Mono P® HR 5/5
Mono P HR 5/20

INSTRUCTIONS

Mono P is a unique anion exchanger designed for chromatofocusing. It offers fast, high resolution separations of proteins and peptides.

Introduction

Mono P is a high performance matrix specially designed for chromatofocusing, a technique separating molecules on the basis of their pI. Mono P is available in two column sizes, HR 5/5 (1 ml) and HR 5/20 (4 ml).

The small column offers very fast chromatofocusing and may also be suitable for anion exchange while the HR 5/20 column offers high selectivity for biomolecules with pI differences of, in some cases, less than 0.02 pH units.

Unpacking

Please check the delivery against this list.

Designation	Code No.	No.supplied
Mono P HR 5/5	17-0611-01	1
or		
Mono P HR 5/20	17-0548-01	1
Wrench	19-7481-01	1
Instructions		1

Quality control tests

To guarantee the quality of each column is efficiency tested. Each media batch undergoes a function test to ensure reproducible results.

Connecting the column to FPLC® System

The columns are supplied with preflanged tubing and tubing connectors at both ends.

1. Connect the shorter preflanged tubing, the outlet, to the detector.
2. Connect the longer preflanged tubing, the inlet, to a valve that can be positioned for sample injection and elution e.g. the GE Healthcare Valve V-7 or Motor Valve MV-7. When using the GE Healthcare Valve PV-7 or the Motor Valve PMV-7, connect the Union, M6 female/1/16" male (Code No. 16-3858-01) between the flanged tubing and the valve.
3. We recommend you to connect a prefilter before the column (see “Spare parts and accessories”).

Connecting the column to HPLC Systems

Columns prepacked with Mono P can be used with any HPLC System if the pump can provide precise and accurate flow at relatively low back-pressures. The column should be connected as described for FPLC System via two unions which adapt 1.8 mm to 1/16" tubing (see “Spare parts and accessories”).

Important before use

The glass columns HR 5/5 and HR 5/20 are stable up to 10 MPa (100 bar, 1500 psi) for short periods. To not alter the packing of the column, run at a maximum back-pressure of 5 MPa (50 bar, 750 psi). Set the pressure limit control accordingly. The best results are achieved with flow rates between 0.5 and 1.5 ml/min.
The gel is supplied in a 24% ethanol-water solution with sulphate as the counter-ion. To equilibrate your column, perform the following steps.

1. Inject 1 ml of 5 M NaOH.
2. Equilibrate with start buffer until the effluent is at the same pH as the start buffer.
3. Make a blank run with the eluent.
4. Repeat steps 1 and 2.

The column is now ready to use for chromatofocusing.

Gel properties

Mono P is a chromatofocusing gel with the same ligands as those on our standard gel PBE 94 to ensure an even buffering capacity over a wide pH range.

52-1624-00
Edition AG

Mono P is based on a beaded hydrophilic resin, MonoBeads®, with one of the narrowest particle size distributions available. The chemistry of the beads was developed at GE Healthcare and the monodispersity was accomplished through an unique process developed by Professor John Ugelstad of SINTEF, Trondheim, Norway.
Mono P has a particle size of 10 µm. The absence of fines gives the packed column a large void volume (40%) and therefore very low back-pressure. The ionic capacity of the gel is 0.15-0.21 mmol/ml. Separation of substances with molecular weights of up to 10^7 have been carried out successfully. Practical protein loading capacity is generally 5-10 mg/ml gel, although the higher capacity refers chiefly to anion exchange. The amount of non-specific adsorption to MonoBeads is negligible and recovery of enzyme activity is normally greater than 80%.

