FACULTY OF MEDICINE
AND HEALTH SCIENCES

DEPARTMENT OF INTERNAL MEDICINE
HIV CURE RESEARCH CENTER

DIGITAL PCR DATA ANALYSIS

HANDS-ON

Dr. Wim Trypsteen

28th November, Gent, Belgium

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OUTLINE

- **Introduction: digital PCR data analysis**
- Threshold determination: ddpcRquant (theory)
- ddpcRquant: overview algorithm
- Hands-on: exercises (droplets at work)

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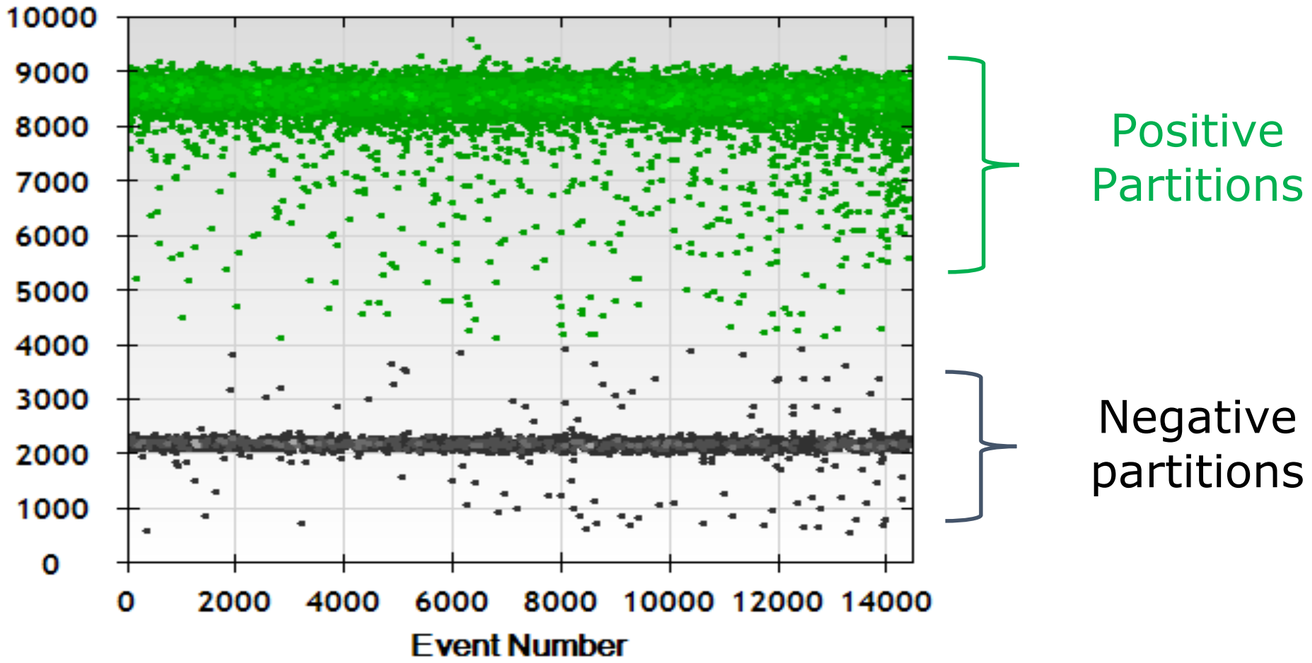
WHAT IS STILL LEFT TO DO: DATA ANALYSIS

– Extract the fluorescence data and quantify sample



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

N_{neg}: number of negative partitions

N: total number of partitions

V_d: volume of partition

D: dilution factor of sample

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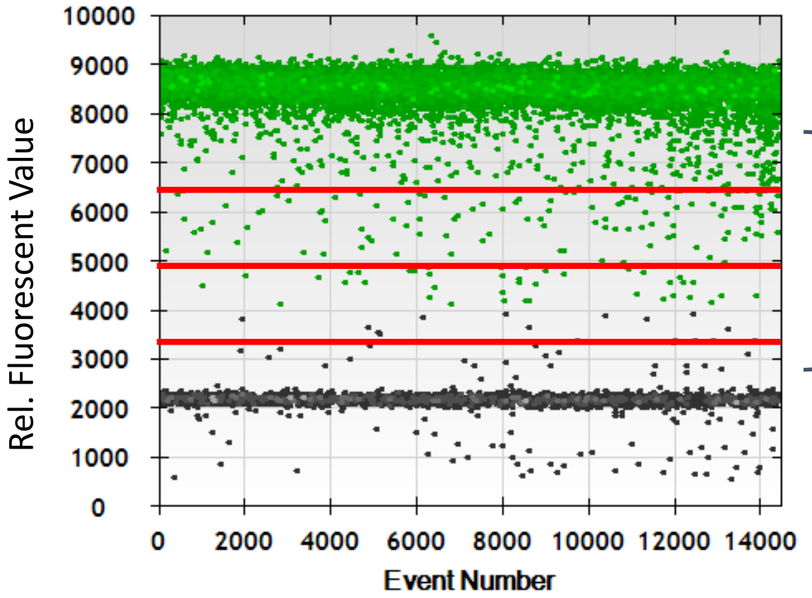
WHAT IS STILL LEFT TO DO: THRESHOLD

– Threshold determination

Treshold affects concentration



Positive Partitions

Rain

Negative Partitions

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

The helping hand: ddpcRquant

A statistical framework for threshold determination

Start from a Negative Template Control and apply the calculated threshold to the samples

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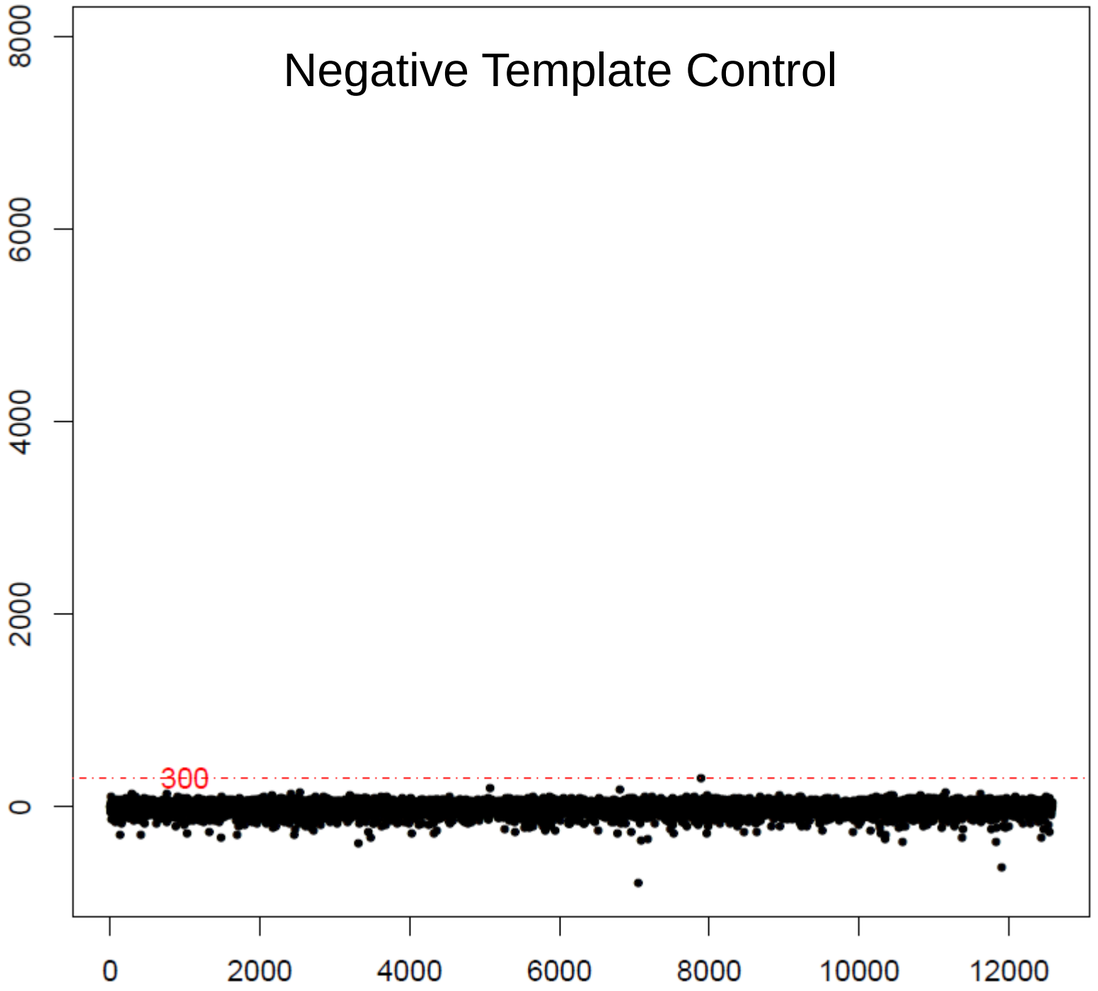
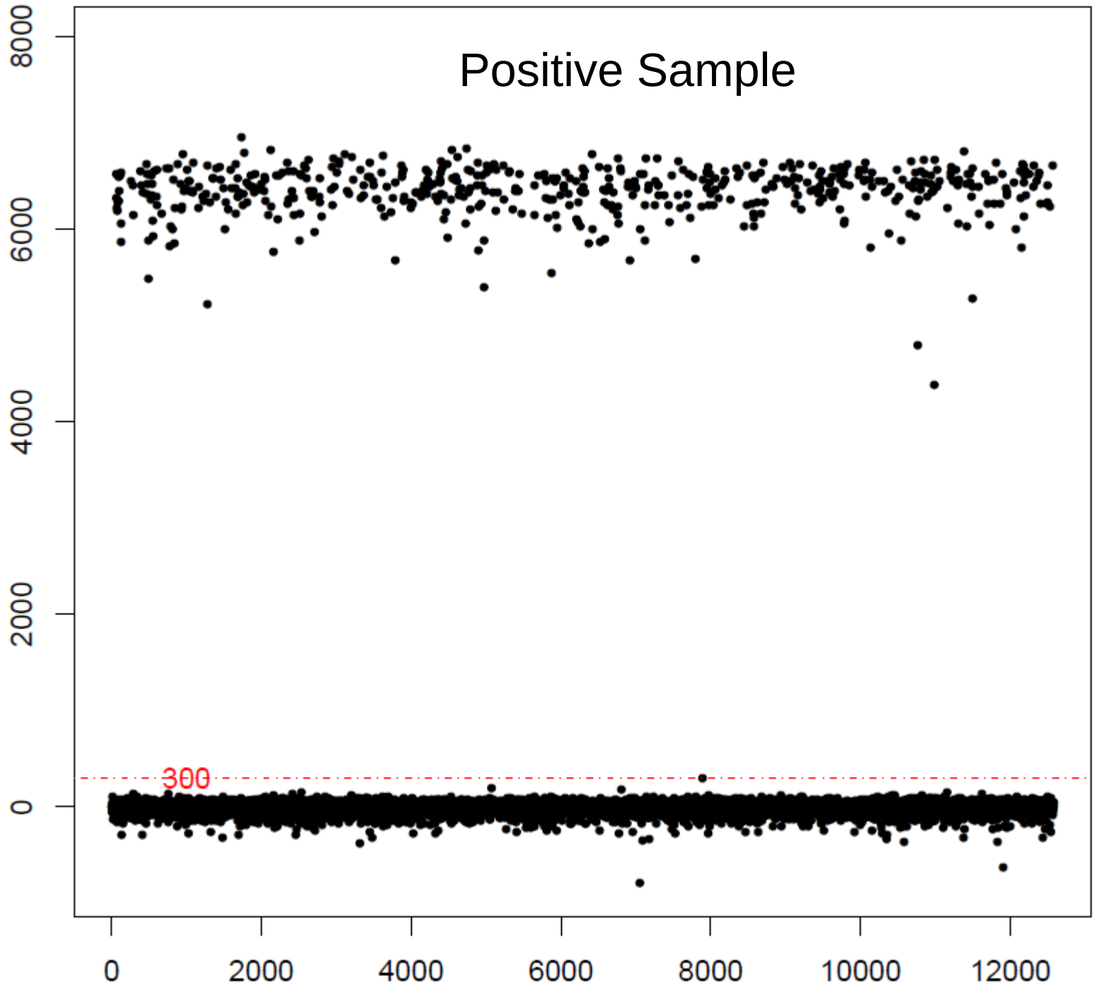
THRESHOLD: FROM NTC TO SAMPLE





