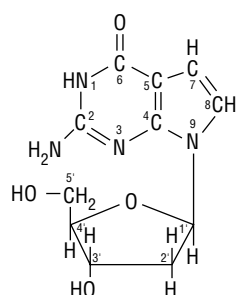
2'-deoxycytidine†



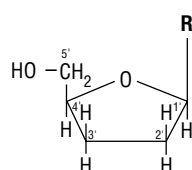
2'-deoxyguanosine\*



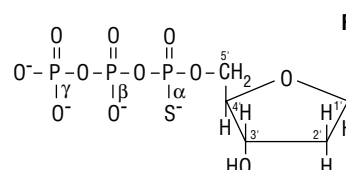
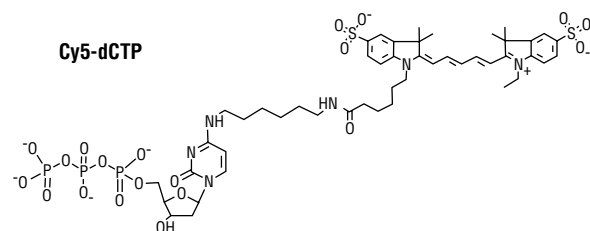
2'-deoxythymidine†



7-deaza 2'-deoxyguanosine\*



2',3'-dideoxynucleoside

 $\alpha$ -thio-deoxynucleoside triphosphate

Cy5-dCTP

## Preparation of nucleotides

Nucleotides may be dissolved in water or dilute buffers at or near pH 7.0. Since laboratory distilled water is often slightly acidic, some addition of dilute sodium hydroxide or ammonia solution may be necessary to raise the pH to 7.0.

## Determining the concentration of nucleotide solutions

The concentration of a nucleotide solution can be determined using the following formula:

$$\frac{\text{Measured absorbance at } \lambda_{\text{max}} \text{ for the nucleotide}}{\text{Absorbance at } \lambda_{\text{max}} \text{ for a 1 M solution of the nucleotide}} = \text{Molar concentration}$$

## Storage of oligonucleotides

Oligonucleotides are stable for years when stored as lyophilized powders at  $-20^{\circ}\text{C}$ . They are less stable in solution but can be kept for several months if stored at  $-20^{\circ}\text{C}$ . Oligonucleotides can be stored in sterile water or dilute buffers at or near pH 7.0.

# Nucleic Acids

## Oligonucleotides / Amidites

### Preparation of oligonucleotides

Oligonucleotides may be dissolved in water or dilute buffers at or near pH 7.0. Since laboratory distilled water is often slightly acidic, some addition of dilute sodium hydroxide or ammonia solution may be necessary to raise the pH to 7.0.

### Determining the molar mass of an oligonucleotide

The molar mass (m) of a DNA oligonucleotide can be calculated from the formula\*:

$$m \text{ (g/mol)} = [(dA \times 312.2) + (dC \times 288.2) + (dG \times 328.2) + (dT \times 303.2)] + [(m \text{ counter-ion}) \times (\text{length of oligo in bases})]$$

The molar mass calculated using this equation must be adjusted for the contribution of the atoms at the 5' and 3' ends of the oligonucleotide. This adjustment is necessary because the weight of the repeat units is calculated for nucleotides located in internal positions within the oligonucleotide.

Phosphorylated oligos, add:  $17 + (2 \times m \text{ counter-ion})$

Nonphosphorylated oligos, subtract:  $61 + (m \text{ counter-ion})$

The molar (atomic) masses of the most common oligonucleotide counter-ions are:

Counter-ion	m (g/mol)
Sodium	23.0
Potassium	39.1
Triethylammonium	102.2

\* The molar mass listed for each nucleotide is the molar mass of that nucleotide incorporated in the oligo.

### Determining the number of micromoles of an oligonucleotide

1. Determine the number of  $\mu\text{g}$  of the oligonucleotide: 1  $A_{260}$  unit of ssDNA = 20–33  $\mu\text{g}$ .
2. Determine the molar mass of the oligonucleotide: see above.
3.  $\mu\text{mol} = \mu\text{g}/m$

### Estimating the concentration of an oligonucleotide

N = number of bases in the oligonucleotide

$$\epsilon_{260} \approx 10^4 \times N \text{ (M/cm)}$$

$$m \approx 324.5 \text{ g/mol} \times N$$

$$\frac{A_{260}}{\epsilon_{260}} \times 10^6 = \text{concentration } (\mu\text{M})$$

$$\text{concentration } (\mu\text{M}) \times m = \text{concentration (ng/ml)}$$

### Determining the specific activity of a probe produced by oligolabeling

$$SA = \frac{(\mu\text{Ci})(2.2 \times 10^9)(F)}{D_i + [(1.3 \times 10^3)(F)(\mu\text{Ci}/S)]}$$

where SA = Specific activity of DNA (dpm/ $\mu\text{g}$ );

$\mu\text{Ci}$  =  $\mu\text{Ci}$  of dNTP in reaction;

F = Fraction of input label incorporated into DNA (acid-precipitable counts/total counts);

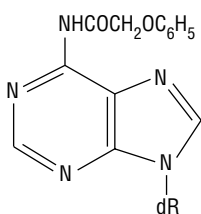
$D_i$  = Mass of input DNA template (in ng);

S = Specific activity of dNTP (in Ci/mmol =  $\mu\text{Ci}/\text{nmol}$ ).

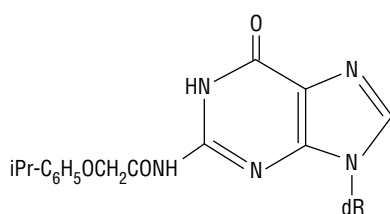
The numerator of this equation is derived by multiplying three factors: the total dpm in the reaction  $[(\mu\text{Ci})(2.2 \times 10^9)]$ ; the fraction of these dpm which were incorporated (F); and a factor to convert the final value for SA from dpm/ng to dpm/ $\mu\text{g}$  ( $10^3$ ).

The denominator represents the total mass of DNA (in ng) at the end of the reaction, equal to the starting mass ( $D_i$ ) plus the mass (in ng) synthesized during the reaction. The latter is calculated from the number of nanomoles of dNMP incorporated  $[(F)(\mu\text{Ci}/S)]$ , multiplied by four times the average molar mass of the four dNMPs  $[(4)(325) = 1.3 \times 10^3]$ .

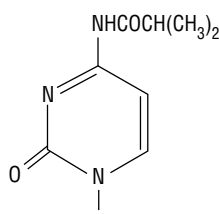
### Structures of PAC amidites



PAC dA phosphoramidite



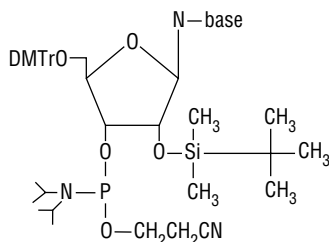
iPr-PAC dG phosphoramidite



IBU dC phosphoramidite

dR=2'-deoxy-5'-O-dimethoxytrityl- $\beta$ -D-erythro-pentofuranosyl-3'-O-( $\beta$ -cyanoethyl-N,N-diisopropylamino) phosphoramidite. Only the amino-protecting groups are different; the 5'-hydroxyl is protected by the dimethoxytrityl group and the phosphoramidite functions are still diisopropylamino and  $\beta$ -cyanoethyl. PAC amidites are used under conditions identical to those used for standard phosphoramidites.

