osteomiR™
Biomarkers of Bone Quality

TechNote TN-02: Analysis of data generated using the osteomiR™ workflow

Part I: Instructions for qPCR analysis of osteomiR™plates:

Roche® LightCycler® 480 II

Part II: Using the osteomiR™ software application for raw data import, quality control analysis and data export

Version 1

Released: December 2016

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1. Introduction

This TechNote guides you through all steps of required for analyzing osteomiR™ qPCR plates on a LightCycler 480 II platform:

1. Setup of a qPCR run on a Roche LightCycler 480 II
2. Export of raw qPCR data
3. Importing of raw qPCR data into the osteomiR™ App provided by TamiRNA

To facilitate the use of the osteomiR™ kit and make your workflow more robust and safe TAmiRNA provides an osteomiR™ specific **Light Cycler 480 II macro**, which is a template that contains all experimental settings for the qPCR run (specified in Annex I) and alleviates the need for programming the LightCycler 480 II yourself.

Macros offer a convenient way of speeding up the creation of an experiment. We have generated the osteomiR™ macro with respective qPCR cycling conditions and microRNA subset information. After initial implementation of the macro on the LightCycler 480 II platform you will only have to click on the macro for starting the experiment, with the proper experimental settings and microRNA subset information already being predefined.

The experimental settings used in the osteomiR™ macro are summarized in Annex I.

The **osteomiR™ App** is a software for standardized analysis of data generated using the osteomiR™ kit and osteomiR™ plates. This software automatically calculates Cq values, performs melting curve (T_m) analysis, allows to check the quality of your samples and subsequently select samples for normalization and data export.

For using the software you will have to upload two types of data: i) the raw qPCR data exported from the Roche LightCycler 480 II as text-file and ii) a file specifying clinical parameters for the respective samples analyzed in .txt format.

An overview of the entire osteomiR™ workflow is given on the next page.

Overview of the osteomiR™ workflow

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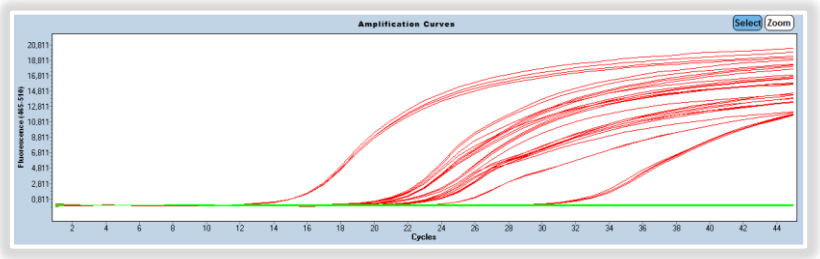
Clinical specification .txt file

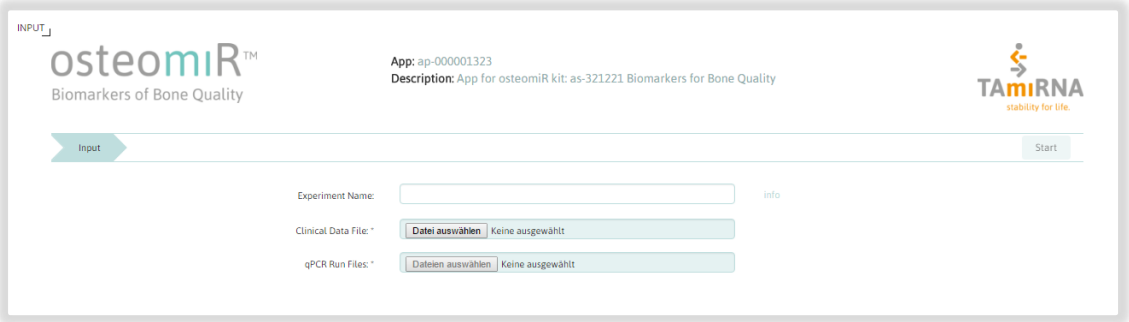
Light Cycler 480 II qPCR run .txt file(s)



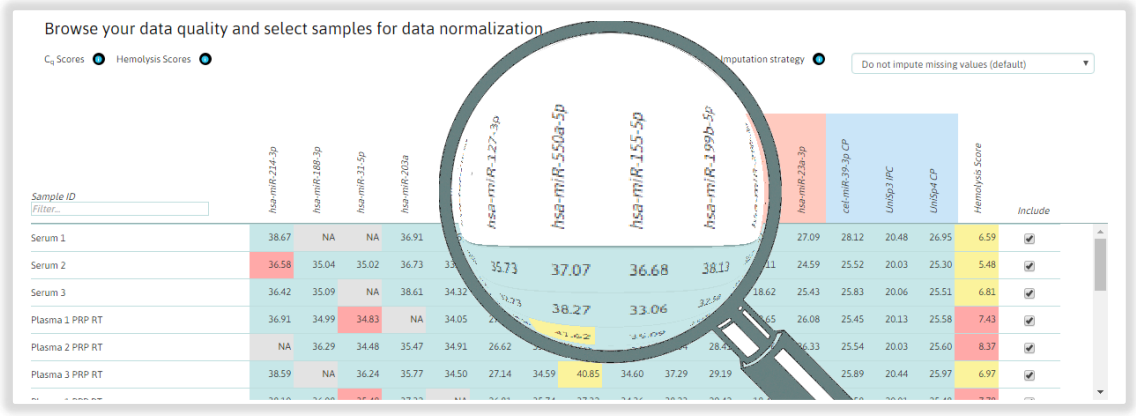
Patient information

Clinical Specification File

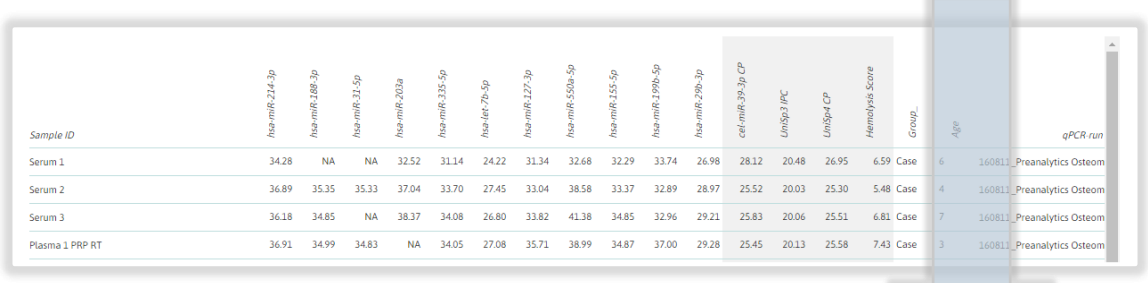
osteomiR™ app / tamirna.platomics.com



Upload qPCR data and clinical specification file



Browse data quality and select samples for normalization



Download normalized data table

2. Roche® LightCycler® 480 II

2.1. Implementation of the osteomiR™ macro on your LightCycler 480 II platform

Upon purchase of the osteomiR™ kit you will receive the osteomiR™ macro via email. The following steps guide you through the implementation and use of the macro.