pos: 544 neg: 12033 tot: 12577 conc: 971.7994 copies/uL stock

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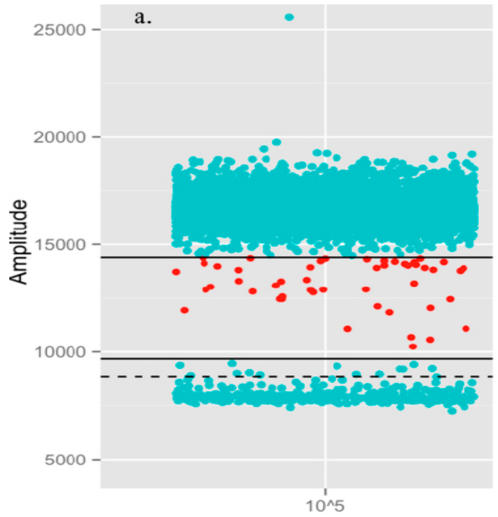
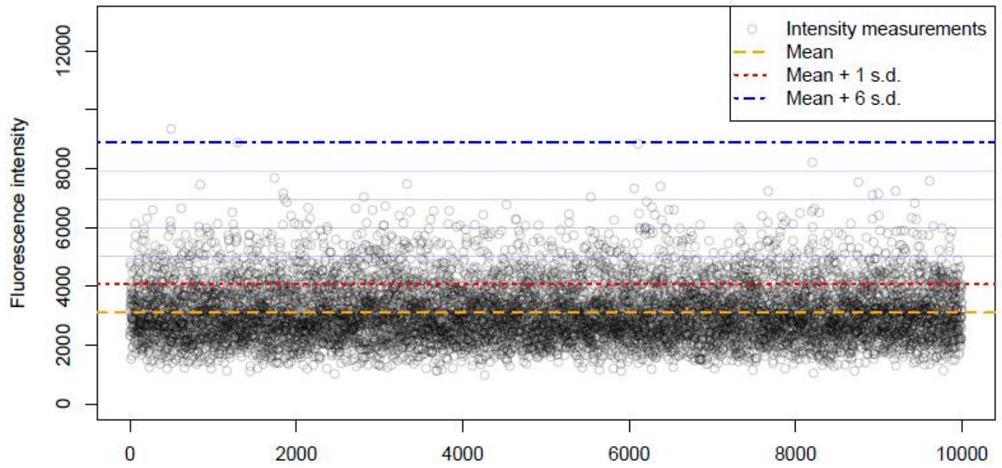
THRESHOLD DETERMINATION

Setting a Threshold: Overview of Methods

- Manually (Software provided by manufacturer)
- Proprietary software: black box algorithm
- Clustering: determine cluster for both positive and negative droplets, discard droplets which are further than a certain distance from cluster centers (Strain et al., 2013; Jones et al., 2014)
- Normal distribution: mean intensity + 6 s.d. = threshold (Dreo et al., 2014)





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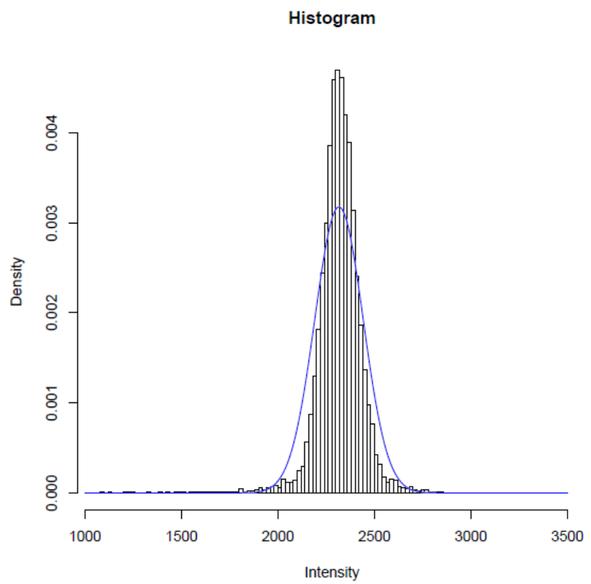
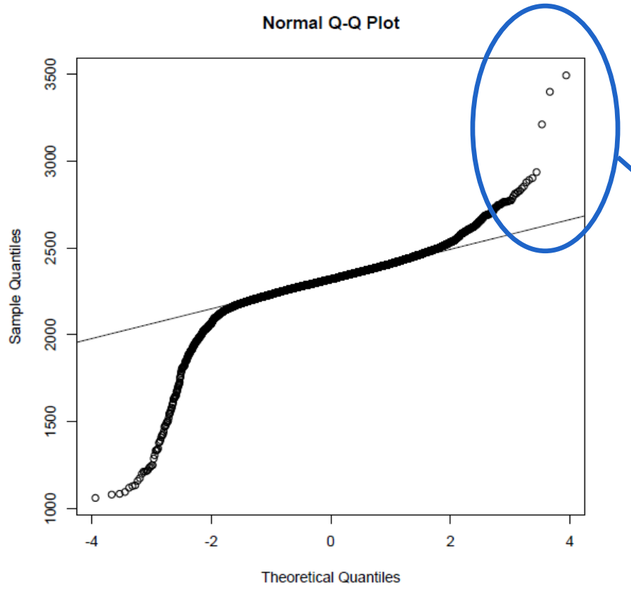
THRESHOLD DETERMINATION





Solution ddpcrquant:
Extreme Value Theory

Intensities are not normally distributed!

