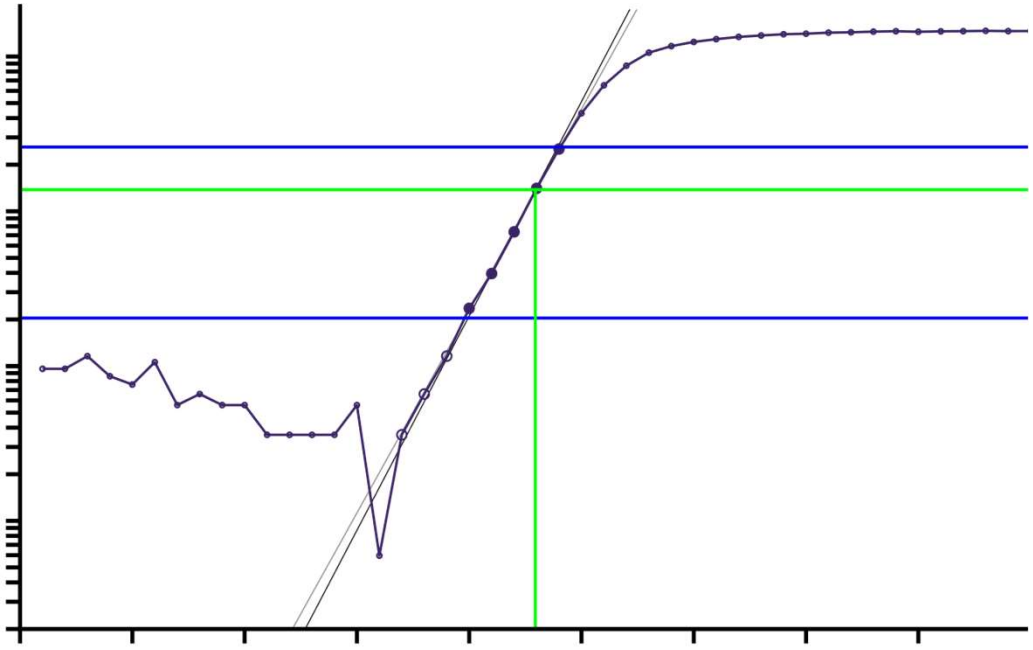
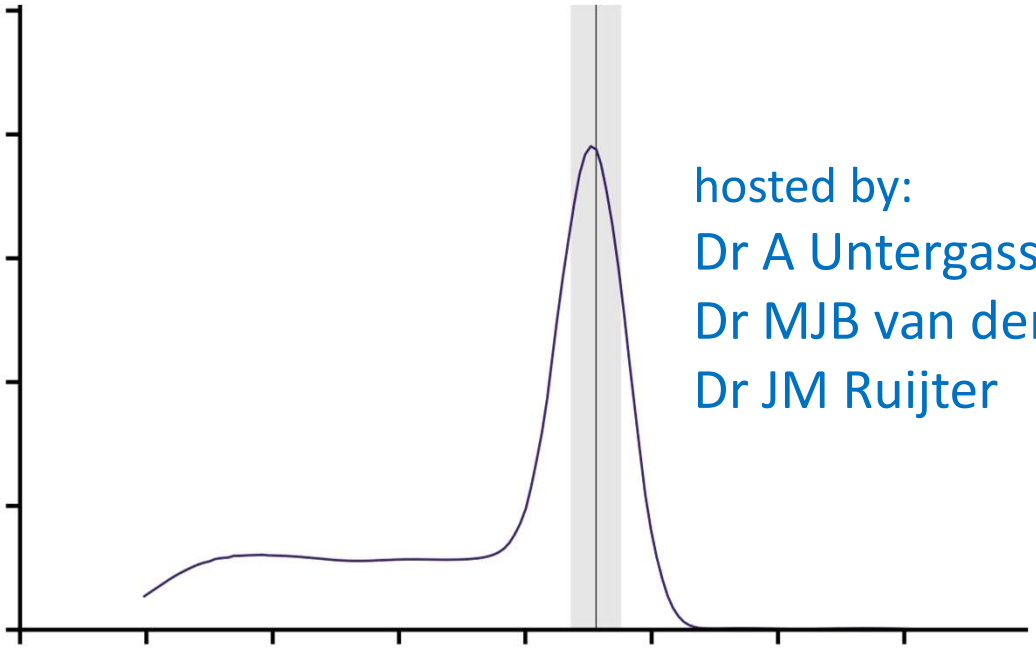
10th Gene Quantification Event qPCR dPCR & NGS
20-24 March 2023



Workshop: Web-based qPCR data analysis.

Unbiased platform-independent analysis of qPCR data
From fluorescent data to publishable graphs



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Workshop: Web-based analysis of qPCR data

Unbiased platform-independent analysis of qPCR data;
from fluorescent data to publishable graphs.

Workshop program, Tuesday March 23, 2023

1: Basics of amplification curve analysis (9.00-10.00)

Theory**:** Basics of amplification curve analysis, with PCR Efficiency determination from the exponential phase and calculation of the efficiency-corrected target quantity (N0) per reaction (with Nq, Cq and mean E).

Hands-on**:** Using RDML-LinRegPCR to get acquainted with the RDML-tools, with RunView and with amplification curve analysis and quality control.

2: Basics of melting curve analysis (10.15-11.15)

Theory: Basics on melting curves analysis and the option to correct N0 with the fraction fluorescence in the correct melting peak.

Hands-on**:** Using RDML-LinRegPCR, to get acquainted with melting curve analysis and learn how to correct quantification data with melting analysis results.

3: Between plate correction (11.30-12.30)

Theory: How to determine the multiplicative factor per plate and remove the systematic between-plate variation.

Hands-on**:** Using RDML-Analyze and InterRunCorrection, to merge the analysis results of different qPCR runs into a multi-run experiment from which the between-plate variation is removed.

4: Import of data into RDML (13.30-14.30)

Theory: RDML structure; from RDML output of the thermocycler to compiling an RDML-dataset from these raw fluorescence data.

Hands-on**:** Using RDML-Edit and RDML-TableShaper to illustrate the file structure, to adapt the format and to create an RDML file using example data with different export formats.

5: Reference gene identification (14.45-15.45)

Theory: The need for reference genes and how to identify reference genes using the geNorm algorithm.

Hands-on**:** Using RDML-LinRegPCR and RDML-geNorm to identify a set of valid reference genes.

6: From qPCR run to results graph and statistics (16.00-17.00)

Theory**:** How to handle technical replicates, how to apply reference genes, how to calculate average expression per group, how to draw a bar graph, how to test difference between groups.

Hands-on**:** Using RDML-LinRegPCR, RDML-Bargraph and RDML-Statistics you will perform the full analysis from raw fluorescent data till the presentation of the results in a publishable graph.

Exercise 1: Inspect data in an RDML file and analyze the amplification curves

In this workshop you will learn to use the web-based version of RDML-Tools (www.gear-genomics.com/rdml-tools) to perform a complete efficiency-corrected analysis of a qPCR experiment with several PCR runs. In 6 steps you will analyze the data with the methods. Each step is introduced with a lecture and consists of a hands-on exercise, in which you analyze provided data sets following this manual.

1. View the data of an RDML file and analyze amplification curves
2. Analyze melting curves
3. Correct for plate variation
4. Import raw data to RDML
5. Identify reference genes
6. Perform relative quantification

Required data files

Before starting the exercises, you have to retrieve the data files. The data files are available as download from LinRegPCR.nl, file **Dataset Workshop Freising.zip**. Unzip this file and save the data files in an easy to reach folder on your desktop.