1. Download the osteomiR™ macro and copy it to the LC 480 II workstation.
2. Start LightCycler480 II software.
3. Enter "username" and "password" to login and proceed to the overview window (Fig. 1)
4. Click on "Navigator" button and select "Import".
5. Go to the folder where you have stored the osteomiR.ixo file you received via email and select it (Fig. 2).
6. An overview window displaying the experimental settings provided with the osteomiR™ macro appears (Figure 5). As only one reporter dye (SYBR green I) is used, options for color compensation on the top left are set to "none". Click on "Save" button , the window shown in Figure 3 will appear. Choose the directory /Root/System Admin/Macros (Fig. 4), confirm with and go back to the Overview window by clicking the "Overview" button .

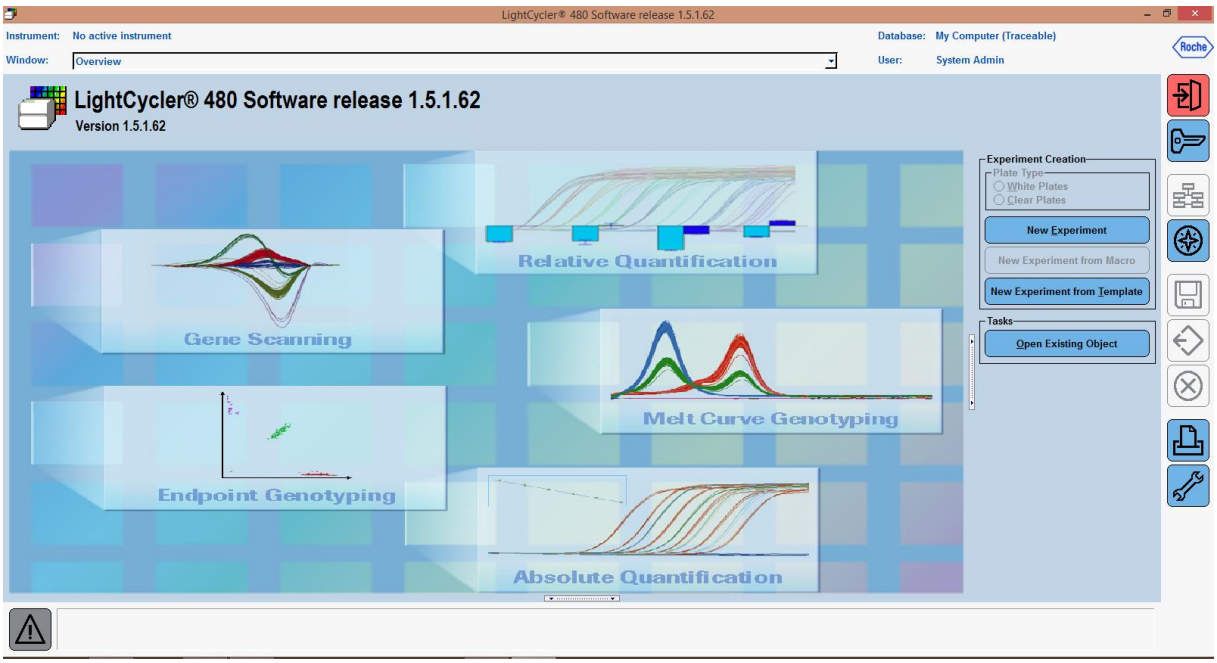


Figure 1: Overview window.

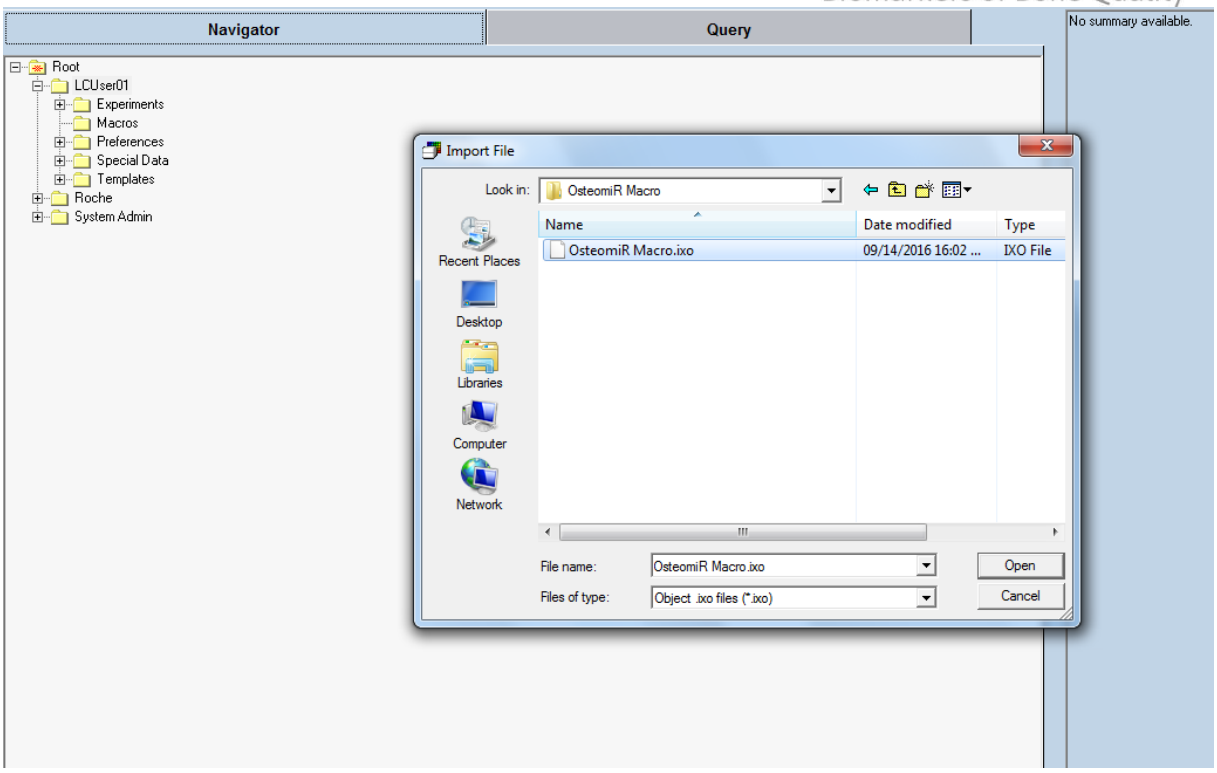


Figure 2: Select osteomiR™ Macro for import.

