MsXelerator™ High Resolution Differential Analysis:
Finding Differences between Sample and Control
- A Quick Starting Guide -

Introduction: this quick starting guide teaches you to quickly find all major and minor differential peaks between a sample and a control/reference. The procedure can be applied to detect both up- and down-regulated peaks. You must be familiar with the Browser and MPeaks Programs. Optionally, you can use the output from differential analysis to be used for peak detection in a large number of samples using MsCompare Peak Matching.
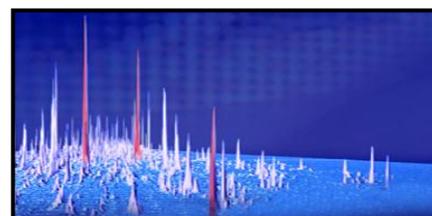
1. **Data Loading/Importing:** start the Browser and Import or open a data file. Remember, the High Resolution Peak Picking algorithm based on the $^{12}C/^{13}C$ Isotopic Pattern uses centroided data. Be sure to convert to centroided data during import if you want to use this algorithm. See High Resolution Peak Picking at the end of this document.

2. **Checking a few Basic Parameters:** using the Browser, you need to determine 3 basic parameters (width of relevant Peaks, accuracy of your instrument and an estimate of the noise level). To Plot Accurate EICs enter the accurate mass value in the m/z box or click any mass peak from the spectrum. Be sure direct accurate plotting is activated (Item under Options). Select or plot a few EIC's of some relevant peaks and measure/estimate the following: the width of the peak in minutes and scans. You can make your scans visible by pressing the **Scans Visible Icon** button. Next use the Peak Width Measurement tool to estimate the width in minutes and scans. Try to measure just above the baseline.
   From the EIC's and the Mass Spectra try to estimate the noise level, e.g. Orbitrap noise level ~ 10.000 counts. Using high resolution EIC plotting decide what mass accuracy is applicable, e.g. for Orbitrap you can typically use +/- 0.005 Da to extract accurate chromatograms.

3. **Run Peak Picking using MPeaks:** from the Browser start MPeaks. Decide on the minimum Peak width in scans, the type of Noise algorithm and the relative size of the peaks that you want to detect. Experiment with the basic parameters. Typically use a peak width smaller than measured in step 2. Next Press the **Run** button. If you are satisfied with the peak list, the nominal masses have to be converted to accurate m/z values in the next step.

4. **Conversion to Accurate Mass List:** from the menu select: Accurate Mass > Accurate Mass Conversion. Use the proper settings for conversion. Depending on the application you may want to keep only the monoisotopic peaks or the largest (deleting the ^{13}C isotopes).
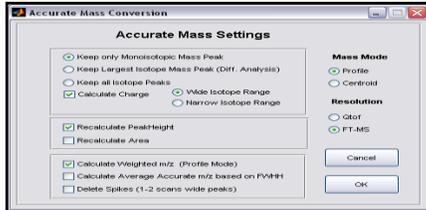
5. **Overlay the Control Sample – Estimate Chromatographic Shift:** Sort the Peak List on Peak Height. Next, from the menu select: Differential Analysis > Overlay Ref/Control. See if peaks are shifted and estimate the shift in minutes (you can use the peak width measurement tool in MPeaks). The sample EIC will be plotted in blue, the control will be plotted as a red trace. To plot the control/reference EIC in mirror mode use: Options > Plot Negative Reference.

Start the Differential Analysis GUI: from the menu select: Differential Analysis > Run. The Diff. Anal. GUI will be shown. **Select the second algorithm (B) and be sure that the option "Check in Accurate Mass Mode" is active.** Define the mass accuracy window (e.g. 0.005 Da.) and Time Window (e.g. 0.25 minutes, depending on the amount of shift observed). As a default we will use a Change Fold Factor of 3. At this moment we want to see results (ratio analysis) for all peaks, so leave the "Delete Reference Peaks" option unchecked.

6. **Run Differential Analysis:** Press the **OK** button and the calculations will start. The algorithm will calculate a ratio (Sample/Control) for all peaks detected in the Sample in accurate EIC mode. The ratios will be returned and added to the result list. Unique Peaks will have very high ratios, up-regulated peaks will have ratios larger than 3. Down-regulated peaks will have ratios smaller than 0.333. Remember that since peak picking was based on the Sample, important down-regulated peaks might have been missed. Therefore, if you want to perform a sensitive Full Down-Regulated Check press **Yes** when this question pops up. A reversed analysis will be performed and unique peaks from the control will be added to the result list (the source of peaks will be marked: Sample or Control).

   Check the final result list for some peaks and decide if the analysis was successful. From the Differential Analysis menu, you can sort the list on ratio or remove peaks that are not up or down-regulated. Peaks that are marked with a star have special meaning. In these cases multiple peaks were detected in the accurate EIC that are closely eluting. Please check these manually.
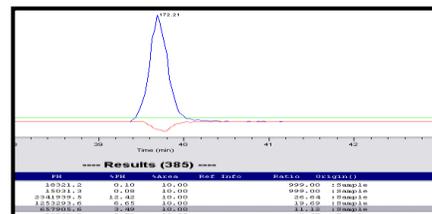
7. **Keeping Differential Peaks Only:** to remove all non-differential peaks from the list, select Menu > Delete Control Peaks. Decide if you want to keep up- **and** down-regulated peaks or only the up-regulated peaks.

8. **Additional Options:** basically the Differential Analysis is done. Please save the results as an MPeaks table using an appropriate comment. You can export the results, edit the list (keep only largest peaks or peaks in certain time window or mass range), start identification, cluster the peaks (which ones are co-eluting), calculate charge states etc. etc.

9. **Run Peak Matching on a Large Number of Samples:** optionally, the current differential peak list can be used to check a large number of samples (Targeted Analysis). This procedure is called Peak Matching. Start MsCompare from the Browser and load or create the Sample List on which to calculate results. From the displayed sample list, click the sample that was used for Peak Picking in MPeaks. *Important:* The differential results should have been saved. From the menu select Peak Matching. Select the result table you want to use. Decide on the allowed shift and mass accuracy. When calculations are done, a table showing results for all samples and all (differential) peaks is shown. You can now use the MsCompare features to find up- and down regulated peaks or apply more advanced statistics. See the MsCompare Manual/Demos.

Alternative: Run Direct High Resolution Peak Picking – Using the IPF II algorithm

1. **High Resolution Peak Picking:** instead of using steps 3 and 4 on the previous page, you may want to use direct high resolution Peak Picking based on the raw MS data. The Peak Picking will be based on the detection of a $^{12}C/^{13}C$ isotope pattern or e.g. a charge 2^+ pattern. From the Menu select: **High Res. Special > High Resolution Peak Picking**. You will need to enter the following information: Time and Mass Range, Isotopic Pattern (select from list), m/z accuracy, typical peak width in scans and minutes, the noise level and the co-elution correlation factor (default 0.8). Uncheck the "Add Second Isotope Peak to Result Table" and the "Mass Weighing" option. Press **Run.** If processing takes a long time, interrupt the algorithm and increase the noise level or use a more specific mass and time window. Check the results.

2. **Continue with step 5 in the Differential Analysis Procedure.**

Full Document References:
1. MsX Differential Analysis two samples 2011.pdf
2. MsX Differential Analysis two Groups.pdf
3. MsX High Resolution Peak Picking using the IPF II algorithm.pdf
4. MsX High Resolution Peak Matching using MsCompare.pdf