Chemical and physical stability

Mono P can be used with aqueous solutions in the pH range 2-12. Aqueous solutions of urea (<3 M), ethylene glycol, C_1-C_4 alcohols, and similar compounds may also be used. For short term use, for example during cleaning, pH stability is 2-14.
Non-ionic detergents can be used but the gel must be equilibrated with the detergent solution before. Anionic detergents should never be used with Mono P since they bind to the gel. Cationic detergents may be used but the ionic strength should be kept low so as not to disturb the developing pH gradient. Dimethyl sulphoxide, dimethyl formamide, formic acid and similar solvents change the separation properties of the gel, and are therefore not recommended. All oxidizing and other reactive substances should be avoided.
The glass columns HR 5/5 and HR 5/20 are stable up to 10 MPa (100 bar, 1500 psi). For best performance, we recommend an operating pressure of up to 5 MPa (50 bar, 750 psi). At a flow rate of 1.0 ml/min with water, in room temperature, a Mono P HR 5/20 column operates at a back pressure of approximately 2.5 MPa (25 bar, 375 psi) while a Mono P HR 5/5 operates at a back pressure of approximately 1 MPa (15 bar, 225 psi).

Choice of elution conditions and pH intervals

In chromatofocusing, a pH gradient is formed on the column by equilibrating the column with start buffer and eluting with Polybuffer® adjusted to a lower pH.
All anion exchangers, including chromatofocusing matrices, should be used with cationic buffers or zwitter-ionic buffers, e.g. histidine. Anionic buffers must be avoided since they bind to Mono P. Similarly anionic detergents, such as SDS, bind to Mono P and should not be used. Cationic or non-ionic detergents (e.g. octylglucoside) may be used.
When separating a substance of known pI, choose a pH interval so that it is eluted after 1/3-1/2 of the pH gradient. It is also best to use narrow pH intervals for maximum resolution.
When separating a substance of unknown pI, start with the pH interval 7-4. If the substance has a pI above 7, it will pass straight through the column. Once recovered it can be run again in a higher pH interval.
Tables 1 and 2 give the start buffers, eluents, total eluent volumes and pre-gradient volumes for various pH intervals in chromatofocusing.

Table 1. Buffer systems for broad pH intervals in chromatofocusing.

pH	Startbuffer	Eluent (100 ml)	Approximate volumes, (ml)			
			Mono P HR5/20		Mono P HR5/5	
			total eluent	pre-gradient	total eluent	pre-gradient
9-7	0.025 M Diethanolamine, pH 9.5, HCl	1.0 ml Pharmalyte 8-10.5, 5.2 ml Polybuffer 96, pH 7.0, HCl	34	7	11	2
9-6	0.025 M Diethanolamine, pH 9.5, HCl or	10 ml Polybuffer 96, pH 6.0, HCl	34	9	19	2
	0.075 M Tris, pH 9.3, CH_3COOH	10 ml Polybuffer 96, pH 6.0, CH_3COOH	30	3	17	2
8-6	0.025 M Triethanolamine, pH 8.3, CH_3COOH	0.21 ml Pharmalyte 8-10.5, 9.0 ml Polybuffer 96, pH 6.0, CH_3COOH	37	7	15	4
8-5	0.025 M Triethanolamine, pH 8.3, iminodiacetic acid*	3.0 ml Polybuffer 96, 7.0 ml Polybuffer 74, pH 5.0, iminodiacetic acid*	47	6	15	3
7-5	0.025 M bis-Tris, pH 7.1, HCl	10 ml Polybuffer 74, pH 5.0,HCl	26	3	13	3
7-4	0.025 M bis-Tris, pH 7.1, iminodiacetic acid*	10 ml Polybuffer 74, pH 4.0, iminodiacetic acid*	46	3	19	3
6-4	0.025 M bis-Tris, pH 6.3, HCl	10 ml Polybuffer 74, pH 4.0, HCl	39	3	16	3

* Use a saturated solution of iminodiacetic acid.

The pre-gradient volume is the approximate volume of eluent which passes through the column before the pH of the effluent begins to change. This information is determined with data from numerous runs performed in our application laboratories. All values are determined at room temperature. For each eluent, Polybuffer and/or Pharmalyte® mixtures are diluted to a volume of approximately 95 ml. This volume is then titrated to the correct pH with the appropriate acid (1-2 M). When the final pH has been reached, distilled water is added to make a total volume of 100 ml. Make sure that the titration is always carried out on a specified maximum volume, e.g. 95 ml, before addition of the final few ml of water.

