ddpcRquant Hands-on

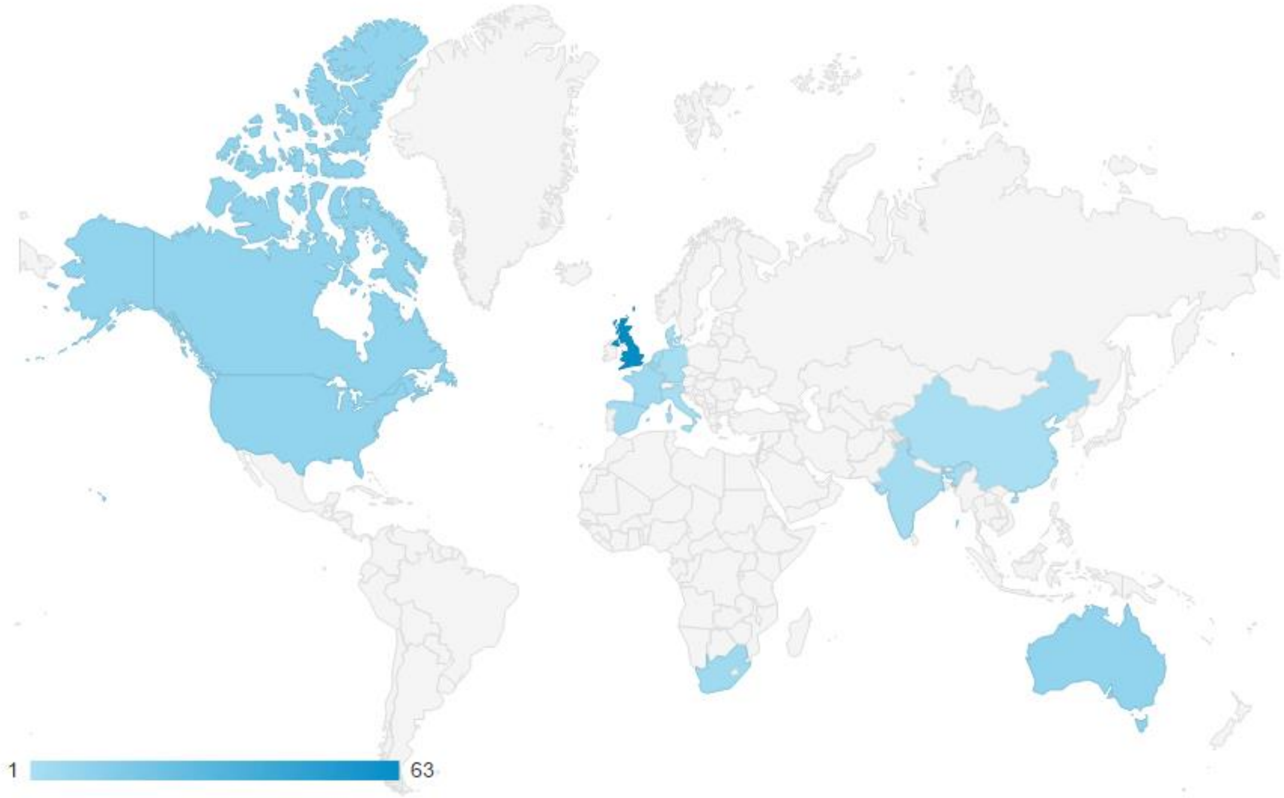
Wim Trypsteen
Matthijs Vynck
Doctoral schools specialist course
1 september 2016, ‘t Pand Ghent

Day 1 (01-09-2016)	Day 2 (02-09-2016)	
08:00-9:30	Introduction to digital PCR *Ward De Spiegelaere*	Assay Design + Hands on training *Jo Vandesompele*
09:30-10:00	coffee break	coffee break
10:00-12:00	Absolute quantification by dPCR: theory + applications (HIV, excision circles, splice variants) *Linos Vandekerckhove* *Ide Smets*	Multitarget applications: CNV, human and mouse examples, genotyping, mutation detection *Jo Vandesompele* *Nicole Hersmus* *Patrick Pauwels*
12:00	Lunch	Lunch
13:00-14:30	Statistical considerations of dPCR and droplet calling methods *Lieven Clement* *Olivier Thas*	RNA quantification by dPCR and normalization tools *Ward De Spiegelaere* *Nicole Hersmus*
14:30-15:00	coffee break	coffee break
15:00-17:00	excercises on Shiny app Wim Trypsteen en Matthijs Vynck	GLMM models for dPCR + Hands on *Lieven Clement* *Olivier Thas*
17:00-17:15	short break	Short break
17:15-18:00	Power analysis for dPCR *Olivier Thas*	Tips and Tricks Minimal reporting guidelines *Ward De Spiegelaere*

ddpcRquant (Global) Goals

GOALS

1. Introduce everyone to the ddpcRquant tool
2. Independent analysis of ddPCR data



Trypsteen et al. Anal Bioanal Chem. 2015 Jul;407(19):5827-34 PMID: 26022094

Outline

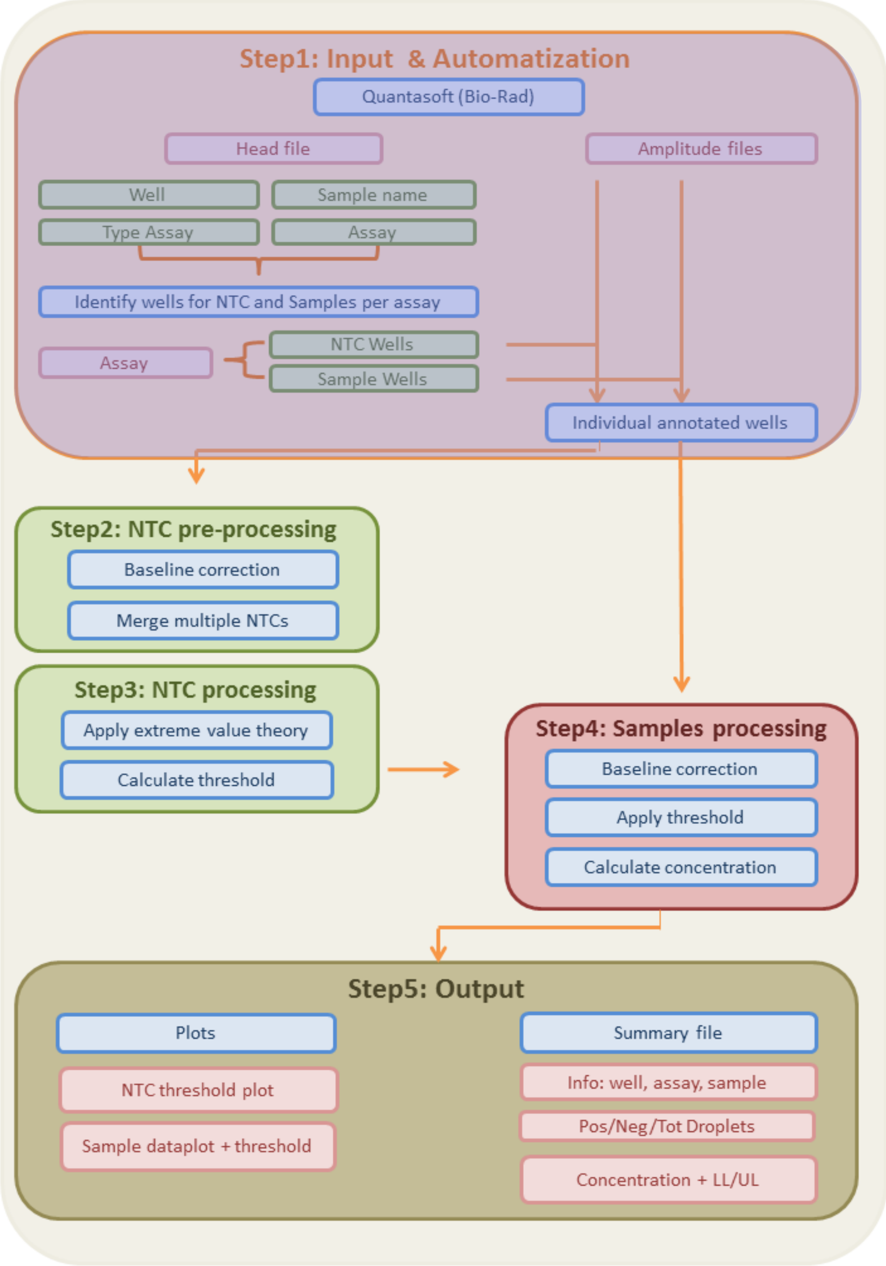
ddpcRquant overview

Annotation Requirements

Demo + DIY Analysis

- Exporting Data from the ddpcr Reader
- Running ddpcRquant
- Exercises part 1 and 2
- Head file options

ddpcRquant



Step1: Input & Automatization

Quantasoft (Bio-Rad)

