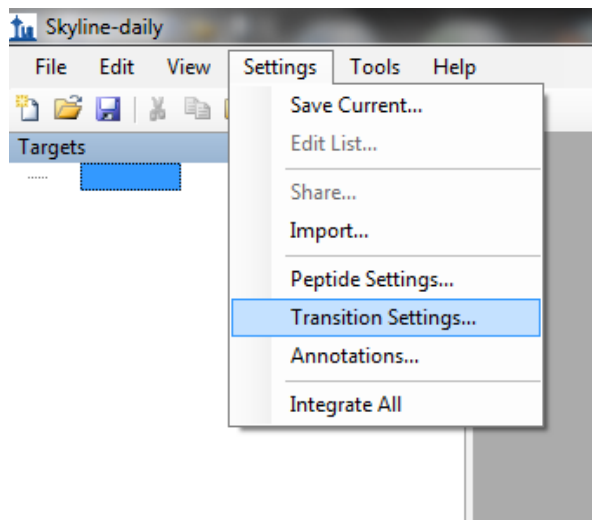


Data Independent Acquisition Using Skyline and the Thermo Q-Exactive

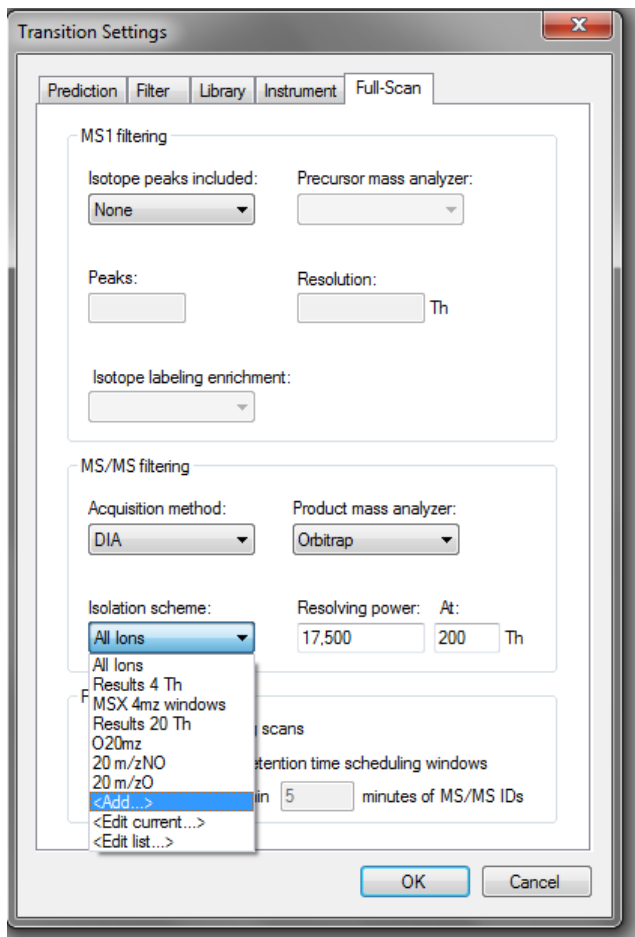
Author: Jarrett Egertson (jegertso <a. t> uw dot edu)

Introduction: This brief tutorial describes how to generate a DIA method using the Skyline Targeted Proteomics Environment suitable for running on the Q-Exactive. Skyline generates an optimized list of isolation windows to cover a m/z range of interest which is then entered into the Q-Exactive method editor. Here, we create a method covering 500-900 m/z with 20 m/z wide isolation windows. At the end of the tutorial, there is a note on how to generate a multiplexed method as in <http://www.ncbi.nlm.nih.gov/pubmed/23793237>. This tutorial was written for use with Skyline 2.1 and Q-Exactive software version 2.2 SP1. Please post any questions, comments, or suggestions on the Skyline support board at <https://skyline.gs.washington.edu/labkey/project/home/support/begin.view>.

