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FAQ – Contents

MetIDQ™

A1: How can the connection to the Oracle database be reestablished?

See document "Database Connection Error - Troubleshooting Guide"

A2: Why did the MS data import to MetIDQ™ fail?

MetIDQ™ requires several Windows® runtime components to process MS data (.wiff, .raw, or .d). Please refer to the MetIDQ™ user manual section 1.1 "MetIDQ™ Requirements "and check if they are all installed.

A3: How can inter-plate data normalizations in MetIDQ™ be performed?

Data normalization is strongly recommended for multi-plate experiments to minimize batch effects and improve reproducibility. To normalize a dataset, perform the following steps:

1. Create a "Report Context" in MetSTAT and make sure the "Condition" is set to "Pending".
2. Link all samples from all kit plates being analyzed in MetSTAT together with the reference sample that is to be used for normalization (biocrates QC or own study pool).
   - The reference sample should have been measured in replicates of three or more on each kit plate. The "Minimum number of replicates for normalization" is defined in the "Settings > MetSTAT".
   - If your own study pool sample is being used for normalization, the MetIDQ™ Sample Barcode of each replicate must be the same.
3. Activate the **Data Normalization** in MetIDQ™ and select the sample to be used for normalization from the dropdown menu.

See document "Normalization for kit results"

A4: How can out of range QC accuracies be interpreted?

The kit performance is usually good even if the accuracy of some metabolites from the QC samples are out of range in MetVAL.

- For metabolites with the Analyte Classification "Quantitative", QC accuracies within the acceptance ranges are expected.
- For metabolites with the Analyte Classification "Relative Quantitative" or "Quantitative with Restrictions" (1-point calibrated metabolites), QC accuracies out of range can occur. Ionization efficiencies among analytes and internal standards are not identical between different instrument types and may result in reported accuracy measurements that are out of range.

Example: AbsAcid does not have its own internal standard (ISTD), but instead is linked to the DHEAS-ISTD. DHEAS and DHEAS-ISTD utilize a less sensitive collision energy (CE) in order to avoid saturating the detector. It is possible that the DHEAS-ISTD signal is reduced over time

since the instrument was last serviced or kit age. Because of this, the concentration of AbsAcid will be reported higher. While this issue is particularly prominent with AbsAcid, it is possible to occur with any analyte that does not have its own ISTD.

Solution: Data normalization can be used to correct for accuracy or variation across plates run at different points in time. The data normalization tools that can be activated in MetSTAT (see MetIDQ™ user manual QC2 vs. Target Normalization and document "Normalization for kit results") and used regardless of number of plates or sample matrix.

- In MetVAL, only a subset of analytes is checked for accuracy, specifically those using a single-point calibration (apart from some LC analytes, all FIA analytes are quantified by a single-point calibration). The subset provides a reasonable representation of the entire panel and is adequate to assess the analytical performance on whole. The single-point calibrated analytes are not quantified against an external standard. A further subset of the single-point calibrated analytes uses an internal standard that is structurally similar but not identical to the analyte being measured. The differing ionization energies from instrument-to-instrument makes it difficult to assign an exact concentration to these analytes in MetVAL. Using Target Value Normalization in MetSTAT can help to correct for these accuracy discrepancies and act as a type of external calibration point.
  Please refer to the document "Normalization for kit results".
- The classification "quantitative" or "relative quantitative" has no impact on the statistical power of your biological interpretation. The results of the relative quantitative compounds have the same analytical quality with respect to precision (reproducibility) as the results of the quantitative compounds.
- If QC values do not meet the expected concentrations given in MetIDQ™ and the accuracies are therefore out of range, how can the kit performance be evaluated?
  1. Check if the QC accuracy values of the compounds that are displayed in MetVAL are consistent over all QC levels and replicates.
  2. Check if the reproducibility (coefficient of variation, CV) of the analytes above LOD in the measured replicates is OK. You can easily do that in Excel after exporting the results from MetIDQ™: CV = (Standard Deviation / Mean) * 100.
  3. If points 1 and 2 are fulfilled, the performance is good, and the kit run was successful.

A5: How can MetIDQ™ error messages be solved?

