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**Protocol status:** Working  
 We use this protocol and it's working

## Using TraceFinder and Excel software to evaluate and report multi-analyte targeted LC-MS data acquired on an ThermoScientific Exploris 240 Orbitrap

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Clinical Mass Spectrometry



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### ABSTRACT

In our lab we focus on targeted analysis and have found that acquiring LC-HRMS data using Xcalibur software and assessing the data using TraceFinder software is the most robust and flexible approach.

This protocol describes our approach to evaluating LC-HRMS data that has been collected in Xcalibur software, on LC-HRMS instruments including ThermoScientific Exploris mass spectrometers. It describes how to build a processing method in TraceFinder and using it to evaluate a full scan analysis data set acquired in Xcalibur software.

The LC-HRMS data set must contain a calibration curve and unknowns analysed as a batch using the same Acquisition method in Xcalibur. The results from TraceFinder are then transferred to Microsoft Excel to summarise the calculated amounts of the analytes of interest in the samples as a final result.

### GUIDELINES

Use methods that have well defined calibration protocols, method details (masses, analyte retention times) and well defined calibration ranges and known units for the calibration ranges. TraceFinder is only successful if the calibration range is well defined, the calibration units are known and the volume of extracted sample is recorded. QCs used across multiple batches are best included in each batch, and give confidence to the results.

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## MATERIALS

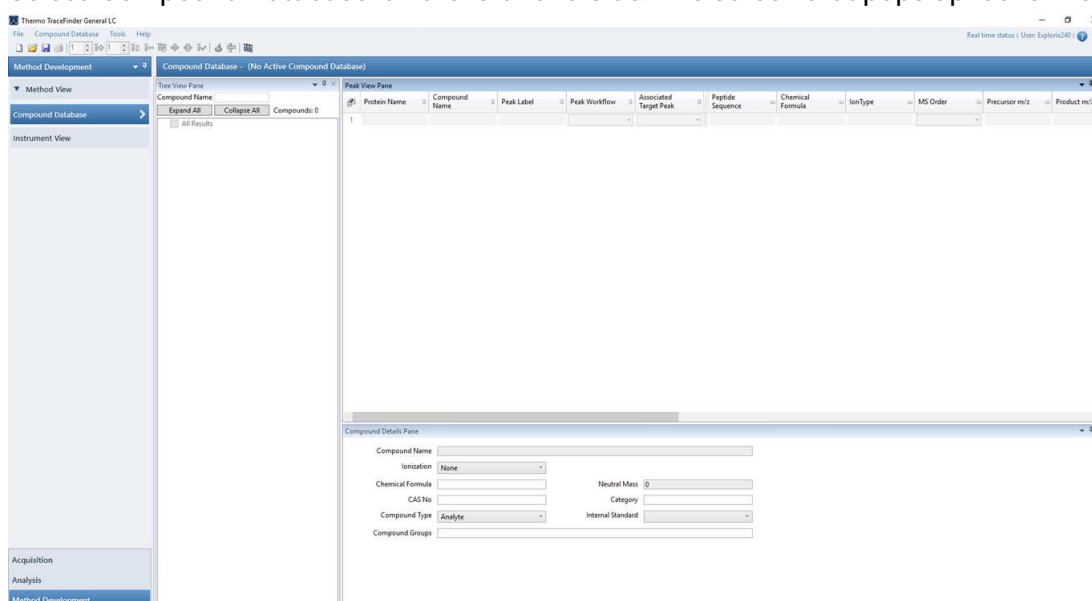
1. Xcalibur Data Acquisition and Interpretation Software 4.5 SP1 (ThermoScientific)
2. Tracefinder 5.1 software license (ThermoScientific)
3. Excel software package (Microsoft)
4. Details of the method used to acquire data in Xcalibur - including retention times, names of analytes, calibration ranges and calibration units and volume (or mass) of sample extracted.

## BEFORE START INSTRUCTIONS

Acquire a dataset in Xcalibur and use the raw file in TraceFinder.

## Creating a compound database in TraceFinder

- 1 In TraceFinder, navigate to the 'Method Development' tab in the bottom left corner of the screen.
- 2 Select 'Compound Database' on the left-hand side. The screen that pops up looks like this:



3 Go to File > New Small Molecule Compound Database  
A new screen pops up where you can give a name to your new compound database.

