

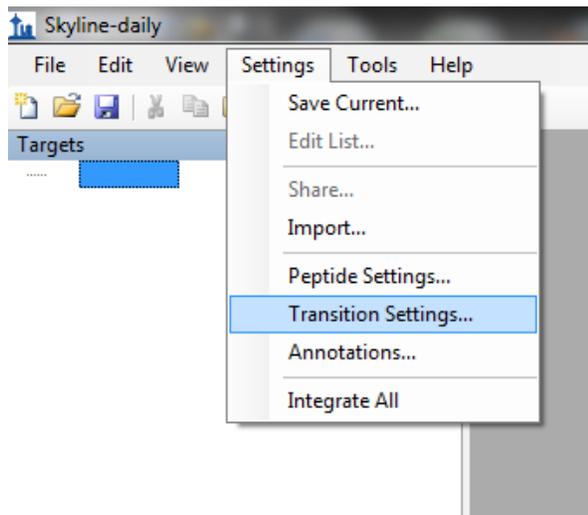
# Data Independent Acquisition Using Skyline and the Thermo Q-Exactive

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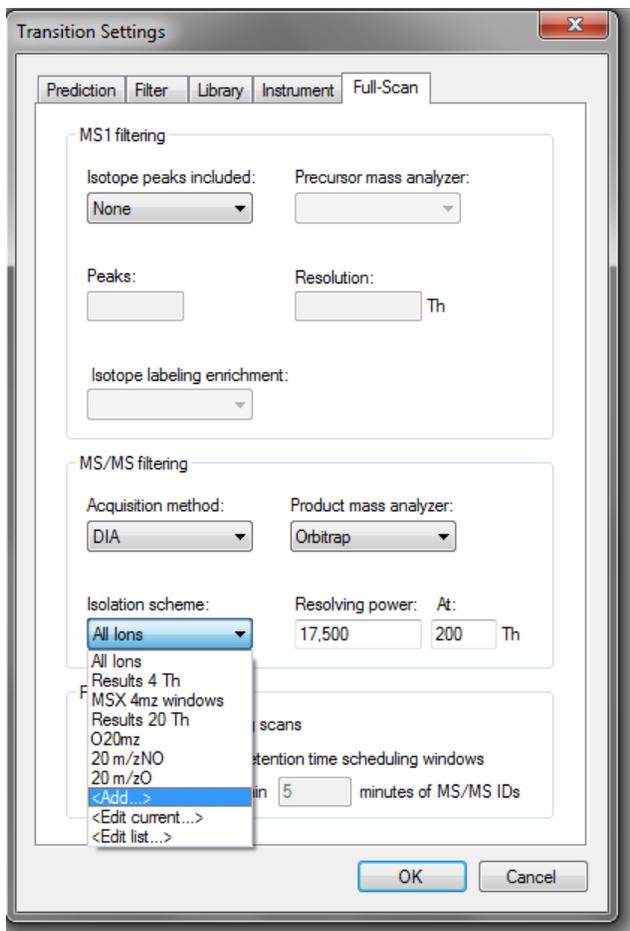
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**Introduction:** This brief tutorial describes how to generate a DIA method using the Skyline Targeted Proteomics Environment suitable for running on the Q-Exactive. Skyline generates an optimized list of isolation windows to cover a  $m/z$  range 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-daily 1.4.1.4756. and Q-Exactive software version 2.2 SP1. The steps are similar for Skyline 1.3, but not exactly the same. Please post any questions, comments, or suggestions on the Skyline support board at <https://skyline.gs.washington.edu/labkey/project/home/support/begin.view>.

