N-Terminal Protein Membrane 4.0 Protein Sequencer Methods

Technical Note

HP 241 Protein Sequencer (N+C)

The N-terminal protein membrane 4.0 sequencer methods implement the Edman degradation chemistry (coupling, cleavage, and conversion) on the Hewlett-Packard column-based protein sequencer. Reagents 1R, 2R, 3R, and the TH-Std are specific for C-terminal sequencing and are not utilized for the N-terminal protein membrane 4.0 sequencer methods.

These methods are suitable for N-terminal sequence analysis of most protein samples in the low nanomole to low picomole range which have been applied onto a Hewlett-Packard Zitex strip. Once dry the strip is loaded into an empty RP/SAX biphasic column. No column preparation is required with this method.

The methods are:

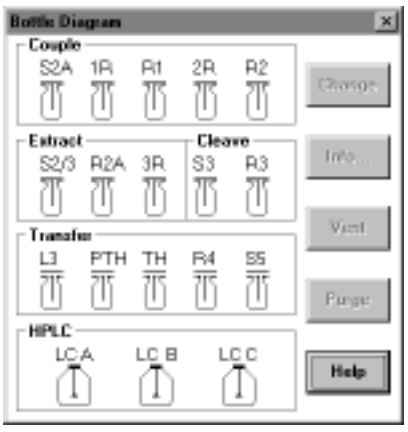
- N-Terminal Protein Membrane 4.0
- N-Terminal Protein Membrane 4.0 (Cycle 1)
- N-Terminal Flask 4.0
- PTH-Std 4.0

The column and flask methods control all of the derivatization/cleavage and conversion reactions, respectively.

The sequence program controls all of the column and flask methods, and well as any cycle exception methods. The N-terminal protein membrane 4.0 sequencer method uses a cycle 1 column exception method.

The cycle 1 method provides a longer initial drying time and a pause to provide time for proper HPLC column equilibration after switching from C-terminal sequencing. This method also performs a double coupling on the N-terminal amino acid. There is no specialized cycle 1 method for the flask.

The PTH Std 4.0 method delivers 10 pmol/100 µl PTH-amino acid standard solution to the on-line HPLC allowing the quantitation and identification of sequenced amino acids. One may choose to run a PTH-standard by using PTH-std 4.0 when scheduling a sample.



The Routine 4.0 sequencer methods require the bottle configuation shown. The reagents and solvents are purchased from Hewlett-Packard. These methods produce less than 1mL/cycle of liquid waste.

S2A	Neat ethyl acetate
1R	Diphenylphosphorylisothiocyanate (DPP-ITC) in toluene/heptane (23:27:50)
R1	Phenylisothiocyanate (PITC) in heptane (3:97)
2R	Pyridine in ethylacetate (2:98)
R2	Diisopropylethylamine (DIEA) in 1-propanol/water (1:3:6)
S2/3	Acetonitrile/toluene (23:77)
R2A	Ocylamine in heptane (3:97)
3R	Potassium trimethylsilanolate (0.1M) in methanol/t-butanol (50:50)
S3	Acetonitrile/toluene (15:85)
R3	Neat trifluoroacetic acid (TFA)
L3	Acetic acid in methanol/water (1:74:25)
PTH	Mixture of PTH amino acids (10 pmol/100 µl) in acetonitrile with DPTU as a marker
TH	Mixture of TH amino acids (50 pmol/100 µl) in acetonitrile
R4	Trifluoroacetic acid (TFA) in water (1:3)
S5	Phosphate buffer (pH 2.9), 0.3% ion pairing reagent

Method details

The steps for the N-terminal protein membrane 4.0 methods are described below. The steps indicate the ranges in volume that are appropriate for the various reagents/solvent deliveries. All sequencer methods are accessed by choosing the top menu item Edit/Method in the Protein Sequencer window. The sequencer column configuration used with the N-terminal protein membrane 4.0 methods consists of an empty reverse-phase (RP) sample column (top) mated with an empty strong anion exchange (SAX) column (bottom).

Method (Column): N-Terminal Protein Membrane 4.0

Total time: 44.9 min

All metering steps deliver the specified reagent or solvent to waste.

Step 2: The delivery of R2A should completely wet the membrane.