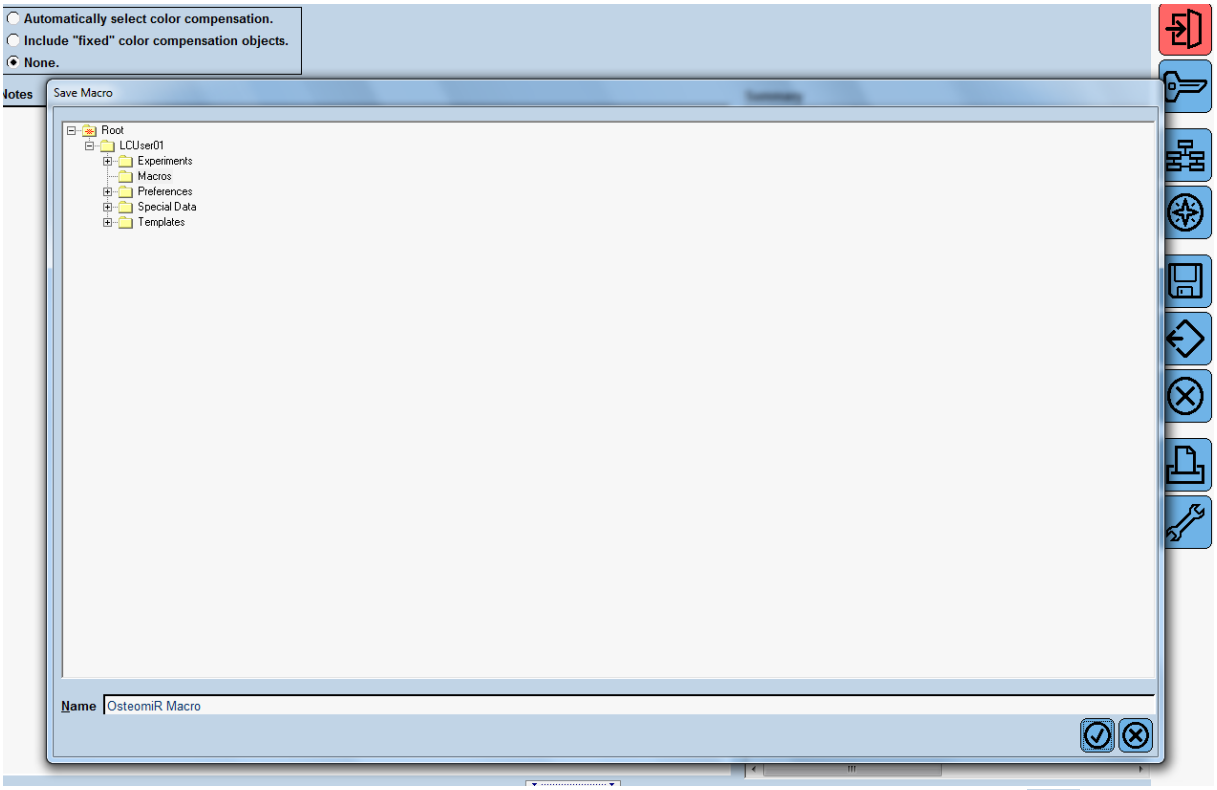


Figure 3: Select a directory (see also Figure 4) for saving the osteomiR™ Macro and confirm with 

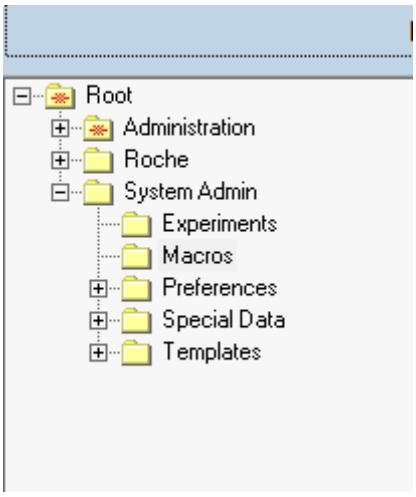


Figure 4: Proposed directory /Root/System Admin/Macros for saving the osteomiR™ macro in the LightCycler480 II software.

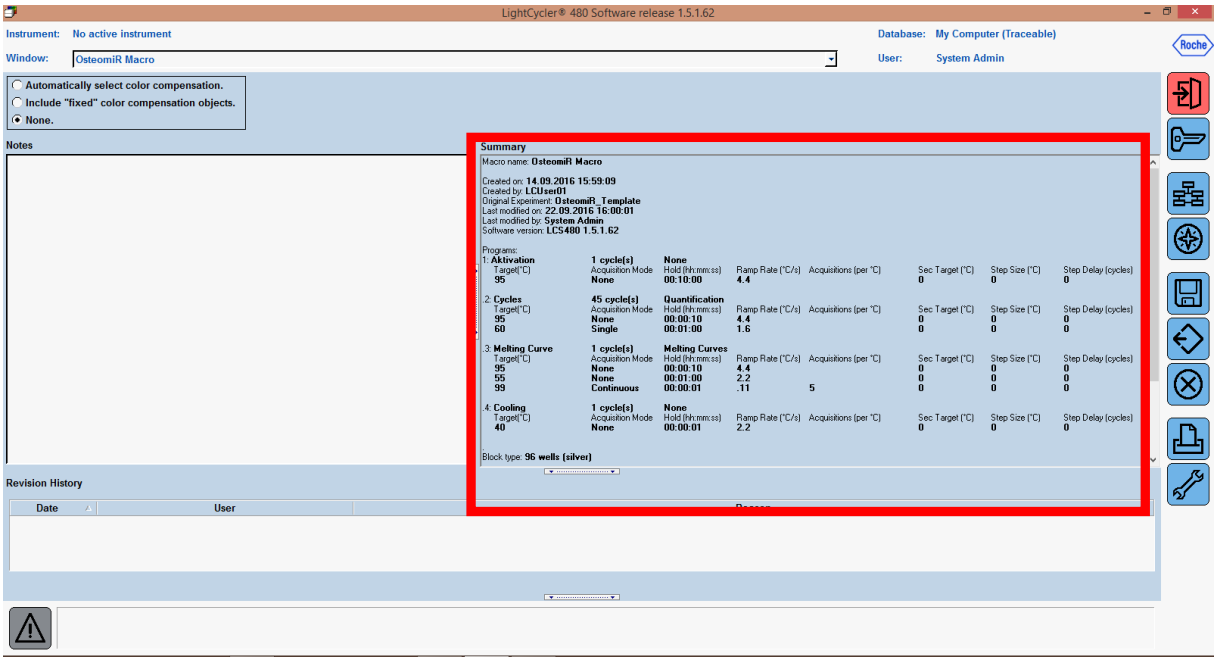


Figure 5: Overview of experimental settings deposited in the osteomiR™ macro (highlighted in red box).

2.2. How to tun the osteomiR™ qPCR experiment and save qPCR data as text-file

Important notes before starting

- Turn on your LightCycler480 II system before proceeding with the following steps.
  - ❖ Wait for the initialization of the instrument to finish – the left LED on the front of the instrument should become a steady green while the right LED should be a steady orange.
  - ❖ Macros can only be selected when the LightCycler480 II system is turned on and initialized.
- As the experiment run automatically starts as soon as the osteomiR™ macro is selected, the qPCR 96-well plate must be prepared **before** proceeding with the following steps.