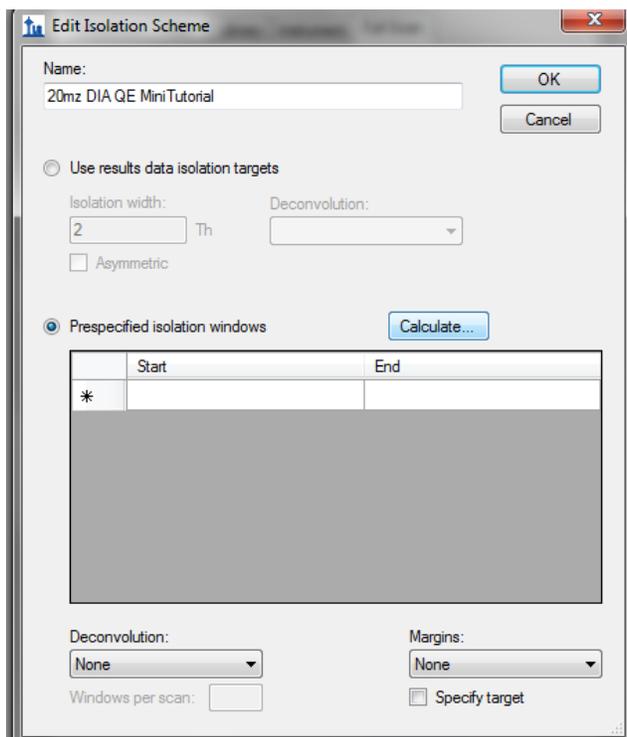
## Generating a DIA Method to Cover 500 – 900 $m/z$ with 20 $m/z$ wide isolation windows.



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*: 500      End *m/z*: 900     

Window width: 20      Overlap:     

Window count: 21

Multiplexed acquisition      Margins: None

Windows per scan:      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: 20mz DIA QE Mini Tutorial     

Use results data isolation targets

Isolation width: 2 Th      Deconvolution:

Asymmetric

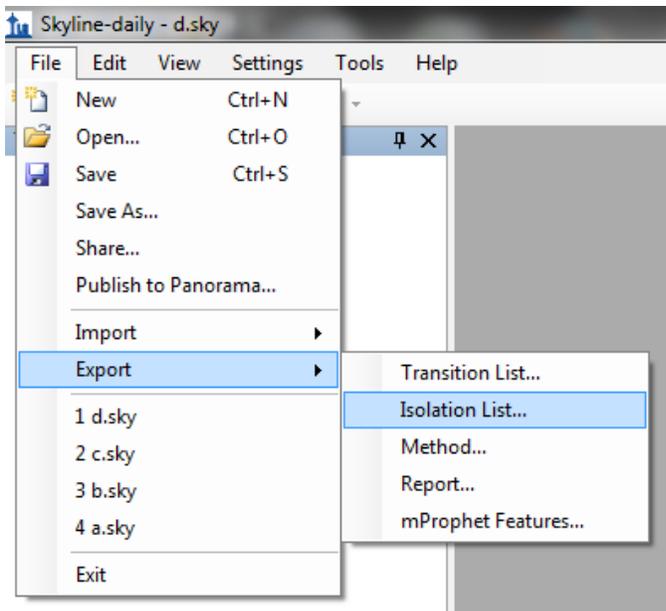
Prespecified isolation windows     

	Start	End	Target
<input checked="" type="checkbox"/>	480.4683	500.4774	490.4728
<input type="checkbox"/>	500.4774	520.4865	510.4819
<input type="checkbox"/>	520.4865	540.4956	530.4910
<input type="checkbox"/>	540.4956	560.5047	550.5001
<input type="checkbox"/>	560.5047	580.5138	570.5092
<input type="checkbox"/>	580.5138	600.5229	590.5183
<input type="checkbox"/>	600.5229	620.5319	610.5274
<input type="checkbox"/>	620.5319	640.5410	630.5365
<input type="checkbox"/>	640.5410	660.5501	650.5456
<input type="checkbox"/>	660.5501	680.5592	670.5547
<input type="checkbox"/>	680.5592	700.5683	690.5638
<input type="checkbox"/>	700.5683	720.5774	710.5729
<input type="checkbox"/>	720.5774	740.5865	730.5820