Table 2. Buffer systems for narrow pH intervals in chromatofocusing.

pH	Start buffer	Eluent (l00 ml)	Approximate volumes (ml)			
			Mono P HR 5/20		Mono P HR 5/5	
			total eluent	pre-gradient	total eluent	pre-gradient
9-8	0.025M Diethanolamine, pH 9.4, HCI	1.0 ml Pharmalyte 8-10.5, 5.2 ml Polybuffer 96, pH 8.0, HCI	28	3	10	3
8.5-7.5	0.025 M Tris, pH 8.8, CH_3COOH	0.11 ml Pharmalyte 8-10.5, 9.5 ml Polybuffer 96, pH 7.5, CH_3COOH	29	4	10	4
8-7	0.025 M Triethanolamine, pH 8.3, HCI	10 ml Polybuffer 96, pH 7.0, HCI	29	5	10	4
7.5-6.5	0.025 M Methylimidazole, pH 7.6, CH_3COOH	10 ml Polybuffer 96, pH 6.5, CH3COOH	27	9	11	6
7-6	0.025 M bis-Tris, pH 7.0, CH_3COOH	9.5 ml Polybuffer 96, 0.5 ml Polybuffer 74, pH 6.0, CH_3COOH	28	10	12	5
6.5-5.5	0.025 M bis-Tris, pH 6.7, CH_3COOH	4.0 ml Polybuffer 96, 6.0 ml Polybuffer 74, pH 5.5, CH_3COOH	23	5	9	3
6-5	0.025 M bis-Tris, pH 6.7, HCI	10 ml Polybuffer 74, pH 5.0, HCI	25	3	10	3
5.5-4.5	0.025 M Piperazine, pH 6.3, HCI or iminodiacetic acid*	10 ml Polybuffer 74, pH 4.5, HCI or iminodiacetic acid*	24	3	10	3
5-4	0.025 M Methylpiperazine, pH 5.7, HCI or iminodiacetic acid*	10 ml Polybuffer 74, pH 4.0, HCI or iminodiacetic acid*	27	7	11	3

* Use a saturated solution of iminodiacetic acid.

Table 3. Recommended buffers for various pH intervals in anion exchange chromatography

pH interval	Buffer	Concentration[1]	Anion[2]	pKa (25 °C)	dpKa[3] / dT(°C)
4.5-5.0	N-methylpiperazine	20 mM	Cl^-	4.75	-0.015
5.0-6.0	piperazine	20 mM	Cl^- $HCOO^-$	5.68	-0.015
5.5-6.0	L-histidine	20 mM	Cl^-	6.15	
5.8-6.4	bis-Tris	20 mM	Cl^-	6.46	-0.017
6.4-7.3	bis-Tris propane	20 mM	Cl^-	6.80	
7.3-7.7	triethanolamine	20 mM	Cl^- OAc^-	7.76	-0.020
7.5-8.0	Tris	20 mM	Cl^-	8.06	-0.028
8.0-8.5	N-methyldiethanolamine	20 mM 50 mM 50 mM	SO_4 Cl^- OAc^-	8.54	-0.028
8.4-8.8	diethanolamine	20 mM at 8.4 50 mM at 8.8	Cl^-	8.88	-0.025
8.5-9.0	1,3-diaminopropane	20 mM	Cl^-	8.64	-0.031
9.0-9.5	ethanolamine	20 mM	Cl^-	9.50	-0.030
9.5-9.8	piperazine	20 Mm	Cl^-	9.82	-0.026
9.8-10.3	1,3-diaminopropane	20 mM	Cl^-	10.62	-0.026

[1] Buffer concentration gradients may improve resolution.

[2] Br- and I- may also be used where CI- is indicated. Results may improve in some cases.

[3] When working at different temperatures, allow for changes in the pKa.