<u>135 NTCs</u>

128 Not likely to follow normal distribution ($p < 0.000001$)

2 Not likely to follow normal distribution ($0.05 > p > 0.001$)

5 Likely to follow normal distribution ($p > 0.05$)

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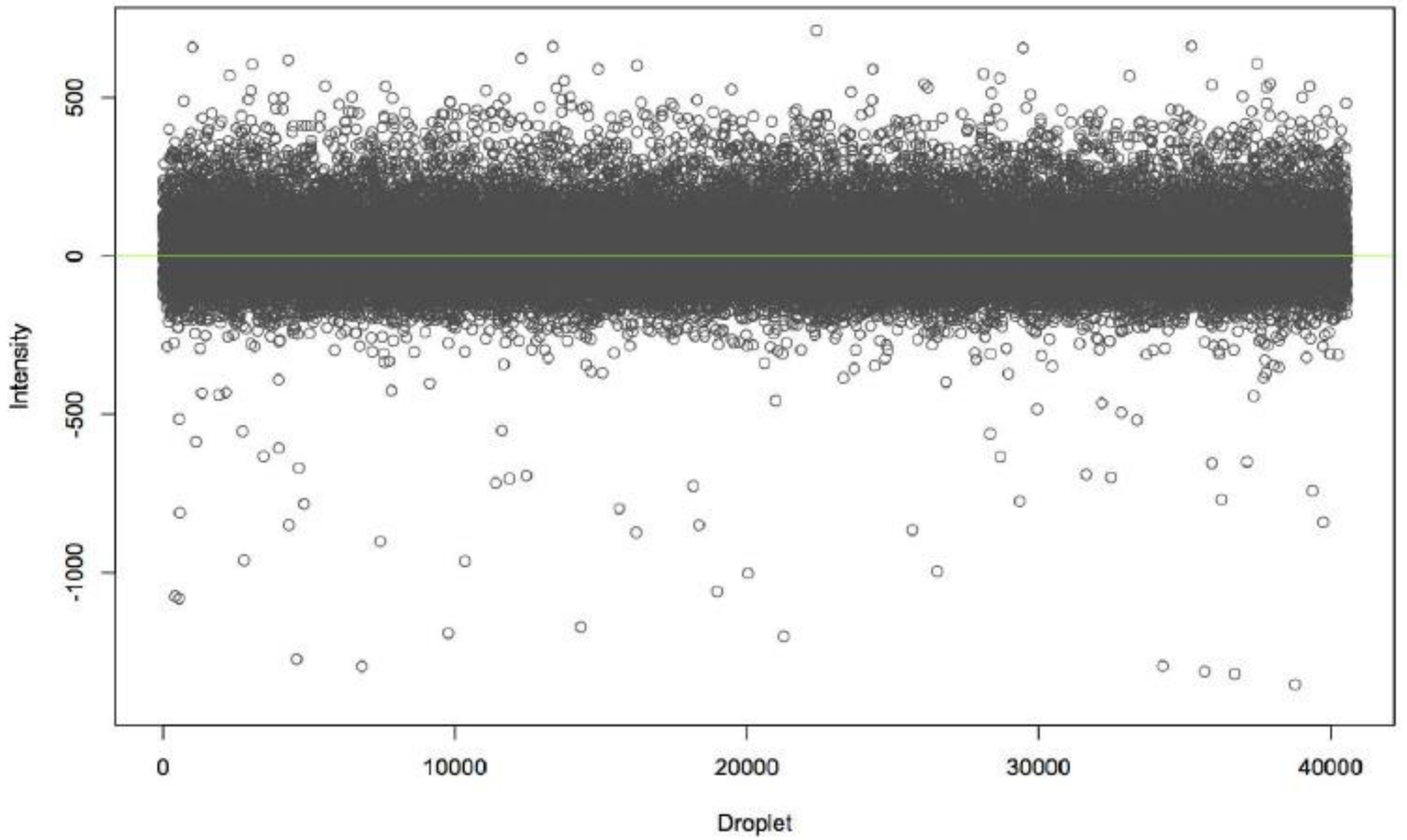
APPLY EXTREME VALUE THEORY



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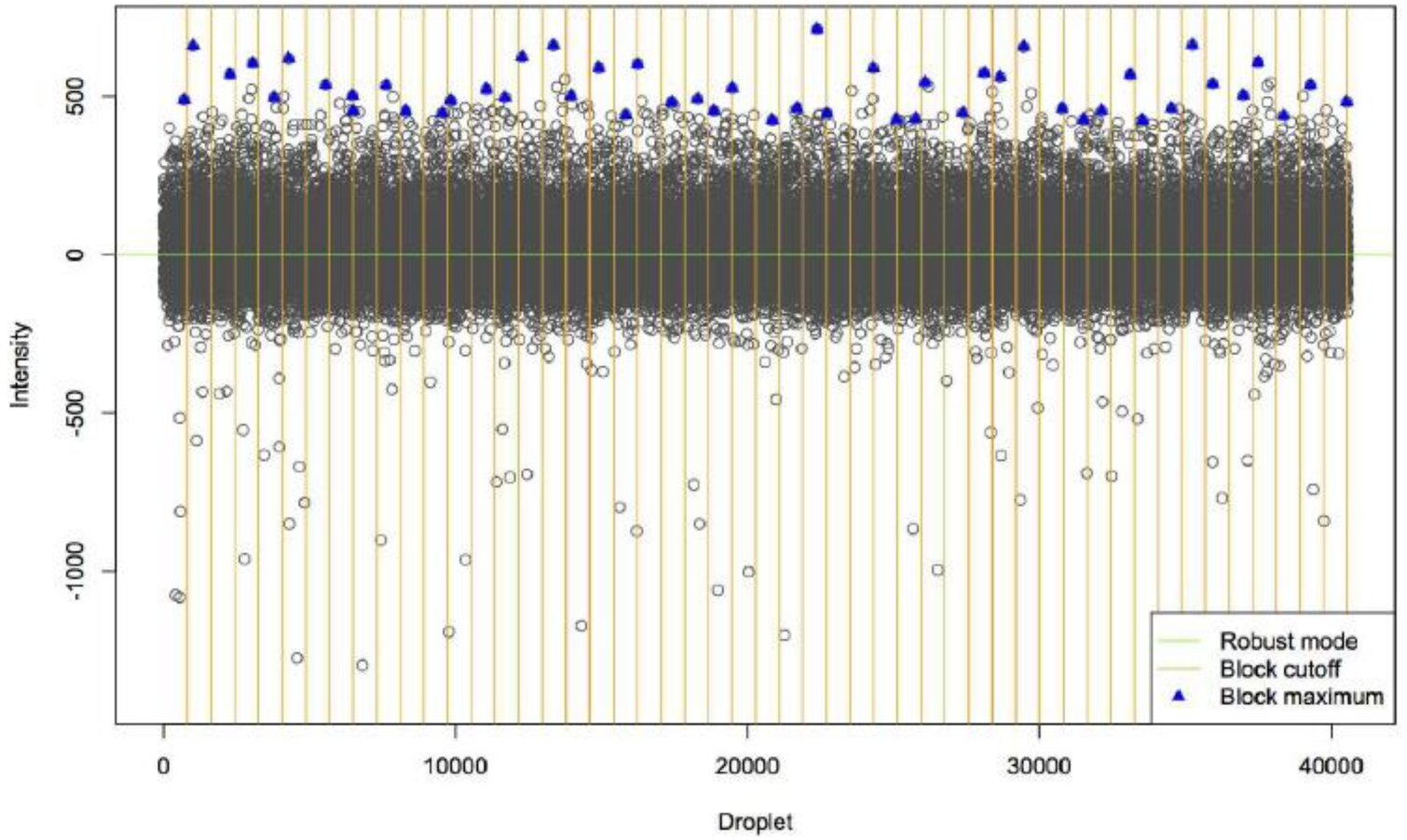
APPLY EXTREME VALUE THEORY



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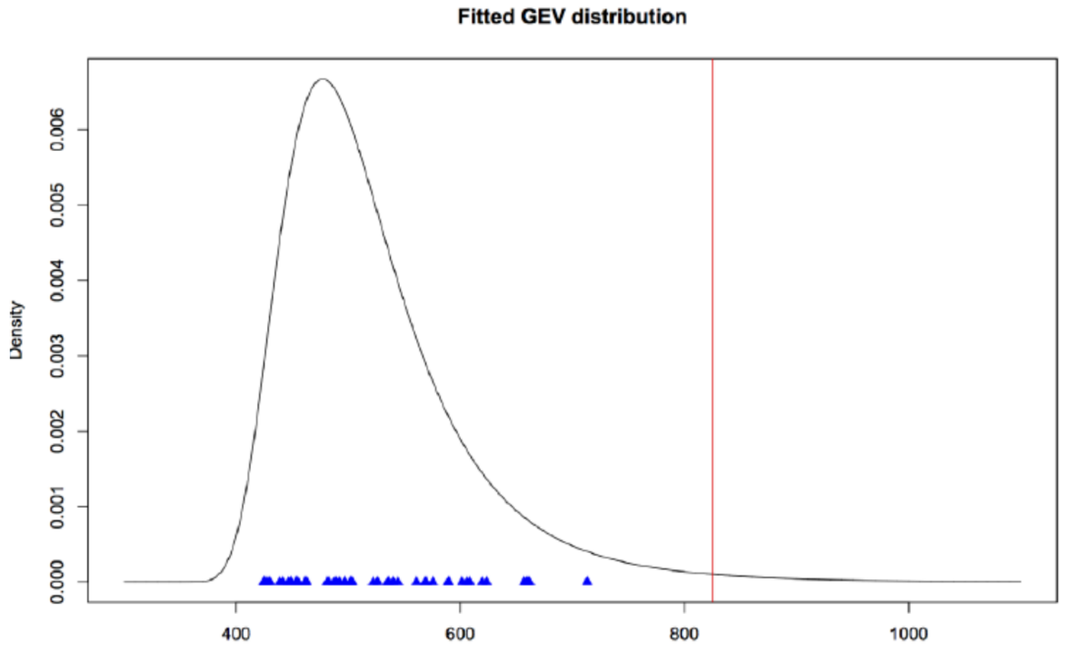
APPLY EXTREME VALUE THEORY

