# **MRM-DIFF** tutorial

#### Introduction

MRM-DIFF is a data processing tool for multiple reaction monitoring (MRM)-based differential analysis. The main target of this application is *'lipidomics'*. The MRM transition, i.e. precursor-product *m*/*z* pair, can be theoretically determined from *in silico* MS/MS database such as LIPID MAPS and LipidBlast. This program hunts every peaks detected by each MRM transition by means of correlation optimized warping (COW) based non-linear alignment. In addition, pooled QC (quality control) data sets will be helpful for the automatic reference file picking and the peak detection method (see manuscript). The features of MRM-DIFF are:

- 1. Every peaks detected by each transition are utilized
- 2. The identification and quantification results can be manually curated by the graphical user interface (GUI).
- 3. It supports all data processing steps and pooled QC data sets would be helpful its processes. Actually, the compound identifications should be performed by means of three or four

transitions for the determination of lipid class and fatty acid compositions. Such targeted analysis can be performed by our reported program, MRMPROBS (see MRMPROBS section).

MRM-DIFF has been developed as the collaborative work among RIKEN, Osaka University, and Reifycs Incorporation.

Hiroshi Tsugawa RIKEN Center for Sustainable Resource Science <u>hiroshi.tsugawa@riken.jp</u>

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MRM-DIFF screenshot

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# Software environments

- Microsoft Windows XP, -Vista, -7 or -8
- .NET Framework 4.0 or later

### Required software programs and files

- Reifycs Analysis Base File Converter (ABF file converter)
   Download link: <u>http://www.reifycs.com/english/AbfConverter/</u>
- MRM-DIFF
   Download link: <u>http://prime.psc.riken.jp/Metabolomics\_Software/MRM-DIFF/index.html</u>
- Reference library (tab-delimited text file)
   Example: <u>http://prime.psc.riken.jp/Metabolomics\_Software/MRM-DIFF/index.html</u>
- Demonstration files
   Download link: <u>http://prime.psc.riken.jp/Metabolomics\_Software/MRM-DIFF/index.html</u>

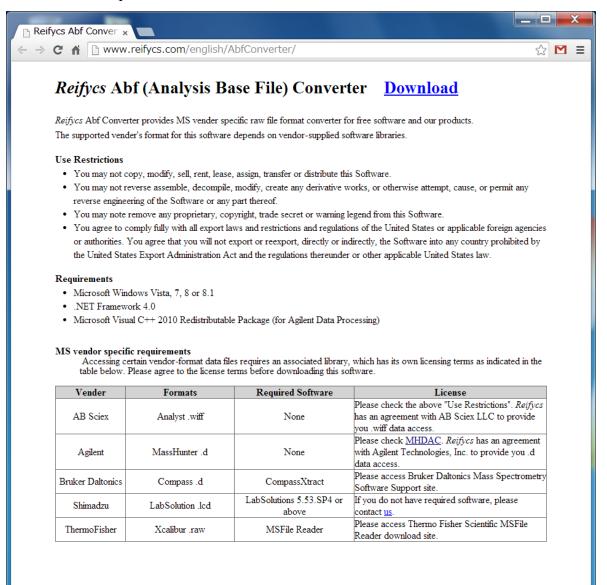
MRM-DIFF can import Analysis Base File (ABF) format data. This program extracts chromatogram data together with the reference library including the name of the target metabolite, its retention-time and amplitude information, and precursor m/z and product m/z. The supported formats for ABF conversion are Shimadzu Inc. (.LCD), Agilent Technologies (.D), AB Sciex (.WIFF), and Thermo Fisher Scientific (.RAW).

MRM-DIFF is also acceptable to mzML format file converted by an open source file translator ProteoWizard. Although our abf converter doesn't accept Waters (.RAW) file due to the license problem yet, MRM-DIFF can import Waters files via mzML.

MRM-DIFF program is implemented as a part of MRMPROBS software. Therefore, please select 'MRM-DIFF project' in the new project window.

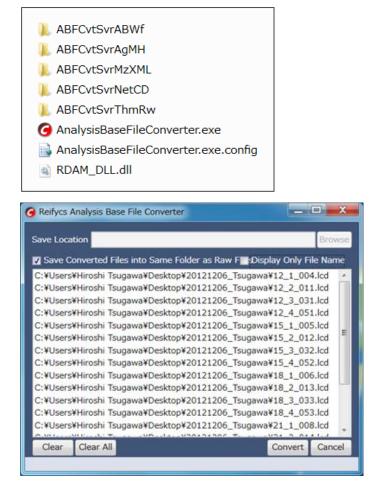
### Downloading the ABF converter from Reifycs Inc.

- 1. Go to http://www.reifycs.com/english/AbfConverter/.
- 2. Check the requirements and license terms, and download the converter.



### File conversion

- 1. Start "AnalysisBaseFileConverter.exe".
- 2. Drag & drop MS vendor files into this program.
- 3. Click "Convert".
- 4. The ABF files are generated in the same directory as the raw data files.



# Reference library for MRMPORBS program (tab-delimited text format)

Four items are required in the library file in tab-delimited format. The first header's name is flexible but the item order should be followed.

20140919_MRMDIFF	_ReferenceLibrary_	40mLC_SerumProje	ect_vs2.txt - Notepad	_ □ >	×
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1 column. Compound name

2 column. Precursor m/z (accurate m/z information is rounded into nominal m/z information)

3 column. Product m/z

4 column. Retention time [min]

*Notes 1:* The compound name should be entered in English one-byte characters.

*Note 2:* Sometimes the tab-delimited file exported from Microsoft Excel includes unexpected hidden trailing columns. These unexpected columns after the 'Retention time' column cannot be handled by MRM-DIFF. You can inspect the exported file by selecting a few rows (see below). If there are selected characters after the last column (Retention time), edit the file in Excel to delete these columns and re-export it again.

