Session 6

qPCR: Validation and Maintenance Issues

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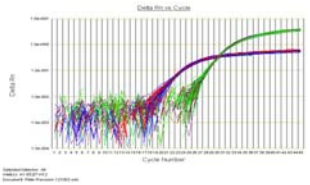
Plate Precision

- To determine precision across 96-well plate
- 4ng template/well – all from same stock of master mix/genomic DNA standard

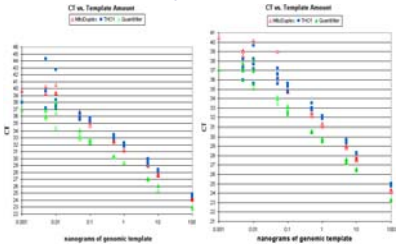


FAM signal (nuTH01)
VIC signal (mtND1)

(all wells shown)

- standard deviation across plate ~0.2 CT
- Or ~15% CV (relative standard deviation)
- no systematic deviations across rows or columns

Sensitivity: Nuclear Assays



- All three assays show adequate precision to down to 50pg of template, though all begin to show significant precision loss at ~50pg.
- At 10 pg, see 20% dropout in nuTH01 duplex but no Quantifiler dropout;
  - at 5 pg, see 80% dropout in nuTH01 duplex and 10% in Quantifiiler.

qPCR Averages and RSD's based on Pooling Single Replicates from the Three Runs

		nuTH01 qPCR		mtND1 qPCR	
Sample	Slot Blot Quantity (ng/μL)	Average Quantity (ng/μL)	RSD (%)	Average Quantity (1000 mt copies/μL)	RSD (%)
A	0.85	0.64 (0.08)	12	130 (79)	62
B	0.80	0.96 (0.10)	10	880 (150)	17
C	0.34	0.53 (0.10)	20	310 (35)	11
D	1.31	1.58 (0.31)	19	430 (31)	7
E	0.97	1.51 (0.17)	11	790 (170)	21
F	0.40*	0.45 (0.05)	12	360 (73)	21

* Sample F represents the calibration standard used for the slot blot quantifications; the value for sample F (0.40 ng/μL) is a defined, rather than a measured quantity.

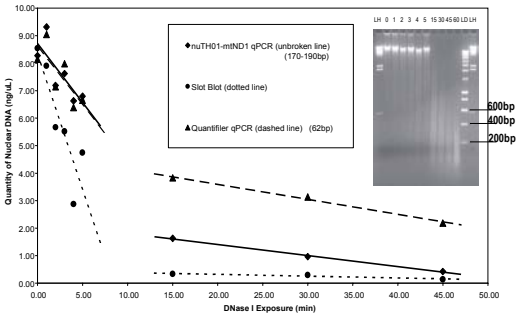
Intra-run Sensivity and Precision Results for nuTH01- mtND1 Duplex Assay

TABLE 2 - *Intra-run sensitivity and precision results for the nuTH01-mtND1 duplex qPCR assay. Results are based on five replicate quantifications of serially diluted Promega Female Genomic DNA standards. Quantities are per 4 μL of sample. Standard deviations are in parentheses.*

	nuTH01 qPCR			mtND1 qPCR	
Input DNA Quantity (ng)	Average Quantity (ng)	RSD (%)	Approx. Input Quantity (mt copies)	Average Quantity (mt copies)	RSD (%)
100	95.8 (7.7)	8.0	1.2×10^5	$8.6 (0.97) \times 10^4$	11
10	10.7 (0.90)	8.4	1.2×10^4	$1.3 (0.15) \times 10^4$	12
5	4.7 (0.088)	1.9	6.1×10^3	$7.6 (0.95) \times 10^3$	12
1	1.2 (0.034)	3.0	1.2×10^3	$1.3 (0.24) \times 10^3$	18
0.5	0.48 (0.057)	12	6.1×10^2	$7.1 (1.4) \times 10^2$	20
0.1	0.10 (0.021)	21	1.2×10^2	$1.0 (0.20) \times 10^2$	20
0.05	0.051 (0.013)	26	6.1×10^1	$7.2 (1.5) \times 10^1$	21
0.01	0.0057* (0.0017)	30*	1.2×10^1	$1.3 (0.21) \times 10^1$	17
0.005	-	-	6.1×10^0	$7.1 (2.0) \times 10^0$	28
0.001	-	-	1.2×10^0	$1.1 (0.39) \times 10^0$	35
0.0001	-	-	1.2×10^{-1}	$1.1 (0.41) \times 10^{-1}$	39

* Based on four replicates, because one quantification dropped out at this template quantity.