Steps 6, 9: The delivery of R1 should completely wet the membrane, but not flood it. The second R1 delivery may require a shorter meter time since the R1 bottle was already pressurized for the first delivery.

Step 11: The volume of R2 delivered by the end of Step 11 should be just enough to wet the membrane.

Step 13: "Flush with S2A" flushes the delivery lines and should not wet the column

Steps 18, 20, 22, 24, 33, 37, 41: The delivery of S2/3 and S3 should completely fill the column, followed by drying.

Step 28: During Step 28, no more than the upper half of the membrane should be wet with R3. The TFA should wick down to wet the whole membrane after delivery.

Step 31: After step 31, the volume of L3 delivered to the flask should be 50 µl (+/- 5 µl)

Step	Description	Primary Time	Temperature
1:	Couple: Meter R2A	5.3	55
2:	Couple: Deliver R2A DOWN (closed)	20.0	55
3:	Couple: Dry column DOWN	60.0	55
4:	Couple: Meter R1	2.0	55
5:	Couple: Deliver R1 DOWN (closed)	20.0	55
6:	Couple: Dry column DOWN	60.0	55
7:	Couple: Meter R1	2.0	55
8:	Couple: Deliver R1 DOWN (closed)	20.0	55
9:	Couple: Dry column DOWN	120.0	55
10:	Couple: Meter R2	10.8	55
11:	Couple: Deliver R2 DOWN	20.0	55
12:	Couple: Dry column DOWN	60.0	55
13:	Couple: Flush with S2A	15.0	55
14:	Couple: React	350.0	55
15:	Couple: Dry column UP	120.0	50
16:	Wash: Purge solvent line		50
17:	Wash: Meter S2/3	17.0	50
18:	Wash: Deliver and dry DOWN (closed)	30.0	50
19:	Wash: Meter S2/3	17.0	50
20:	Wash: Deliver and dry DOWN (closed)	30.0	50
21:	Wash: Meter S2/3	17.0	50
22:	Wash: Deliver and dry DOWN (closed)	30.0	50
23:	Wash: Meter S2/3	17.0	50
24:	Wash: Deliver and dry DOWN (closed)	30.0	50
25:	Wash: Dry column DOWN	150.0	50
26:	Cleave: Purge cleavage line		50
27:	Cleave: Meter R3	3.2	50
28:	Cleave: Deliver R3 DOWN	30.0	50
29:	Cleave: React	350.0	50
30:	!Extract: Dry column DOWN to flask	120.0	50
31:	!Extract: Deliver L3 to flask	7.0	50
32:	!Extract: Meter S3	19.0	50
33:	!Extract: Deliver solvent UP (closed)	15.0	50
34:	!Extract: Dry column UP to flask	5.0	50
35:	!Extract: Evaporate flask	50.0	50
36:	!Extract: Meter S3	19.0	50
37:	!Extract: Deliver solvent UP (closed)	15.0	50
38:	!Extract: Dry column UP to flask	5.0	50
39:	!Extract: Evaporate flask	50.0	50
40:	!Extract: Meter S2/3	19.0	50
41:	!Extract: Deliver S2/3 UP (closed)	15.0	50
42:	!Extract: Dry column UP to flask	5.0	50
43:	!Extract: Evaporate flask	20.0	50
44:	Extract: Dry column DOWN to waste	120.0	55

Method (Column): N-Terminal Protein Membrane 4.0 (Cycle 1)
Total time: 77.0 min

Step 1: Cycle 1 begins with an extended dry to remove any residual solvent from the sample loading process. There is also a pause to provide time for proper HPLC column equilibration after switching from C-terminal sequencing

Step 4: The delivery of R2A should completely wet the membrane.

Steps 7, 10, 18: The delivery of R1 should completely wet the membrane, but not flood it. The second and third R1 delivery may require a shorter meter time since the R1 bottle was already pressurized for the first delivery.

Step 13, 21: The volume of R2 delivered by the end of Step 13 and 21 should be just enough to wet the membrane. The second R2 delivery may require a shorter meter time since the R2 bottle was already pressurized for the first delivery.

Step 23: "Flush with S2A" flushes the delivery lines and should not wet the column

Steps 28, 30, 32, 34, 43, 47, 51: The delivery of S2/3 and S3 should completely fill the column, followed by drying.