Head file

- Well
- Sample name
- Type Assay
- Assay

Identify wells for NTC and Samples per assay

Assay
- NTC Wells
- Sample Wells

Amplitude files

Individual annotated wells

Step2: NTC pre-processing

- Baseline correction
- Merge multiple NTCs

Step3: NTC processing

- Apply extreme value theory
- Calculate threshold

Step4: Samples processing

- Baseline correction
- Apply threshold
- Calculate concentration

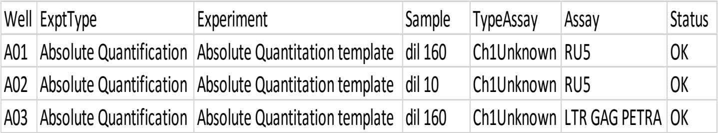
Step5: Output

Plots
- NTC threshold plot
- Sample dataplot + threshold

Summary file
- Info: well, assay, sample
- Pos/Neg/Tot Droplets
- Concentration + LL/UL

HEAD file

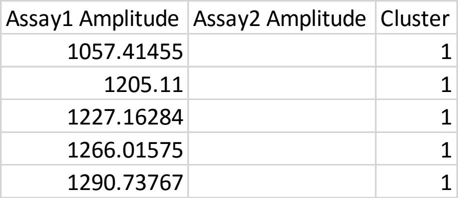
- Summary of the experiment
- Contains annotation info (NTC,..)



Well	ExptType	Experiment	Sample	TypeAssay	Assay	Status
A01	Absolute Quantification	Absolute Quantitation template	dil 160	Ch1Unknown	RU5	OK
A02	Absolute Quantification	Absolute Quantitation template	dil 10	Ch1Unknown	RU5	OK
A03	Absolute Quantification	Absolute Quantitation template	dil 160	Ch1Unknown	LTR GAG PETRA	OK

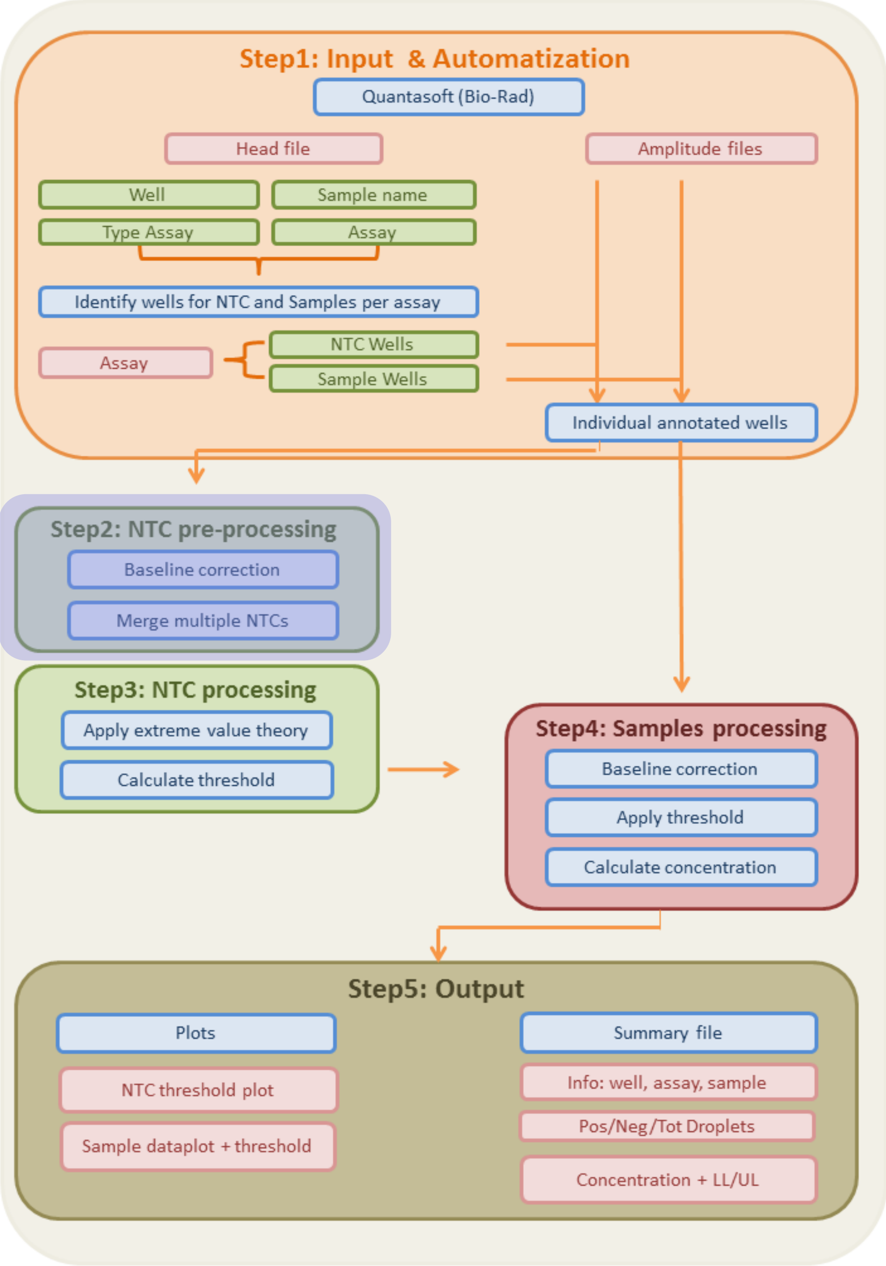
AMPLITUDE files

- Individual well files with the fluorescent intensity information (droplets)



Assay1 Amplitude	Assay2 Amplitude	Cluster
1057.41455		1
1205.11		1
1227.16284		1
1266.01575		1
1290.73767		1

ddpcRquant



Step1: Input & Automatization

Quantasoft (Bio-Rad)