4 Build your compound database as seen in this example:

Compound Name	Chemical Formula	Peak Label	Polarity	Adduct	Retention Time (min)	Peak Workflow	Associated Target Peak	MS Order	Precursor m/z	Product m/z
Compound 1	C <sub>2</sub> H <sub>5</sub> SO	T1: 46.0432	Positive	Hydrogen	1.00	TargetPeak	-	ms1	0	0
Compound 2	C <sub>2</sub> H <sub>6</sub>	T1: 31.0543	Positive	Hydrogen	2.00	TargetPeak	-	ms1	0	0
Compound 3	C <sub>2</sub> H <sub>6</sub>	T1: 46.0777	Positive	Hydrogen	3.00	TargetPeak	-	ms1	0	0
Internal Std	[13]C <sub>2</sub> H <sub>6</sub>	T1: 49.0877	Positive	Hydrogen	2.50	TargetPeak	-	ms1	0	0

- Enter each individual compound that should be analysed + all internal standards in the method and enter the chemical formula, the polarity, the adduct and the expected retention time for each of the compounds.

Select each individual compound in the Tree View Pane on the left hand side and fill in the 'Compound Details Pane' on the bottom:

Compound Name	Chemical Formula	Peak Label	Polarity	Adduct	Retention Time (min)	Peak Workflow	Associated Target Peak	MS Order	Precursor m/z	Product m/z
Compound 3	C <sub>2</sub> H <sub>6</sub>	T1: 46.0777	Positive	Hydrogen	3.00	TargetPeak	-	ms1	0	0

- For the internal standard(s), select 'Internal Standard' for compound type in the lower tab and fill in the ISTD concentration as 1.
- For the analytes, select 'Analyte' for compound type in the lower tab.

5 Save the compound database.  
Go to File > Save Compound Database.

## Creating a processing method in TraceFinder

6 Go to the 'Method View' tab on the left hand side.  
Select File > New > Master method...

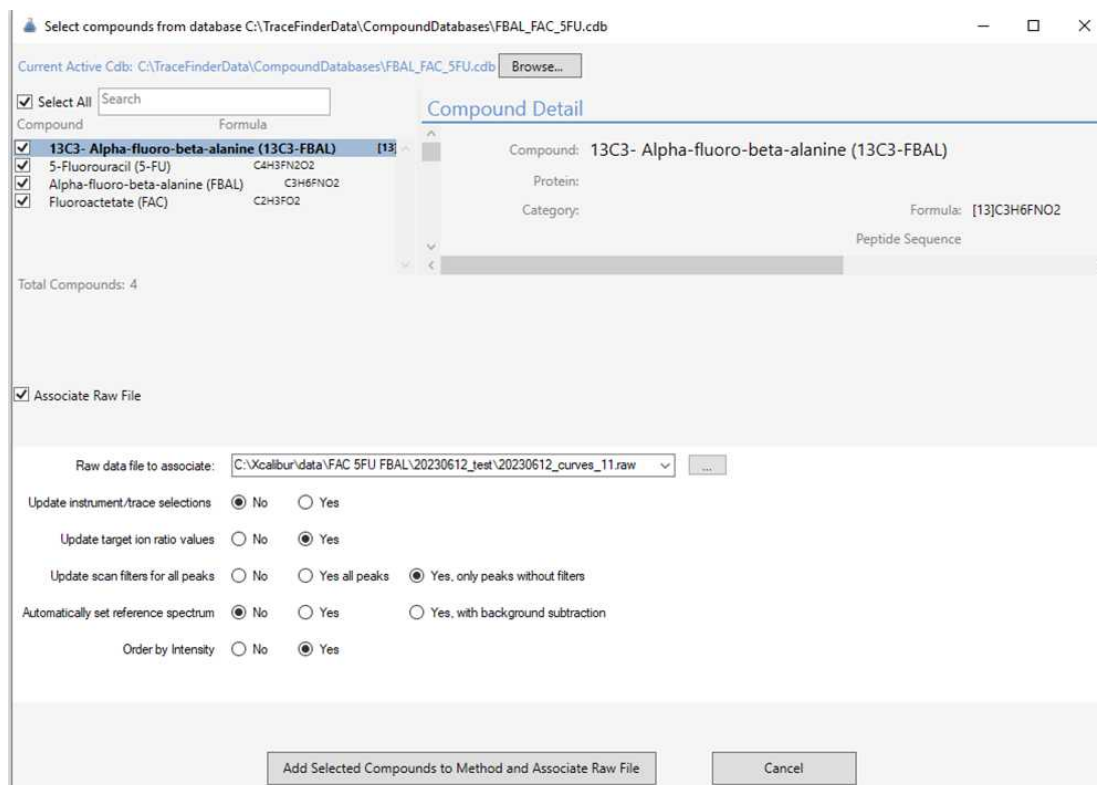
7 The following screen will pop up:

Select Workflow Template	
<input type="radio"/> Quan using Method Forge	<b>Method Forge</b> Performs peak detection against a raw data file. Performs library lookup if requested.
<input type="radio"/> Quan by importing an Xcalibur Processing Method	<b>Import Xcalibur Processing Method</b> Imports a previously created processing method, finding configured compounds and reference spectra.
<input type="radio"/> Quan - blank method	<b>Create blank method</b> Associate a raw data file and manually select peaks.
<input checked="" type="radio"/> Quan by Selecting compounds from CDB	<b>Select Compounds from a compound database</b> Creates a blank master method and displays the configured compound database, allowing compound selection.
<input type="radio"/> Target Screening method	<b>Create a target screening method.</b>
<input type="radio"/> Unknown Screening method - Only	<b>Create an unknown screening method.</b> This method will not have quan or target screening elements.