1a. View the data of an RDML file

In this short exercise you open RDML file **example_2_tm_annotated.rdml** using RDML-Edit, examine the data and learn about data types.

1. Start your internet browser
2. Go to www.gear-genomics.com/rdml-tools
3. Open RDML-Edit and go to the “Main” tab
4. Load the file **example_2_tm_annotated.rdml** by
    a. Select the file after pressing the “Select” button (Note that the language on the Select button depends on the language of your personal setting).
    b. Press the “Upload File” button.
5. Go to the “Experiments” tab and scroll down to inspect the contents of the file.
    a. How many experiments are in the file?
    b. How many runs does each experiment include?
    c. What is the number of reactions in each run?
6. Go to the “Samples” tab. Scroll through the samples.
7. Go to the “Targets” tab.
8. Change the melting temperature of “ANF” to 83.8°C.
    a. Activate the Edit Mode by clicking on the green button on the top left.
    b. Scroll to the target “ANF” (do NOT change ANFe and ANFa).
    c. Press the Edit button to edit this target.
    d. Change the melting temperature.
    e. Press “Save Changes” at the bottom of the list of target “ANF”.

1b. Analyze the amplification curves of Run 1

In this exercise you analyze the amplification data in the RDML file using RDML-LinRegPCR and check the quality of the amplification curves.

When you did not follow exercise 1a, you first have to:

1. Start your internet browser
2. Go to www.gear-genomics.com/rdml-tools
3. Open RDML-LinRegPCR.
4. Go to the “Main” tab.
5. Select the file **example_2_tm_annotated.rdml**
6. press the “Upload File” button.

otherwise start at.

5. Go to the “Experiments” tab. In each Run you find the **BLUE** link to “Analyze Run in LinRegPCR”.
6. Scroll to “Run 1” and click its “Analyze Run in LinRegPCR” link.

RDML-LinRegPCR will open in a new window and start in “Runview”.
To inspect and analyze the amplification curves you

7. If the “RunView” tab was not activated automatically, switch to the “RunView” tab.

The top line of the page shows which Experiment “Experiment” and Run “Run 1” are selected. The drop-down box at Run shows the runs in this experiment

a. How many runs are present in this file?

The top part of the page shows which data are shown and how the data are displayed, the table shows that plate lay-out and graph show the amplification curves. In each well you see the sample name, target and Cq value. Colors indicate technical replicates which have the same sample name.

a. How many technical replicates of each sample and target are present?
b. Confirm that no Cq are present in the plate setup above the curves, as the file only contains raw fluorescence data.

Below the table you see the amplification curves based on the raw fluorescence, thus before subtraction of the fluorescence baseline. The curves are shown on a logarithmic Y-axis.

a. Confirm that amplification data are present (curves with increasing fluorescence with increasing cycles on the X-axis).
b. What is the approximate fold-increase of fluorescence values between the first cycles and the plateau?
c. Is this what you expect for qPCR data?

The plate view and the amplification curves are linked.

a. Click on an amplification curve (it becomes a thick black line) and look up which well it belongs to (well with a thick black border).

   b. Repeat this with a curve that does not reach the plateau. What is the input in this well?

8. On the top of the page, switch the “Data Source” to “Meltcurve Raw Data”.
   a. Confirm that melting data are present. The curves show decreasing fluorescence with increasing temperature on the X-axis.

You will now analyze the amplification curves:

9. Switch back to “Data Source” “Amplification raw data”.
10. Go to the “LinRegPCR” tab,
11. Set “Update RDML Data:” to yes
12. Click “Run LinRegPCR”.

As the interface warned you, this analysis may take a few minutes. The program estimates a fluorescence baseline for each reaction, sets a Window-of-Linearity for each target and a quantification threshold for the whole run. The intersection of the amplification curve with the quantification threshold is the Cq value per reaction. It then calculates a mean PCR efficiency per target. With the quantification threshold, the mean efficiency per target and the Cq value per reaction the program calculates the N_0 (starting concentration or target quantity) per reaction.
During all those steps the program performs quality controls on the individual amplification curves (Figure 1) and on the parameters derived from these curves (Figure 2).
Because you did set Update RDML data to yes, the Cq values are now added in the plate lay-out shown in the Runview. The amplification curves shown in the graph on this page are now the baseline-corrected amplification curves.

13. Switch to the “Runview” tab and confirm that the Cq values are present in the plate setup.
14. Scroll down to the amplification curves graph
    a. What is the approximate fold-increase in fluorescence of the amplification curve between the first cycles and the plateau?
    b. Is this what you expect?

The Runview interface allows you to display the wells and curves in different colors to facilitate the identification of deviating amplification curves.

15. At the top of the “RunViews” page, set “Select Sample by” to “Sample” and choose one of the samples from the drop-down box. In the plate lay-out the wells of the select samples are shown. The graphs show their amplification curves.
    a. How many targets are shown?
    b. How many technical replicates are present per target?
    c. What is the difference in Cq values between the replicates per target?
    d. Is the difference between Cq values the same for different targets.
16. Set “Select Sample by” to Target and choose Ppia as the target to show.
    a. Which wells are shown?
    b. Which curves are shown?
    c. Do you see deviating curves?
    d. Click on the curve shown in orange and check the well in the plate lay-out

e. Click on another mQ well to compare their curves.

17. Set “Select Sample by” to Sample and choose Adult_5 as the sample to show.
    a. Which wells are shown?
    b. Which curves are shown?
    c. Do you see deviating curves?

16. Switch to the “LinRegPCR” tab and double-click on row 29, well C5 of the results table. This action will open the “RunView” tab and display the amplification curve of this well. Examine the graph:
    a. The dots are the individual measured fluorescence values.
    b. The big dots are part of the exponential phase, which is linear on the logarithmic scale of this graph.
    c. The blue lines indicate the limits of the window of linearity (W-o-L).
    d. The filled big dots were used to calculate the individual PCR efficiency.
    e. The individual PCR efficiency is indicated by the thin grey line.
    f. The mean PCR efficiency is indicated by the thin black line.

    Note that this reaction was selected as an example because the difference between individual and mean PCR efficiency is relatively big.