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/soPC 15:1 (sn1)		84.1 7.23	
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soPC 20:0 (sn1)	552.4	84.1 16.67	
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			>

Good example (no unexpected column)

Bad example (there are unexpected columns)

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### Starting MRM-DIFF

Note that again, MRM-DIFF is run as a part of MRMPROBS program. Therefore, the assembly name, i.e. EXE file name, is 'MRMPROBS.exe'.

- 1. Starting up your project
- 2. Importing Abf files
- 3. Setting parameters
- 4. Running the software (1-2 min / sample)

\*The tutorial uses 37 demonstration files and the lipid reference library which are downloadable from the above link. The common measurement conditions of the demonstration files were as follows.

Liquid chromatography: total 45 min run per sample with InertSustain C18:  $2.1 \times 150$  mm, 3 µm (GL sciences Co.).

Mass spectrometer: MRM method with positive and negative ion mode. Target metabolite number: 284 Total transitions: 284

The detail of experimental conditions is downloadable at the MRM Database section (ODS-lipids).

http://prime.psc.riken.jp/Metabolomics Software/MrmDatabase/index.html

# Starting up your project

- 1. File  $\rightarrow$  New project.
- 2. Chose a project type (select the bottom one for this demonstration).

	Project option 🛛 🗖 🗖	×
Project	t type	
0	) MRMPROBS: key index:: metabolite name (abf)	
0	) MRMPROBS: key index:: MRM transition (abf, mzML)	
0	) MRMPROBS: key index:: Function (mzML)	
۲	MRMDIFF (abf, mzML)	
	Next Cance	el

### **Importing Abf files**

		MRMPROBS: new project wind	wob				
oject folder path	Select						
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nalysis file paths	Select						
	File path	File name	Туре	Class ID	Analytica	Included	
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F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_02_Serum Human_02	Sample	Human serum	3	~	1
F:\140507_MRME	OIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_02_Serum Human_03	Sample	Human serum	4	~	1
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F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_03_Serum mix	QC	Pooled QC	7	>	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_04_Serum Human_06	Sample	Human serum	8	1	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_04_Serum Human_07	Sample	Human serum	9	1	1
F:\140507_MRME	OIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_04_Serum Human_08	Sample	Human serum	10	~	1
F:\140507_MRME	OIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_04_Serum Human_09	Sample	Human serum	11	>	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_04_Serum Human_10	Sample	Human serum	12	>	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_05_Serum mix	QC 🗸	Pooled QC	13	1	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_06_Serum Mouse_01	Sample	Mouse serum	14	~	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_06_Serum Mouse_02	Standard	Mouse serum	15	~	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_06_Serum Mouse_03	QC	Mouse serum	16	1	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_06_Serum Mouse_04	Sample	Mouse serum	17	~	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_06_Serum Mouse_05	Sample	Mouse serum	18	>	1
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_07_Serum mix	QC	Pooled QC	19	~	1
F:\140507_MRME	)IFFER_LipidsData\131122_	131122_LCMS_Lipidomics_08_Serum Mouse_06	Sample	Mouse serum	20	~	1
F:\140507_MRME	)IFFER_LipidsData\131122_	131122_LCMS_Lipidomics_08_Serum Mouse_07	Sample	Mouse serum	21	~	]
F:\140507_MRME	DIFFER_LipidsData\131122_	131122_LCMS_Lipidomics_08_Serum Mouse_08	Sample	Mouse serum	22	>	]
F:\140507 MRME	DIFFER LipidsData\131122	131122 LCMS Lipidomics 08 Serum Mouse 09	Sample	Mouse serum	23	✓	~
					Next	Cance	ł

#### Note:

- We recommend that the project folder be made for each batch experiment. In the MRM-DIFF project, three folders (raw, processed, aligned) and one file (\*.mth) are generated. They should be included in the same directory.
- The file name should be entered in half-width alphanumeric symbols.
- Select the file type of each file from "Sample", "Standard", and QC". QCs must be
  required for MRM-DILL program to perform chromatogram alignment- and peak
  detection methods as well as LOESS-based normalization method. If you don't have
  pooled QC data sets, use the wild type (control) sample data sets as QCs. What matters
  is to select the (biological or technical) replicate data sets. In such case, the LOESSCubic spline normalization will not work well, but the others such as chromatogram
  alignments and peak detections will be fine.
- Decide the class ID used for the color labels.
- The analytical order and class ID can be changed after data processing.

### **Setting parameters**

R R	eference option	– 🗆 🗙
Analysis parameters Advance: MR	MDIFF	
Library: F:\140507_MRMDIFFE	R_LipidsData\20140919_MRMDIFF_I	Refere Select
Peak detection prameters		
Smoothing method:	Linear weighted moving aver $\ arphi$	
Smoothing level:	2	scan
Minimum peak width:	5	scan
Minimum peak height:	100	amplitude
Peak detection prameters		
Retention time tolerance:	0.1	min
Amplitude tolerance:	15	%
Minimum posterior:	70	%
		1
Create new library		
Sample file for library edit:	v	
	Finis	h Cancel
	rinis	Cancer
-	-f	- 🗆 X
Analysis parameters Advance: MF	Reference option	
Advanced mode		
Peak alignment parameter		_
Column type:	ODS ~	
Segment size:	0.5	
Min slack parameter:		scan
Max slack parameter:	1	scan
Border limit:	Constant ~	
	Finis	h Cancel

Select '20140919\_MRMDIFF\_ReferenceLibrary\_40mLC\_SerumProject.txt' and set the above parameters for this demonstration.

# [Recommended]

Peak detection Smoothing method: linear weighted moving average. Smoothing level: 1-2 Minimum peak width: 3-5 Minimum peak height: 50-100

Peak identification

Retention time tolerance: As long as the reverse phase or hydrophilic interaction chromatography LC are used, 0.1-0.2 min is recommended. Amplitude tolerance: non-meaningful Minimum posterior: 50-70.