Deconvolution: None      Margins: None

Windows per scan:       Specify target

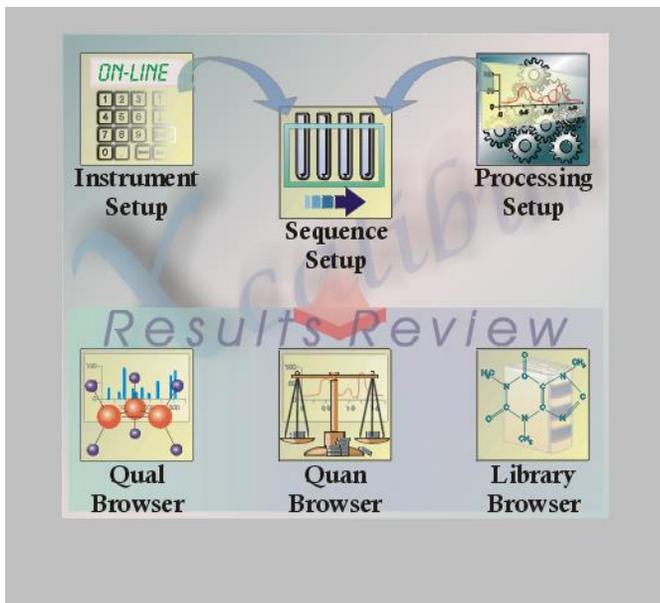
In the Edit Isolation Scheme window, there will be a list of isolation windows. The list has 21 isolation windows, but the first window is from 480.4683 – 500.4774 *m/z*. This window is technically needed to comprehensively cover the *m/z* range from 500-900 *m/z* with optimized edges, but is not really necessary because it hardly overlaps that range. I usually select the first row, and delete it, to have a total of 20 isolation windows. Click **OK**.



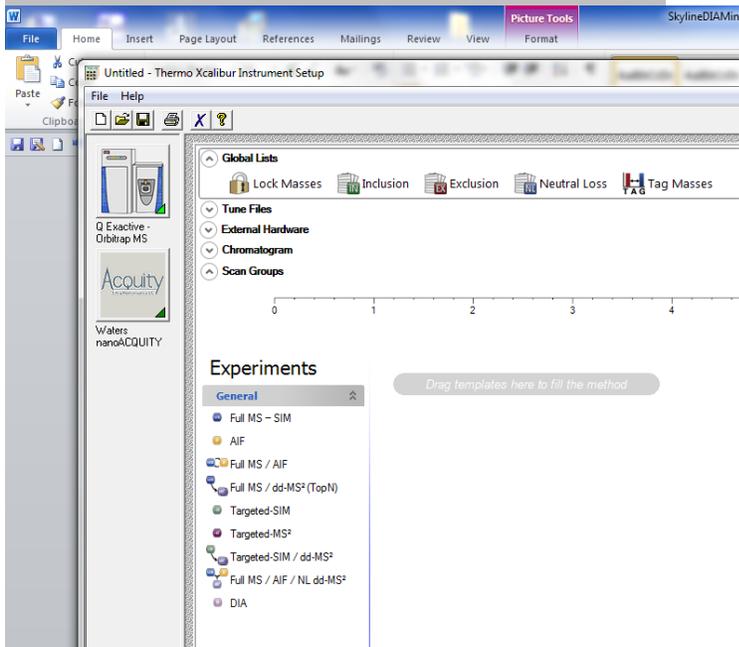
Select **File** and then click **Export** and **Isolation List...** Once you do this, Skyline will ask for a filename to which the isolation list will be saved. This file is a .csv file that can be opened in Excel.

A screenshot of an Excel spreadsheet. The 'File' menu is open, showing options like Home, Cut, Copy, Paste, and Format. The spreadsheet shows a list of numbers in column A, with the first cell A1 highlighted. The numbers are: 510.4819, 530.491, 550.5001, 570.5092, 590.5183, 610.5274, 630.5365, 650.5456, 670.5547, 690.5638, 710.5729, 730.582, 750.5911, 770.6002, 790.6093, 810.6183, 830.6274, 850.6365, 870.6456, 890.6547. The cell A1 is highlighted in yellow.