- **`ORA-12952: The request exceeds the maximum allowed database size of X GB.`**
  The storage limit of the Oracle XE database has been reached. You must delete raw data files stored in the database to create more room or purchase a full Oracle database license.
- **`An error occurred because of insufficient main memory: MetIDQ™ could not perform the last task because there is insufficient main memory left…`**
  The memory limit was exceeded during exporting the results table in MetSTAT. The report context is too large to submit in its current form. Reduce the size by unchecking "Export comments to Excel files" (that mostly helps already), "Export Status Information", or export as CSV or TXT format.
- **`Plate run barcode # not found in the database.`**

You are attempting to import a sample set that was not registered in the current database. There are two ways to solve this. Both methods are also described in the MetIDQ™ user manual section 4.1.1.

- From the MetIDQ™ database where the worklist was created, export the Project or Submission as a .metidq file. Import this file on the other database.
- Reconstruct the plate from the raw data files. In MetLIMS > Projects, create or navigate to the desired submission. Choose "Import Worklists" and navigate to the folder where the raw data is stored. A new worklist will be created based on the structure present in the raw data file names. Be aware that creating a plate this way will prevent further data exchange if the original plate was created in a different database.

- `Data import failed due to sensitivity issues. Please contact customer support.`
  p400 users only. The latest database patch has not yet been applied. Apply the latest patch from the USB included with your kit (MetIDQ™ and Oracle\Patches) or contact biocrates support for the latest version.
- Data import failed in MetCONC:
  - `Incorrect file type selected`: After clicking "Add Files", ensure that the correct file type is selected at the bottom of the file browser window according to your instrument manufacturer.
  - FIA: `Proper files for converting could not be found.`
    LC: `Import of result file failed!`
    The correct Microsoft runtime libraries are not installed properly on the PC. From the MetIDQ™ USB stick, locate the folder "MetIDQ™ and Oracle\Installation Files\software_requirements". Install the *NET_framework* and all five *vcredist* executables.

A6: How can a backup or transfer of MetIDQ™ data be performed?

- **Export MetIDQ™ file:** Projects or Submissions can be exported in MetLIMS > Projects by selecting the desired Project or Submission and clicking "Export Project" or "Export Submission" button above the Project Tree panel. The entirety of the contents will be saved to a .metidq file that can be imported into another database (identical MetIDQ™ version on both computers is required). This file will contain all samples and data that has already been processed. For additional information, refer to section 4.1.1 of the MetIDQ™ user manual.
- **Recreating plate from raw data files:** In MetLIMS > Projects, create or navigate to the desired submission. Choose "Import Worklists" and navigate to the folder where the raw data is stored. A new worklist will be created based on the structure present in the raw data file names. For additional information, refer to section 4.1.1 of the MetIDQ™ user manual.
- **MetIDQ™ database backup and restoration:** The entire database can be backed up or restored from the login screen of MetIDQ™. Refer to section 7.6 of the MetIDQ™ user manual for detailed instructions.

Kit preparation

B1: Why do the extracts appear cloudy?

Answer: For AbsoluteIDQ® p180, p400 HR and MxP® Quant 500 kits

When adding water to the methanolic sample extracts to make the LC plate, cloudiness can occur due to residues of pyridine and phenyl isothiocyanate from the derivatization solution. If that happened, perform the following steps:

1. Extend the shaking time at 600 rpm to 20-30 min.
2. If the extracts are still cloudy, leave the plate standing for 15 min at room temperature and shake for another 15 min at 600 rpm.
3. Afterwards, run the plate, even if the extracts are still a bit cloudy. We found that the cloudiness has no impact on the kit performance or on the quality of the data. Also, we did not observe any wastage of hardware parts.

B2: What are reasons why the extracts did not go through the filters or the volumes in the capture plate are low / inconsistent?

Inconsistent or lack of volume in the filter plate may occur for several reasons. Below are some suggestions:

1. Water or another solvent was used for making the extraction solvent instead of Methanol. Perform the following steps:
   a. Transfer the extracts back to the filter plate.
   b. Dry the samples under nitrogen.
   c. Dissolve again using pure methanol (volume according to extraction step in user manual).

2. The pressure manifold was not set up correctly.
   a. Check that the pressure manifold settings are correct according to the instructions. **see document "Instructions using pressure manifolds for 96-well plates with biocrates kits"**
   b. Put the plate back in the manifold and try again.
   c. If the problem persists, increase the low flow pressure up to 30 psi

3. Droplets of liquid remaining on the underside of the filter plate. It is normal if a small amount of liquid is on the underside of the filter. Internal standards will account for small volume discrepancies. Take care that there is no cross contamination into other wells when removing the top filter plate. If large droplets remain on the underside of the filter plate:
   a. Put the plate back into the pressure manifold and run at a higher pressure (30 psi).
   b. While the filter and capture plate are still secured together by tape, tap the plate lightly on the table to shake the droplet loose.