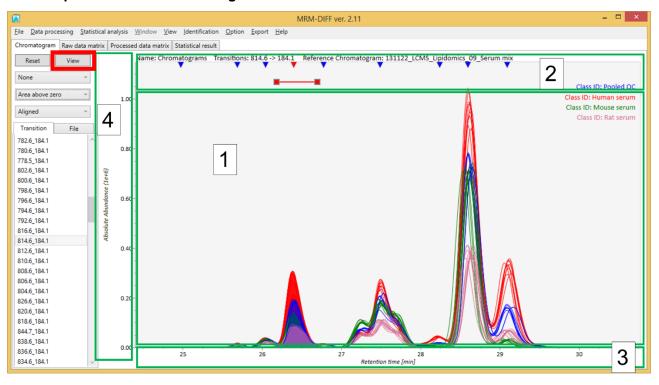
#### Advance: MRMDIFF

Column type: select a type used in your experiment.

Segment size: add the average peak width in your data sets.

Others: Min slack, max slack, and border limit are automatically determined from the column type. These settings are derived from our pre-experiment. For the classical correlation optimized warping (COW), select 'Constant' for the border limit and in such case, max slack parameter is not meaningful.

### **MRM-DIFF** viewer



Mouse operation in the chromatogram viewer

#### View mode

- Chromatogram window: drag holding left click → chromatogram scroll, drag holding right click
   → chromatogram zoom.
- ②. Detected window: left double-click the reverse triangle  $\rightarrow$  change the focused peak
- ③. Retention time window: drag holding right click  $\rightarrow$  warping on retention time range.
- (4). Intensity window: drag holding right click  $\rightarrow$  warping on intensity range.

#### Edit mode

- Right click and drag on un-detected peak area → detect new peak. Right click and drag on detected peak area → delete detected peaks.
- ②. Left click and drag on the peak edge [red square]  $\rightarrow$  change the location of the peak edge.

### **Tool button**

File Data processing Statistical analysis Window View Identification Option Export Help

- File: start new project, open existing project, save as a project, and save the project.
- Data processing: for data re-processing per file, per metabolite, or in all data sets.
- Statistical analysis: data normalization and statistical analysis.
- Window: non-meaningful in MRM-DIFF project.
- View: change focused chromatograms.
- Identification: Manual curation for identification results.
- Option: re-define class ID and analytical order, choose the internal standard, decide "include" or "exclude" data for statistical analysis.
- Export: The result is exported in tab-delimited text format.
- Help: show version information.

# Tab

Chromatogram Raw data matrix Processed data matrix Statistical result

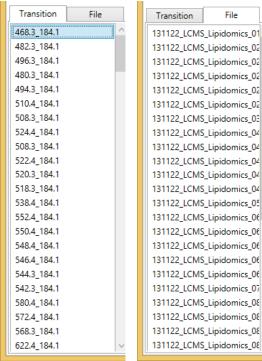
- Chromatogram: All data manipulation tasks are performed here.
- Raw data matrix: Not used in MRM-DIFF project.
- Processed data matrix: Not used in MRM-DIFF project.
- Statistical result: The result of statistical analysis is shown here.

### Button

Reset	View
None	v
Height	v
Aligned	Ŷ

- Reset: Reset the display range of chromatograms.
- View: If you push this "View" button, the chromatogram viewer is changed to "Edit" mode. In the "Edit" mode you can modify the peak edge and detect new peaks manually
- None: The properties of detected peaks are shown in this ComboBox. You can confirm isotopic ions and identified lipids.
- Height: You can set the quantification mode. The default is set by peak height. Instead, you can change it to area mode. By using the "All" option, the quantification mode is reflected = implemented in all files and all metabolites.
- Aligned: You can see raw chromatograms as well as just smoothed chromatograms.

# List Box



If you double-click a transition name, the chromatograms are generated in the chromatogram viewer. If you double-click a file name, the reference chromatogram used for alignments and peak detections is highlighted.

# Details on the MRMPROBS function

# File menu

- New project: used for creating a new project.
- Open project: used for opening an existing project. Make sure that \*.mth file, raw folder, and processed folder are included in the same directory.
- Save as: use to save as a new file.
- Save: use to overwrite an existing project.

## Data processing menu

Data re-processing can be done by newly optimized parameters in this option. Reprocessing is also performed per transition. Also, in MRM-DIFF program, you can re-set the compound library and the identifications can be only done by checking '*Re-identification processing only*'.

🔀 Data re	e-processing window	- 🗆 🗙						
Analysis parameters Advance: MRI	MDIFF							
Peak detection parameters								
Smoothing method:	Linear weighted moving aver $\   \sim$							
Smoothing level:	2	scan			Data re-	processing window		×
Minimum peak width:	5	scan	Analysis pa	rameters Ad	ivance: MRM	DIFF		
Minimum peak height:	100	amplitude	Advan	ced mode				
Compound identification parameters			Peak align	ment parame	ter			
Retention time tolerance:	0.1	min	Reference	e file:		Automatic	Ŷ	
Amplitude tolerance:	15	%	Segment	Segment size:			0.5	min
Minimum posterior:	70	%	Min slac	Min slack parameter:			1	scan
Re-analysis targets			Max slac	k parameter:			1	scan
Target transition:	258.9 -> 79.1	~	Border li	imit:		Constant	Ŷ	
Data re-processed metabolite:	G1P	v	Re-identifu	cations				
Data re-processed file:	All	~	File path:	F:\140507_N	IRMDIFFER_L	ipidsData\20140919_MRM	DIFF_R	Select
			🗌 Re-ide	entification pr	rocessing onl	у		
	Finish	n Cancel					Finish	Cancel

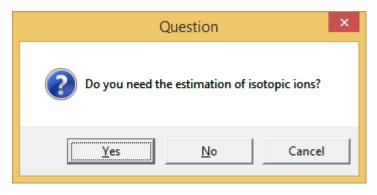
### Statistical analysis menu

#### Normalization setting

At first, you can set properties of aligned peaks and files. In the file properties (left), you can reset file type, class ID, or analytical order. If you clear the check box of the "Included" column, the corresponding data are no longer used in the statistical analysis. In the alignment properties (right), you can set internal standard information for each aligned peak. Please make sure to assign "Annotated peak name" in the "internal standard" column.