OK Cancel

Select the option 'Quan by Selecting compounds from CDB' and click on 'OK'.

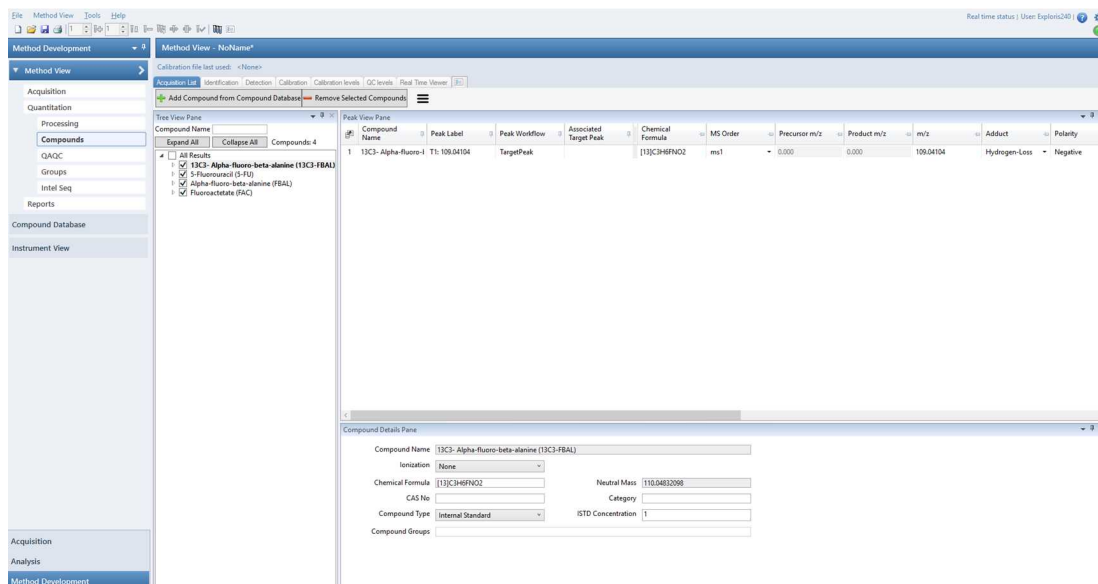
8 The following screen will pop up:



- Using the browse function, select your prepared compound library.
- Tick the 'Select All' and 'Associate Raw Datafile' boxes.
- Select a datafile from a mid-high standard on the calibration curve.
- Use the settings as displayed in the screenshot above.
- Click on 'Add Selected Compounds to Method and Associate Raw File'

9 Save your Master Method by selecting File > Save as...

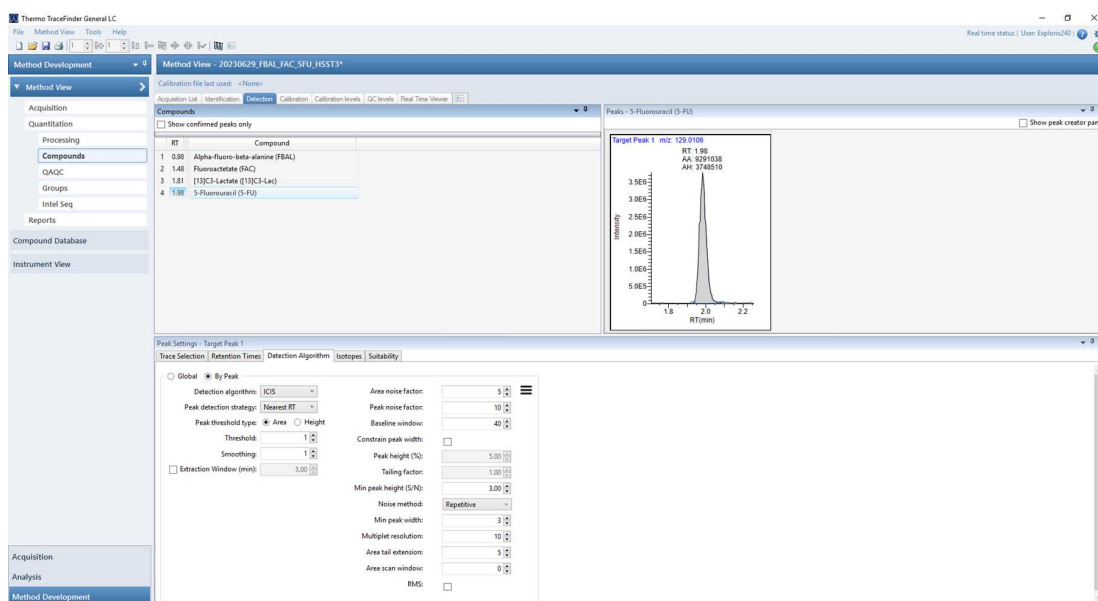
10 Navigate to the 'Compounds' tab on the left hand side. The screen should look like this:



You are currently in the 'Acquisition List' tab on top. Here:

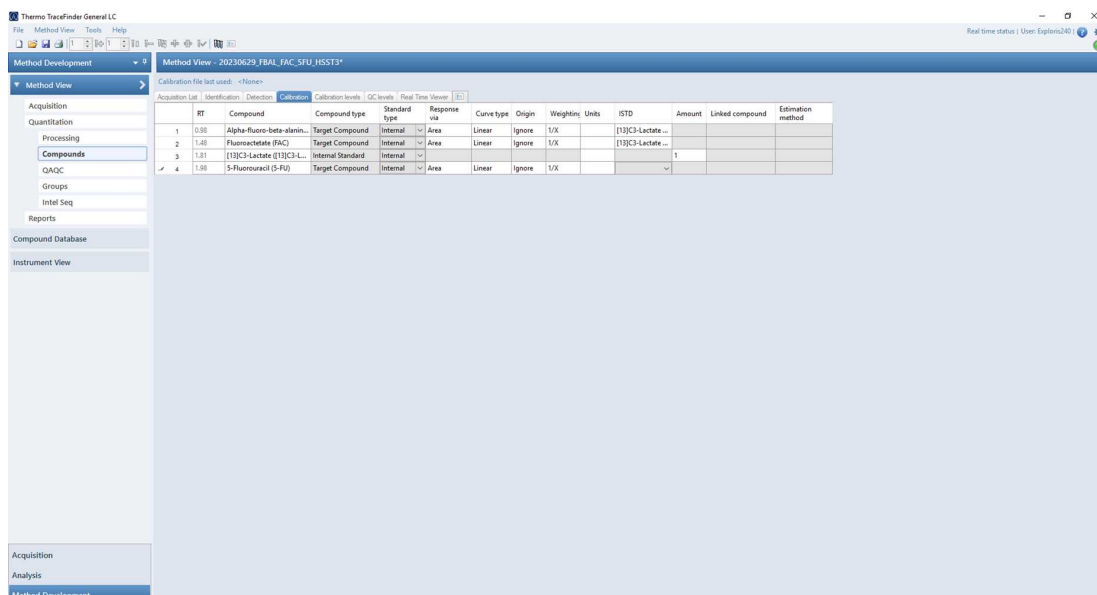
- Select the each individual compound and check that the chemical formula, the polarity, the adduct and the expected retention time are all correct.
- For the analytes, select which internal standard should be used for each individual analyte.

11 Navigate to the 'Detection' tab on top. The screen should look like this:



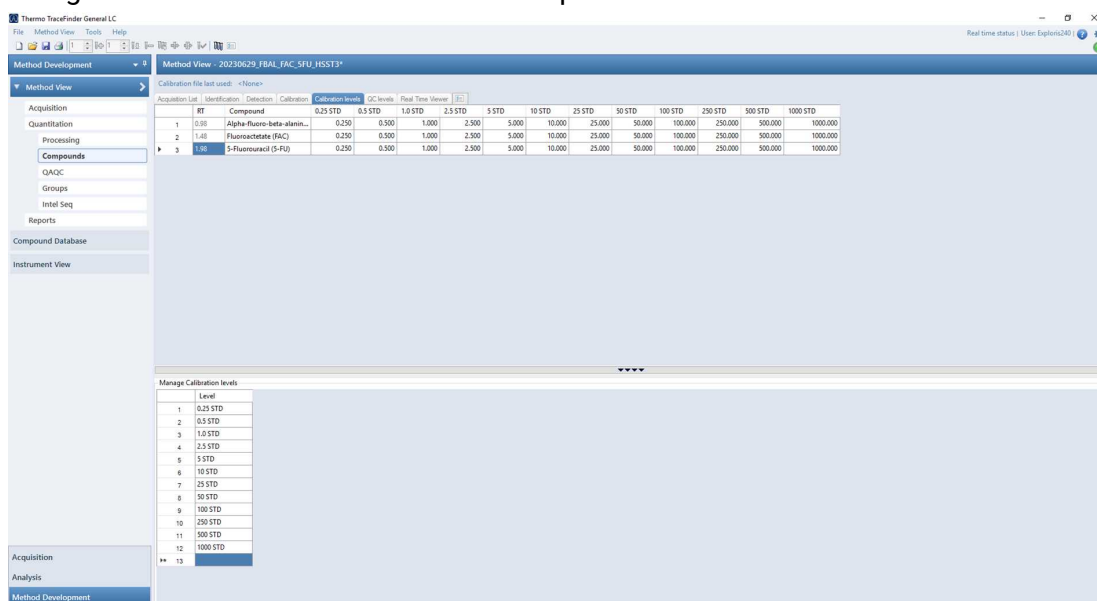
Here, you can alter the parameters for peak detection in your method, such as the expected retention times, the retention time window, the detection algorithm used and more.