Always follow the GEV distribution



GEV(μ, σ, ξ)

$$\frac{1}{\sigma}\, t(x)^{\xi+1} e^{-t(x)},$$

where

$$t(x) = \begin{cases} \left(1 + \left(\frac{x-\mu}{\sigma}\right)\xi\right)^{-1/\xi} & \text{if } \xi \neq 0 \\ e^{-(x-\mu)/\sigma} & \text{if } \xi = 0 \end{cases}$$

This function takes 3 parameters: location, scale and shape to be modelled based on the extremes

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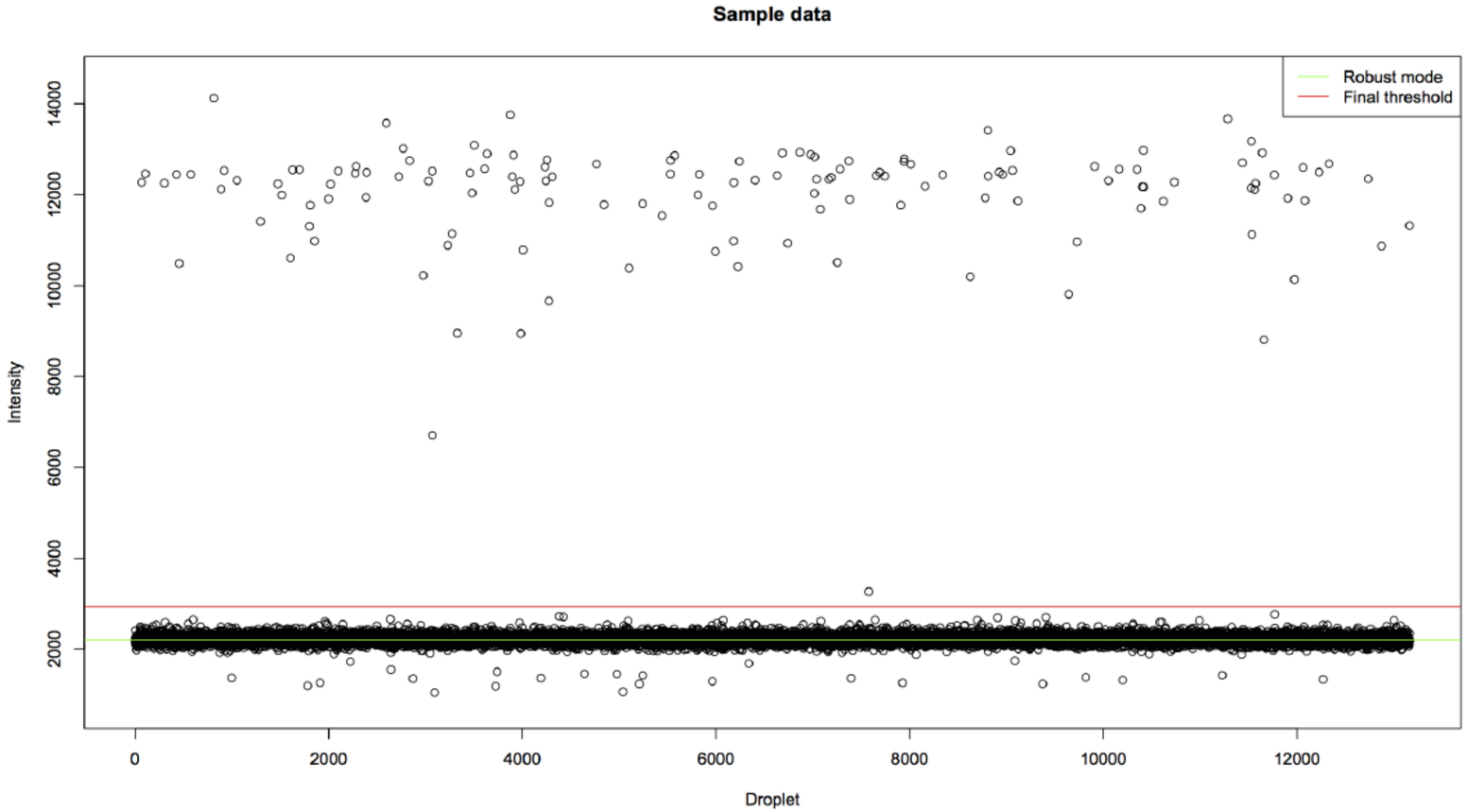
APPLY EXTREME VALUE THEORY



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- **ddpcRquant: overview algorithm**
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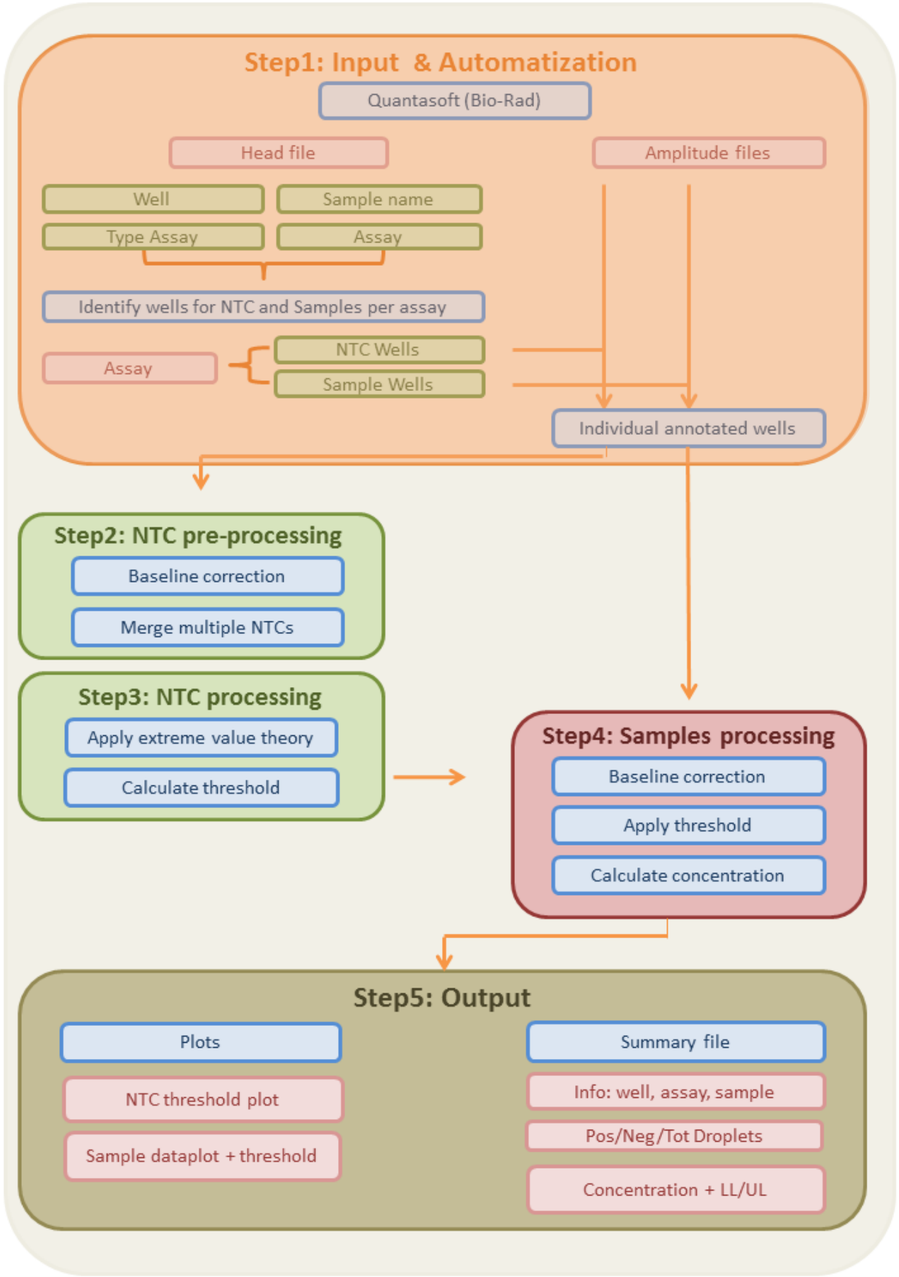
DDPCRQUANT



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
1057.41455
1205.11
1227.16284
1266.01575
1290.73767