1. Start LightCycler480 II software and enter "Username" and "password" to log in and proceed to the Overview window (Figure 1).
2. Put the prepared osteomiR™ 96-well plate into the LightCycler480 II instrument.
3. Select "New Experiment from Macro". The window shown in Figure 6 will appear.
4. Select the osteomiR™ macro.
5. The window shown in Figure 7 appears- Choose a directory for the experiment to be saved and proceed with  , the qPCR run starts.
6. Replace pre-annotated sample names (Patient 1 to Patient 6) by your own sample annotation. **Please remember the sample names must be matched with the clinical specification file.**
7. After the run is finished, the window shown in Figure 8 opens up. You can now save the generated data as .txt file by clicking on  . **The window shown in Figure 9 will appear for choosing a directory for saving the qPCR data and importantly choosing the required data format as .txt.**
   - ❖ There is no need in analyzing the Cq values (by using e.g. Second Derivative Maximum method) in the LightCycler 480 II software, as the osteomiR™ app does this by using the raw data of the amplification curves by applying the Second Derivative Maximum method.
   - ❖ The same applies for Melting Curve analysis- Melting curves are computed and can be checked when the data is plugged into the osteomiR™ software.

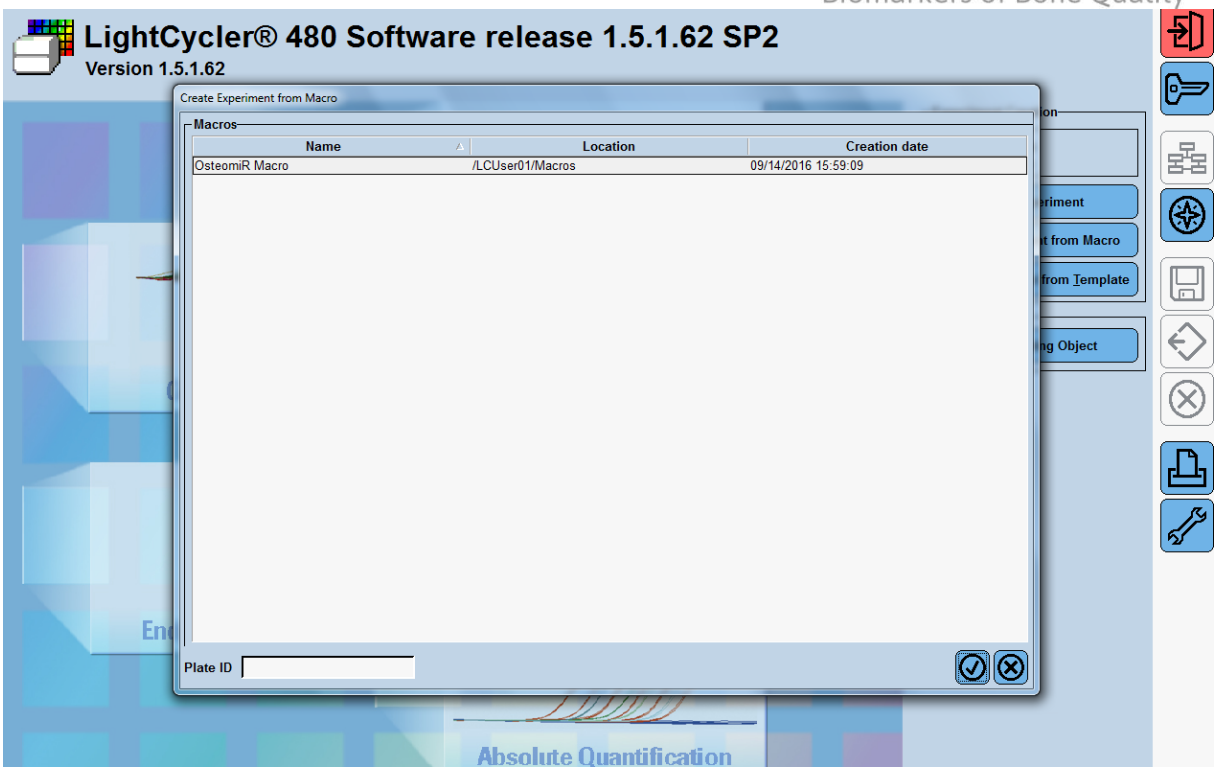


Figure 6: Start the osteomiR™ Macro by selecting it and confirm with 

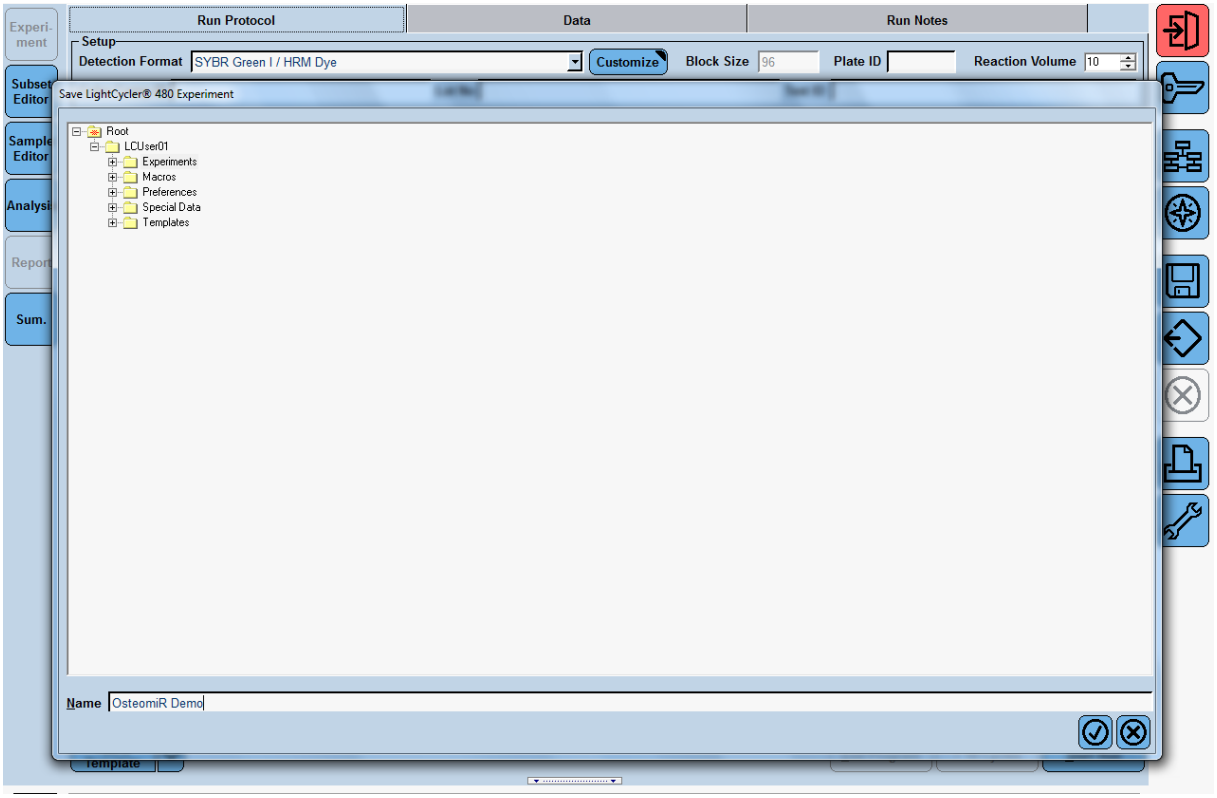


Figure 7: Choose a directory for your osteomiR™ experiment to be saved and proceed with 