Head file

Amplitude files

Well

Sample name

Type Assay

Assay

Identify wells for NTC and Samples per assay

Assay

NTC Wells

Sample Wells

Individual annotated wells

Step2: NTC pre-processing

Baseline correction

Merge multiple NTCs

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Apply extreme value theory

Calculate threshold

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Apply threshold

Calculate concentration

Step5: Output

Plots

NTC threshold plot

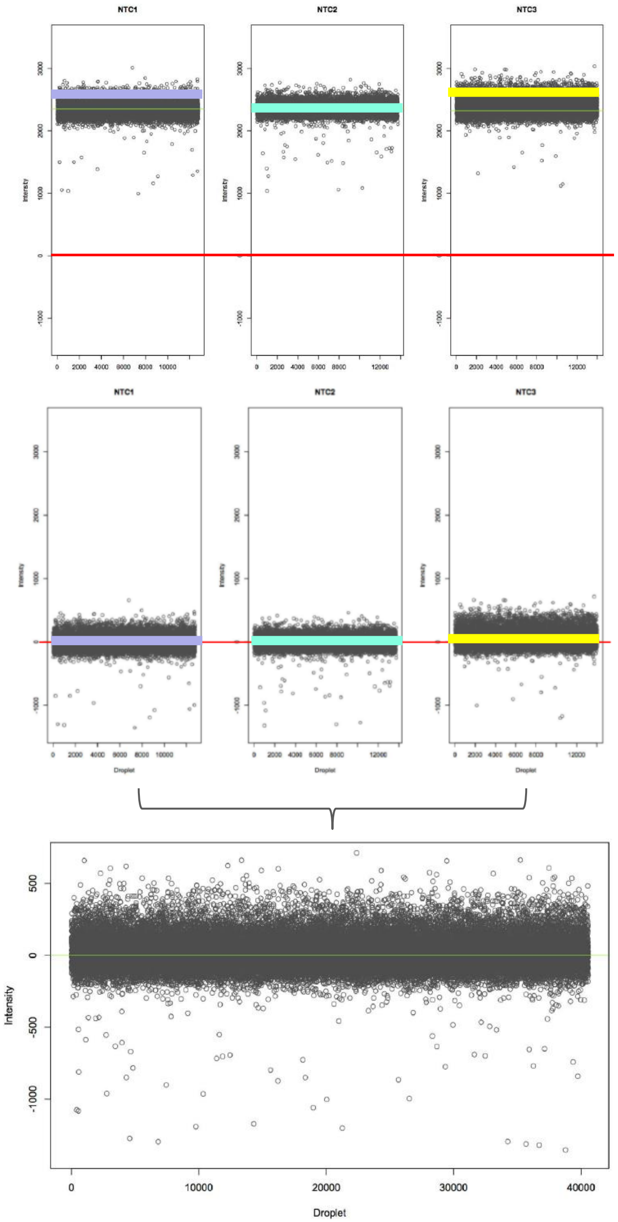
Sample dataplot + threshold

Summary file

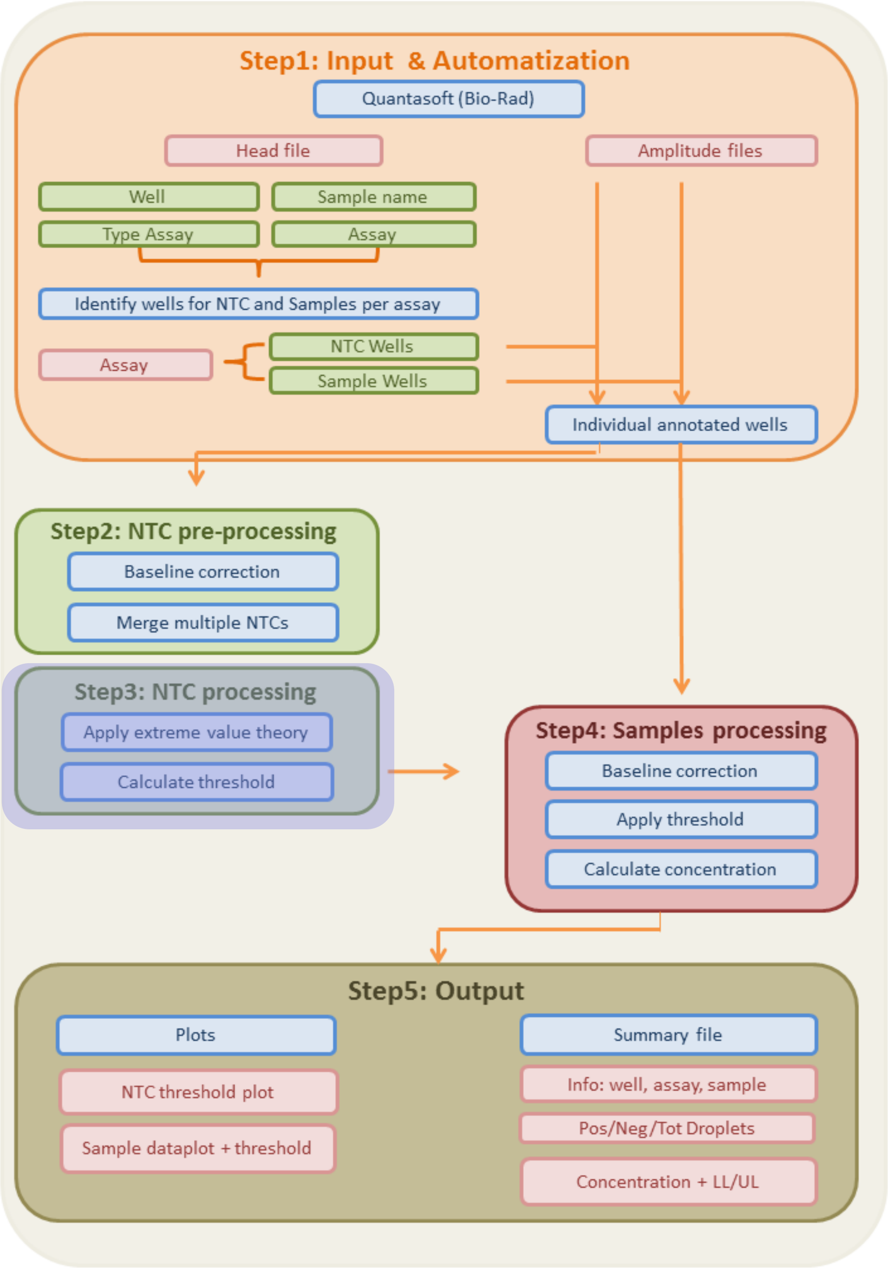
Info: well, assay, sample

Pos/Neg/Tot Droplets

Concentration + LL/UL



ddpcRquant



Step1: Input & Automatization

- Quantasoft (Bio-Rad)
- Head file
  - Well
  - Sample name
  - Type Assay
  - Assay
- Identify wells for NTC and Samples per assay
- Assay
  - NTC Wells
  - Sample Wells
- Amplitude files
- Individual annotated wells

Step2: NTC pre-processing

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- Merge multiple NTCs

Step3: NTC processing

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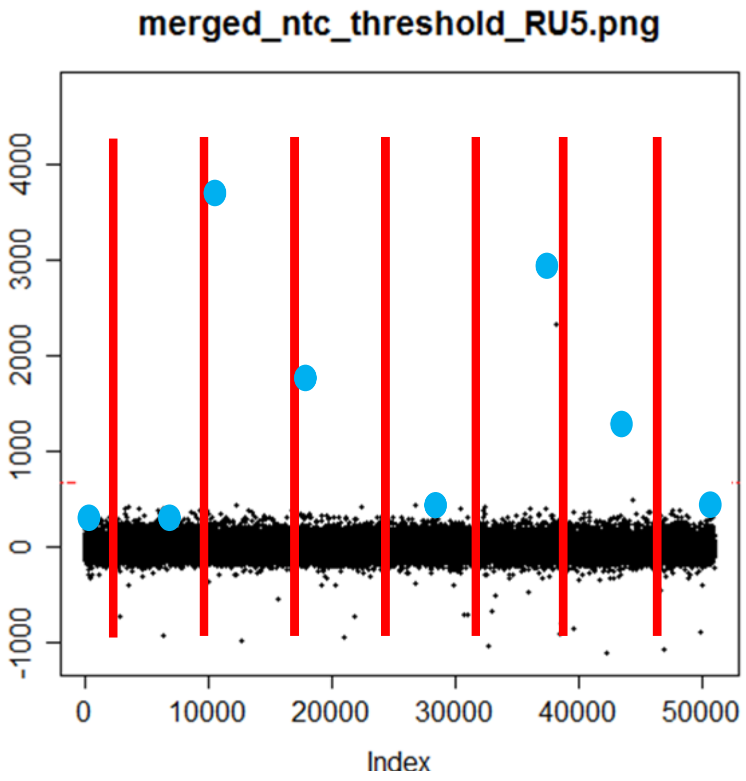
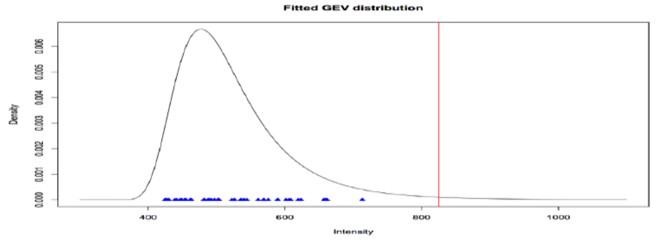
  - Concentration + LL/UL

Fisher-Tippett theorem: The distribution of block maxima is given by the Generalized Extreme Value distribution (GEV)

