Manual for SEC-MALS
（Waters Alliance - Wyatt DAWN HELEOS II (2023.1.27 revised)）

For staff：
**Setting up HPLC on the day before. (1~5)**

For Users：
Setting up HPLC. (6~9), Experiment.（10~15）

1. Setting up HPLC（the day before）

1.1 Connection between PC and equipment.

Connect HPLC's hub and the PC for HPLC (Fujitsu) with grey LAN cable.

Connect the hub for MALS/RI and the LAN port on the motherboard side of the PC for MALS/RI with black LAN cable.

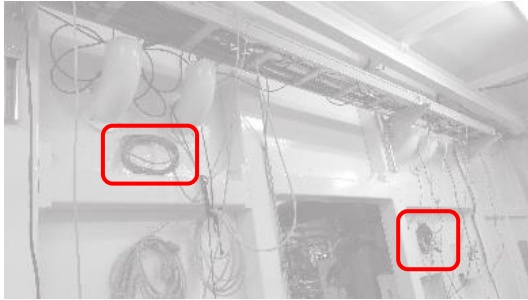Hub for MALS/RI

LAN port (HPLC)

LAN port (MALS/RI)
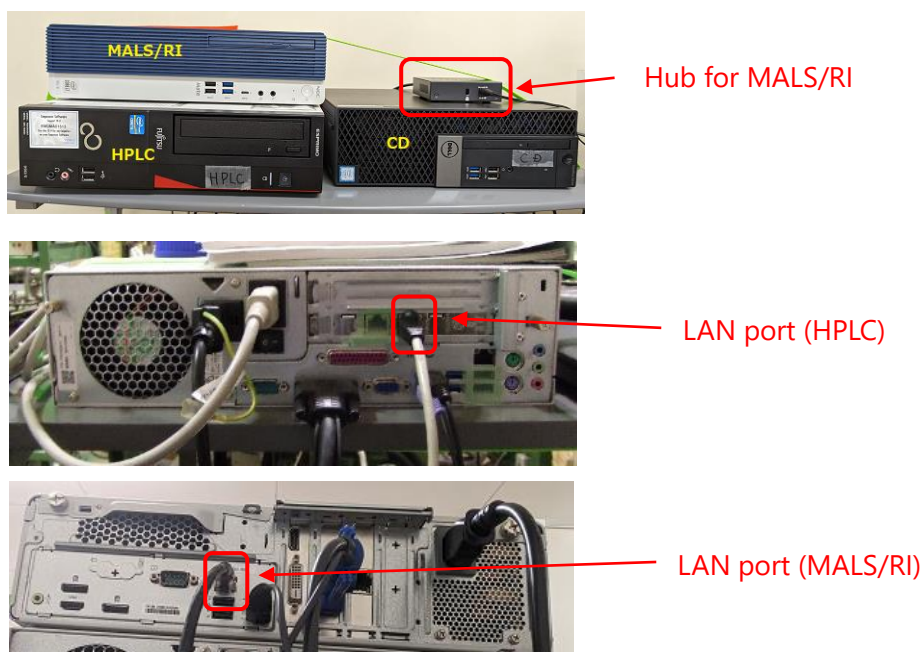
1.2 [When HPLC is installed in the BL-15A2 experimental hatch] Connecting the system to BL network.

Connect the BL LAN cables (which are tagged "Hub2") to the LAN port of the PC for HPLC and the LAN port of the USB-LAN converter of the PC for RI, respectively.

[LAN cables tagged "Hub2" are near the hatch door on the BL-16 side @15A2 (there are

1.3 Checking the glass bottles for waste liquid.

If glass bottle for waste liquid under the desk is full, replace it.

　(If it does not fall under any other waste classification, such as heavy metals or acid, etc.)

Dispose waste liquid in the polyethylene tank for hydrous organic solvent in the chemistry laboratory.

1.4 Check the volume of 10% ethanol in the glass bottle on Alliance (e2695). If little, change new one.

1.5 Connecting the power supply cable.

Connect the power supply cables of the two desktop PCs (HPLC, MALS/RI) to the OA tap for the PC under the table.

And then connect the two OA taps (one for the PC and one for the equipment), to the power supply.

1.6 [**In case of starting the HPLC only.]⇒Normally start at 1.7**

・Power on e2695 (The unit with LCD panel (The switch is on the left side.)

・Power on 2489 (UV/Vis)

・Wait for several minutes to system initialization.

e2695 (The switch is on the left side)
MALS
2489(UV/Vis)
RI
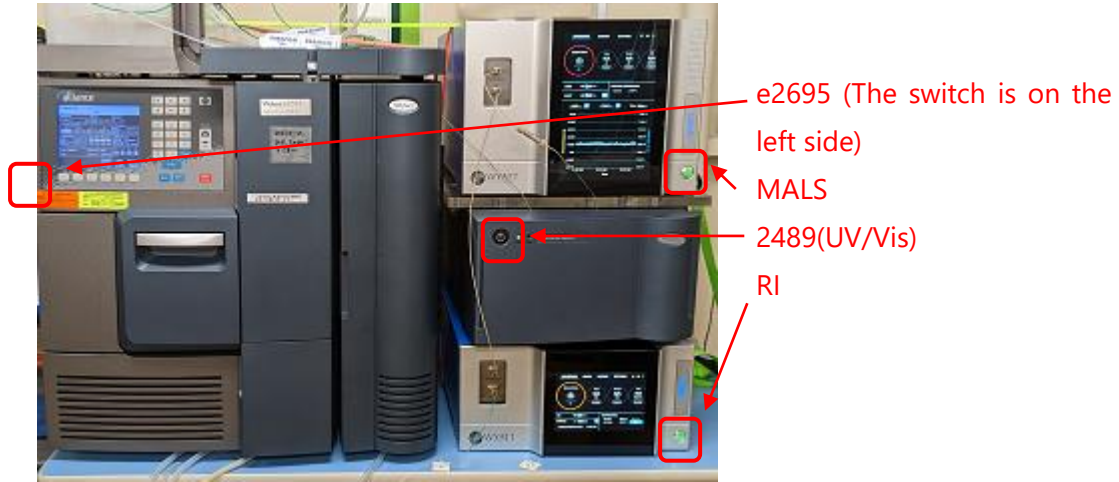
1.7 [In case of starting all system.]

HPLC

・（**Always first**）Power on PC for HPLC.

User name: Administrator, password: hplc

After logging in, wait until the OS starts up and access to the HDD settles down.

・Power on e2695 (The unit with LCD panel (The switch is on the left side.)

・Power on 2489 (UV/Vis)

MALS・RI

・Power on PC for RI

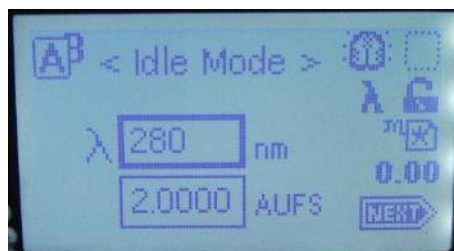User name and password：none（As of Jan 2023）

・Power on MALS and RI

1.8 Because the UV lamps is consumable part, turn off the UV lamps when preparing the day before the experiment.

・Open the 2489 door (A)

・Press Shift →1 on the keypad (B) and Press Shift →1 again (C).
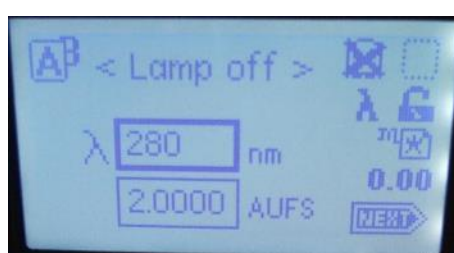
If you want to turn on the UV lamp, press Shift →1 and then press Shift →1 again.

(A)

(B)   (C)

2. Equilibration with MilliQ.

2.1 Check the volume of 10% ethanol (the bottle with the clear tube labeled "Seal Wash In" in green) on the tray at the top of HPLC. If it is less than 400 ml, replace a 10% ethanol bottle. Set the bottle, insert the "Seal Wash In" line into the bottle, and cover the mouth of the bottle with Parafilm. Also, make sure that the filter at the end of the line is dipped in the bottom of the bottle.
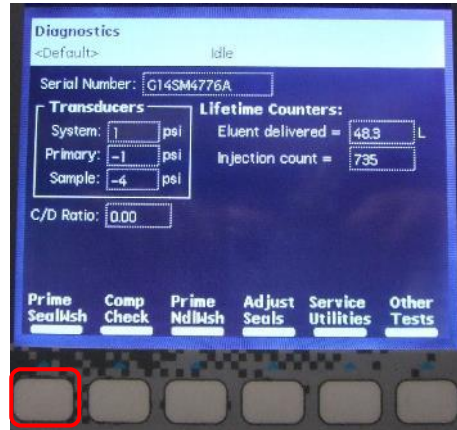
2.2 [If there is air bubble in "Seal Wash In" tubing, perform a "Prime Seal Wash".]
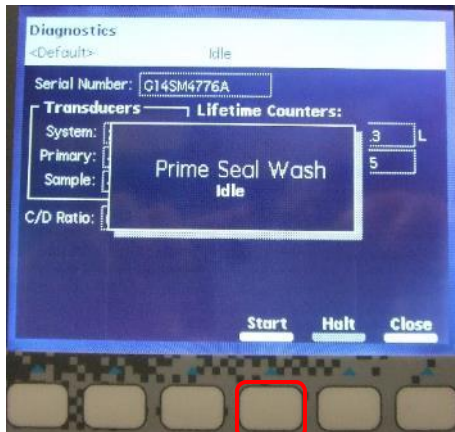
On the LCD panel of the e2695
・From the Main screen, press the Diagnostics button to move to Diagnostics (A).
・Select "Prime SealWsh" button (B) and press the Start button (C).
・Connect a syringe with a tubing adapter (stored in Waters clear plastic case (E) under PC desk) to the plunger seal wash waste tubing (1/8" diameter clear tubing (D) stuck in the waste bottle).
・Pull the plunger   until washing solution comes out from the end of the tube **(Do not push.).**
・press "Halt" to stop (F) after solution comes out.
・press "Close" (G).
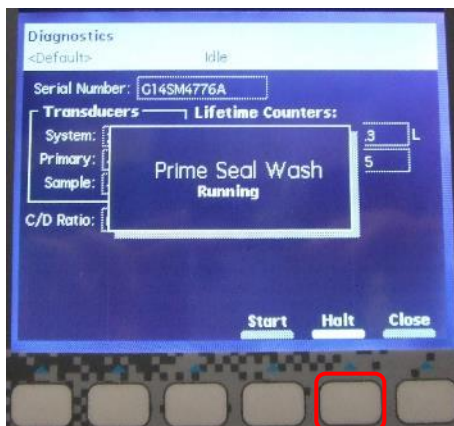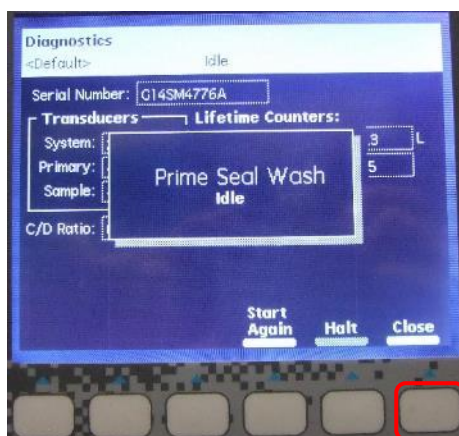
(A)

(B)

(C)

(D)

(E)

(F)

(G)

2.3 Set a MilliQ bottle on the tray at the top of HPLC, insert two lines (A (yellow label) and needle washing (green)) in 20% ethanol bottle to MilliQ bottle, and cover the mouth of the bottle with Parafilm. (As a rule, MilliQ is made and degassed on that day.) Also, make sure that the ends of each line is dipped into the bottom of the MilliQ bottle.

2.4 Perform a "Prime Seal Wash".
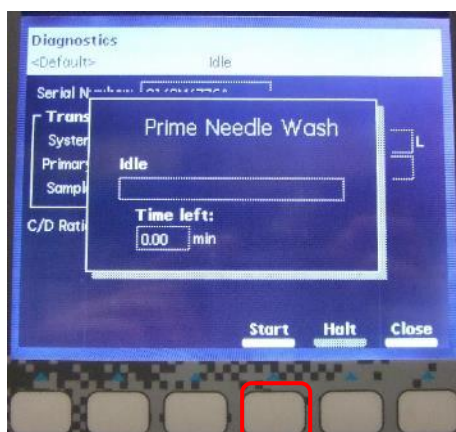On the LCD panel of e2695
   ・From the Main screen, press the Diag button (A) to move to Diagnostics.
   ・Select " Prime NdlWsh" button and press "Start" button (B).
   ・If no solvent comes out from yellow waste tube outlet, press the Start Again button (C).
   ・If solvent comes out from yellow waste tube outlet, press Close (D), and then press "Exit" to return to the Main screen.
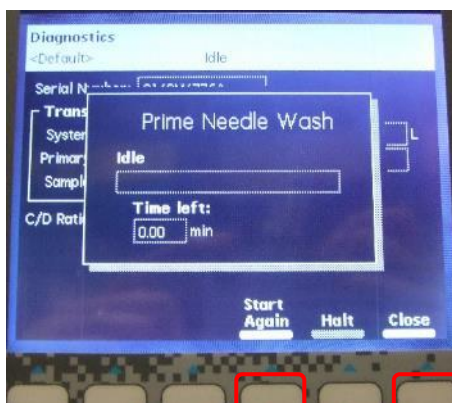

(A)
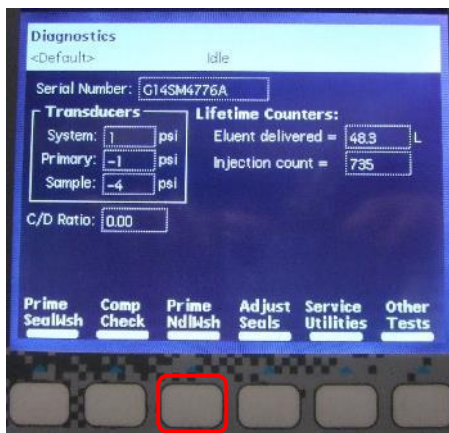
(B)

(C)   (D)

2.5 Check the volume of 50% ethanol bottle on the tray at the top of HPLC (the B, C, and D lines are dipped in 50% ethanol bottle). If the ends of each tube are not completely submerged in solution, replace 50% ethanol bottle. (The 50% ethanol should be degassed.)
Set 50% ethanol bottle, insert B, C, and D lines into the bottle, and cover the mouth of the bottle with Parafilm. Make sure that the ends of each line are at the bottom of the bottle.

