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We used the NuGO Standard Operating Procedure (SOP) number 40 produced by the University of Copenhagen. Details of the SOP are available via the web link:

<http://www.nugo.org/frames.asp?actionID=38790&action=loginFromPP>

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Data analysis of metabolomics profiling data by Markerlynx

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To be updated every second year	Prepared by: (sign/date) LDRA	Approved by: LDRA

1. Clinical biochemistry

Data handling

2. Principles

2.1 Principle of analysis

Data analysis by alignment of chromatograms in the time and mass dimensions

2.2 Principle of measurement

Data are ordered by alignment into small time/mass buckets

3. Measurement area and accuracy of analysis

3.1 Measurement area

Not relevant

3.2 Accuracy of analysis

Accuracy below 20% CV

4. Reference area

To be updated with reference to metabolomics standard experience.

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5. Sample preparation and handling

Preparation for analysis

1. The raw data files are transferred to the processing computer. Usually lockmass-corrected, centroided data files. Up to 600 files have been processed successfully but the vendor does not guarantee seamless processing of more than approx. 300 samples.
2. Make sure data files for blank samples and urine mix samples are included.

6. Apparatus/utensils

- Raw data files to be processed
- A Windows XP (32bit) based computer, the faster the better, with 16 MB RAM
- Waters Masslynx software ver. 4.1 (updated 2007) including Markerlynx.

7. Chemicals/reagents

none

8. Solvents

none

9. Safety

none

10. Check material

Not relevant

11. Procedure

The operator must be familiar with the software.

1. If necessary modify method according to actual needs, selecting the mass range of interest and a mass tolerance of 0.05 Da. Usually also de-isotope data automatically and select noise reduction level 3. PABA may be added in the internal standard list, if urine samples from volunteers given PABA have been selected. This may help alignment further. Threshold should be 20-100. Use higher values for reanalyses if >12.000 markers have been detected.
2. In the sample list add the following additional columns: Sample group, sample gender, sample text Mass A.
3. Make sure Masslynx has loaded the correct project. In the 'Process samples' window select project folder, sample list and samples.
4. Select 'detect peaks' as the only process and press run.
5. Following processing (depending on size of data files and computing power) the 'view results' windows opens. Immediately save as a markerlynx file in a folder named markerlynx in the correct project.
6. Visually inspect each chromatogram by running through them with the arrow down key. Deselect any empty or other obviously odd chromatograms.

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7. Select 'Processing', then only select 'collect markers' and run.
8. Immediately save the file again after this step. The number of markers is usually several thousands. Avoid in excess of 10-12.000, otherwise go back to step 1.
9. Now select RT-mass pairs corresponding to standards mixture compounds and inspect alignment over the entire sample set. Blanks should be empty and standards should have similar levels ($\pm 20\%$) throughout with no obvious time trends. Otherwise change method and reprocess. The compounds should have below 3ppm mass error.
10. Also samples analyzed repeatedly should have similar concentration levels. If samples are systematically different by more than 20% between repeated analyses this may be caused by bad alignment. Go back to raw data files and integrate the relevant peaks. If they do not differ there, alignment is not acceptable and the data analysis should be repeated with altered parameters for integration parameters, RT or mass alignment.
11. Sample groups (if known) should now be marked with different colours, using the sample group column and samples can now be sorted by group.
12. If known contrasting markers exist, localize them by mass and check that only the correct samples have the marker values expected. A clear visual contrast between groups indicates good data quality. Outliers may be identified and checked as 'excluded' from further analysis.
13. Save again.
14. Now check blanks and standards as 'excluded' and select processing by PCA. Usually mean center, normalize and scale by Pareto scaling as a first choice.
15. Save again after processing. Check that groups are fairly well differentiated by PCA analysis in the scores plot. If so, search for new markers can be initiated by selecting markers in the loadings plot corresponding to the differences in direction of the samples in the scores plot. Visually inspect that these markers contrast between groups. Record RT-mass pairs for subsequent metabolite identification (**SOP to be prepared**).

12. Calculation and reporting

Under development

13. Time schedule

Not relevant

14. Price of material

Programme updates approx. 10-20€/analysis day

15. Waste disposal

Send bad data to cyberspace

16. Sources of error / error detection / advice

See step 15 in the procedure (11)

17. References

To be included

18. Appendices

None