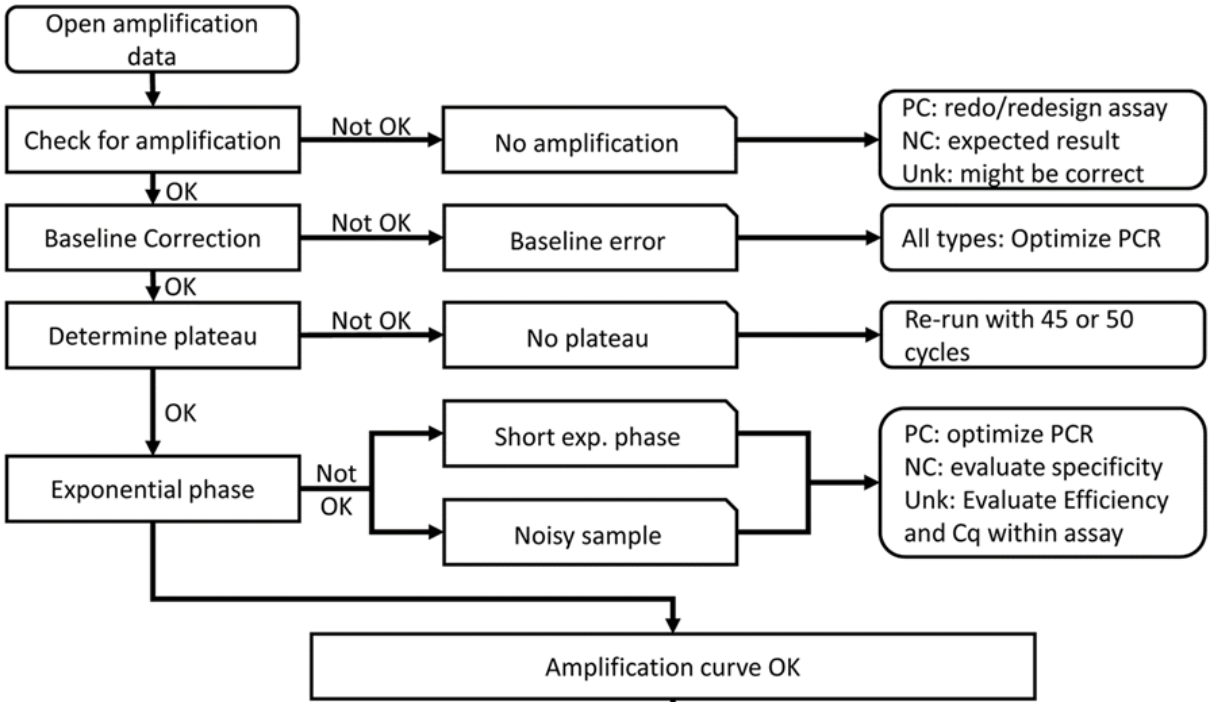

Figure 1. Quality control criteria applied to the individual amplification curve during the baseline correction and W-o-L setting steps. Unk: sample with unknown gene expression; PC: positive control; NC: negative control

We will now have a look at the analysis results table and the quality control criteria that the program applied as shown in Figure 1 and Figure 2. For this task you have to switch between the “LinRegPCR” tab and the “RunView” tab. Start with the “LinRegPCR” tab and scroll through the Results table. Deviating reactions are indicated by an orange cell in one of the columns.

17. For each row with colored cell, or an entry in the column “note”, double-click on the row of the table. This will high-light the well in the plate lay-out and show its amplification curve.

a. Which step in the quality control Figures has led to this exclusion or note?
b. How does the graph of the amplification curve show the detected deviation?
c. Are there excluded reactions in this run?

Finally, you have to save the result of the LinRegPCR analysis of Run 1 to the RDML file:

18. Go to the “LinRegPCR” tab
19. Press “Save RDML” to save the RDML file that now contains Cq and N_0 values.

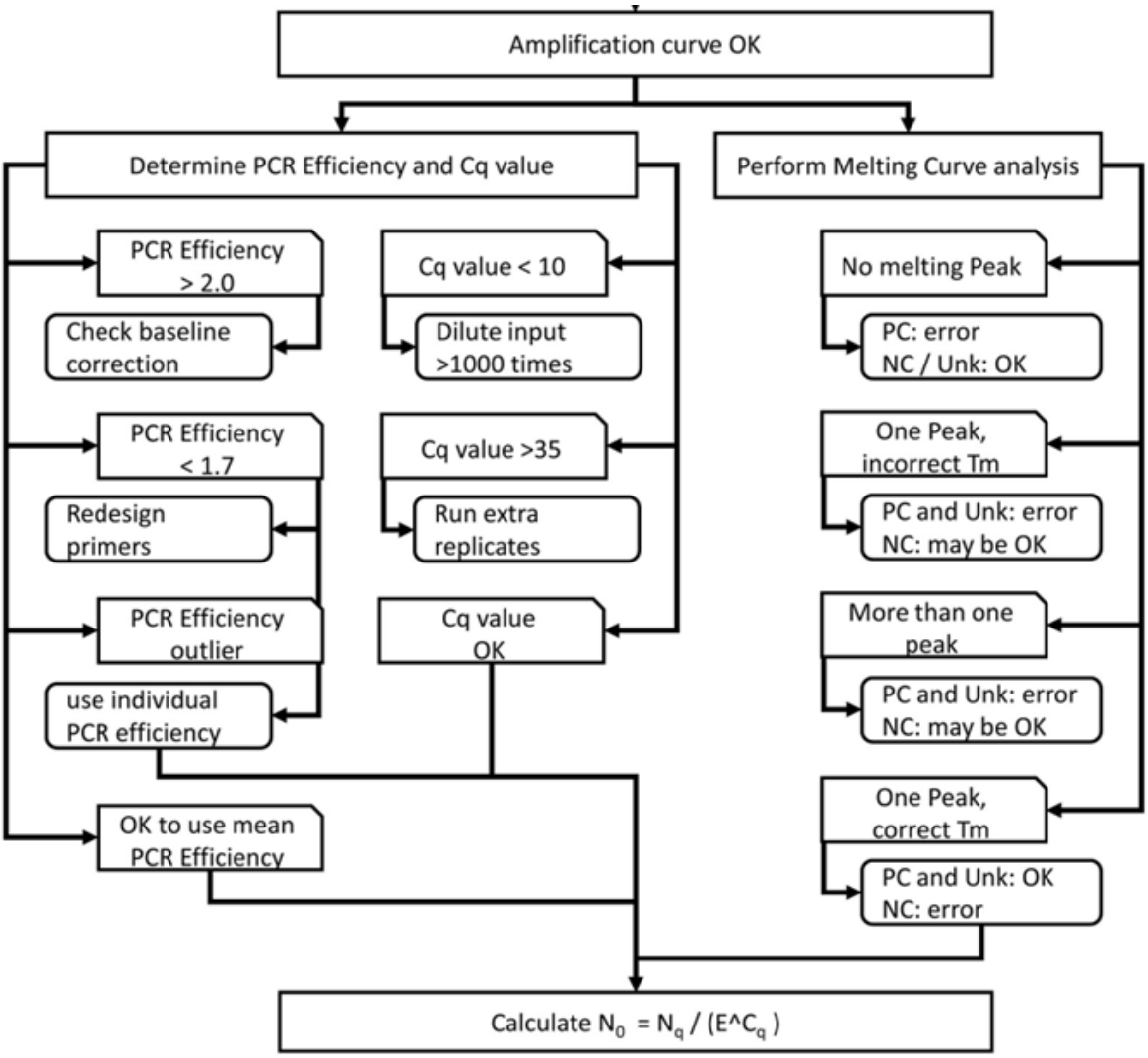

Figure 2. Quality control criteria applied to the PCR efficiency values and Cq values observed in the individual amplification curve. The criteria applied to the melting curve analysis results will be discussed in part 2 of the workshop. Unk: sample with unknown gene expression; PC: positive control; NC: negative control

1c. Analyze the amplification curves of Run 2

As you saw when you inspected this RDML file, the experiment consisted of 3 runs. In this first exercise we also have to analyze Run 2.

1. Go to the “RunView tab”
2. Select Run 2
3. Go to the “LinRegPCR” tab,
4. Set “Update RDML Data:” to yes
5. Click “Run LinRegPCR”.
6. Go back to the “RunView” tab

In the graphs you now see some amplification curves that deviate from what you saw in Run 1.

7. Repeat step 17 of exercise 1b to examine the deviating amplification curves in Run 2.

The LinRegPCR interface has a number of build-in selection criteria and, on the top of the “LinRegPCR” tab, gives the user a number of options to include or exclude deviating amplification curves from of the analysis, either partly (no inclusion in the mean PCR efficiency per target) or completely (no Cq and N_0 reported). In most cases the default settings are the best.

