Introduction of a time-saving protocol: Reduction of qPCR-cycles in the Detection step

Scope

The final step of the Olink protocol, the Detection step, quantifies DNA reporters for each biomarker using high throughput real-time qPCR on the Fluidigm Biomark System (Figure 1, step 3).

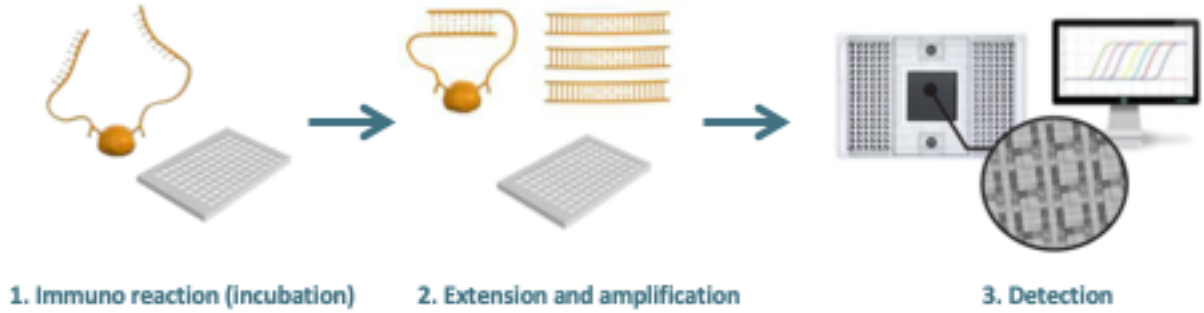

Figure 1
Overview of the three core steps in the Proximity Extension Assay (PEA) technology

The current PCR protocol consists of 40 amplification cycles and has a total run time of 2 h 10 min. Since this step, next after the overnight incubation, is the most time-consuming step of the reaction, Olink started an investigation to find out whether a shortening of this protocol would be possible.

An evaluation of the key features for the negative control samples run on each sample plate; blank buffer samples that represent background values with high Ct, showed that the maximum Ct value measured during validation experiments was 30.4 (see Table 1). Based on this information, an investigation on the possibility to reduce the number of PCR-cycles to 35 and thereby save 10 min run time was initiated.

Table 1 Key features of negative controls, based on Ct-values, measured in 14 different Olink Panels

Parameter	Ct-value
Mean	20.8
Median	21.0
Min	9.1
Max	30.4

Investigation and results

A thorough two-step validation including 14 Olink Panels was performed:

1. Evaluation of the performance of negative control samples run at 35 vs 40 qPCR cycles
2. Statistical evaluation of the results from different sample types run at 35 vs 40 qPCR cycles

1. Evaluation of negative control samples

The data from the initial investigation of negative control values measured during Olink's validation runs (Table 1) show that negative controls never amplified later than Ct 30.4. It was therefore

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concluded unlikely that a reduction from 40 to 35 cycles would reduce assay sensitivity nor give rise to failed data points (due to potential impact on the shape of the amplification curve).

To stress the analysis, *in-silico* experiments simulating experiments run with 35 amplification cycles were generated and compared with the results from the corresponding runs with 40 amplification cycles.

A total number of 21,120 data point pairs were analysed. The results showed no increase in number of failed data points when shortening the PCR program with 5 cycles.

2. Statistical evaluation of samples run at 35 and 40 amplification cycles

To test the assumptions made in the first part of the evaluation, experiments using different sample types were run using 35 and 40 PCR cycles and the results were evaluated statistically.

A representative statistical evaluation using the Olink® NEUROLOGY panel is presented below.

Experimental design:

One incubation and extension reaction was performed. The same extension product was thereafter run six times through the detection step:

- Three IFCs were run using 40 qPCR cycles on the Fluidigm Biomark
- Three IFCs were run using 35 qPCR cycles on the Fluidigm Biomark

Samples:

Four different sample types were run in 20 replicates each:

1) Negative control
2) Pooled plasma
3) A prepared sample with low antigen concentrations (1ng/mL)
4) A prepared sample with high antigen concentrations (10ng/mL)

Statistical analysis:

The following statistical tests, evaluations were performed:

Accuracy	T-test for each sample type, comparing mean NPX for each assay for 35 vs 40 cycles.
Precision	F-test for each sample type, comparing variance for each assay between 35 and 40 cycles.
Precision	Coefficient of variation (CV%) within plates (Intra CV) and between plates (Inter CV) calculated for each sample type separately
Multiple testing	Multiple testing adjustment was done using the Bonferroni approach, significance was defined as adjusted p-value < 0.05
Other parameters	Distribution of NPX values, number of samples passing QC and detectability

Results:

The results from the statistical evaluation of data generated using 35 and 40 qPCR-cycles, respectively, are summarized in Table 2. Equal results were obtained with both protocols and the small variations detected between the protocols fall within the expected inter run variability for two runs using the same PCR protocol.