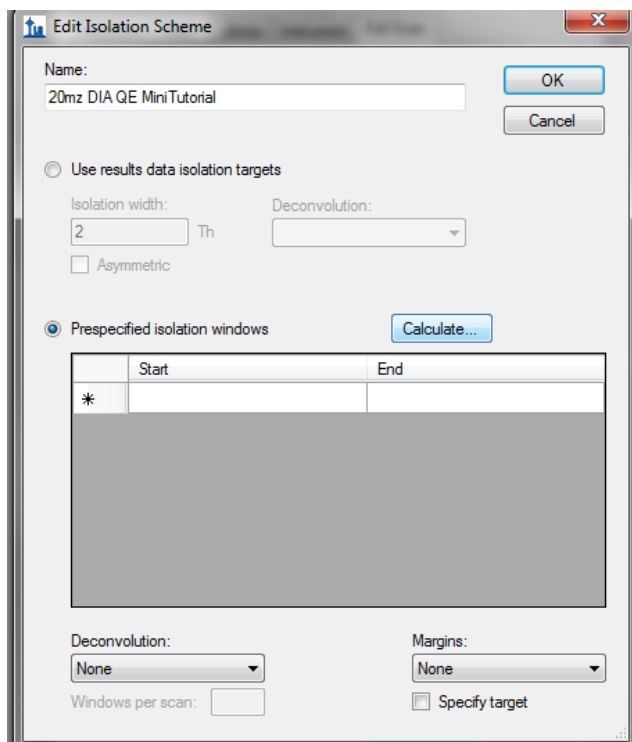
Generating a DIA Method to Cover 500 – 900 m/z with 20 m/z wide isolation windows.



Click on **Settings** and then **Transition Settings...**



Select the **Full-Scan** tab in the Transition Settings window. Set **Acquisition Method** to **DIA**, **Product mass analyzer** to **Orbitrap**, and resolving power. Click the drop down box under **Isolation Scheme** and click on **<Add...>**



Enter a name for the isolation scheme in the box labeled **Name**:

Select **Prespecified isolation windows** and click **Calculate...**

Calculate Isolation Scheme

Start m/z: End m/z:

Window width: Overlap: %

Window count: 20

Multiplexed acquisition
 Windows per scan:

Margins:

Margin width:

Optimize window placement Generate target

Enter a **start m/z** and **end m/z** for the precursor m/z range you would like to analyze by DIA. I use 500 – 900 with a **Window width**: of 20 m/z . Click the checkbox for **Optimize window placement**, and **Generate target**. Optimize window placement avoids placing edges of the isolation windows in regions where peptides are likely to occur. Generate target causes Skyline to output the center of each isolation window along with the start and end. Click **OK**.

Edit Isolation Scheme

Name:

Use results data isolation targets

Isolation width: Th Deconvolution:

Asymmetric

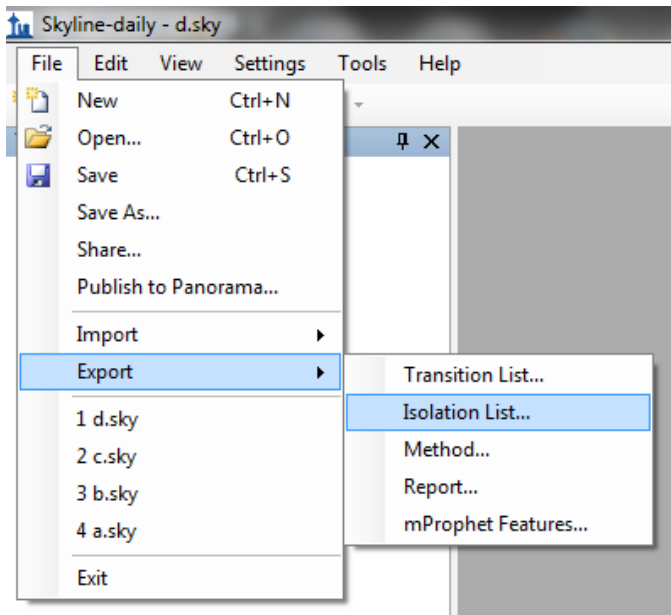
Prespecified isolation windows

	Start	End	Target
▶	500.4774	520.4865	510.4819
	520.4865	540.4956	530.4910
	540.4956	560.5047	550.5001
	560.5047	580.5138	570.5092
	580.5138	600.5229	590.5183
	600.5229	620.5319	610.5274
	620.5319	640.5410	630.5365
	640.5410	660.5501	650.5456