Finally, you have to save the result of the analysis of Run 2 to the RDML file:

8. Go to the “LinRegPCR” tab
9. Press “Save RDML” to save the RDML file that now contains Cq and N_0 values.

Exercise 2a. Analyze the melting curves

In this exercise you analyze the melting curves in the RDML file **example_2_tm_annotated.rdml** using RDML-LinRegPCR.

1. If you do not have it still open from the previous exercise, start your internet browser, go to www.gear-genomics.com/rdml-tools, start RDML-LinRegPCR, select and upload the file **example_2_tm_annotated.rdml** by "Selecting" it on the "Main" tab and pressing the "Upload File" button.
2. On the "RunView" tab select Run 1
3. Select Data Source: "Meltcurve Raw Data"
4. Have a look at the melting curve graphs at the bottom of the page
    a. Note the annotation of the X-axis.
    b. Are these the curves that you expected?
5. Go to the "Melting Curve Analysis" tab,
6. Set "Update RDML Data:" to yes
7. Click "Run Meltcurve".

The program now performs a number of steps to smooth and normalize (= scales between 1 and 0) the melting curves and then calculates the negative first derivative to determine the temperature at which the melting of the dsDNA is fastest. This temperature is referred to as the melting temperature (Tm)

8. Scroll through the Results. Note the presence of an "expected melting temperature" column.
    a. Where does the expected melting temperature (Tm) come from?
    b. Why is the expected melting temperature important?
    c. Look at the differences in the values in the column "peak temp".
    d. Double click on row 7 (well A7); this will open this reaction in the tab "RunView".
9. Now the reaction in well A7 (Adult_1, SCX) is displayed. Check the curves graph:
    a. The first derivative of the fluorescence values is displayed.
    b. What is indicated by the thin black vertical line?
    c. What is indicated by the grey box around the thin black line?
10. Scroll to the top left. In the drop-down box at Data Source you can follow the processing steps from raw melting data to smoothed, normalized and first derivative data and
    a. Step through the processing steps and explain the changes on the graph.
11. Set the Data Source to "Meltcurve – First Derivative"
12. Set "Select Sample by" to "Show All"
    a. Now all melting peak curves are displayed and several groups can already be distinguished.

Different targets have a different Tm, and the same target has the same Tm in different samples, provided that the same master mix, machine and melting protocol is used.

13. Set "Select Sample by" to "Target" and choose the target "Ppia"

   a. Now you see only all melting peaks observed for the Ppia target and the vertical black line indicating the expected melting temperature.
      i. Are there reactions with an deviating Tm?
   b. Click on the curve which does not have a peak.
      i. To which well does it belong?
      ii. Is the missing peak a problem?
14. Repeat step 13 for every target in the run.
   a. Are there targets that with deviating melting curves?
15. Set “Select Sample by” to “Sample” and choose sample “mQ”
   a. Check the curves of these negative control reactions.
      i. Are there issues to consider when reporting about these data?
16. Save the RDML file on the “LinRegPCR” or the “Melting Curve Analysis” tab. The RDML file now includes the conclusions from the melting curve analysis.

As you saw when you inspected this RDML file in the first exercise, this experiment consisted of 3 runs. In this second exercise we also have to analyse Run 3.

1. Go to the “RunView tab”
2. Select Run 3
3. Go to the “Melting Curve Analysis” tab,
4. Set “Update RDML Data:” to yes
5. Click “Run MeltCurve” and wait for the Results to appear.
6. Go back to the “RunView” tab

In the graphs you now see some melting curves that deviate from what you saw in Run 1 and what you expected.

7. Use the display tools and the Melting Curve Results table and Figure 2 of the first exercise to examine the melting curve analysis results.
   a. Determine which wells, samples and targets show deviating melting peaks
   b. What has the program decided to do with those reactions and why?.

The Melting Curve interface has a number of build-in selection criteria and gives the user a number of options to include or exclude deviating peaks from the analysis, The most important of these are the True Peak Tolerance and the Artifact Peak Tolerance. The True Peak Tolerance defines how far the melting peak can deviate from the expected Tm of the expected correct product to still be considered the correct peak. Similarly, the Artifact Peak Tolerance determines how far artifact peaks should be apart to be scored as different artifacts. A low Artifact Peak Tolerance results in more columns in the results table, each with a specific artifact Tm.

Exercise 2b. Correction of the amplification curve analysis results the melting curve analysis results

In this exercise you will analyze the RDML file **example_LCGreen_raw.rdml** with amplification and melting curve data using RDML-LinRegPCR. The data in this file result from an experiment in which the input of the PCR consisted of a mixture of two targets: the correct product (CRE) and an artefact that is amplified with the same primer pair. So, in most reactions there are two melting peaks. The presence of this second amplification product affects the quantification of the correct product because the artifact contributes to the observed fluorescence and therefore an earlier Cq is determined. As explained in the lecture on melting curve analysis, the fraction

fluorescence in the melting peak of the correct product can be used to correct the observed N0 results. This correction requires the PCR to be monitored with a saturating DNA binding dye, e.g. LCGreen or EvaGreen.

1. Start RDML-Edit and load the file **example_LCGreen_raw.rdml** by Selecting it on the “Main” tab and pressing the “Upload File” button.
2. This file only contains raw data. The correction only works with saturating dyes. Therefore, this property has to added:
    a. Activate the edit mode by pressing the “Enable Edit mode” button on the top left.
    b. Go to the “More…” tab.
    c. Scroll to the “Dyes” section.
    d. Click the “Edit” button in the dye “Lcgreen” field.
    e. Change the “Fluorescence Reporter” field to “saturating DNA binding dye”.
    f. Press “Save Changes” at the bottom of the field.
3. Go to the “Targets” tab, set the melting temperature of “CRE” to 90.2°C and press “Save Changes” at the bottom of the list.
4. Go to the “Experiments” tab, go to the field “Run with Artifacts” and click the blue link “Analyze Run in LinRegPCR”.

The program opens RDML-LinRegPCR. The amplification curve analysis with LinRegPCR has to be run before you run the melting curve analysis. Therefore:

5. Go to the “LinRegPCR” tab, switch “Update RDML Data:” to yes and click the “Run LinRegPCR” button.
    a. In the “RunView” tab you now see that all amplification curves have a similar slope, indicating equal PCR efficiencies for all reactions.
6. Go to the “Melting Curve Analysis” tab, switch “Update RDML Data:” to yes and click the “Run Meltcurve” button.
    a. The melting curve results table shows that in most of the reactions two or more melting peaks are observed: the expected product with a melting peak at 90.2°C and a second peak with a Tm between 82.5 and 83.3°C or lower
7. Go to the “RunView" and inspect the melting peak graphs.
    a. Set “Data Source” to “Meltcurve – Raw Data” and look at the graph.
    b. Do you notice the ‘shoulder’ in all reactions, except the upper set of curves?
    c. Set “Data Source” to “Meltcurve – First Derivative”
    d. Set “Select Sample by” to Sample and display the different samples. The name of the samples gives the percentage C and the percentage A in the input mixture.
    e. Note the relative size of the correct peak (Tm = 90.2°C) and the other peaks.

A saturating DNA binding dye allows the correction of observed N_0 values with the fraction of fluorescence in the correct peak. Because you defined LCgreen as a saturating dye (step2), pressing the “Run Meltcurve” button has created the “Corrected Data” tab. The program has already performed the correction.

8. Go to the “Corrected Data” tab.
9. Scroll through the table to see how the Cq and N_0 values are corrected using the fraction fluorescence in the correct peak.

a. Given the mixtures of C and A in the input (column 'sample'), did you expect the values in the column 'correction factor'?
b. Are the changes in Cq and N_0 in the direction that you expected?

