Data Independent Acquisition Using Skyline and the Thermo Q-Exactive

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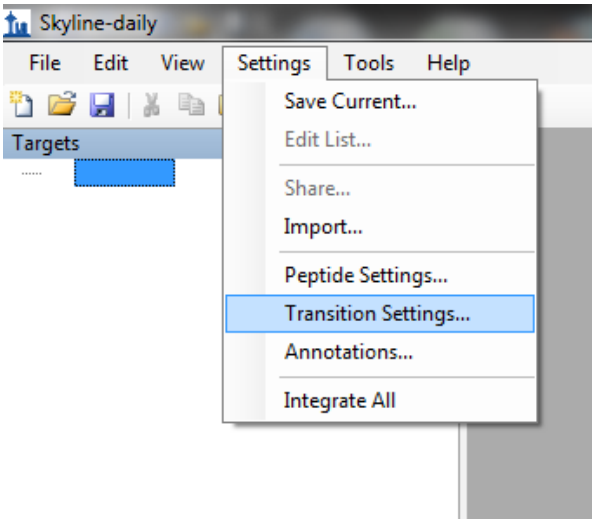
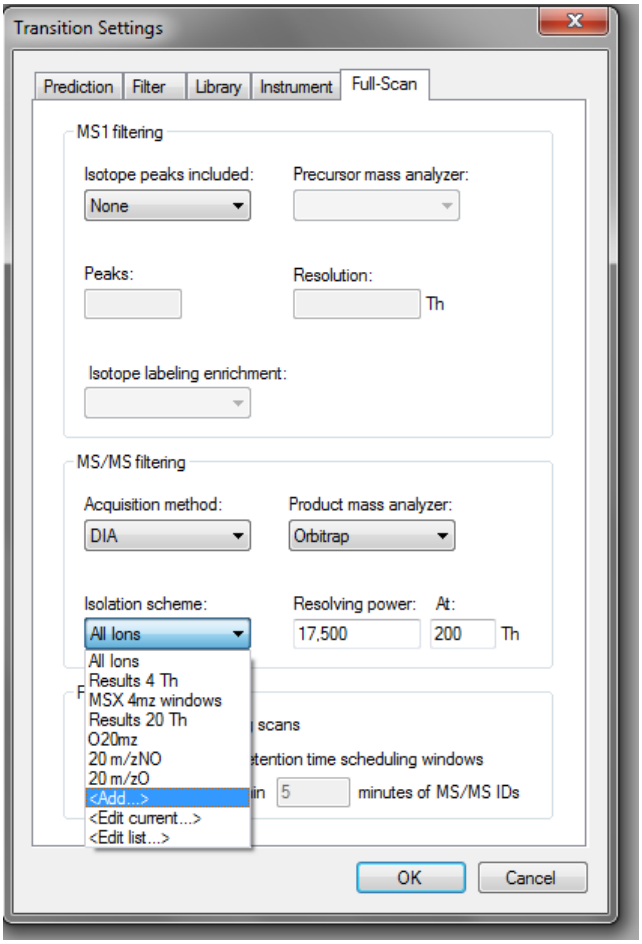
Introduction: This brief tutorial describes how to generate a DIA method using the Skyline Targeted Proteomics Enivronment suitable for running on the Q-Exactive. Skyline generates an optimized list of isolation windows to cover a *m/z* range of interest which is then entered into the Q-Exactive method 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.

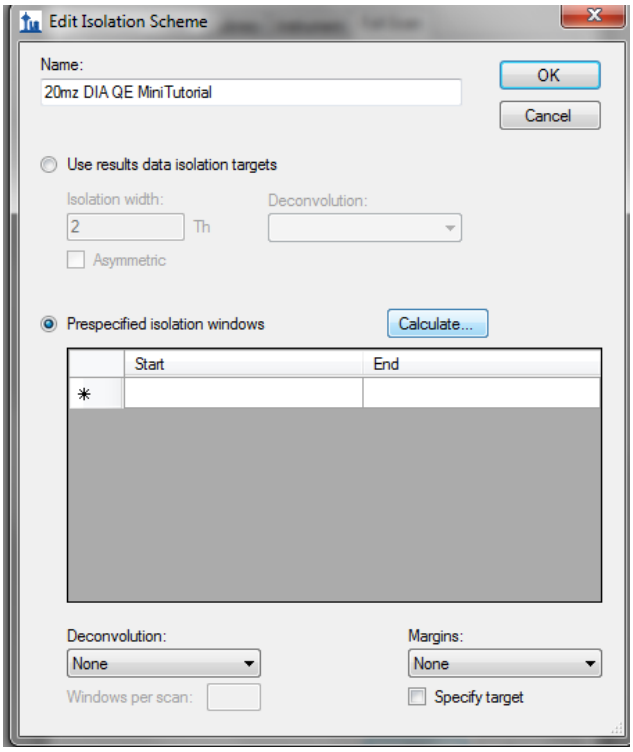
Generating a DIA Method to Cover 500 – 900 *m/z* with 20 *m/z* wide isolation windows.