4. It is also possible that some amount of volume was lost during the shaking step or when transporting the plate. You can check the wells with lower volume in the capture plate to see if the remaining volume is still in the top filter plate. If the top filter plate is empty, then it's likely that the volume was spilled at some point during the preparation. **Take extra care during shaking and carrying the plate that nothing is spilled when the wells are uncovered!**

B3: Why are there signals in the blank or in the zero samples, why are analytes in the zero samples above LOD?

The blank well A1 does not contain any analytes or internal standards. The zero samples only contain internal standards, but no analytes. If there are still peaks the following may have occurred:

1. A sample or internal standard mix was accidentally loaded.
2. Spillover when loading the extraction solvent onto the kit plate, e.g. the dispensing speed of the repeater was too high.
3. Spillover when shaking the plate after loading the extraction solvent.
4. Cross-contamination by residues at the bottom of the filter plate when removing the filter plate from the capture plate.
5. Cross-contamination when transferring the extracts to make the LC and FIA plates.
    - To make sure the contamination comes from the plate and not from the system, inject a blank from an autosampler vial to cross-check.

B4: How long can the kit plates be stored?

- Before preparation: Please refer to the specific expiration date stamped on your kit plate.
    - Plate shelf life at -80°C:
        - **p180**, **p400 HR**, **Quant HR Xpress**, **Bile Acids kits**: 12 months
        - **Quant 500 kit**: 6 months
        - **Stero17 kit**: 3 months
- **After preparation:** Plates should be run immediately after preparation for best results. If unforeseen complications arise (e.g. instrumental issues) that prevent the plate form being run immediately:
    - **Plate can be run within 48h:** Store plates at 4°C
    - **Plate cannot be run within 48h:** Contact biocrates support!

B5: Why are QC accuracies consistently low or high for all analytes?

If the QC accuracies are consistent for all measured analytes, but differ significantly from the expected values (i.e. all metabolites for any particular QC sample are at 50% accuracy), the following may have occurred:

- **Pipetting issue:** More or less than 10 µL of the QC sample was transferred to the plate. An air bubble in the pipette tip, pipette tip missed the center of the filter paper, or excess liquid was attached to the outside of the tip. It could also be that the internal standard mix was not transferred correctly (not applicable to the MxP® Quant 500 kit).
- **Reconstitution issue:** The QC vials were not resuspended in the correct volume. For instance, if 200 µL were used instead of 100 µL, the accuracies would be around 50%.
- **Mixed QC samples:** The QC samples were not pipetted in the correct order.
- **Calibration standard issue:** Check both the FIA and LC parts of the kit. If the problem only occurs in the LC part of the kit, it is likely related to a problem with the calibration curves or calibration standards might have been mixed up during pipetting onto the kit plate (e.g. Cal3 to Cal4 well and vice versa).

LC-MS performance

C1: What should FIA peaks look like and how can the peak shape be improved?

- The best peak shapes are obtained by avoiding unnecessary dead volumes (caused by valves or connection parts) and connecting the injector directly to the mass spectrometer. For this, red PEEK tubing is recommended.
- The FIA peaks must be located approximately in the middle of the data acquisition window, rather earlier than later. It is important that the peaks are not cut off or running out of the window.
- The peaks must not show "satellite" or large shoulder peaks.
- The peaks must have a defined beginning and ending without interruptions. They must not show huge valleys in between or be too jagged (stable spray).
- Jagged peaks are typically caused by an instable electrospray. In that case, rinse at high flow rate using different solvents or replace the ESI electrode.
- Huge and broad valleys can be caused by ion suppression. In that case, dilute the samples further.
- Refer to the user manual for more information and SST criteria.

C2: How should the LC-MS system be maintained for optimal performance?

