FACULTY OF MEDICINE
AND HEALTH SCIENCES

DEPARTMENT OF INTERNAL MEDICINE
HIV CURE RESEARCH CENTER

DIGITAL PCR DATA ANALYSIS

HANDS-ON

Drs. Wim Trypsteen

22nd November, Gent, Belgium

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OUTLINE

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

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DIGITAL PCR LANDSCAPE

- Plethora of platforms (Bio-Rad is market leader)

Bio-Rad

Raindance (Bio-Rad)

Formulatrix

JN Medsys

Stilla

Applied Biosystems

Fluidigm

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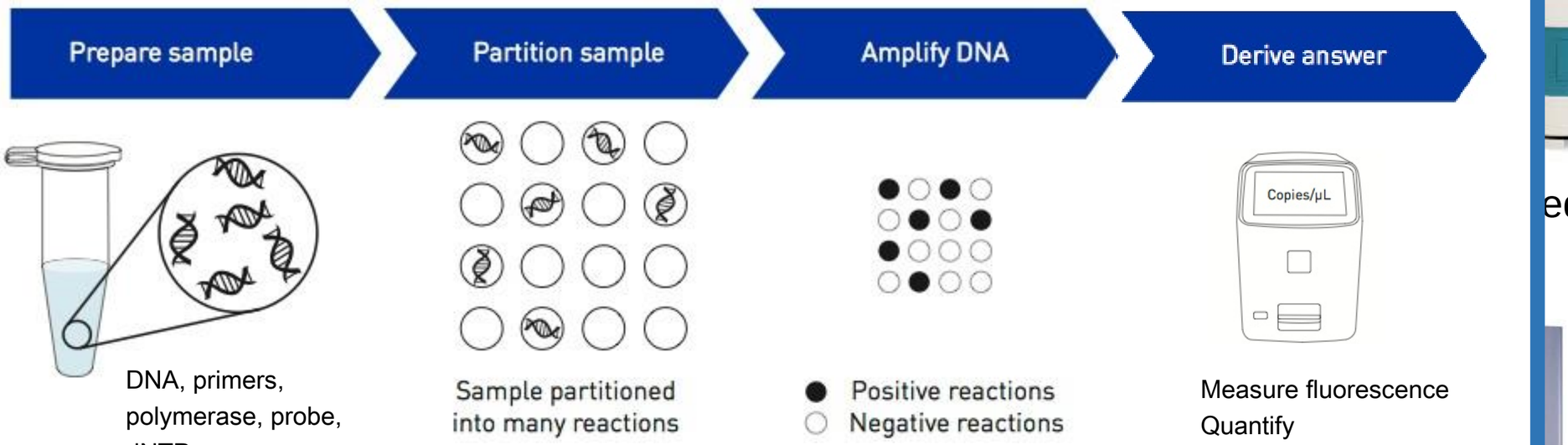
DIGITAL PCR LANDSCAPE

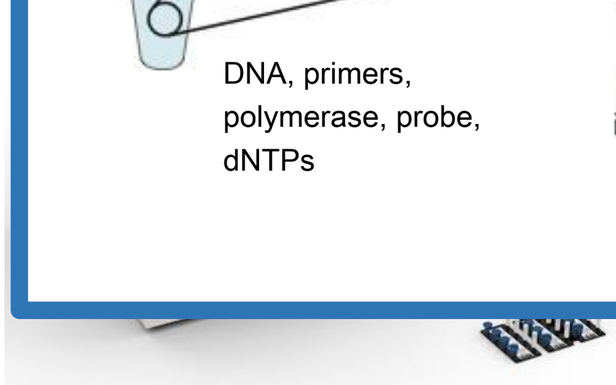
– Plethora of platforms (Bio-Rad is market leader)

Bio-Ra

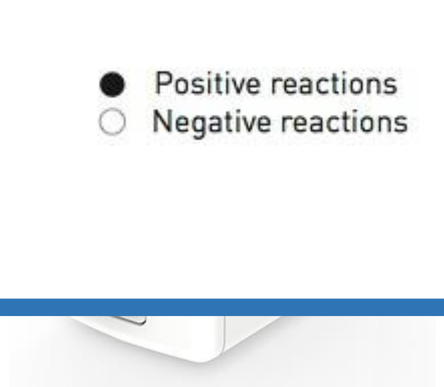
edsys

They all are based on the same principle

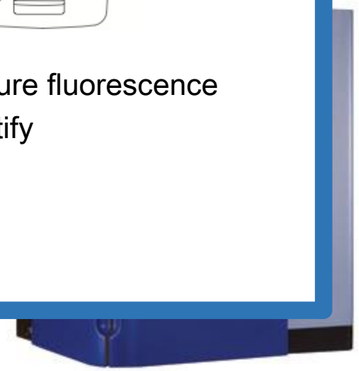




Stilla



Applied Biosystems



Fluidigm

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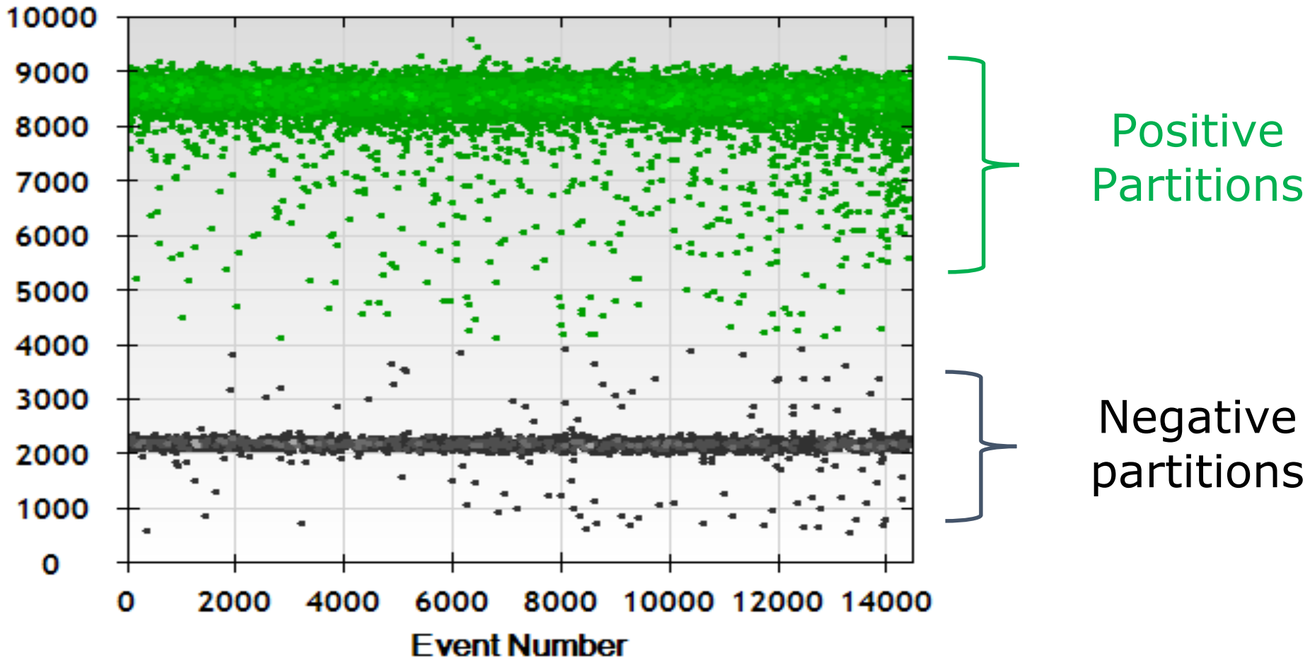
WHAT IS STILL LEFT TO DO: DATA ANALYSIS