**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 Masses

Tune Files

External Hardware

Chromatogram

Scan Groups

Full MS - SIM

DIA

time (min)

Experiments

General

- Full MS - SIM
- AIF
- Full MS / AIF
- Full MS / dd-MS<sup>2</sup> (TopN)
- Targeted-SIM
- Targeted-MS<sup>2</sup>
- Targeted-SIM / dd-MS<sup>2</sup>
- Full MS / AIF / NL dd-MS<sup>2</sup>
- DIA

Properties

Properties of the method

- Global Settings
  - use lock mass: 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

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 for configuring mass spectrometry methods. On the left, a 'Scan Groups' section shows a timeline from 0 to 10 minutes with two scan types: 'Full MS - SIM' and 'DIA'. Below this is an 'Experiments' list with various scan configurations. On the right, two property panels are visible. The top panel, 'Properties of the method', shows 'Global Settings' (use lock masses: best, Chrom. peak width (FWH): 15 s) and 'Time' (Method duration: 10.00 min). The bottom panel, 'Properties of DIA', shows 'General' settings (User Role: Advanced, Runtime: 0 to 10 min, Polarity: positive, In-source CID: 0.0 eV, Default charge state: 2) and 'DIA' settings (Microscans: 1, Resolution: 17,500, AGC target: 1e6, Maximum IT: auto, Loop count: 10, MSX count: 1, MSX isochronous ITs: on, Isolation window: 20.0 m/z, Fixed first mass: -, NCE: 30.0, Stepped NCE: -, Spectrum data type: Centroid).

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. Note that the optimal NCE may vary based on sample.

## 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. The 'Settings' menu is open, displaying options: 'Save Current...', 'Edit List...', 'Share...', 'Import...', 'Peptide Settings...', 'Transition Settings...' (highlighted), 'Annotations...', and 'Integrate All'. The 'Targets' list is visible on the left side of the interface.

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

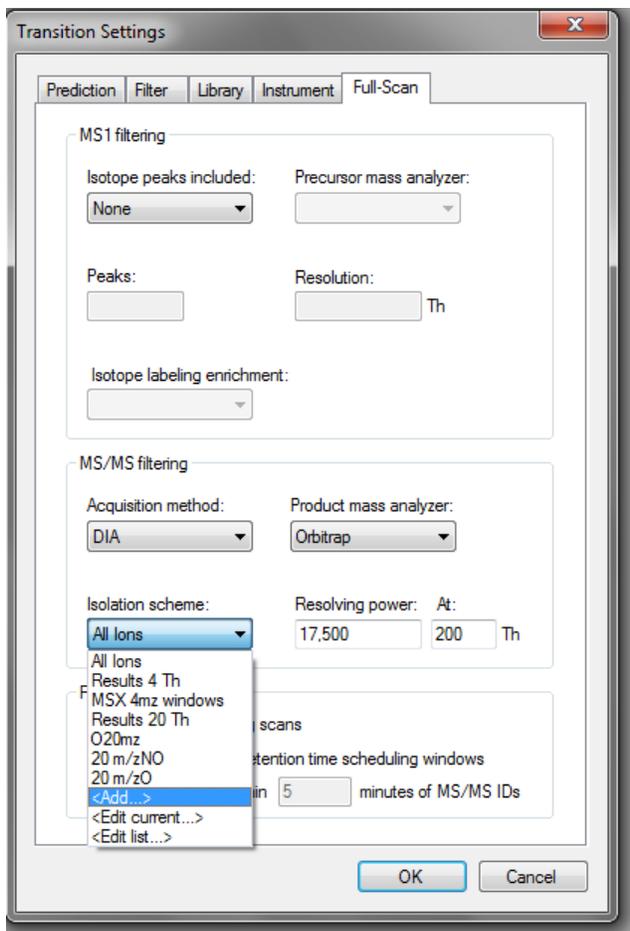
Method match tolerance m/z:  Th

Firmware transition limit:       Firmware inclusion limit:

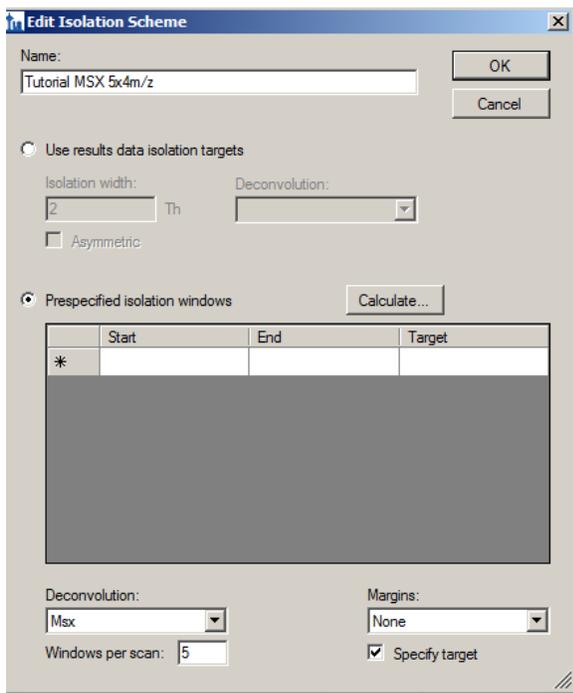
Min time:  min      Max time:  min

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**.  
 Select **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

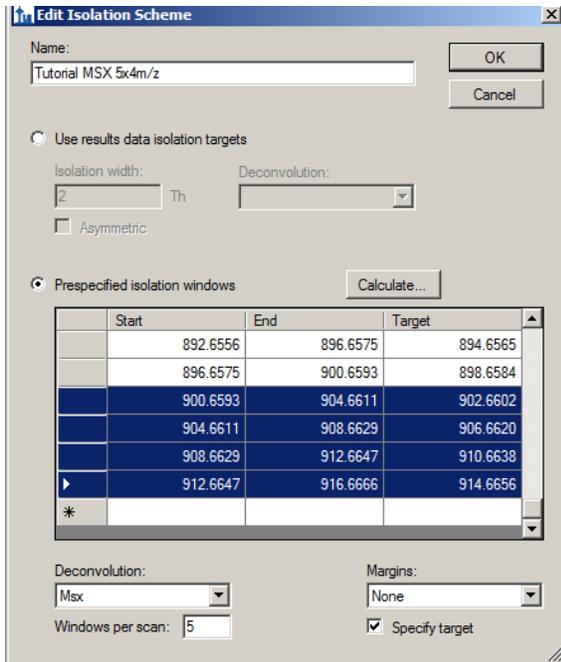
	Start	End	Target
▶	496.4756	500.4774	498.4765
	500.4774	504.4792	502.4783
	504.4792	508.4810	506.4801
	508.4810	512.4828	510.4819
	512.4828	516.4847	514.4837
	516.4847	520.4865	518.4856
	520.4865	524.4883	522.4874
	524.4883	528.4901	526.4893

Deconvolution:  Margins:

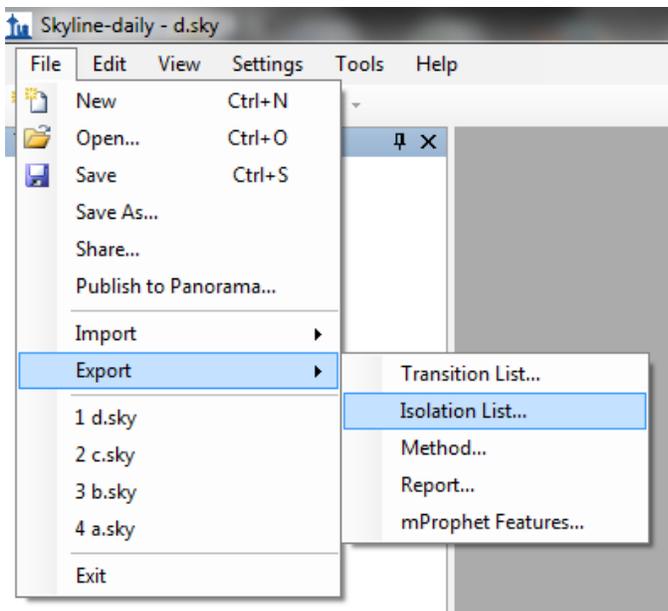
Windows per scan:   Specify target

OK Cancel

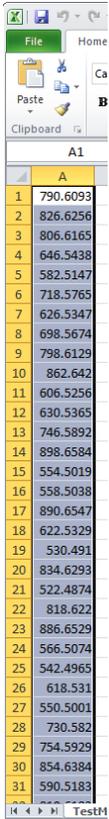
Some of the windows generated will be outside of the defined *m/z* range. The first window only barely overlaps with the 500-900 *m/z* range to be covered. Delete this window, by **selecting the first row** and pressing **Delete**.



