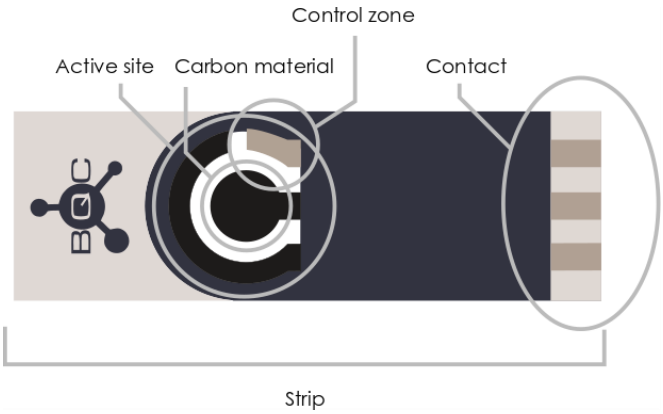
MOST COMMON MISTAKES WHILE USING THE e-BQC DEVICE: RESULTS WITH LOW REPRODUCIBILITY, ACCURACY AND ERROR MESSAGES

The e-BQC is a very sensitive device that uses electrochemistry to measure antioxidant capacity. This means that there are some factors to take in account and good practices to minimize the variation when doing replicates, since they can have an important impact on reproducibility and accuracy. These factors are based on three main keystones:

e-BQC STRIPS:

The measurement takes place on the strips, so it is very important to take good care of them. The strips have several parts as can be seen below, to which the text refers.



a. **Do not touch the strips with bare hands**: our hands contain natural oils and sweat that can alter the surface and give wrong measurements. Even the lowest amount will contribute to these alterations.
   i. Oils will act as a barrier, avoiding the sample to be in direct contact with the surface and in addition preventing the electron transfer, which will result in lower antioxidant capacity than expected.
   ii. Sweat contains high amounts of uric acid, which is a very potent antioxidant, so even in low amounts it can contribute to the total antioxidant capacity of the sample and give false positives.

   Recommendation: Always use **clean** gloves to handle the strips. **Never**, even with clean gloves, touch the active site, where the sample is disposed on.

b. **Do not reuse the same strip**: The strips are clean surfaces, when they are used, the voltage applied may cause irreversible binding of some substances to the inert carbon material and so interfering with the measurements.

   Recommendation: Always use **new** strips for each measurement. **Never,** even for replicates, use the same strip to measure.

c. **Avoid scratches**: scratches remove a part of the carbon material that is present in the strip, and so, diminishing the surface that is in contact with the sample and the electron transfer is difficulted. One very common way to scratch the strips is when disposing and extending the drop with the pipette tip.

Recommendation: **Maintain** the strips in the **container** until used to avoid damage. **Avoid rubbing** the strips against themselves or any other material. **Do not use** any strip that seems damaged. When pipetting the samples, **do not touch** the surface of the strip with the tip.

d. **Protect the strips from the dust**: Dust particles have many components that may interfere with the measurement, either by diminishing the active surface or by adding interferant substances that are not present in the original sample.

Recommendation: **Maintain** the strips in the **container** until used in a clean space.

SAMPLE:

Some samples will present more reproducibility than others. This will depend on their composition which will determine their physico chemical properties and suitability to measure with the e-BQC device:

a. Aqueous/Lipidic composition: One of the most important factors that affect the measurement is the ratio of hydrophilic and hydrophobic components. Organic solvents as well as lipidic matrixes are not suitable for the measurement with the e-BQC since the electron transfer is from low to inexistent depending on their concentration.

Recommendation: If your sample is extracted in organic diluents, remove them and dilute in an aqueous buffer. If not possible, dilute your sample with an aqueous buffer at least to a 1:2 ratio. Depending on the strength of the organic solvent you might need a higher dilution factor.

b. pH: The pH will affect the state of the antioxidant groups, specially hydrogen atoms.

Recommendation: To avoid this effect, some samples may need to standardize the pH to a value considered **biologically relevant**. This can be simply done by diluting the sample in a buffer. **Do not** compare samples with very different pH values.

c. Heterogeneity: Some samples may be heterogeneous depending on their components. Typically, the most problematic ones are those that contains lipids, dividing the sample in two phases. This makes that different parts of the sample, will have different proportion of components, and so affecting the reproducibility of the results.