- 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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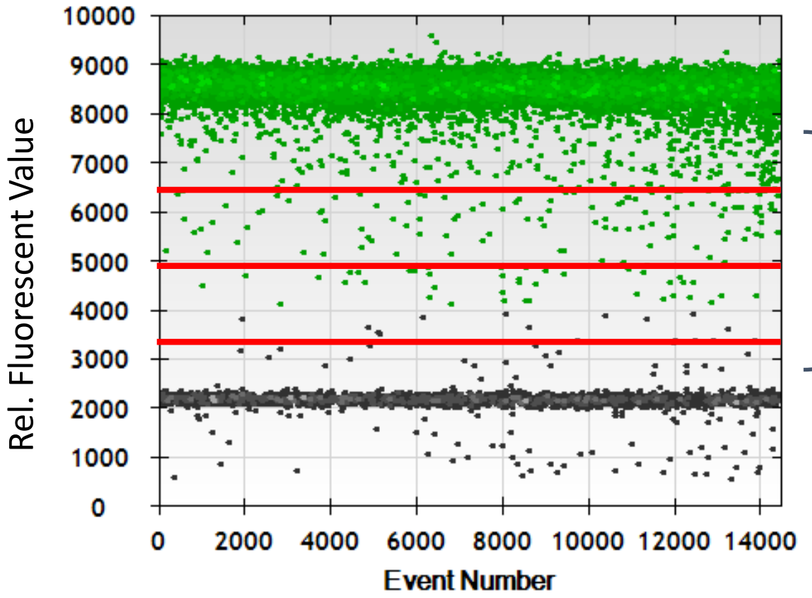
WHAT IS STILL LEFT TO DO: THRESHOLD

– 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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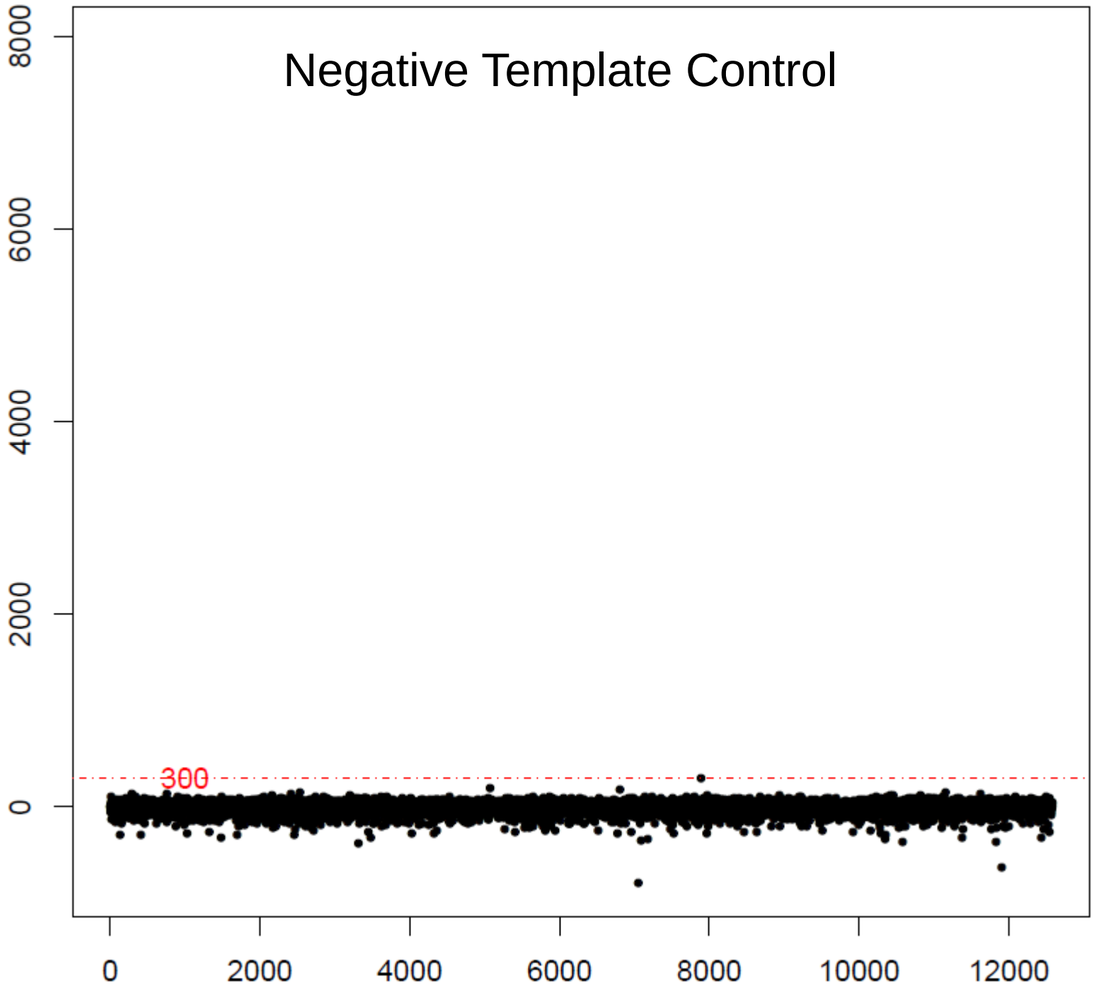
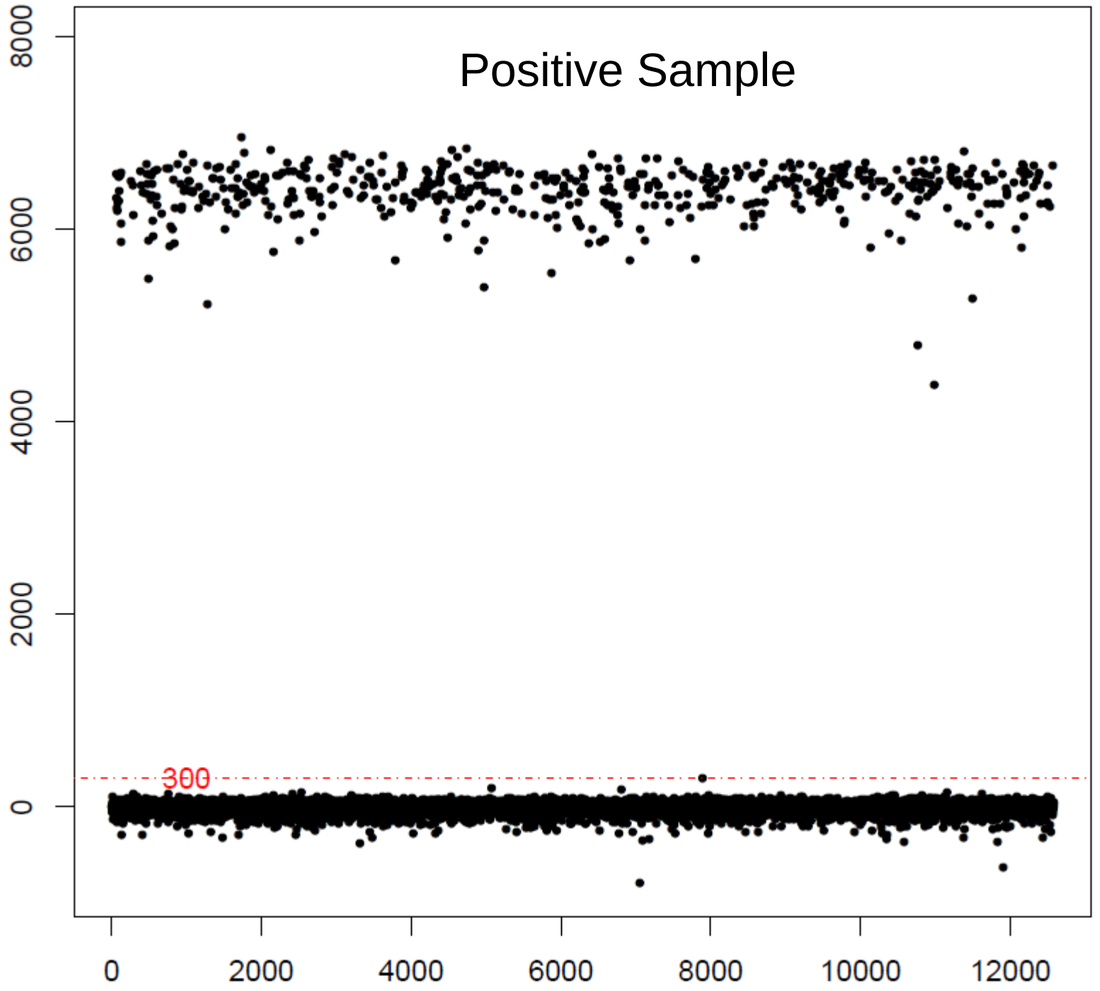
THRESHOLD: FROM NTC TO SAMPLE