File name	File type	Class ID	Analytical order	Included		Annotated peak	Internal standard
31122_LCMS_Lipidomics_	QC	Pooled QC	1	<ul> <li>Image: A start of the start of</li></ul>	$\sim$	468.3_184.1_6.34	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	2	<ul> <li>Image: A start of the start of</li></ul>		468.3_184.1_7.03	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	3	<ul> <li>Image: A start of the start of</li></ul>		468.3_184.1_10.51	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	4	<ul> <li>Image: A start of the start of</li></ul>		482.3_184.1_7.88	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	5	Image: A start of the start		482.3_184.1_8.65	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	6	<b>\</b>		482.3_184.1_12.2	622.4_184.1_17.7
131122_LCMS_Lipidomics_	QC	Pooled QC	7	Image: A start of the start		496.3_184.1_8	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	8	<ul> <li>Image: A start of the start of</li></ul>		496.3_184.1_8.47	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	9	<ul> <li>Image: A start of the start of</li></ul>		496.3_184.1_9.17	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	10	Image: A start of the start		496.3_184.1_9.58	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	11	<b>\</b>		496.3_184.1_10.4	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Human serum	12	Image: A start of the start		480.3_184.1_5.82	622.4_184.1_17.7
131122_LCMS_Lipidomics_(	QC	Pooled QC	13	<b>v</b>		480.3_184.1_6.49	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	14	<b>v</b>		480.3_184.1_7.23	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	15	Image: A start of the start		480.3_184.1_11.86	622.4_184.1_17.7
131122_LCMS_Lipidomics_	Sample	Mouse serum	16	<ul> <li>Image: A start of the start of</li></ul>		480.3_184.1_15.05	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	17	<b>v</b>		494.3_184.1_7.29	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	18	Image: A start of the start		494.3_184.1_7.66	622.4_184.1_17.7
131122_LCMS_Lipidomics_(	QC	Pooled QC	19	<b>&gt;</b>		494.3_184.1_8	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	20	<b>\</b>		494.3_184.1_8.67	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	21	Image: A start of the start		510.4_184.1_10.83	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	22	<ul> <li>Image: A start of the start of</li></ul>		510.4_184.1_11.29	622.4_184.1_17.7
131122_LCMS_Lipidomics_S	Sample	Mouse serum	23	✓	$\sim$	510.4_184.1_11.64	622.4_184.1_17.7

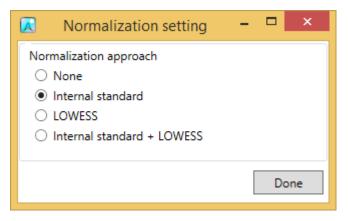
Then, in this demonstration, please choose 'Yes' from the below message.



Select '20140919\_MRMDIFF\_FormulaLibrary\_40mLC\_SerumProject.txt' as shown in below. The compound names, which should be the same as the identified name in the MRM-DIFF program, and formulas are utilized to estimate the peak abundance from isotopic ion. Moreover, the MRM-DIFF program can also estimate the isotopic abundances from unknown peaks as 'alkane'. Checking '*Also consider unknown peaks*' is to consider it.