## Structure of riboamidites



Chemical structure of riboamidites. TBDMS, t-butyldimethylsilyl, protects the 2'-hydroxyl group. Protecting groups for the amine groups in the base are as follows: Phenoxyacetyl (PAC) for A; Acetyl for C isopropyl-phenoxyacetyl (iPr-PAC) for G; and none for U.

## Preparation of polynucleotides

Single-stranded polymers may be dissolved in water or dilute buffers at or near pH 7.0. Since laboratory distilled water is often slightly acidic, some addition of dilute sodium hydroxide or ammonia solution may be necessary to raise the pH to 7.0.

Double-stranded polynucleotides should be dissolved in an appropriate salt-buffer mixture (application-dependent) to maintain the double-strand configuration.

## Solubility of polynucleotides

Sometimes it is difficult to rehydrate a polymer. If you have trouble getting a polynucleotide into solution, try one of the following procedures:

- Agitate on a vortex mixer.
- Gently heat at 40 to 50°C for 5 to 10 min.
- Add dilute NaOH or NH<sub>3</sub> to bring the solution to pH 7.0.
- For DNA polymers, dissolve in alkaline solution, then titrate to pH 7.0.

Polymers rich in guanosine are most difficult to dissolve. For these polynucleotides, the following may be necessary:

- Heat at 90 to 100°C for 2 to 3 min. This may be necessary to break up aggregates of a G-containing polymer after the polymer has been dissolved. After heating at 100°C, cool slowly to reaction temperature (usually 37°C).
- Sonicate mildly for 30 s.

## Storage of polynucleotides

Polynucleotides are stable for years when stored as lyophilized powders at -20°C. Polymers are less stable in solution but can be kept for several months if stored at -20°C. Polymers can be stored in sterile water or dilute buffers at or near pH 7.0. To maintain the polymer's double-stranded configuration, 100 mM NaCl should be added to the storage buffer.

# Nucleic Acids

## Polynucleotides

### Extinction coefficients for common polynucleotides

Product	$\lambda$ -max (nm)	$\epsilon$ -max (l/mol $\times$ cm $\times$ bp or base)	pH	Reference
<b>DNA homopolymers</b>				
Poly(dA)	257	8,600	7.0	1
Poly(dC)	274	7,400	7.0	2
		6,800	7.0	7
Poly(dT)	264	8,520	7.0	2
<b>DNA duplexes</b>				
Poly(dA)-Poly(dT)	260	6,000	7.5	3
Poly(dG)-Poly(dC)	253	7,400	7.5	3
Poly(dI)-Poly(dC)	245	5,300	8.0	4
<b>DNA alternating copolymers</b>				
Poly(dA-dT)-Poly(dA-dT)	262	6,600	6.4	5
Poly(dI-dC)-Poly(dI-dC)	251	6,900	7.0	6
Poly(dG-dC)-Poly(dG-dC)	254	8,400	7.5	3
Poly(dA-dC)-Poly(dG-dT)	258	6,500	7.5	3
<b>RNA homopolymers</b>				
Poly(A)	258	9,800	7.0	7
Poly(C)	269	6,200	7.0	7
Poly(U), K+	260	9,350	7.0	7

For an approximation of quantity, use the conversion factors (see pages 138 and 660), under Spectrophotometric quantitation of DNA or RNA.

#### References

1. Chamberlin, M. J., *Fed. Proc.* **24**, 1446 (1965).
2. Tso, P. O. et al., *Biochemistry* **5**, 4153 (1966).
3. Wells, R. D. et al., *J. Mol. Biol.* **54**, 465 (1970).
4. Inman, R. B. et al., *J. Mol. Biol.* **8**, 452 (1964).
5. Inman, R. B. et al., *J. Mol. Biol.* **5**, 172 (1962).
6. Grant, R. C., *JACS* **90**, 4474 (1968).
7. GE Healthcare, unpublished results.

## Polynucleotides / Vector Sequencing Primers

## Composition of common polynucleotides

	Poly(dI-dC)-Poly(dI-dC)	Poly(I)-Poly(C)	Poly(rA)-Oligo p(dT) <sub>12-18</sub>	Poly(rC)-Oligo p(dG) <sub>12-18</sub>
<b>Composition</b>	This product is a DNA alternating copolymer composed of two Poly(dI-dC) strands annealed to each other. The sequence of each strand is an alternating sequence of ICICIC... The product is believed to be primarily double-stranded, although some single-stranded regions may be present. The product is supplied in the sodium salt form.	A duplex RNA polymer composed of a Poly(I) strand and a Poly(C) strand. The product is believed to be primarily double-stranded in nature, although some single-stranded regions may be present. The product is supplied as the potassium salt.	Poly(rA)-Oligo p(dT) <sub>12-18</sub> is a template-primer with a Poly(rA) backbone and annealed Oligo p(dT) <sub>12-18</sub> . The ratio of the number of As and Ts is one to one. (Note, there is a much higher molar ratio of Oligo p(dT) <sub>12-18</sub> to Poly(rA). The 1:1 ratio refers to the ratio of each nucleotide present.) The template-primer is supplied as a lyophilized solid, sodium salt.	Poly(rC)-Oligo p(dG) <sub>12-18</sub> is a template-primer with a Poly(rC) backbone and annealed Oligo p(dG) <sub>12-18</sub> . The ratio of the number of Cs and Gs is one to one. (Note, there is a much higher molar ratio of Oligo p(dG) <sub>12-18</sub> to Poly(rC). The 1:1 ratio refers to the ratio of each nucleotide present.) The template-primer is supplied as a lyophilized solid, sodium salt.
<b>Conversions</b>	1 A <sub>260</sub> unit is ~ 50 µg of polymer. Note: The OD/mg (units/mg) value shown on the Certificate of Analysis is simply a measure of the residual salt present in the lyophilized nucleotide mixture and should not be used to convert the mass of lyophilized product to a number of absorbance units.	1 A <sub>260</sub> unit is ~ 50 µg of polymer.	1 A <sub>260</sub> unit is ~ 50 µg of polymer. Note: The OD/mg (units/mg) value shown on the Certificate of Analysis is simply a measure of the residual salt present in the lyophilized nucleotide mixture and should not be used to convert the mass of lyophilized product to a number of absorbance units.	1 A <sub>260</sub> unit is ~ 50 µg of polymer. Note: The OD/mg (units/mg) value shown on the Certificate of Analysis is simply a measure of the residual salt present in the lyophilized nucleotide mixture and should not be used to convert the mass of lyophilized product to a number of absorbance units.
<b>Extinction coefficient</b>	The extinction coefficient is also known as molar absorptivity. $6.9 \times 10^3$ (l/mol × cm × bp) at 251 nm (pH 7.0) (1). 1. Grant, R. C., JACS 90, 4474 (1968).	The extinction coefficient is also known as molar absorptivity. <b>Poly(C):</b> $6.2 \times 10^3$ (l/mol × cm × base) at 269 nm (pH 7.0) <b>Poly(I):</b> $10.2 \times 10^3$ (l/mol-cm-base) at 248 nm (pH 7.0)	The extinction coefficient is also known as molar absorptivity. <b>Poly(rA):</b> $9.8 \times 10^3$ (l/mol × cm × base) at 258 nm (pH 7.0) (1). 1. GE Healthcare, unpublished results.	The extinction coefficient is also known as molar absorptivity. <b>Poly(rC):</b> $6.2 \times 10^3$ (l/mol × cm × base) at 269 nm (pH 7.0) (1). 1. GE Healthcare, unpublished results.
<b>Length in base pairs</b>	Observed length range 951–8009 bp. The average length of the Poly(dI-dC)-Poly(dI-dC) can be calculated by dividing the average molecular mass of the lot by the molecular mass of a base pair (using Na <sup>+</sup> as the counter ion), which is 647 g/mol.	No length information is provided on this product since the two strands are synthesized independently and then annealed together.	Observed length range [Poly(rA)]: 286–428 bases. The average length of the poly(rA) can be calculated by dividing the average molecular mass of the lot by the molecular mass of A (using K <sup>+</sup> as the counter ion) which is 367 g/mol.	Observed length range [Poly(rC)]: 277–414 bases. The average length of the poly(dG) can be calculated by dividing the average molecular mass of the lot by the molecular mass of G (using K <sup>+</sup> as the counter ion) which is 383 g/mol.
<b>Primer-template ratio</b>	Not applicable.	Not applicable.	The ratio of the number of As and Ts is one to one. (Note, there is a much higher molar ratio of Oligo p(dT) <sub>12-18</sub> to Poly(rA). The 1:1 ratio refers to the ratio of each nucleotide present.)	The ratio of the number of Cs and Gs is one to one. (Note, there is a much higher molar ratio of Oligo p(dG) <sub>12-18</sub> to Poly(rC). The 1:1 ratio refers to the ratio of each nucleotide present.)