10. Save the corrected results table by pressing "Save table as CSV" on the top right of the page
11. Save the RDML file on the "Main" tab. It now includes the corrected Cq and N_0 values.

This CSV file can be imported into Excel to draw a graph of the corrected N0 values (Figure 3a and 3b). To facilitate their interpretation the mean observed and corrected N0 values in sample A_0_C_100 were set to 100. Comparison of the graphs shows that the corrected N0 data (Figure 3b) reflect the expected 100-75-50-25-0 series of values whereas the observed data (Figure 3a) show a large over-estimation of the C content in the mixtures.

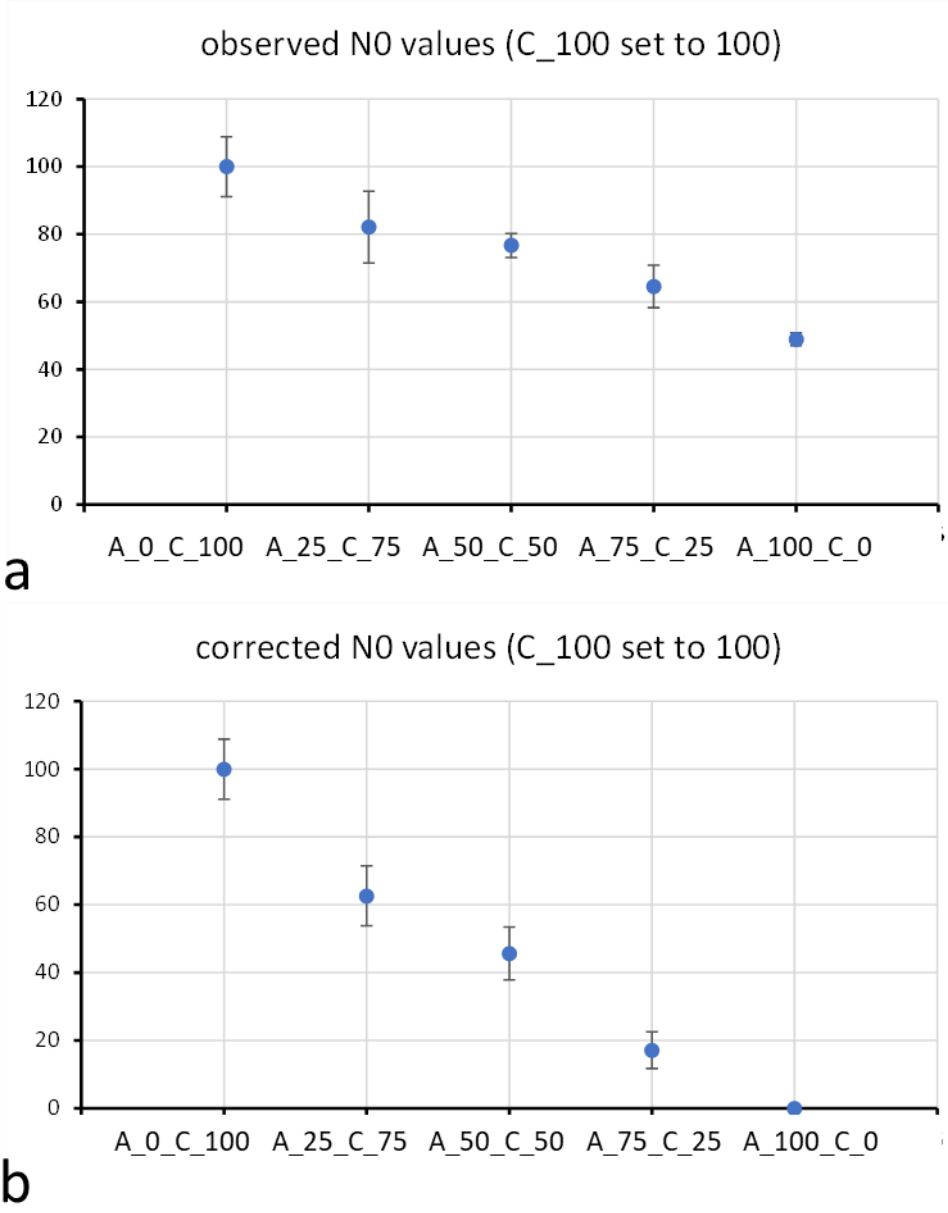

Figure 3. Observed N0 values (a) and corrected N0 values (b) after amplification and melting curve analysis of the data in the **example_LCGreen_raw.rdml** file.

3. Combine both runs

In this exercise you will learn how to combine the LinRegPCR results that were saved for each of the runs in the **example_2_tm_annotated.rdml** file into a single results sheet, as if the 3 runs were one large plate. As shown in the lecture, for unknown reasons different qPCR plates, run under standardized conditions, can show a multiplicative difference between plates. To combine the 3 runs in the experiment we will need RDML-AnnotationEdit and RDML-Analyze.

1. Start your internet browser and go to https://www.gear-genomics.com/rdml-tools/
2. Start RDML-Analyze
3. Select and Upload the file **example_3_linregpcr.rdml**
4. Go to the tab “InterRunCorrection”, switch “Update RDML Data:” to yes and click “Run InterRunCorrection”.

The pink “Error Plate” warning tells you that the program “could not fix all matrix gaps”. Why is the inter-run correction not working?

To answer this question we have to check that there is overlap in conditions (samples and targets) between the runs.

5. Return to RDML-Tools
6. Start RDML-Edit
7. Select and Upload the **example_3_linregpcr.rdml** RDML file.
8. Scroll down to Run 1 and click the blue “View Run in RunView”
9. Check that on each of the Runs there are Cq values displayed in the plate layout.
    a. If not, run LinRegPCR to analyze the amplification curves in this run.
10. Inspect which samples are present on each of the plates.
    a. Note that Run 1 and Run 3 only have Adult and Embryo samples, respectively, whereas Run 2 contains Adult and Embryo sample
    b. Is this enough overlap between runs?

There is overlap in sample origin (Adult and Embryo), but the program only “sees” different sample names. And these are different between plates. As no samples overlap, we need to create an annotation including all embryo samples in one group and all adult samples in another. Note that we need overlap between sample groups, not necessarily overlap in individual samples.

11. Return to RDML-Tools
12. Start RDML-AnnotationEdit
13. Select and Upload the **example_3_linregpcr.rdml** RDML file.
14. Go to the tab “AnnotationEdit”
15. Scroll down to the “Create New Annotation” field.
16. Fill the “property” field with “group” and the “value” field with “embryo”.
17. Select all embryo samples and click once on the button “Create Annotations”
    - Seemingly nothing happens, but when you scroll down you see that the embryo samples did get the annotation “embryo”.
18. Press the “Deselect All Samples” button
19. Fill the “property” field with “group” and the “value” field with “adult”.
20. Select all adult samples and click once on the button “Create Annotations”.

- Scroll down to check that the annotation has been assigned correctly.

21. Save by pressing the RDML file button on the top right of the page.

As the assignment annotations groups Adult and Embryo are attached to the samples, this annotation is applied to all 3 runs in the experiment. Therefore, based on this annotation the Run 2 now serves to provide overlap between Run 1 and Run 3. We can now determine the between-plate difference and remove it.

22. Start your internet browser,
23. Start RDML-Analyze
24. Select and Upload the **example_3_linregpcr.rdml** RDML file.
25. Go to the tab “InterRunCorrection”
26. Select overlap “Same Annotation” and the sel. Annotation “group”.
27. Set “Update RDML Data:” to yes
28. Click the “Run InterRunCorrection” button.