Click on **Settings** and then **Transition Settings…**

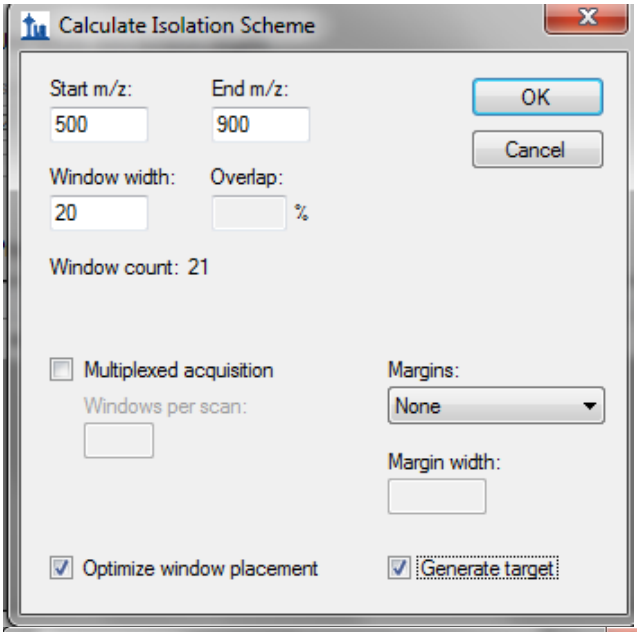


Select the **Full-Scan** tab in the Transition Settings window. Set **Acquisition Method** to **DIA**, **Product mass analyzer** to **Orbitrap**, and resolving power. Click the drop down box under **Isolation Scheme** and click on **<Add…>**

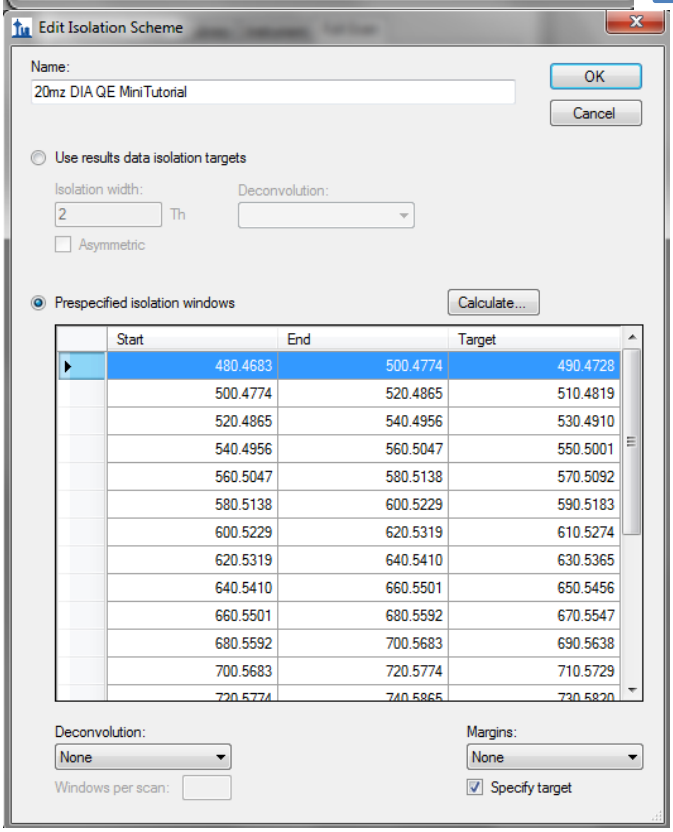


Enter a name for the isolation scheme in the box labeled **Name:**

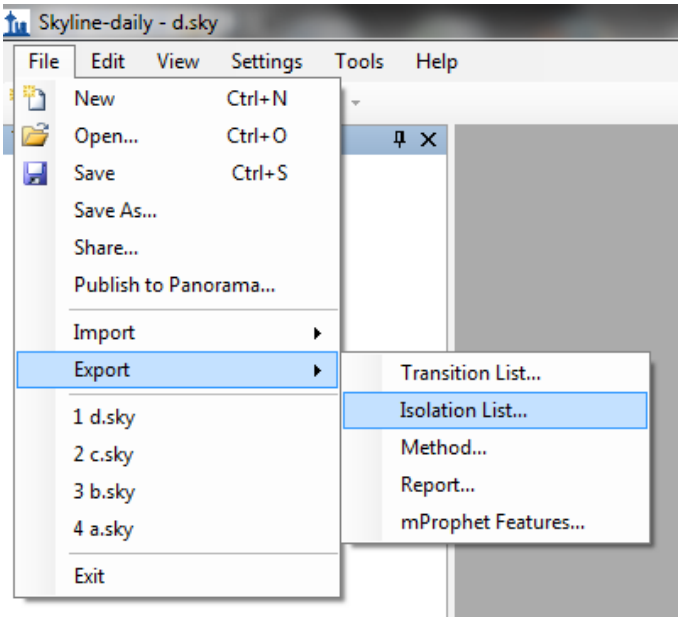
Select **Prespecified isolation windows** and click **Calculate…**



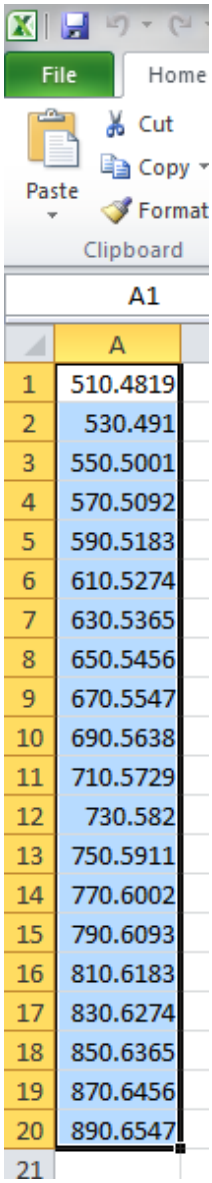
Enter a **start *m/z*** and **end *m/z*** for the precursor *m/z* range you would like to analyze by DIA. I use 500 – 900 with a **Window width:** of 20 *m/z*. Click the checkbox for **Optimize window placement**, and **Generate target**. Optimize window placement avoids placing edges of the isolation 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**.