Deconvolution: Margins:

Windows per scan: Specify target

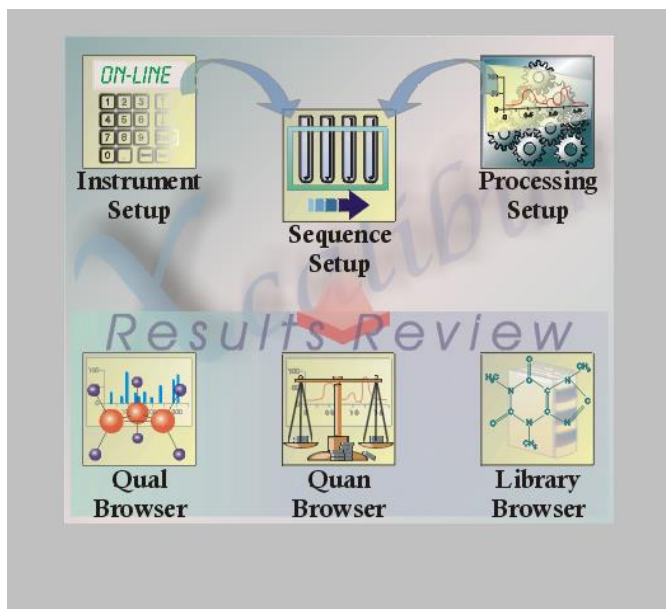
In the Edit Isolation Scheme window, there will be a list of isolation windows. The list has 20 isolation windows. Click **OK**.



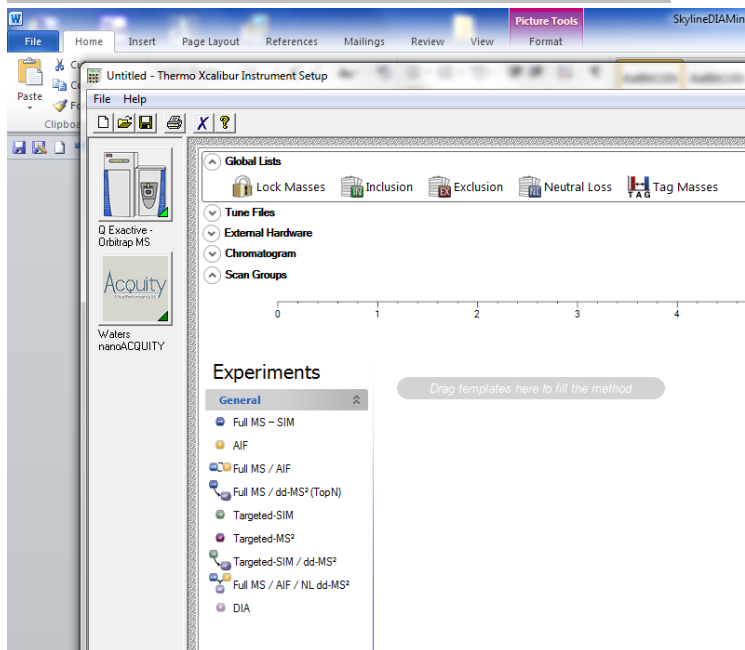
Select **File** and then click **Export** and **Isolation List...** Once you do this, Skyline will ask for a filename to which the isolation list will be saved. This file is a .csv file that can be opened in Excel.

	A1
	A
1	510.4819
2	530.491
3	550.5001
4	570.5092
5	590.5183
6	610.5274
7	630.5365
8	650.5456
9	670.5547
10	690.5638
11	710.5729
12	730.582
13	750.5911
14	770.6002
15	790.6093
16	810.6183
17	830.6274
18	850.6365
19	870.6456
20	890.6547
21	

Open the saved .csv isolation list file in Excel (or use OpenOffice or notepad if you do not have Excel) and **highlight the column of numbers**. These numbers are the centers of the isolation windows generated by Skyline. Press **Ctrl + C** or select **Edit -> Copy** to copy the window centers to the clipboard.