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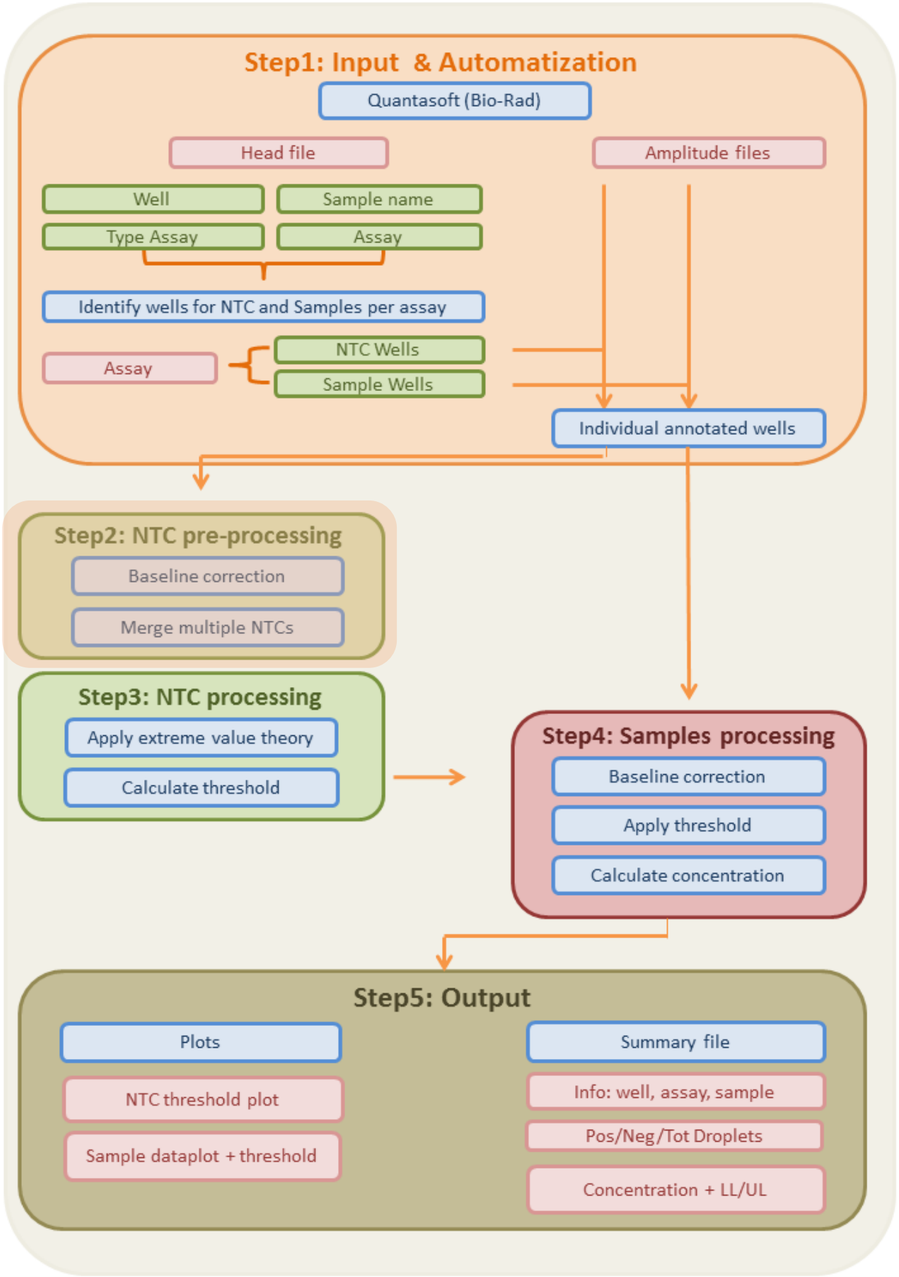
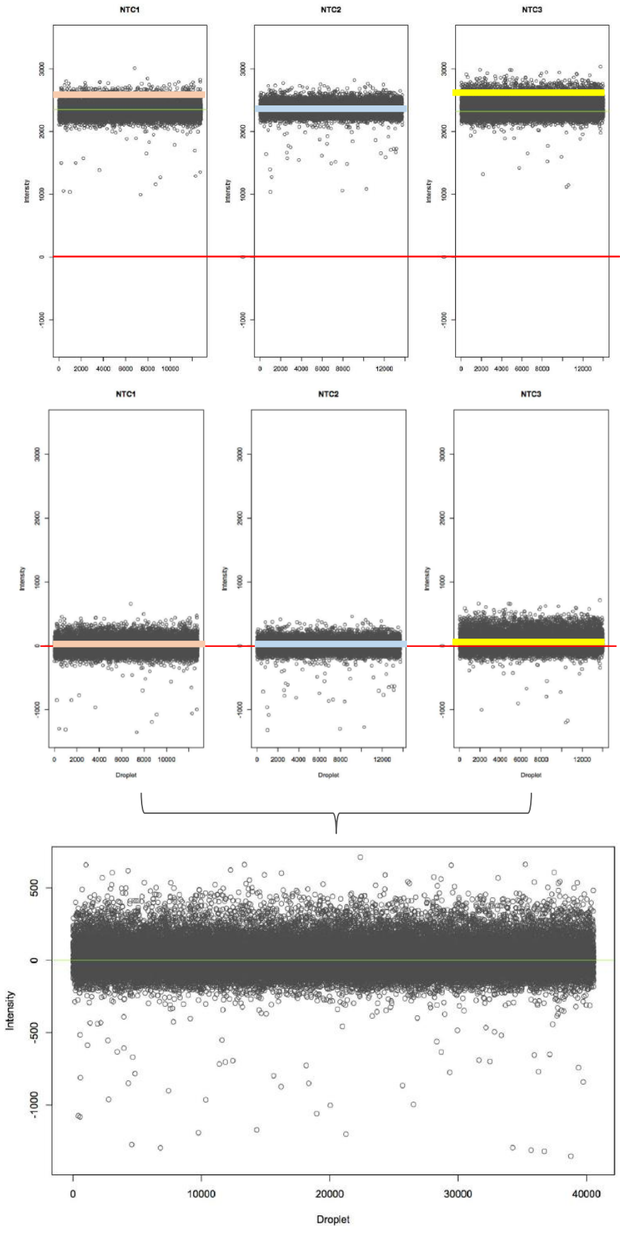
DDPCRQUANT





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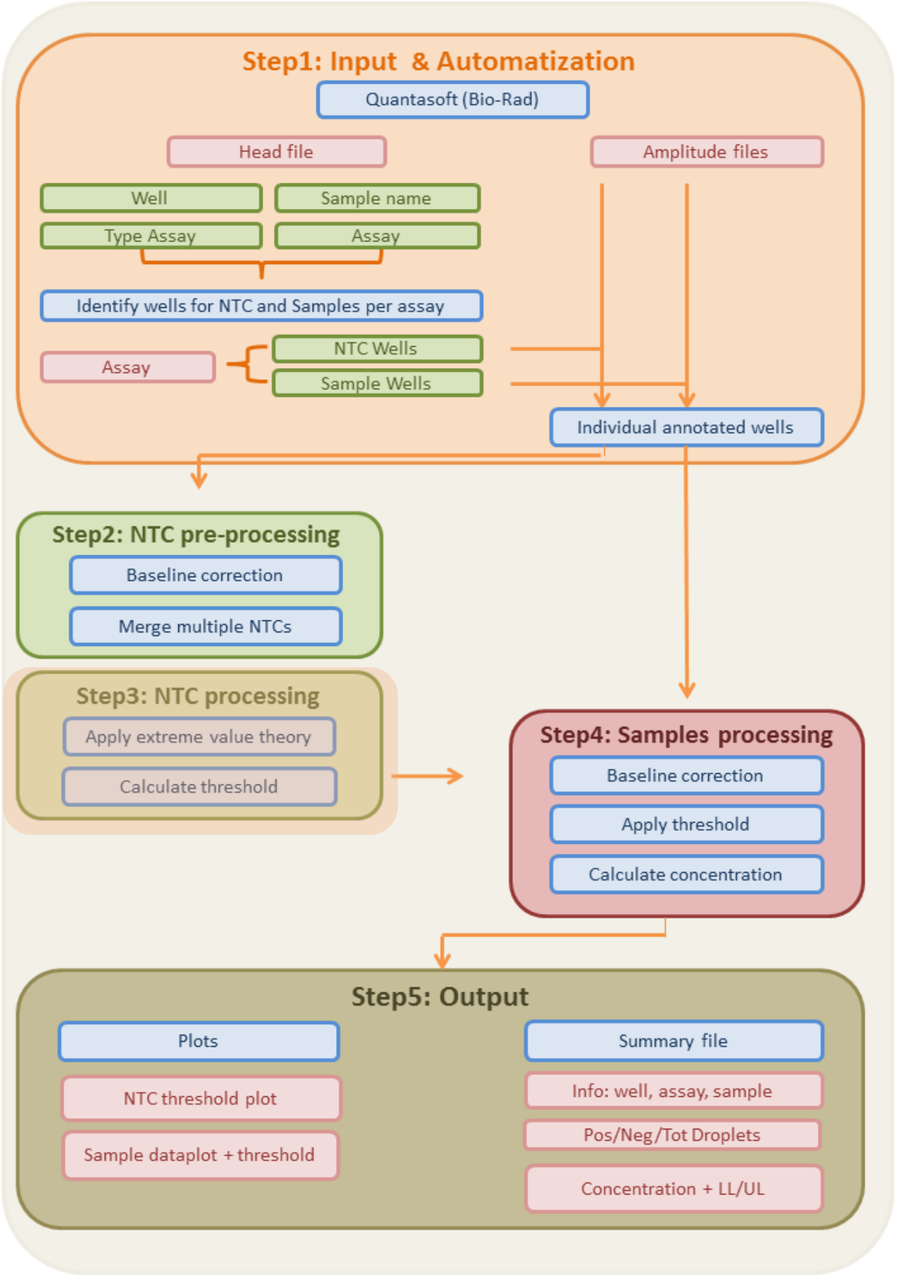
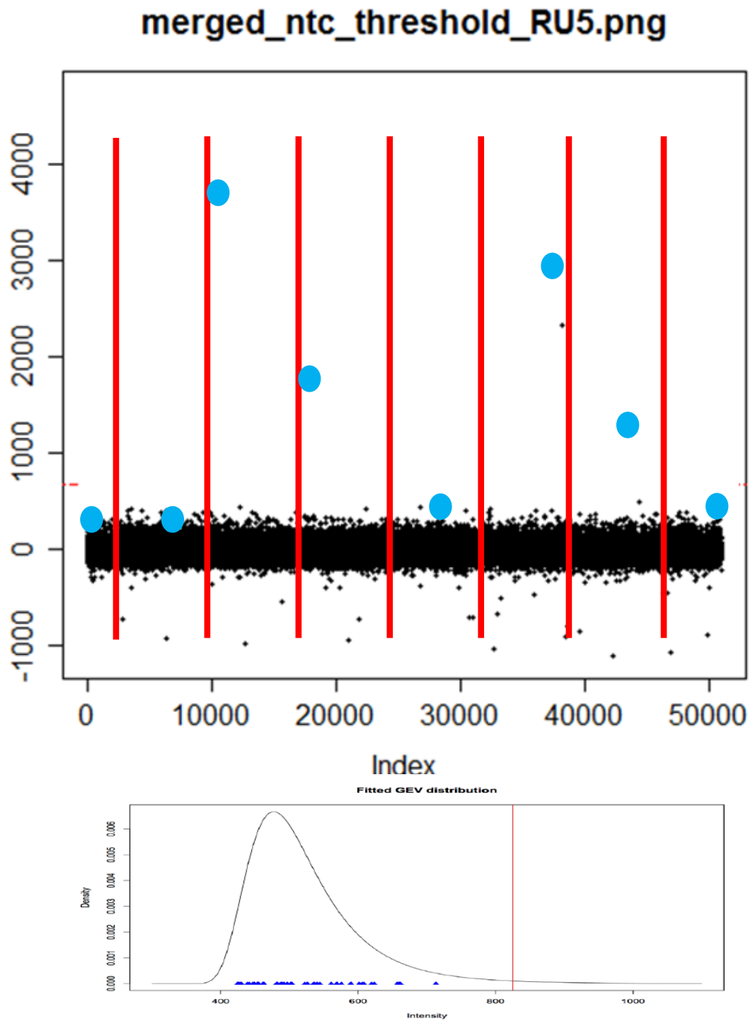
DDPCRQUANT