2.6 Perform "Wet prime" A, B, C, and D lines each at 3 ml/min, 0.5 min. (If the latest use was recent (approx. 10 days before), perform line A only.).
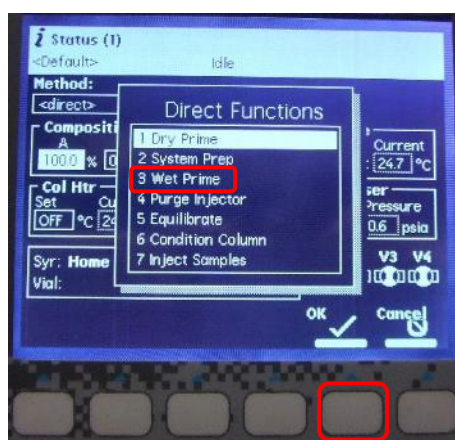
On the LCD panel of the e2695
   ・Press the Menu/Status button to go to the Status screen (A).
   ・Change to "100%" in "A" of "Composition"   (B) with the cursor keys.
   ・Press "Direct Function" button and select "3 Wet Prime" (C).
   ・Enter 3 ml/min, 0.5 min, and press OK button to start Wet Prime (D).
If necessary, perform the B, C, and D lines as well. (A~D lines must be filled with solvent.)

(A)

(B)

(C)

(D)

2.7 After wet prime, keep the flow rate at 0 ml/min for 5 min to equilibrate the solvent in the in-line degasser.

2.8 Perform "Wet prime" A line with MilliQ at 3 ml/min, 5min. (Not necessary for B, C, and D).

On the LCD panel of the e2695

・ Press the Menu/Status button to go to the Status screen.

・ Change to "100%" in "A" of "Composition" with the cursor keys.

・ Press "Direct Function" button and select "3 Wet Prime".

・ Enter 3 ml/min, 5 min, and press OK button to start Wet Prime.

2.9 After wet prime, enter flow rate in the Status screen (A), and press enter to start pumping (B). Increase the flow rate in a gradual manner (e.g., 0->0.2->0.4 ~ 0.5~1ml/min) to replace the solvent in the detectors.
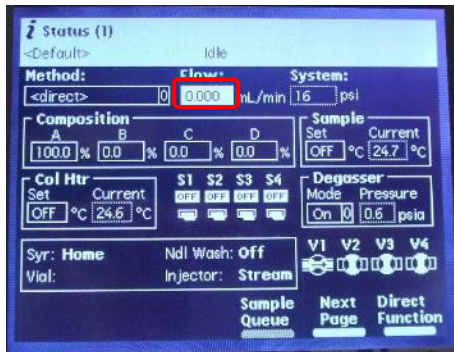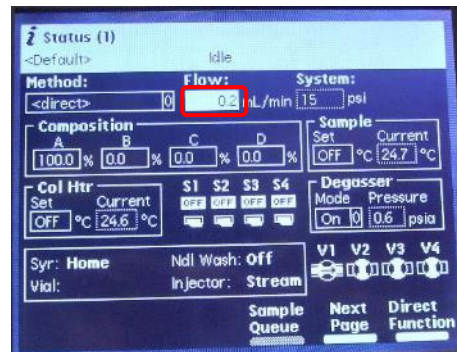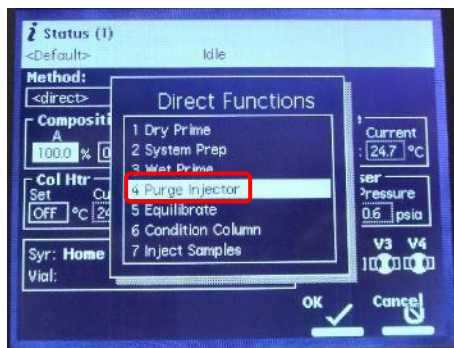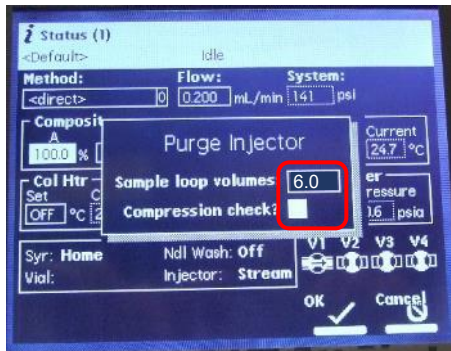
(A)   (B)

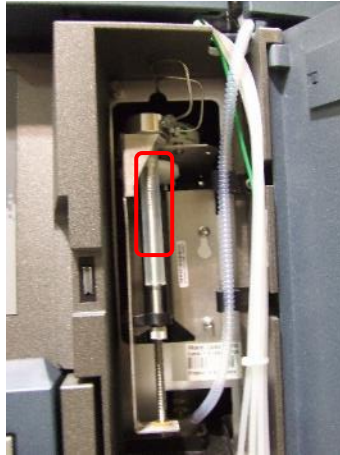2.10   Perform "Purge injector".

On the LCD panel of the e2695,

・Press "Direct Function" button and select "4 Purge Injector" (A).

・Confirm Sample loop volumes 6.0 and Uncheck Compression Check (B).

・Press "OK" button.

・After "Purge Injector", check no air bubbles in the syringe of the sample injector (C).

・If there is any air bubble, repeat "purge injector" with flicking the syringe.

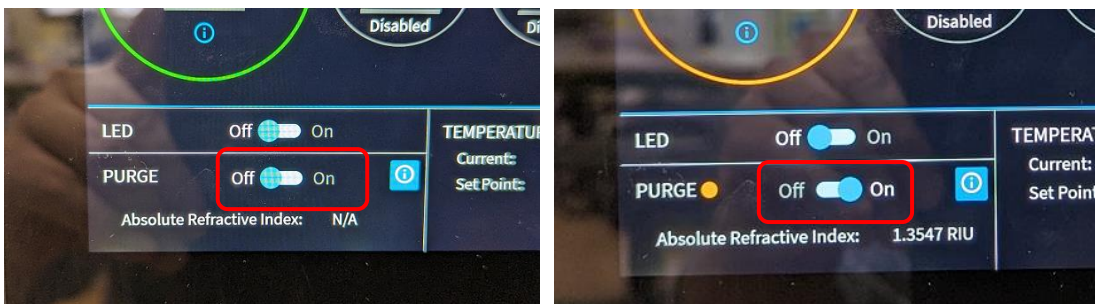(A)   (B)

(C)

2.11   Purge the RI detector Optilab.

With the e2695 pumping solution to the detector, switch to "OFF→ON" at "PURGE" on the front touch panel of the Optilab unit.

***The temperature setting for RI is 25°C. Do not change it!**

2.12   When users connect their own columns by themselves, we will hand over the system which is equilibrated with MilliQ. Decrease the flow rate (about 0.05 to 0.1 ml/min). Be careful not to run out of MilliQ.

3.   Connection with Column and equilibration with MilliQ

3.1 Set flow rate lower (e.g.,0.2 ml/min for Superdex200 increase 10/300)

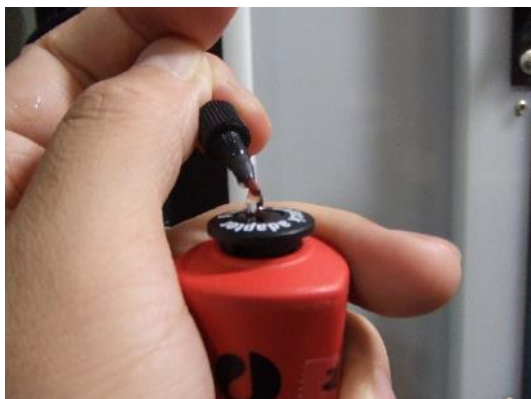3.2 Remove the locking pin while pushing down on the spring-loaded cap of the "Storage/Shipping device."
Release the cap, and then unscrew "Storage/Shipping device".

- spring-loaded cap
- locking pin
- Column inlet
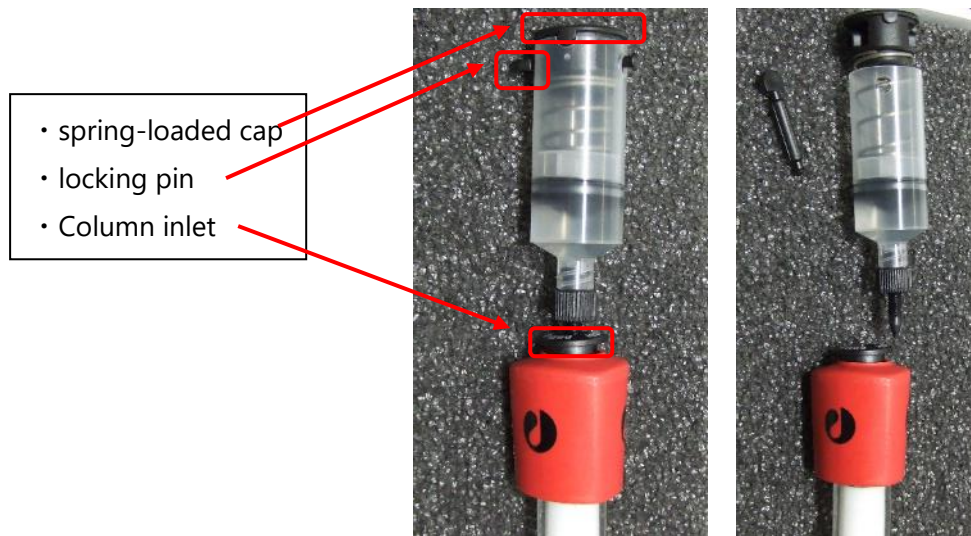
3.3 Disconnect the union part in the column heater.

3.4 Unscrew the column outlet plug. And fill the column inlet with MilliQ drop-to-drop.

3.5 Connect the column inlet to the orange PEEK tube. Screw the black fitting while lightly pushing down the tube, and after connecting, pull the PEEK tubing to make sure it does not come off.

3.6 Wait until the column outlet is filled with solution, then connect the column outlet to the HPLC metal tubing. Screw the fitting while pressing down metal tubing lightly. After connecting, pull the metal tubing to make sure it does not come off.

3.7 Install the column in the column holder so that it flows from the bottom to the top.

3.8 Wipe the inlet and outlet with a wiping paper, and check no leak.

3.9 Set flow rate higher (e.g., 0.3 ml/min for Superdex200 increase 10/300 [while changing from 20%ethanol to MilliQ.) and equilibrate with at least 3 CV MilliQ (4 or more CV is better).

3.10 In case hand over the system and column which are equilibrated with MilliQ. Decrease the flow rate (about 0.05 to 0.1 ml/min). Be careful not to run out of MilliQ.

4.   Equilibration with buffer.

4.1 (Only if user requests in advance.)

   After equilibration with MilliQ in step 3.10, perform equilibration with buffer (refer to step 6.) After equilibration with buffer, decrease the flow rate (about 0.05-0.1 ml/min) until user use SEC-MALS system. Be careful not to run out of buffer.
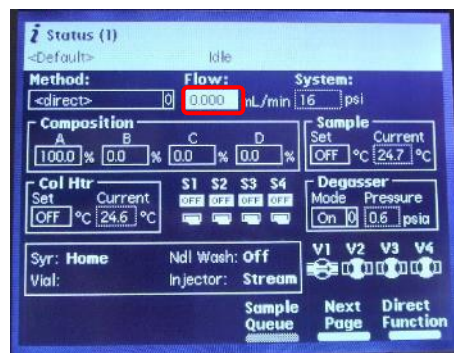
5. Setting up HPLC and preparation. (In case column is already connected.)

5.1 If the PC is not started, HPLC need to be restarted (if both of PCs and system are running, proceed step5.7.)

Set the flow rate to 0 ml/min on the status screen (A) of e2695's LCD panel and stop the pump (B). Wait for 1~2 minutes until the pressure drops completely.
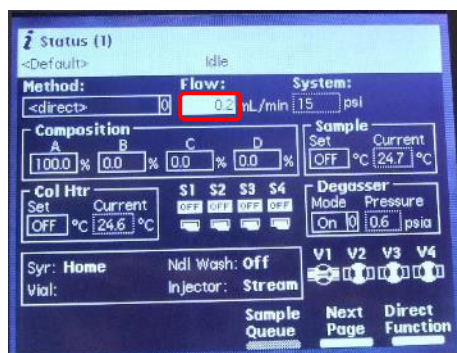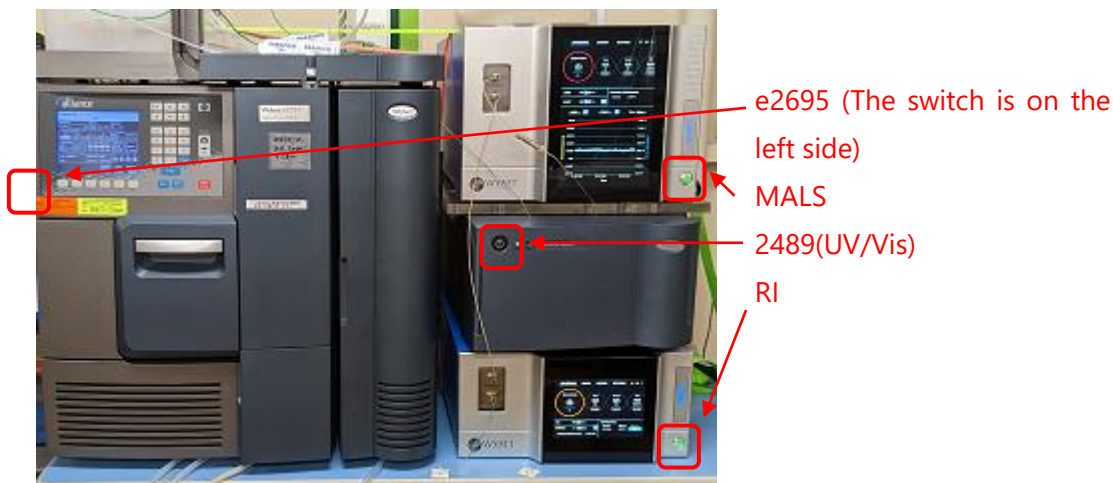

    A   B

5.2 Switch off e2695 and 2414. Leave MALS/RI on.

e2695 (The switch is on the left side)
MALS
2489(UV/Vis)
RI

5.3 Starting HPLC and MALS

HPLC

・（**Always first**）Power on PC for HPLC.