The program determines the correction factors per plate and applies them to the results of the individual runs.

29. Check the correction factors and the output.
30. Save the RDML file.
31. Check on the tab “RunView” to see the calculated results.
32. Tick the boxes for “raw Cq” and “raw N_0” to see the values before correction.

This exercise concentrates on the RDML format which was originally developed for the exchange of qPCR data, together with all information needed to repeat, and reproduce, a qPCR experiment.

4a. Export RDES files from RDML

In this exercise you get to know the RDES format. This is a file format that was recently developed to enable the distribution and sharing of raw fluorescence data. Whereas the full RDML format encompasses all information to describe and reproduce a qPCR experiment, the RDES format is restricted to the raw fluorescence data annotated with well, target sample and dye information. These exercises are to familiarize you with this format.

In the first part of this exercise, you will extract the from the RDML file that we have been using in this workshop into an RDES file. Just to familiarize you with this format.

1. Start your internet browser and go to https://www.gear-genomics.com/rdml-tools/,
2. Start RDML-Edit and go to the “Main” tab
3. Load the file **example_2_tm_annotated.rdml** by
    - Selecting the file after pressing the “Choose File” button
    - Pressing the “Upload File” button.
4. Go to the “Experiments” tab.
5. Go to Run 1 and press the button “Export Amplification Data”.
    - The RDML-Tools export data to files that will be placed in the Downloads folder of your computer.
    - Go to your Downloads folder, rename the file to “amplification_data.csv” and move it the folder you created for your data files.
6. Stay in Run 1 and press the button “Export Melting Data”.
    - Go to your Downloads folder, rename the file to “melting_data.csv” and move it the folder you created for your data files.
7. Open the saved files in a spreadsheet program, like Calc or Excel
    - Start your spreadsheet program and drop the CSV file on an empty sheet.
    - The CSV files use points as decimal separators. Take care that this decimal point is recognized by your spreadsheet program. Easy check: the fluorescence values all contain a decimal separator.
    - Evaluate the column headers. What information is included in the RDES format?
    - Why is the columns Cq empty?
8. What is the difference between the amplification and the melting data?
9. Close Excel before you start with exercise 4b.

4b. Create RDES files from a known spreadsheet format

However, in most case you will start the analysis of your data with a file exported by your qPCR machine. These files differ between machines. In this exercise you import the file **course_plate_1.txt** which is a text file with the amplification and melting curve fluorescence values of a rum exported by the LightCycler 480. You will use this file to create an RDML file using RDML-TableShaper and RDML-Edit.

1. Start your internet browser,
2. Open the RDML-Tools (www.gear-genomics.com/rdml-tools)
3. Start RDML-Tableshaper
4. Select and Upload the **course_plate_1.txt** file into RDML-TableShaper

The file contains amplification and melting curve data and is in a table format with one row per well/cycle/temperature (repeated wells in column SamplePos) and Cycle (column Cycle#). Scroll through the table and note that after 45 cycles, the Cylce# column changes to 1 whereas the temperature (column Temp) starts to rise. At that row, the column Prog# changes from 2 to 3, which is from the amplification program to the melting program. At row 197, the SamplePos column changes to A2 and the Prog# to 2 and we are looking at amplification data again. This goes on for all used wells in the plate. But TableShaper only shows the first 500 rows.

First extract the Amplification data:

5. Go to the “Modify” tab and under “Load predefined setting” choose “Roche LCS480 Amplification v1.5.0.39” from the drop-down list. These are the predefined settings for importing amplification data from the LightCycler text export into RDML.

After loading these predefined settings the program has automatically extracted the Amplification data from the uploaded TXT file.

6. Go to the Save tab.

The table at the bottom of the screen shows the amplification data in a row per well and column per cycle format. You recognize the RDES format that we studied in exercise 4a.

7. On the top right of the Save tab click “Save table file in RDES format (.tsv)”
   - You find the saved file “course_plate_1_amplification.tsv” in your Downloads folder
   - Transfer the file to your data folder.

Now extract the Melting curve data:

8. In RDML-TableShaper go back to the “Modify” tab and select under “Load predefined settings” choose “Roche LCS480 Meltcurve v1.5.0.39”

The table at the bottom of the Save tab now shows the melting curve data in a well per row and temperature per column format

9. Go to the Save tab and click “Save table file in RDES format (.tsv)”
    - You find the saved file “course_plate_1_melting.tsv” in the download folder
    - Transfer the file to your data folder

Both files only contain the Well (column 1) and amplification or melting curve information (column 7 and further). The other leading columns just have a header and default values. To complete the RDES format for analysis with RDML-LinRegPCR the columns 2 till 6 have to be filled. The annotation of the reactions for this experiment is given in “**course_annotation.xlsx**“

10. Start Excel, create an new sheet and drop the **course_1_amplification.tsv** file that you saved in step 5 on this empty sheet.
11. In Excel, also open the file “**course_annotation.xlsx**”
    - Note that in this file there are empty rows for, e.g. wells A15 till A24.
    - In the **course_1_amplification.tsv** file there are fluorescence values for these wells. However, these values do not increase in later cycles. They are just background fluorescence of empty wells.
12. Complete columns 2 to 6 of the **course_1_amplification.tsv** file by copying columns 2 to 6 from sheet Plate_1 of **course_annotation.xlsx** file into the **course_1_amplification.tsv** file, thus replacing these columns in this file.
13. In Excel, save the completed **course_1_amplification** data sheet with a new name “**course_1_amplification_completed**” but keep the Tab-delimited format (replace the file format txt with tsv)
14. Repeat steps 8 till 11 for the file **course_plate_1_melting.tsv** that you saved in step 7 and save the file as **course_plate_1_melting_completed.tsv** in step 11.

4c. Create an RDML file from the created RDES files

This would be the place to start when your qPCR machine exports RDES files. Unfortunately, this is not the case, yet.

1. In your internet browser, go to www.gear-genomics.com/rdml-tools, and open RDML-Edit,
2. Click the “create new file” button
    - The program switches to the “Experiments” tab and is in Edit mode. However, when you see the button “Enable Edit Mode” you have to press it
3. If needed: Press the “Enable Edit Mode” button to allow yourself to add your data to the experiment.

An RDML file can contain multiple experiments and each experiment can contain multiple runs. For now we only have one run (course_plarte_1) to add to this file.

4. Go to the "Experiments" tab and create a new experiment by clicking on "Create New Experiment".

5. Give a name for this experiment, e.g. “course plates” and press “Save Changes”.
6. Create a run by clicking the "New Run" button in this new experiment.
7. Name the run “Plate 1” by replacing the default “New Run”
8. Set the correct plate setup (in this case “384 Well Plate”)
9. At “Import Amplification RDES”, select the amplification data file that you have created from **course_1_amplification_completed** in the previous exercise.
10. At “Import Melting RDES”, select the melting data file that you have created from **course_1_amplification_completed** in the previous exercise.
11. Save the run by pressing "Save Changes".

RDML-Edit now converts the file into RDML format, creates the ‘header’ items of the RDML file and removes the empty wells and the wells without complete annotation See the Pink message block and check that this was done correctly. You see that various elements of the RDML format are created (Targets, Dyes etc.) and the empty wells are removed.