Open XCalibur and click **Instrument Setup**



Select **Global Lists** and click **Inclusion**

Method Editor - Inclusion List

	Mass [m/z]	Formula [M]	Species	CS [z]	Polarity	Start [min]	End [min]	NCE
1	510.48192				Positive			
2	530.49102				Positive			
3	550.50011				Positive			
4	570.50921				Positive			
5	590.51830				Positive			
6	610.52740				Positive			
7	630.53649				Positive			
8	650.54559				Positive			
9	670.55468				Positive			
10	690.56378				Positive			
11	710.57287				Positive			
12	730.58197				Positive			
13	750.59106				Positive			
14	770.60016				Positive			
15	790.60925				Positive			
16	810.61835				Positive			
17	830.62744				Positive			
18	850.63654				Positive			
19	870.64563				Positive			
20	890.65473				Positive			
21					Positive			

Paste the values copied to the clipboard and click **OK** on the window that pops up and saying 20 rows will be added. Click **Done**.

Untitled - Thermo Xcalibur Instrument Setup

File Help

Global Lists: Lock Masses, Inclusion, Exclusion, Neutral Loss, TAG Tag Masses

Tune Files

External Hardware

Chromatogram

Scan Groups

Full MS - SIM
DIA

time (min) 0 1 2 3 4 5 6 7 8 9 10

Properties

Properties of the method

- Global Settings
 - use lock masses: best
 - Chrom. peak wi 15 s
- Time
 - Method duration 10.00 min

Properties of Full MS - SIM

- General
 - User Role Standard
 - Runtime 0 to 10 min
 - Polarity positive
- Full MS - SIM
 - Resolution 35,000
 - AGC target 1e6
 - Maximum IT 55 ms
 - Scan range 490 to 910 m/z

Experiments

General

- Full MS - SIM
- AIF
- Full MS / AIF
- Full MS / dd-MS² (TopN)
- Targeted-SIM
- Targeted-MS²
- Targeted-SIM / dd-MS²
- Full MS / AIF / NL dd-MS²
- DIA

Full MS

DIA

Add a **Full MS-SIM** and **DIA** scan event to the method. This is done by clicking and dragging the scan event name from the list of **Experiments** and dropping the event onto the grey timeline bar just to the right of where it says **Experiments**. Click the **Full MS** scan event that was just dragged over. The properties that I use for this scan event are displayed on the right.

The screenshot displays a software interface with several components:

- Global Lists:** A sidebar on the left containing expandable sections for Global Lists, Tune Files, External Hardware, Chromatogram, and Scan Groups.
- Chromatogram:** A horizontal bar at the top showing two scan groups: 'Full MS - SIM' and 'DIA' over a 10-minute time scale.
- Experiments:** A list on the left showing various scan configurations like 'Full MS - SIM', 'AIF', 'Full MS / AIF', etc.
- Properties of the method:** A panel on the right showing settings for 'Global Settings' (e.g., use lock masses: best) and 'Time' (Method duration: 10.00 min).
- Properties of DIA:** A detailed panel on the right showing 'General' settings (User Role: Advanced, Runtime: 0 to 10 min) and 'DIA' settings (Microscans: 1, Resolution: 17,500, AGC target: 1e6, Loop count: 10, etc.).
- Diagram:** A central diagram with two boxes labeled 'Full MS' and 'DIA' under a horizontal bar, with arrows pointing down to each box.

Click on the **DIA** scan. The properties that I use are shown on the right. The **Loop Count** of 10 means that an MS scan will be taken every 10 MS/MS (DIA) scans.

Generating a Multiplexed Method

A multiplexed method with 5 4 m/z wide isolation windows per scan covering 500-900 m/z is generated.

NOTE (10/6/2014) – Importing data takes longer for a multiplexed method. If >100,000 transitions are going to be extracted from the data, the import can take hours per file. One user reported 20 hrs for importing a 220Mb file with ~150,000 transitions. Files with <50,000 transitions should import in 10-30 minutes with retention time filtering enabled.