= Block maxima (extremes) follow this family of distributions

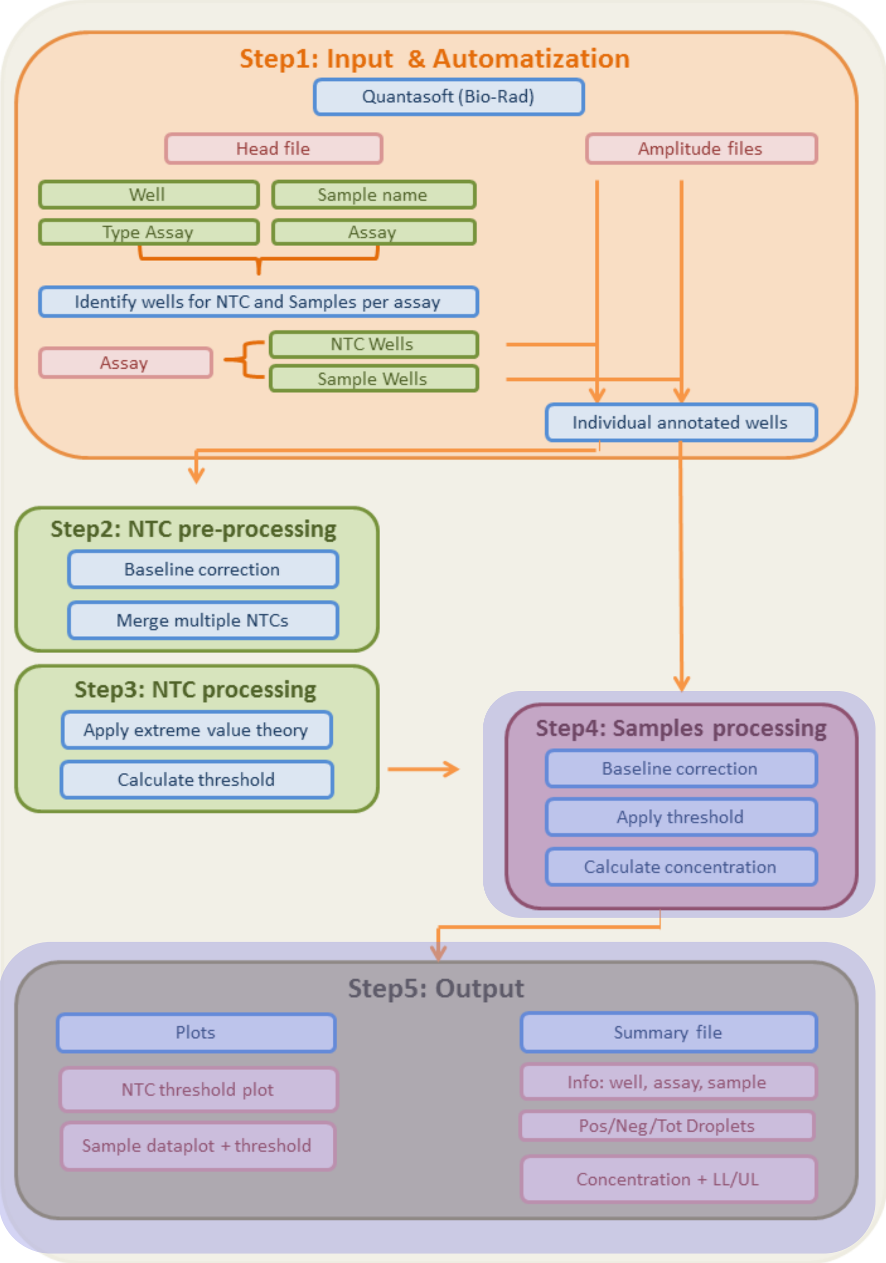


merged_ntc_threshold_RU5.png



Fitted GEV distribution

ddpcRquant



Step1: Input & Automatization

Quantasoft (Bio-Rad)

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Sample name

Type Assay

Assay

Identify wells for NTC and Samples per assay

Assay

NTC Wells

Sample Wells

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NTC threshold plot

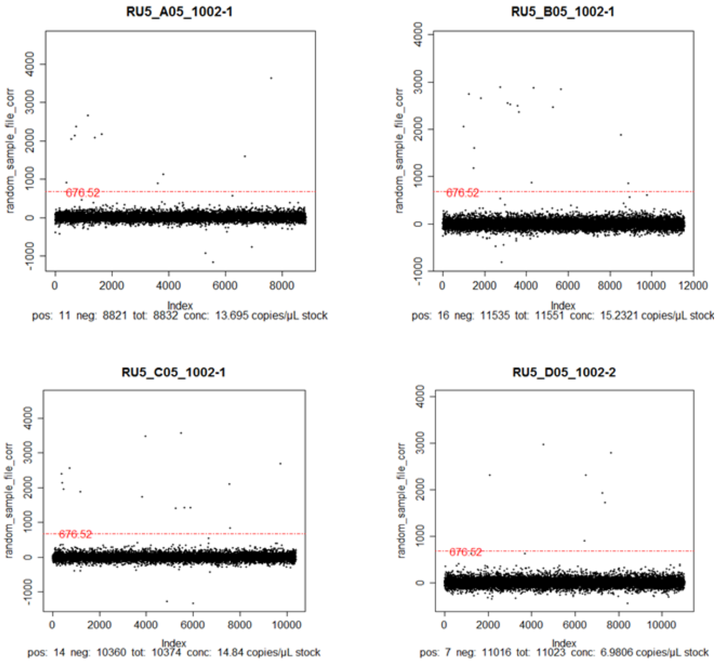
Sample dataplot + threshold

Summary file

Info: well, assay, sample

Pos/Neg/Tot Droplets

Concentration + LL/UL



$$C = -\ln\left(\frac{Nneg}{N}\right) * \frac{1000}{Vd} * D$$

D: Template/Mix

	Well	assay	name	type	positive droplets	negative droplets	total droplets	concentration	lowerCI	upperCI
1	merged	2 LTR	merged_NTC	ntc	1	19376	19377	0.5671	0.0666	4.8298
2	E01	2 LTR	gDNA 1	sample	116	10957	11073	115.7274	91.1503	146.887
3	F01	2 LTR	gDNA2	sample	163	10391	10554	171.0427	139.8199	209.1717
4	G01	2 LTR	plas 1	sample	1	12425	12426	0.8844	0.1038	7.531
5	H01	2 LTR	plas 2	sample	9	7758	7767	12.7409	5.5316	29.3334

Limitations

ddpcRquant is made for 1-D analysis (Bio-Rad qx100/qx200)

- 1 channel at a time
- If 2 channels per well => one after the other will be analyzed

Use the NTCs to calculate a single threshold

- Whether the extreme value measured in the NTC is a real or false positive value is beyond the scope of ddpcrquant

ddpcRquant uses a predefined annotation

- Throws most of the errors when using the tool
- R functions available but webtool GUI

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Annotation Requirements

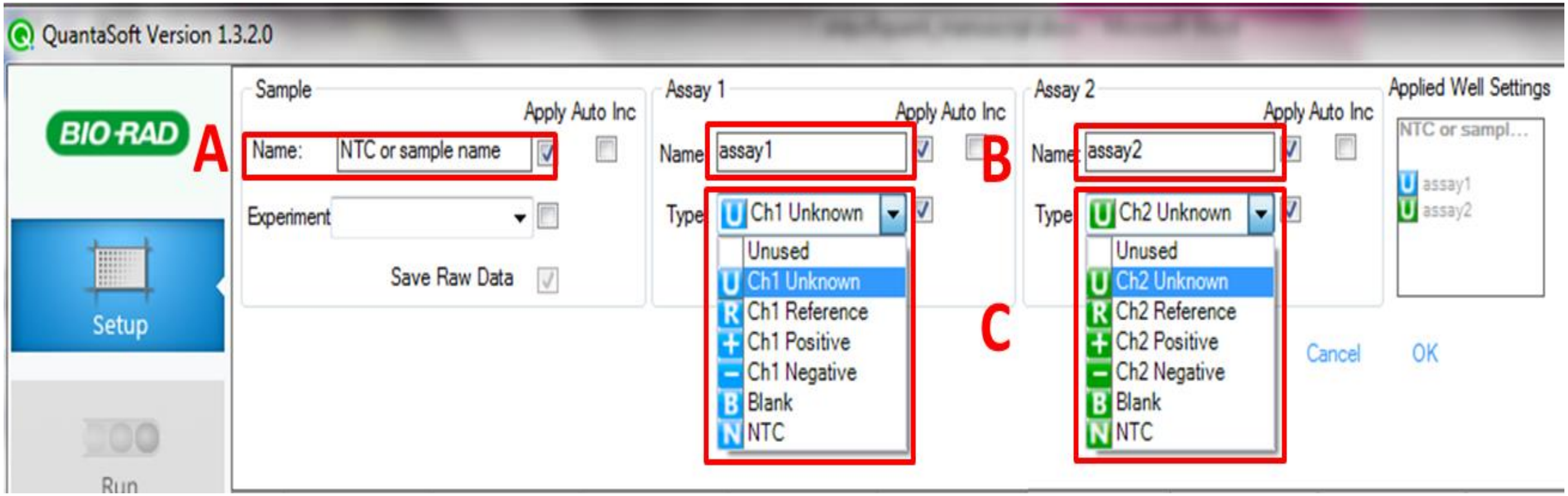
Annotation before run starts: Quantasoft