User name: Administrator, password: hplc

After logging in, wait until the OS starts up and the access to HDD settles down.

・Power on e2695 (The unit with LCD panel (The switch is on the left side.)

・Power on 2489 (UV/Vis)

MALS・RI (If they are turned off.)

・Power on PC for RI

User name and password：none（As of Jan 2023）

・Power on MALS and RI

5.4 If HPLC is started up on the same day, UV lamp will come up ON automatically. Proceed step5.5.

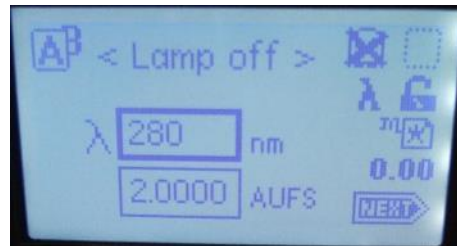If HPLC has been started up the day before, UV lamp should be OFF.

Turn the UV lamp ON.

Open the 2489 door (A) and press Shift →1 on the keypad (B). Press Shift →1 again to turn the UV lamp ON (C).

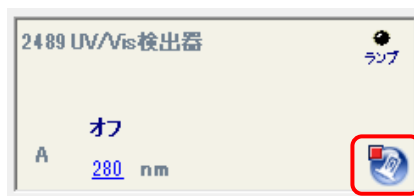(Wait for at least 30 min to warm-up.)

A

B

C

When Empower3 is running on HPLC PC, the lamp can be turned On/Off from 2489 UV/Vis detector column.
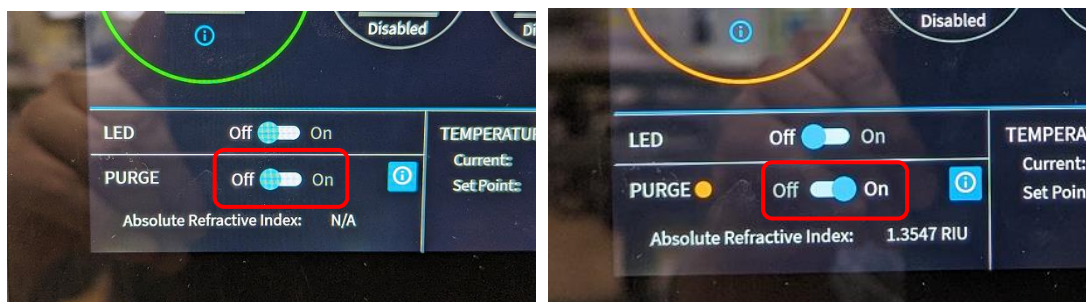
5.5 Purge the RI detector Optilab.

If Optilab has been started on the previous day, it should have already been purged, so leave it as it is. If Optilab is started up on the same day, start purging.

With the e2695 pumping solution to the detector, switch to "OFF→ON" at "PURGE" on the front touch panel of the Optilab unit.

*The temperature setting for RI is 25°C. Do not change it!

5.6 Set buffer bottle on the tray at the top of HPLC, insert A (yellow label) line in MilliQ bottle to buffer bottle, and cover the mouth of the bottle with Parafilm. (Buffer must be filtered and degassed on that day.) Also, make sure that the ends of line is dipped into the bottom of buffer bottle.

5.7 Perform "Wet prime" A line each at 5.0 ml/min for 3.0 min.

※If the high pressure error occurs, the flow rate should be reduced, such at 3.0 ml/min for 5.0 min (Total15 ml flow is needed.)
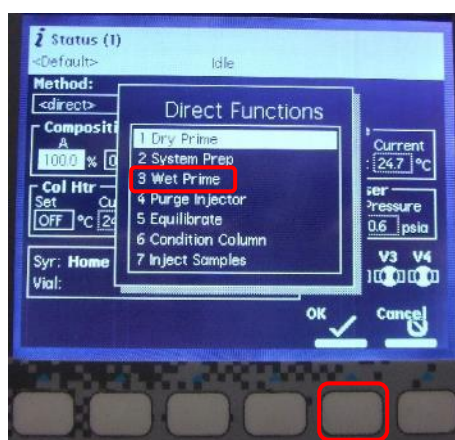
On the LCD panel of the e2695
・Press the Menu/Status button to go to the Status screen (A).
・Change to "100%" in "A" of "Composition" (B) with the cursor keys.
・Press "Direct Function" button and select "3 Wet Prime" (C).
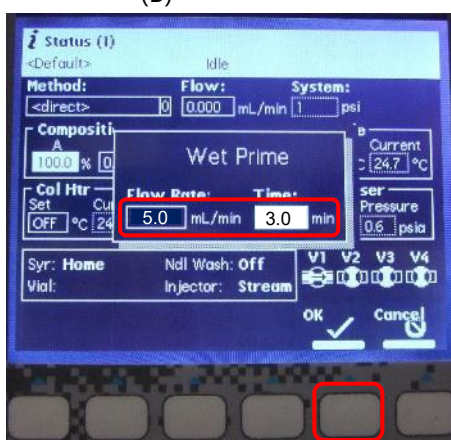・Enter 5.0 ml/min, 3.0 min, and press OK button to start Wet Prime (D).

(A)

(B)

(C)

(D)

5.8 ~~After wet prime, keep the flow rate at 0 ml/min for 5 min to equilibrate the solvent in the~~
~~in-line degasser.~~

5.9 Perform "Purge injector".

On the LCD panel of the e2695,

・Press "Direct Function" button and select "4 Purge Injector" (A).

・Confirm Sample loop volumes 6.0, Uncheck Compression Check (B) and Press "OK"
button

・repeat "Purge injector" again.

・After "Purge Injector", check no air bubbles in the syringe of the sample injector (C).

・If there is　any air bubble, repeat "purge injector" with flicking the syringe.
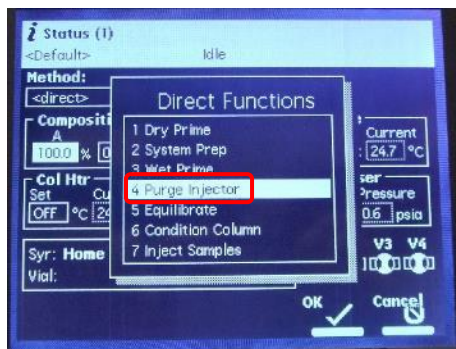
(A)
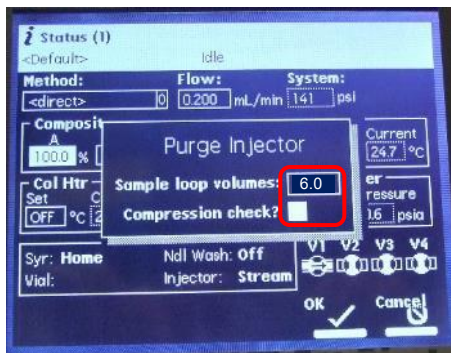
(B)
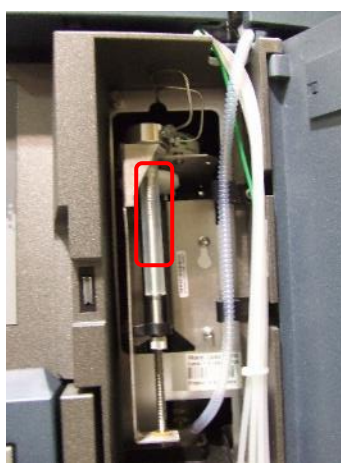
(C)

5.10   Start pumping to A line and equilibrate the column by increasing the flow rate in 0.1 ml/min increments (e.g., Superdex 200 increase 10/300, ~0.5 ml/min). Equilibration should be performed with a sufficient amount of buffer.

6 Setting up HPLC and preparation.（**In case users connect their own columns by themselves.**）

6.1 HPLC is already started and equilibrated with MilliQ.

6.2 Set flow rate 0.2ml/min on the LCD panel of e2695 (e.g., For Superdex200 increase 10/300)

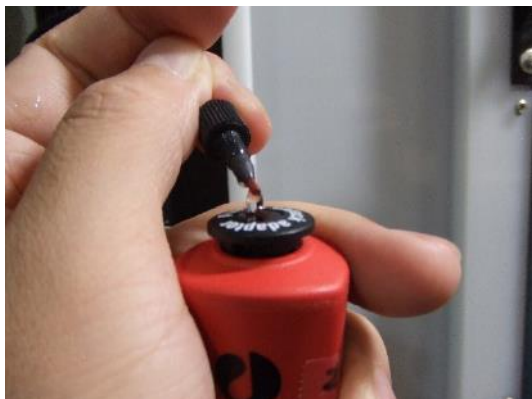6.3 Remove the locking pin while pushing down on the spring-loaded cap of the "Storage/Shipping device."
Release the cap, and then unscrew "Storage/Shipping device".

・spring-loaded cap
・locking pin
・Column inlet
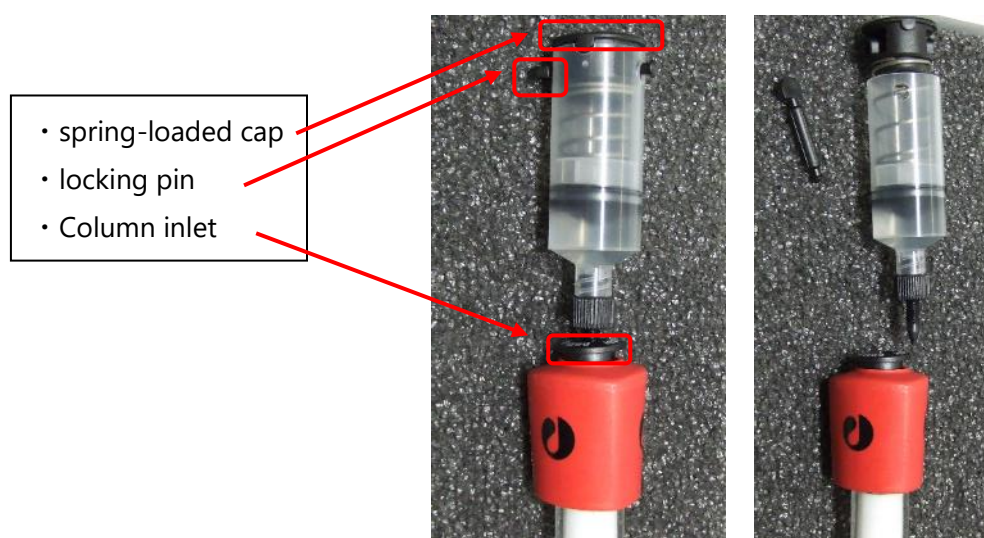
6.4 Disconnect the union part in the column heater.

6.5 Unscrew the column outlet plug. And fill the column inlet with MilliQ drop-to-drop.

6.6 Connect the column inlet to the orange PEEK tube. Screw the black fitting while lightly pushing down the tube, and after connecting, pull the PEEK tubing to make sure it does not come off.

6.7 Wait until the column outlet is filled with solution, then connect the column outlet to the HPLC metal tubing. Screw the fitting while pressing down metal tubing lightly. After connecting, pull the metal tubing to make sure it does not come off.

6.8 Install the column in the column holder so that it flows from the bottom to the top.

6.9 Wipe the inlet and outlet with a wiping paper, and check no leak.

6.10   Set flow rate higher (e.g., 0.3 ml/min for Superdex200 increase 10/300 [while changing from 20%ethanol to MilliQ.) and equilibrate with at least 3 CV MilliQ (4 or more CV is better).

6.11   After equilibration with MilliQ, Set the flow rate to 0 ml/min on the status screen (A) of e2695's LCD panel and stop the pump (B). Wait for the pressure to drop completely (for 1~2 minutes).

6.16   Set buffer bottle on the tray at the top of HPLC, insert A (yellow label) line in MilliQ bottle to buffer bottle, and cover the mouth of the bottle with Parafilm. (Buffer must be filtered and degassed on that day.) Also, make sure that the ends of line is dipped into the bottom of buffer bottle.

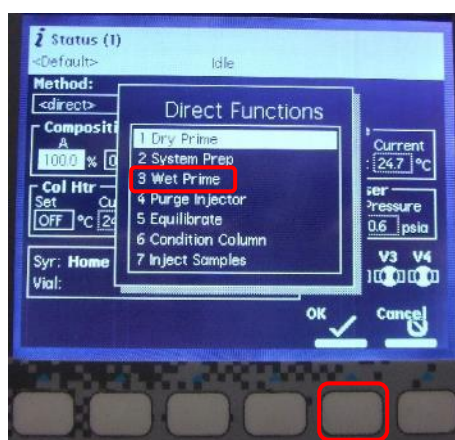6.17   Perform "Wet prime" A line each at 5.0 ml/min for 3.0 min.

On the LCD panel of the e2695
・Press the Menu/Status button to go to the Status screen (A).
・Change to "100%" in "A" of "Composition"   (B) with the cursor keys.
・Press "Direct Function" button and select "3 Wet Prime" (C).
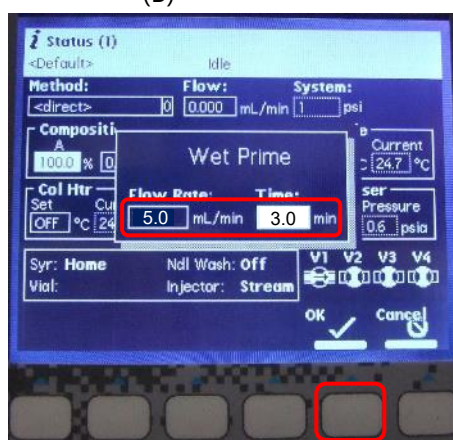・Enter 5.0 ml/min, 3.0 min, and press OK button to start Wet Prime (D).

(A)

(B)

(C)

(D)

~~6.18   After wet prime, keep the flow rate at 0 ml/min for 5 min to equilibrate the solvent~~
~~in the in-line degasser.~~

6.19   Perform "Purge injector".

On the LCD panel of the e2695,

・Press "Direct Function" button and select "4 Purge Injector" (A).

・Confirm Sample loop volumes 6.0, Uncheck Compression Check (B) and Press "OK"
button

・repeat "Purge injector" again.