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



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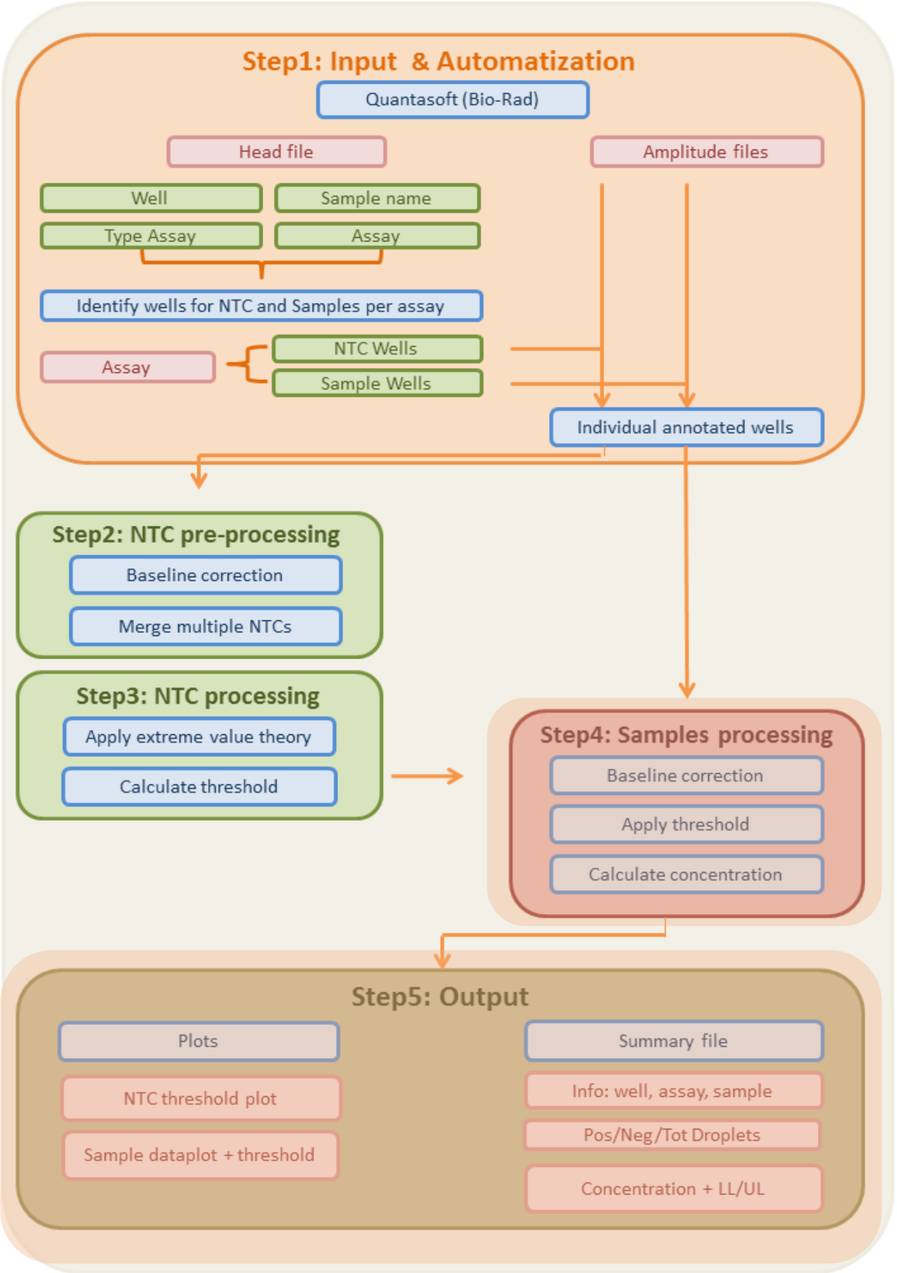
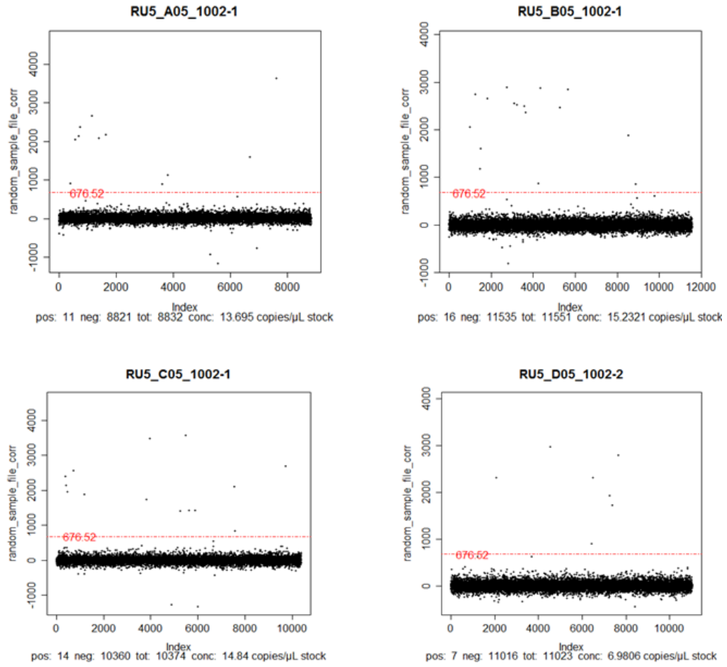
DDPCRQUANT