## Vectors and sequencing primers

Vector	Reverse		Universal	
	Binding region (1)	Use (2)	Binding region (1)	Use (2)
pUC18	481–465	D	379–395	D
pUC19	481–465	D	379–395	D
pSL1180	3219–3203	D	2857–2873	S/D
M13mp18	6204–6220	D	6306–6290	S/D
M13mp19	6204–6220	D	6306–6290	S/D
pEZZ 18			2990–2974	S/D

- The binding region is given in the 5'–3' orientation.
- S/D = The primer may be used for single- or double-stranded sequencing.  
D = The primer may be used for double-stranded sequencing only.

## Choosing a sequencing primer

The distance between the binding region and the target sequence must be considered when choosing a sequencing primer. For example, in pUC19, the multiple cloning site is located from 396 to 452. The distance from each of the primers to the EcoR I and Hind III sites is given below:

Cloning site	Cloning site location	Distance of primer binding site from cloning site	
		Reverse	Universal
EcoR I	396	65	1
Hind III	447	18	52

If an insert is cloned into the EcoR I site, the universal primer should not be used. However, this primer could be used for an insert cloned into the Hind III site.



## Electrophoresis of Nucleic Acids / Electrophoresis and Detection of Proteins

## Effective range of separation of DNAs in polyacrylamide gels\*

Acrylamide (% [w/v]) <sup>a</sup>	Effective range of separation (bp)	Xylene cyanol FF <sup>b</sup>	Bromophenol blue <sup>b</sup>
3.5	1000–2000	460	100
5.0	80–500	260	65
8.0	60–400	160	45
12.0	40–200	70	20
15.0	25–150	60	15
20.0	6–100	45	12

<sup>a</sup> *N,N'*-methylenebisacrylamide is included at 1/30th the concentration of acrylamide.

<sup>b</sup> The numbers given are the approximate sizes (in nucleotide pairs) of fragments of double-stranded DNA with which the dye co-migrates.

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## Recommended acrylamide concentrations for protein separation

Separation size range ( $M_r \times 10^3$ )	% Acrylamide in resolving gel
36–205	5%
24–205	7.5%
14–205	10%
14–66*	12.5%
14–45*	15%

\* The larger proteins fail to move significantly into the gel

## Recommended gel concentrations for complete semi-dry transfer of proteins from a 0.75 mm thick gel (1)

$M_r (\times 10^3)$	% Acrylamide
< 20	≤ 20%
< 36	≤ 15%
14–66	≤ 10%
29–150	≤ 5%

## References

1. Smejkal, G. and Gallagher, S., *BioTechniques* **16**, 197 (1994).

## Characteristics of total protein stains

Stain	Detection limit	Nitrocellulose	Nylon	PVDF	Comments
Ponceau S	1–2 µg	+	-	+	reversible
Amido Black*	1.5 µg	+	-	+	permanent; low background
Coomassie Blue*	1.5 µg	+	-	+	permanent; high background
India Ink	100 ng	+	-	+	permanent
Biotin-avidin	30 ng	+	+	+	permanent; fades with time
Colloidal gold	3 ng	+	-	+	permanent

\* Anionic stains.

For the permanent stains, samples of interest should be run in duplicate with half of the membranes stained by one of the methods described above and the other half used for immunodetection.

# Electrophoresis / Chromatography

## Electrophoresis and Detection of Proteins / General

### Protein marker characteristics

Protein	Source	Use*	Native M <sub>r</sub> (×10 <sup>3</sup> )	Subunit M <sub>r</sub> (×10 <sup>3</sup> )	pI at 24°C
α-2 macroglobulin	bovine plasma	S	170	170	
α-lactalbumin	bovine milk	S	14.4	14.4	
actin	bovine muscle	S	43	43	5.0, 5.1
albumin	bovine serum	N, S	66	66	5.4, 5.5, 5.6
amyloglucosidase	<i>Aspergillus niger</i>	I			3.50
aprotinin	bovine pancreas	S	6.5	6.5	
β-galactosidase	<i>Escherichia coli</i>	S	116	116	
β-lactoglobulin A	bovine milk	I	35	18.4	5.30
carbonic anhydrase	bovine erythrocyte	S, I	30	30	5.85
carbonic anhydrase	human	I		35	6.55
catalase	bovine liver	N	232	60	5.40
conalbumin	egg white	I		76	6.0, 6.3, 6.6
creatine phosphokinase	rabbit muscle	I	81	40	7.00
cytochrome c	equine heart	I	12.4	12.4	10.25
ferritin	horse spleen	N	440	220	4.23, 4.38, 4.50
				18.5	
GAPDH	rabbit muscle	I	140	36	8.3, 8.5
glucose oxidase	<i>Aspergillus niger</i>	I	160		4.15
glutamate dehydrogenase	bovine liver	S	53	53	
insulin A chain	bovine	S	2.5	2.5	
insulin B chain	bovine	S	3.5	3.5	
lactate dehydrogenase	beef heart	N	140	36	
lentil lectin	lentil	I		50	8.15, 8.45, 8.65
lysozyme	egg white	S		14.4	
myoglobin	equine heart	S, I		17.5	6.85, 7.25
myosin	rabbit muscle	S	220	220	
ovalbumin	egg white	S	45	45	4.7
pepsinogen	porcine stomach	I			2.8
phosphorylase b	rabbit muscle	S	97	97	
thyroglobulin	hog thyroid	N	669	335	
transferrin	human	S	76	76	
triose phosphate isomerase	rabbit	S	53	26.5	5.8, 6.2, 7.0, 7.7, 8.0
trypsin inhibitor	soybean	S, I	20.1	20.1	4.55
trypsinogen	bovine pancreas	I		24.0	9.3