Select the last four isolation windows, which also don't overlap with the range of interest, and **delete** them. Click **OK**.



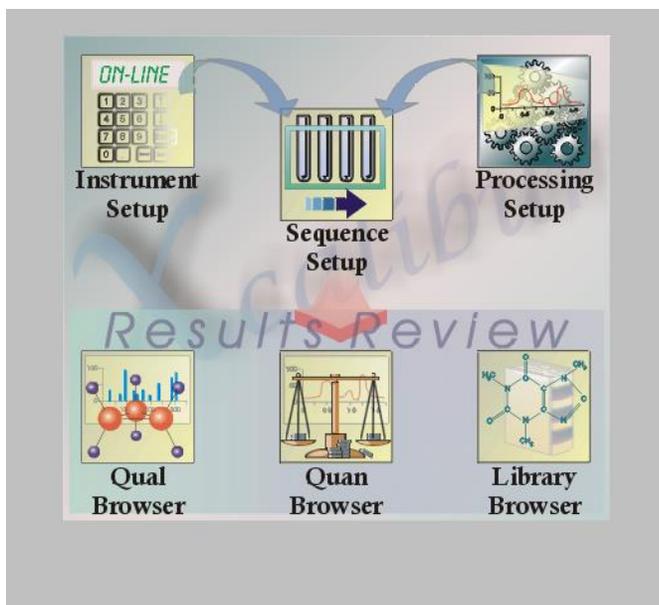
Select **File** and then click **Export** and **Isolation List...** Once you do this, Skyline will ask for a filename to which the isolation list will be saved. This file is a .csv file that can be opened in Excel.



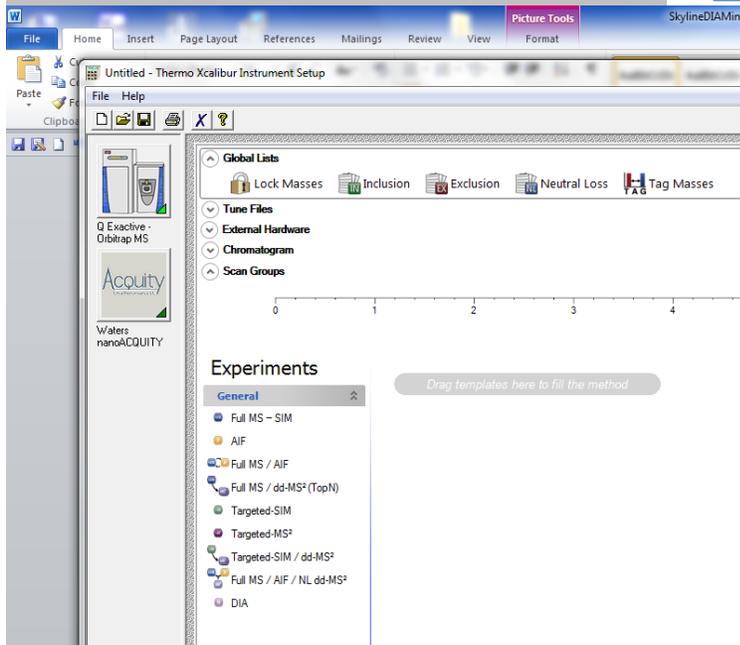
The image shows a screenshot of an Excel spreadsheet. The spreadsheet has a single column labeled 'A' and 31 rows. The numbers in column A are: 790.6093, 826.6256, 806.6165, 646.5438, 582.5147, 718.5765, 626.5347, 698.5674, 798.6129, 862.642, 606.5256, 630.5365, 746.5892, 898.6584, 554.5019, 558.5038, 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. The spreadsheet interface includes a ribbon with 'File' and 'Home' tabs, a 'Clipboard' section with 'Paste' and 'Clipboard' options, and a status bar at the bottom showing 'K 4 y N TestM'.