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

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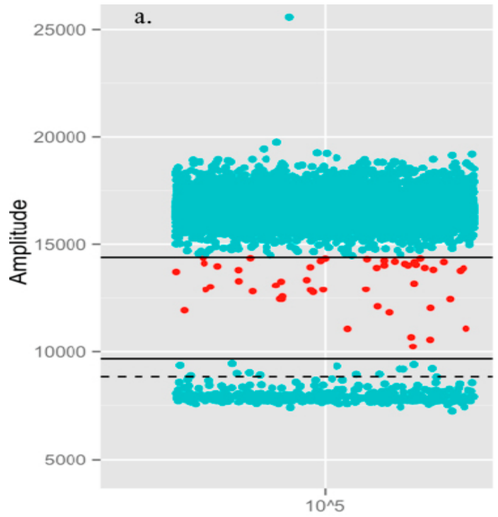
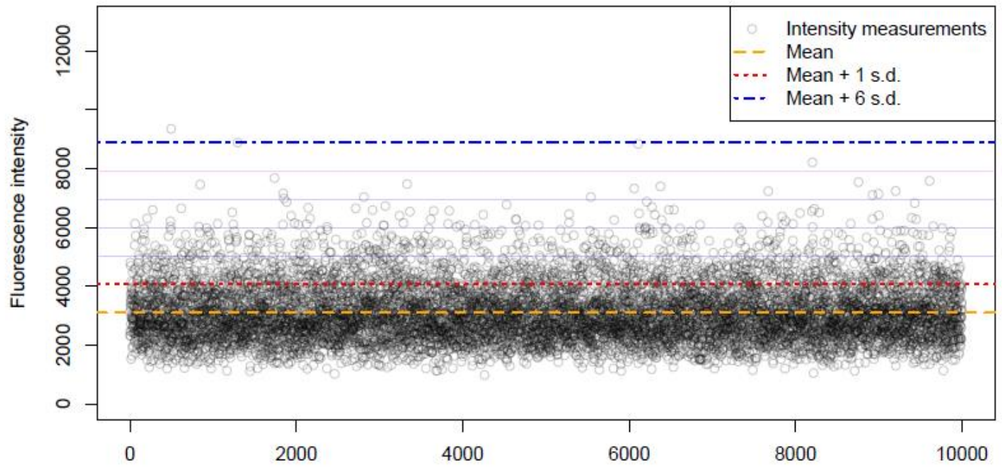
THRESHOLD DETERMINATION

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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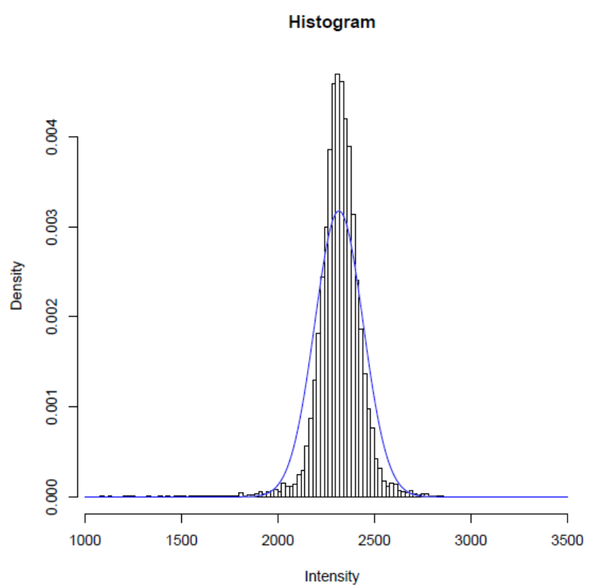
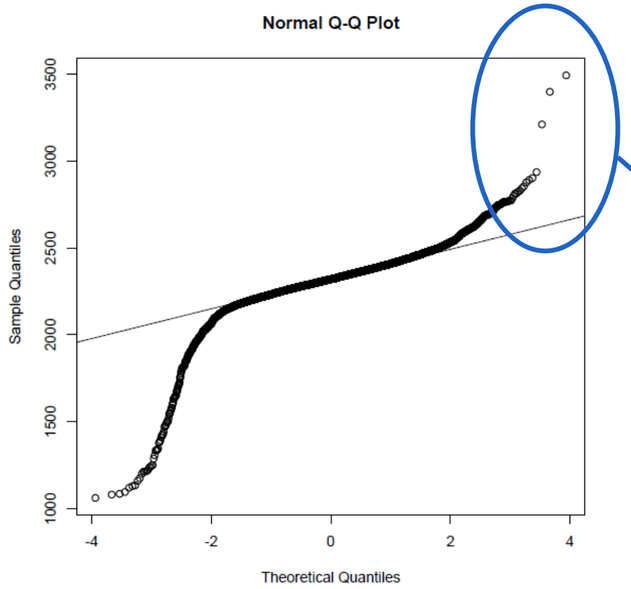
THRESHOLD DETERMINATION