・After "Purge Injector", check no air bubbles in the syringe of the sample injector (C).

・If there is   any air bubble, repeat "purge injector" with flicking the syringe.
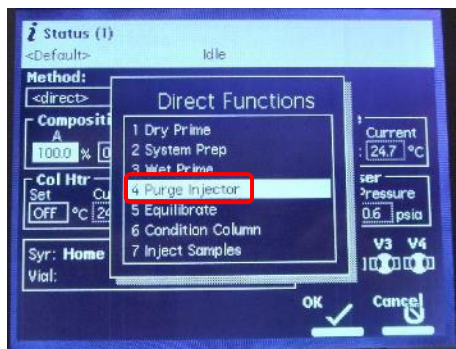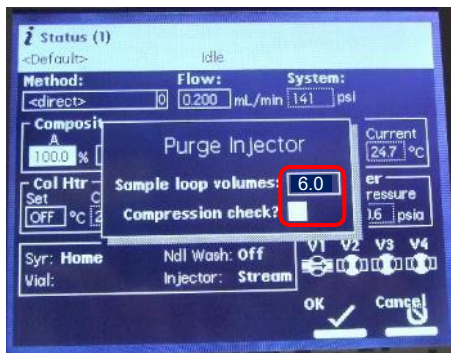
(A)   (B)
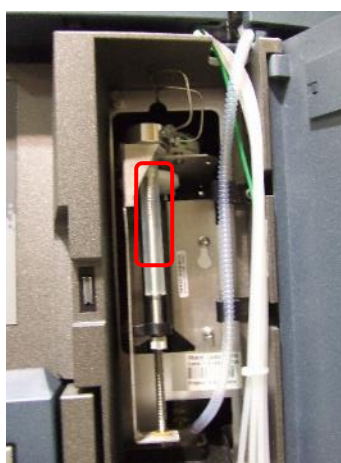

(C)

6.20   Start pumping to A line and equilibrate the column by increasing the flow rate in
        0.1 ml/min increments (e.g., Superdex 200 increase 10/300, ~0.5 ml/min). Equilibration
        should be performed with a sufficient amount of buffer.

7   Starting MALS control software.

Switch to MALS PC with KVM switch. [1: MALS, 2: HPLC]

7.1 Start Astra 8.1.2 (Control and data analysis software for MALS).
    Click Astra 8.1.2 icon on desktop.
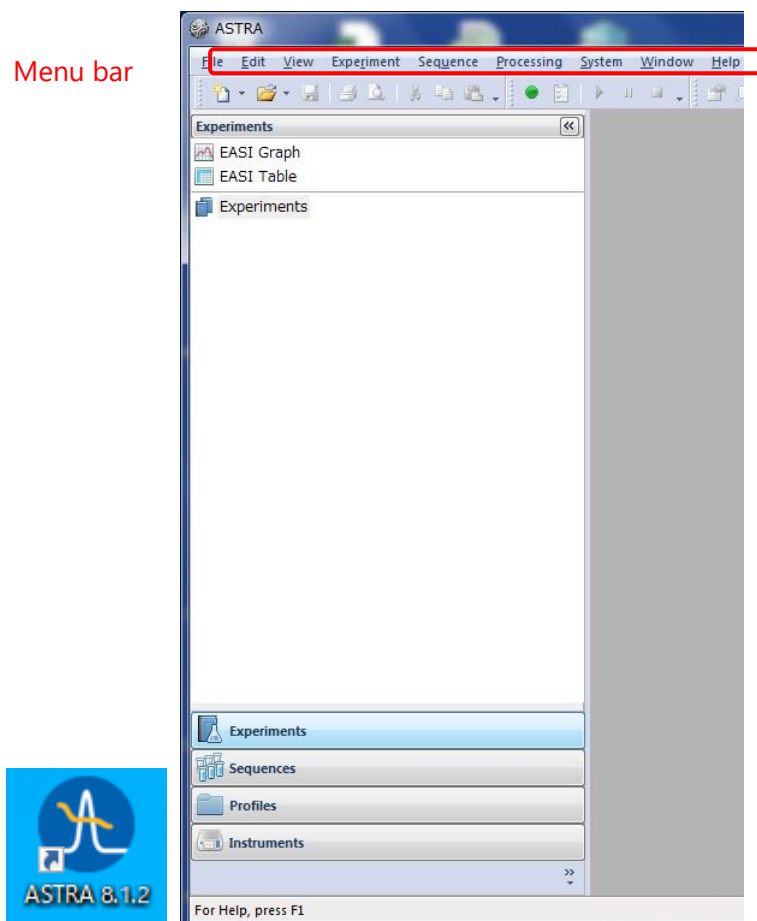
7.2 Check the connection MALS and PC.
    ・Select [System] → [Instruments] from the menu bar.
    ・Confirm there are [DAWN] and [OptiLab] in Instruments window.

        DAWN HELEOS II：WYATT-3909D3 – DAWN
        Optilab：WYATT-3950OP1
    [If not, click [Refresh]. If they still don't appear, restart Astra 8.1.2.]
        ・Click on [DAWN] to highlight it and click [View] to open Diagnostic Manager
        window.

・Click the Instruments column, select DAWN, and click the [Start Monitoring"]
button to check if the signal is acquired.
　・Click the [Stop Monitoring] button to stop signal acquisition.
　・Click [Close] to close Diagnostic Manager and Instruments windows.

8　Starting HPLC control software.

8.1 Start Empower3（HPLC control software).
Switch to HPLC PC with KVM switch.
Click Empower3 icon on desktop.

8.2 Enter [User ユーザー名] : system and [password パスワード] : manager in login window,
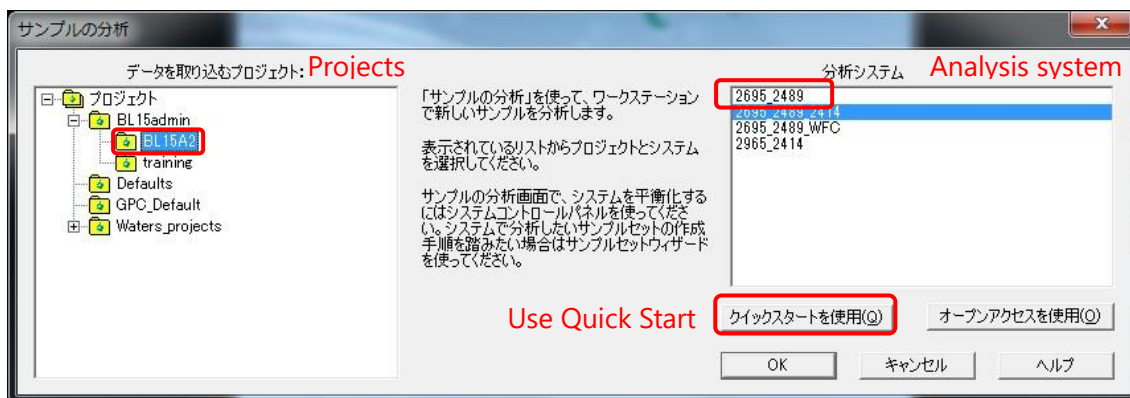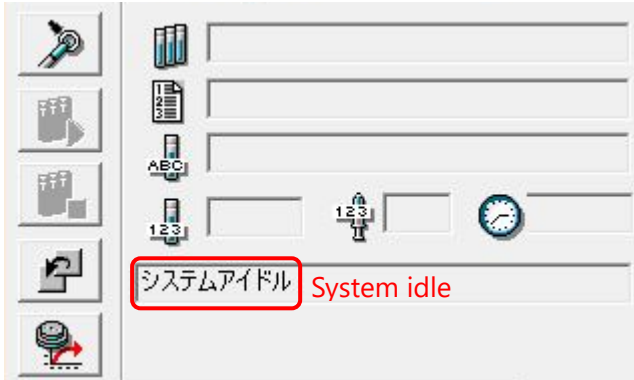and click [OK].

8.3 On Empower server window サーバーウインドウ, click [sample analysis サンプルの分
析].

Sample analysis

8.4 In the [sample analysis サンプルの分析] window, select [projects データを取り込むプロジェクト: BL15A2], [analysis system 分析システム: 2695_2489], and click [Use Quick Start クイックスタートを使用].

Projects / Analysis system / Use Quick Start

8.5 If [System Idle システムアイドル] appears in the message box at the bottom of [Quick Start クイックスタート] window, the HPLC system has started successfully. After startup, proceed to step 10.

System idle
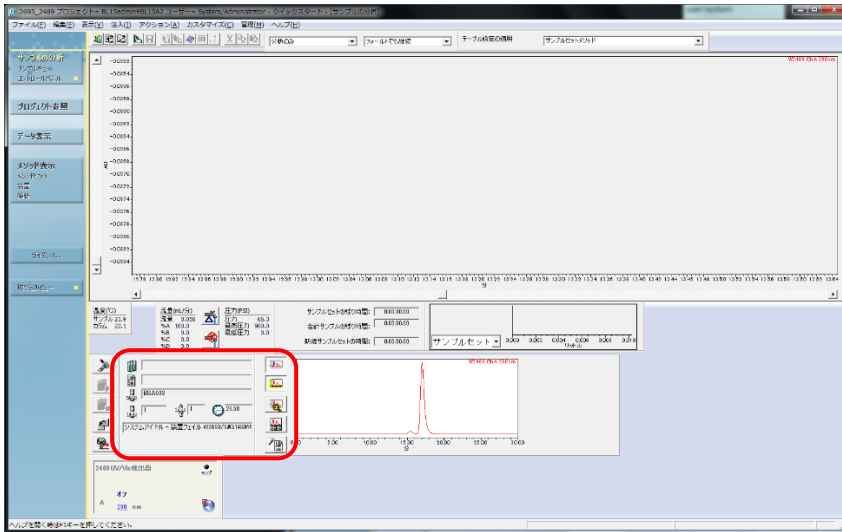
8.6 If a message such as a [communication error 通信エラー] appears in the message field at the bottom of [Quick Start クイックスタート] window, the HPLC system should be restarted. (Step 8.7~8.10)
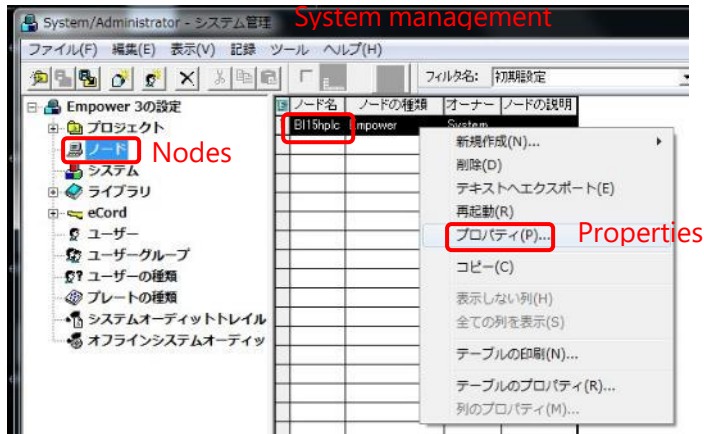
Close [Quick Start クイックスタート] window and click [Configure System システムを構成] in [Empower Server サーバー] window.

Configure System

8.7 Select [node ノード] from the left side of [System Management システム管理] window, right-click [Bl15hplc] from the central table and select [Properties プロパティ(P)].

8.8 Check [OK?] column of [device 装置] tab in [properties プロパティ] window of node Bl15hplc. If both of W2690/5 and W2489 are [Yes], they are connected. [W410 and WFCIII (Fraction collector) should be [No]],
If not, click [Scan devices 装置のスキャン] to re-scan.
And then confirm that the two devices are [Yes], and click [OK].

8.9 Click OK in 　[properties プロパティ] window of node Bl15hplc, close 　[System Management システム管理] window, and start over from step 8.3.

8.10 　 If Empower3 does not start up normally after executing step 8.7~8.9, close Empower3 and start over from step 8.1.
If Empower3 still does not start successfully, restart PC and all HPLC units.

　Exit Procedure
　Confirm that HPLC is not working.

Exit (log out) Empower3

Shut down the PC

Switch off devices (e2685, 2489(UV/Vis))

Startup procedures

Power on PC for HPLC.

After logging in, wait until the OS starts up and access to the HDD settles down.

Power on e2695 (The unit with LCD panel (The switch is on the left side.)

Power on 2489 (UV/Vis)

Restart Empower3 (step8.1~)

e2695 (The switch is on the left side)

MALS

2489(UV/Vis)

RI
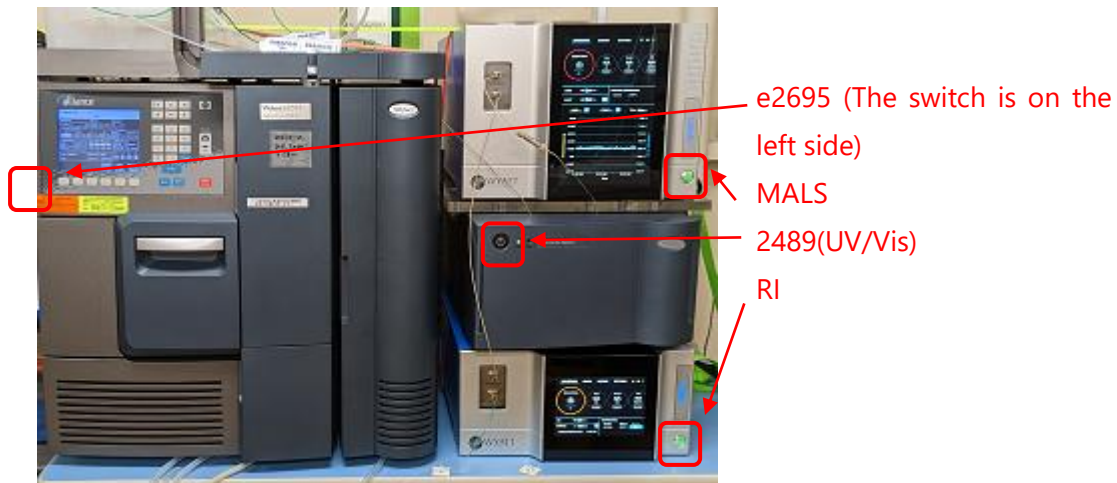
9 Setting up of Empower3

9.1 Selection [Method set メソッドセット]

Click [Method Set メソッドセット] on the left side of Quick Start window (A).