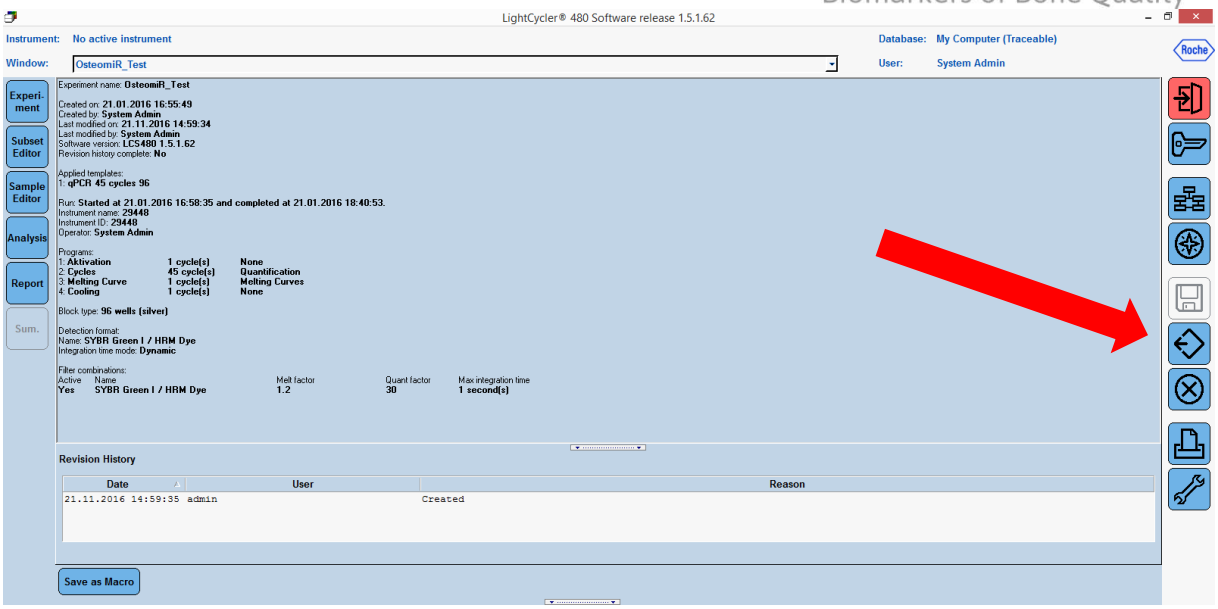


Figure 8: Window indicating finish of the qPCR run- data ready for export as .txt file. Red arrow indicates the necessary button for proceeding with data export 

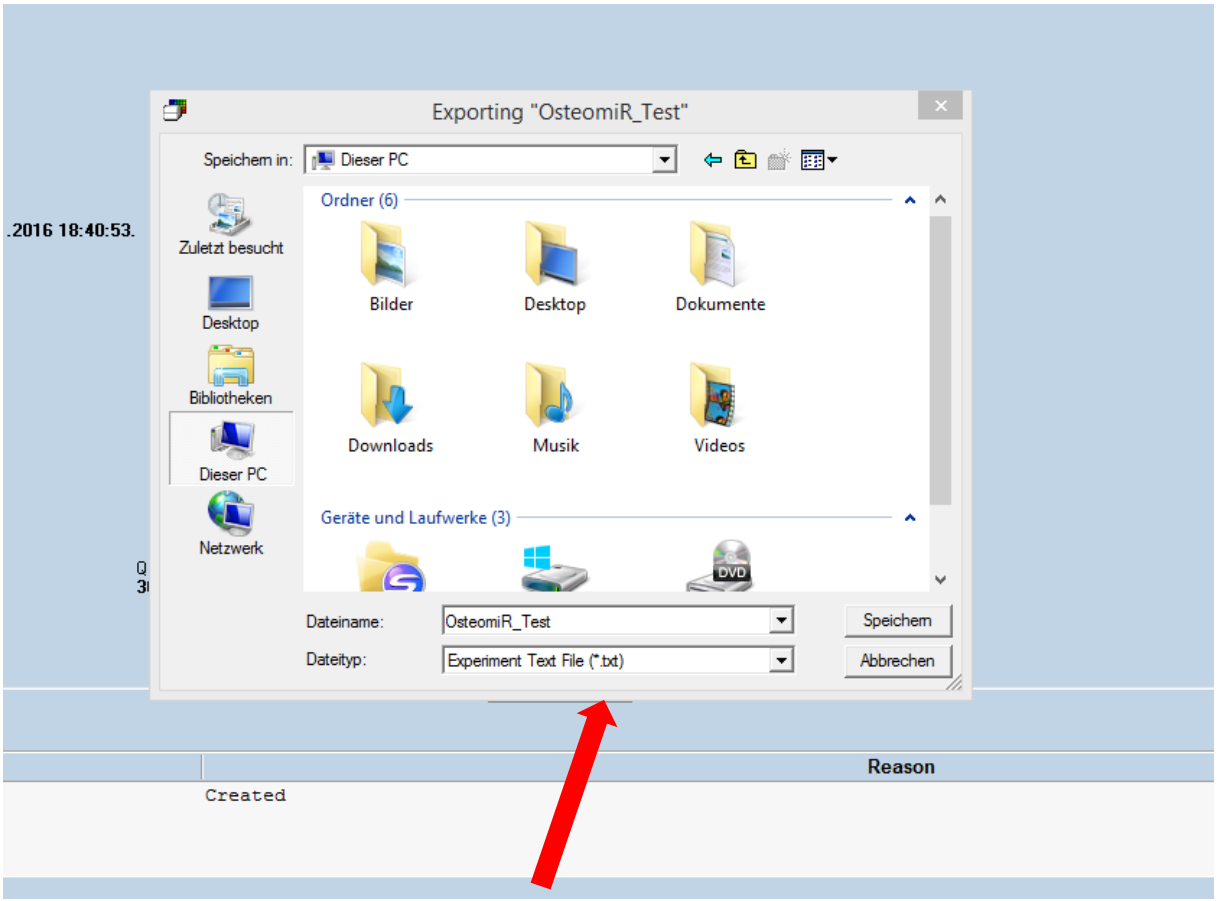


Figure 9: Choosing a directory for saving the generated qPCR data and the required data format as .txt (indicated by red arrow).

3. osteomiR™ App

3.1. Creation of the clinical data file

3.1.1. Create clinical data file in Excel

For using the osteomiR™ app, a clinical data file matched to the samples analyzed on the osteomiR™ 96-well plate(s) needs to be provided in addition to the qPCR run .txt files.