12 Navigate to the 'Calibration' tab on top. The screen should look like this:



- Check that the information in this tab is correct.
- Select 'Linear' as the curve type and '1/X' as the weighting.
- Again, select the appropriate internal standard for each analyte.

## 13 Navigate to the 'Calibration levels' tab on top. The screen should look like this:



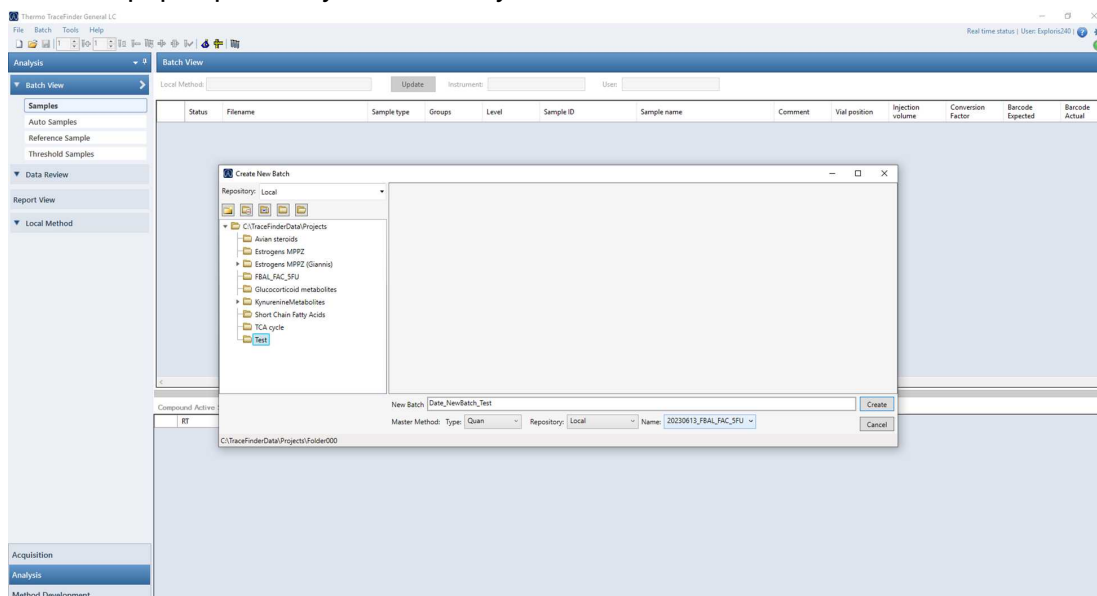
- Enter the names of all calibration levels in the 'Manage Calibration levels' tab at the bottom of the screen.
- For each individual analyte, fill in the concentration associated with each calibration level.