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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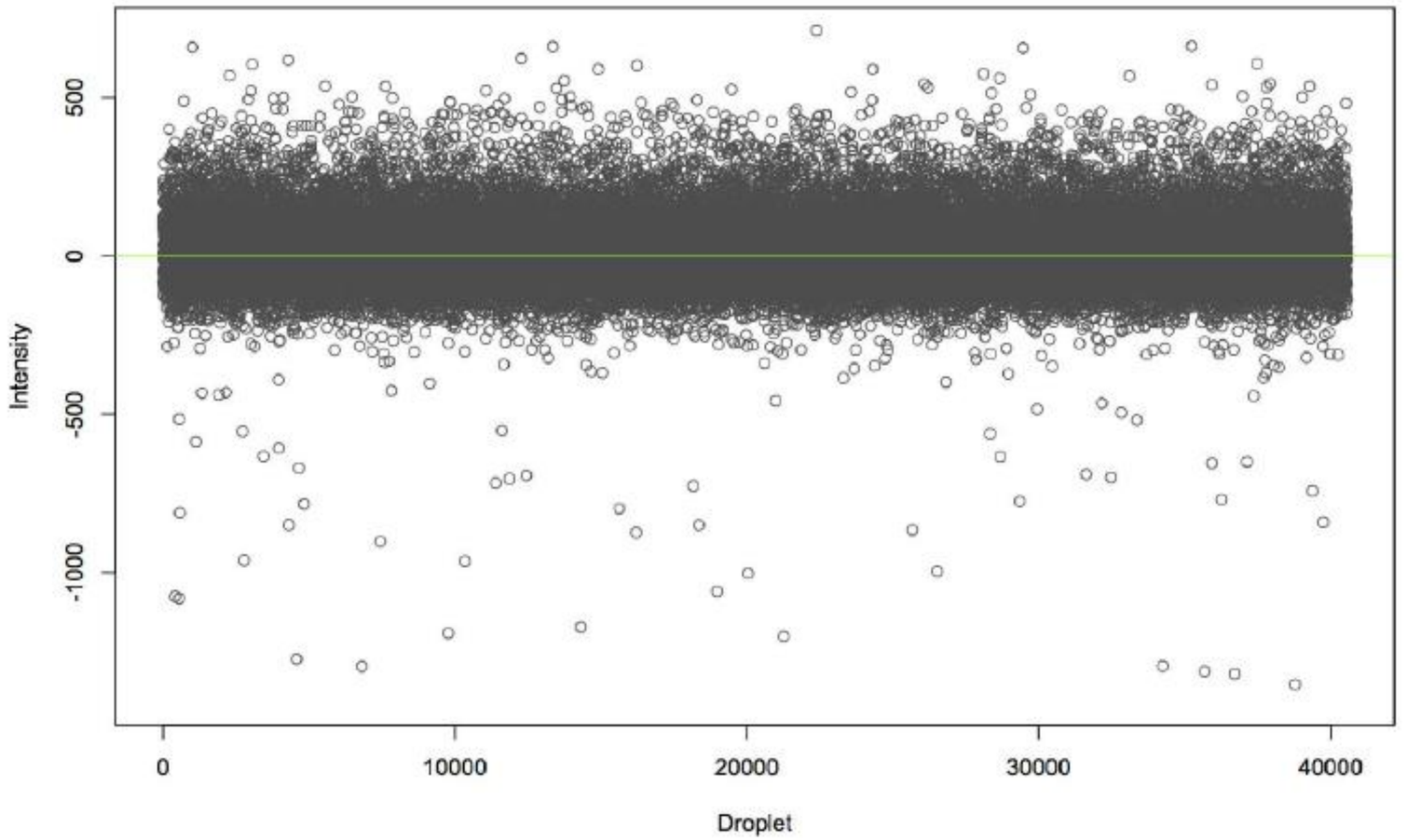
APPLY EXTREME VALUE THEORY



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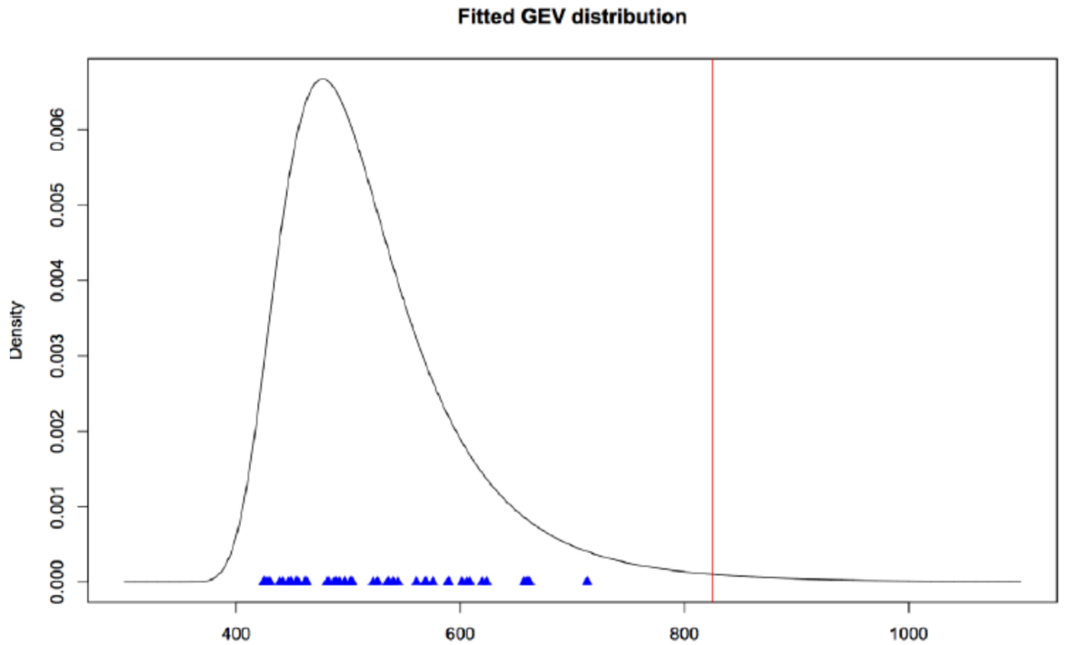
APPLY EXTREME VALUE THEORY

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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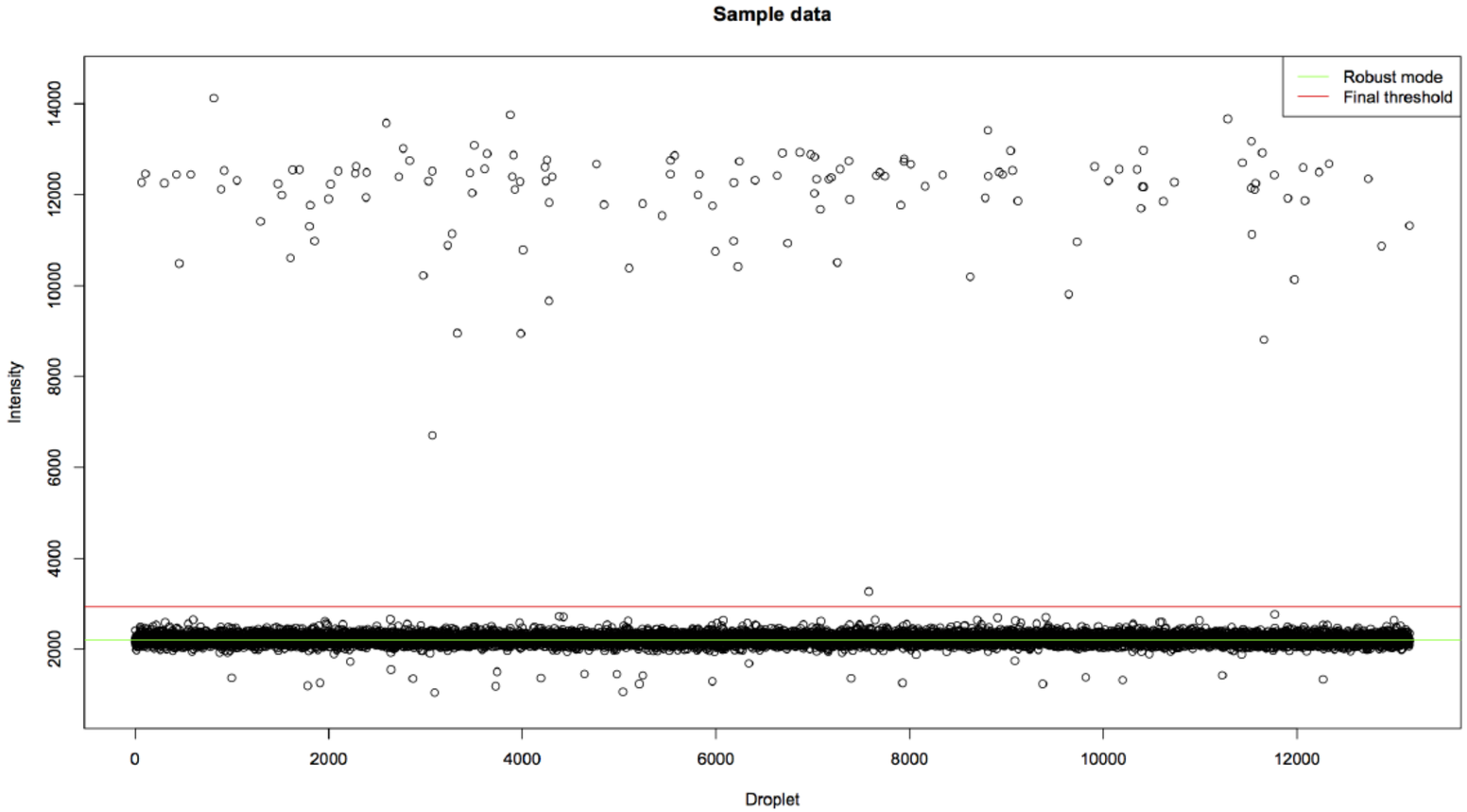
APPLY EXTREME VALUE THEORY