	A	B	C	D
1	Sample ID	Age	Group	
2	Patient 1	77	Case	
3	Paitent 2	53	Control	
4	Patient 3	68	Case	
5	Patient 4	44	Control	
6	Paitent 5	59	Case	
7	Patient 6	81	Case	
8	Patient 7	66	Case	
9	Patient 8	69	Case	
10	Patient 9	71	Case	
11	Patient 10	58	Case	
12	Paitent 11	55	Control	
13	Patient 12	68	Case	
14	Patient 13	69	Case	
15	Paitent 14	72	Control	
16	Patient 15	63	Case	
17	Patient 16	61	Control	
18	Paitent 17	59	Case	
19	Patient 18	42	Control	
20				

The Excel screenshot on the left (Figure 10) gives an example of how the clinical data file needs to be prepared- in this case shown for the analysis of three qPCR run .txt files and accordingly 18 samples.

In the first column (column A) provide the sample IDs.

Important note: The sample IDs provided in the clinical data file need to be exactly matched with the sample IDs assigned in the LightCycler 480 II software (the sequence of samples in the clinical specification file and in the qPCR run file does NOT matter).

In the second column (column B) enter the age of the respective patients.

In the third column (column C) define whether the sample analyzed is regarded as Case or Control sample.

Figure 10: Example Excel sheet for creating the Clinical Specification File.

3.1.2. Save clinical data file as .txt file

For importing the clinical specification file into the osteomiR™ app, the file needs to be saved as .txt file. For this purpose choose the required .txt format when clicking on *Saving as* in Excel as shown in Figure 11 below (indicated by red box).

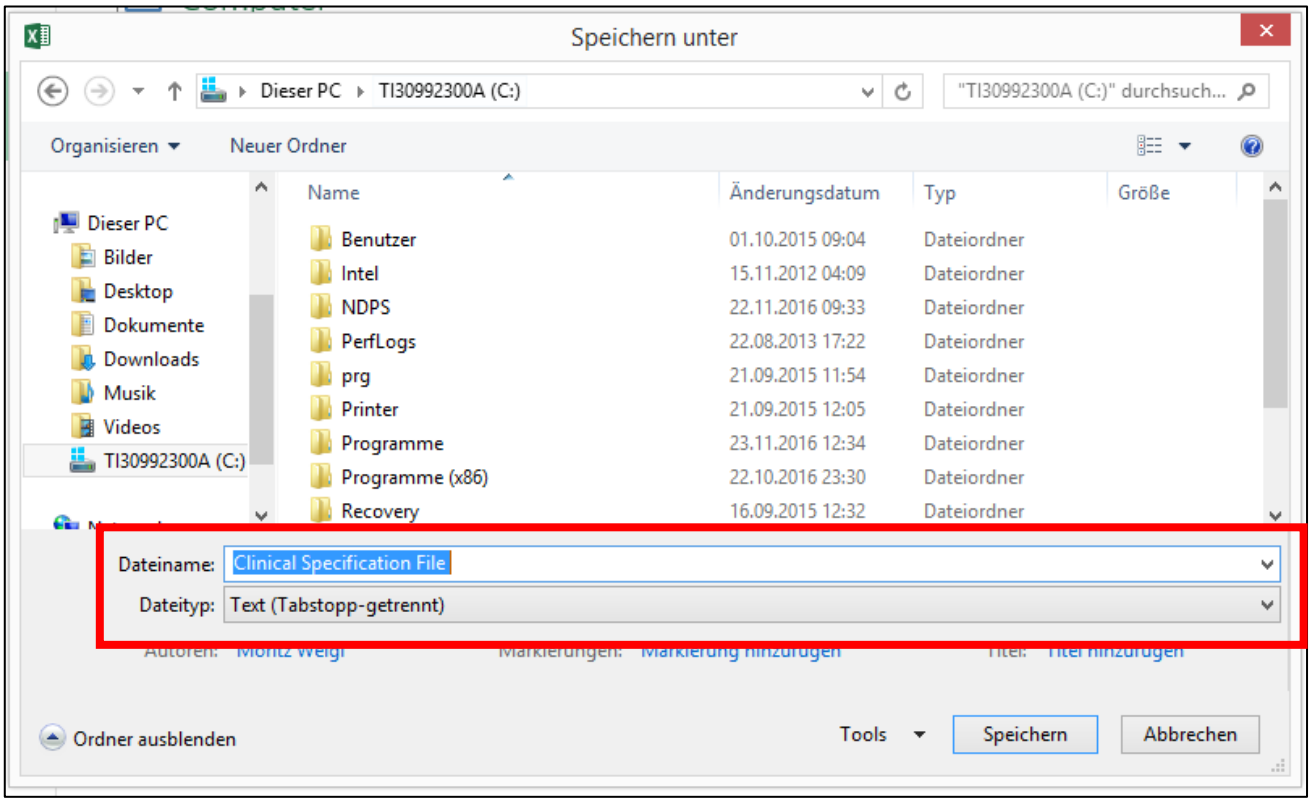


Figure 11: Save Clinical Specification File as .txt file (selection of .txt format is shown in red box, English: "tab-delimited").

3.2. Data import into the osteomiR™ App

1. Open Google Chrome and go to *tamirna.platomics.com* and log in via your user ID + password to access the Overview window (Figure 13).
    a. Important Note: For full functionality please use Google Chrome to access the osteomiR™ App.
2. On the left side find the Workspace strip, click on *Project* and select *osteomiR* (Figure 14).
3. Choose an experiment name and upload the clinical data file (Figure 15).
4. Upload the qPCR run .txt file(s). You can also upload and analyze data from multiple qPCR runs, be careful to annotate all samples from the qPCR plates in the clinical specification file (Figure 16).
    a. Be aware that it is very important that sample names from the qPCR run .txt files (derived from the LightCycler 480 II software) and sample names specified in the clinical data file exactly match.
5. Click Start and wait for the quality control table to be generated (Figure 17).

3.3. Quality Control and Data normalization

1. Browse the quality of your data by checking the Cq values of osteomiR microRNAs and spiked control oligonucleotides. Clicking on Cq values accesses amplification and melting curves.
    a. Mouse-over C_q Scores Hemolysis Scores will give you information on how to interpret Cq values regarding quality parameters.
2. Select samples on the right strip *Include* (Figure 12) destined for further analysis and normalization.

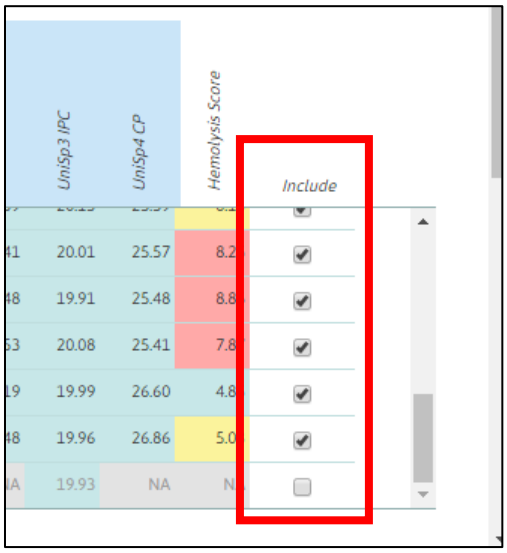


Figure 12: Red box indicates strip for selection of samples destined for further analysis.