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

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

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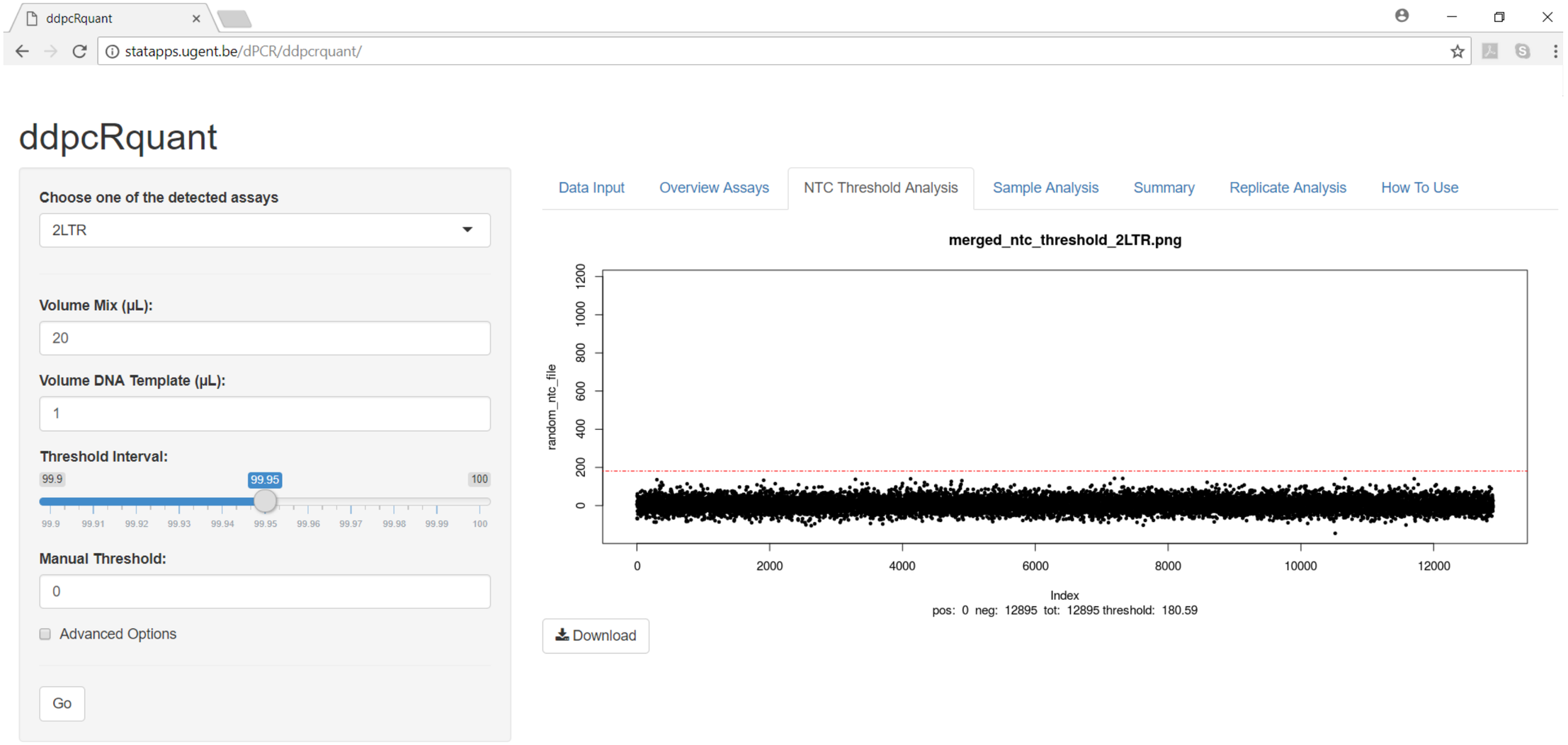
WEBTOOL DDPCRQUANT



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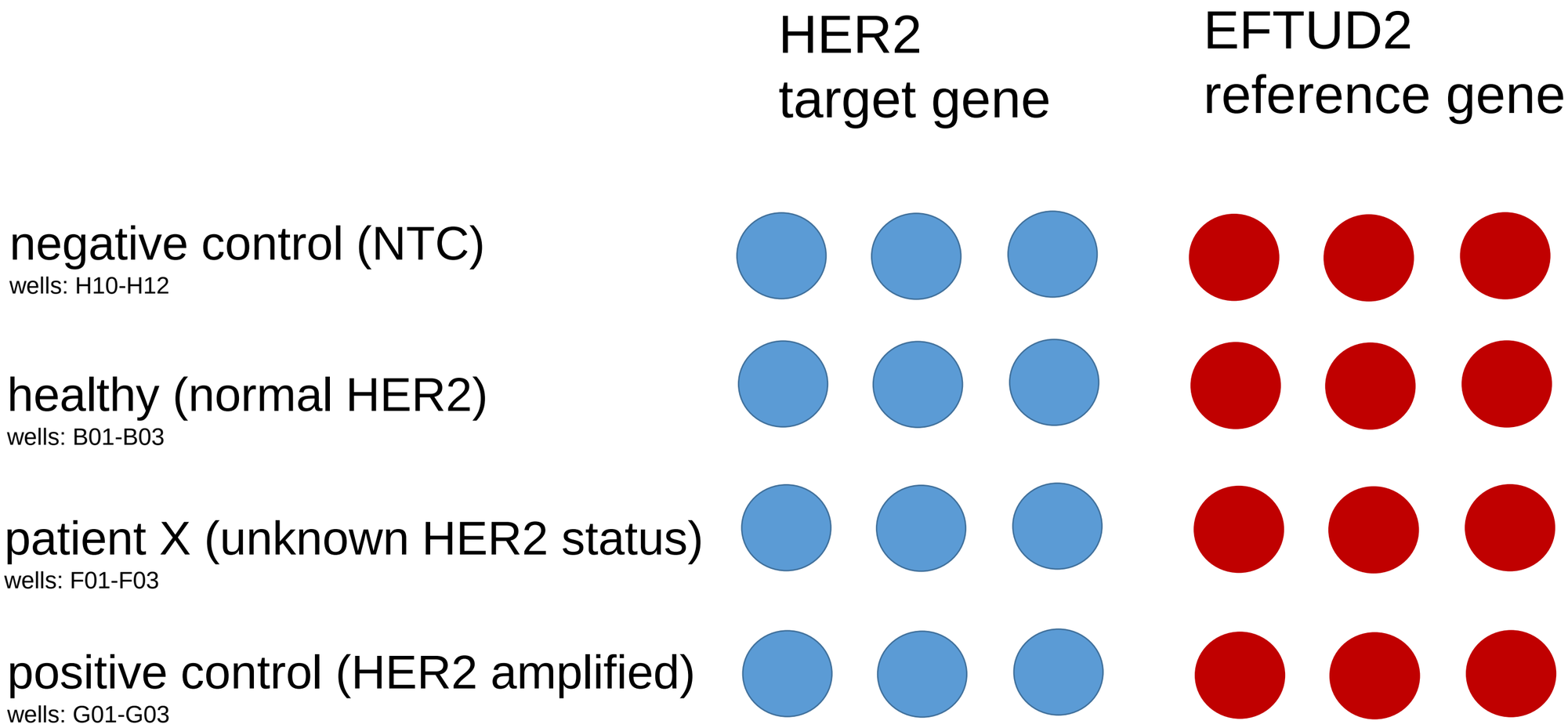
QUESTION FROM THE CLINIC

- Patient X suffers from breast cancer. Can you determine the possibility of a curative intervention for Patient X with Herceptin.
  - Herceptin can be given if the breast cancer tumors are positive for amplification of the HER2 gene and boost survival of breast cancer patients

- A digital PCR test was designed to resolve this question using DNA from the tumor
  - duplex digital PCR (two targets in the same reaction)
    - HER2 target gene
    - EFTUD2 reference gene (located on the same chromosome arm) for normalization

EXPERIMENTAL SETUP OF DIGITAL PCR RUN



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DIGITAL PCR DATA ANALYSIS

- Open Rstudio and exercise_digital_pcr.R script: follow instructions

- Perform ddpcRquant analysis to determine threshold and absolute quantification per well for HER2 and EFTUD2

- Use GLMM to perform
  - absolute quantification per sample for HER2 and EFTUD2 (combining replicates)
  - ratio or CNV calculation of HER2/EFTUD2 (combining replicates)

- for GLMM work will be divided in 3 groups
  - group1: healthy sample
  - group2: unknown sample (patient X)
  - group3: positive control sample
  - all: report the ratio with 95% CI