\* Indicates protein intended use: S, SDS PAGE; I, IEF; N, non-denaturing PAGE.

### Protein detection substrates

Method	Substrate	Sensitivity	Stability of result	Restrictions
Horseradish peroxidase (HRP)	4CN,	200–500 pg	Poor	Azide,
	DAB/NiCl <sub>2</sub> , TMB	200–500 pg	Good	endogenous
	ECL	1 pg	Excellent	peroxidase
	ECL Plus	0.1 pg	Excellent	
	ECL Advance	0.01 pg	Excellent	
Alkaline phosphatase (AP)	NBT/BCIP	100 pg	Good	endogenous
	ECF	5 pg amplified	Excellent	phosphatases
Colloidal Gold		100 pg	Good	none
		5 pg if amplified		

### Common conversions

1 kb of DNA	= 333 amino acids of coding capacity
	= 37 000 g/mol
270 bp DNA	= 10 000 g/mol
1.35 kb DNA	= 50 000 g/mol
2.70 kb DNA	= 100 000 g/mol

Average molecular weight of an amino acid = 120 g/mol.

### Mass of protein to mole of protein

m (g/mol)	1 µg	1 nmol
10000	100 pmol; 6 × 10 <sup>13</sup> molecules	10 µg
50000	20 pmol; 1.2 × 10 <sup>13</sup> molecules	50 µg
100000	10 pmol; 6.0 × 10 <sup>12</sup> molecules	100 µg
150000	6.7 pmol; 4.0 × 10 <sup>12</sup> molecules	150 µg

## Concentration of protein to absorbance of protein

Protein	A <sub>280</sub> for 1 mg/ml
IgG	1.35
IgM	1.20
IgA	1.30
Protein A	0.17
Avidin	1.50
Streptavidin	3.40
Bovine Serum Albumin	0.70

## Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C

Values calculated according to *Protein Purification*, R. K. Scopes (Springer-Verlag, New York), Third Edition, p. 346, 1993. For a review of precipitation techniques in protein purification see Precipitation techniques. Englund, S. and Seifter, S., *Meth. Enzymol.* **182**, 285 (1992).

Starting percent saturation	Final percent saturation to be obtained																	
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100	
	Amount of ammonium sulfate to add (grams) per liter of solution at 20°C																	
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761	
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723	
10	57	85	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685	
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647	
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609	
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571	
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533	
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495	
40					0	31	63	96	130	166	202	241	281	322	365	410	457	
45						0	31	64	98	132	169	206	245	286	329	373	419	
50							0	32	65	99	135	172	210	250	292	335	381	
55								0	33	66	101	138	175	215	256	298	343	
60									0	33	67	103	140	179	219	261	305	
65										0	34	69	105	143	183	224	267	
70											0	34	70	107	146	186	228	
75												0	35	72	110	149	190	
80													0	36	73	112	152	
85														0	37	75	114	
90															0	37	76	
95																0	38	

## General

### Converting flow rates from linear flow rates (cm/h) to volumetric flow rates (ml/min) and vice versa

It is often convenient when comparing results for columns of different sizes to express flow rates in linear flow rate (cm/h). However, flow rates are usually measured in volumetric flow rate (ml/min). To convert between linear and volumetric flow rate use one of the formulae below.

#### From volumetric flow rate (ml/min) to linear flow rate (cm/h)

$$\begin{aligned}\text{Volumetric flow rate (ml/min)} &= \frac{\text{Linear flow rate (cm/h)}}{60} \times \text{column cross sectional area (cm}^2\text{)} \\ &= \frac{Y}{60} \times \frac{\pi \times d^2}{4}\end{aligned}$$

where

Y = linear flow rate in cm/h

d = column inner diameter in cm

*Example:*

What is the volumetric flow rate in an XK 16/70 column (i.d. 1.6 cm) when the linear flow rate is 150 cm/h?

Y = linear flow rate = 150 cm/h

d = inner diameter of the column = 1.6 cm

$$\begin{aligned}\text{Volumetric flow rate} &= \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ ml/min} \\ &= 5.03 \text{ ml/min}\end{aligned}$$

#### From linear flow rate (cm/h) to volumetric flow rate (ml/min)

$$\begin{aligned}\text{Linear flow rate (cm/h)} &= \frac{\text{Volumetric flow rate (ml/min)} \times 60}{\text{column cross sectional area (cm}^2\text{)}} \\ &= Z \times 60 \times \frac{4}{\pi \times d^2}\end{aligned}$$

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

*Example:*

What is the linear flow rate in a Tricorn 5/50 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

Z = volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

$$\begin{aligned}\text{Linear flow rate (cm/h)} &= 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \\ &= 305.6 \text{ cm/h}\end{aligned}$$