To make shallower gradients within the same pH interval, the dilution of Polybuffer and/or Pharmalyte is increased. When diluted eluents are used, proteins are eluted in larger volumes; therefore total volumes and pre-gradient volumes also increase.

The use of Mono P HR 5/5 as an anion exchanger

The Mono P HR 5/5 column can be used both for chromatofocusing and anion exchange. (Mono P HR 5/20 is less suitable for anion exchange since run times will be rather long.)
The distinction between Mono P and Mono Q® is that Mono P is a weak anion exchanger and Mono Q is a strong anion exchanger. This means that Mono P has a buffering capacity, and the amount of charge it carries will vary with pH. Consequently, its ionic capacity will also vary with pH. Mono Q displays negligible buffering capacity; it has a constant amount of charge and therefore constant ionic capacity, regardless of pH.
Table 3 gives recommended buffers for various pH intervals in anion exchange. 20 ml is a reasonable gradient volume for ion exchange.

Eluent and sample preparation

Water should be of Milli-Q* or corresponding quality.
Use HPLC grade solvents, salts and buffers. Degas and filter all buffers through a 0.22 µm sterile filter. Either centrifuge (10 000 x *g* for 10 min) or filter samples through a 0.22 µm filter. Be sure to select a solvent resistant filter if samples are dissolved in organic solvents. Store all eluents in closed bottles.
The samples should be fat-free. Turbid solutions can decrease the column lifetime.
When possible, dissolve the sample in start buffer. Buffer exchange is easily accomplished by gel filtration with Sephadex® G-25, e.g. using the Fast Desalting Column HR 10/10 or PD-10 columns.
Note: Careful handling of solutions and samples increases the lifetime of the column considerably.

Increased back-pressure

If back-pressure increases, perform the following steps in sequence until normal pressures are obtained.

1. Check the flanges of the tubings and reflange or exchange assemblies if damaged.
2. Turn the red adjusting ring on the top adaptor half a turn counter clockwise. (The adaptor should still be close to the gel bed, but without pressing against it.) Reverse the flow direction and pump 2 ml of buffer at 0.5 ml/min. Return to normal flow direction and run for 2-6 min at 1 ml/min. Readjust the top adaptor.
3. Check the filters (Filter HR 5) and change them if dirty (see “Spare parts and accessories”).
4. If the problem persists, clean the column according to the procedure described under “column cleaning”.

Column cleaning

The following observations indicate that column washing may be necessary.

- increased back-pressure**
- colour change at the top of the column
- loss of resolution
- decreased sample recoveries

It is best to avoid these problems by washing the column routinely (e.g. every fifth or tenth run) following steps 1-6 below. More frequent washing may be necessary if complex samples are applied. Steps 1-8 are guidelines. Your knowledge about possible contamination should be used to evaluate which cleaning method is suitable.

1. Connect the column inlet to the detector. Set the sensitivity to 2.0 AUFS.
2. Make sure there is no space between the gel and adaptor. Start a reversed flow at a rate of 0.25-0.50 ml/min. Carry out steps 3-6 in sequence, ensuring each time that the monitored peaks are identical in size before proceeding to the next step.
3. Inject 500 µl 2 M NaCl solution. Rinse with water or buffer.
4. Inject 500 µl 2 M NaOH solution. Rinse with water or buffer.
5. When performing chromatofocusing, repeat step 4 until the pH stabilizes at start conditions.
6. Inject 500 µl 75% acetic acid or 1% TFA. Rinse with water or buffer and equilibrate.
7. If the column performance is still not restored, try leaving the column overnight in a solution of approximately 1 mg/ml pepsin, 0.1 M acetic acid and 0.5 M NaCl. (Instead, you may try other enzymes, e.g. DNase, depending on the contamination) For enzymatic washing, the solution must be at a temperature of 37 °C (98.6 °F).
   After the enzymatic cleaning, perform the chemical scrubbing (steps 1-6) again.
8. As a last attempt to restore performance, suspend 2-3 mm of the gel top and remove it with a pasteur-pipette. Adjust the adaptor to eliminate the space above the gel bed.

