# Designing a DIA Method

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Background

Data independent acquisition (DIA) is a series of compromises between comprehensive detection and selective quantitation where the goal is to detect as many peptides as possible, but in a way that maintains quantitative rigor. With modern mass spectrometers you can only scan at a maximum rate of 10-20 Hz without sacrificing spectrum quality. An additional constraint is that at least 6-8 measurements are required to describe a quantitative peak (**Figure 1**).
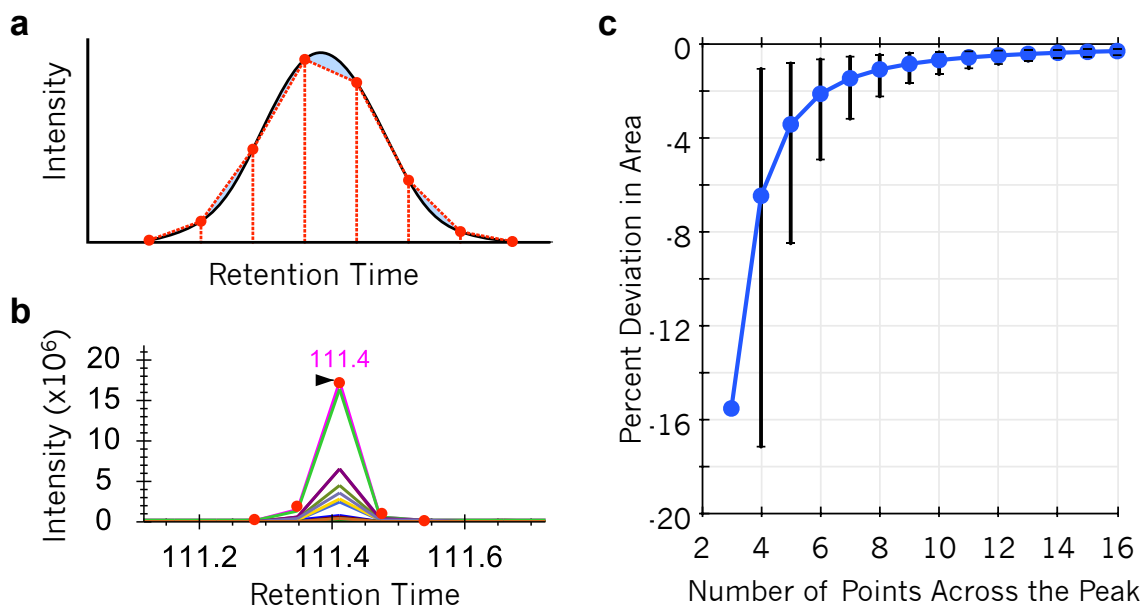
Figure 1: (**a**) Error (shaded in blue) in trapezoidal quantitation (red dashed lines) should cancel when measuring a Gaussian peak (black solid line) with 8 points across the peak. (**b**) However, trapezoidal quantitation can produce poor measurements with only 5 points (or fewer). (**c**) The average percent deviation with 95% error bars in actual/calculated area at different number of points across a Gaussian peak.

DIA methods make several compromises to manage these constraints. In particular, instead of trying to monitor all peptides, the measurement range is typically limited to measure peptides in a restricted precursor window (e.g. 400-1000 m/z). In addition, precursor isolation windows are widened such that multiple peptides are usually isolated together and co-fragmented, resulting in higher interference.

In general we recommend structure windowing methods assuming 10 measurements across an average peak so that regions of the chromatogram with narrower peaks will not be underrepresented. We can calculate the necessary cycle time and windowing scheme from the average peak width and maximum scan rate. If we assume peak widths of 25 seconds and 10 Hz (typical for many quadrupole orbitraps such as the Thermo Fusion, QE, QE+, or QE-HF), we require cycle times of 2.5 seconds, resulting in 25 windows per cycle. This allows for 25x 24 m/z-wide precursor isolation windows to cover 600 m/z in windowing range (400-1000 m/z). However, if we use a faster scanning instrument that can achieve 20 Hz (typical for the Thermo Lumos, QE-HFX, and many TOFs), we can use up to 50 windows per cycle. This allows for 50x 12 m/z-wide precursor isolation windows to cover the same 600 m/z in windowing range.