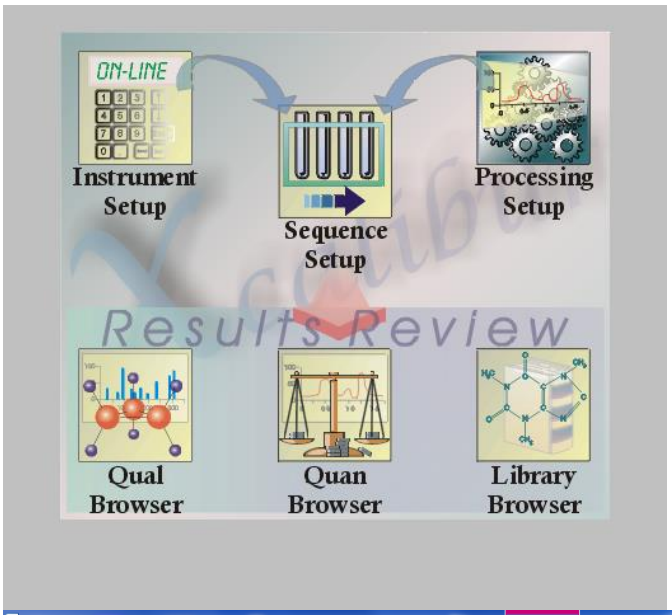
In the Edit Isolation Scheme window, there will be a list of isolation windows. The list has 21 isolation windows, but the first window is from 480.4683 – 500.4774 *m/z*. This window is technically needed to comprehensively cover the *m/z* range from 500-900 *m/z* with optimized edges, but is not really 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.**



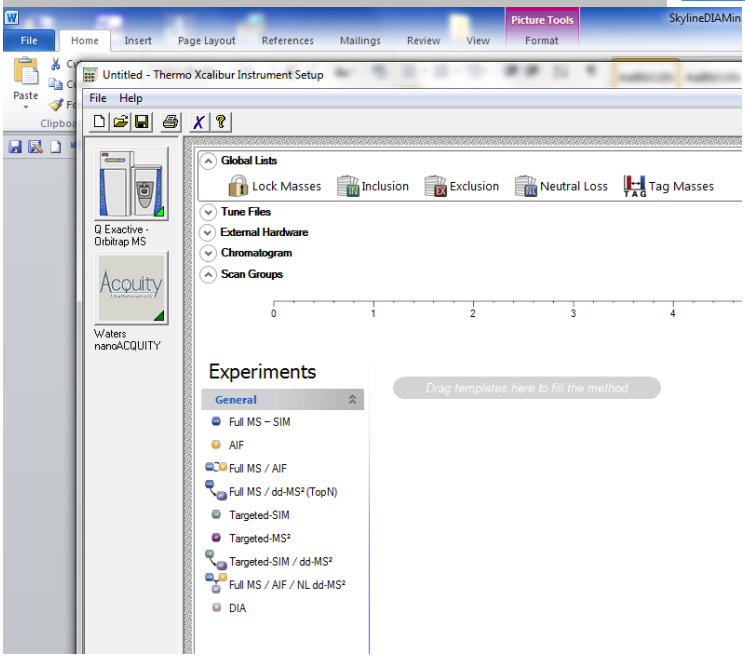
Select **File** and then click **Export** and **Isolation List…** Once you do this, Skyline will ask for a filename to which the isolation list will be saved. This file is a .csv file that can be opened in Excel.



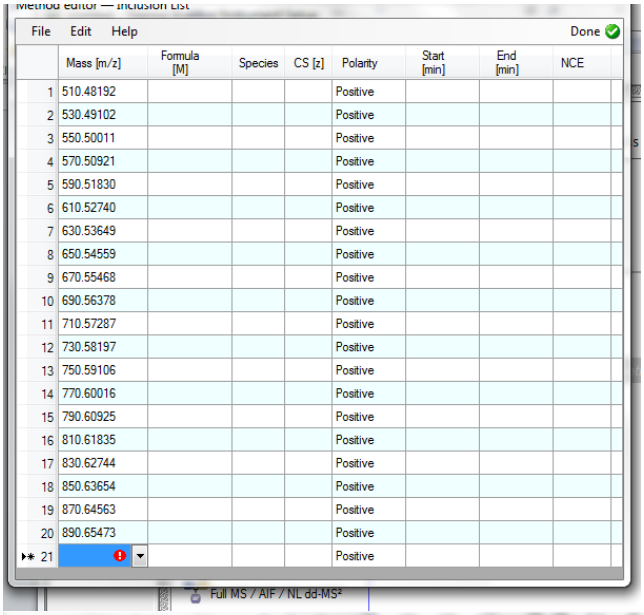
Open the saved .csv isolation list file in Excel (or use OpenOffice or notepad if you do not have Excel) and **highlight the column of numbers**. These numbers are the centers of the isolation windows generated by Skyline. Press **Ctrl + C** or select **Edit -> Copy** to copy the window centers to the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