In the [Instrument Method 装置メソッド] column,

Select [20230117_Glass_Column] from the pull-down menu when using glass-cased columns such as GE (Cytiva) Superdex increase (C),
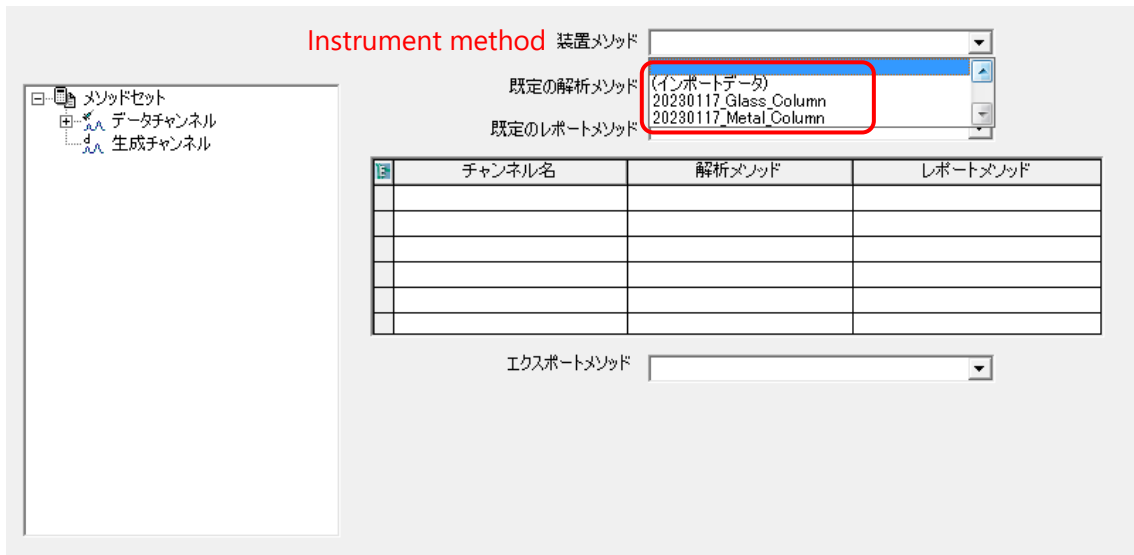
Select [20230117_Metal_Column] when using metal-cased columns such as Shodex/Nacali/Wyatt.

And click [Device 装置] (D). At that time, a confirmation window will appear, and ask if you want to save the untitled method set, select [No いいえ(N)] (E).
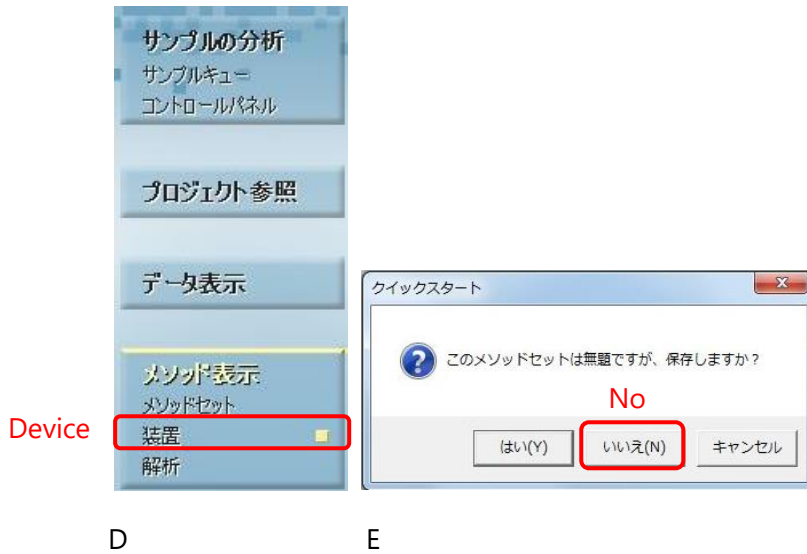
A

B

Method set

C

Instrument method

D   E
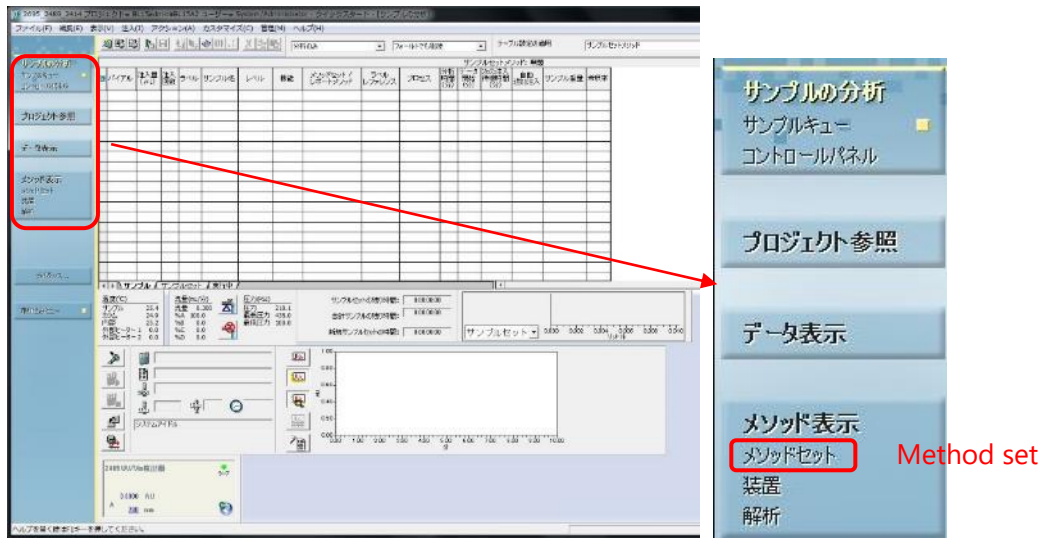
9.2 Setting flow rate on 2690

Click [W2690/5] icon. Click the [Flow rate 流量] tab and set the flow rate.

· Set the [maximum pressure limit 最大圧力(psi (I)] to 500 psi for glass columns and about 1000 psi for metal columns [1 MPa = 145 psi].
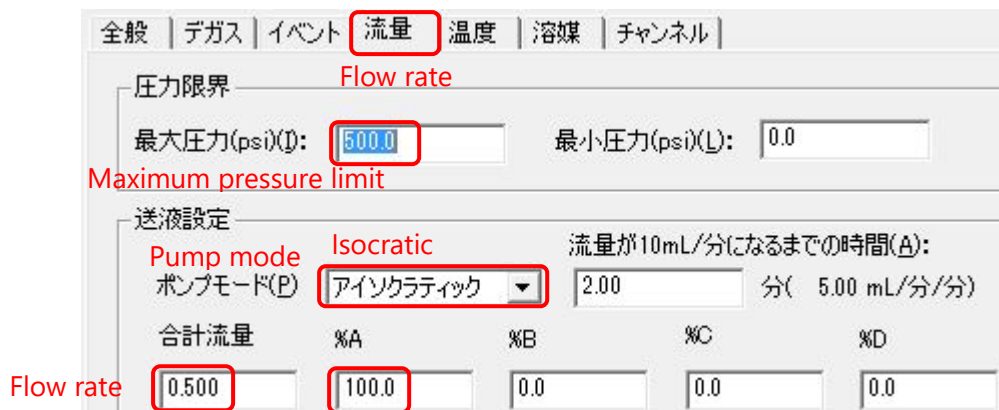
(Check the pressure limit of column from the column manual.)

· [Pump mode ポンプモード(P)]: [Isocratic アイソクラティック].

· [Total flow rate 合計流量]: 0.4~0.6 ml/min max for Superdex 200 increase 10/300, 0.04~0.06 ml/min for Superdex 200 increase 3.2/300, 0.5~1.0 ml/min for metal columns (they are dependent on buffer viscosity.)

　　· %A: 100 (only A line is used in SEC-MALS)
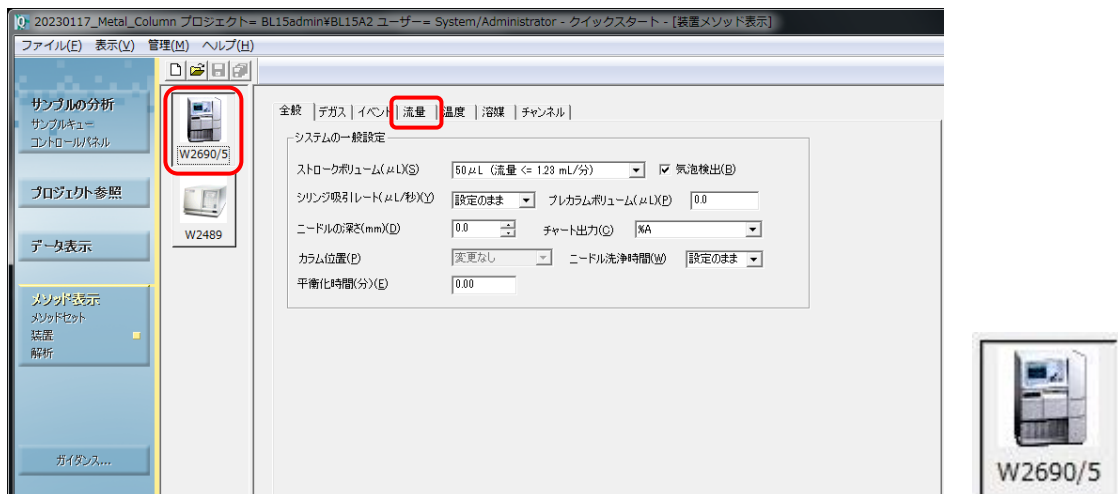
9.3 Click on the save icon (blue FD icon) after changing conditions.
Once saved, the save icon will turn gray. Note that if you do not click the save icon, the modified conditions will not be executed.

9.4 Other settings for e2690 (basically, users need not change. Check for wrong value.)

[General 全般] tab
・[Stroke volume ストロークボリューム(ul)(S)]：50 ul (流量 <= 1.23 ml/分)
・[bubble detection 気泡検出(B)]：Put a check mark.
・[Syringe aspiration rate シリンジ吸引レート(ul/秒)(Y)]：[default 設定のまま]
・[Needle depth ニードルの深さ(mm)(D)]：0.0 mm (For Water's Total recovery vials)
・[Chart output チャート出力(C)]：%A
・[Column position カラム位置(P)]：[default 変更なし]
・[Needle wash time ニードル洗浄時間(W)]：[default 設定のまま]
・[Equilibration time 平衡化時間(分)(E)]：0.00
[degas デガス]tab
・[degas mode デガスモード(M)]：[ON オン]
[Event イベント]tab
Not for use.
[Temperature 温度]tab
・[Control column temp カラム温度を制御する(C)]：Do not put a check mark.
・[Control Sample temp サンプル温度を制御する(S)]：Do not put a check mark.

[Solvent 溶媒]tab

Not for use.

[Channel チャンネル]tab

Not for use.

General
全般 デガス イベント 流量 温度 溶媒 チャンネル

システムの一般設定

Stroke volume
ストロークボリューム(μL)(S)   50μL (流量 <= 1.23 mL/分)

Bubble detection
気泡検出(B)

Syringe aspiration rate
シリンジ吸引レート(μL/秒)(Y)   設定のまま   プレカラムボリューム(μL)(P)   0.0

Needle depth
ニードルの深さ(mm)(D)   0.0

Chart output
チャート出力(C)   %A

Column position
カラム位置(P)   変更なし

Needle wash time
ニードル洗浄時間(W)   設定のまま

Equilibration time
平衡化時間(分)(E)   0.00

全般 デガス イベント 流量 温度 溶媒 チャンネル

Degas
スパージ

スパージ (mL/分)(R):   A 0.0   B 0.0   C 0.0   D 0.0

デガス

Degas mode
デガスモード(M):   オン

全般 デガス イベント 流量 温度 溶媒 チャンネル

Temperature
カラム温度

Control column temp
カラム温度を制御する(C)

カラム設定温度(℃)(O):   -1.0   カラム温度許容範囲 (+/-℃)(T):   5.0

サンプル温度

Control sample temp
サンプル温度を制御する(S)

サンプル設定温度(℃)(A):   -1.0   サンプル温度許容範囲 (+/-℃)(M):   5.0

9.5 Settings for 2489(UV/Vis detector) (basically, users need not change. Check for wrong value.)

Click [W2690/5] icon.

[Wavelength mode 波長モード]：[single シングル]

[Channel A チャンネル A]tab

・[Lamp ON ランプオン]：Put a check mark.

・[Wave length 波長]：280

・[Data mode データモード]：[Absorbance 吸光度]

・[Sampling rate サンプリングレート]：1

・[Auto zero at injection 注入時にオートゼロ]：Put a check mark.

・[Time constant タイムコンスタント]：[default 標準]

・[Auto zero at changing wavelength 波長変更時にオートゼロ]：[keeping baseline ベースラインを維持

[Analogue output アナログ出力]tab

Not in use.

[Event イベント]tab

Not in use.