3. Click Start Normalization to normalize the selected samples, the normalized data table is generated (Figure 18).
4. The normalized data table can now be downloaded as .xls file by clicking on Download

Figure 13: Overview window of osteomiR™ software.

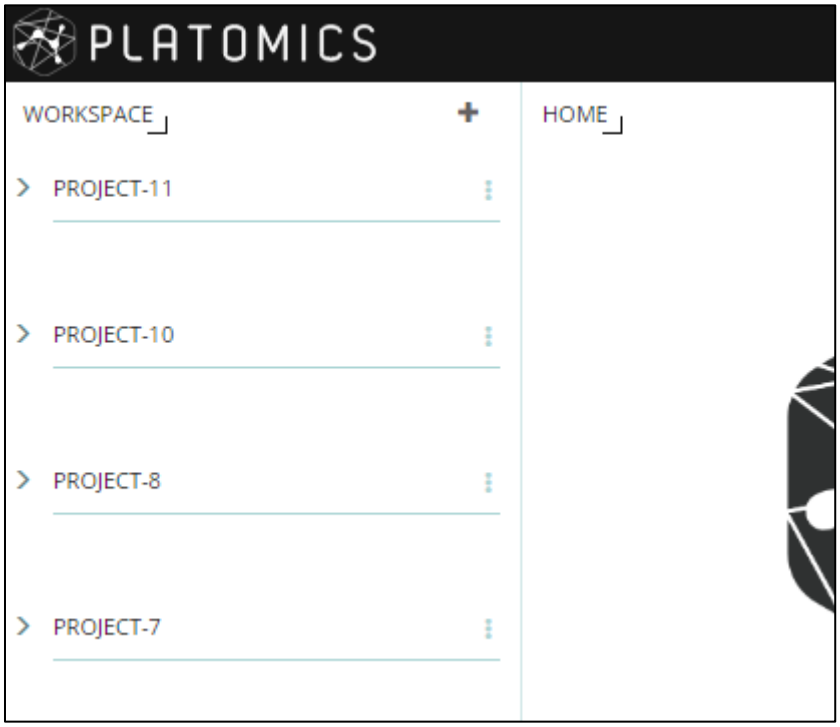


Figure 14: Zoom of workspace strip in Overview window. Click on Project and select osteomiR™ for proceeding to the data upload interface.

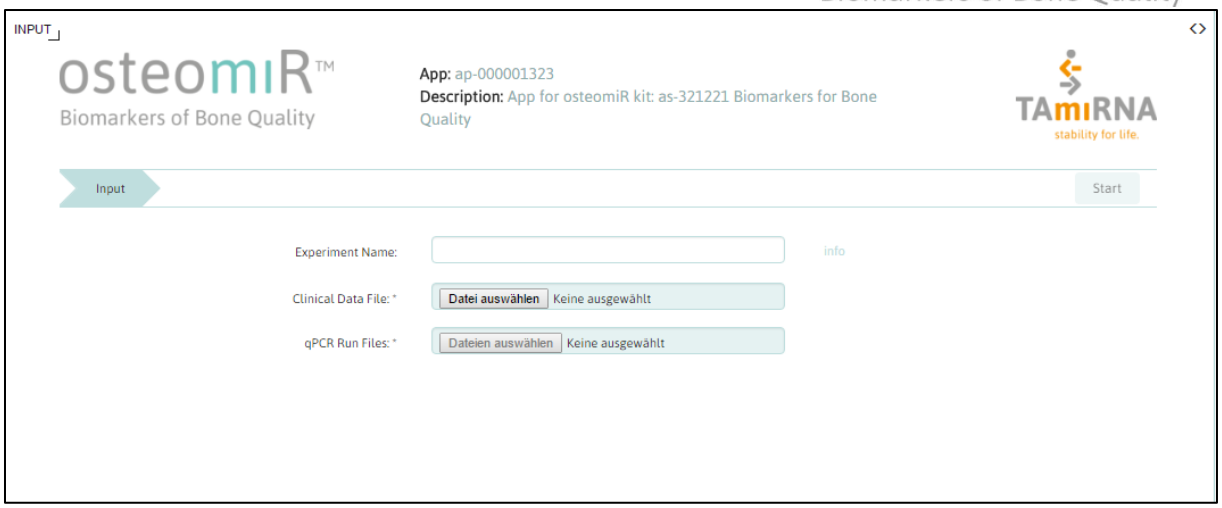


Figure 15: Choose an experiment name and upload the clinical data .txt file + qPCR .txt files.

