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| Plate –  Experimental Factor | Lievens et.al’s remarks on the results | Additional remarks from our observations |
| 1 - qPCR validated  conditions | - More than two populations amplified for acp and cru  - High amount of rain for TC1507 and M88017 | - Negligible amount of rain for few targets  - Moderate amount of rain for few targets |
| 2 - Primer  concentration | - Significant increase in peak separation at higher primer concentrations | - No effect for some targets |
| 3 - Two-fold dilution  series | - Rain is concluded to contain target sequence but does not amplify at the same efficiency as the distinct positives | - Moderate amount of rain at higher concentrations for both targets |
| 4 - PCR Enhancers | - No effect for both targets | - More than two populations amplified for one replicate of M88017 |
| 5 - Two-fold dilution  and Cycles | - Rain is decreased at higher cycles for both targets |  |
| 6 - Sonication | - Significant decrease in rain for M88017  - No effect for TC1507 |  |
| 7 - Annealing  temperature | - Significant increase in peak separation at higher temperatures for TC1507 and GTS4032  - Increased rain at higher temperatures  - No effect for cru and MON1445 | - High presence of rain for some targets  - More than two populations amplified for acp and cru  - Two populations overlap for GTS4032 |
| 9 - dPCR optimized  parameters | - Optimal peak separation, template DNA concentration, and at most 2.5% rain\* for all targets. | - More than two populations amplified for one replicate of M88701  - Moderate amount of rain for some targets |

Table 2 : This study’s observations are based on the figures of Lieven’s paper, and the produced scatter plots and density plots of their dataset. \*Rain was defined here as fluorescence not within some standard deviations away from the two population means.

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| Plate – Experimental  Factor | Levels | | DNA Targets | | | |
| 1 - qPCR validated  conditions | N/A | | acp | hmg | | M88017 |
| cru | le1 | | M88701 |
| GT73 | M1445 | | M89788 |
| GTS4032 | M810 | | TC1507 |
| 2 – Primer  concentration | 150 | 450 | acp | hmg | | M88017 |
| cru | le1 | | M88701 |
| 300 | 600 | GT73 | M1445 | | M89788 |
| GTS4032 | M810 | | TC1507 |
| 3 - Two-fold dilution  series | Conc 8000 | Conc 1000 | M88701 | | TC1507 | |
| Conc 4000 | Conc 500 |
| Conc 2000 | Conc 250 |
| 4 - PCR Enhancers | Enhancer NA (none) | | M88701 | | TC1507 | |
| Enhancer DMSO2% | |
| Enhancer Trehalose0.2M | |
| 5 - Two-fold dilution  and Cycles | Conc 8000 Cycles 45 | | M88701 | | TC1507 | |
| Conc 8000 Cycles 60 | |
| Conc 8000 Cycles 75 | |
| Conc 8000 Cycles 90 | |
| Conc 4000 Cycles 45 | |
| Conc 4000 Cycles 60 | |
| Conc 4000 Cycles 75 | |
| Conc 4000 Cycles 90 | |
| 6 – Sonication time | 0 | 9 | M88701 | | TC1507 | |
| 3 | 12 |
| 6 | 15 |
| 7 - Annealing  temperature | 62 | 58.4 | acp | hmg | | M88017 |
| 61.6 | 57.3 | cru | le1 | | M88701 |
| 60.9 | 56.5 | GT73 | M1445 | | M89788 |
| 59.8 | 56 | GTS4032 | M810 | | TC1507 |
| 9 - dPCR optimized  parameters | N/A | | GT73 | M1445 | | M89788 |
| GTS4032 | M810 | | TC1507 |
| hmg | M88017 | |  |
| le1 | M88701 | |  |

Table 1 : **Levels of the design factors per plate and the DNA targets examined.** Plates 1 and 9 have constant design parameters for each DNA target, the other plates explore varying levels of the given design parameter(s). Conc refers to the diluted template DNA concentration (copies/\(\micro L\), and not the target DNA concentration. Primer concentration is measured in nM, sonication time is measured in seconds; and annealing temperature is in degree Celsius. Plate 8 was omitted since it was a digital touchdown variation of acp from plate 7.

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| Method | Algorithm | Droplet classification rule | Additional Input |
| Bio-Rad  QuantaSoft | (Undisclosed) | (Undisclosed) |  |
| Cloudy | Iterative parameter estimation of \(\mu\_p)\) and \(\sigma\_p)\) using observations within \(\hat{\mu}\_p \pm a \cdot \hat{\sigma}\_p\) at each iteration. | Negative if fluorescence is \(< \hat{\mu}\_{neg}+1.5 \cdot a\_{neg} \cdot \hat{\sigma}\_{neg}\) |  |
| ddpcRquant | Extreme Value theory to estimate negative population | Negative if fluorescence is less than the average of one hundred 0.995 percentiles of extreme values sampled | NTC sample *(optional)* |
| definetherain | K-means clustering to cluster 2 populations | Negative if fluorescence is \(< \hat{\mu}\_{neg} + 3 \cdot \hat{\sigma}\_{neg}\); positive if fluorescence is \(> \hat{\mu}\_{pos} + 3 \cdot \hat{\sigma}\_{pos}\); otherwise, droplet is rain. | Positive Control sample |
| Umbrella | Non-parametric approach to estimate a 2-component mixture density | Negative if the droplet’s probability given the negative population is \(> 80\%\) | NTC sample |
| EM-T /  EM-skewT | Expectation Maximization to estimate a G-component mixture density | Droplet population membership is where its probability given a population is highest. |  |

Table 3: List of methods, and its algorithm, droplet classification rule, and required input. NTC stands for No-Template-Control.

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| Method | Accessibility | Included in Performance Evaluation? |
| Bio-Rad QuantaSoft | Paid Desktop software  <https://www.bio-rad.com/en-ph/sku/1864011-quantasoft-software-regulatory-edition?ID=1864011> | No  (tool is unavailable to researcher) |
| Cloudy | R  <https://github.com/Gromgorgel/ddPCR/blob/master/Cloudy-V2-05.R> | Yes |
| ddpcRquant | R and Free web app  <https://ddpcrquant.ugent.be/ddpcrquant_functions_qx100.R> | Yes, only in simulated data (required NTC samples input are not available in Lievens’ dataset) |
| definetherain | Free web app  <http://www.definetherain.org.uk/> | No  (required positive control samples input are not available) |
| Umbrella | R  <https://github.com/statOmics/umbrella/blob/master/1D/Umbrella_1d_V1.R> | Yes |

Table 4: List of methods to be compared for the real dataset of Lieven’s et. al and the simulated dataset.