Important cleaning notes:

- Frequent cleaning actions and rinsing methods are strongly recommended to minimize the risk of instrument contaminations or performance loss.
- For optimal time-efficiency, clean and rinse the instruments routinely between the kit runs while a new kit is prepared (see recommendations below).
- Monitor test sample intensities each time you run the SST and perform troubleshooting when you observe significant signal decrease. Clean and rinse system again if required.
- Check immediately after each plate run if all injections have worked and if the peaks are visible and have a good shape. In case of missing peaks or bad shapes, troubleshoot and reinject the plate or affected samples.
- The instrument requires a full service at least every 6 months when the kit is used in high throughput.
    - Please be aware that performance-related issues that cannot be resolved by instrument cleaning are not within biocrates' responsibility and must be handled by the user or the LC-MS engineer!

Cleaning actions after every plate run (LC or FIA):

- Rinse the column for 60 min using wash solvent according to user manual (after LC plate only).
- Install 10% methanol (in water) as mobile phase A and wash solvent as mobile phase B. Rinse entire LC-MS system:
  - 15 min at 100% A offline (to waste, MS switched off)
  - 15 min at 100% B offline (to waste, MS switched off)
  - 15 min at 100% A online (to source/ESI needle, MS switched on)
  - 15 min at 100% B online (to source/ESI needle, MS switched on)
- Keep the wash solvent as mobile phase B, transfer some wash solvent to autosampler vial, and perform 10 blank injections using 100% B.

Before FIA run:

- Install FIA mobile phase for the assay, purge and flush the entire LC-MS system with FIA mobile phase at 1 mL/min until the pressure is stable.
- Perform another 10 blank injections using FIA mobile phase as blank solvent. Perform more blank injections to decrease background noise if necessary. Continue with SST according to user manual.

Before LC run:

- Install mobile phases A and B for the assay, purge and perform another 10 blank injections using 50% methanol as blank solvent.
- Perform more blank injections to decrease background noise if necessary. Continue with SST according to user manual.

After every 2 kits:

- Rinse the autosampler for 20 min with 10% methanol (in water) to get rid of salts, followed by another 20 min with wash solvent to get rid of lipids (in the same way as the entire LC-MS system above)
- Clean Curtain Plate or Cone according to manufacturer's instructions.
- Wipe the Orifice (without removing the Orifice plate) using a foam rod, first with water, followed by isopropanol and methanol.
- Wipe the tip of the ESI electrode using a foam rod, first with water, followed by isopropanol and methanol.

After every 5 kits or more:

- Replace ESI electrode after every 5 kits.
- Perform instrument front-end cleaning (Orifice and QJet or Q0) after every 15-20 kits.

C3: What could cause a loss in sensitivity or absence of peaks?

Please be aware that performance-related issues that cannot be resolved by instrument cleaning are not within biocrates' responsibility and must be handled by the user or the LC-MS engineer. Based on our experience, however, we are happy to share some suggestions:

- Sample preparation:
  - Make sure the testmix/test sample has been reconstituted or diluted correctly.
  - Make sure there was no air in the pipette tip when transferring sample to another vial.
  - Make sure the testmix/test sample used was not expired.
- Instrumental setup: double-check all method parameters and make sure the analytical column and mobile phases were prepared and installed correctly.
- Connections: check for leaks and retighten all connections.
- Injection needle: the injection needle might not be correctly adjusted or draws air. Adjust positioning or replace the needle.
- Injector: check for leaks or contaminations in the injector valve and rotor seal. Clean or replace the valve.
- Divert valve: set to waste instead of MS. Check setting at valve and in method.
- ESI electrode: leak, contamination, or defective. Reassemble ESI probe, clean with different solvents at high flow rate, or replace ESI needle.
- MS: perform instrument front-end cleaning (Orifice, QJet, quadrupole, ion transfer tube etc.). Tune and calibrate the instrument.

C4: Why are the retention times shifted?

If the retention times are unstable across a plate or shifting over time:

- Make sure the mobile phases are purged and there are no bubbles in the system.
- Make sure no air is drawn or injected by the autosampler.
- Make sure the entire LC-MS system is well conditioned and the column is sufficiently equilibrated.

Otherwise, check the following parts with your instrument engineer:

- Degasser
- Mixing chamber (needs to be replaced?)
- Intake/outlet valve at pump head
- Seals at pump head
- Replace the analytical column (it may have reached the end of its lifespan)

C5: What can be done if AbsoluteIDQ® p400 HR kit does not meet SST criteria from the manual?

The SST criteria given in the manual are based on our Q Exactive Focus connected to a Vanquish UHPLC system (used for kit development and validation). The sensitivity of another Q Exactive instrument, as well as of another Q Exactive Focus can be different, depending on the instrument condition. The following ideas can help to improve the instrument performance. Otherwise please check with your Thermo engineer.