Table 2 Summary of statistical parameters evaluated in the comparison between 35 and 40 qPCR-cycles

Parameter	35 vs 40 cycles
Number of samples passing	No difference
Detectability	No difference
Accuracy	No difference
Precision (variance)	Lower variance (higher precision) for all samples
Precision (intra CV%)	No difference
Precision (Inter CV%)	Lower inter CV (-2 percentage points) for all samples*

**Please note that intra and inter CVs may differ more than 2 percentage points between sample plates run with the same PCR program*

Figure 2 demonstrates the high correlation between 35 and 40 qPCR-cycles, using the pooled plasma sample as an example (left). As a reference, the correlation between two different plates run at 40 qPCR-cycles is shown (right).

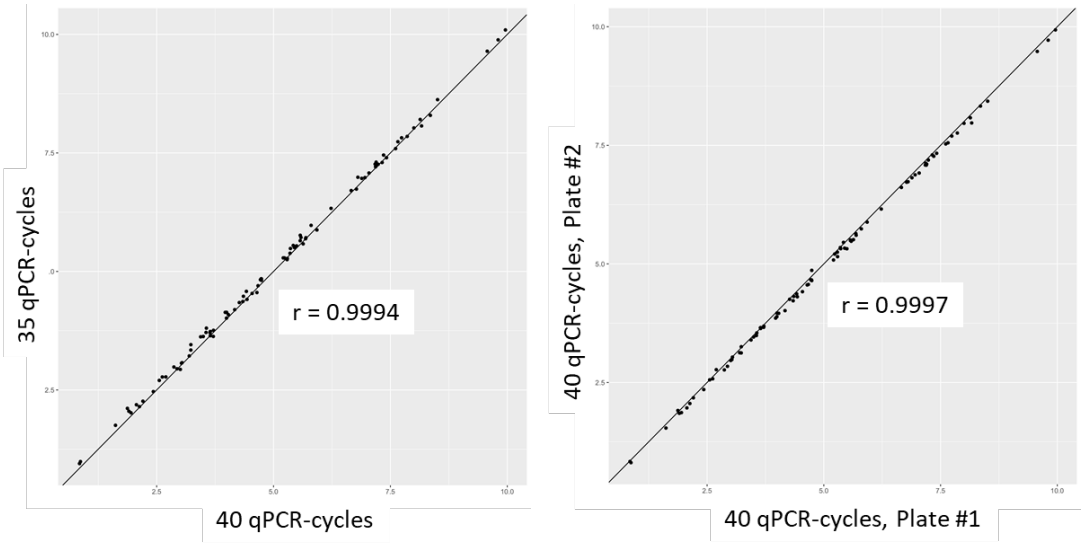

Figure 2 Correlation plots comparing NPX-values for pooled plasma samples between 1) 35 and 40 qPCR-cycles and 2) two runs using the same number of qPCR cycles (40).

Conclusions

The results generated using the modified protocol with 35 qPCR-cycles are comparable to the results generated using the protocol with 40 qPCR-cycles. This modification saves 10 min for each run, which in a daily workflow with 3 runs saves a total of 30 min.

Olink will implement this modification in all runs using the Fluidigm Biomark and our recommendation to our customers is to use the same approach.

FAQs

- *Do I have to change to 35 cycles?*
  The change is not mandatory but recommended for time saving purposes. All runs in-house at Olink (RnD, QC and Analysis Service) will change to the new protocol.
- *How do I change to 35 cycles?*

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Please see the Fluidigm Biomark Files section for instructions and the PCR-program setup file.

- *Can I switch to 35 cycles in the middle of a project/study?*
  Yes, but we recommend changing the PCR program between two projects/studies.
- *Can I combine data from projects run on 35 and 40 cycles, respectively?*
  Yes, data from projects run using the two different PCR-program can be combined in the same manner as two projects run at different time points using the same PCR-program.

For questions and further information, please contact support@olink.com.

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