* Milli-Q is a registered trademark of Millipore Corp.

** Please check the filters first.

Column equilibration

For chromatofocusing

1. Inject 1 ml of 5 M NaOH.
2. Equilibrate with start buffer until the effluent is at the same pH as the start buffer.
3. Make a blank run with the eluent.
4. Repeat steps 1 and 2.

The column is now ready to use.

For anion exchange on Mono P HR 5/5

1. Wash with 5 ml of start buffer (low ionic strength).
2. Change to the desired counter-ion by washing with 10 ml of eluent B (high ionic strength).
3. Equilibrate with start buffer until the base-line is stable.

The column is now ready to use.

Note: When you use detergents, longer equilibration times may be necessary.

Sample application

The most convenient and reproducible sample application is via the GE Healthcare valves V-7, PV-7, MV-7 or PMV-7. Make sure the sample is recently filtered or centrifuged before applying it to the column. Practical protein loading capacity is around 5-10 mg/ml gel depending on the sample, the conditions and the resolution needed.

Sample elution

In chromatofocusing, the pH gradient is self-generated by the interaction of the eluent with the charged groups on the gel. Monitoring the pH of the column effluent gives the approximate gradient volume (see Tables 1 and 2), and the pH at which the proteins are eluted. In ion exchange chromatography, the salt gradient is made by mixing an increasing volume of buffer containing a high salt concentration with the start buffer. The best results are achieved with flow rates between 0.5 and 1.5 ml/min.

Column re-equilibration

To re-equilibrate the column for chromatofocusing, inject 1 ml of a 1-2 M solution of the sodium salt having the same counter-ion as the start buffer. Equilibrate with start buffer until the pH of the effluent is stable.

To re-equilibrate the column for anion exchange, inject 1 ml of a 1-2 M solution of the elution salt and equilibrate with the start buffer.

If changing pH interval or counter-ions, follow the column equilibration procedure mentioned previously (see section “Column equilibration”).

Checking the column packing

A well packed column is essential for high performance chromatography, especially in isocratic techniques. For best performance, make sure there is no space between the top adaptor and the gel bed (adjust the adaptor by clockwise rotation of the red adjusting ring on the top of the column), and that the column is clean (see section “Column cleaning”).

If you suspect column packing to be the cause of reduced resolution, run a sample and note the shape of the sample zone. Haemoglobin (Sigma type 4, human) is a good test protein, since it is coloured and readily available.

1. Dissolve the haemoglobin, 10 mg/ml, in 20 mM diethanolamine, pH 8.5.
2. Apply approximately 100 µl of the haemoglobin solution to the column.
3. Elute with a 20 mM diethanolamine solution containing 1 M NaCl.

Note the shape of the protein zone. When injected it should be a narrow, horizontal band at the top of the column. When eluted with 100% eluting buffer, the zone should move down the column as a band.

If the band is wavy, diffuse or not horizontal during elution, adjust the top adaptor and, if not already done, clean the column.

Efficiency test

After column maintenance procedures the efficiency of the column should be checked. Column efficiency, expressed as plates per metre (H^{-1}), is estimated using following equation:

$H^{-1} = 5.54 * (V_R/w_h)^2 * (1000/L)$

L =bed height (mm)

V_R =peak retention (elution) volume (ml)

w_h =peak width at half peak high (ml)

H^{-1} =number of theoretical plates/metre

Mono P HR 5/5

Sample: 25 µl of cytidine 5´-monophosphate, 0.1 mg/ml
Eluent: 0.01 M diethanolamine, pH 8.9 (H_2SO_4)
Flow rate: 0.5 ml/min
Detector: UV-M, 254 nm, 0.2 AUFS
Chart speed: 2 cm/min

Mono P HR 5/20

Sample: 20 µl of triglycine, 0.2 mg/ml
Eluent: 0.005 M NH4Cl, pH 9.9
Flow rate: 0.5 ml/min
Detector: UV-M, 214 nm, 0.2 AUFS
Chart speed: 3 cm/min

The number of theoretical plates per metre (H^{-1}) should be more than 25 000.