## Elution conditions for some chromatographic techniques used in protein and peptide purification

Technique	Typical starting conditions	Typical elution scheme	Typical final conditions	Comments	Page
Gel filtration	Any aqueous buffer solution, salt concentration preferably > 100 mM.	Constant eluent composition.	Same as starting conditions.	Any elution buffer can be used if the target is stable.	page 504
Anion exchange	Aqueous buffer, pH in range 4–10, concentration < 50 mM.	Linear gradient of increasing salt concentration.	Starting conditions plus 1 M NaCl.	pH usually above pI of target protein. GuHCl and anionic detergents cannot be used.	page 518
Cation exchange	Aqueous buffer, pH in range 3–8, concentration < 50 mM.	Linear gradient of increasing salt concentration.	Starting conditions plus 1 M NaCl.	pH usually below pI of target protein. GuHCl and cationic detergents cannot be used.	page 518
Hydrophobic interaction	Aqueous buffer containing 1.5 M ammonium sulfate.	Linear gradient of DECREASING salt concentration.	Starting conditions minus ammonium sulfate.	Presence of detergents during HIC may cause unsatisfactory results.	page 542
Chromatofocusing	Aqueous buffer, pH in range 9–6, concentration 25 mM.	Self-generated linear gradient of decreasing pH.	Solution of Polybuffer at 1–3 pH units below start pH.	Start pH above pI of target protein. Final pH below pI of target protein. Salts, including GuHCl, and anionic detergents cannot be used.	page 539
Reversed phase	TFA, 0.05–0.1%, acetonitrile, 1–5%, in water.	Linear gradient of increasing concentration of organic solvent.	TFA, 0.05–0.1% in acetonitrile.	All solvents should be de-gassed.	page 553
Covalent	Phosphate buffer, pH 7–8, containing NaCl, 0.5–1 M and EDTA, 1 mM.	Step gradient of reducing agent concentration.	Start buffer plus l-cysteine, 25 mM.	All solutions should be de-gassed. Avoid reducing agents in the start buffer. Avoid heavy metal ions.	page 477
Metal chelate affinity	Phosphate buffer, pH 7–8, containing NaCl, 0.5 M.	Linear gradient of imidazole concentration.	Binding buffer plus imidazole, 20–40 mM.	Avoid chelating agents (EDTA, EGTA, citrate) in the binding buffer. Tris tends to reduce binding.	page 491
<b>Affinity chromatography</b>					
His-tagged proteins on Ni <sup>2+</sup> - charged media	Phosphate, 20 mM, pH 7.4–7.6, NaCl, 0.5 M, imidazole, 20–40 mM.	Step or linear gradient of charged imidazole concentration.	Binding buffer containing imidazole, 20–40 mM.	Gua-HCl, 6 M, or urea, 8 M, may be used to improve solubility.	page 413
Glutathione transferase (GST) tagged proteins on immobilized glutathione	PBS, pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na <sub>2</sub> HPO <sub>4</sub> , 1.8 mM KH <sub>2</sub> PO <sub>4</sub> .)	Step gradient with increased glutathione concentration.	Glutathione, 10 mM; Tris-HCl, 50 mM; pH 8.0.		page 442
Monoclonal or polyclonal IgG on immobilized Protein A/Protein G	Sodium phosphate, 20 mM, pH 7.0 or Tris buffer, 50 mM, pH 8.6.	Step pH gradient.	Glycine buffer, 100 mM, pH 3*.	IgGs from different species or from different sub-classes within the same species typically elute at different pHs.	page 456
MBP-tagged proteins on MBPTrap HP	Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4	Step or linear gradient of increased maltose concentration.	Binding buffer containing 10 mM maltose.	Regeneration and cleaning of the column is fast and easy done using 0.5 M NaOH.	page 447
Antigens on immobilized antibodies (immuno-affinity chromatography)	Phosphate buffer, pH 7–8, containing NaCl, 0.5 M.	Step pH gradient, usually decreasing.	Glycine buffer, 50 mM, pH 2.5–3, containing NaCl, 0.5 M*.	GuHCl or urea may be used for elution in combination with low pH. These harsh conditions will usually reduce yields of biologically active material.	page 456
Glycoproteins on immobilized lectins	Aqueous buffer, pH 7–8. Metal ions, e.g. Ca <sup>2+</sup> , Zn <sup>2+</sup> or Mn <sup>2+</sup> .	Step gradient of sugar concentration.	Start buffer plus competing sugar, 100–200 mM.	Different competing sugars are used for different lectins.	page 489
General: specific elution	Aqueous buffer, pH 7–8.	Step gradient of concentration of competing binding agent.	Start buffer plus competing agent.	Different affinity systems will usually require different competing agents.	page 451
General: nonspecific elution	Aqueous buffer, pH 7–8.	Step pH gradient, usually decreasing.	Glycine buffer, 50 mM, pH 2.5–3, containing NaCl, 0.5 M*.	GuHCl or urea may be used for elution in combination with low pH. These harsh conditions will usually reduce yields of biologically active material.	page 451

\* Collect fractions in tubes containing 60–200 µl, 1 M Tris, pH 9 to help reduce activity losses after elution.

# Chromatography

## General / Gel Filtration

### Pressure conversion

1 bar = 1 atm = 14.5 psi = 0.1 MPa.

### Antimicrobial agents for chromatography

Aqueous buffer solutions will often support growth of microorganisms commonly found in laboratories. Since it is difficult to clean columns that have become heavily contaminated with microorganisms, precautions should be taken to avoid growth in the column or in buffer vessels. Always use fresh buffer solutions and cover the buffer vessels to keep out dust, spores and other particles. If a column will not be used for more than a couple of days, it should be equilibrated with the bacteriostatic agent described in the manual supplied with the separation medium or prepacked column. We recommend ethanol or sodium hydroxide for general use. These agents are effective, inexpensive and do not cause disposal problems.

#### Ethanol 20%

Many chromatography media from GE Healthcare are supplied as a suspension containing 20% ethanol. Ethanol, 20%, can also be used as an alternative to NaOH for storing chromatography media under bacteriostatic conditions.

#### Sodium hydroxide

Sodium hydroxide, 0.01 M, is an effective bacteriostatic agent. At higher concentrations (0.5–1.0 M) it is an effective sanitizing agent for contaminated columns. For the most frequent contaminants in chromatographic systems, such as gram-negative bacteria, a good bactericidal effect is reached even at concentrations as low as 0.01 M NaOH. Treatment with sodium hydroxide inactivates endotoxins (e.g. lipopolysaccharides) and will, in many cases, solubilize substances precipitated on the column. Its low toxicity is an advantage that reduces the risk of sample contamination. Sodium hydroxide is not recommended for storage of Sephacryl HR.

### Darcy's Law

Sephadex G-10, G-15, G-25 and G-50 may be assumed to behave as rigid spheres in gel filtration and therefore obey Darcy's Law, i.e.

$$U = K \times \Delta P \times L^{-1} \quad (1)$$

where U is the linear flow rate expressed in cm/h ( $\text{ml} \times \text{cm}^{-2} \times \text{h}^{-1}$ ),  $\Delta P$  is the pressure drop over gel bed expressed in  $\text{cm H}_2\text{O}$ , L is the bed height expressed in cm and K is a proportionality constant that depends on the properties of the bed material and the eluent. Assuming an eluent with viscosity of 1 cP, one can write

$$U = K_0 \times \Delta P \times L^{-1} \quad (2)$$

where  $K_0$  is the "specific permeability" depending on the particle size of the gel beads and their water regain. Observe that the flow rate is proportional to the pressure drop over the bed and, assuming a constant pressure head, inversely proportional to the bed height. Notice that (to a good approximation) the flow rates are independent of the column diameter.

Flow rates at viscosities greater than 1 cP can be obtained by using the relationship: flow rate inversely proportional to viscosity. At first sight, it would appear that high eluent viscosities lead to poor flow rates but the operating pressure can be increased to compensate for the viscosity effect. Temperature influences the viscosity of the eluent. Lower flow rates are obtainable, for a given pressure head, in a cold room than at room temperature.

Theoretical flow rates (not maximum) can be calculated from equation (2) by inserting values for  $\Delta P$  and L. Specific permeabilities are given in the table below.