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SOLUTION

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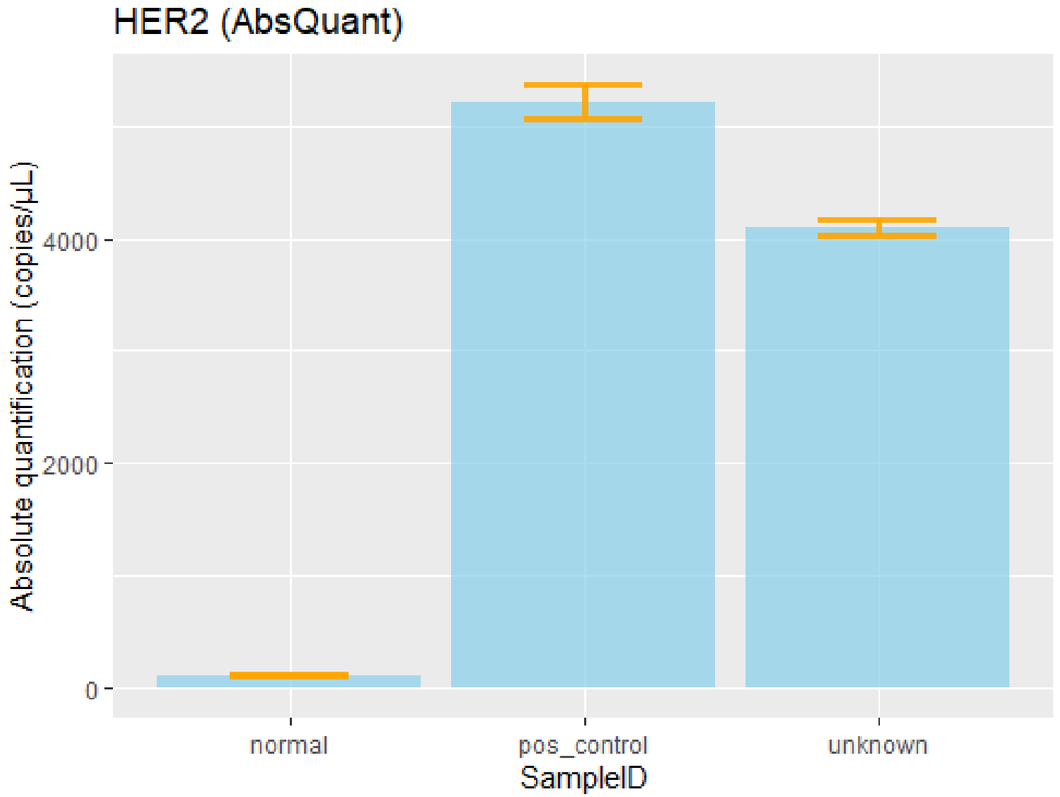
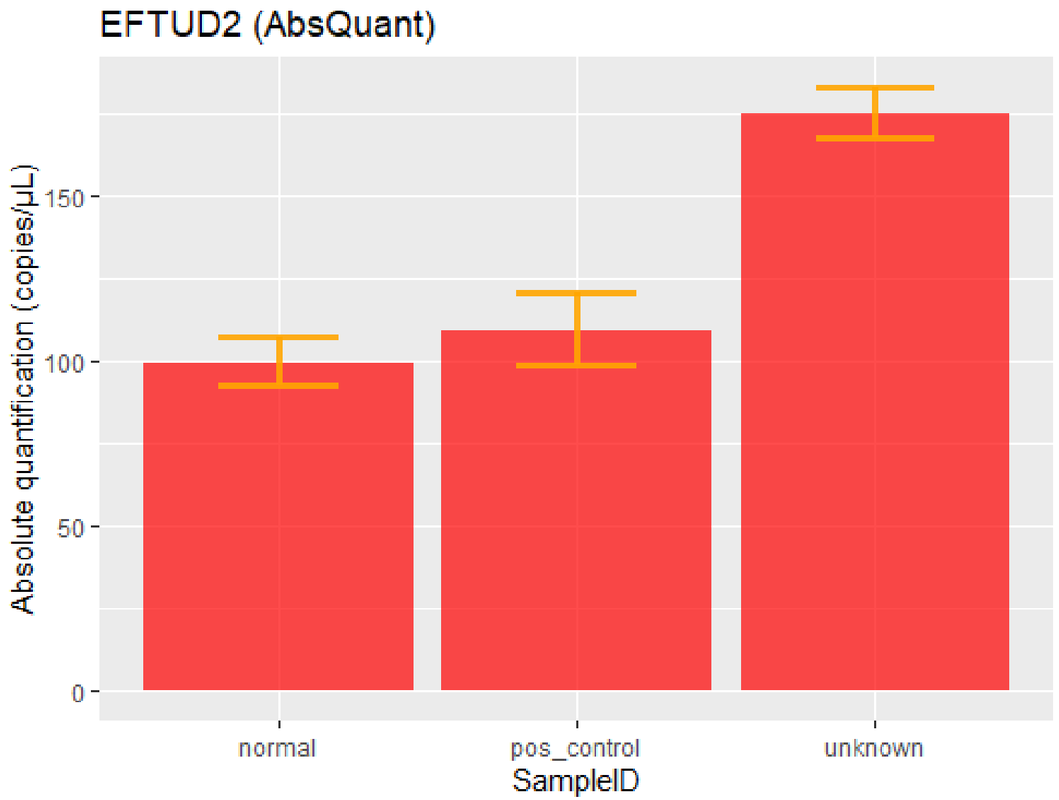
GLMM ABSOLUTE QUANTIFICATION



115.6
[112.2-119.2]

5211.3
[5063.0-5364.0]

4097.8
[4029.5-4167.4]



99.8
[93.0-107.2]

109.5
[99.2-120.8]

175.3
[167.8-183.2]

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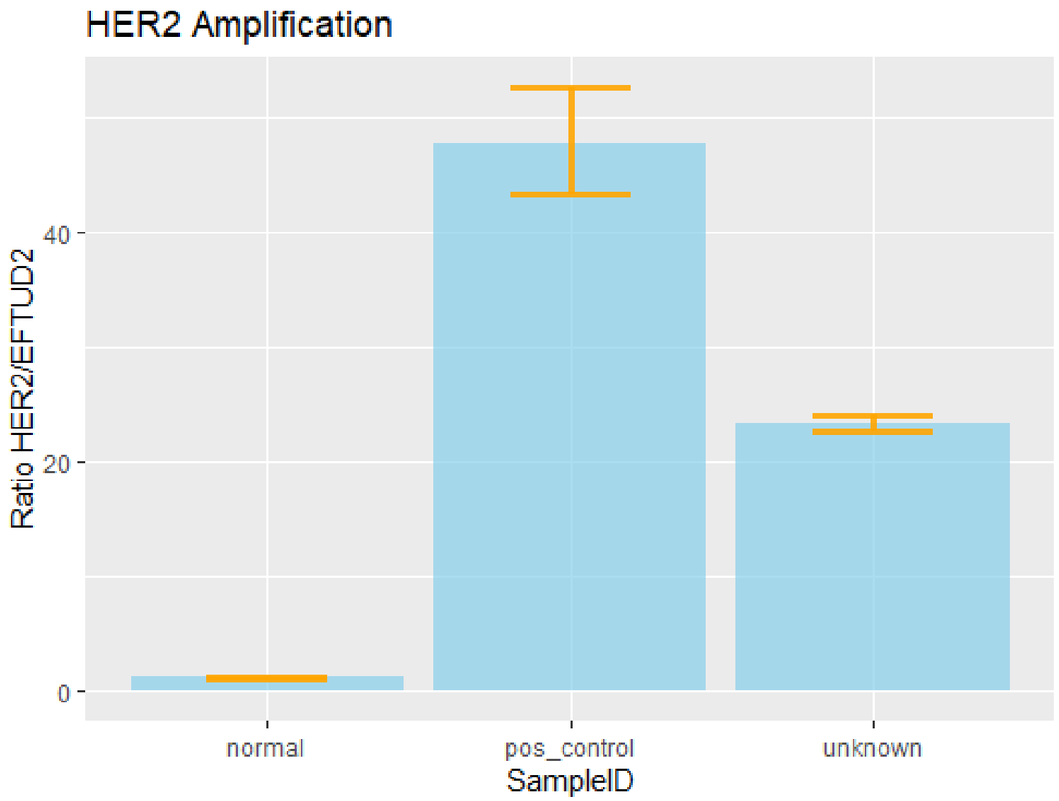
HER2 AMPLIFICATION IN RATIO (GLMM)



1.16	47.8	23.4
[1.08-1.25]	[43.34-52.66]	[22.68-24.12]

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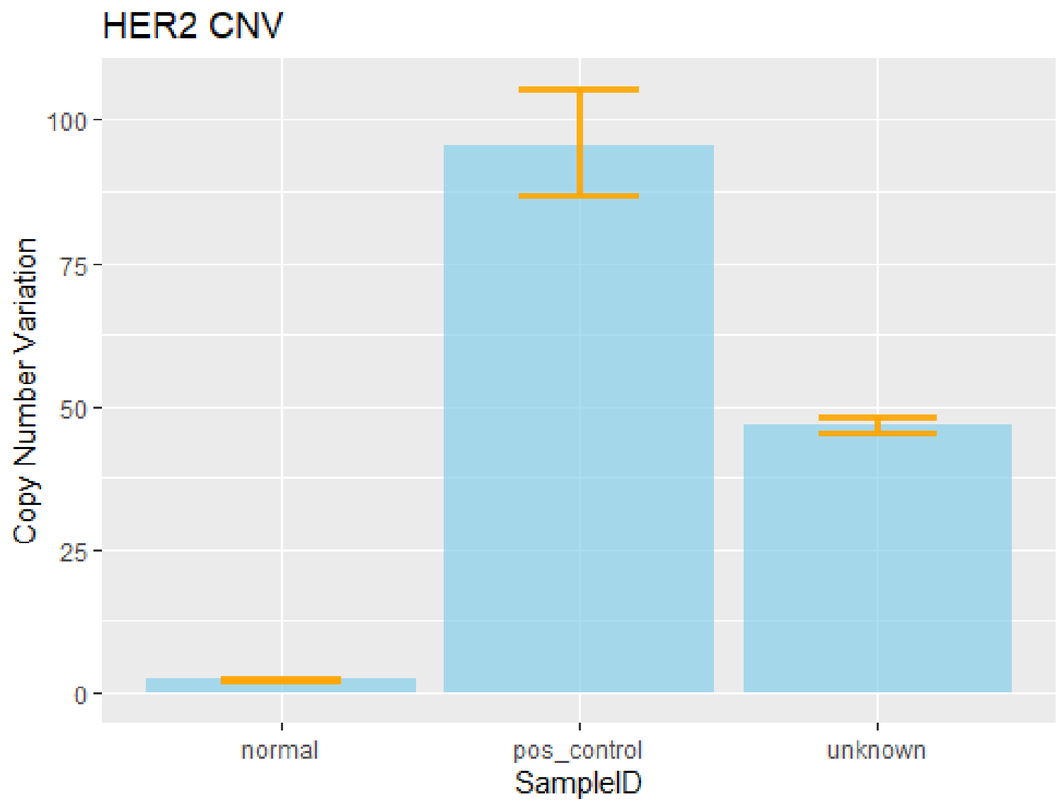
HER2 AMPLIFICATION IN CNV (GLMM)



2.32
[2.16-2.49]

95.54
[86.68-105.32]

46.78
[45.37-48.24]

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Contact Information
wim.trypsteen@ugent.be

More info on ddpcRquant:
Trypsteen et al. Anal Bioanal Chem. 2015 Jul;407(19):5827-34. PMID: 26022094.
www.ddpcrquant.ugent.be
More info on GLMM:
Vynck et al. Biomol Detect Quantif. 2016 Sep; 9: 1–13. PMID: 27551671.

More info on the web tools: www.dpcr.ugent.be

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