Recommendation: Always try to work with homogeneous samples. If not possible, try to **separate interferent** substances by purifying your sample or **diluting** it to minimize this effect. You may also find that your sample presents a matrix effect, so check the Matrix effect section below. Use higher volumes to measure, since it will take a more representative sample of components.

d. Conductivity: Conductivity is one of the most important factors to take in account, the mobility of the electrons in the media will determine the success of the measurement. Higher ion content will favour this movement while low amount (like on ddH_2O) will give an ERROR while measuring with the device.

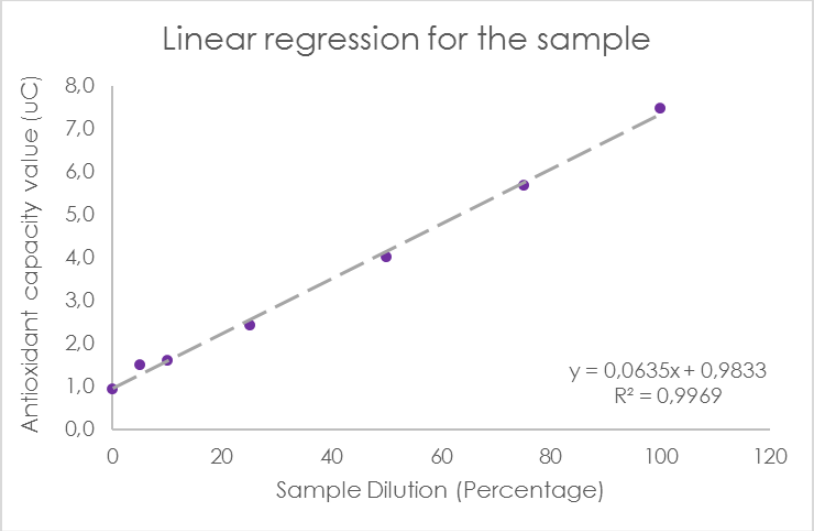
Recommendation: Samples with low conductivity will require **dilution** with a buffer to improve this electron movement. **Check** the content of organic solvents/lipidic compound in your sample.

e. **Viscosity:** Samples with high viscosity are more difficult to work with and in general, have low conductivity. Some samples, like protein extracts, may have DNA impurities, which will result in low reproducibility and low values of antioxidant capacity. This can be visualized easily in the liquid as a denser mass.

Recommendation: To make the samples more appropriate to measure with the device, dilute them with a buffer.

f. **Matrix effect:** The matrix effect is the sum of different factors as heterogeneity and also the relation of molecules in biological environments with complex components. This will make difficult the electron movement as well as worsen the reproducibility of the results.

Recommendation: One solution to this problem is **diluting** the sample. This matrix effect will make that when multiplying the value of a solution with the dilution factor, it will not be the value of the original sample. For this reason, a **calibrate** is recommended. The lower the slope of the calibrate, the higher the matrix effect. In this graphic below a calibrate for a milk sample is plotted.



g. **High protein content:** The proteins are adsorbed easily by the active site and for this reason, tend to give more disperse results.

Recommendation: **Avoid** letting the sample in the strip for a long time, measure immediately as the drop is disposed. **Do not** reuse the strip, even for replicate measurements. For protein extracts, use a buffer to **dilute** and **test different concentrations** to optimize your results.

h. **Interferant components:** Some antioxidants are used in the protocols of sample treatment of obtention like for example urea.

Recommendation: **Check** the presence of exogenous or added antioxidants in the sample that may not be of interest for the test.

Antioxidants are very sensitive substances that because of their special properties, are oxidized with ease. For this reason, another factor to take in account is the sample handling:

i. **Light exposure:** Light exposure will excite electrons and facilitate the antioxidant oxidation.

Recommendation: **Keep** your samples in **opaque containers** and avoid direct lighting.

j. **Oxygen exposure:** Bubble generation, rough vortexing and leaving containers opened for a prolonged time will cause reactive oxygen species to be generated and oxidize the antioxidants in the sample.

Recommendation: **Handle** your samples with care. **Do not** mix by vortexing at high speeds, instead, use inversion and mixing with the pipette. **Avoid bubbles** when disposing the drop, one way to do that is not pulsing the second stop of the pipette.

k. **Temperature of storage:** High temperatures damage the antioxidants present in the samples, giving low antioxidant activities.

Recommendation: **Store** samples at low temperatures (between -80°C and -20°C) to ensure prolonged stability. Before measuring, let the samples **reach room temperature**. Do not compare results of samples with different temperatures**. Avoid** multiple freeze-thaw cycles, instead, use aliquots.

l. **Time of storage:** Inevitably, antioxidant activity will decrease with the time of storage, even at -80 °C.