Path:       C:\Users\tensa_000\Documents\\MRM-DIFF paper\20140922_References\2014         Also consider unknown peaks:       Image: Construction of the second of th	Molecular formula setting for isotopic peaks estin	nation –	
Image: Control of the second	Path: C:\Users\tensa_000\Documents\MRM-DIFF paper\20140922	_References\201	4 Select
20140919_MRMDIFF_FormulaLibrary_40mLC_SerumProject.bt         Image         Control           UsePC         14:0         (sr2)         (22H46N07P           UsePC         14:0         (sr2)         (22H46N07P           UsePC         15:0         (sr2)         (22H46N07P           UsePC         15:1         (sr1)         (22H46N07P           UsePC         15:1         (sr1)         (22H46N07P           UsePC         16:10         (sr2)         (22H46N07P           UsePC         16:11         (sr2)         (sr2)         (sr2)           UsePC         16:11         (sr2)	Also consider unknown peaks: 🗹		
Lot Dot 3 2, interface         Control 10, interface         Control 10, interface           Name         Formula         C22446N07P         Interface           VisePC 14:0 (an)         C22446N07P         Interface         Interface           VisePC 14:0 (an)         C23448N07P         Interface         Interface           VisePC 15:0 (an)         C23448N07P         Interface         Interface           VisePC 16:0 (an)         C23448N07P         Interface         Interface           VisePC 16:0 (an)         C23448N07P         Interface         Interface           VisePC 16:0 (an)         C2445N07P         Interface         Interface           VisePC 16:1 (an)         C2445N07P         Interface         Interface           VisePC 17:1 (an)         C255E0N07P         Interface         Interface           VisePC 17:1 (an)         C255E0N07P         Interface         Interface           VisePC 18:1 (an)         C255E0N07P         Interface         Interface           VisePC 18:1 (an)         C255E0N07P         Interface         Interface           VisePC 18:1 (an)         C265E0N07P         Interface         Interface           VisePC 18:1 (an)         C265E0N07P         Interface         Interface           VisePC 18:1		Finish	Cancel
Name         Formula           1xsePC 14:0 (ar2)         C22H46N07P           1xsePC 15:0 (ar2)         C22H48N07P           1xsePC 15:0 (ar2)         C22H48N07P           1xsePC 15:0 (ar2)         C22H48N07P           1xsePC 15:0 (ar1)         C2H48N07P           1xsePC 15:0 (ar1)         C2H52N08P           1xsePC 15:0 (ar1)         C2H52N07P           1xsePC 15:0 (ar1)         C2F52N07P           1xsePC 15:0 (ar1)         C2F58N07P           1xsePC 15:0 (ar1)         C2F58N07P           1xsePC 15:0 (ar1)         C2F58N07P           1xsePC 15	🖸 20140919_MRMDIFF_FormulaLibrary_40mLC_SerumProject.txt 😑 🗖 💌		
IysePC 14:0 (srC)         C22H46N07P           IysePC 15:0 (srC)         C32H48N07P           IysePC 15:0 (srC)         C33H48N07P           IysePC 15:0 (srC)         C33H48N07P           IysePC 16:0 (srC)         C3H48N07P           IysePC 16:0 (srC)         C3H48N07P           IysePC 16:0 (srC)         C3H48N07P           IysePC 16:0 (srC)         C2H50N07P           IysePC 16:1 (srC)         C2H48N07P           IysePC 16:1 (srC)         C2H48N07P           IysePC 16:1 (srC)         C2H48N07P           IysePC 16:1 (srC)         C2H52N07P           IysePC 17:1 (srC)         C2F52N07P           IysePC 18:1 (srC)         C2F62N07P           IysePC 18:1 (srC)         C2F63N07P           IysePC 18:1 (srC)         C2F63N07P           IysePC 18:1 (srC)         C2F65N07P           IysePC 18:1 (srC)         C2F65N07P           IysePC 18:1 (srC)         C2F65N07P           IysePC 18:1 (srC)         C2F65N07P           IysePC 20:1 (srC)         C2F65N07P			
PC 31:1; PC(15:0/16:1) C39H70H08P PC 31:1p; PC(16:0p/16:0) C39H76H07P PC 32:0; PC(16:0/16:0) C40H80N08P PC 32:1; PC(14:0/18:1 or 16:0/16:1) C40H78N08P PC 32:2; PC(14:0/18:2 or 16:1/16:1) C40H76N08P	lysoPC 14:0 (sn1)         C22H46N07P           lysoPC 15:0 (sn2)         C23H48N07P           lysoPC 15:0 (sn1)         C23H48N07P           lysoPC 16:0 (sn2)         C23H48N07P           lysoPC 16:0 (sn2)         C23H48N07P           lysoPC 16:0 (sn2)         C24H50N07P           lysoPC 16:0 (sn2)         C24H50N07P           lysoPC 16:1 (sn1)         C24H48N07P           lysoPC 16:1 (sn2)         C24H48N07P           lysoPC 16:1 (sn1)         C24H48N07P           lysoPC 16:1 (sn2)         C24H48N07P           lysoPC 16:1 (sn2)         C24H48N07P           lysoPC 16:1 (sn2)         C24H48N07P           lysoPC 17:0 (sn1)         C25H52N07P           lysoPC 17:0 (sn1)         C25H50N07P           lysoPC 18:0 (sn2)         C26H54N07P           lysoPC 18:0 (sn2)         C26H54N07P           lysoPC 18:1 (sn2)         C26H52N07P           lysoPC 18:1 (sn1)         C26H50N07P           lysoPC 18:2 (sn2)         C26H50N07P           lysoPC 18:3 (sn2)         C26H50N07P           lysoPC 18:3 (sn2)         C26H50N07P           lysoPC 18:3 (sn2)         C28H50N07P           lysoPC 18:3 (sn2)         C28H50N07P           lysoPC 20:0 (sn2)         C28H50N07P		
FC 32:2; FC(14:0/16:2 OF 16:1/16:1) C40H76N06F	PC 31:1; PC(15:0/16:1) C39H/6N08P PC 31:1p: PC(16:0p/16:0) C39H/6N07P		
X	FG 32:2; FG(14:0/16:2 OF 10:1/16:1) G40H/0N06F		

Finally, select a normalization approach.



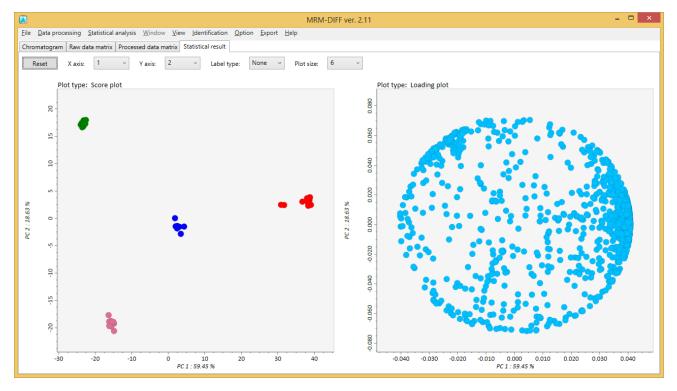
### Note:

- None: after implementation of the missing value approach, the values of the raw data matrix are stored in the processed data matrix.
- Internal standard: after implementation of the missing value approach, the value divided by the internal standard value set in the "Option menu" is stored in the processed data matrix.
- LOESS: after implementation of the missing value approach, the signal intensities of each metabolite are normalized with the QC samples information by means of loess/cubic spline.
- Internal standard + LOESS: After internal standard normalization, loess/cubic spline based normalization is performed.

# Statistical analysis setting

	Statistical analysis setting 😑 🗖 🗙					
Principal o	component analysis					
Maxir	mum principal component:	5				
Scale	method:	Auto scale 🗸 🗸				
Trans	form method:	None v				
		Finish				

You can do principal component analysis. Add the calculated number of the principal components and choose the scale and transform method.



# **Identification menu**

You can manually correct identification result. This option may be useful to check internal standards which are not included in the reference library.

Identified metabolite property setting         -         ×					
Precursor m/z	Product m/z	Retention time [min]	Identified metabolite		
572.4	184.1	11.92	lysoPC 22:4 (sn1)		
568.3	184.1	8.69	lysoPC 22:6 (sn2)		
568.3	184.1	9.33	lysoPC 22:6 (sn1)		
568.3	184.1	12.91	Not annotated		
568.3	184.1	13.7	Not annotated		
622.4	184.1	11.72	Not annotated		
622.4	184.1	14	Not annotated		
622.4	184.1	17.7	); PC(12:0/12:0) (Internal standard)		
622.4	184.1	21.8	Not annotated		
622.4	184.1	22.34	Not annotated		
706.5	184.1	9.61	Not annotated		
706.5	184.1	14.59	Not annotated		
706.5	184.1	16.4	Not annotated		
706.5	184.1	20.75	Not annotated		
706.5	184.1	21.62	Not annotated		
706.5	184.1	21.84	Not annotated		
706.5	184.1	22.14	Not annotated		
706.5	184.1	22.89	Not annotated		
706.5	184.1	23.17	Not annotated		
706.5	184.1	23.39	PC 30:0; PC(14:0/16:0)		
706.5	184.1	23.86	Not annotated		
706.5	184.1	25.13	Not annotated		
704.5	184.1	12.73	Not annotated		
	L		Finish Cancel		