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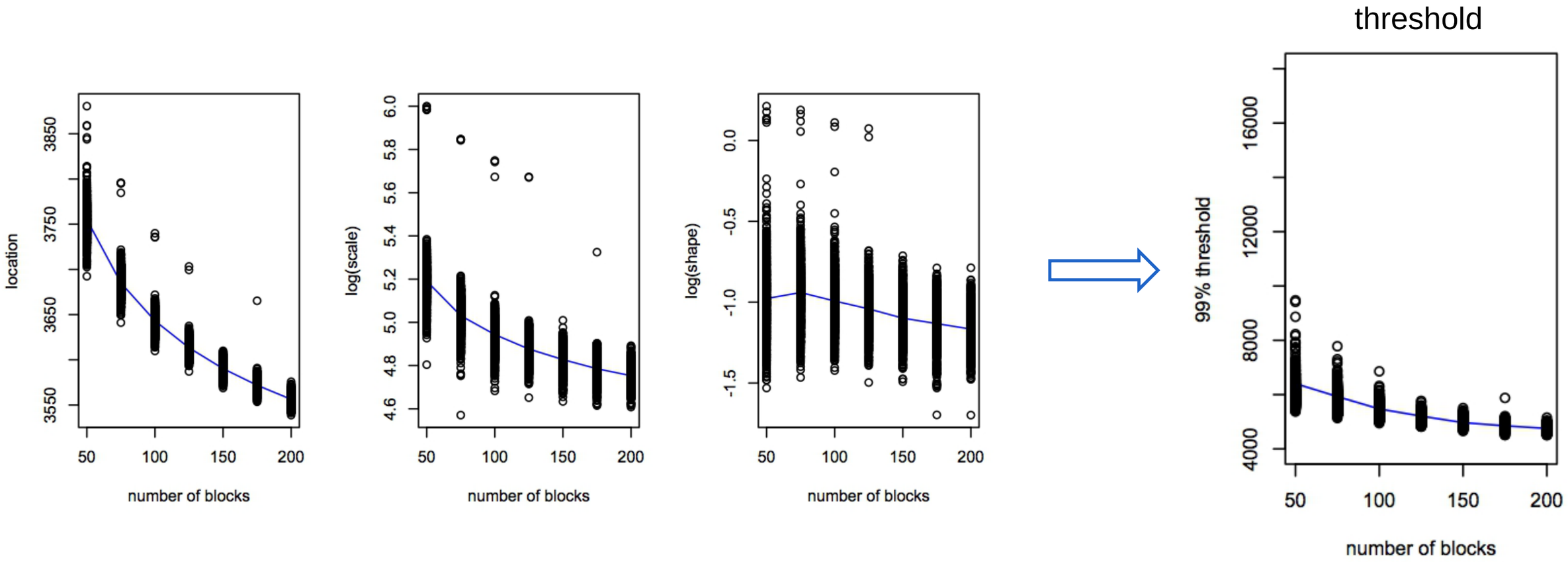
TRADE-OFF VARIANCE VS BIAS



- More blocks = more values => less variance in threshold estimation
- Too much blocks => not looking at the extremes of the distribution (threshold will be too low and fall within negative population)

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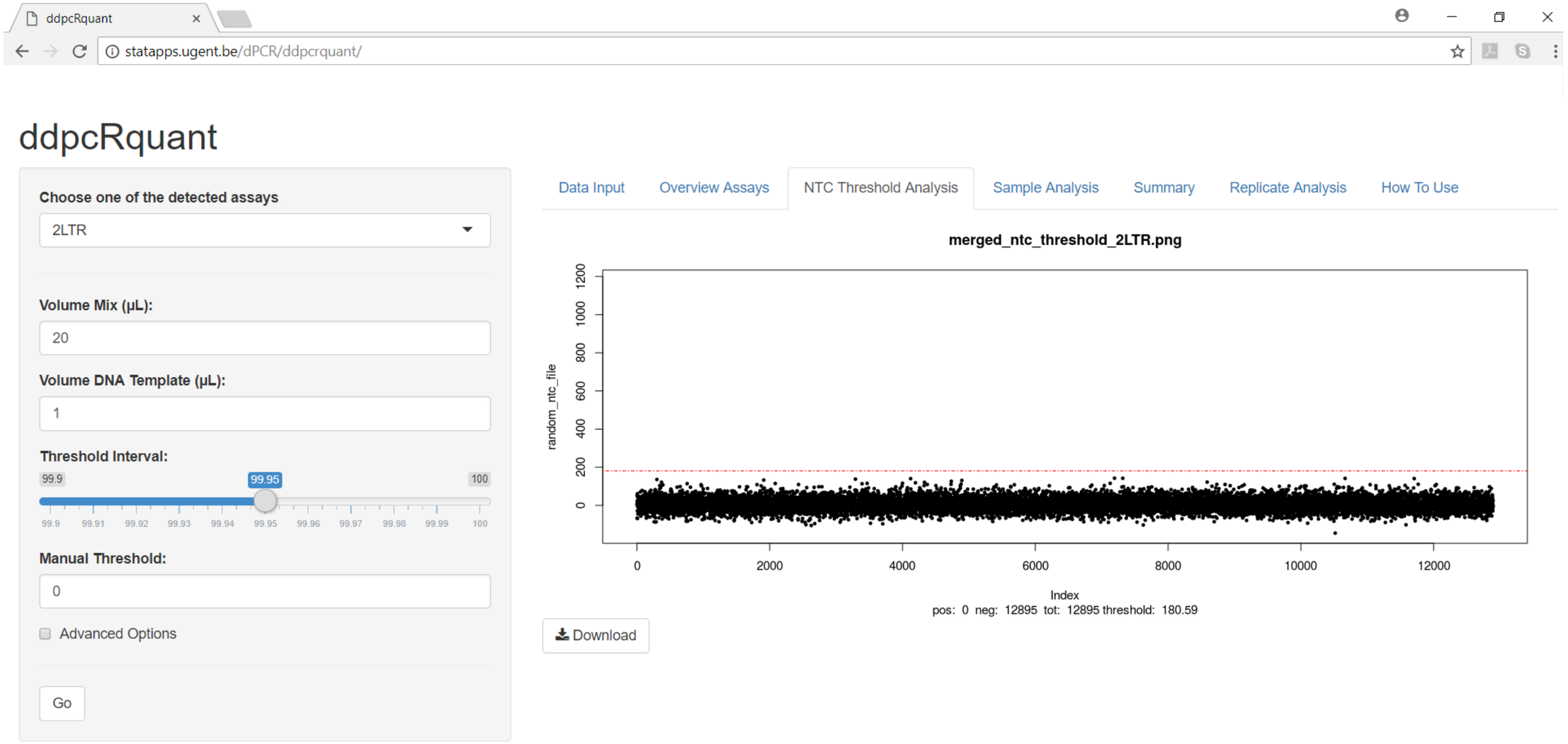
WEBTOOL DDPCRQUANT