9.6 Click on the save icon (blue FD icon) after changing conditions.

Once saved, the save icon will turn gray. Note that if you do not click the save icon, the modified conditions will not be executed.
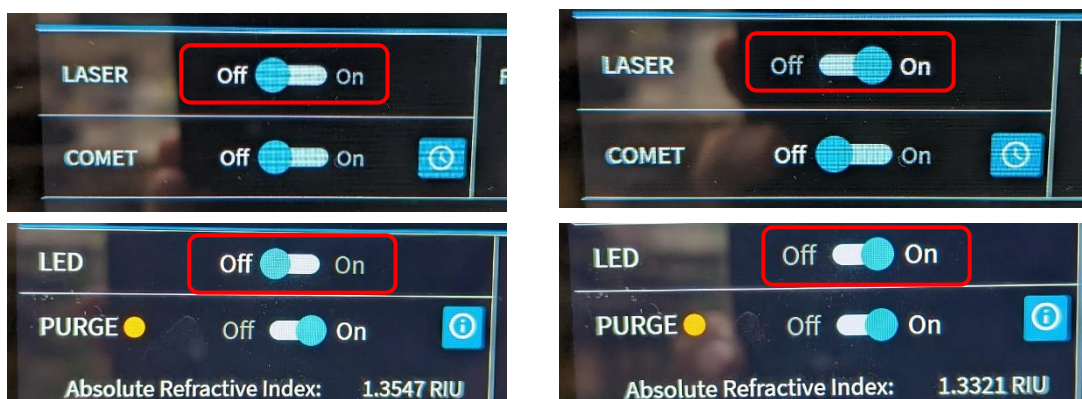

9.7 Click [Control panel コントロールパネル]. And then a confirmation window will appear and ask if you want to save the untitled method set, select [No いいえ(N)] (E).
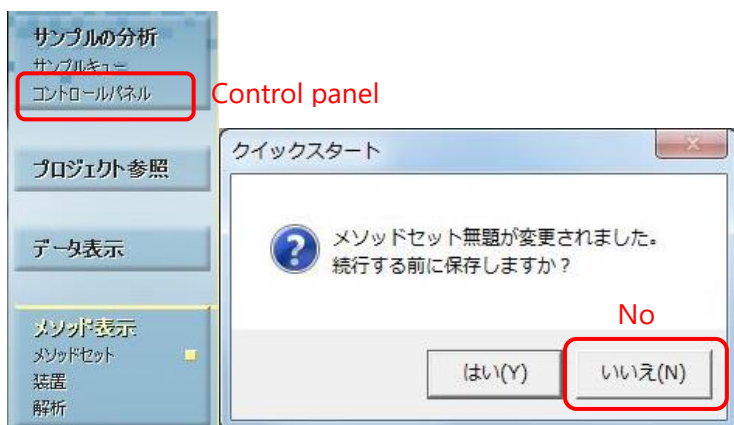
10 Setting of MALS(Astra8.1.2)

10.1   Turning on laser of MALS and LED of RI.

Touch to switch LASER from OFF to ON on the touch panel of the MALS (DAWN) unit and touch to switch LED from OFF to ON on the touch panel of the RI (Opitilab) unit.


10.2   Starting COMET(Ultrasonic cleaning for MALS cell)

Touch COMET ON on the touch panel of the MALS unit to start COMET.

[Perform COMET while the solution is flowing in the HPLC (e.g. during equilibration), for about 10 minutes]. Click the light blue clock symbol to set the COMET time on a timer.

Set [Run for:], for example 10 min and press OK to run COMET for 10 minutes.

To stop COMET, touch OFF.
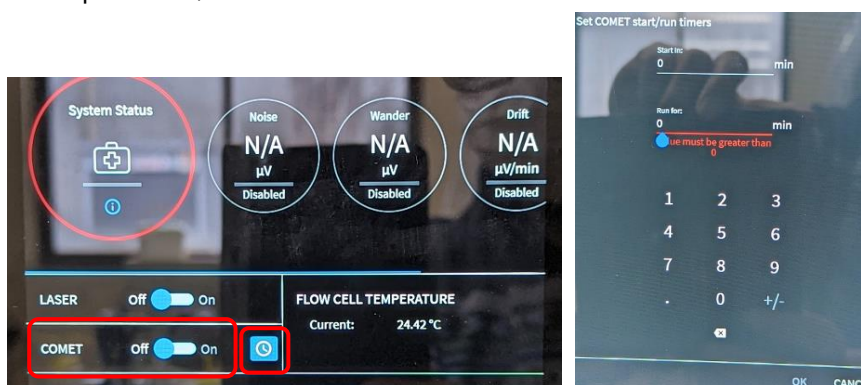
10.3   Creating Method.

Select [File] → [New] → [Experiment from Method] from the menu bar to open the [New from Existing] window (A).
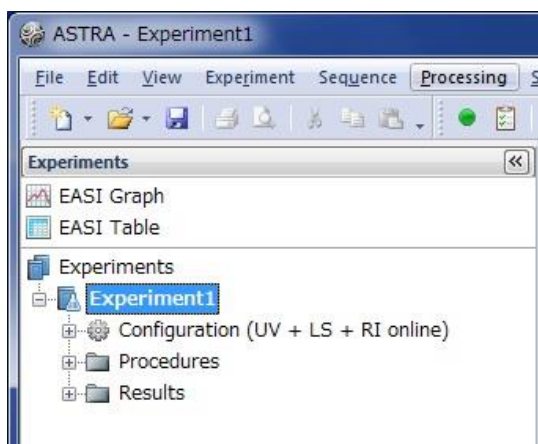
Click [SEC_MALS_default_Method_20230120_1] in the Methods folder [Normally this folder is opened, 20230120] to select it and click [Create] (B). (As of 27 January 2023. If there is a posting at the bottom of the computer screen or elsewhere to select a more

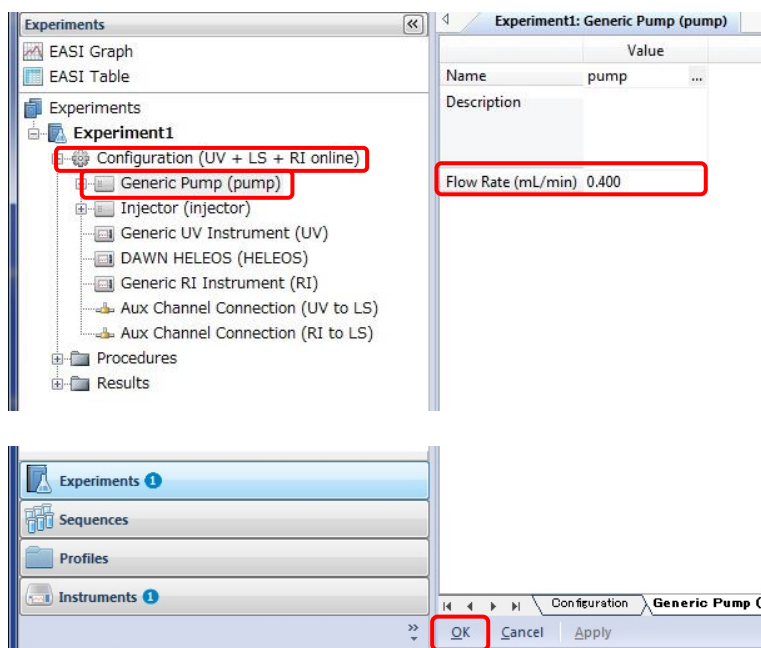recent date, use that one.) A new Experiment is created (C).
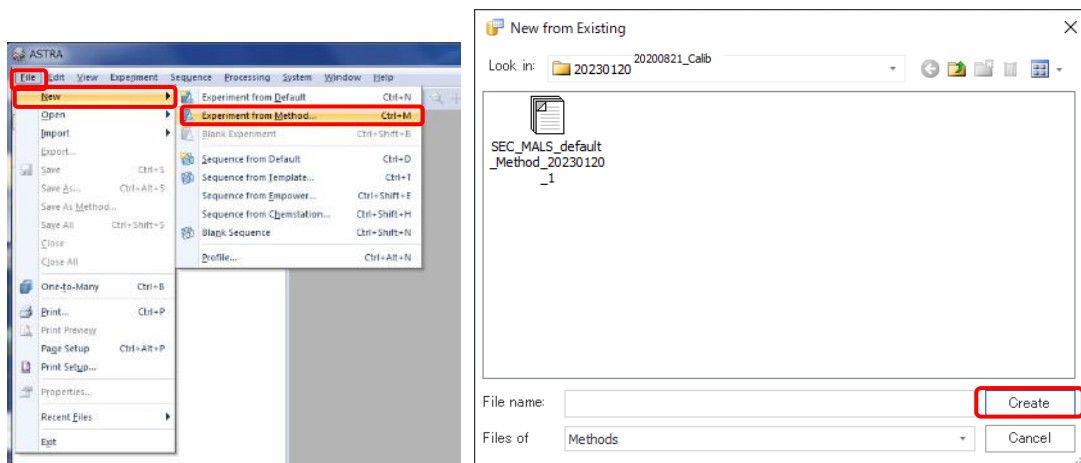
(A)   (B)

(C)

10.4   Modifying parameters: setting flow rate.

Double-click [Configuration]→[Generic pump] in the workspace to display [Generic pump] tab, enter the flow rate to be used for the measurement in the [Flow rate] field and click [OK] at the bottom of [Generic pump] tab.

10.5   Modifying parameters: setting the refractive index of the solvent

Double-click [Configuration]→[Generic pump]→[Solvent] in the workspace to display solvent tab.

The refractive index of solvent is left as water for normal aqueous buffers (up to 500 mM NaCl is OK).
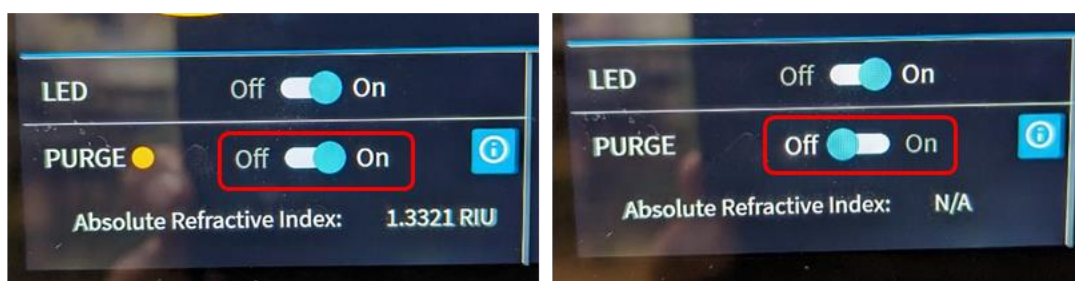
*If buffer includes Glycerol and so on, the appropriate Refractive Index should be entered. For information on how to check the value, please refer to the following page.
If user use specific buffer regularly, Specific solvent profiles could be created. For more information on how to add Solvent profiles, please refer to the SHOKO Science manual on the following page.

It is possible to select Water once and re-enter values later for re-analysis.
If you have entered new refractive index, click [OK] at the bottom of solvent tab.

***How to measure refractive index value of the solvent.**

- The column and pathway is fully equilibrated with buffer and the RI instrument has been set to [PURGE ON] (left-hand figure).
- If PURGE is on, a value is displayed in the 'Absolute Refractive Index' of OptiLab panel (1.3321 RIU in the left-hand diagram). This value is the Refractive Index value of the solvent.
- In step 10.5, a standard value for Water is set, but if you want to change it to match your buffer, rewrite this value. User can save your buffer as a new profile and load it. (See next page for information on creating and loading profiles).
- If PURGE is switched off, the refractive index are not displayed. Therefore, the refractive index value of the buffer is unknown during the sample measurement and should be checked immediately before the sample measurement.

10.6　　Modifying parameters: setting the sample injection volume

Double-click [Configuration]→[Injector] to display the injector tab.

Enter the injected sample volume in the Injected Volume field. (This is used to calculate the recovery rate not the molecular weight.) If you change the value, click [OK] at the bottom of the injector tab.
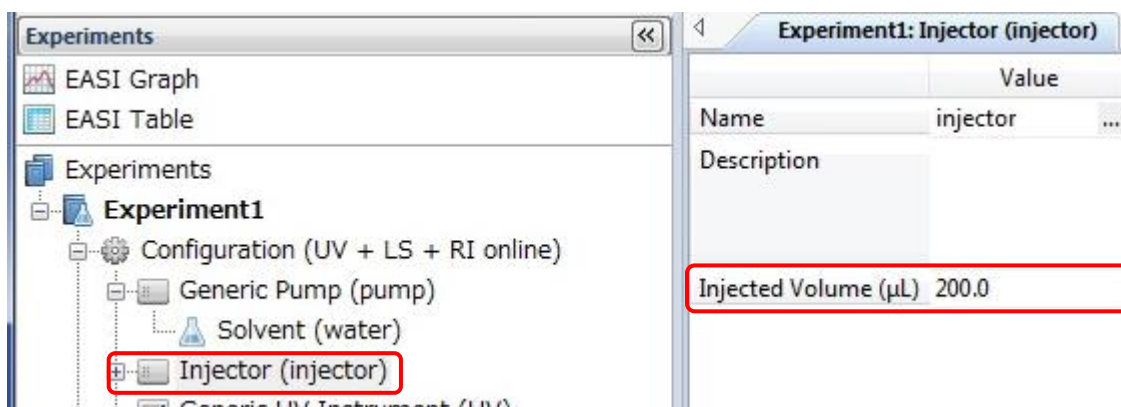

10.7　　Modifying parameters: setting sample information

Double-click [Configuration]→[Injector]→[Sample] to display the sample tab.

・Name: sample name [Do not select by clicking […] button on the right].

・Description: Comment field (e.g. buffer conditions).

・dn/dc: Default value is 0.185 for proteins. [User can enter the literature values or experimental values,

・A2: second virial coefficient. If unknown, leave as is.

・UV Extinction Coefficient: UV absorption coefficient. If unknown, leave as is. [e.g. 0.667 (ml/(mg-cm)) for BSA.]

・Concentration: Sample concentration If unknown, leave as is (not use for molecular weight determination).