- Make sure the Ring Position is B.
- Clean or replace the ion transfer tube.
- Clean the S-lens.
- Was the customized calibration successful?
- Check if the ESI needle is in the correct position and tight (the inner needle can sometimes slip out at higher flow rates and is then no longer in the optimal position). Clean or replace the ESI electrode if necessary.
- The spray voltage that we give in the protocol gave best results during kit development on our Q Exactive Focus. However, you can try other spray voltages. You can go up to 4 kV and compare if the intensities are getting better.
- Check gas supply.
- Perform instrument service.
- See other FAQ sections: 0, 0.

C6: What is the expected lifespan of the biocrates column?

The lifespan of the column depends heavily on the number and type of samples being run, as well as the storage conditions between plates (column should be stored in pure LC-MS grade acetonitrile). Running plasma samples, the column system can normally be used for 15-20 kits (1200-1600 samples, MxP® Quant 500). Dirtier sample matrices (e.g. urine or fecal samples) may reduce the lifespan. Usually a minimum of 10 kits (800 samples, MxP® Quant 500) can be run per column irrespective of sample type. Regardless of the number of runs, chromatography should always be evaluated immediately after every LC run. As the column ages, the early eluting peaks will begin to broaden and show peak fronting, shoulder peaks, or in worse cases double peaks. Retention times of the early eluting compounds may also begin shifting earlier. Late eluting peaks are not as affected by column age. Some of these issues may also be a result of a leak somewhere in the system. Loosen and retighten all fittings and perform test injections before replacing the column.

If the peaks becoming jagged, it may be a result of instability in the electrospray (ESI electrode, gas flow, contamination, etc.) rather than chromatography related.

General FAQs

G1: What software is required to run the kits and how is the data processing handled?

Sample registration, plate layout creation, quantification, technical validation, and results formatting are handled by biocrates' MetIDQ™ software. The data collection is controlled by the instrument manufacturer's software using acquisition methods provided on the USB stick that comes with the kit. It is not required that MetIDQ™ being installed on the same computer as the instrument.

MetIDQ™ contains tools for data normalization and analysis using StatPack and MetaboINDICATOR™ (latter only applicable for MxP® Quant 500 kit). Exported data can be formatted for direct uploaded in MetaboAnalyst or use in R with a provided script. Additionally, biocrates offers a statistical analysis service and interpretation package performed by our in-house experts.

G2: What kinds of samples can be used with the kits?

biocrates kits have been specifically validated for use with plasma and serum. However, a wide variety of other sample matrices can be analyzed using the kits. Essentially any samples with endogenous metabolites can be analyzed with the correct preparation.

For most kits, protocols for the following matrices are available:

- Tissue (links to SOPs/App Notes)
- Urine
- Fecal
- Cell culture
- Dried blood spots (DBS)
- Cerebrospinal fluid (CSF)

If you are interested in other matrices, please contact biocrates support!

G3: What kinds of plasma anticoagulants are compatible with biocrates kits?

biocrates kits were validated with EDTA and heparin plasma only. Citrate plasma may also be used, but performance may be reduced. The most important aspect is to ensure that all plasma samples within one study are collected in the same way and using the same anticoagulant. For a summary of various anticoagulants and their effect on metabolomics samples, please refer to the following study: https://doi.org/10.1007/s11306-012-0450-4

G4: How are the lipids reported?

The lipids are annotated according to head group, total number of carbons in the chains, and total number of double bonds in the chains. For example, PC(16:1/20:5) becomes PC(36:6).The lipid analysis in the AbsoluteIDQ® p180, p400 HR and MxP® Quant 500 kits does not provide specific information regarding either the positions or chain lengths of the fatty acid residues linked to each lipid's backbone. Consequently, the reported signal is a sum of several isobaric/isomeric lipids. For more detailed information please refer to the list of lipids for each kit: AbsoluteIDQ® p180, p400 HR, MxP® Quant 500 (available on request).

G5: What other laboratory equipment and chemicals are required to run the kits that is not provided by biocrates?

While the biocrates kits come with all the standards and quality controls necessary, some other additional solvents and lab equipment are required to successfully prepare and run a plate. Please contact biocrates support for a detailed list of requirements and recommendations.