## 14 Save your Master Method by selecting File > Save.

# Using TraceFinder to process Xcalibur acquired LC-MS data

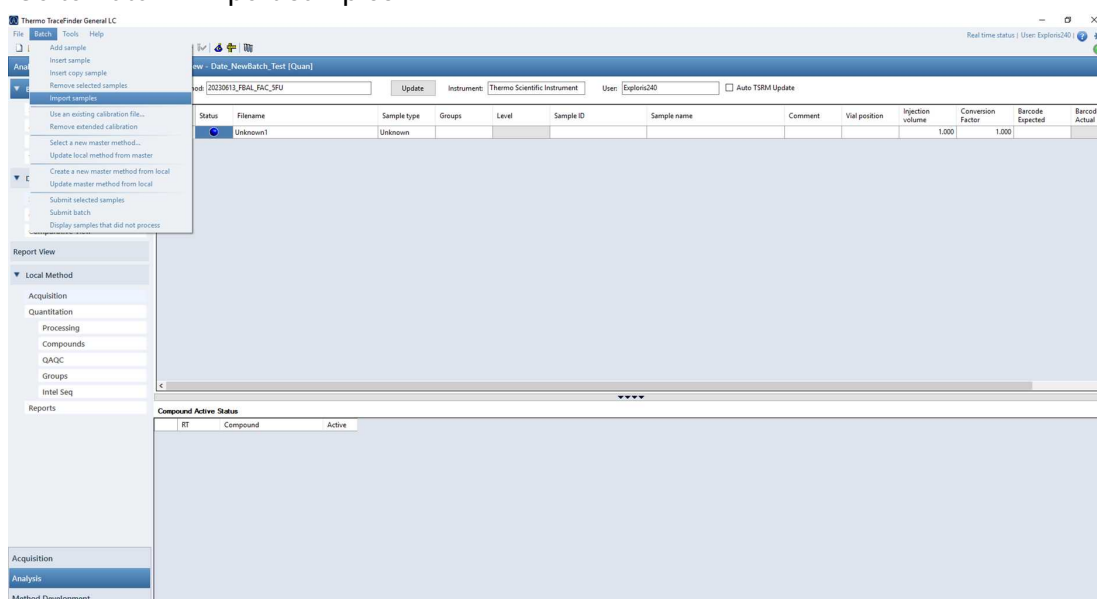
15 In TraceFinder, navigate to the 'Analysis' tab in the bottom left corner of the screen.

16 Select File > New > Batch  
A screen pops up where you can save your new batch.



Select a folder, give the new batch a name, select the Master Method you designed and select 'Create'.

17 Go to Batch > Import samples





- Browse to the SLD file of the data set that you want to process.
- All samples in the SLD file will be imported into Tracefinder.
- Delete the topmost sample which is labeled 'Unknown' by right mouse click > remove selected samples.
- Select the correct calibration level for each of the standards.
- At this point of processing, 'sample status' should be blue.

Batch View - 20230613\_FBAL\_FAC\_S-FU\_V2 (Quan)

Local Method: 20230613\_FBAL\_FAC\_SFU\_V2 Update Instrument: Thermo Scientific Instrument User: Explorer240 Auto TSM Update

Status	Filename	Sample type	Groups	Level	Sample ID	Sample name	Comment	Vial position	Injection volume	Conversion Factor	Barcode Expected	Barcode Actual
1	20230612_curves_01	Unknown						GH12	5.000	1.000		
2	20230612_curves_02	Unknown						GH12	5.000	1.000		
3	20230612_curves_03	Unknown				Double Blank		GA1	5.000	1.000		
4	20230612_curves_04	Unknown		0	0	0 STD (FAC)		GB1	5.000	1.000		
5	20230612_curves_05	Unknown		0.25	0.25	0.250 STD (FAC)		GC1	5.000	1.000		
6	20230612_curves_06	Unknown		0.5	0.5	0.500 STD (FAC)		GD1	5.000	1.000		
7	20230612_curves_07	Unknown		1	1	1.000 STD (FAC)		GE1	5.000	1.000		
8	20230612_curves_08	Unknown		2.5	2.5	2.500 STD (FAC)		GF1	5.000	1.000		
9	20230612_curves_09	Unknown		5	5	5.000 STD (FAC)		GH1	5.000	1.000		
10	20230612_curves_10	Unknown		10	10	10.000 STD (FAC)		GH1	5.000	1.000		
11	20230612_curves_11	Unknown		25	25	25.000 STD (FAC)		GA2	5.000	1.000		
12	20230612_curves_12	Unknown		50	50	50.000 STD (FAC)		GB2	5.000	1.000		
13	20230612_curves_13	Unknown		100	100	100 STD (FAC)		GC2	5.000	1.000		
14	20230612_curves_14	Unknown		250	250	250 STD (FAC)		GD2	5.000	1.000		
15	20230612_curves_15	Unknown		500	500	500 STD (FAC)		GE2	5.000	1.000		
16	20230612_curves_16	Unknown				Double Blank		GF2	5.000	1.000		
17	20230612_curves_17	Unknown				Double Blank		GH2	5.000	1.000		
18	20230612_curves_18	Unknown				Double Blank		GA3	5.000	1.000		
19	20230612_curves_19	Unknown				Double Blank		GB3	5.000	1.000		
20	20230612_curves_20	Unknown		0	0	0 STD (FAC)		GB3	5.000	1.000		

Compound Active Status

RT	Compound	Active

18

Batch View - 20230613\_FBAL\_FAC\_S-FU\_V2 (Quan)

Local Method: 20230613\_FBAL\_FAC\_SFU\_V2 Update Instrument: Thermo Scientific Instrument User: Explorer240 Auto TSM Update