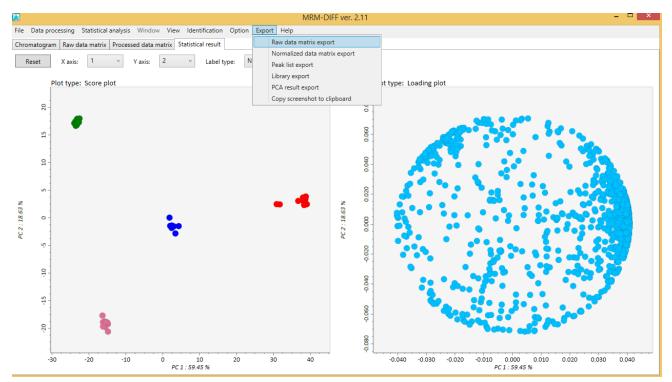
# **Option menu**

You can set properties of files. You can reset file type, class ID, or analytical order. If you clear the check box of the "Included" column, the corresponding data are no longer used in the statistical analysis.

		File property setting		
File name	File type	Class ID	Analytical order	Included
131122_LCMS_Lipidomics_01	QC	Pooled QC	1	✓
131122_LCMS_Lipidomics_02	Sample	Human serum	2	✓
131122_LCMS_Lipidomics_02	Sample	Human serum	3	✓
131122_LCMS_Lipidomics_02	Sample	Human serum	4	✓
131122_LCMS_Lipidomics_02	Sample	Human serum	5	✓
131122_LCMS_Lipidomics_02	Sample	Human serum	6	✓
131122_LCMS_Lipidomics_03	QC	Pooled QC	7	✓
131122_LCMS_Lipidomics_04	Sample	Human serum	8	✓
131122_LCMS_Lipidomics_04	Sample	Human serum	9	✓
131122_LCMS_Lipidomics_04	Sample	Human serum	10	✓
131122_LCMS_Lipidomics_04	Sample	Human serum	11	✓
131122_LCMS_Lipidomics_04	Sample	Human serum	12	✓
131122_LCMS_Lipidomics_05	QC	Pooled QC	13	✓
131122_LCMS_Lipidomics_06	Sample	Mouse serum	14	✓
131122_LCMS_Lipidomics_06	Sample	Mouse serum	15	✓
131122_LCMS_Lipidomics_06	Sample	Mouse serum	16	✓
131122_LCMS_Lipidomics_06	Sample	Mouse serum	17	✓
131122_LCMS_Lipidomics_06	Sample	Mouse serum	18	✓
131122_LCMS_Lipidomics_07	QC	Pooled QC	19	✓
131122_LCMS_Lipidomics_08	Sample	Mouse serum	20	✓
131122_LCMS_Lipidomics_08	Sample	Mouse serum	21	✓
131122_LCMS_Lipidomics_08	Sample	Mouse serum	22	✓
131122_LCMS_Lipidomics_08	Sample	Mouse serum	23	✓

### Export menu

A tab-delimited text file can be exported for a raw data matrix, a processed data matrix, the updated library, detected peak information detail, and PCA results. Moreover, the PCA result can be exported by some image formats.



# Appendix A: how to obtain appropriate file conversion of the Shimadzu .lcd file. Suitable method file (.lcm)

Although you can do a content change of the .lcd file after LC-QqQ/MS (MRM) analysis, it is very useful to construct a suitable method file (.lcm format file) for the successful file convert of the MRMPROBS software.

🔱 Offline Editor	(WINDOWSXP-Instrument1-System Administrator) - [Method Editor - Tsugawa_1201017_25min_60event.lcm]	
🗐 Eile Edit View	Method Instrument Acquisition Tools Window Help - 🗗 🗙	
i 🗋 🖄 🗟 😹		
X		
Main Method Editor	Offline	
Method Editor	Instrument Parameters View Normal Advanced End Time: 25.00 min	
$\odot$	MS Interface Data Acquisition LC Time Prog. Pump Column Oven Controller Autosampler AutoPurge	
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Single Hun	MRM(+) [Product Ion Scar(+)] [Precursor Ion Scar(+)] [Neutral Loss Scar(+)] [SIM(+)] [Scar(+)]	
12		
Batch Queue	Use CID Gas Settings     Attenuation	
Ducingucuc	Type Event# +- Compound Name mz Time (0.287 min - 19.084 min)	
	MRM 1 - Arginine 173.10-131.20,173.1	
	MRM 3 Theanine 173.00-155.25,173.	
	Key 4 - Common 45 - Common 45 000-133 01 4900 - Software, the compound name should be	
	MRM 6 6 - Utiline 243.00≥110.15,243.00	
	MRM 7 - Trymine 125.00-42.05 MRM 8 - Incoine 267.00-135.15, 267.00 made just by ASCII format.	
	MRM 8 - hotele 257 00-135 15, 267 00 Interesting 20 20 Interesting	
	MRM 10 - Thyndia 21, 10-42,05,24.	
	MRM V Acq. Tige: 2.33 - 4.33 min _ compound Name: juanine	
	Ch Precursor m/z Product m/z Pause Time (msec) Dwell Time (msec) 01 Pre Bias(V) CE 03 Pre Bias(V) 👌	
	Ord         150.00         633.10         1.0         15.0         12.0         20.0         24.0           Ord         150.00         66.15         1.0         15.0         12.0         21.0         11.0	
	Chi 150.00 108.00 1.0 15.0 12.0 18.0 19.0 MRM transitions should h	be
		<i>J</i> C
	Event Time: 0.048 sec Q1 Resolution: Unit V Advanced Settings	
	Event Time: 0.048 sec QI Resolution: Unit Advanced Settings Constructed for one metabolite.	
		1
	• The completely same precursor an	ıd
	product <i>m/z</i> pair cannot h	be
	acceptable in the file converter.	
	T	