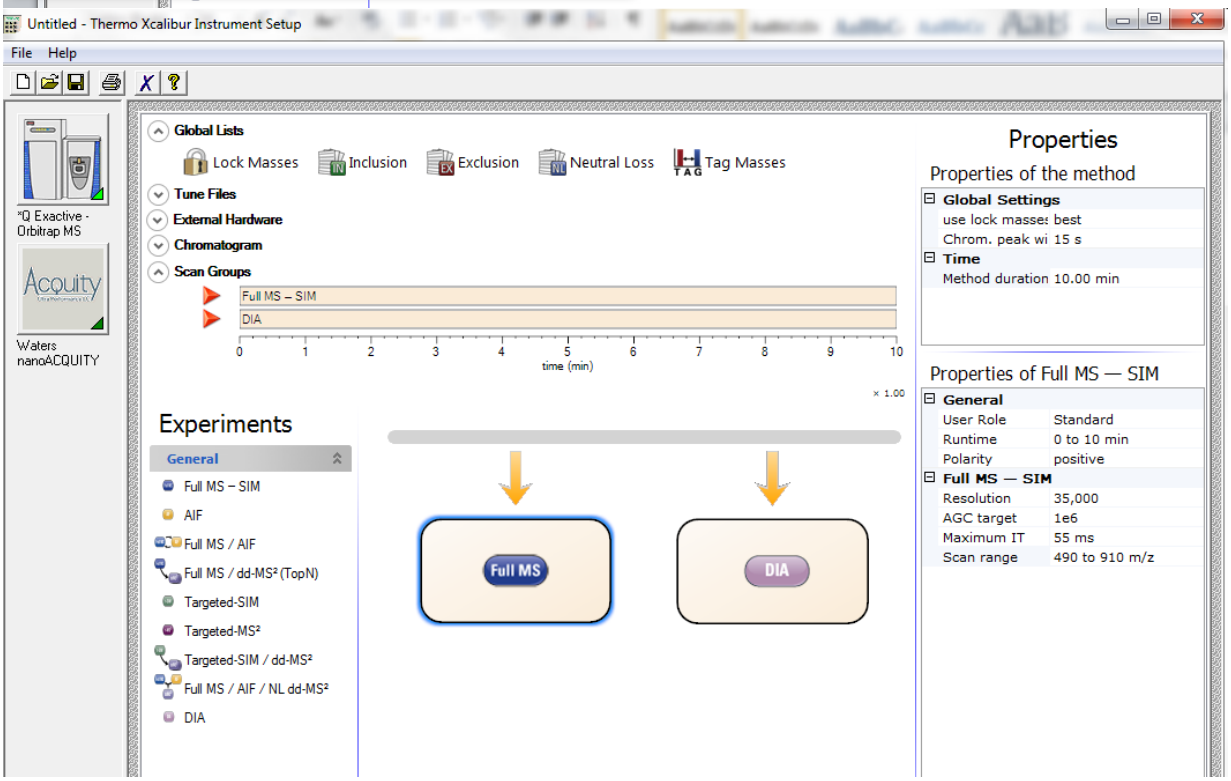
Open XCalibur and click **Instrument Setup**



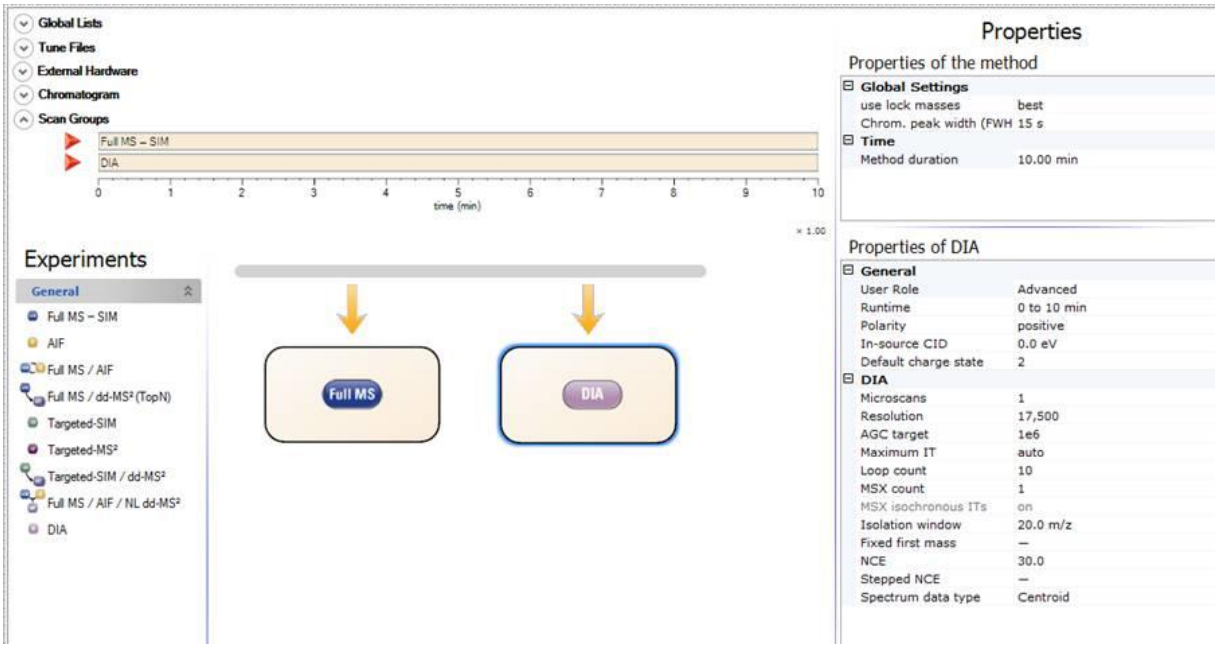
Select **Global Lists** and click **Inclusion**



Paste the values copied to the clipboard and click **OK** on the window that pops up saying 20 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.