The screenshot shows the Skyline-daily software interface with the 'Settings' menu open. The menu options are:

- Save Current...
- Edit List...
- Share...
- Import...
- Peptide Settings...
- Transition Settings...** (highlighted)
- Annotations...
- Integrate All

Click on **Settings** and then **Transition Settings...**

Transition Settings [X]

Prediction | Filter | Library | **Instrument** | Full-Scan

Min m/z: Th Max m/z: Th

Dynamic min product m/z

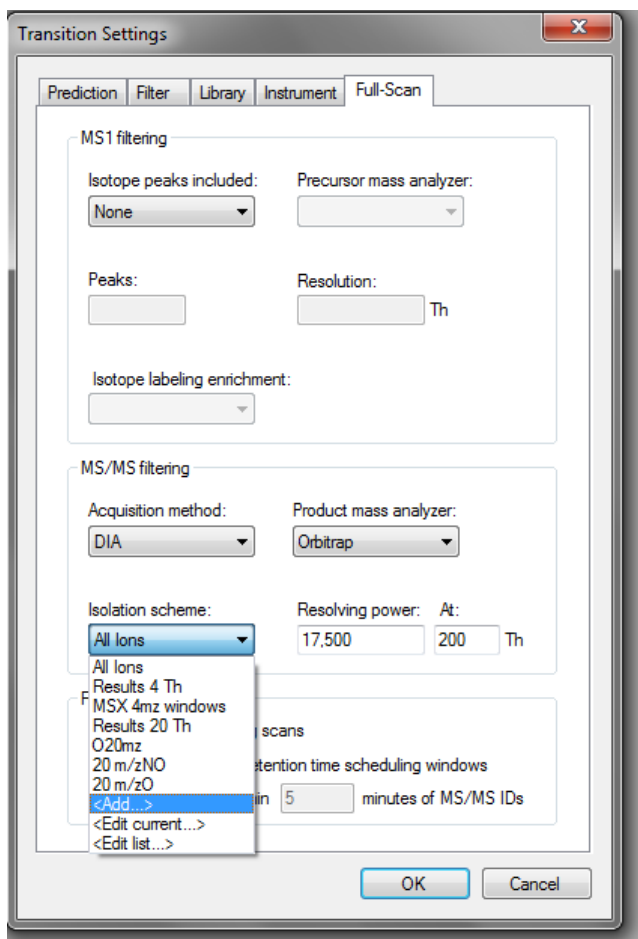
Method match tolerance m/z: Th

Firmware transition limit: Firmware inclusion limit:

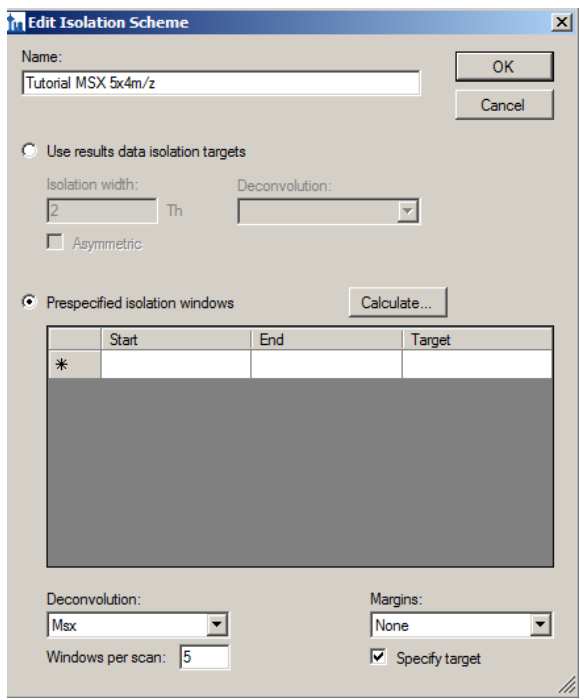
Min time: min Max time: min

OK Cancel

Click on the **Instrument** tab and enter **5000** in the box titled **Firmware inclusion limit**



Click on the **Full-Scan** tab, set **Acquisition Method** to **DIA**, **Product mass analyzer** to **Orbitrap**, and under **Isolation scheme** click **<Add...>**



Enter a name for the isolation scheme in the box that says **Name**, select **Prespecified isolation windows**, under **Deconvolution** select **Msx** and in **Windows per scan** enter **5**. Select **Specify target** and click **Calculate...**

Calculate Isolation Scheme

Start m/z: End m/z:

Window width: Overlap: %

Window count: 105

Multiplexed acquisition
 Windows per scan:

Margins:

Margin width:

Optimize window placement Generate target

OK Cancel

Enter **500** as the **Start m/z** and **900** as the **End m/z**. Window width should be **4**.
 Selecte **Multiplexed acquisition** with **5 windows per scan**. Select **Optimize window placement** and **Generate target**.
 Click **OK**.