Function test

An alternative to the efficiency test to check column performance is the function test described in figure 1 and 2.

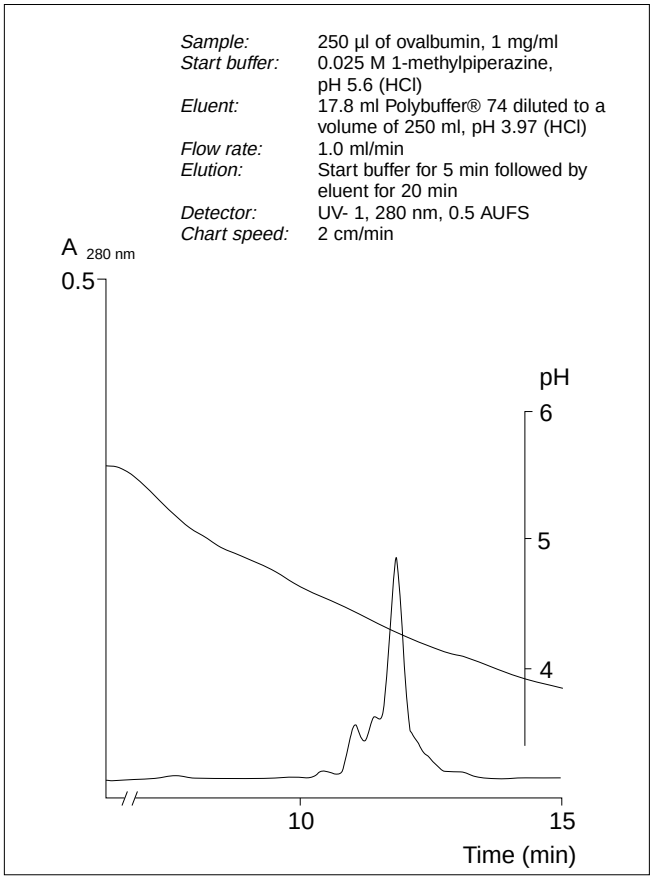


Fig. 1. Typical chromatogram from a function test of Mono P® HR 5/5.

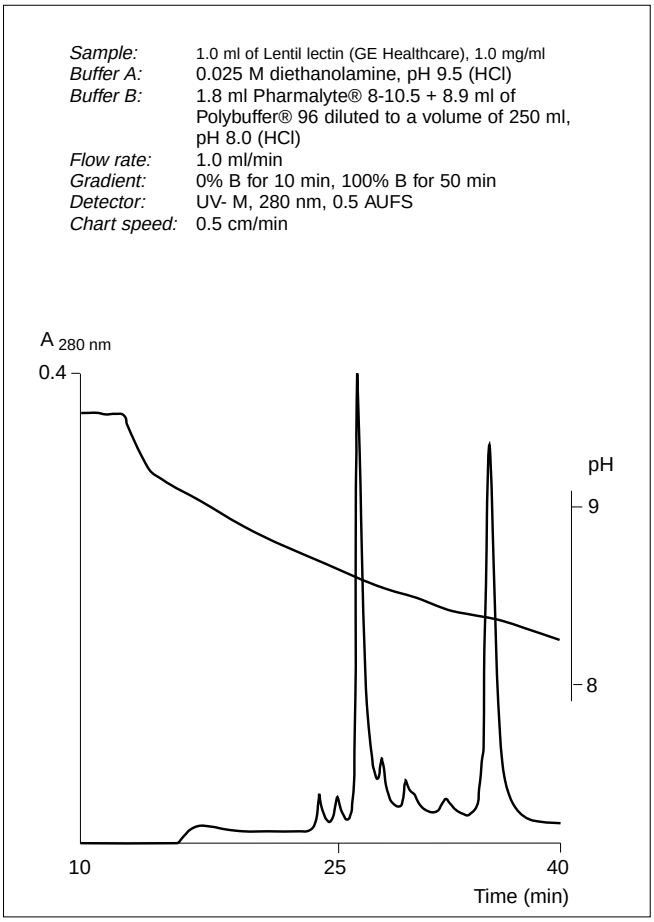


Fig. 2. Typical chromatogram from a function test of Mono P® HR 5/20.