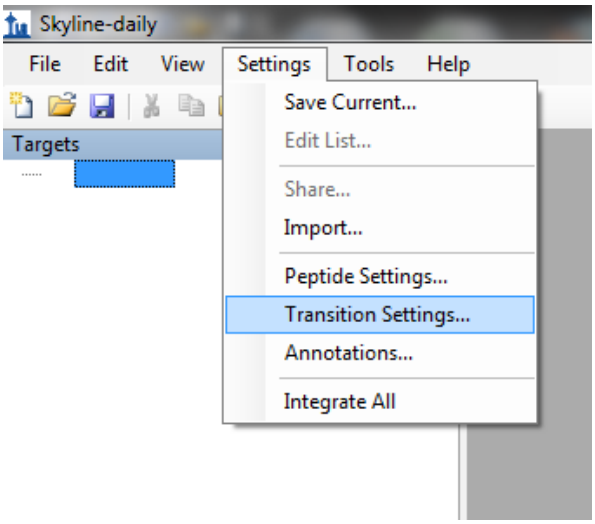


Click on the **DIA** scan. The properties that I use are shown on the right. The **Loop Count** of 10 means that an MS scan will be taken every 10 MS/MS (DIA) scans. Note that the optimal NCE may vary based on sample.

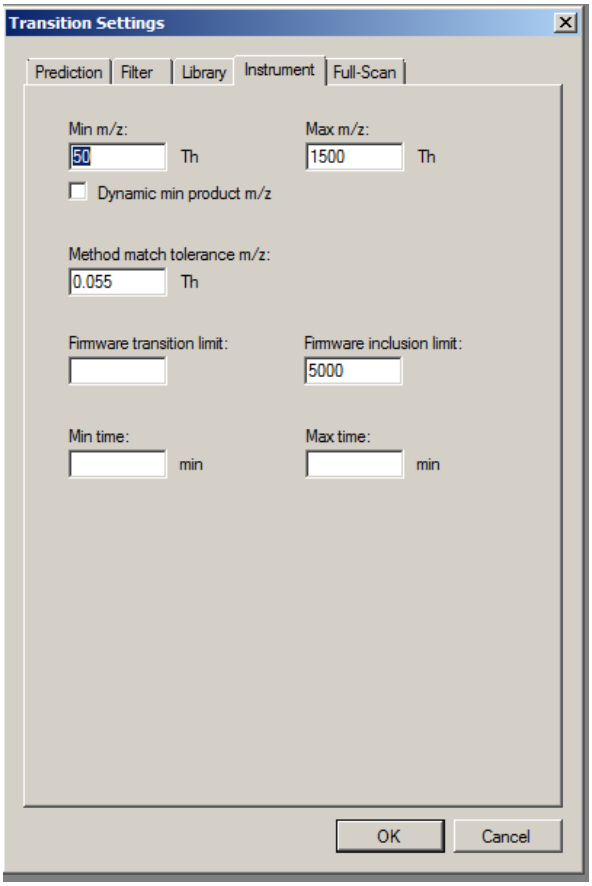
Generating a Multiplexed Method

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

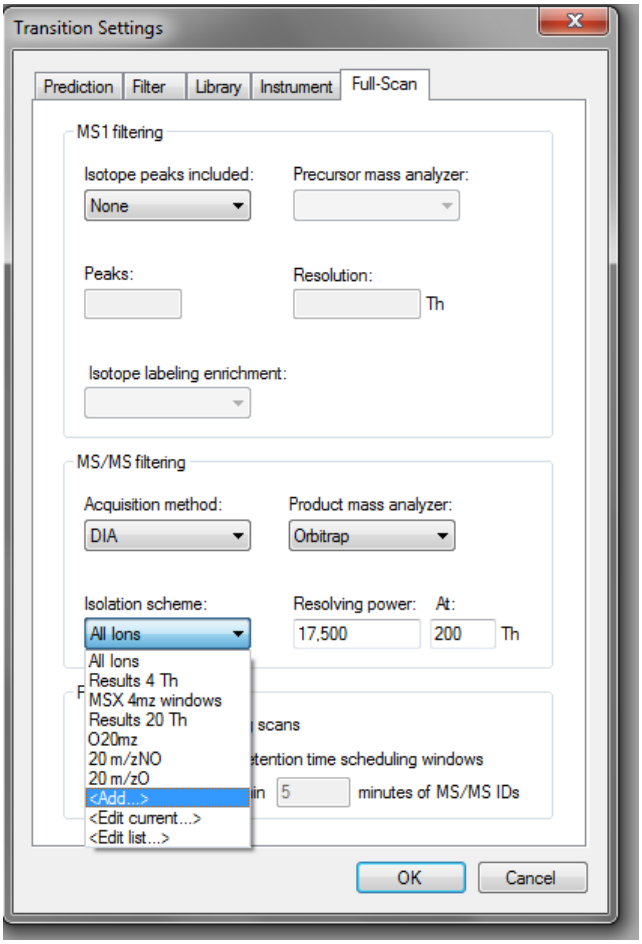
NOTE (10/6/2014) – Importing data takes longer for a multiplexed method. If >100,000 transitions are going to be extracted from the data, the import can take hours per file. One user reported 20 hrs for importing a 220Mb file with ~150,000 transitions. Files with <50,000 transitions should import in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



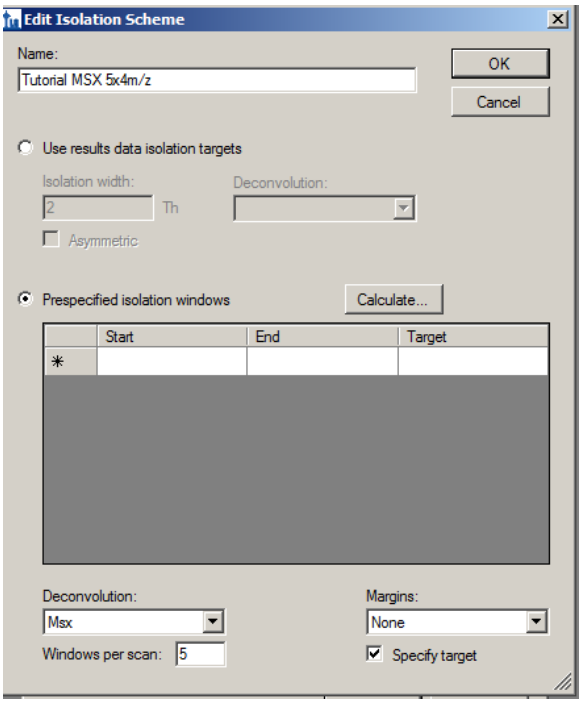
Click on **Settings** and then **Transition Settings…**