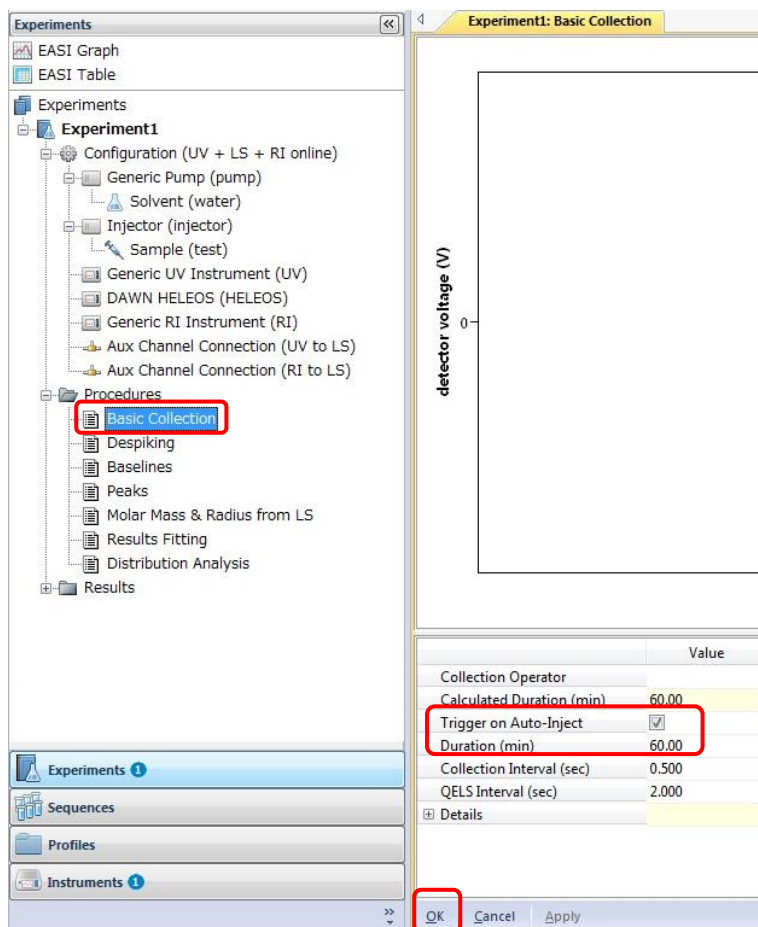If the values are changed, click [OK] at the bottom of the sample tab.
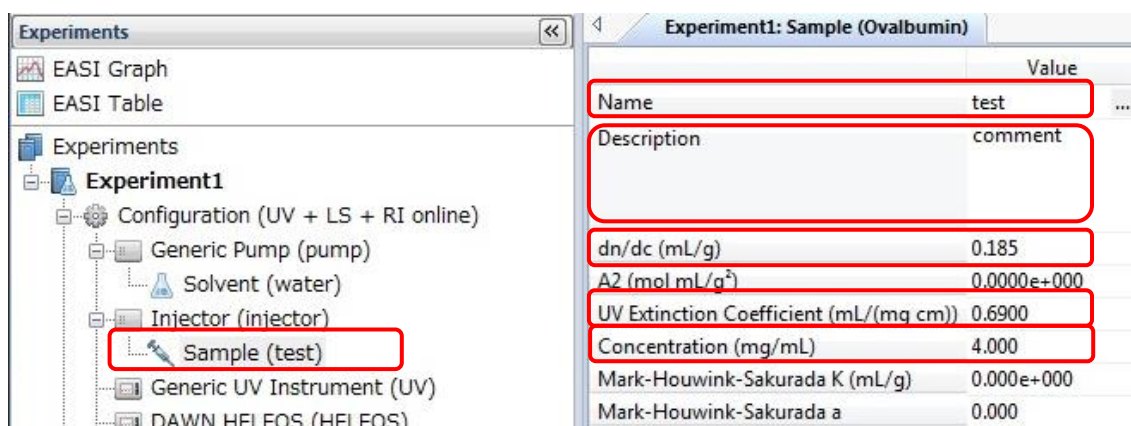
10.8　Modification of Procedures parameters

Double-click [Procedures]→[Basic Collection] to display the Basic Collection tab.

　　　・Trigger on Auto-Inject: Put a check mark.

　　　・Duration: Data acquisition time in min (enter the same value as the HPLC duration).

After checking and correcting, press [OK].

11 Performing measurements (i): MALS

11.1   Stop the PURGE of the RI detector (Optilab). It is automatically stopped when the measurement is started, but it involves pressure fluctuations, it is more appropriate to stop it in advance to check that the pressure is stable before starting the measurement. **The temperature setting of the RI is 25 °C and should not be changed!**
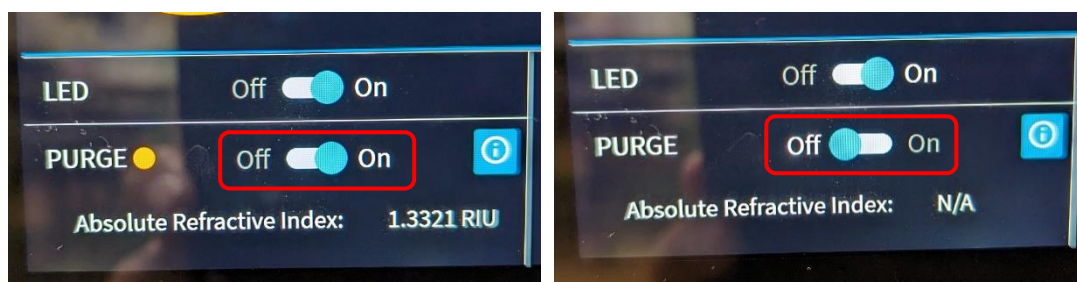

11.2   Stop COMET of MALS if it is running. [It automatically stops when the measurement starts, so there is no problem if you forget.] Touch COMET OFF on the touch panel of the MALS unit.
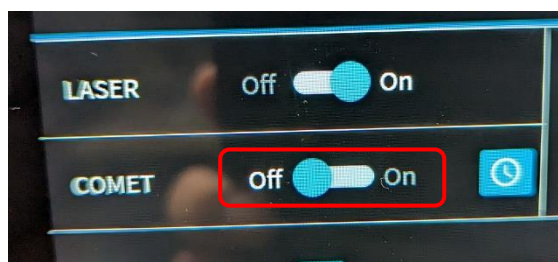
11.3   Starting MALS.

Clicking on the Run button. After a short wait, the message [Waiting for auto-injection signal] is displayed and the system waits for the start signal from the HPLC. If multiple experiments are open, click on the experiment you want to run, highlight it and then run it (it is safer to close other experiments that you do not want to run).

Waiting for auto-injection signal
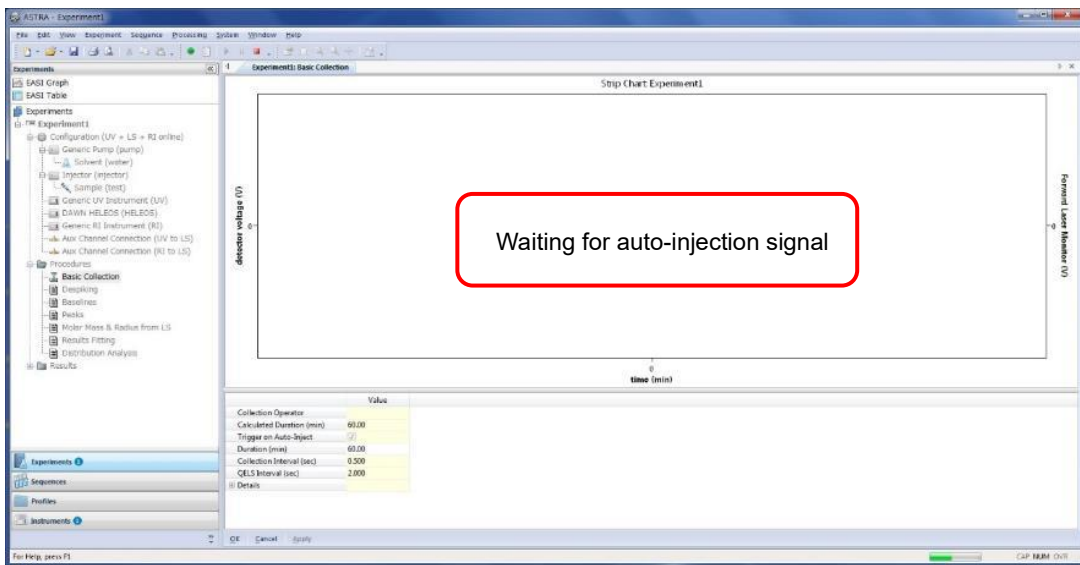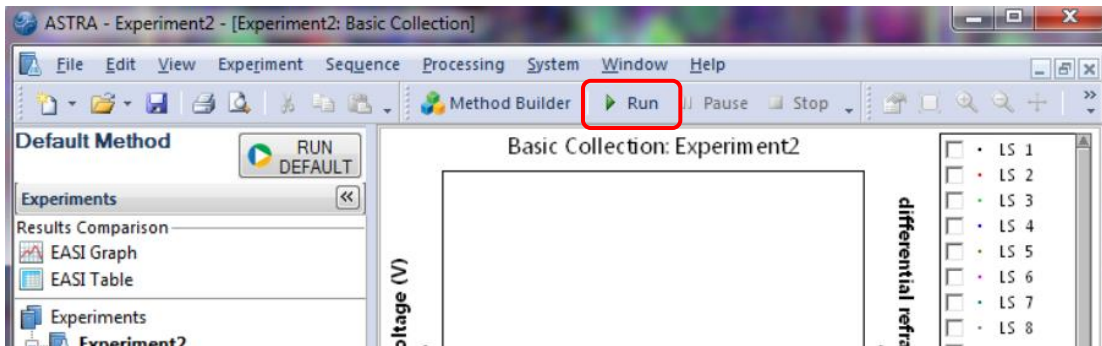
12   Performing measurements (ii): setting the sample vial

12.1   Dispensing samples into vials.

Sample should be supernatant after centrifugation (15 krpm, 10 min or more) or filtered.

Dispense [**injected volume + 10ul or more**] into the glass vial (waters Total Recovery (P/N: 186000385c)).

Avoid getting bubbles in the narrow part of the vial as sample is sucked up from a few mm above the bottom. It is easier to dispense with a yellow tip. If bubbles get in the vial, they can be shaken out.

12.2   Placing the vial in the autosampler. (See step 13.2)

Open the autosampler door (A), press the A button on the LCD screen (B) and bring the carousel A towards you (C); set the vial in position 1 (D) and close the autosampler door.


(A)

(B)

(C)


(D)

13 Performing measurements (iii): HPLC

13.1 　　　Checking the baseline.

Click [Setup Baseline Monitor セットアップ・ベースラインモニター] icon to open the [Setup for Equilibrium/System Monitor 平衡/システムモニターのセットアップ] window.

Select [20230117_Glass_Column or 20230117_Metal_Column, which are edited in step 9] as the [instrument method 装置メソッド], click [Setup セットアップ], and then click [Equilibrate/Monitor 平衡/モニター] to start the pump running under the method conditions and monitoring. Check that the flow rate and maximum pressure have changed as your planning and that the pressure is below the maximum value. Check that the light scattering intensity and RI values are stable on the graphs on the panel of each unit.