Storage and prevention of microbial growth

Before storing the column for long periods of time, wash it through sequentially with 5 ml 0.5 M Na_2SO_4, 10 ml of water and 10 ml of 20% ethanol. The column should be stored between 4 and 30 °C.

Further information

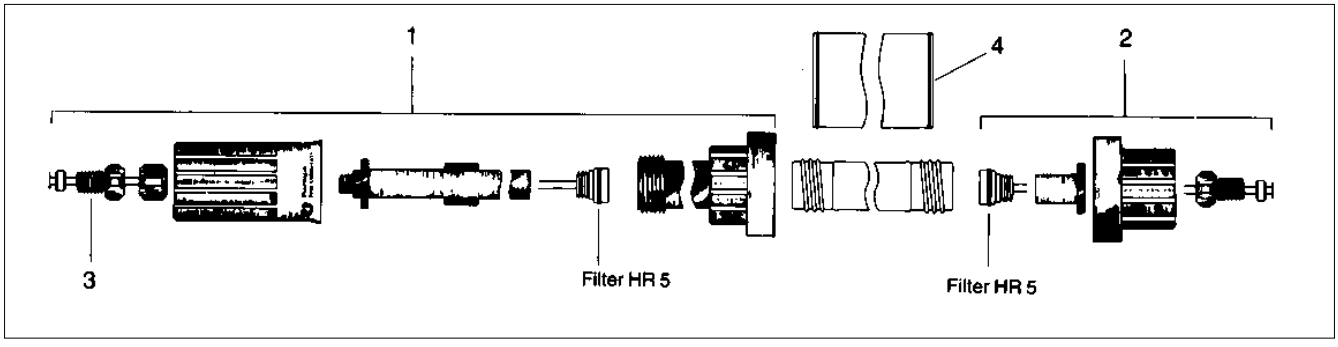
For further information please contact your local GE Healthcare representative.

Spare parts and accessories

Pos.	Designation	Code No.	No. per pack
1	Top assembly HR 5	18-6352-01	1
	Filter HR 5	18-6353-01	10
2	Bottom assembly HR 5	18-6351-01	1
3	Tubing connectors*	19-7476-01	5
4	Protective jacket HR 5/5	19-7447-01	1
	5/20	19-7448-01	1
	Capillary tubing (o.d. 1.8 mm, i.d. 0.5 mm)	19-7477-01	2 m
	Prefilter	19-5084-01	1
	Filters+O-rings (prefilter)	19-5082-01	5+2
	Flanging/Start-Up kit		
	120V	19-5079-01	1
	220V	19-5090-01	1
	Sample loops 1ml, 2ml	18-5897-01	1 of each
	Superloop 10 ml	19-7585-01	1
	Superloop 50 ml	19-7850-01	1
	Superloop 150 ml	18-1023-83	1
	Union, M6 female/1/16" female, stainless steel (Waters** compatible)	18-3405-01	2
	(Swagelok** compatible)	18-3406-01	2
	Union, M6 female/1/16" female, titanium (Valco** compatible)	18-3859-01	1
	Union, M6 female/1/16" male, plastic (Valco**compatible)	18-3858-01	5
	Fast Desalting Column HR 10/10	17-0591-01	1
	PD-10	17-0851-01	30

* You need the Flanging/Start-Up kit to attach new tubing connectors.

** Waters is our abbreviation for the fittings produced by Millipore Corp. Swagelok is a registered trademark of the Crawford Fitting Company. Valco is a trademark of Valco Instrument Co. Inc.



GE imagination at work

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