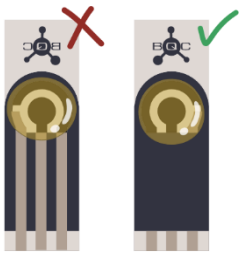
Recommendation: As far as possible, **use** freshly obtained samples. If not possible, store at low temperatures (between -80°C and -20°C) and measure the sooner the better. **Do not use** samples that have been stored for a long time or inappropriately stored.

USAGE:

After the sample and strips handling, a correct usage should be ensured for reproducibility and accuracy.

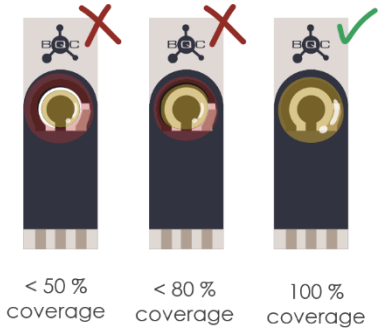
a. **Wrong strip position:** The two sides of the strip are not equivalent and using it wrong will lead to an ERROR message.

Recommendation: **Follow** the scheme below:



b. **Inadequate coverage:** One of the most common sources of error is poorly strip coverage. The strip is formed for the above explained parts that are equally key for a reliable measurement that should be correctly covered.

Recommendation: **Follow** the scheme below as a guide for a good coverage, all the surface inside of the blue zone should contain sample. In yellow can be seen the sample drop and in red the surface without sample. **Ensure** that the control zone is completely covered, preferably, start with this part to fill the active site of the strip.



c. **Too low sample volumes:** The device measures only the part of the sample that is in direct contact with the surface. For this reason, different sample volumes can be used as long as the surface is correctly covered. With too low sample volumes, is very difficult to reach this coverage and so ERROR messages or incorrect measurements could be displayed.

Recommendation: **Never** use less than 30 µL of sample. A starting volume could be 50 µL. Depending on the **viscosity**, more volume should be used. If there is no sample volume limitation, 100 µL is recommended, which ensures complete strip coverage. The volume could be adapted to the necessities.

d. **Not changing tips:** As well as with other experiments, not changing tips between samples could lead to low reproducibility.

Recommendation: **Always** change tips between samples to avoid cross-contamination.

e. **Using different sample volumes:** While the sample volume is not important for the measurement, it is key for comparison. Higher sample volume will ensure a more representative drop from the original sample, so ensuring that the representativeness is equal for each sample will be important for the statistical premise and especially important with heterogeneous samples.

Recommendation: **Be consistent** with the volume chosen for all the tests that will be compared lately. For heterogeneous samples, **use** higher volumes.

f. **Scratching the active site:** As mentioned earlier, scratching the active site can remove the carbon surface and result in wrong values.

Recommendation: **Maintain** the strips in the **container** until used to avoid damage. **Avoid rubbing** the strips against themselves or any other material. **Do not use** any strip that seems damaged. When pipetting the samples, **do not touch** the surface of the strip.

g. **Bubbles in the drop:** the oxygen contained in the bubbles not only means oxidation of the antioxidants, but also wrong volumes applied to the sample.

Recommendation: **Avoid bubbles** when disposing the drop, one way to do that is not pulsing the second stop of the pipette. This will cause the same amount of volume not to be dispensed but it will be the same error for every measurement.

h. **Working surface:** Vibrations and movement of the surface in which the device is on could affect the measurement due to the sensitiveness of the method since it facilitates the renovation of the layer in contact with the active site, giving wrong antioxidant capacity values.

Recommendation: **Ensure** that the surface in which you are working is smooth, horizontal and stable. **Do not** hold the device while measuring. **Avoid** measuring in a place where the activity of other equipment could cause vibrations, for example close to a working centrifuge. **Do not** touch the table while the measurement is being done.

i. **Misleading statistics:** When analyzing the data, the calculation of several statistics is of interest, especially the standard deviation. This standard deviation is usually referred to the mean as the relative standard deviation. Since the Q_1 value is always lower than the Q_2 value (less antioxidants belonging to the first category) the mean of the Q_1 will always be lower than the Q_2 with the same standard deviation. This way, when calculating the relative standard deviation, it will seem that it is greater for the Q_1 than for Q_2.

Recommendation: **Do not use** the relative standard deviation as a reliable statistic for the measurement of parameters with very different means, instead use non-relative statistics.