12. Go to the “Experiments” tab.
    - How many reactions were added to the run "Plate 1"?
    - In the run description you find the link to “Analyze Run in LinRegPCR”.
    - Click this link and RDML-LinRegPCR will open in “Runview”.
13. Investigate the amplification curves of the raw fluorescence data of the amplification and the melting curves.
    - Note that the data are NOT yet analyzed, the switch to RDML-LinRegPCR was just a quick short-cut to open RunView and inspect the file contents.

14. Return to RDML-Edit
15. Go to the "Main" tab (below the huge pink message) and save the RDML file by hitting “Save File”.
    - You find the saved file in your Downloads folder with the default file name **data.rdml**.
    - Rename the file to “**course_plate_1.rdml**” and transfer it to your data folder.
16. Open the newly created “**course_plate_1.rdml**” in RDML-Validate to confirm that the created file is a valid RDML file.

Exercise 5: RDML and web-based geNorm:

Because biological and clinical samples always differ in size and composition, the results of a qPCR analysis are mostly expressed, or normalized, with respect to the expression of a set of reference genes. As discussed in the lecture, these reference genes have to be validated for the different experimental conditions present in the design of the complete experiment.

This exercise consists of two parts. Firstly, the import of data in an unknown table format into RDML and secondly, the identification of reference genes with the web-based version of geNorm which is part of RDML-Analyze. The total exercise consists of the following steps

a) Create a text file from Excel data
b) Reshape the data with RDML-TableShaper and create an RDML file
c) Add annotation to the RDML file
d) Analyze the amplification curves with LinRegPCR
e) Identify the best set of reference targets with web-based geNorm

In this exercise you start with the file **geNorm_exercise.xlsx**, export the data to a text file, and then create an RDML file using RDML-TableShaper and RDML-Edit WITHOUT using predefined settings as you did in exercise 4b.

Exercise 5a. Create two text files from the Excel data file

1. Open the file **geNorm_exercise.xlsx** in Excel
2. Select the sheet “geNorm_exercise”
3. Save this sheet as txt file by selecting “Save as”.
   - Rename to **geNorm_exercise_data.txt**
   - Choose the export type “Text (tab delimited)”
   - Make a note of the file extension (.txt or .csv).
   - Accept the Warnings of Excel that data (on other sheets) may be lost.
4. Select the sheet “tissue_annotation”.
5. Save this sheet as txt file by selecting “Save as”.
   - rename this file “**geNorm_exercise_annotation.txt**”.

Exercise 5b. Reshape the data with RDML-TableShaper and create an RDML file

1. Start your internet browser and go to https://www.gear-genomics.com/rdml-tools/
2. Start RDML-TableShaper” and go to the “Load” tab
3. Select and upload the TXT (or CSV) “**geNorm_exercise_data.txt**” file that you saved in exercise 5a, step 3.
4. Check that you have the correct column separator (On the “Load page, you should see separate columns in the “Table after import” field).
5. Switch to the "Modify" tab.
6. In the field "Settings to reshape the table" behind "Select PCR type" choose to import amplification data by selecting “Amplification” from the drop-down box

You recognize the RDES format with columns for every variable and rows for each reaction. However, in this case, the user added some columns therefore hampering the direct import of these data into RDML.

7. Check the table format in the “Table after reshaping” field at the bottom of the page. The table shows separate columns with rows of information for each reaction followed by, in column 14 and further, the fluorescence data per well. We can leave “Keep table shape” unmodified.

For the correct entries in the “Settings to extract the fluorescence data block” fields, you always have to refer to the “Table after reshaping” at the bottom of the screen. Identify the correct columns for each entry (well, sample etc.) in this table. Leave the field open when the information is not present in the file. Not all entries have to be given. The following steps tell you which fields have to be given.

8. Under “Settings to extract the fluorescence data block” give the following entries:
   a) Check the number of heading rows without data by looking at the table below. In this case there is 1 row with titles per column. So: at “Number of heading rows to ignore” enter: 1.
   b) There are no rows to ignore between values: at “Number of rows to ignore between values” enter: 0
   c) Provide the number of heading columns without fluorescence. In this case, including the “well” column, there are 14 columns. at “Number of heading columns to delete” enter: 14
   d) Set the checkbox "Replace all commas with dots" if your decimal separator is a comma like in European data formats. When you see commas in the fluorescence values in the table, keep this box checked, otherwise, un-check.
9. Under “Settings to extract data” you have to give the columns of the table that give information on the samples and targets per well. Give the following entries:
   a) At “Extract cycle data (Amp) … from row” enter: 1
   b) At “Extract well information from column” enter: 1
   c) At “Extract sample information from column” enter: 2
   d) At “Extract sample type information from column” enter: 3
   e) At “Extract target information from column” enter: 4
   f) At “Extract target type information from column” enter: 5
   g) At “Extract dye information from column” enter: 6
10. Press the "Save Settings JSON file" button. This will tell the program to use the settings and to convert the imported table into a proper RDES format.
    - When you expect that in the future you will encounter an Excel file with the same format you have to go to your Downloads folder and move the “TableShaper_settings.json” file to your data folder. For this exercise this in not needed.

The results of the conversion can be seen on the “Save” tab of RDML-TableShaper:

11. Switch to the "Save" tab
12. Click the Button “384 Well Plate” to select the correct plate format.
13. Scroll down to check that there are no missing data in the table.
    - When everything is green, you succeeded in converting the data.
14. Scroll up and press the button “Create RDML”.
15. RDML-Edit opens up, go to the Experiments tab to check how many reactions are present in the first Run of the first Experiment (this should be 207).
16. Do NOT close the RDML-Tools but continue with exercise 5c.

Exercise 5c: Add annotation to an RDML file

The dataset that you imported consists of samples from different types of experiments that can be performed with tissue from embryonic and adult heart. Although we prefer to have a single set of reference genes for all heart research, we already know that that is not going to happen. Therefore, we need to add annotation to the samples to indicate to which kind of experiment the sample belongs. The experiments are “development”, “infarction” and transverse aortic constriction, abbreviated as “tac”. Instead of assigning this annotation by hand, in this exercise we will use a spreadsheet approach to import the annotation.

The second sheet in the **geNorm_exercise.xlsx** file contains a list of all tissues in the RDML file and 3 columns with an entry “Y” when the tissue belongs to the experiment “development”, “infarction” or “tac”, respectively.

1. Go back to the “Samples” tab of RDML-Edit,
2. Press “Enable Edit Mode” to allow changes to the RDML file.
3. At “Import Annotation Data” select the file “**geNorm_exercise_annotation.txt”** that you created in exercise 5a.
4. Press the “Import Annotation” button.

When you scroll down on the Samples tab of RDML-Edit you can see that for each of the tissue the annotation to development, infarction and/or tac has been added.

Exercise 5d: Analyze the amplification curves with LinRegPCR

The input for the reference gene validation with geNorm are the N0 values that reflect the expression of the candidate reference genes in each of the tissues in the dataset. Therefore, we need to run LinRegPCR to determine these N0 values.

1. In RDML-Edit: go to the “Experiments” tab,
2. In the Run 1 field click the blue link “Analyze Run in LinRegPCR”.
3. RDML-LinRegPCR opens up, go to the LinRegPCR tab, set “Update RDML data” to yes and press “Run LinRegPCR”.
4. Click on "Save RDML"
    a. You find the saved file **data.rdml** in your Downloads folder
    b. Rename the file to **geNorm_data.rdml** and transfer it to your data folder

Exercise 5e. Identify the best set of reference targets with web-based geNorm.

In this exercise you open the open the previously generated file **geNorm_data.rdml**. When you failed in the previous steps you can open the provided file **geNorm_LinRegPCR.rdml.** We will now analyze the reference genes in this file using RDML-Analyze.