Step 38: During Step 38, no more than the upper half of the membrane should be wet with R3. The TFA should wick down to wet the whole membrane after delivery.

Step 41: After step 41, the volume of L3 delivered to the flask should be 50 µl (+/- 5 µl)

Step	Description	Primary Time	Temperature
1:	Wash: Flush with S2A	15.0	55
2:	Couple: Dry column DOWN	300.0	55
3:	Couple: Meter R2A	5.3	55
4:	Couple: Deliver R2A DOWN (closed)	20.0	55
5:	Couple: Dry column DOWN	60.0	55
6:	Couple: Meter R1	2.0	55
7:	Couple: Deliver R1 DOWN (closed)	20.0	55
8:	Couple: Dry column DOWN	60.0	55
9:	Couple: Meter R1	2.0	55
10:	Couple: Deliver R1 DOWN (closed)	20.0	55
11:	Couple: Dry column DOWN	120.0	55
12:	Couple: Meter R2	10.8	55
13:	Couple: Deliver R2 DOWN	20.0	55
14:	Couple: Dry column DOWN	60.0	55
15:	Couple: React	300.0	55
16:	Couple: Dry column UP	120.0	55
17:	Couple: Meter R1	2.0	55
18:	Couple: Deliver R1 DOWN (closed)	20.0	55
19:	Couple: Dry column DOWN	60.0	55
20:	Couple: Meter R2	8.1	55
21:	Couple: Deliver R2 DOWN	20.0	55
22:	Couple: Dry column DOWN	60.0	55
23:	Couple: Flush with S2A	15.0	55
24:	Couple: React	300.0	55
25:	Couple: Dry column UP	120.0	50
26:	Wash: Purge solvent line		50
27:	Wash: Meter S2/3	17.0	50
28:	Wash:Deliver and dry UP (closed)	30.0	50
29:	Wash: Meter S2/3	17.0	50
30:	Wash:Deliver and dry UP (closed)	30.0	50
31:	Wash:Meter S2/3	17.0	50
32:	Wash:Deliver and dry UP (closed)	30.0	50
33:	Wash:Meter S2/3	17.0	50
34:	Wash:Deliver and dry UP (closed)	30.0	50
35:	Wash:Dry column UP	150.0	50
36:	Cleave: Purge cleavage line		50
37:	Cleave: Meter R3	3.2	50
38:	Cleave: Deliver R3 DOWN	30.0	50
39:	Cleave: React	350.0	50
40:	!Extract: Dry column DOWN to flask	120.0	50
41:	!Extract: Deliver L3 to flask	7.0	50
42:	!Extract: Meter S3	19.0	50
43:	!Extract: Deliver solvent UP (closed)	15.0	50
44:	!Extract: Dry column UP to flask	5.0	50
45:	!Extract: Evaporate flask	50.0	50
46:	!Extract: Meter S3	19.0	50
47:	!Extract: Deliver solvent UP (closed)	15.0	50
48:	!Extract: Dry column UP to flask	5.0	50
49:	!Extract: Evaporate flask	50.0	50
50:	!Extract: Meter S2/3	19.0	50
51:	!Extract: Deliver S2/3 UP (closed)	15.0	50
52:	!Extract: Dry column UP to flask	5.0	50
53:	!Extract: Evaporate flask	20.0	50
54:	Extract: Dry column DOWN to waste	120.0	55

Method (Flask): N-Terminal Flask 4.0

Total time: 34.6 min

Step 1: During Step 1, the liquid in the flask partially evaporates but must not dry down completely (10 - 20 µl remains). The flask will continue to dry during the first part of Step 2.

Step 2: During Step 2, the volume of R4 delivered to the flask should be approximately 70 µl (+/- 5 µl)

Step 4: By the end of Step 4, the flask will have been dry for 100-150 seconds.

Step 5: By the end of Step 5, 70 µl (+/- 5 µl) of L3 will have been delivered to the flask

Step 6: By the end of Step 6, the flask will have been dry for 75-100 seconds

Step 7: The delivery of L3 may not be visible

Step 8: After Step 8, the volume of L3 and S5 in the flask should be 75-80 µl. If adjustment is needed, it should be done by adjusting the metering time of S5, leaving L3 unchanged.