Status	Filename	Sample type	Groups	Level	Sample ID	Sample name	Comment	Vial position	Injection volume	Conversion Factor	Barcode Expected	Barcode Actual
1	20230612_curves_01	Matrix Blank						GH12	1.000	1.000		
2	20230612_curves_02	Matrix Blank						GH12	1.000	1.000		
3	20230612_curves_03	Matrix Blank				Double Blank		GA1	1.000	1.000		
4	20230612_curves_04	Cal Top		0 STD	0	0 STD (FAC)		GB1	1.000	1.000		
5	20230612_curves_05			0.25	0.25	0.250 STD (FAC)		GC1	1.000	1.000		
6	20230612_curves_06			0.5	0.5	0.500 STD (FAC)		GD1	1.000	1.000		
7	20230612_curves_07			1	1	1.000 STD (FAC)		GE1	1.000	1.000		
8	20230612_curves_08			2.5	2.5	2.500 STD (FAC)		GF1	1.000	1.000		
9	20230612_curves_09			5	5	5.000 STD (FAC)		GH1	1.000	1.000		
10	20230612_curves_10			10	10	10.000 STD (FAC)		GH1	1.000	1.000		
11	20230612_curves_11			25	25	25.000 STD (FAC)		GA2	1.000	1.000		
12	20230612_curves_12			50	50	50.000 STD (FAC)		GB2	1.000	1.000		
13	20230612_curves_13			100	100	100 STD (FAC)		GC2	1.000	1.000		
14	20230612_curves_14			250	250	250 STD (FAC)		GD2	1.000	1.000		
15	20230612_curves_15			500	500	500 STD (FAC)		GE2	1.000	1.000		
16	20230612_curves_16					Double Blank		GF2	1.000	1.000		
17	20230612_curves_17					Double Blank		GH2	1.000	1.000		
18	20230612_curves_18					Double Blank		GA3	1.000	1.000		
19	20230612_curves_19					Double Blank		GB3	1.000	1.000		
20	20230612_curves_20			0	0	0 STD (FAC)		GB3	1.000	1.000		

Compound Active Status

RT	Compound	Active

- Right click on one of the samples and select 'Map raw files to samples...'
- Select all raw files which appear in the windows file explorer window that pops up.
- The raw files should now be linked to each individual sample in the batch.
- At this point of processing, 'sample status' should turn yellow.

Batch View - 20230613\_FBAL\_FAC\_5-FU\_V2 [Quan]\*

Local Method: 20230613\_FBAL\_FAC\_5FU\_V2 Update Instrument: Thermo Scientific Instrument User: Exploris240 Auto TSPM Update

Status	Filename	Sample type	Groups	Level	Sample ID	Sample name	Comment	Val position	Injection volume	Conversion Factor	Barcode Expected	Barcode Actual
1	20230612_curves_01	Matrix Blank						GH12	5.000	1.000		
2	20230612_curves_02	Matrix Blank						GH12	5.000	1.000		
3	20230612_curves_03	Matrix Blank						GA1	5.000	1.000		
4	20230612_curves_04	Cal Std		0 STD	0	0 STD (FAC)		GB1	5.000	1.000		
5	20230612_curves_05	Matrix Blank		0.25 STD	0.25	0.250 STD (FAC)		GC1	5.000	1.000		
6	20230612_curves_06	Cal Std		0.5 STD	0.5	0.500 STD (FAC)		GD1	5.000	1.000		
7	20230612_curves_07	Cal Std		1.0 STD	1	1.000 STD (FAC)		GE1	5.000	1.000		
8	20230612_curves_08	Unknown		2.5 STD	2.5	2.500 STD (FAC)		GF1	5.000	1.000		
9	20230612_curves_09	Cal Std		5 STD	5	5.000 STD (FAC)		GG1	5.000	1.000		
10	20230612_curves_10	Cal Std		10 STD	10	10.0 STD (FAC)		GH1	5.000	1.000		
11	20230612_curves_11	Cal Std		25 STD	25	25.0 STD (FAC)		GA2	5.000	1.000		
12	20230612_curves_12	Cal Std		50 STD	50	50.0 STD (FAC)		GB2	5.000	1.000		
13	20230612_curves_13	Cal Std		100 STD	100	100.0 STD (FAC)		GC2	5.000	1.000		
14	20230612_curves_14	Cal Std		250 STD	250	250.0 STD (FAC)		GD2	5.000	1.000		
15	20230612_curves_15	Cal Std		500 STD	500	500.0 STD (FAC)		GE2	5.000	1.000		
16	20230612_curves_16	Unknown						GF2	5.000	1.000		
17	20230612_curves_17	Unknown						GG2	5.000	1.000		
18	20230612_curves_18	Unknown						GH2	5.000	1.000		
19	20230612_curves_19	Unknown						GA3	5.000	1.000		
20	20230612_curves_20	Unknown			0	0 STD (FAC)		GB3	5.000	1.000		

Compound Active Status

RT	Compound	Active

19 Submit your batch for processing by clicking on the following icon at the top of the screen:



The following screen should pop up:

Batch View - 20230613\_FBAL\_FAC\_5-FU\_V2 [Quan]\*

Local Method: 20230613\_FBAL\_FAC\_5FU\_V2 Update

User name: 20230613-Exploris240

Samples: 143

Workflow steps to perform:

- ☐ Acquire data
- ☒ Process data
- ☐ Create reports

Submit as a priority sequence?