1. Start RDML-Analyze, select and upload the **geNorm_data.rdml** file that you saved in the exercise 5d.
    - When you failed in the previous steps you can open the provided file **geNorm_LinRegPCR.rdml.**
2. Navigate to the “geNorm” tab and click “Run geNorm”.
3. Save the M-values and V-values graphs to your folder

4. Interpret the results of geNorm:
    - How many reference genes are required?
    - What can you conclude from the M-values?

The large number of reference genes that is required indicates that the candidate reference genes are not stable in the tissues included in this dataset. As we saw in the annotation, this is because these tissues are derived from three types of cardiovascular experiments: heart tissues obtained after experimental infarction, after transverse aortic constriction (tac) and tissues harvested during development. This may be the reason for the instability of the candidate reference genes.

5. On the geNorm tab set: Select Samples – “By Annotation”, Sel. Annotation – the subset you want to analyze and Sel. Value – “Y”.
    - Perform this analysis for “development”, “infarction” and “tac”.
6. Compare the results of the three geNorm analyses.

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Exercise 6. Perform relative quantification

The term relative quantification refers to the common approach in qPCR to express the observed gene expression in a number or technical replicate measurements of each biological sample with respect to the geometric mean expression of a set of reference genes that were validated for the specific type of experiment and also measured in that sample. This expression value is commonly referred to as the normalized expression.

The relative quantification procedure thus consists of a number of steps:

1. Handling the technical replicates and performing a quality check on these replicates.
2. Applying the normalization based the geometric mean of the reference genes.
3. Calculating the mean expression per experimental group.
4. Drawing a bar graph of the mean, with SD or SEM as error bar.
5. Do the appropriate statistical test between groups in the experiment.

Steps 1-3 are performed by RDML-Analyze and the results per step are displayed in its results table. This table can be imported into RDML-BarGraph to perform step 4. The results of step 5, the between-group statistics, are at the bottom of the relative quantification results table.

1. Start your internet browser, and go to https://www.gear-genomics.com/rdml-tools/
2. Start RDML-Analyze
3. On the “Main” tab, select and upload the file **example_4_interrun.rdml**
4. Go to the “RunView” tab, if not opened automatically
   Confirm that the amplification curves are already analyzed
   - Are there Cq values in the plate layout?
   - Are the amplification curves baseline corrected?
5. At “Select Annotation” choose “Tissue”.
6. Check the box “Annotation”
   In the plate lay-out the program now displays the annotation per well
   - Which groups are distinguished in each run?
7. At “Run” select “Run 3”
8. Check the box “Excluded”
   - Which wells are excluded and why?

   Note: when there is only the text “Excluded:” the reaction is NOT excluded
9. Check the box “corr Plate”
   In the plate lay-out the program now displays the between-run correction factor: corr P
   - What is this factor for Run 1, Run 2 and Run 3?
   - Use a calculator to multiple these numbers. What result do you expect?

The corrected N0 values (corrected between-runs) are used to perform the calculation of the mean expression per target and per biological sample (mean of technical replicates) and, with this expression per sample, the normalized expression per tissue sample. This normalized expression is then used to calculate the mean expression per group in the experiment.

10. Switch to the “RelativeQuantification” tab.
11. Select “Overlap” by “Same Annotation”.
12. At the “Sel. Annotation” drop-down box select “Tissue”.
13. Press the button “Run RelativeQuantification”

The program has now performed the different steps required in the relative quantification and has listed the results in the table on this page. The first part of the table, labelled “Technical Replicates” shows the calculations performed on the technical replicates per biological sample: calculation of the mean N0 and its Standard Deviation per sample.

14. Scroll through the Results in the “Technical Replicates” section:
    - Which target types are present in the dataset?
    - What entries do you see in the “Error” column?
    - Can you explain the reason for this error?
    - What does the Note “Tec. Rep. CV > 0.3” indicate?

How to address these samples? A coefficient of variation (CV) of more than 0.3 means that the technical replicates are too far apart. However, with a low number of replicates, it will be hard to decide which reaction is deviating. Therefore, it is important to always make notes of issues that occurred while preparing the qPCR plate. If you had made a note that you were not sure of your pipetting actions with this well, you can decide to remove a deviating reaction from the analysis and to re-analyze the whole run. If not, it is your data and they should be included. In this example there were no notes in the lab-log, so no replicates were removed despite the CV > 0.3 notes.

15. Scroll through the Results till you reach the “Reference Genes” section:
    - Confirm that all reactions have both reference genes.
    - Note that the geometric mean of the expression of the two reference genes is used as the normalization factor.

The normalized expression of each of the targets of interest in each of the tissues is calculated by dividing the mean expression (first section of the Results) by the normalization factor (second section of the Results).

16. Scroll through the Results till you reach the "Relative Expression” section:
    - Why do some reactions have a normalized expression of -1.0?
      Hint: check the error column in the “Technical Replicates” section.

In the “Expression per Annotation” section of the table the relative expression per sample and target of interest is averaged per tissue group to determine for each target the mean expression per tissue. The program chooses the appropriate statistical test depending on the choice made at the top of the page (default: non-parametric) and the number of groups in the dataset (Figure 4).

17. Scroll through the Results in the “Expression per Annotation” and the “Statistics” sections:
    - Can you explain why this statistical test was done?

- Why do the tests for ANFa and ANFe fail?

When the design of your experiment is more complicated that just 2 or more groups in a one-way design, and thus requires more sophisticated statistical tests, you have to save the results table from RDML-Analyze to a CSV file (see buttons on top of the page) which you can import into all spreadsheet programs and in most statistical packages.

	Parametric (normal distribution)	Non-parametric (normal distribution not sure)
Two groups	Tests based on mean and SD t-test test statistic: t = diff / SE(diff) compare to t-distribution	Tests based on ranks Mann-Whitney test test statistic: U compare to Z-distribution
More groups	ANalysis Of VAriance (ANOVA) test statistic: F = Var-between / Var-within compare to F-distribution	Kruskal-Wallis test test statistic: T compare to Chi^2 distribution
	NOTE: when only two groups: $t = \sqrt{(F)}$	NOTE: When only two groups: $U = \sqrt{(T)}$

Figure 4. Choice of the appropriate statistical test.

18. Click on the blue link to RDML-BarGraph at the bottom of the page.

The program opens RDML-BarGraph and displays a bar graph combined with a column-scatter graph

19. Evaluate the bar graph.
    - Why do you see no bars for the SCX, ANFa and ANFe genes?
20. Switch back to “RDML-Analyse” and scroll to the “Expression per Annotation” section.
    - Compare the relative expression values of SCX with those of the cTnI gene.

- What is the fold-difference in expression between those genes?
- Can you visualize such a fold-difference on a linear scale?
- How would you solve this problem?

21. Switch back to “RDML-BarGraph”
22. Scroll a bit down and change on the Y-axis the “image scale” to logarithmic.
    The graph now shows bars; remember that you can interpret a logarithmic axis as a fold-difference axis.
    - What do the white dots mean?
    - Why is there an empty space in each tissue?
    - Feel free to modify the graph as you like.
      You can save your preferred graph formatting settings as a JSON file for future use. (see Save and Load JSON file buttons at the top of the page)

When you are not satisfied with the formatting options that RDML-BarGraph gives you, you can:

23. Save the image as SVG file
    - Install InkScape on your computer.

    a. Open the SVG file in Inkscape
    b. Remove the numbers on the Y-axis that overlap.
    c. Feel free to modify the graph as you like.

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All PDFs of these manuscripts are available in the literature.zip file.

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