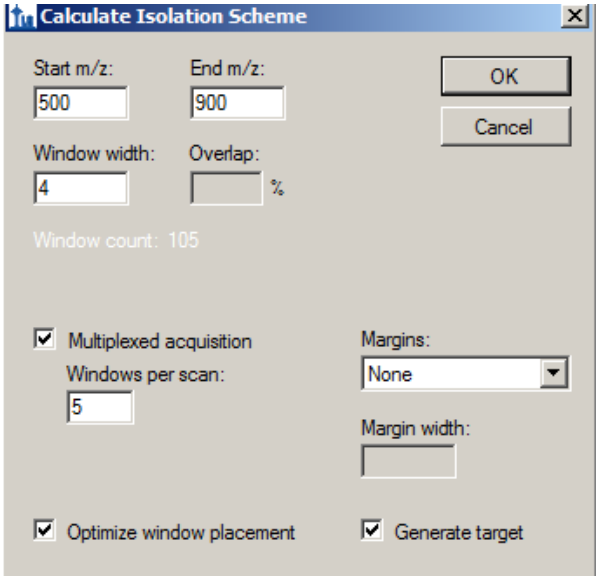
Click on the **Instrument** tab and enter **5000** in the box titled **Firmware inclusion limit**



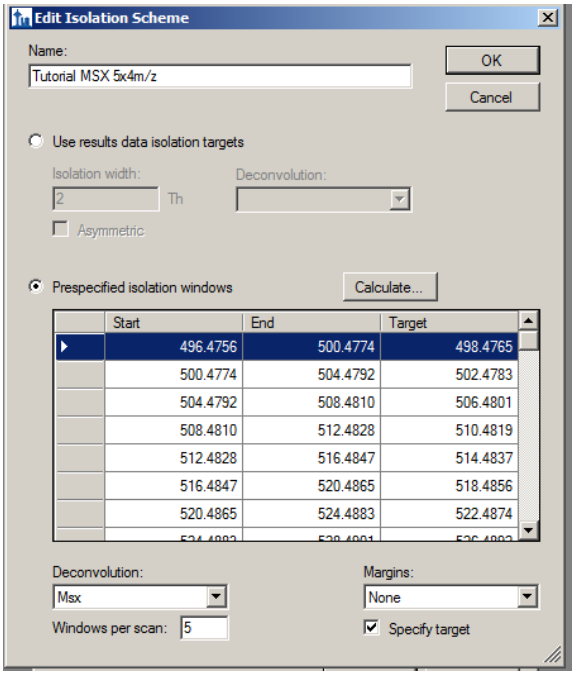
Click on the **Full-Scan** tab, set **Acquisition Method** to **DIA**, **Product mass analyzer** to **Orbitrap**, and under **Isolation scheme** click **<Add…>**



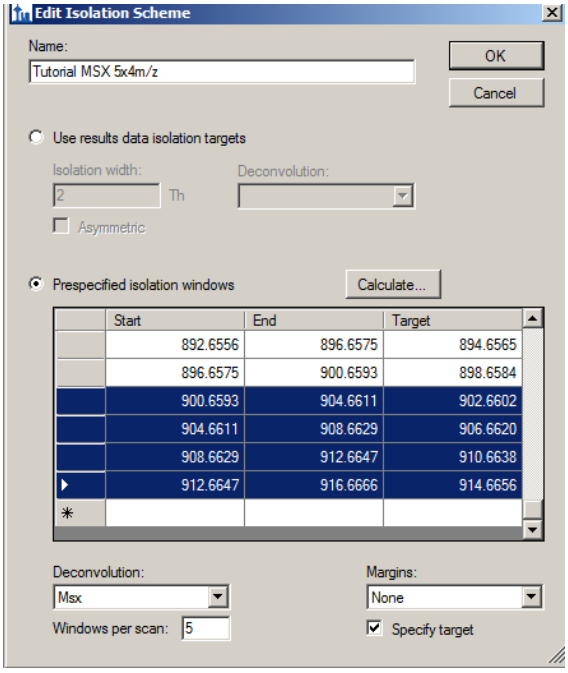
Enter a name for the isolation scheme in the box that says **Name**, select **Prespecified isolation windows**, under **Deconvolution** select **Msx** and in **Windows per scan** enter **5**. Select **Specify target** and click **Calculate…**



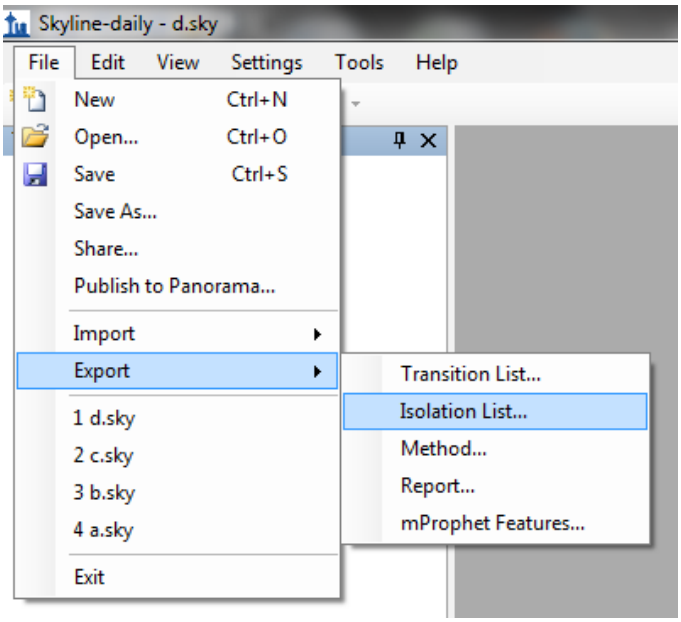
Enter **500** as the **Start m/z** and **900** as the **End m/z**. Window width should be **4.** Selecte **Multiplexed acquisition** with **5 windows per scan**. Select **Optimize window placement** and **Generate target**. Click **OK.**



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**.