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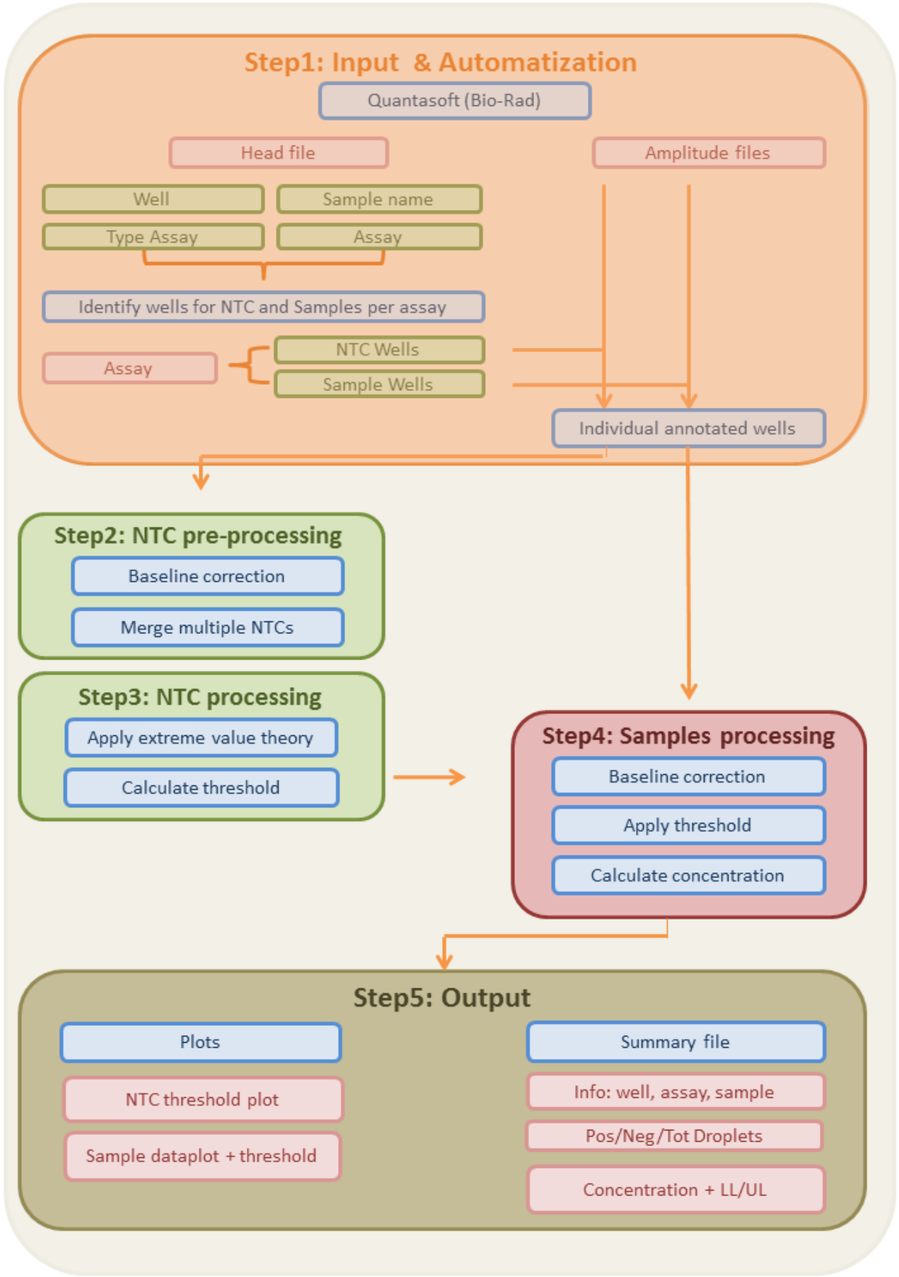

HEAD file

- 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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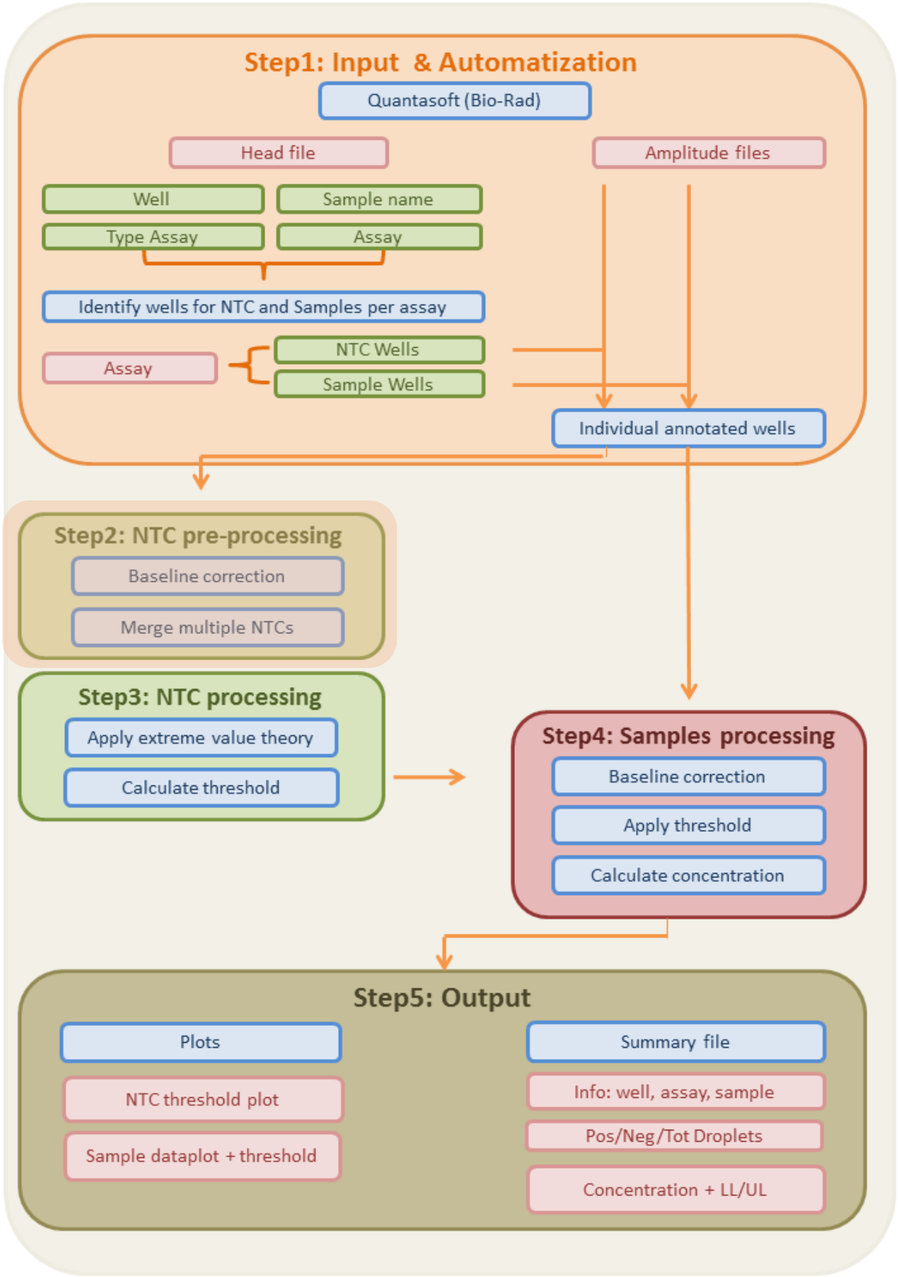