Setting up a windowing scheme with Skyline

Skyline makes it easy to set up a regularly spaced windowing scheme. After downloading and installing Skyline 4[1] navigate to the "Settings/Transition Settings…" menu option to bring up the Transition Settings dialog (**Figure 2**).
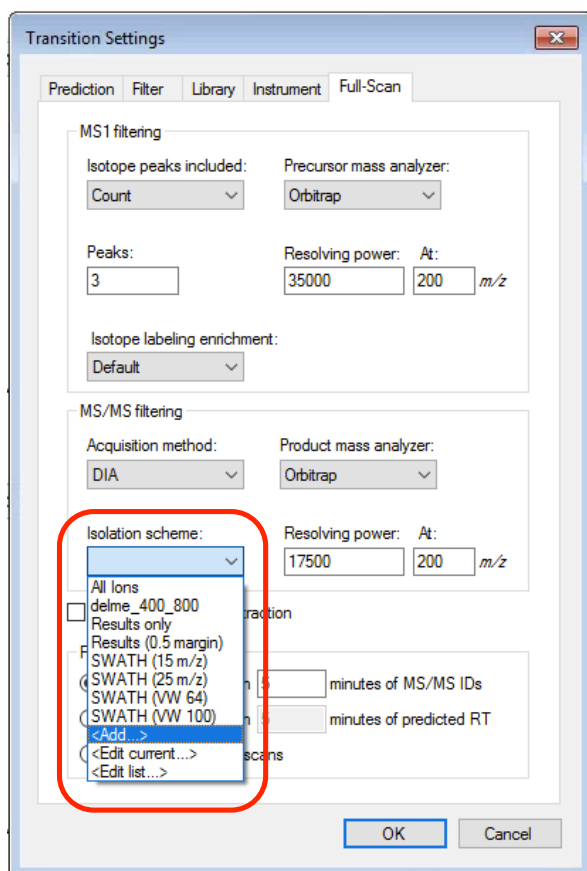
Figure 2: Adding a new isolation scheme with Skyline.

Set the "Acquisition method" to DIA, and "<Add…>" a new "Isolation scheme". Here you can select "Prespecified isolation windows" and "Calculate…" a new windowing scheme (**Figure 3a**). In the Calculate dialog (**Figure 3b**) specify the start and stop m/z range (typically 500-900 m/z or 400-1000 m/z), and the calculated precursor isolation window width (e.g. 24 or 12 m/z). Skyline will automatically calculate the total window count. We recommend using "None" or "Overlap" deconvolution for normal and overlap window placement, respectively. We highly recommend selecting "Optimize window placement" to sit the windows in regions between nominal m/z values where peptides are unlikely to exist. Some older instruments (e.g. Thermo Fusion or QE) have quadrupole geometries that are not optimized for DIA. In certain circumstances these instruments may benefit from small (<0.5 m/z) margins added to the window width. At this time Skyline cannot be used to generate variable width windows.
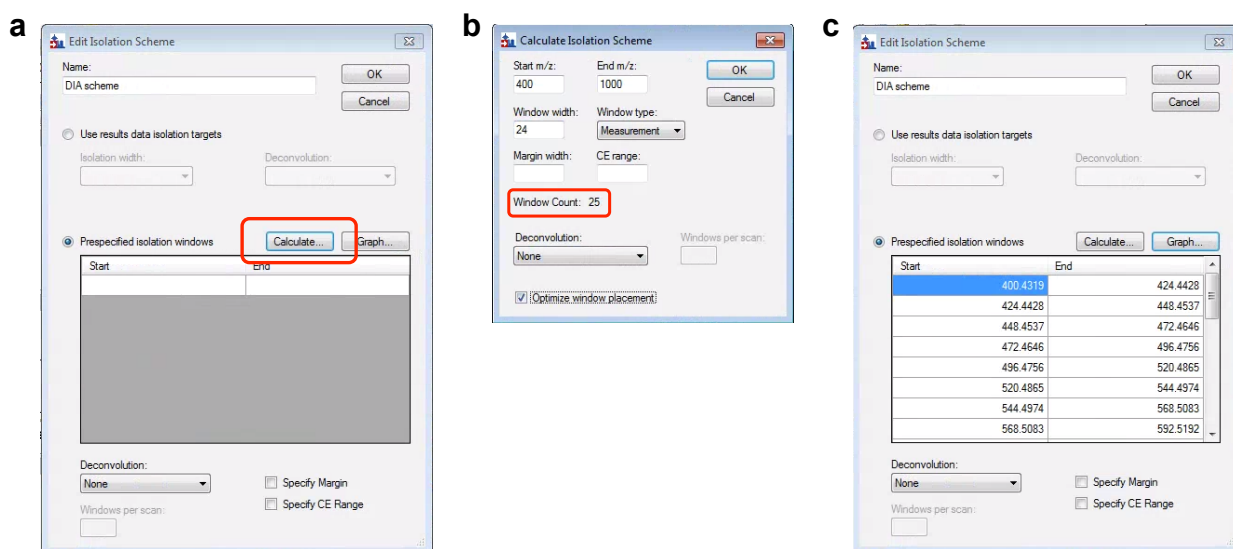
Figure 3: Calculating a new isolation scheme with Skyline. (**a**) The Isolation Scheme dialog. (**b**) The Calculate Isolation Scheme dialog. (**c**) An automatically generated isolation scheme.