Quantifications of DNase-Degraded DNA Samples: DNA Quantity Depends Upon Quantification Method



PROCEDURE FOR PERFORMANCE CHECK OF ABI 7500 qPCR INSTRUMENTS

- the evaluation of standard curves based on results from a standard DNA dilution series in each run
- the inclusion of a negative qPCR amplification control in each run
- the examination of the fluorescence intensities of the ROX
- passive reference signal for selected samples in each run.

Routine Maintenance Procedures

- monthly background calibration, with optical calibration (including assessment of possible block contamination and cleaning, if necessary)
- annual regions of interest (ROI) calibration and pure dye calibration, followed by an instrument performance check

Routine Maintenance Procedures

Follow the *Protocol for the nuTH01-nuCSF-IPC Triplex qPCR Assay* to perform a qPCR run on a standard DNA dilution series and at least one negative control sample (TE^{-4}). Analyze data from the run according to the interpretation guidelines in the protocol.

- B*oth* nuTH01 and nuCSF standard curves give: -4.0 ≤ slope ≤ -2.9 *and* $R^2 \geq 0.98$
- Negative amp control gives "<LQ" for *both* nuTH01 and nuCSF assays
- Non-inhibited IPC amplification curves are seen for all samples

Print

- standard curve (landscape format) for nuTH01 portion of assay
- standard curve (landscape format) for nuCSF portion of assay
- output from "qPCR Casework-Degradation Triplex" spreadsheet
- amplification curves (linear ΔRn vs. cycle) for all standards and TE^{-4} for nuTH01 portion of assay (all curves in one landscape plot)
- amplification curves (linear ΔRn vs. cycle) for all standards and TE^{-4} for nuCSF portion of assay (all curves in one landscape plot)
- amplification curves (linear ΔRn vs. cycle) for all standards and TE^{-4} for IPC portion of assay (all curves in one landscape plot)

ANNUAL MAINTENANCE PROCEDURES

Perform ***in the following sequence***:

- Regions of Interest (ROI) Calibration
- Background Calibration (with Optical Calibration) from *Procedure for Monthly Maintenance of ABI 7500 qPCR Instruments*
- Spectral Dye Calibration
- Performance Check qPCR Run

ROI CALIBRATION

The data gathered during the ROI calibration allows the SDS software to map the positions of the wells on the sample block so that during instrument operation the software can associate increases in fluorescence with specific wells of the reaction plate. Since the 7500 instrument uses a set of optical filters to separate the fluorescent energy gathered during runs, a calibration image is generated for each individual filter to account for minor differences in the optical path.

BACKGROUND CALIBRATION (WITH OPTICAL CALIBRATION)

The background calibration is performed as part of the monthly maintenance of the Applied Biosystems 7500 Real-Time PCR System. A background run measures the level of ambient fluorescence in the 7500 instrument. During the calibration run, the 7500 instrument performs continuous reads for 10 minutes at 60 °C of a background plate containing either PCR buffer or de-ionized water. Afterwards, the 7500 software averages the spectrum recorded during the run and extracts the resulting spectral component to a calibration file. The software then uses the calibration file during subsequent runs to remove the background signal from the run data. The last portion of the background calibration procedure includes an "optical" calibration that is required for the 7500.

PURE DYE CALIBRATION

A pure dye calibration consists of a set of runs during which the SDS software collects spectral data from a series of dye standards. The software stores the spectral information for the pure dye standards in the pure spectra run file, a calibration file located in the SDS directory. After the run, the software stores the pure spectra data to characterize pure dyes used on the instrument.

DECONTAMINATION OF THE SAMPLE BLOCK

Clean the contaminated wells of the sample block using a small volume of deionized water:

a. Pipet a small volume (~100 µL) of deionized water into each contaminated well. (Use a new tip for each well)

b. Pipet the water up and down several times to rinse the well

c. Pipet the water to a waste beaker

MONITORING LAMP STATUS

To determine whether the halogen lamp has enough electrical current, select **Instrument ►Lamp Status/Replacement**. In the Lamp Status/Replacement dialog box, the "Lamp Current" field indicates a percentage figure for the electrical current. The "Condition:" field indicates one of the following conditions:

Good - lamp is functioning well, no need to replace the lamp bulb at this time

Failed - lamp bulb needs to be replaced

Change Soon - lamp bulb usage is above 2000 hours, replace bulb soon.