Specific permeabilities of Sephadex G-types:

Sephadex type	Permeability K
Sephadex G-10	19
Sephadex G-15	18
Sephadex G-25 Superfine	9
Sephadex G-25 Fine	30
Sephadex G-25 Medium	80
Sephadex G-25 Coarse	290
Sephadex G-50 Superfine	13.5
Sephadex G-50 Fine	36
Sephadex G-50 Medium	145
Sephadex G-50 Coarse	400

Calculation of flow rates in other less rigid gels is somewhat more complicated since Darcy's Law is not applicable. Not only is the flow rate dependent upon the factors already mentioned but also on the column diameter. Wider columns do not allow as high a pressure and linear flow rate ( $\text{ml} \times \text{cm}^{-2} \times \text{h}^{-1}$ ) as narrower ones. These gels do not have a linear relationship between pressure and flow; exceeding the maximum recommended values can lead to gel compression, reduction in flow rate and loss of resolution.

### Molecular weight standards for gel filtration of proteins

Protein	Molecular Weight ( $M_r$ )	Stokes' Radius (Å)	Source
Ribonuclease A	13 700	16.4	bovine pancreas
Chymotrypsinogen A	25 000	20.9	bovine pancreas
Ovalbumin	45 000	30.5	hen egg
Albumin	66 000	35.5	bovine serum
Aldolase	158 000	48.1	rabbit muscle
Catalase	232 000	52.2	bovine liver
Ferritin	440 000	61.0	horse spleen
Thyroglobulin	669 000	85.0	bovine thyroid

## Recommended sample volumes for fractionation by gel filtration

The values given may be used as a starting point. Higher resolution may be obtained at the expense of peak height by using smaller sample volumes. If the components of interest are well separated, as in group separations like desalting or buffer exchange, larger sample volumes may be used.

Medium	Recommended sample volume (as percent of total bed volume)
Sephadex	2-5 %
Sepharose	2-5 %
Sephacryl HR	1-2 %
Superdex prep grade	1-2 %
Superose prep grade	1-2 %
Superdex	0.5 %
Superose	0.5 %

## Volatile buffer systems used in ion exchange chromatography

pH range	Buffer system	Counter-ion	pK-values for buffering ions*
3.3-4.3	Formic acid	H <sup>+</sup>	3.75
3.3-4.3; 4.8-5.8	Pyridine/formic acid	HCOO <sup>-</sup>	3.75; 5.25
3.3-4.3; 9.3-10.3	Trimethylamine/formic acid	HCOO <sup>-</sup>	4.75; 9.81
4.3-5.8	Pyridine/acetic acid	CH <sub>3</sub> COO <sup>-</sup>	4.75; 5.25
4.3-5.3; 9.3-10.3	Trimethylamine/acetic acid	CH <sub>3</sub> COO <sup>-</sup>	4.75; 9.81
3.3-4.3; 8.8-9.8	Ammonia/formic acid	HCOO <sup>-</sup>	3.75; 9.25
4.3-5.3; 8.8-9.8	Ammonia/acetic acid	CH <sub>3</sub> COO <sup>-</sup>	4.75; 9.25
5.9-6.9; 9.3-10.3	Trimethylamine/carbonate	CO <sub>3</sub> <sup>2-</sup>	6.35; 9.81
5.9-6.9; 8.8-9.8	Ammonium bicarbonate	HCO <sub>3</sub> <sup>-</sup>	6.35; 9.25
5.9-6.9; 8.8-9.8	Ammonium carbonate/ammonia	CO <sub>3</sub> <sup>2-</sup>	6.35; 9.25
5.9-6.9; 8.8-9.8	Ammonium carbonate	CO <sub>3</sub> <sup>2-</sup>	6.35; 9.25
4.3-5.3; 7.2-8.2	N-Ethylmorpholine/acetate	HCOO <sup>-</sup>	4.75; 7.72

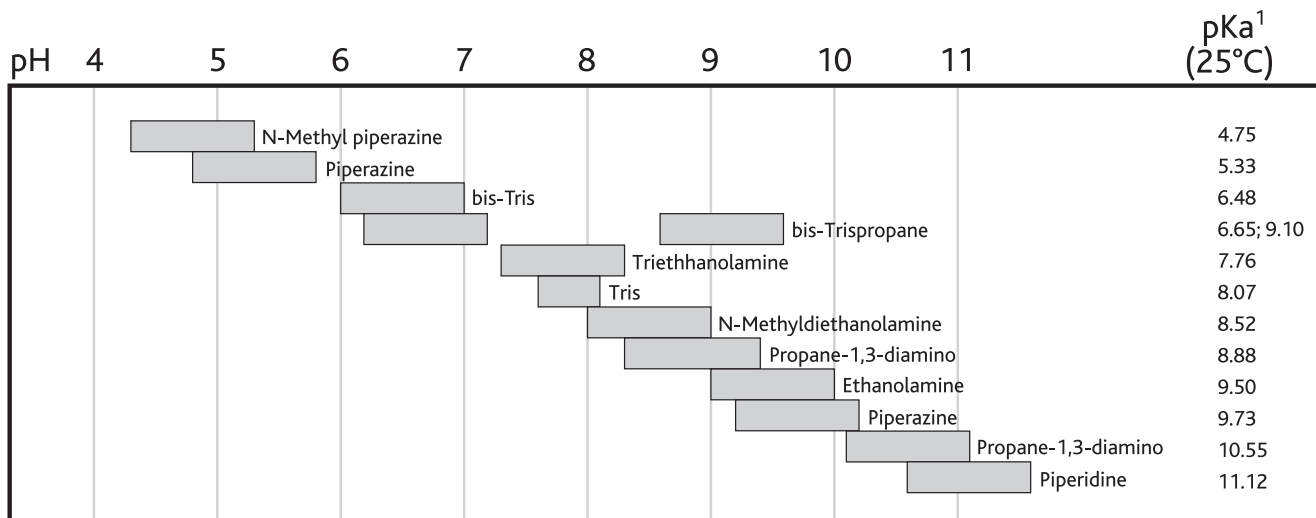
\* Ref: Handbook of chemistry and physics, 83<sup>rd</sup> edition, CRC, 2002-2003.

# Chromatography

## Ion Exchange Chromatography

### Buffers for anion exchange chromatography

Recommended buffer substances for anion exchange chromatography.