Annotation after run

- Change headfile in text format reader
  notepad, noteblock, NOT excel

- Use qx100/qx200 template

Annotation Requirements



QuantaSoft Version 1.3.2.0

Sample
Name: NTC or sample name ☑
Experiment
Save Raw Data ☑

Assay 1
Name: assay1 ☑
Type: Ch1 Unknown ☑

Assay 2
Name: assay2 ☑
Type: Ch2 Unknown ☑

Applied Well Settings
NTC or sampl...
U assay1
U assay2

Before the ddPCR run: Annotation & Input Info

SampleName: NTC/ntc can only be in the name of the negative control

AssayName: different assay names required

Type: Select Ch1Unknown or Ch2Unknown (nothing else at the moment)

WellNumber: automatically

This info will be stored in the **Head file**

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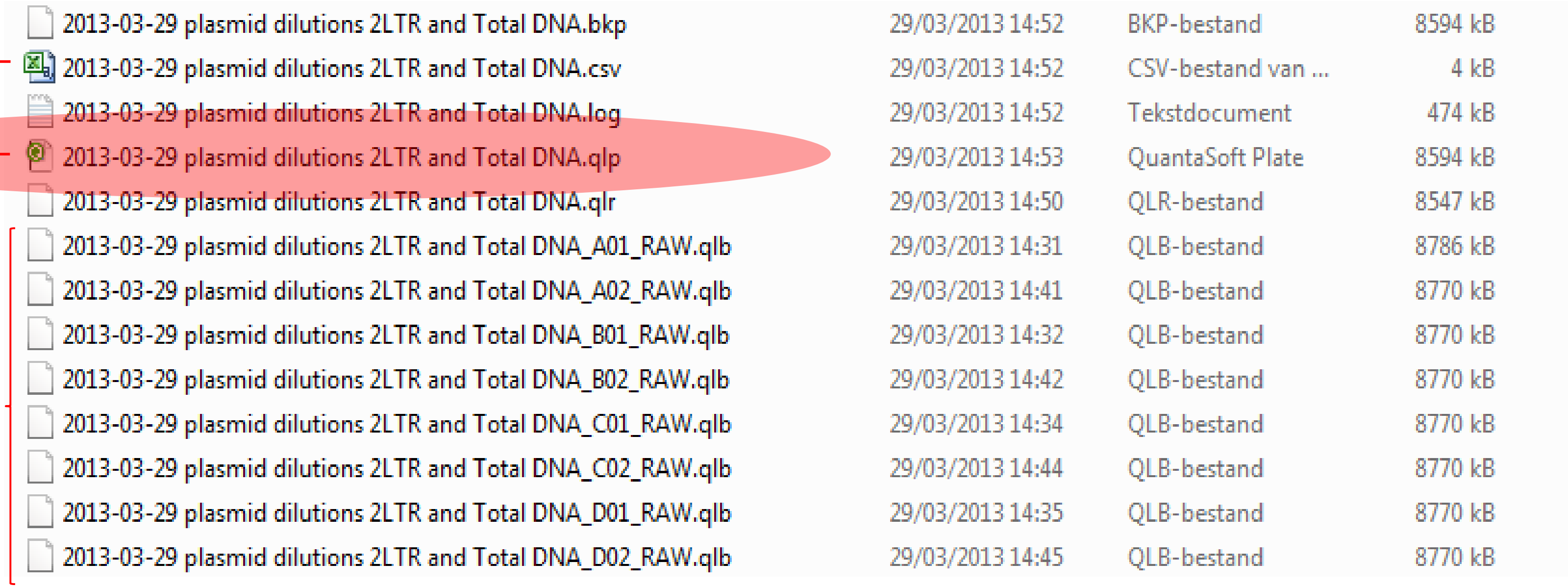
Exporting Data from Quantasoft

After ddPCR run: Load ddPCR data into Quantasoft



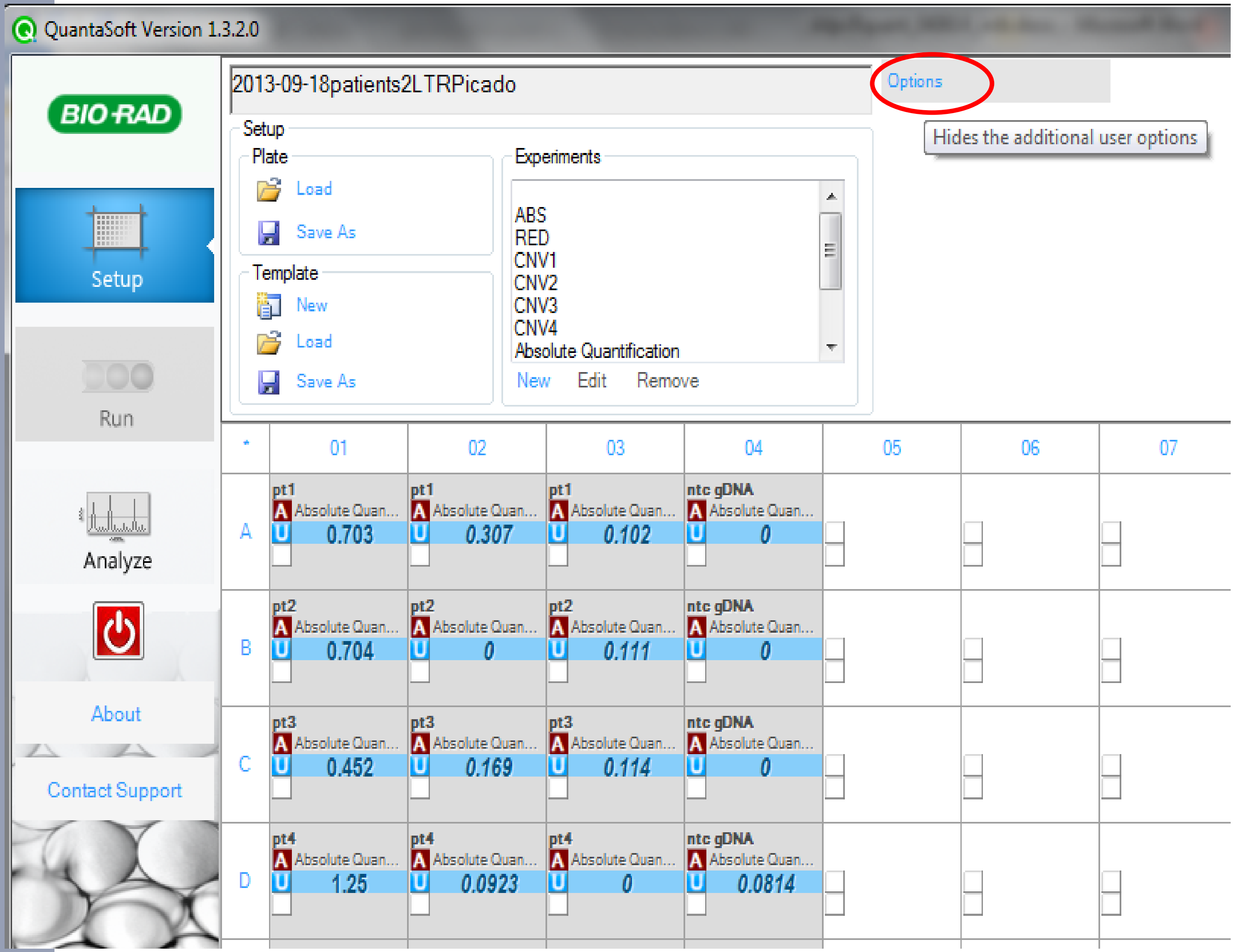
head ←

QS ←

Raw data

2013-03-29 plasmid dilutions 2LTR and Total DNA.bkp	29/03/2013 14:52	BKP-bestand	8594 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.csv	29/03/2013 14:52	CSV-bestand van ...	4 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.log	29/03/2013 14:52	Tekstdocument	474 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.qlp	29/03/2013 14:53	QuantaSoft Plate	8594 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.qlr	29/03/2013 14:50	QLR-bestand	8547 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_A01_RAW.qlb	29/03/2013 14:31	QLB-bestand	8786 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_A02_RAW.qlb	29/03/2013 14:41	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_B01_RAW.qlb	29/03/2013 14:32	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_B02_RAW.qlb	29/03/2013 14:42	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_C01_RAW.qlb	29/03/2013 14:34	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_C02_RAW.qlb	29/03/2013 14:44	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_D01_RAW.qlb	29/03/2013 14:35	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_D02_RAW.qlb	29/03/2013 14:45	QLB-bestand	8770 kB