1. Event name and channel (MRM transitions) rule.

#### 2. Update compound table

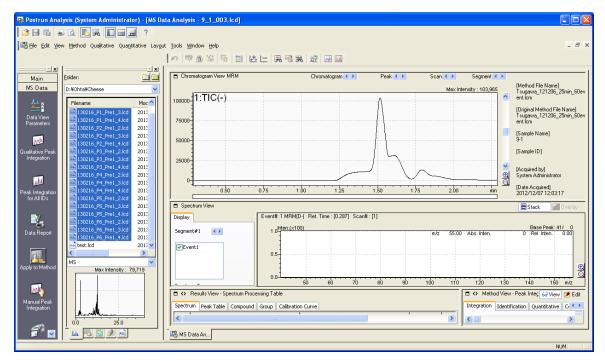
After the method construction of MRM transitions, you should update the compound table m/z by the MRM event. If you can analyze the samples by using the updated method file, you do not have to perform any other tasks for the stable file convert.

🔱 Offline Edito	litor (WINDOWSXP-Instrument1-System Administrator) - [Method Editor - Tsugawa_1201017_25min_60event.lcm]	
🗐 Eile Edit Viev	Yew Method Instrument Acquisition Iools Window Help	_ 8 ×
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X	A Data Processing Parameters(M5)	
Main	MS Data View Parameters	
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$\odot$	Advanced End Time : 25.00 min System Suitability Settings mp Column Oven Controller Autosampler AutoPurge	
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Start Single Run	Add MRM event	
	Ugdate MRM event time by compound table (+) Neutral Loss Scan(+) SIM(+) Scan(+)	
11111	Updgte compound table m/z by MRM event	
Batch Queue	Update display sequence by compound table	
	Export Compound Table und Ilame m/z Time (0.287 min - 19.084 min)	
	Import Compound Table 242.00>109.15, 242.0	
	MRM 3 - Theanine 173.00×155.25, 173.	
	MRM 5 - 2-Amino adipic acid 160.00>11	
	MRM 6 - Uridine 243.00>110.15, 243.00 MRM 7 - Trymine 125.00>42.05	
	MMM 7 11/mile 12300-12516 MRM 8 - Inosine 287.00-12515, 287.00	
	MRM 9 - Guanosine 282.10×150.20,28 MRM 10 - Trymidine 241.10×42.05.241.	
	MRM Acq. Time: 2.33 - 4.33 min Compound Name: Guanine	
	Ch Precursor m/z Product m/z Pause Time (msec) Dwell Time (msec) 01 Pre Bias(V) CE 03 Pre Bias(V) 🔨	
	Ch1         150.00         133.10         1.0         15.0         12.0         20.0         24.0           Ch2         150.00         66.15         1.0         15.0         12.0         21.0         11.0	
	C12         1500         06.15         1.0         15.0         12.0         21.0         11.0           C13         150.0         108.00         1.0         15.0         12.0         18.0         19.0	
	Ch4	
	Eyent Time: 0.048 sec Q1 Resolution: Unit V Advanced Settings	
	Q3 Resolution: Unit	
		<u>~</u>
	S/ Method Editor	
Update compound ta	id table m/z by MRM/SIM event.	Free NUM

# You can check the updated table by Method->Data Processing Parameters->Compound tab.

ntegratio		Quantitative	compound Group			stom QC Check	
ID#	Name	Туре	m/z	Ref. Ions	Ret. Time	Conc.(1)	Conc.(2) 🔨
1	Arginine	Target	173.10>131.20	173.10>156.20	0.001	1	
2	Cytidine	Target	242.00>109.15	242.00>42.00	0.001	1	
3	Theanine	Target	173.00>155.25	173.00>84.20	0.001	1	
4	Guanine	Target	150.00>133.10	150.00>66.15	0.001	1	
5	2-Amino adipic	Target	160.00>116.15	160.00>142.15	0.001	1	
3	Uridine	Target	243.00>110.15	243.00>200.25	0.001	1	=
7	Thymine	Target	125.00>42.05		0.001	1	
3	Inosine	Target	267.00>135.15	267.00>108.00	0.001	1	
9	Guanosine	Target	282.10>150.20	282.10>133.15	0.001	1	
10	Thymidine	Target	241.10>42.05	241.10>151.10	0.001	1	
11	Shikimate	Target	173.00>93.15	173.00>73.15	0.001	1	
12	Glycerate	Target	105.00>75.15	105.00>59.10	0.001	1	
13	G6P	Target	258.90>97.05	258.90>79.05	0.001	1	
14	Lactate	Target	89.00>43.10	89.00>45.05	0.001	1	
15	PIPES	Target	301.00>193.25	301.00>221.25	0.001	1	
16	R5P	Target	229.10>97.05	229.10>79.05	0.001	1	
17	S7P	Target	288.90>97.10	288.90>59.10	0.001	1	
18	F6P	Target	258.90>97.10	258.90>169.00	0.001	1	
19	a-Glycerophosph		171.10>79.10	171.10>96.90	0.001	1	
20	G1P	Target	258.90>79.10	258.90>97.10	0.001	1	
21	GAP	Target	168.90>97.10	168.90>87.15	0.001	1	
22	E4P	Target	198.90>97.20	198.90>79.00	0.001	1	
23	Orotate	Target	155.00>111.15	155.00>42.05	0.001	1	
24	Ru5P	Target	229.00>97.10	229.00>78.95	0.001	1	
25	b-Glycerophosph		170.90>79.10	170.90>97.10	0.001	1	
26	CMP	Target	322.00>79.10	322.00>97.10	0.001	1	
27	NAD	Target	662.10>540.10	662.10>408.15	0.001	1	
28	DHAP	Target	168.90>97.10	168.90>79.10	0.001	1	×
<							>

3. If your data (.lcd) were not collected by a suitable method described above, you can improve the .lcd file by using the method file modified in the above way. After the construction of the modified method file, please open "Postrun Analysis" of LabSolutions.