	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

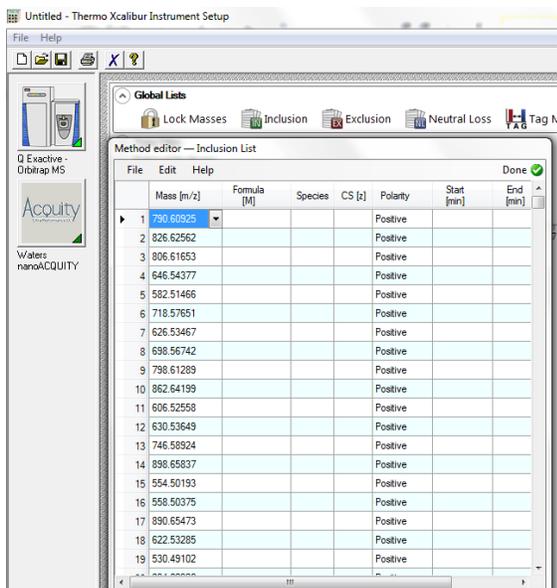
**Open the saved .csv isolation list file in Excel** (or use OpenOffice or notepad if you do not have Excel) and **highlight the column of numbers**. These numbers are the centers of the isolation windows generated by Skyline. There should be 5,000 windows in total, and they should be in random order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



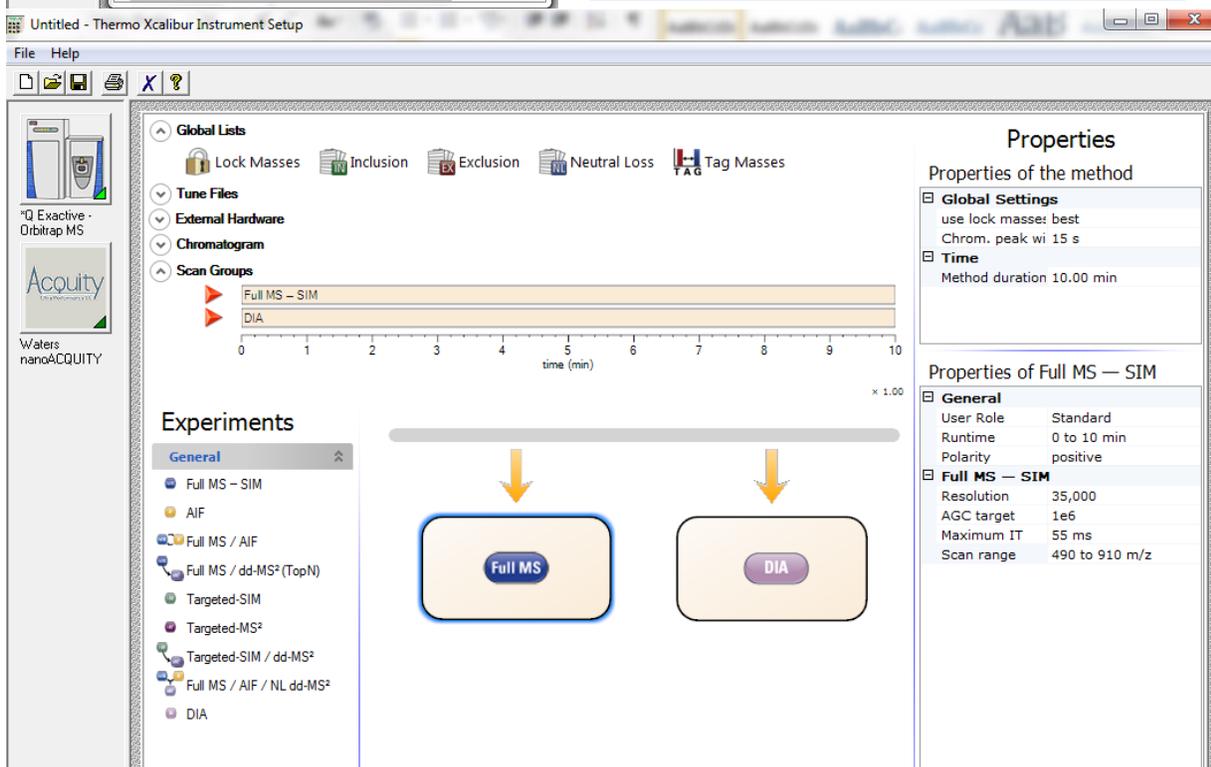
Open XCalibur and click **Instrument Setup**



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.