Setup Baseline Monitor

Instrument method
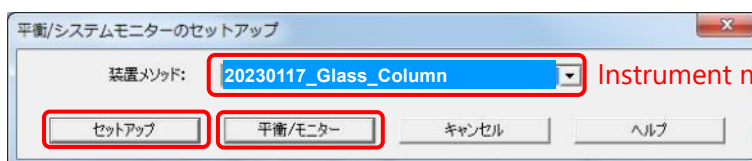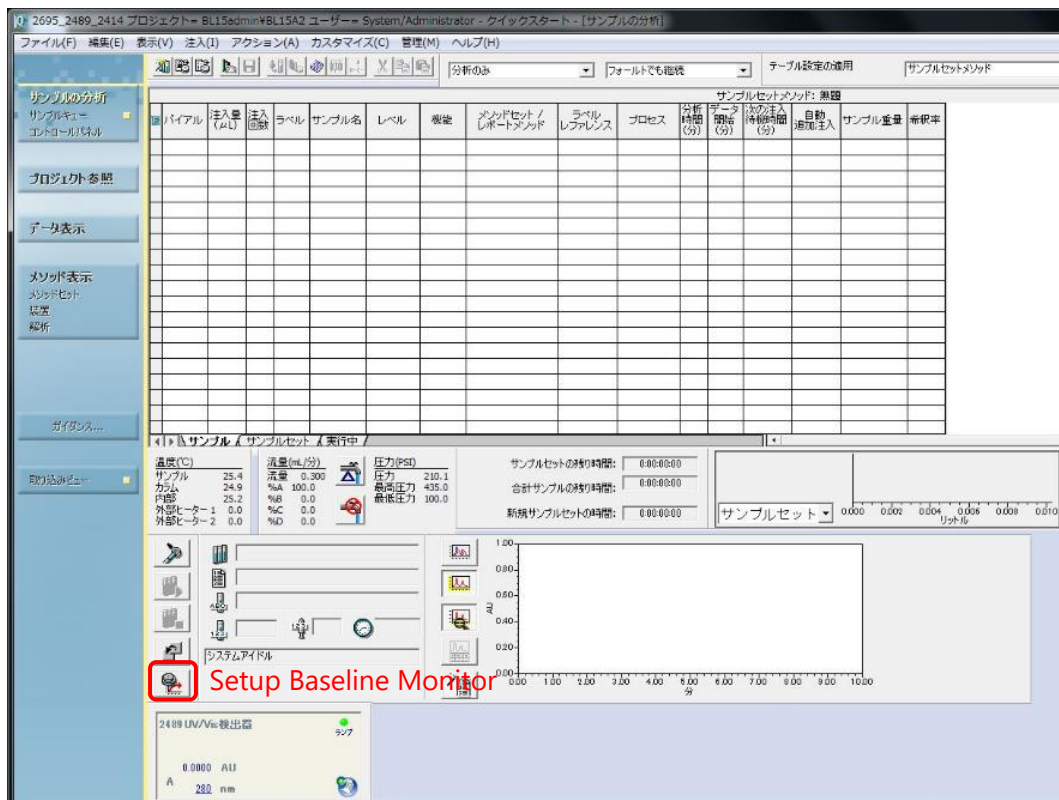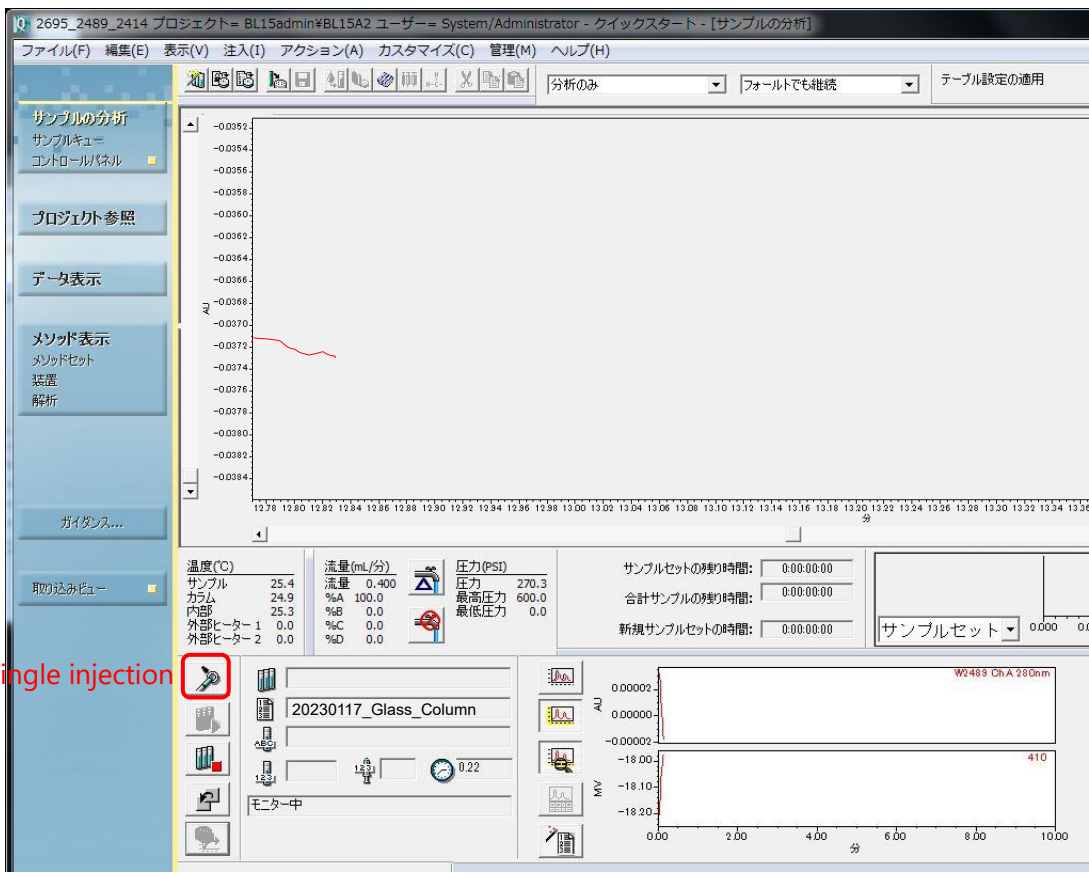
Setup　　　Equilibrate/Monitor
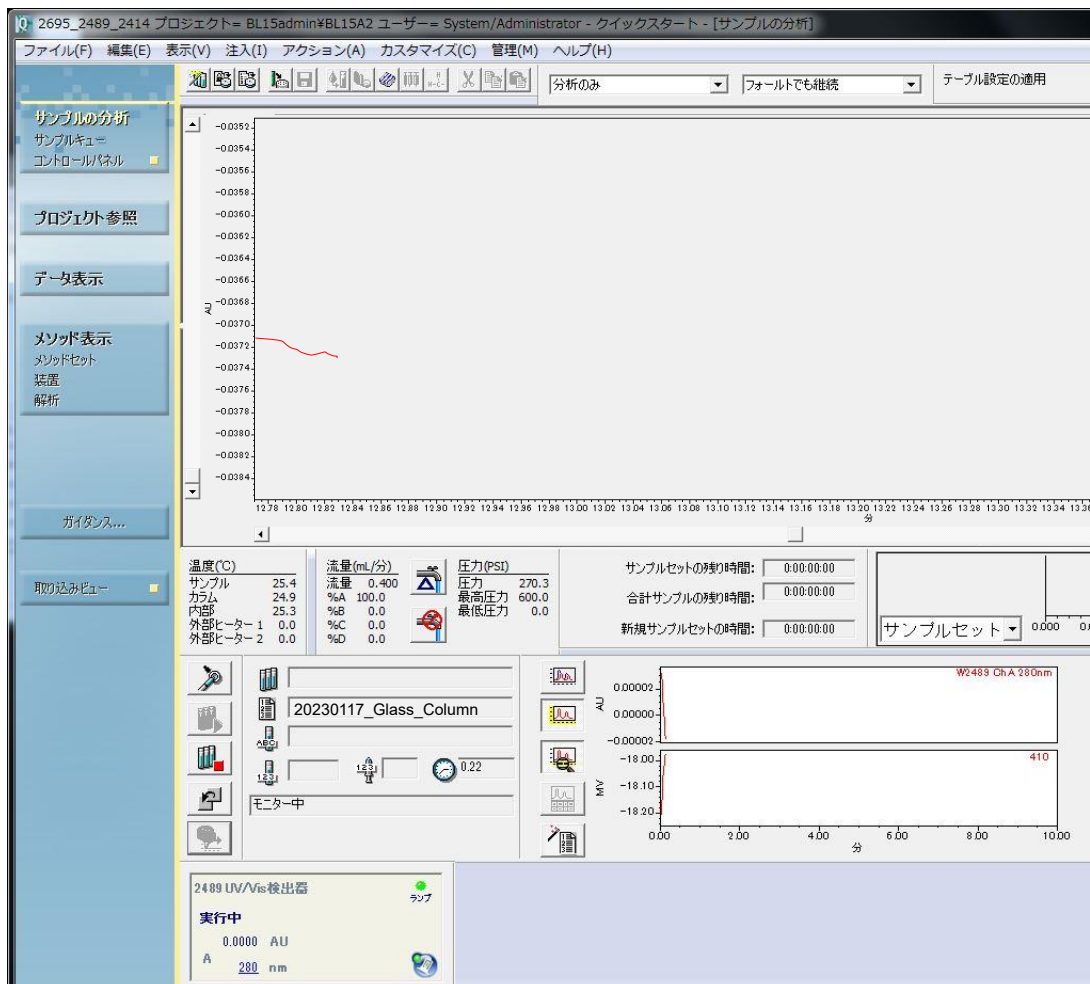
13.2　　Setting sample vials.

Set the sample vial in the carousel, see step 12.2.

13.3　　Setting parameters for sample injection.

Click the [Single injection シングル注入] button to open the [Parameters for single injection シングル注入のパラメータ指定] window and set each parameter as follows.

・[sample name サンプル名]：sample name

・[function 機能]：select [inject unknown sample 未知試料注入.]

・[Method set メソッドセット]：select the method set which are selected at step 13.1.

・[Vial バイアル]：enter No. of vial [e.g. #1 in carousel A->1.]

・[Volume 注入量]：sample volume for injection (ul)

・[Time 分析時間]：duration time (min)

Single injection

Sample name サンプル名: test12345

Function 機能: 未知試料注入

Method set メソッドセット: 20230117_Glass_Column

Vial バイアル: 1 □ 各注入の後に増加させる

Volume 注入量: 100.0 オプション... メソッド作成

Time 分析時間: 60.00 □ 注入後にダイアログを開いたままにする

Inject 注入 キャンセル ヘルプ(H)

13.4   Starting injection.

Click [Inject].

It takes about 1 minute until the injection. When the measurement has started, [Analysis in progress シングルインジェクション-分析中] is displayed and the elapsed time is also displayed (clock symbol).

When switched to the MALS PC by KVM, "Waiting for auto-injection signal" disappears on ASTRA 8.1 and the graphs are plotted. Green is UV, red is light scattering intensity (LS11 = detector 11 (90° scattering angle)) and blue is differential refractive index. As the detectors are arranged in series, they are displayed in the following order: UV, light scattering intensity, differential refractive index.
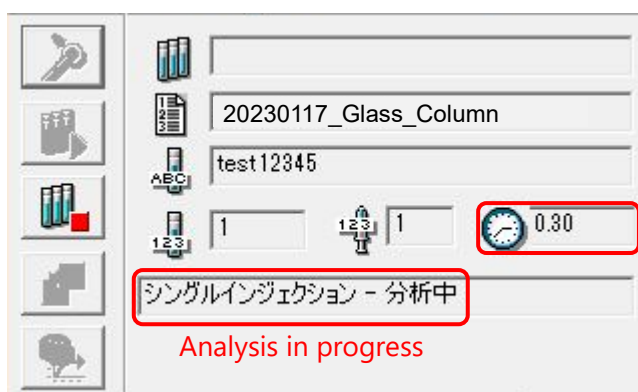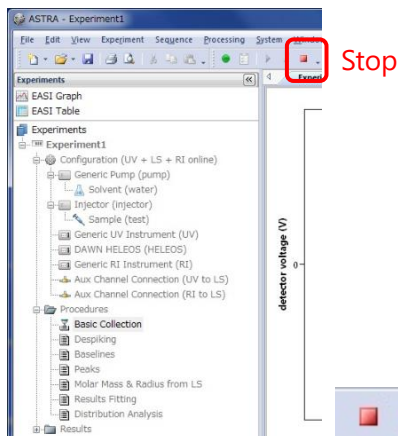

13.5   After measurement

MALS: data acquisition is stopped after the duration time.

HPLC: data acquisition is stopped after the duration time, but note that the flow continues under the conditions which are set in the method set.
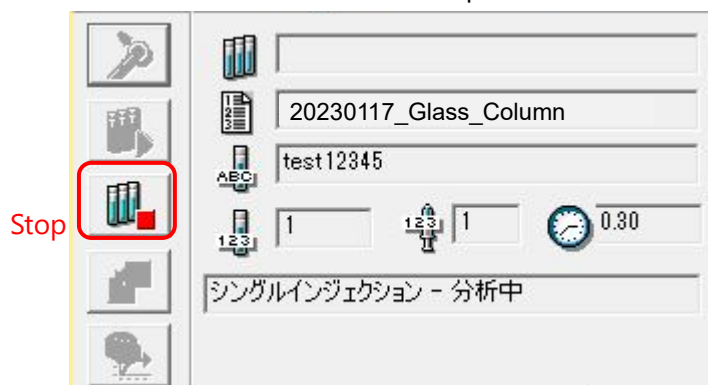
13.6   Aborting measurement

Stop MALS and HPLC separately.

[MALS]: click on the stop icon (red square) in the menu bar of the Astra screen.

Stop

[HPLC]: click on the [Stop] icon on the Empower3 screen and select the interruption method from the window that opens.

Stop

To stop the HPLC flow, click on the [Change flow rate] button (triangle on the tap) of the flow rate display to open the Change flow rate window, enter 0 in the flow rate area and click OK. Alternatively, click on the [Stop flow] button (No symbol on the tap).
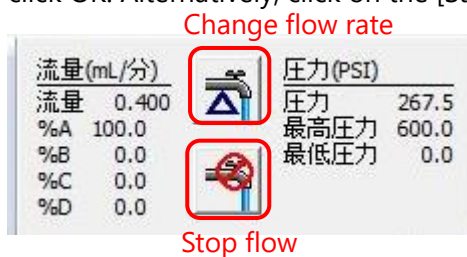
Change flow rate

Stop flow

As the duration time for MALS and HPLC cannot be changed during the experiment, it might be better to set a longer time and stop after the elution of peaks.

13.7   Next experiment

Re-equilibrate the column and repeat procedures after step 9. Depending on the situation, perform COMET for MALS and PURGE for RI. If the buffer is changed, repeat procedures after step 6.16.

14 After SEC-MALS experiments.

14.1   HPLC

・Replace the solvent in the system and column from buffer to MilliQ (see step 3).

・Replace the solvent in the system and column from Milli Q to 20% Et-OH (see step 3).

・Disconnect the column (see step 4).

・Turn off UV/Vis lamp, because of lamp's life time (see step 1.8).

It can be switched on and off in the [2489UV/Vis 検出器 detector] field in the bottom of the Empower3 window.
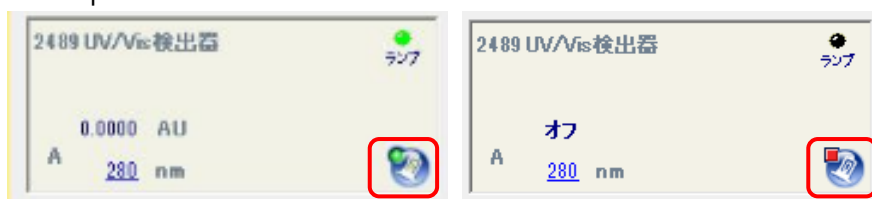
・Close Empower 3, shut down HPLC PC and switch the HPLC unit off.

・Dispose waste liquid (if it does not fall under any other waste classification , such as heavy metals or acid ,etc.) Dispose waste liquid in the polyethylene tank for hydrous organic solvent in the chemistry laboratory.

14.2   MALS/RI

・Turn off LASER of MALS and LED of RI(Optilab).
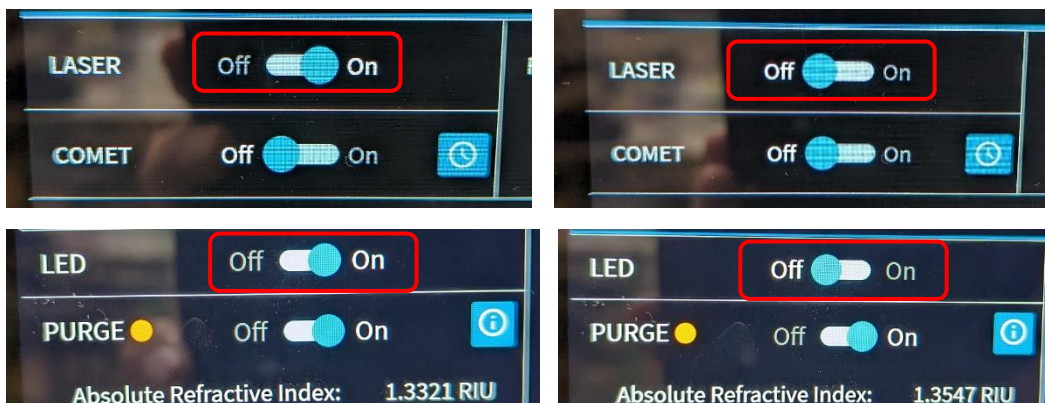
・execute COMET (see step 10.1) and PURGE (see step 11.1).

・Replace the solution in MALS and RI with 20% ethanol by HPLC operation.

・Close Astra 8.1.2, shut down the MALS PC and switch off the MALS/RI.

Appendix 1 Solvent lines

There are 6 solvent lines in HPLC.

Solvent A (yellow), B (blue), C (red), D (green): B, C and D (50% Et-OH) are not used.

Needle wash（green tube）: Needle washing solution

Seal Wash In（green label）: Pomp seal washing solution（10% Et-OH）

※Solutions of Solvent A and Needle wash tubes could be changed, respectively.

※Before changing solution bottles, Stop HPLC pump!

(1) For storage

20%Et-OH: Solvent A (yellow), Needle wash（green tube）

(2) Milli Q

Milli Q Water: Solvent A (yellow), Needle wash（green tube）

(3) For experiment

Buffer: Solvent A (yellow)

Milli Q Water: Needle wash（green tube）

	Solvent A (yellow)	**Needle wash (green tube)**	Seal Wash In (green)	B, C, D
Storage	**20%Et-OH**	**20%Et-OH**	10%Et-OH	50%Et-OH
Milli Q	**Milli Q**	**Milli Q**	10%Et-OH	50%Et-OH
Buffer	**Buffer**	**Milli Q**	10%Et-OH	50%Et-OH