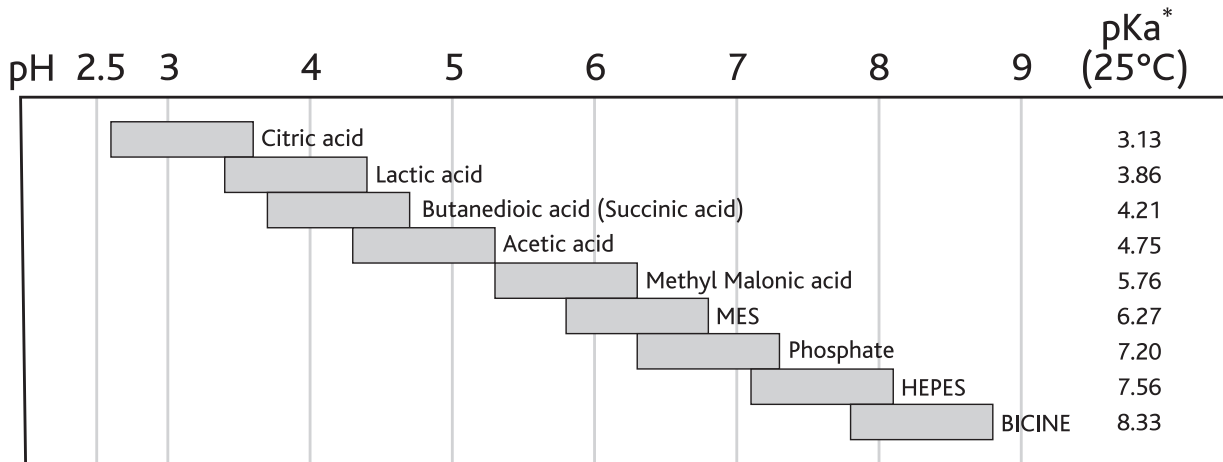
pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25°C)*	d(pKa)/dT (°C)
4.3–5.3	N-Methylpiperazine	20	Cl <sup>-</sup>	4.75	-0.015
4.8–5.8	Piperazine	20	Cl <sup>-</sup> or HCOO <sup>-</sup>	5.33	-0.015
5.5–6.5	L-Histidine	20	Cl <sup>-</sup>	6.04	
6.0–7.0	bis-Tris	20	Cl <sup>-</sup>	6.48	-0.017
6.2–7.2; 8.6–9.6	bis-Trispropane	20	Cl <sup>-</sup>	6.65; 9.10	
7.3–8.3	Triethanolamine	20	Cl <sup>-</sup> or CH <sub>3</sub> COO <sup>-</sup>	7.76	-0.020
7.6–8.6	Tris	20	Cl <sup>-</sup>	8.07	-0.028
8.0–9.0	N-Methyldiethanolamine	20	SO <sub>4</sub> <sup>2-</sup>	8.52	-0.028
8.0–9.0	N-Methyldiethanolamine	50	Cl <sup>-</sup> or CH <sub>3</sub> COO <sup>-</sup>	8.52	-0.028
8.4–9.4	Diethanolamine	20 at pH 8.4 50 at pH 8.8	Cl <sup>-</sup>	8.88	-0.025
8.4–9.4	Propane-1,3-diamino	20	Cl <sup>-</sup>	8.88	-0.031
9.0–10.0	Ethanolamine	20	Cl <sup>-</sup>	9.50	-0.029
9.2–10.2	Piperazine	20	Cl <sup>-</sup>	9.73	-0.026
10.0–11.0	Propane-1,3-diamino	20	Cl <sup>-</sup>	10.55	-0.026
10.6–11.6	Piperidine	20	Cl <sup>-</sup>	11.12	-0.031

\* Ref: Handbook of chemistry and physics, 83<sup>rd</sup> edition, CRC, 2002-2003.

## Ion Exchange Chromatography / Chromatofocusing

## Buffers for cation exchange chromatography

Recommended buffer substances for cation exchange chromatography.



pH interval	Substance	Conc. (mM)	Counter-ion	pKa* (25°C)	d(pKa)/dT (°C)
1.4–2.4	Maleic acid	20	Na <sup>+</sup>	1.92	
2.6–3.6	Methyl Malonic acid	20	Na <sup>+</sup> or Li <sup>+</sup>	3.07	
2.6–3.6	Citric acid	20	Na <sup>+</sup>	3.13	-0.0024
3.3–4.3	Lactic acid	50	Na <sup>+</sup>	3.86	
3.3–4.3	Formic acid	50	Na <sup>+</sup> or Li <sup>+</sup>	3.75	+0.0002
3.7–4.7; 5.1–6.1	Butanedioic acid (Succinic acid)	50	Na <sup>+</sup>	4.21; 5.64	-0.0018
4.3–5.3	Acetic acid	50	Na <sup>+</sup> or Li <sup>+</sup>	4.75	+0.0002
5.2–6.2	Methyl Malonic acid	50	Na <sup>+</sup> or Li <sup>+</sup>	5.76	
5.6–6.6	MES	50	Na <sup>+</sup> or Li <sup>+</sup>	6.27	-0.0110
6.7–7.7	Phosphate	50	Na <sup>+</sup>	7.20	-0.0028
7.0–8.0	HEPES	50	Na <sup>+</sup> or Li <sup>+</sup>	7.56	-0.0140
7.8–8.8	BICINE	50	Na <sup>+</sup>	8.33	-0.0180

\* Ref: Handbook of chemistry and physics, 83<sup>rd</sup> edition, CRC, 2002-2003.

## Buffer systems for broad pH intervals in chromatofocusing

Start buffer				Eluent (final volume 100 ml) <sup>†</sup>			
pH range	Buffer to use	Adjust to pH	Acid to use	Polybuffer or Pharmalyte type to use		Adjust to pH	Acid to use
9–7	Diethanolamine, 0.025 M	9.5	HCl	1.0 ml Pharmalyte 8–10.5 5.2 ml Polybuffer 96		7.0	HCl
9–6	Diethanolamine, 0.025 M or Tris, 0.075 M	9.5	HCl	10 ml Polybuffer 96		6.0	HCl
				10 ml Polybuffer 96			
8–6	Triethanolamine, 0.025 M	8.3	CH <sub>3</sub> COOH	0.21 ml Pharmalyte 8–10.5 9.0 ml Polybuffer 96		6.0	CH <sub>3</sub> COOH
8–5	Triethanolamine, 0.025 M	8.3	Iminodiacetic acid*	3.0 ml Polybuffer 96 7.0 ml Polybuffer 74		5.0	Iminodiacetic acid*
7–5	bis-Tris, 0.025 M	7.1	HCl	10 ml Polybuffer 74		5.0	HCl
7–4	bis-Tris, 0.025 M	7.1	Iminodiacetic acid*	10 ml Polybuffer 74		4.0	Iminodiacetic acid*
6–4	bis-Tris, 0.025 M	6.3	HCl	10 ml Polybuffer 74		4.0	HCl

\* Use a saturated solution of iminodiacetic acid.

<sup>†</sup> In the eluent recipes, dilute the Polybuffer and/or Pharmalyte mixtures with distilled water to a volume of approximately 95 ml. Titrate this volume to the correct pH with the listed acid (1–2 M). When the final pH is reached, add distilled water to make a total volume of 100 ml.

For shallower gradients within the same pH interval, increase the total volume. Make sure that the titration is always carried out on a maximum volume before adding the final few milliliters of water. When more dilute eluents are used, proteins are eluted with increased volumes, therefore pregradient and total volumes increase also.