After selecting the analysis files (.lcd) push the "Apply to Method" button.

Save Method	As				? 🛛
Savejn: 🗀	Tsugawa 💌	G	ø	ø	<b></b>
🔂 Tsugawa_1	201017_25min_60event.lcm				
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Save as <u>t</u> ype:	LC Method File (*.lcm)	•	~		Cancel

Select the modified method file and improve your .lcd file including the compound table m/z. If you can do this, the file (.lcd) is successfully converted by Reifycs Inc. software.

👫 Postrun Ana	lysis (System Administrator) - [MS	Data	ta Analysis - 9_1_003.lcd)	
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Qualitative Peak Integration			the file convert from .lcd file to .abf file.	
1				
Peak Integration for AIIIDs			Spectrum View	
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e,			Segment#1 ()	
Data Report				
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7			9 Quancere Target 2821015030 2821015315 0.001 10 Thymchine Target 241104245 2411015110 0.001	
Create Compound Table Wizard	K		11 3hkmate Target 17200/2015 17200/2015 0001 12 Olycenste Target 10500/2015 16 10500/601 0 0001	
r aute wizaru	Max Intensity : 0		13 06P Tweet 25809705 25809705 0000	
6a.	Max meristry: 0		15 PPPES Target 3010019325 010002225 0000 10 F8P Target 222103716 0000	
Create SIM Table			17 S7P Tureet 2880/0710 2880/5810 0.001 18 F6P Tureet 26830/2710 2880/5810 0.001	
Lieate SIM Lable			19        040vcrspharph Tareet         171.10: 751.0         171.10: 550         0.001           30         01 P         Tareet         126800: 751.0         268800: 971.0         0.001	
			21 GAP Tweet 168300710 168300710 10001 22 E4P Tweet 168007070 16800710 0001	
			33         O-traine         Target         15500/1115         15500/205         0.001           24         R_ASP         Target         22900/7310         22900/735         0.0001           25         Ib-Officereprintph/Target         1790/7210         1790/7210         0.0001	
			25         b=Olycersphosph Target         1708/07/10         0.001           26         CMP         Target         32200/9710         0.001           27         NeD         Target         42200/9710         0.0001	
	50 100			
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#### 4. File convert

Conditions: You can convert from .lcd files to .abf files on your computer by installing LabSolutions software. "TTFLDataExportVer5.dll" of LabSolutions ver. 5.53 SP4 or later is required for the file convert. Check the "TTFLDataExportVer5.dll" (Program Files (or \*86)>LabSolutions) file property. If the file size is less than 577,536 bytes, contact Shimadzu Inc. for a file change.

After "AnalysisBaseFileConverter.exe" is opened, drag and drop the .lcd files to this converter.

G Reifycs Analysis Base File Converter	
	🚱 😡 🗢 🕌 « Ionpair_LC-MS 🔸 20121206_Tsugawa 📼 🍫 Search 20121206_Tsugawa 🔎
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✓ Save Converted Files into Same Folder as Raw F	Favorites Name Date modified Type
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Clear Clear All Convert Cancel	I items selected Show more details

Push the "Convert" button. The ABF format files will be generated in the same folder as the .lcd files.

# Appendix B: mzML file conversion via ProteoWizard.

#### **Required software and file**

• MSConvert Download link: http://proteowizard.sourceforge.net/

#### **Download ProteoWizard**

- 1. Select download type: Windows installer (includes vendor reader support) is recommended.
- 2. Read license agreements and download the proteowizard.

proteowizard	info • download user docs • dev docs • contact ProteoWizard Downloads	
	Get ProteoWizard (3 steps)         Step 1, Choose Download Type:         Windows installer (includes vendor reader support)         For End Users         Windows installer (includes vendor reader support)         Macintash (No vendor reader support)         Junux 64 bit (NO vendor reader support)         Mudows tar.bz2 (includes vendor reader support)         Unixed 54 bit (NO vendor reader support)         Windows tar.bz2 (includes vendor reader support)         Vindows tar.bz2 (includes vendor reader support)         Vindows factors (NO Barker or T2D support)         Tot For Developers         Fred       Source, bjam build (includes vendor reader support)         red       Source, bjam build (INO 3rd party reader support (e.g. vendors, mz5))         Source, GUU Autotools build (No 3rd party reader support (e.g. vendors, mz5))         Source Compassitiat         Thermo-Scientifie MSFileReader Library         Waters Raw Data Access Component Library         Waters Raw Data Access Component Library         Waters Raw Data Access Component Library         Waters Covers Core ProteoWizard Tools and Library         Version 2.0. January 2004         Step 3, Click below to agree to Licenses and get ProteoWizard         Iagree to the licensing terms, download ProteoWizard	

(http://proteowizard.sourceforge.net/downloads.shtml)

#### Setup ProteoWizard

- 1. Follow the wizard windows. (Maybe you don't miss it.)
- 2. "SeeMS" should be also imported.

#### Convert the vendor's MS file to mzML via ProteoWizard

- 1. Open the MSConvertGUI.exe.
- 2. Select "List of Files".
- 3. Select the vendor's file via "Browse" button.
- 4. In the "Options", never check any additional compression including "Use numpress linear compression", "Use numpress short logged float compression", and "Use numpress short positive

integer compression". Each of binary encoding precision is available.

5. Click "Start" button.

P MSConvert	
List of Files File of file names File:     Add Remove  C:¥Users¥Hiroshi Tsugawa¥Desktop¥Agilent GC-QqQM C:¥Users¥Hiroshi Tsugawa	About MSConvert Filters Levels: 1 –
Utput Directory: C:¥Users¥Hiroshi Tsugawa¥Desktop¥Agiler Browse	Add Remove Filter Parameters
Options Output format: mzML  Extension: Binary encoding precision:  64-bit  32-bit	
Write index:       Image: Compression in the index index in the index index in the index	
Use these settings next time I start MSConvert	Start