Edit Isolation Scheme

Name:

Use results data isolation targets
 Isolation width: Th Deconvolution:

Asymmetric

Prespecified isolation windows

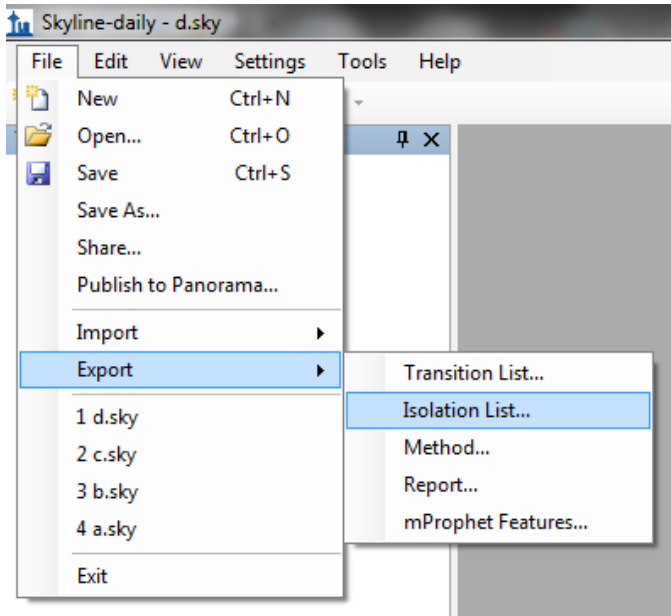
	Start	End	Target
▶	500.4774	504.4792	502.4783
	504.4792	508.4810	506.4801
	508.4810	512.4828	510.4819
	512.4828	516.4847	514.4837
	516.4847	520.4865	518.4856
	520.4865	524.4883	522.4874
	524.4883	528.4901	526.4892
	528.4901	532.4919	530.4910

Deconvolution: Margins:

Windows per scan: Specify target

OK Cancel

An isolation list containing 100 isolation windows is generated. Click **OK**.

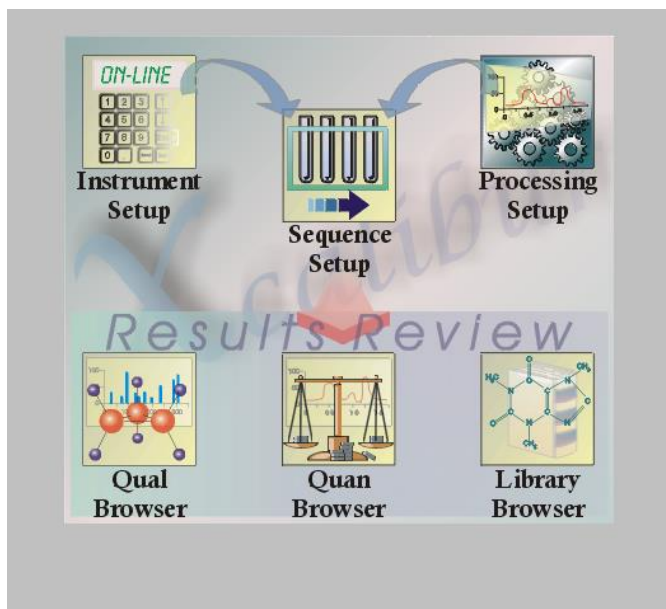


Select **File** and then click **Export** and **Isolation List...** Once you do this, Skyline will ask for a filename to which the isolation list will be saved. This file is a .csv file that can be opened in Excel.