The resulting isolation scheme (**Figure 3c**) can be exported or copy and pasted directly into method editors.

Recommended DIA Windowing Schemes

In **Table 1** we list some recommended settings for common Thermo instruments, as well as the Gillet *et al*[2] recommendation for ToF instruments. Please note: any scheme you use for experiments should be tested on your hardware! These recommendations are just starting points and should be treated as such. For convenience, **Table 2** contains three pre-computed windowing schemes from 400-1000 m/z suitable for instruments scanning at approximately 10 Hz: 25x 24 m/z windows

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[2] Gillet LC, Navarro P, Tate S et al. Mol Cell Proteomics. 2012;11:O111.016717.

(**Table 2a**), 25x variable-width windows (**Table 2b, 2c**), and 51x 24 m/z overlapped windows (**Table 2d, 2e**).

**Table 1:** Recommended starting points for DIA parameter settings.

	QE	QE+	QE-HF	QE-HFX	Fusion	Lumos	ToF
Start m/z	400	400	400	400	400	400	400
Stop m/z	1,000	1,000	1,000	1,000	1,000	1,000	1,200
Window Width	24	24	24	12	24	12	variable
Margin Width	0 or 0.2	0	0	0	0 or 0.2	0	0.5
Overlapping	Yes	Yes	Yes	Yes	Yes	Yes	No
Resolution	17,500	17,500	30,000	15,000	30,000	15,000	
Ion Inject Time	60	60	60	25	60	25	

Table 2: Precursor isolation window center m/zs for normal windows (**a**), variable width windows (**b**) and deltas (**c**), and overlapping windows (**d, e**).

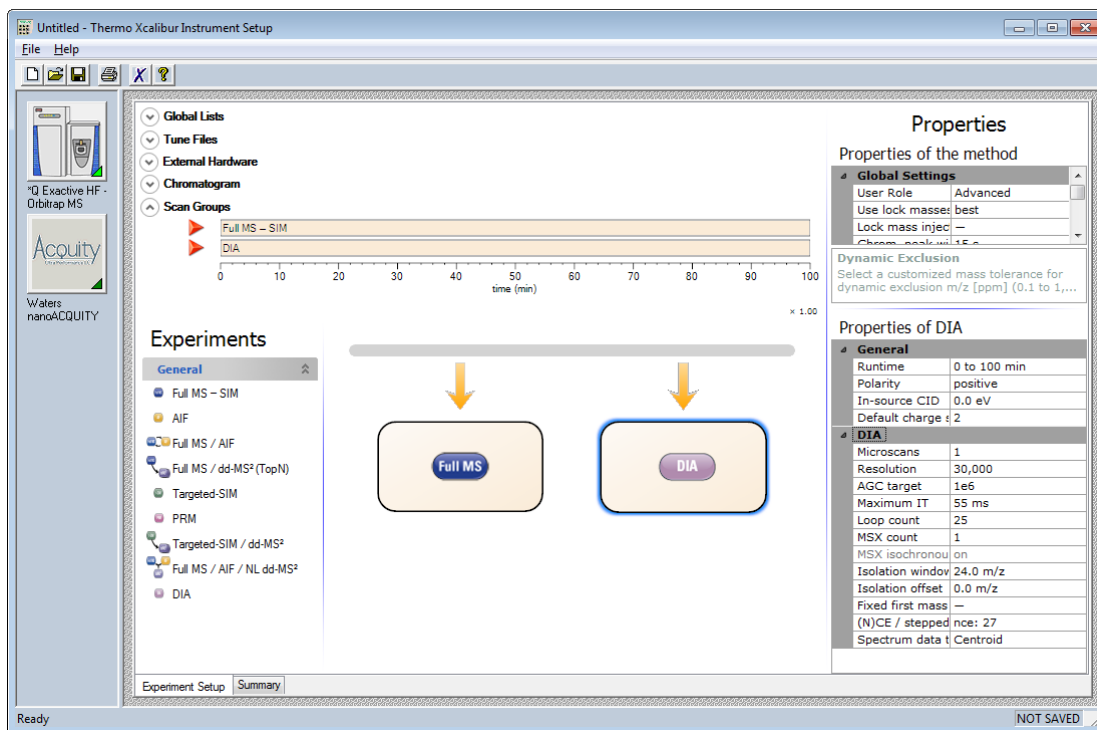| Scan	Normal Windows (a)	Variable Width Windows (b)	Variable Window Deltas (c)	Overlap Windows Cycle 1 (d)	Overlap Windows Cycle 2 (e)
1	412.44	415.70	31.4	400.43	412.44
2	436.45	444.05	25.3	424.44	436.45
3	460.46	467.40	21.4	448.45	460.46
4	484.47	487.75	19.3	472.46	484.47
5	508.48	506.75	18.7	496.48	508.48
6	532.49	524.90	17.6	520.49	532.49
7	556.50	542.20	17.0	544.50	556.50
8	580.51	558.65	15.9	568.51	580.51
9	604.52	574.60	16.0	592.52	604.52
10	628.54	590.30	15.4	616.53	628.54
11	652.55	605.70	15.4	640.54	652.55
12	676.56	621.65	16.5	664.55	676.56
13	700.57	638.15	16.5	688.56	700.57
14	724.58	655.45	18.1	712.57	724.58
15	748.59	673.85	18.7	736.58	748.59
16	772.60	693.10	19.8	760.60	772.60
17	796.61	713.20	20.4	784.61	796.61
18	820.62	734.10	21.4	808.62	820.62
19	844.63	756.65	23.7	832.63	844.63
20	868.64	780.85	24.7	856.64	868.64
21	892.66	807.25	28.1	880.65	892.66
22	916.67	836.70	30.8	904.66	916.67
23	940.68	870.25	36.3	928.67	940.68
24	964.69	911.20	45.6	952.68	964.69
25	988.70	967.00	66.0	976.69	988.70
26				1000.70	