Exporting Data from Quantasoft



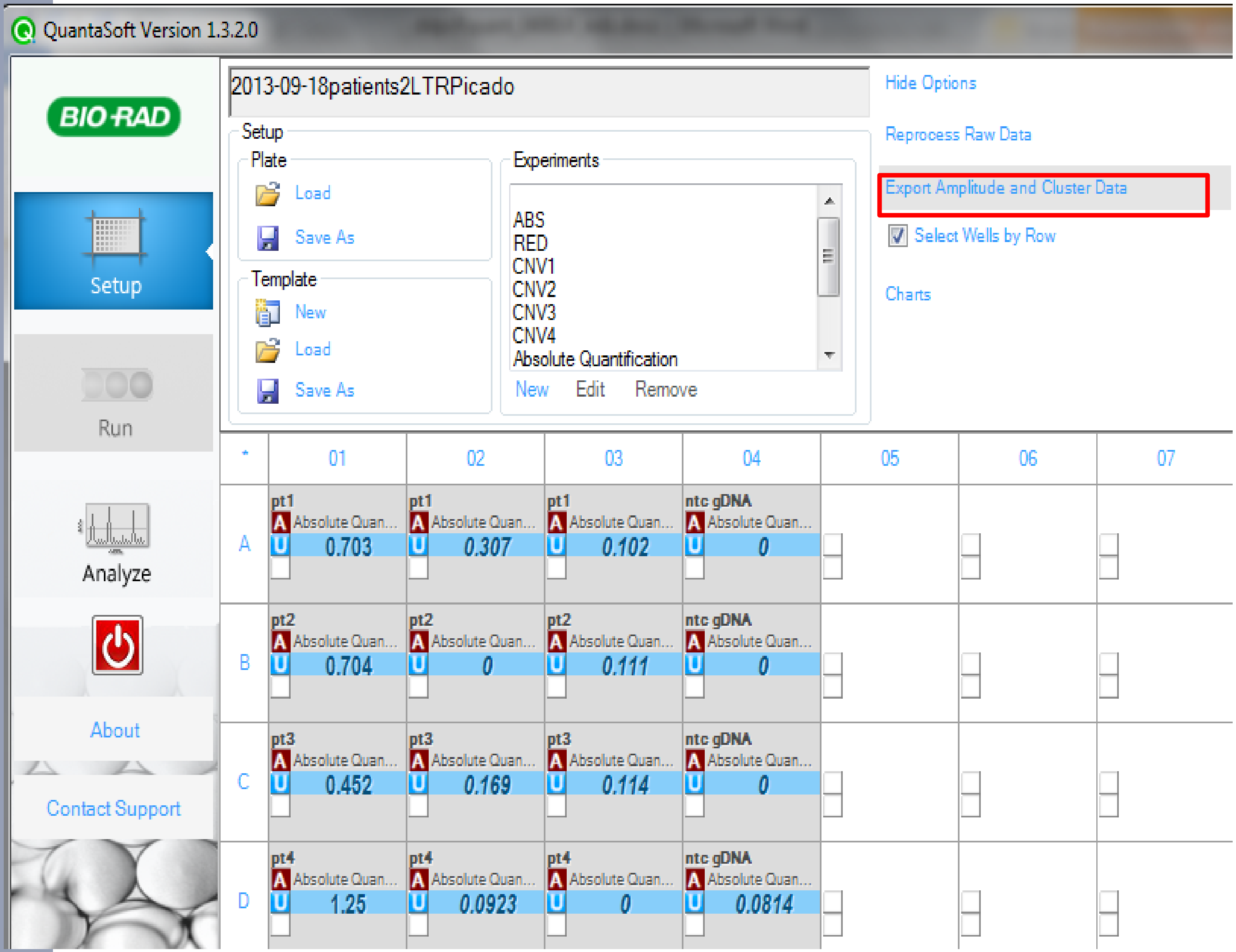
2013-09-18patients2LTRPicado

Hides the additional user options

Experiments:
- ABS
- RED
- CNV1
- CNV2
- CNV3
- CNV4
- Absolute Quantification

*	01	02	03	04	05	06	07
A	pt1 A Absolute Quan... U 0.703	pt1 A Absolute Quan... U 0.307	pt1 A Absolute Quan... U 0.102	ntc gDNA A Absolute Quan... U 0			
B	pt2 A Absolute Quan... U 0.704	pt2 A Absolute Quan... U 0	pt2 A Absolute Quan... U 0.111	ntc gDNA A Absolute Quan... U 0			
C	pt3 A Absolute Quan... U 0.452	pt3 A Absolute Quan... U 0.169	pt3 A Absolute Quan... U 0.114	ntc gDNA A Absolute Quan... U 0			
D	pt4 A Absolute Quan... U 1.25	pt4 A Absolute Quan... U 0.0923	pt4 A Absolute Quan... U 0	ntc gDNA A Absolute Quan... U 0.0814			

Exporting Data from Quantasoft



2013-09-18patients2LTRPicado

☑ Select Wells by Row

Experiments:

- ABS
- RED
- CNV1
- CNV2
- CNV3
- CNV4
- Absolute Quantification

*	01	02	03	04	05	06	07
A	pt1 A Absolute Quan... U 0.703	pt1 A Absolute Quan... U 0.307	pt1 A Absolute Quan... U 0.102	ntc gDNA A Absolute Quan... U 0			
B	pt2 A Absolute Quan... U 0.704	pt2 A Absolute Quan... U 0	pt2 A Absolute Quan... U 0.111	ntc gDNA A Absolute Quan... U 0			
C	pt3 A Absolute Quan... U 0.452	pt3 A Absolute Quan... U 0.169	pt3 A Absolute Quan... U 0.114	ntc gDNA A Absolute Quan... U 0			
D	pt4 A Absolute Quan... U 1.25	pt4 A Absolute Quan... U 0.0923	pt4 A Absolute Quan... U 0	ntc gDNA A Absolute Quan... U 0.0814			

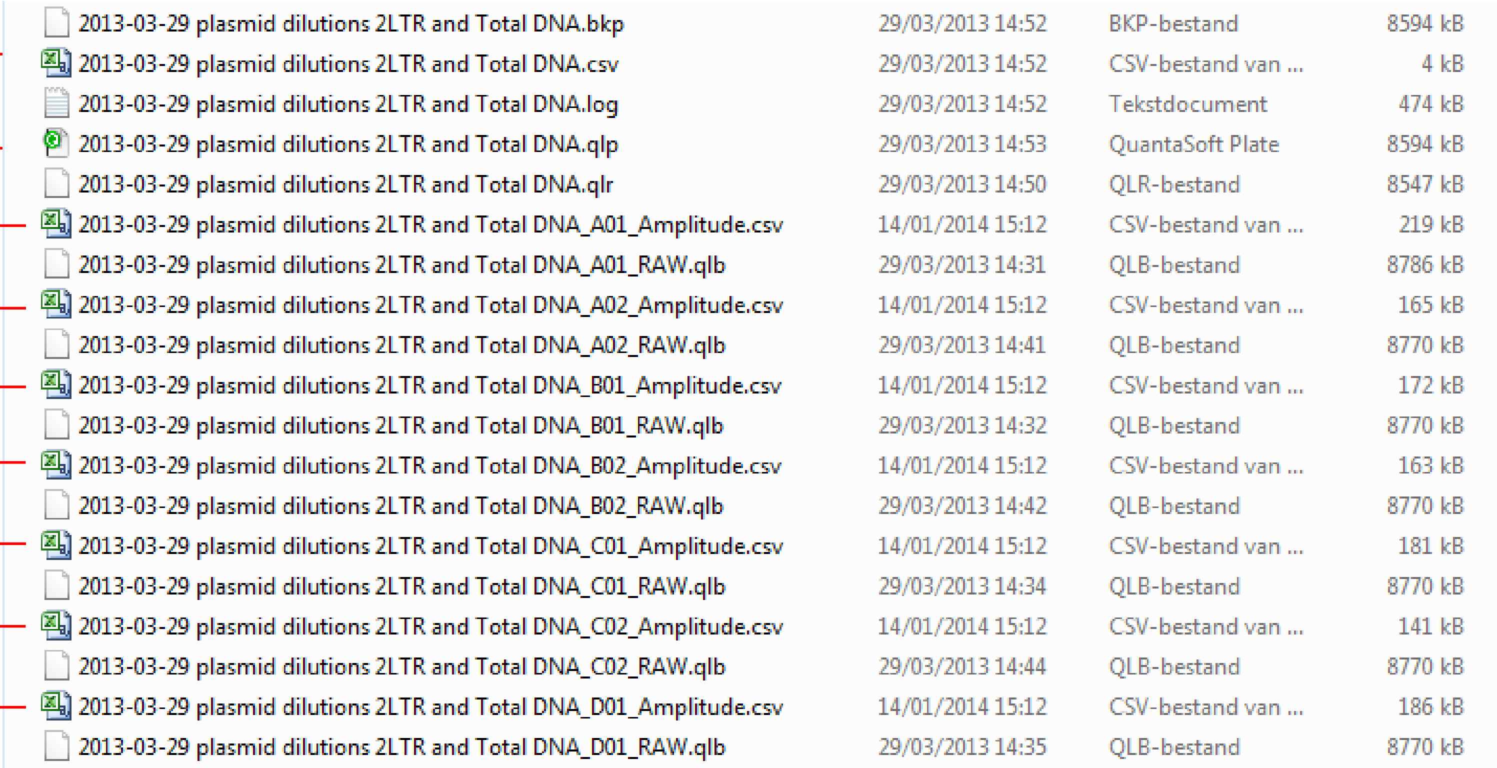
Exporting Data from Quantasoft

After exporting the folder should look like:

head

QS

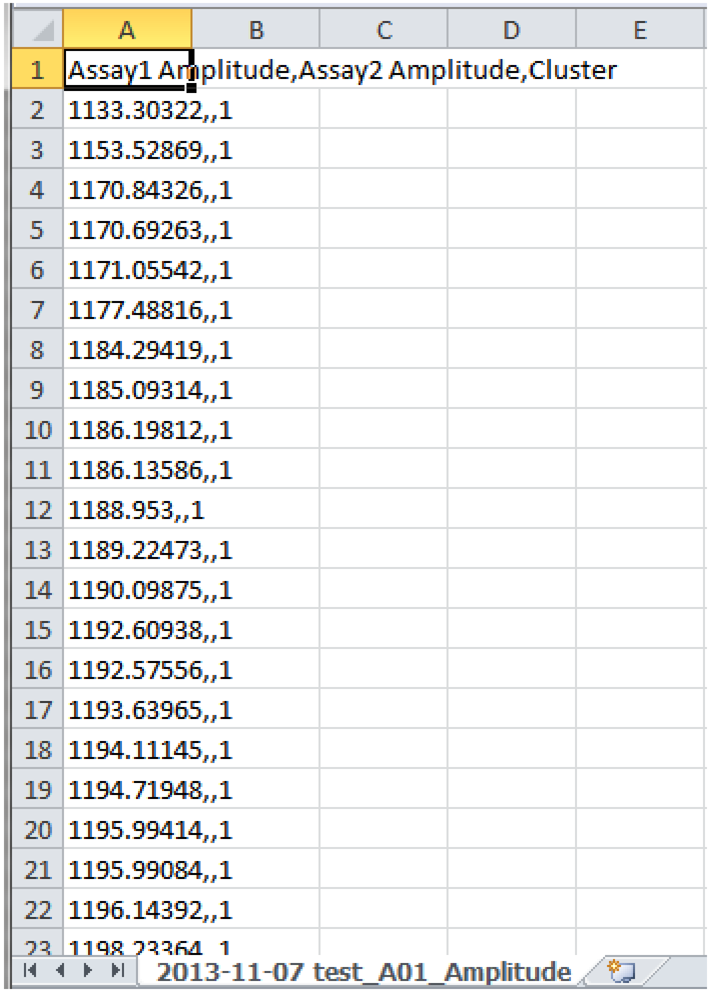
Amplitude csv



2013-03-29 plasmid dilutions 2LTR and Total DNA.bkp	29/03/2013 14:52	BKP-bestand	8594 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.csv	29/03/2013 14:52	CSV-bestand van ...	4 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.log	29/03/2013 14:52	Tekstdocument	474 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.qlp	29/03/2013 14:53	QuantaSoft Plate	8594 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA.qlr	29/03/2013 14:50	QLR-bestand	8547 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_A01_Amplitude.csv	14/01/2014 15:12	CSV-bestand van ...	219 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_A01_RAW.qlb	29/03/2013 14:31	QLB-bestand	8786 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_A02_Amplitude.csv	14/01/2014 15:12	CSV-bestand van ...	165 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_A02_RAW.qlb	29/03/2013 14:41	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_B01_Amplitude.csv	14/01/2014 15:12	CSV-bestand van ...	172 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_B01_RAW.qlb	29/03/2013 14:32	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_B02_Amplitude.csv	14/01/2014 15:12	CSV-bestand van ...	163 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_B02_RAW.qlb	29/03/2013 14:42	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_C01_Amplitude.csv	14/01/2014 15:12	CSV-bestand van ...	181 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_C01_RAW.qlb	29/03/2013 14:34	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_C02_Amplitude.csv	14/01/2014 15:12	CSV-bestand van ...	141 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_C02_RAW.qlb	29/03/2013 14:44	QLB-bestand	8770 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_D01_Amplitude.csv	14/01/2014 15:12	CSV-bestand van ...	186 kB
2013-03-29 plasmid dilutions 2LTR and Total DNA_D01_RAW.qlb	29/03/2013 14:35	QLB-bestand	8770 kB

Exporting Data from Quantasoft

This is how the csv file should look like



	A	B	C	D	E
1	Assay1 Amplitude,Assay2 Amplitude,Cluster				
2	1133.30322,,1				
3	1153.52869,,1				
4	1170.84326,,1				
5	1170.69263,,1				
6	1171.05542,,1				
7	1177.48816,,1				
8	1184.29419,,1				
9	1185.09314,,1				
10	1186.19812,,1				
11	1186.13586,,1				
12	1188.953,,1				
13	1189.22473,,1				
14	1190.09875,,1				
15	1192.60938,,1				
16	1192.57556,,1				
17	1193.63965,,1				
18	1194.11145,,1				
19	1194.71948,,1				
20	1195.99414,,1				
21	1195.99084,,1				
22	1196.14392,,1				
23	1198.23364,,1				

On the first row:
3 names seperated by 2 commas

Next rows:
3 numerical values separated by 2 commas

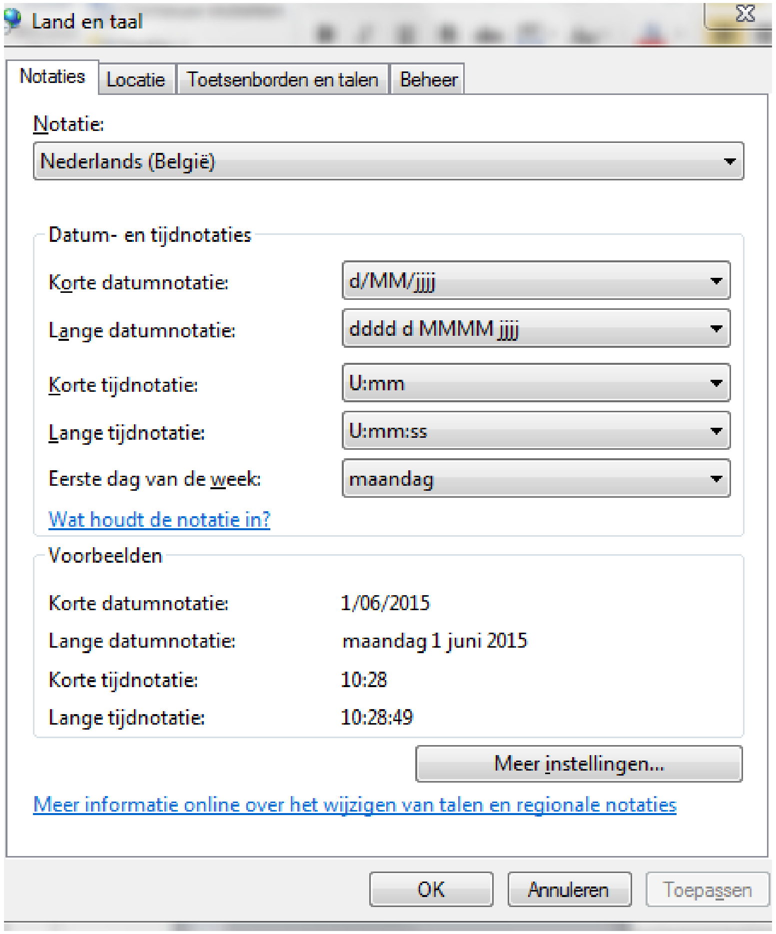
Depending on the measured channels 1 or 2 decimal numbers

Cluster number

Exporting data from Quantasoft

Decimal sign needs to be “ . “

Control Panel > Regional and Language options > Format tab



Notatie: Nederlands (België)

Datum- en tijdnotaties

Korte datumnotatie: d/MM/jjjj

Lange datumnotatie: dddd d MMMM jjjj

Korte tijdnotatie: U:mm

Lange tijdnotatie: U:mm:ss

Eerste dag van de week: maandag

Wat houdt de notatie in?