# Chromatography

## Chromatofocusing / Affinity / Hydrophobic Interaction

### Buffer systems for narrow pH intervals in chromatofocusing

Start buffer				Eluent (Final volume 100 ml) <sup>†</sup>		
pH range	Buffer to use	Adjust to pH	Acid to use	Polybuffer or Pharmalyte type to use	Adjust to pH	Acid to use
9–8	Diethanolamine, 0.025 M	9.4	HCl	1.0 ml Pharmalyte 8–10.5 5.2 ml Polybuffer 96	8.0	HCl
8.5–7.5	Tris, 0.025 M	8.8	CH <sub>3</sub> COOH	0.11 ml Pharmalyte 8–10.5 9.5 ml Polybuffer 96	7.5	CH <sub>3</sub> COOH
8–7	Triethanolamine, 0.025 M	8.3	HCl	10 ml Polybuffer 96	7.0	HCl
7.5–6.5	2-Methylimidazole, 0.025 M	7.6	CH <sub>3</sub> COOH	10 ml Polybuffer 96	6.5	CH <sub>3</sub> COOH
7–6	bis-Tris, 0.025 M	7.0	CH <sub>3</sub> COOH	9.5 ml Polybuffer 96 0.5 ml Polybuffer 74	6.0	CH <sub>3</sub> COOH
6.5–5.5	bis-Tris, 0.025 M	6.7	CH <sub>3</sub> COOH	4.0 ml Polybuffer 96 6.0 ml Polybuffer 74	5.5	CH <sub>3</sub> COOH
6–5	bis-Tris, 0.025 M	6.7	HCl	10 ml Polybuffer 74	5.0	HCl
5.5–4.5	Piperazine, 0.025 M	6.3	HCl or Imino-diacetic acid*	10 ml Polybuffer 74	4.5	HCl or Imino-diacetic acid*
5–4	N-Methylpiperazine, 0.025 M	5.7	HCl or Imino-diacetic acid*	10 ml Polybuffer 74	4.0	HCl or Imino-diacetic acid*

\* Use a saturated solution of iminodiacetic acid.

<sup>†</sup> In the eluent recipes, dilute the Polybuffer and/or Pharmalyte mixtures with distilled water to a volume of approximately 95 ml. Titrate this volume to the correct pH with the listed acid (1–2 M). When the final pH is reached, add distilled water to make a total volume of 100 ml.

For shallower gradients within the same pH interval, increase the total volume. Make sure that the titration is always carried out on a maximum volume before adding the final few milliliters of water. When diluted eluents are used, proteins are eluted with increased volumes; therefore pregradient and total volumes increase also.

### Major coupling techniques used in affinity chromatography

Type of ligand	Chemical group on ligand	Chemical group on gel matrix	Chemical bond formed between ligand and medium	Coupling medium	Page
Protein, peptide, amino acid	amino	cyanate ester	isourea	CNBr-activated Sepharose	474
		N-hydroxysuccinimide	peptide	NHS-activated Sepharose	474
		carboxyl	peptide	ECH Sepharose 4B	475
		epoxy	alkylamine	Epoxy-activated Sepharose 6B	476
	carboxyl	amino	peptide	EAH Sepharose 4B	476
	thiol	thiol	mixed disulfide	Thiopropyl Sepharose 6B	477
		2-dipyridyl disulfide	mixed disulfide	Activated Thiol Sepharose 4B	477
epoxy		thioether	Epoxy-activated Sepharose 6B	476	
Sugar	hydroxyl	epoxy	ether	Epoxy-activated Sepharose 6B	476
	amino	carboxyl	peptide	ECH Sepharose 4B	475
		N-hydroxysuccinimide	peptide	NHS-activated Sepharose	474
		epoxy	alkylamine	Epoxy-activated Sepharose 6B	476
carboxyl	amino	peptide	EAH Sepharose 4B	476	
Polynucleotide	amino	cyanate ester	isourea	CNBr-activated Sepharose	474
	mercurated base	thiol	mercaptide	Thiopropyl Sepharose 6B	477
Coenzyme, cofactor, antibiotic, steroid, other small ligands	amino, carboxyl,	various	various	AC gels with spacer arm	473
	thiol or hydroxyl				

### The Hofmeister series

The Hofmeister series describes the relative effects of some anions and cations in precipitating proteins and in promoting interactions in hydrophobic interaction chromatography (HIC). Anions and cations on the left are most effective in precipitating proteins or causing them to bind to HIC media ("salting-out"). Those on the right are most effective in solubilizing proteins ("salting-in"). Note that not all combinations of anion and cation produce soluble salts.

← Increasing precipitation ("salting-out") effect

Anions: PO<sub>4</sub><sup>3-</sup> > SO<sub>4</sub><sup>2-</sup> > CH<sub>3</sub>COO<sup>-</sup> > Cl<sup>-</sup> > Br<sup>-</sup> > NO<sub>3</sub><sup>-</sup> > ClO<sub>4</sub><sup>-</sup> > I<sup>-</sup> > SCN<sup>-</sup>

Cations: NH<sub>4</sub><sup>+</sup> > Rb<sup>+</sup> > K<sup>+</sup> > Na<sup>+</sup> > Cs<sup>+</sup> > Li<sup>+</sup> > Mg<sup>2+</sup> > Ca<sup>2+</sup> > Ba<sup>2+</sup>

Increasing chaotropic ("salting-in") effect →

### Suitable organic components for the mobile phase in reversed phase chromatography

Solvent*	Comments
Acetonitrile	Good UV-transparency, low viscosity
Ethanol	Viscous, rather less effective eluent than acetonitrile
Methanol	Quite viscous, half to two-thirds eluting power of acetonitrile
2-propanol	Very viscous, adequate UV-transparency. More powerful eluent than acetonitrile.
1-propanol	Very viscous, adequate UV-transparency. More powerful eluent than acetonitrile.
Isopropanol	Very viscous, adequate UV-transparency. More powerful eluent than acetonitrile.
n-Propanol	Very viscous, adequate UV-transparency. More powerful eluent than acetonitrile.

\* HPLC grade solvents should always be used to prepare eluents for RPC.

### Some suitable buffers for reversed phase chromatography of peptides

Eluent*	Concentration	Comments
<b>Volatile, low pH</b>		
Trifluoroacetic acid (TFA)	≤ 0.3%	Forms ion pairs, low UV-absorbance
Pentafluoropropionic acid (PFPA)	≤ 0.3%	Forms ion pairs, more hydrophobic than TFA
Heptafluorobutyric acid (HFBA)	≤ 0.3%	Forms ion pairs, more hydrophobic than TFA
Perchloric acid	≤ 0.3%	
Ammonium acetate	10–50 mM	
<b>Non-volatile, low or neutral pH</b>		
Phosphoric acid	10–50 mM	Less hydrophobic than TFA. Adjust to chosen pH with NaOH pKa <sub>1</sub> = 2.12, pKa <sub>2</sub> = 7.21

\* HPLC grade chemicals should always be used to prepare eluents for RPC.