Setting up a DIA instrument method

Here we will use the Thermo QE-HF method editor, but most of these settings are transferable to other platforms. Starting with a new method, we recommend adding a precursor scan followed by DIA MS/MS scans (**Figure 4**). While precursor scans are not required for detection or quantitation, we find they are useful for troubleshooting experiments while acquiring data. We recommend setting the resolution and maximum ion inject time to a lower value than for a typical DDA experiment to limit the time collecting MS scans. Also, we recommend only scanning through the precursor isolation range of the experiment. Here for a 400-1000 m/z experiment we use a precursor scan of 395-1005 m/z. Since these windows are not being used for quant, we recommend collecting MS survey scans in centroid mode to help keep file sizes down.
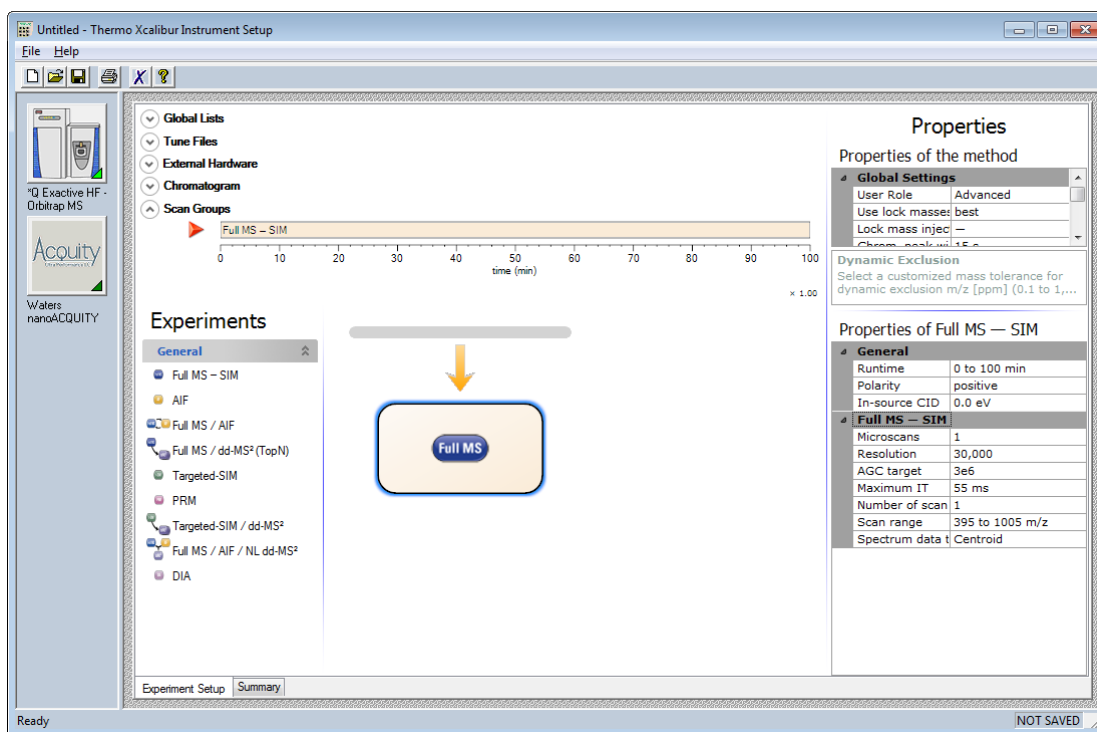
Figure 4: Setting up a QE-HF "Full MS - SIM" scan.