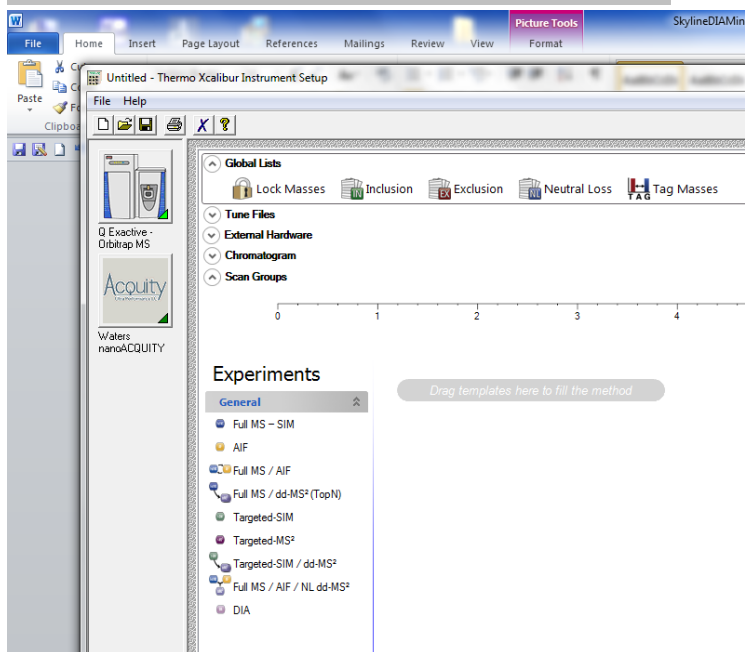
A screenshot of an Excel spreadsheet. The active cell is A1. The spreadsheet contains a list of 31 numerical values in column A, ranging from 790.6093 to 590.5183. The values are: 790.6093, 826.6256, 806.6165, 646.5438, 582.5147, 718.5765, 626.5347, 698.5674, 798.6129, 862.642, 606.5256, 630.5365, 746.5892, 898.6584, 554.5019, 558.5038, 890.6547, 622.5329, 530.491, 834.6293, 522.4874, 818.622, 886.6529, 566.5074, 542.4965, 618.531, 550.5001, 730.582, 754.5929, 854.6384, 590.5183.

	A
1	790.6093
2	826.6256
3	806.6165
4	646.5438
5	582.5147
6	718.5765
7	626.5347
8	698.5674
9	798.6129
10	862.642
11	606.5256
12	630.5365
13	746.5892
14	898.6584
15	554.5019
16	558.5038
17	890.6547
18	622.5329
19	530.491
20	834.6293
21	522.4874
22	818.622
23	886.6529
24	566.5074
25	542.4965
26	618.531
27	550.5001
28	730.582
29	754.5929
30	854.6384
31	590.5183

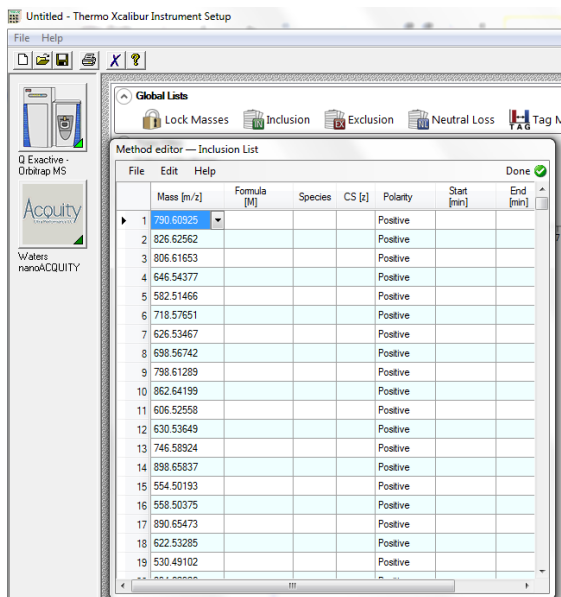
Open the saved .csv isolation list file in Excel (or use OpenOffice or notepad if you do not have Excel) and **highlight the column of numbers**. These numbers are the centers of the isolation windows generated by Skyline. There should be 5,000 windows in total, and they should be in random order as shown (left). Press **Ctrl + C** or select **Edit -> Copy** to copy the window centers to the clipboard.