TEST Delivery protocol:
To check the delivery volume of L3 and S5, copy steps 7, 8, 9, and 10 to the clipboard and run from the clipboard. At the end of Step 10, remove the flask from the heating chamber. Carefully unscrew the flask cap and measure the delivered volume using a syringe. Be careful that some of the delivered volume is not retained on the tube sides or top of the flask.

Step 11: Before injection the injector loop should be filled, leaving the solvent visible in both the inlet and outlet lines of the injector loop. The time for Step 11 may have to be adjusted in order to optimize the delivery.

Step	Description	Primary Time	Temperature
1:	Convert: Evaporate	40.0	70
2:	Convert: Deliver R4	29.0	70
3:	Convert: React	400.0	70
4:	Convert: Evaporate dry	400.0	70
5:	Convert: Flush with L3	15.0	70
6:	Convert: Evaporate dry L3	140.0	70
7:	Convert: Deliver L3 dripwise	2.0	60
8:	Convert: Deliver S5 dripwise	12.0	60
9:	Convert: Mix and equilibrate	15.0	60
10:	Convert: Solubilize	20.0	60
11:	Convert: Fill loop and inject	2.0	60
12:	Convert: Empty flask	30.0	60
13:	Convert: Flush with L3	85.0	45
14:	Convert: Mix	20.0	45
15:	Convert: Empty flask	30.0	45
16:	Convert: Deliver R4 wash	40.0	45
17:	Convert: Empty flask	30.0	45
18:	Convert: Deliver R4 wash	40.0	45
19:	Convert: Empty flask	30.0	45
20:	Convert: Dry waste line	60.0	45
21:	Convert: Dry vent line	60.0	45

Method (Flask): PTH Std 4.0
Total time: 50.3 min

This PTH Std 4.0 method is appropriate with any of the N-terminal sequence programs. Ten picomoles of PTH standard are delivered to the flask in this method.

Step 1: Step 1: Cycle 1 begins with a pause to provide time for proper HPLC column equilibration after switching from C-terminal sequencing.

Step 4: Step 4 should add 100 µl (+/- 2 µl) of PTH-standard to the dry flask

TEST Delivery protocol:
To check the delivery volume of the PTH-standards, copy the steps in the PTH Std Test 4.0 method to the clipboard and run from the clipboard. At the end of test program, remove the flask from the heating chamber. Carefully unscrew the flask cap and measure the delivered volume using a syringe. Be careful that some of the delivered volume is not retained on the tube sides or top of the flask

Step 6: The delivery of L3 may not be visible

Step 7: After Step 7, the volume of L3 and S5 in the flask should be 75-80 µl. If adjustment is needed, it should be done by adjusting the metering time of S5, leaving L3 unchanged.

TEST Delivery protocol:
To check the delivery volume of L3 and S5, copy steps 6, 7, 8, and 9 to the clipboard and run from the clipboard. At the end of Step 9, remove the flask from the heating chamber. Carefully unscrew the flask cap and measure the delivered volume using a syringe. Be careful that some of the delivered volume is not retained on the tube sides or top of the flask.

Step	Description	Primary Time	Temperature
1:	Convert: Empty flask	120.0	60
2:	Convert: Dry waste line	200.0	60
3:	Convert: Dry vent line	200.0	60
4:	Convert: Deliver PTH Std with L3 purge	6.9	60
5:	Convert: Evaporate dry standard	180.0	60
6:	Convert: Deliver L3 dripwise	2.0	60
7:	Convert: Deliver S5 dripwise	12.0	60
8:	Convert: Mix and equilibrate	15.0	60
9:	Convert: Solubilize	20.0	60
10:	Convert: Fill loop and inject	2.0	60
11:	Convert: Empty flask	30.0	60
12:	Convert: Flush with L3	120.0	45
13:	Convert: Mix	20.0	45
14:	Convert: Empty flask	30.0	45
15:	Convert: Deliver R4 wash	40.0	45
16:	Convert: Empty flask	30.0	45
17:	Convert: Deliver R4 wash	40.0	45
18:	Convert: Empty flask	30.0	45
19:	Convert: Dry waste line	250.0	45
20:	Convert: Dry vent line	250.0	45

**Printed in the U.S.A. November 1997
5966-2773E**