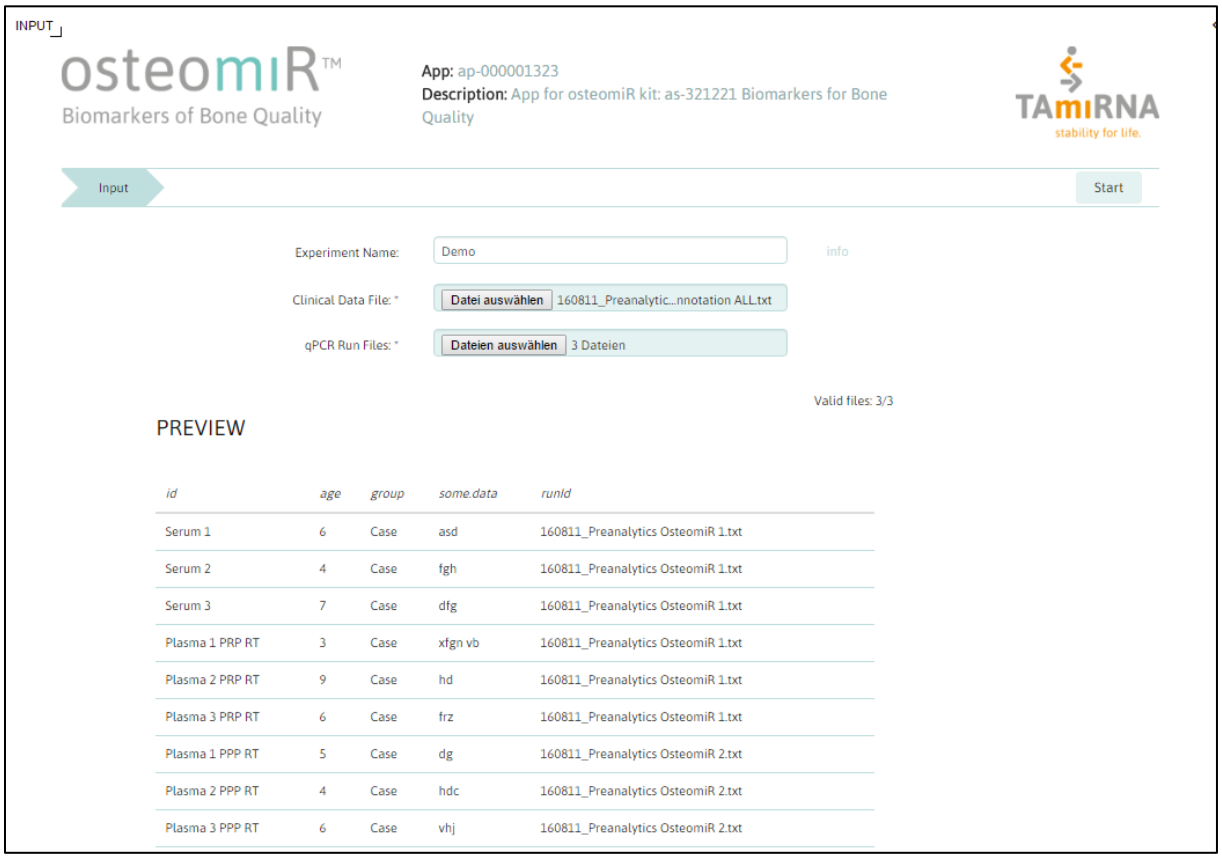


Figure 16: Uploaded clinical data .txt file + assigned qPCR .txt files ready for analysis.

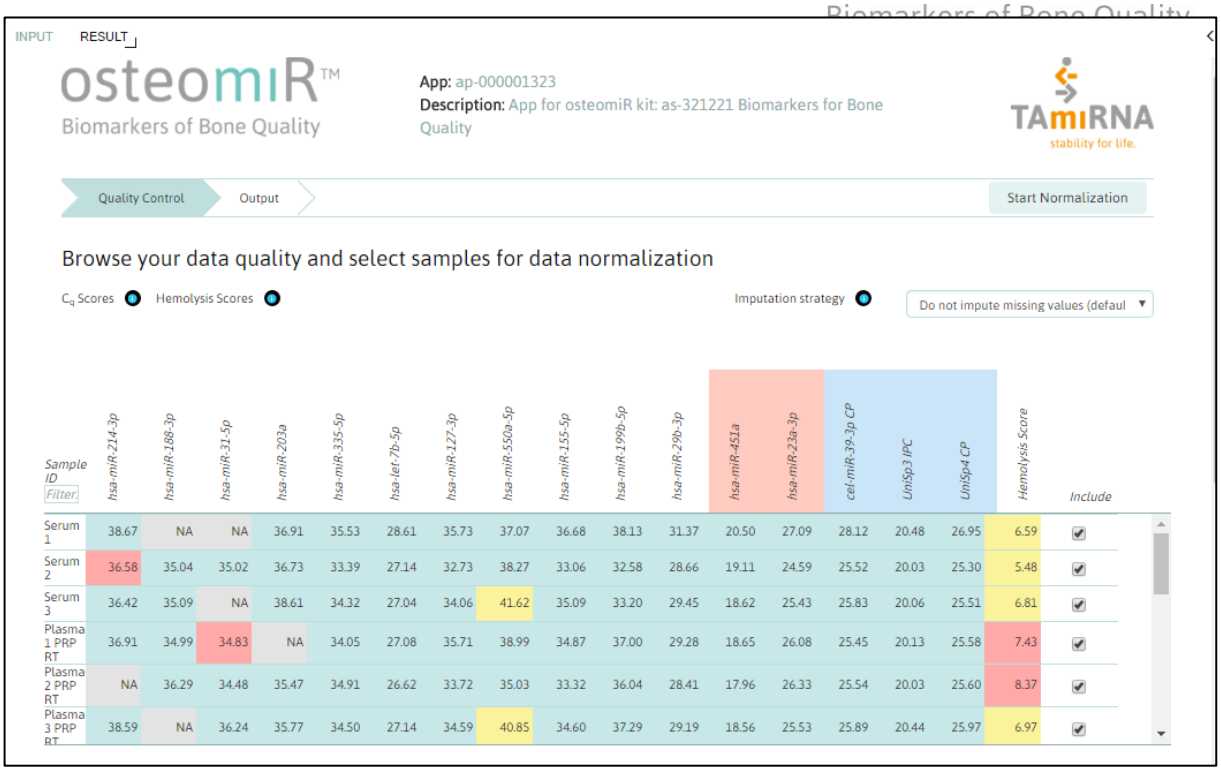


Figure 17: Quality Control data table.

Figure 18: Table with normalized data, ready for download as .xls Excel file.

FAQ on the use of the osteomiR™ app

What happens in case of pipetting errors e.g. accidental mix up of wells in the lab?

At the moment you will have to exclude the whole sample from further analysis with the osteomiR™ app. We will eventually implement a "Flag as error" possibility for single wells, though it has to be emphasized that this might lead to negative effects on normalization and if at all should only be done for Research Use Only experiments.

The bottom line is that accurate pipetting and analysis of all osteomiR™ microRNAs constitutes a prerequisite for making quality data driven assumptions.

Why are some of the Cq values in the downloaded raw/normalized data tables displayed as dates when viewed in Excel?

The Cq values use dots " . " as comma separator. This problem relates to your Excel settings and appears when you have chosen comma " , " as comma separator. Please change your settings to using a dot " . " as comma separator- then this problem should not occur anymore.

Once I have uploaded qPCR run .txt files or the clinical specification file on the upload interface (Figure 15), can I remove/change the files destined for analysis in case of miss-clicking before proceeding and pressing Start?

Yes. Just select "Select Data" again, choose the respective files for analysis and the prior uploaded files will be replaced by those now chosen.

Do I have to use a specific browser for optimal function of the osteomiR™ app?

Yes. Please only use Google Chrome. Using Internet Explorer, Mozilla Firefox or other browsers might lead to functional problems with the software.

Is it possible to analyze data from multiple osteomiR™ 96-well plates at once?

Yes. As long as the clinical specification file matches to the qPCR data you can upload and analyze multiple 96-well plates by simply choosing more qPCR .txt files on the upload interface (Figure 15).

Annex I: Specification of the qPCR protocol deposited in the osteomiR™ macro

Setup parameters

- Detection format: SYBR Green I / HRM Dye
- Block Type: 96
- Plate ID (optional)
- Reaction Volume: 10µl

Four program lines are specified in the osteomiR™ experimental protocol

1. Heat activation

   Analysis mode: None
   Cycles: 1

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisition (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
95	None	00:10:00	4.4		0	0	0

2. Cycles

   Analysis mode: Quantification
   Cycles: 45

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisition (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
95	None	00:00:10	4.4		0	0	0
60	Single	00:01:00	1.6		0	0	0

3. Melt curve

   Analysis mode: Melting curves
   Cycles: 1

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisition (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
95	None	00:00:10	4.4		0	0	0
55	None	00:01:00	2.2		0	0	0
99	Continuous	00:00:01	0.11	5	0	0	0

4. Cooling

   Analysis mode: None
   Cycles: 1

Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisition (per °C)	Sec target (°C)	Step size (°C)	Step delay (cycles)
40	None	00:00:01	2.2		0	0	0