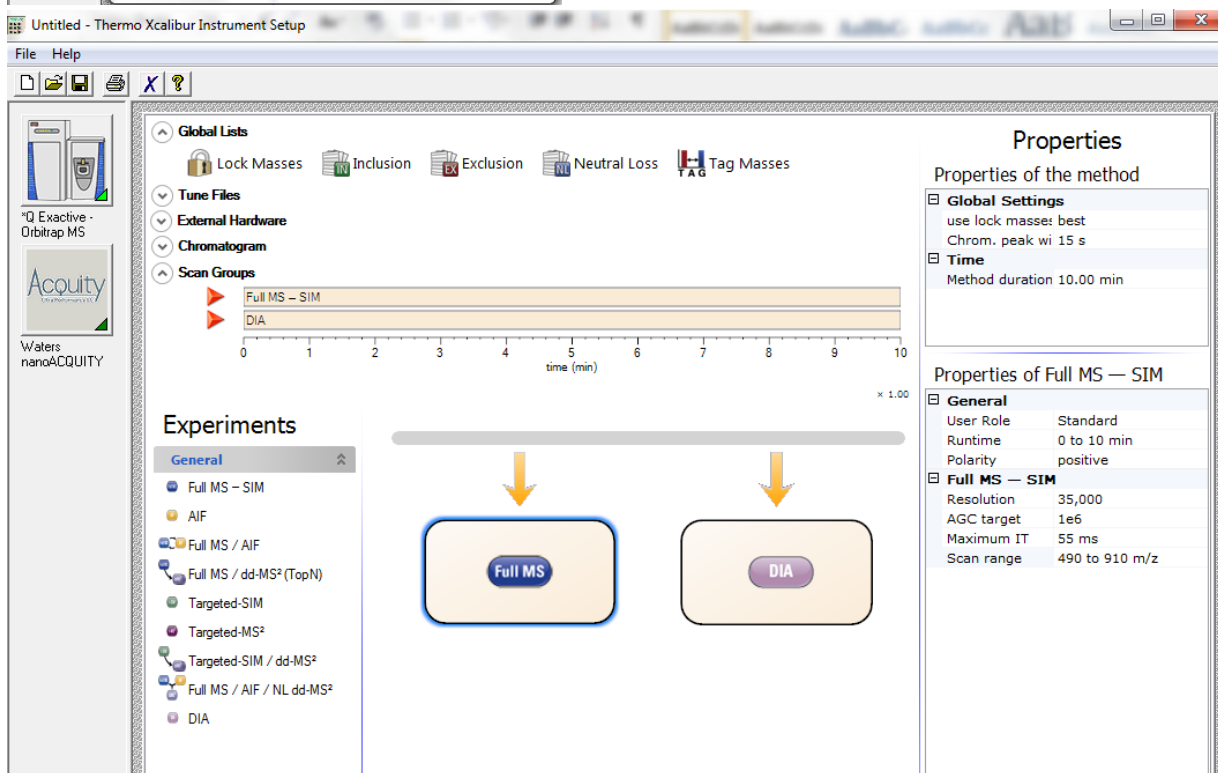
Open XCalibur and click **Instrument Setup**



Select **Global Lists** and click **Inclusion**



Paste the values copied to the clipboard and click **OK** on the window that pops up saying 5000 rows will be added. Click **Done**.



Add a **Full MS-SIM** and **DIA** scan event to the method. This is done by clicking and dragging the scan event name from the list of **Experiments** and dropping the event onto the grey timeline bar just to the right of where it says **Experiments**. Click the **Full MS** scan event that was just dragged over. The properties that I use for this scan event are displayed on the right.

Click on the **DIA** scan. The properties that I use are shown on the right. The **Loop Count** of 10 means that an MS scan will be taken every 10 MS/MS (DIA) scans.

Edits:

8/21/2013 – Changed the settings screenshot for the DIA scan settings in the non-multiplexed case. Changes are User Role is “Advanced” instead of “Standard”. Maximum IT is “auto” instead of 55 ms, NCE is now 30.0 instead of 25 and Spectrum data type is “Centroid” to save space.

10/6/2014 – Noted that importing a lot of transitions with multiplexed data may take a long time.