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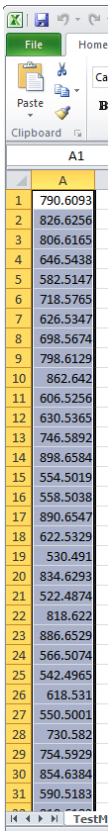
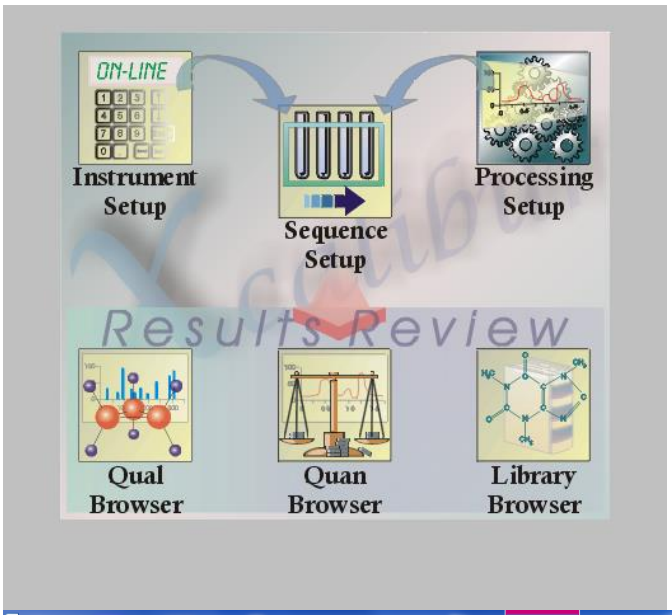


Open the saved .csv isolation list file in Excel (or use OpenOffice or notepad if you do not have Excel) and **highlight the column of numbers**. These numbers are the centers of the isolation windows generated by Skyline. There should be 5,000 windows in total, and they should be in random order as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



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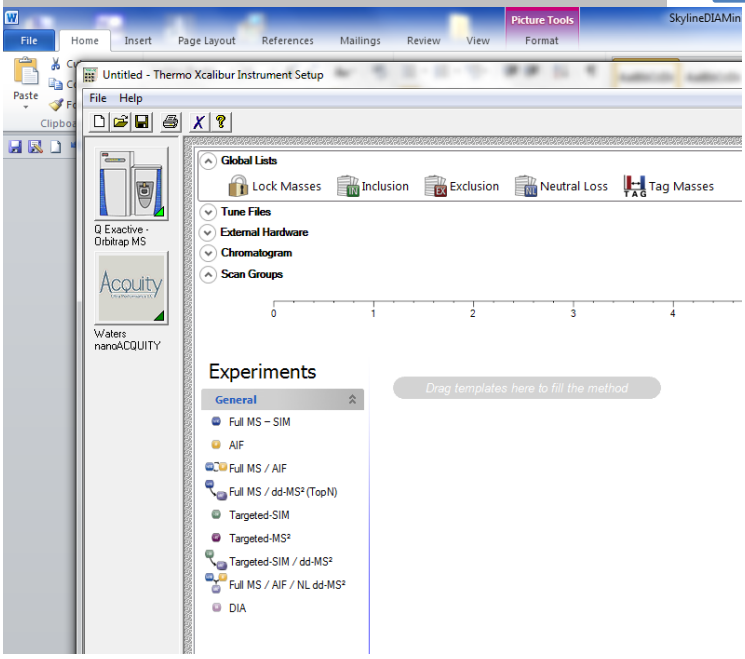

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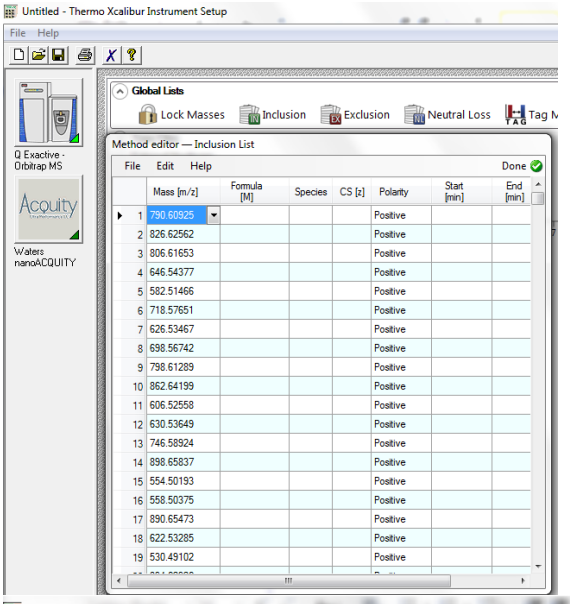