For DIA scans (**Figure 5**), we set the default charge to 2 and a complementary collision energy setting (NCE) to what you typically use for DDA. In general we set automatic gain control (AGC) target intensity to a high enough value such that most MS/MS spectra are acquired to the maximum ion inject time (max IT) duration, ensuring regular scans time differences between cycles throughout the experiment. AGC should not be set so high that ion coalescence is a concern. Again, collecting scans in centroid mode helps keep file sizes down. Full settings for both precursor and fragment scans are shown in **Figure 6**.

Figure 5: Setting up QE-HF "DIA" scans.

Figure 6: Typical QE-HF properties for DIA.

Finally, we add the inclusion list from Skyline (**Figure 7**). When generating overlapping windows, a bug in Skyline causes it to add an unnecessary extra window outside the range. This window should be deleted before saving the method.
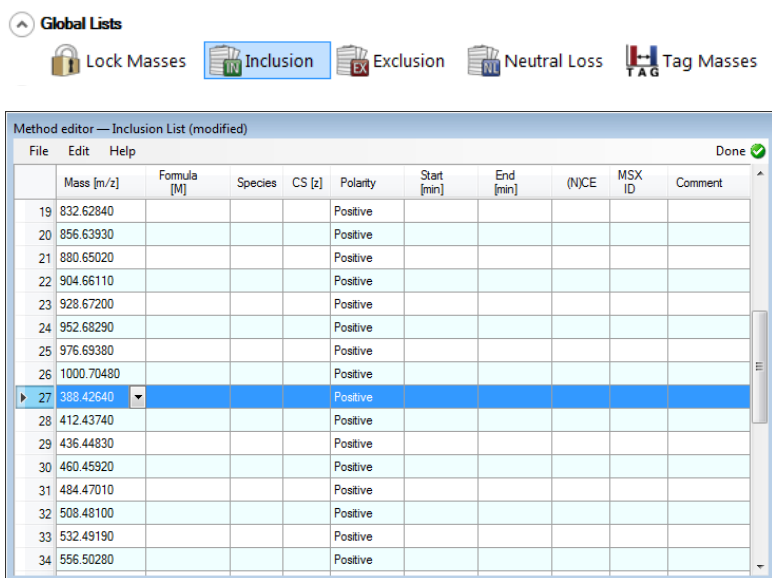
Figure 7: Adding the inclusion list from Skyline. The unnecessary window (center at 388 m/z) added by the Skyline overlapping routine is selected.

Collecting chromatogram libraries

Chromatogram libraries are generated from a small collection of narrow-window DIA experiments, rather than from DDA like typical spectrum libraries. To generate chromatogram libraries, we typically acquire between 4 and 12 gas-phase fractionated runs, in a 4-6x 100 m/z or 8-12x 50 m/z configuration. These fractions are tiled to cover the entire precursor isolation window in our wide-window DIA experiments, for example 400-500 m/z, 500-600 m/z, 600-700 m/z, 700-800 m/z, 800-900 m/z, and 900-1000 m/z. Since the windows do not generally share peptides, retention time consistency between runs is of utmost importance. Consequently, we recommend collecting several conditioning runs of the same sample type on your column before collecting your chromatogram library. A great way to test chromatogram libraries on your instrument is to use the following acquisition order:

1. typical DDA
2. wide-window DIA (400-1000 m/z)
3. typical DDA
4. wide-window DIA (400-1000 m/z)
5. typical DDA
6. wide-window DIA (400-1000 m/z)

7. narrow-window DIA (400-500 m/z)
8. narrow-window DIA (500-600 m/z)
9. narrow-window DIA (600-700 m/z)
10. narrow-window DIA (700-800 m/z)
11. narrow-window DIA (800-900 m/z)
12. narrow-window DIA (900-1000 m/z)