- ☐ Priority Sequence
- ☐ Need Available Batch
- ☒ Need Available Sample

Click Acquire data above to edit device settings

Post-run system state: On

Programs: Pre-acquisition

OK Cancel

- Click on 'OK' and all data should now be processed according to your master method.
- At this point of processing, 'sample status' should turn green.

## Using TraceFinder to evaluate Xcalibur acquired LC-MS data

**20** In TraceFinder, go to the 'Compound View' tab on the left hand side.

**21** Here, each analyte can be evaluated in each sample.

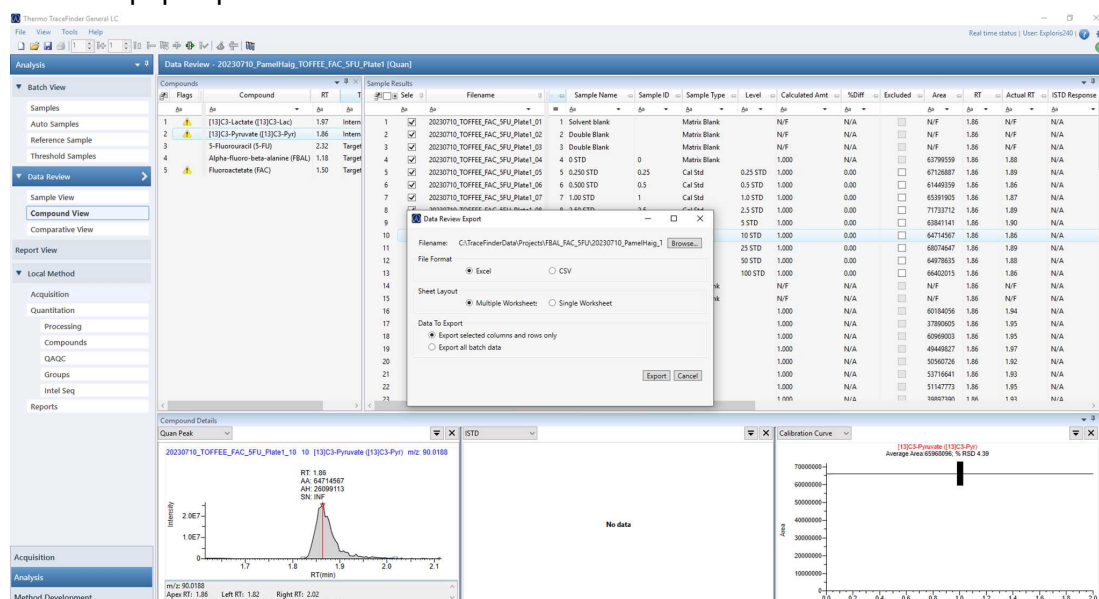
- Check that all peaks have integrated and that there are peak areas for each analyte - reassign retention time if it is not quite picking the peak and visually assess peak area and retention time to ensure that the correct peaks have been integrated.
- Check the calibration curves for accuracy (<20%) and exclude those points that are outwith. Ensure there are a minimum of 6 points in each calibration curve and that they have a regression coefficient  $R > 0.99$ .

**22** Once you are satisfied with all calibration curves and the data of each sample, go to File > Save... to save your results.

## Transferring TraceFinder alphanumeric data into Excel to sum...

**23** Go to File > Export data to > CSV or Excel...

**24** A screen pops up:



- Select 'Excel' as the file format.
- Select 'Multiple Worksheets' as the sheet layout.
- Select 'Export selected columns and rows only' as data to export.

**25** Open the exported Excel file. All analytes should be sorted in individual tabs. Create a new tab and call it 'Summary'.

**26** Copy the sample names from one of the analyte tabs, and paste into the summary tab. Then return to the Analyte tab and copy the '[Analyte name] ng' column. Repeat this copying of the [Analyte name] ng column until all analytes in the excel file, have been copied. This results in a Summary table that is the major results of the analysis in terms of concentration.

**27** If the calibration curve units are ng and the sample extracted has been a volume, add a column ahead of all analyte concentrations, include the volume extracted for each sample and then use this column to calculate the ng/mL amount by dividing the 'calculated amount' by the volume (uL) and multiply by 1000 to give the ng/mL amount of each analyte.

**28** Save the excel file in the appropriate folder.