Voorbeelden

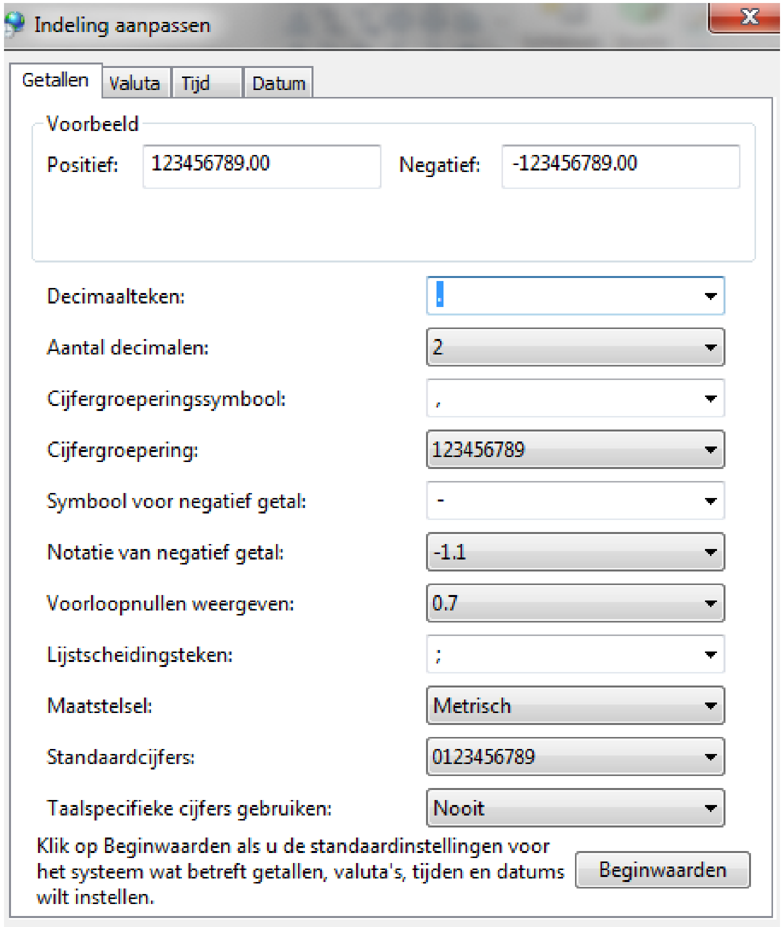
Korte datumnotatie: 1/06/2015

Lange datumnotatie: maandag 1 juni 2015

Korte tijdnotatie: 10:28

Lange tijdnotatie: 10:28:49

Meer informatie online over het wijzigen van talen en regionale notaties



Voorbeeld

Positief: 123456789.00

Negatief: -123456789.00

Decimaalteken: .

Aantal decimalen: 2

Cijfergroeperingssymbool: ,

Cijfergroepering: 123456789

Symbool voor negatief getal: -

Notatie van negatief getal: -1.1

Voorloopnullen weergeven: 0.7

Lijstscheidingsteken: ;

Maatstelsel: Metrisch

Standaardcijfers: 0123456789

Taalspecifieke cijfers gebruiken: Nooit

Klik op Beginwaarden als u de standaardinstellingen voor het systeem wat betreft getallen, valuta's, tijden en datums wilt instellen.

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- Exporting Data from the ddpcr Reader
- **Running ddpcRquant**
- Exercises part 1 and 2
- Head file options

Running ddpcRquant

Where to find the tool and additional info:

1. Browse to http://www.ddpcrquant.ugent.be
    - Click on the ddpcRquant webtool link (demo)
2. See also the Quick ddpcRquant analysis guide

What you will need today:

1. There is a preloaded demo dataset
2. Download the exercises folder from the course dropbox

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Exercises part 1

1. **Perform the demo analysis (set to qx100!)**
2. **Use the demo dataset and set the threshold to a manual defined threshold for assay RNA**
    1. Download the plots, summary table and summary parameters

Exercises part 2

1. **Quantasoft analysis in following folder exercises_part2**
    1. Open in Quantasoft (qlp)
    2. Go to Analyze (left side panel)
        1. Select all samples (*) + sort on target + select all RU5 + update
        2. Go to 1-D Amplitude
        3. Auto Analyze with individual and combined wells => What is wrong?
            1. Individual wells?
            2. Combined wells?
        4. Select wells C01 and E03 and look in ch1 => What is wrong?
        5. Write down the concentration of C01

    3. Export the amplitudefiles
        1. Go to Setup > options > export amplitude and cluster data
        2. Select folder

Exercises part 2

2. Upload the amplitudefiles + headfile to ddpcRquant

3. Run analysis with standard settings

Write down the concentration of sample C01 (0.5 vs 0.28) => reason?

4. Run analysis with a 99.99 threshold

What happens with the threshold? Why?

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Head file options

Annotation before run starts: Quantasoft

Annotation after run

- Change headfile in text format reader:
  notepad, noteblock, NOT excel
- Use qx100/qx200 template

Head file options

Open following headfile:

1. **Find some missing or wrong annotation and change it according to the annotation rules**

   6 mistakes

2. **Upload head & amplitude and look at overview**

Demo: Make template

1. **Make the demo template yourself**

   Wells: A03,B03,G03,H03

   Sample: test1,test1,NTC,NTC

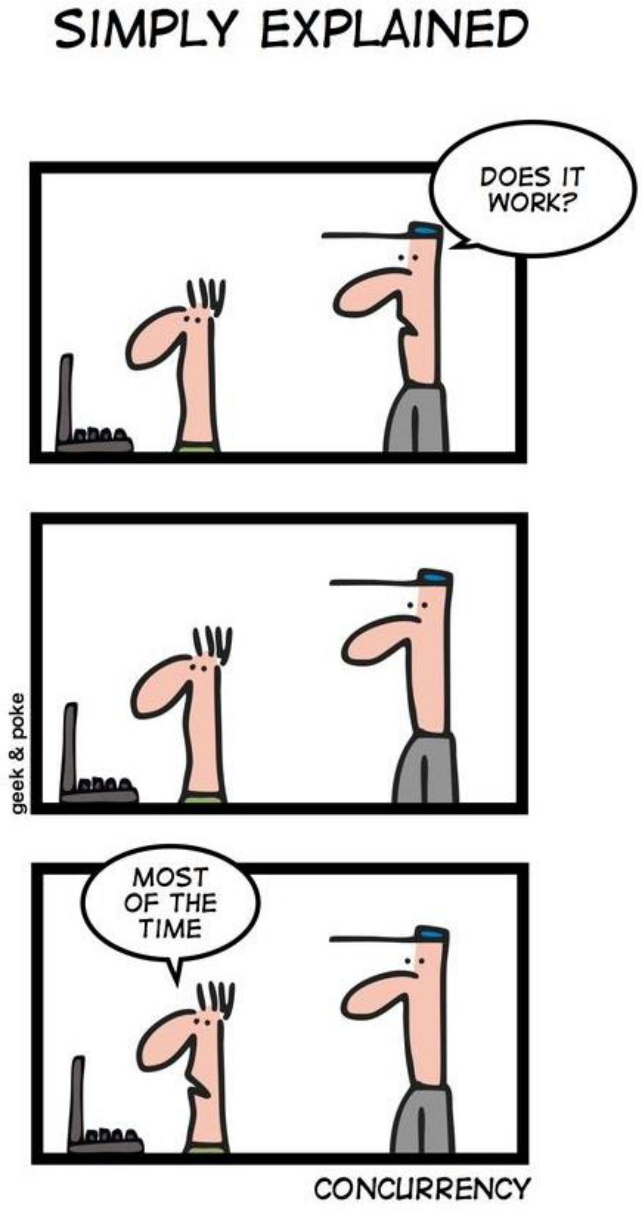
   TypeAssay: Ch1Unknown

   Assay: RPP30

2. **Upload template & amplitudefiles to ddpcRquant**

   make sure the template file is uploaded first (sort folder before upload)

Hope it works most of the time!



Good luck with future analysis!

Troubleshooting + ddPCR advice:

Wim.trypsteen@ugent.be