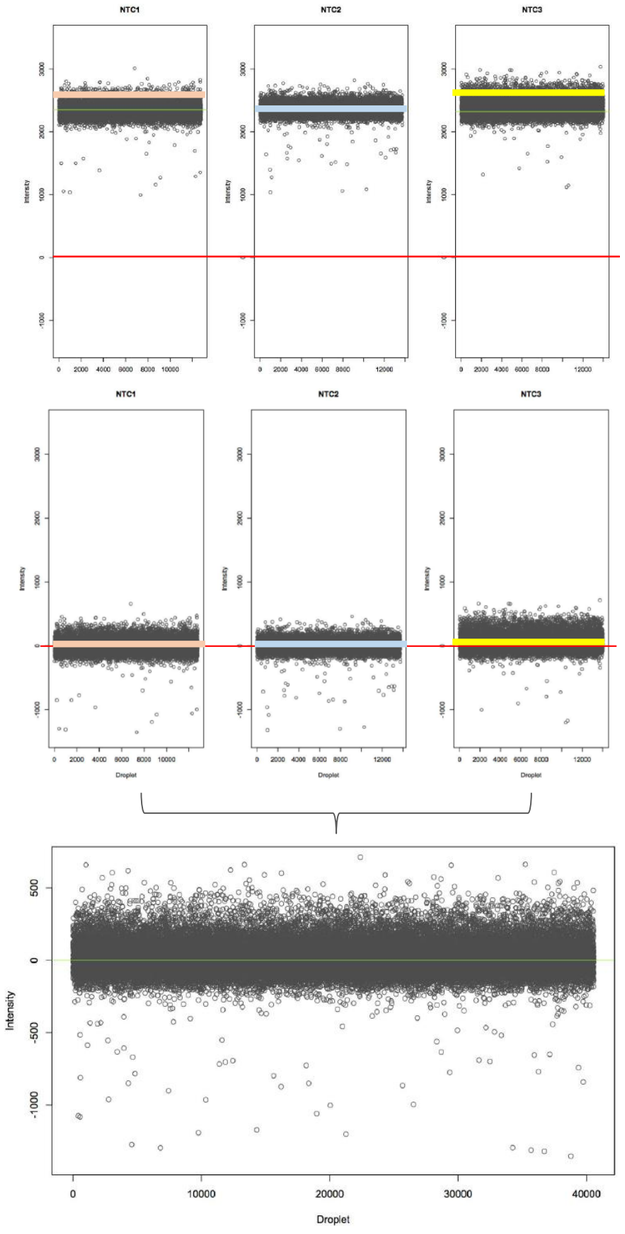

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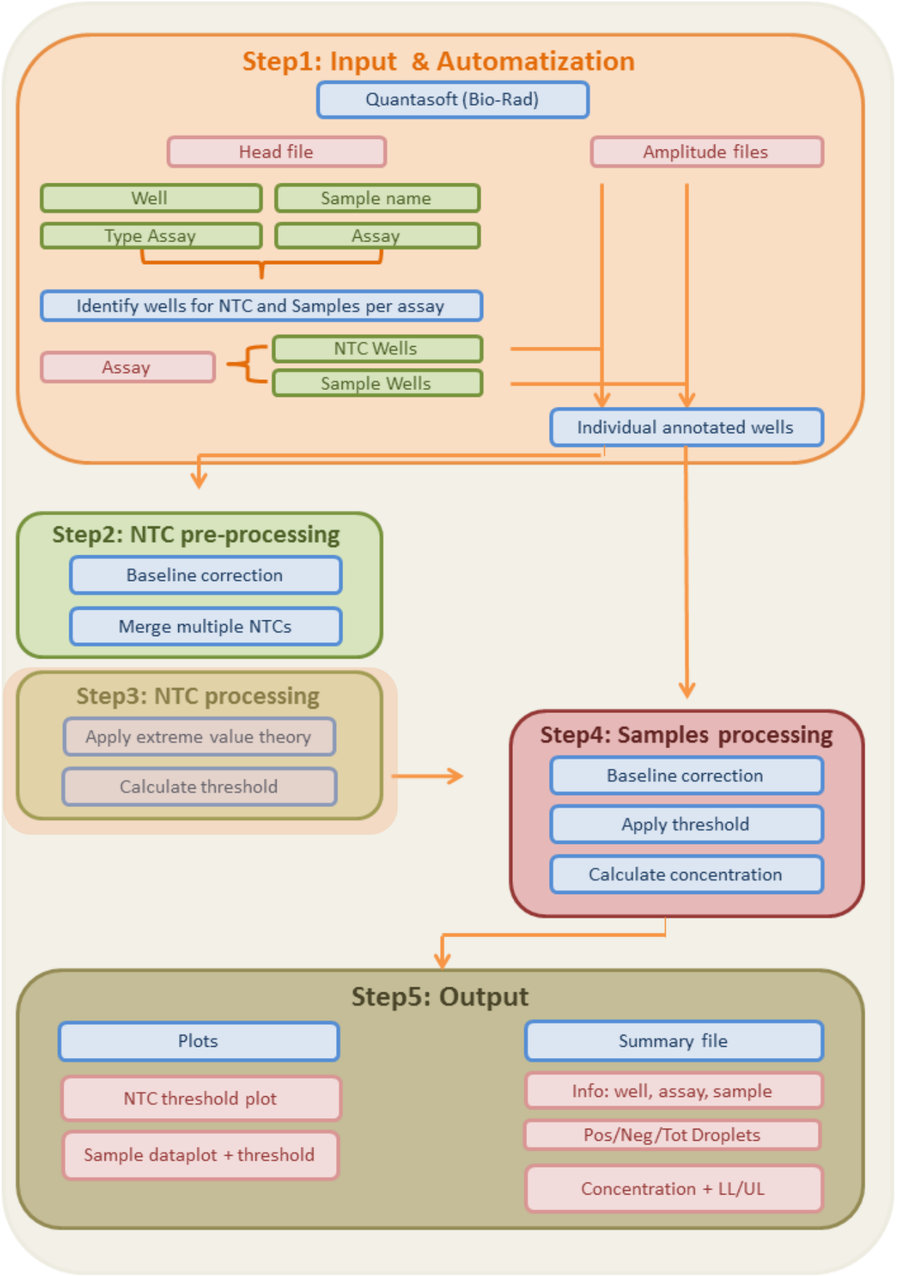
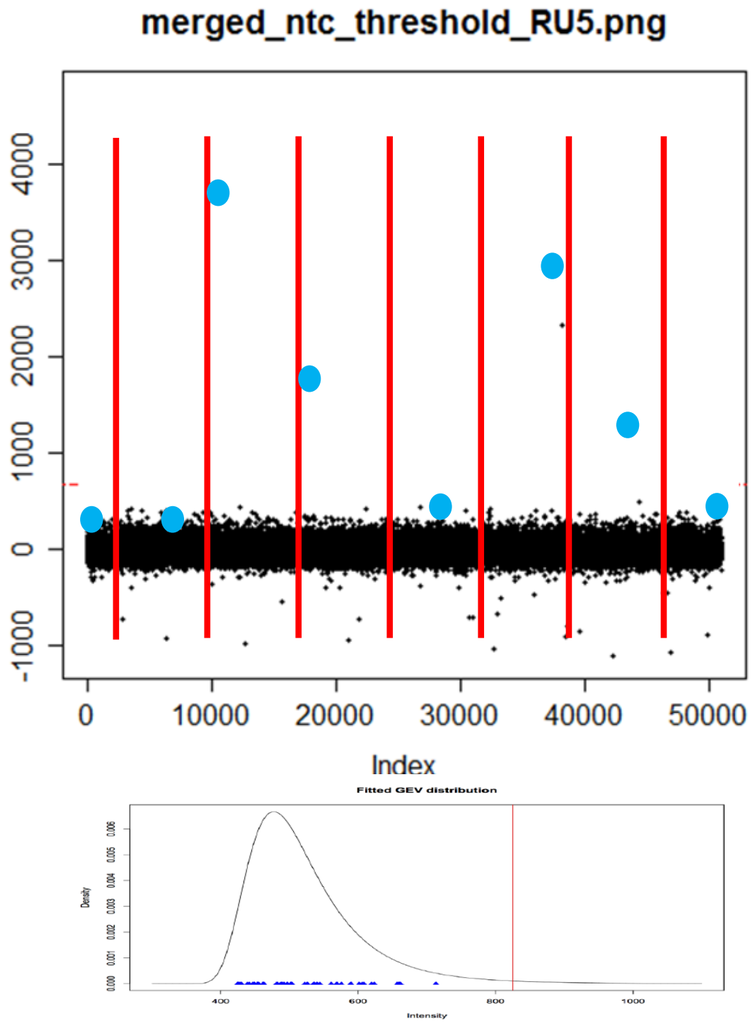