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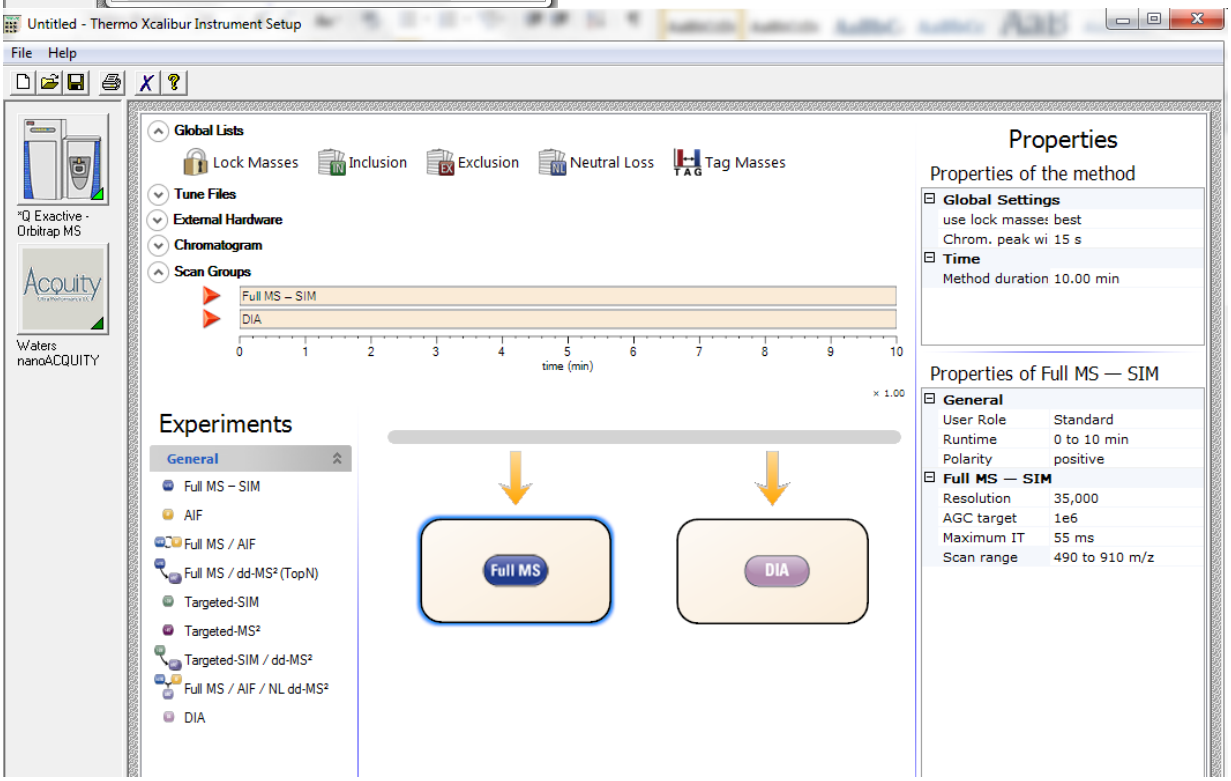

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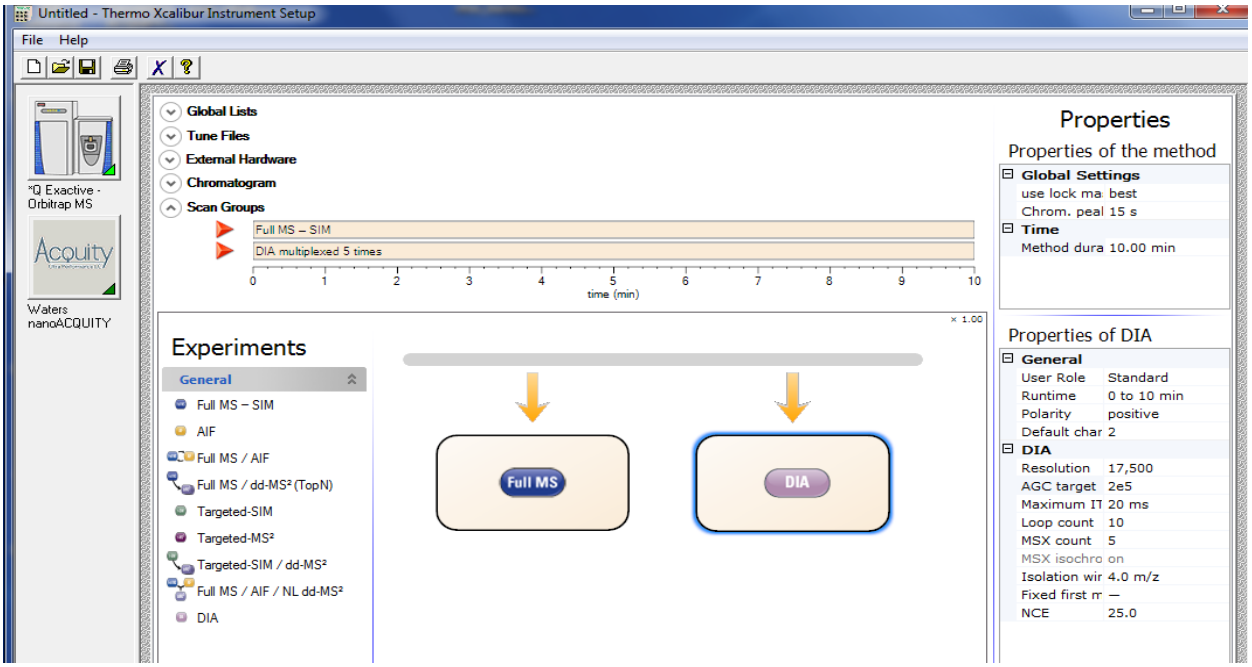

Click on the **DIA** scan. The properties that I use are shown on the right. The **Loop Count** of 10 means that an MS scan will be taken every 10 MS/MS (DIA) scans.

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.