Fisher-Tippett theorem: The distribution of block maxima is given by the Generalized Extreme Value distribution (GEV)

= Block maxima (extremes) follow this family of distributions



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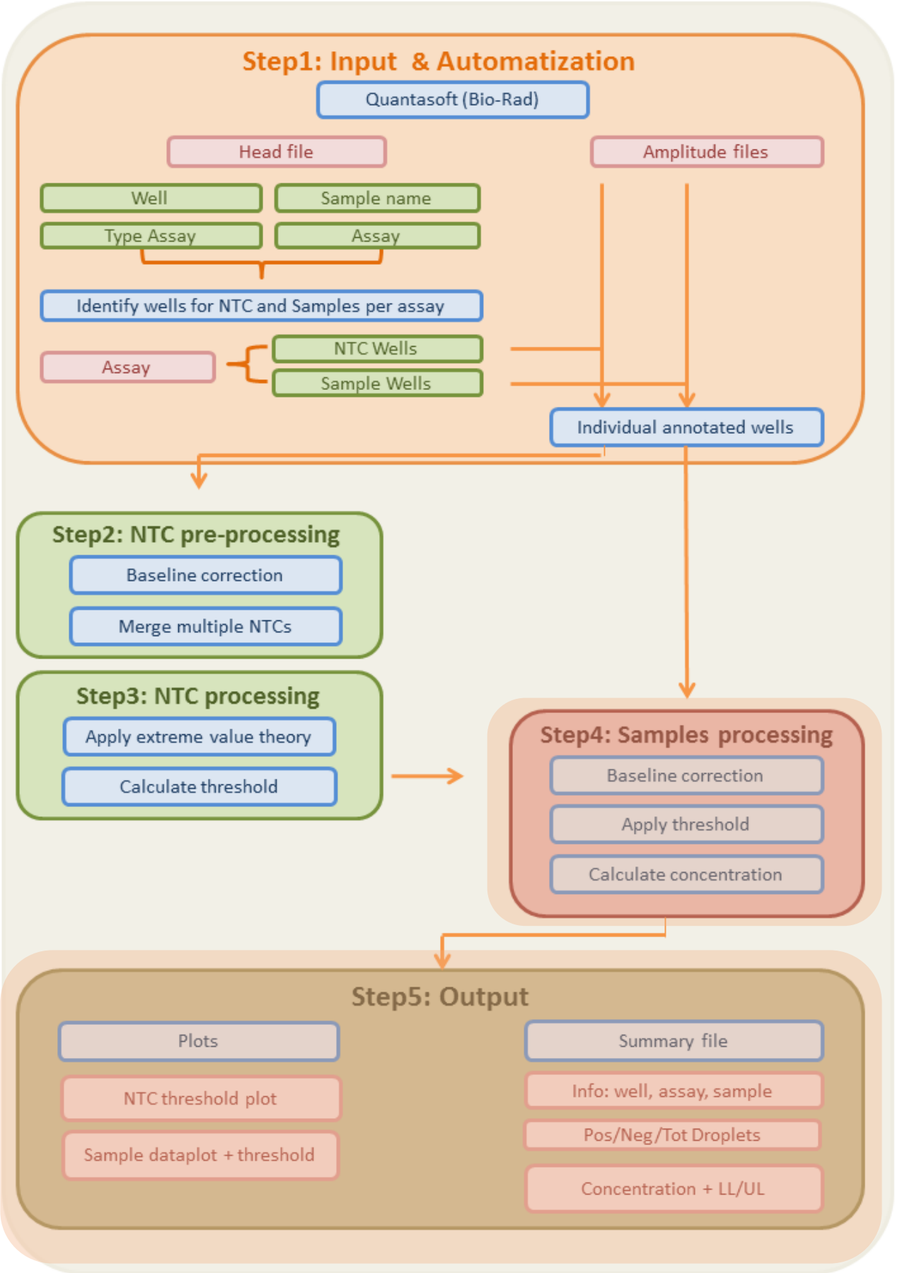

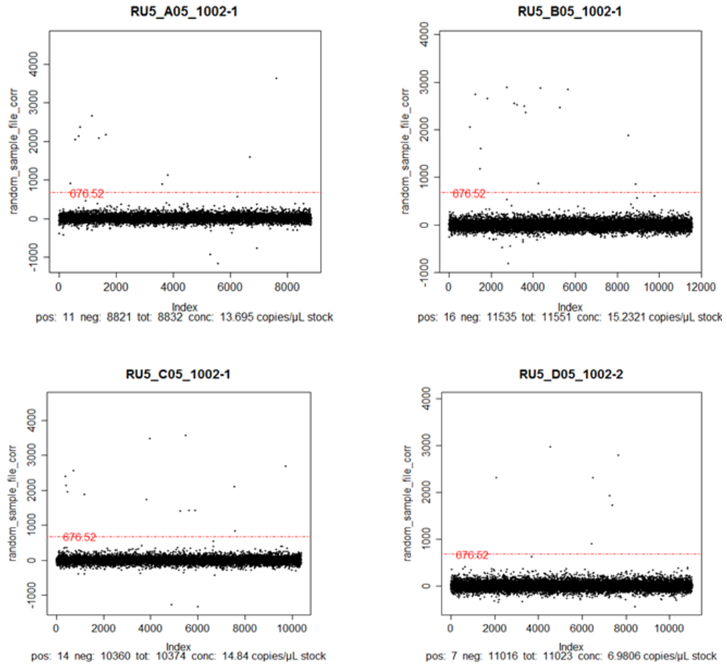

$$C = -\ln\left(\frac{Nneg}{N}\right) * \frac{1000}{Vd} * D$$

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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DEMO ANALYSIS

- Demo analysis

- Run analysis with a 99.99 threshold

  => What happens with the threshold? Why?

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EXERCISE 1: HIV RESERVOIR

- Calculate the size of the HIV reservoir in an infected patient
- If you know the following:
  - HIV DNA assay uses 4 µL DNA input (total vol 20µL)
  - The quantification by ddpcRquant is for the stock solution (this is a volume of 50 µL)
  - The stock solution is isolated DNA from 1 mL of blood (average blood in human is 5L)

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EXERCISE 2: HIV VIRAL LOAD

- Based on the previous result, the patient was put on therapy and is in follow-up. HIV RNA was measured at week 0 (time of diagnosis and therapy start) and week 10
  - HIV RNA is a marker for ongoing infection in the body
- Calculate the HIV RNA, if you know the following:
  - HIV RNA assay uses 2 µL cDNA input (total volume 20)
- How effective is the therapy?

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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

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