Optical Calibration Certificate

On site Calibration

Certificate nr. OPT14012503-00062

Applicant

Institute / Client

Department

Address

Zip / City

Country

On site location

Contact

Address

Room

Zip / City

Country

Phone

E-mail

Calibrated instrument (DUT, Device Under Test)

Manufacturer	Applied Biosystems
Instrument	7500 Fast Real-Time PCR System
Instrument serial number	275010522
Block type	96 x 0.2ml Fast
Block position	Single
Block serial number	750S6021664
Internal reference	

Additional information can be found on the last page of this certificate

Calibration Unit (CU) and method

The Calibration Unit (CU) contains calibrated temperature sensors. These temperature sensors measure the temperature at different prescribed points in the sample block of the qPCR thermocycler and are monitored through an ITS-90 traceable measurement system. Measurements are made on all temperature sensors simultaneously at several temperature levels. More details are described in quality system procedures of BV.

The CU is positioned in the qPCR thermocycler and a specific qPCR protocol program is started.
CU temperature sensors are in communication with calibrated LED light generating elements (LGE). The conditioned LGE's, located in the upper layer of the calibration unit, have a known spectrum and intensity with a range from 400 to 660 nm. During the calibration of the DUT the CU is emulating, depending on the measured temperature, a typical Absolute Quantification qPCR profile and an amplicon melt.

qPCR instruments

qPCR instruments can detect small amounts of fluorescence and/or light from either a wide spectrum and/or a defined smaller spectrum. The detection spectrum may vary between 400 and 720 nm. The optical calibration unit (CU) is positioned in the qPCR instrument (DUT) and a specific qPCR-protocol with a number of selected dyes is started. The Optical unit (CU) monitors the cycles of the qPCR thermocycler (DUT) while dynamically measuring the temperature of the cycler block. Depending on the measured cycle the Calibration Unit (CU) provides a calibrated intensity of light with a known spectrum towards the optics and optical detector of the qPCR thermocycler.

Instrument settings

Instrument in fast mode and heated lid on. The reaction volume is set to 10 µl and the instrument is set to maximum ramp rate.

Non-conformities

No non-conformities reported.

Environmental conditions (average)

Ambient	N/A
Relative humidity	N/A

Protocol

Pre-heat protocol
30 °C for 60 seconds
95 °C for 60 seconds
30 °C for 60 seconds

Protocol
30 °C for 60 seconds
95 °C for 180 seconds
30 °C for 120 seconds
90 °C for 180 seconds
50 °C for 180 seconds
70 °C for 180 seconds
60 °C for 180 seconds
30 °C for 60 seconds

Quantification Protocol
85 °C for 10 seconds
60 °C for 30 seconds
Repeat 32 times

Results

The calibration results are given on following pages of this certificate.

Calibration and measurement capability (CMC)

0.1°C (k =2) based on an idealized thermocycler block at a given point in time

Uncertainty

0.12°C (k =2)
The reported uncertainty of measurement is based on the standard uncertainty of measurement multiplied by a coverage factor k = 2, which for a normal distribution corresponds to a coverage probability of approximately 95%. The standard uncertainty has been determined in accordance to 'Guide to the expression of uncertainty in measurement' (GUM).

Calibration date

12-Aug-2014

Report date

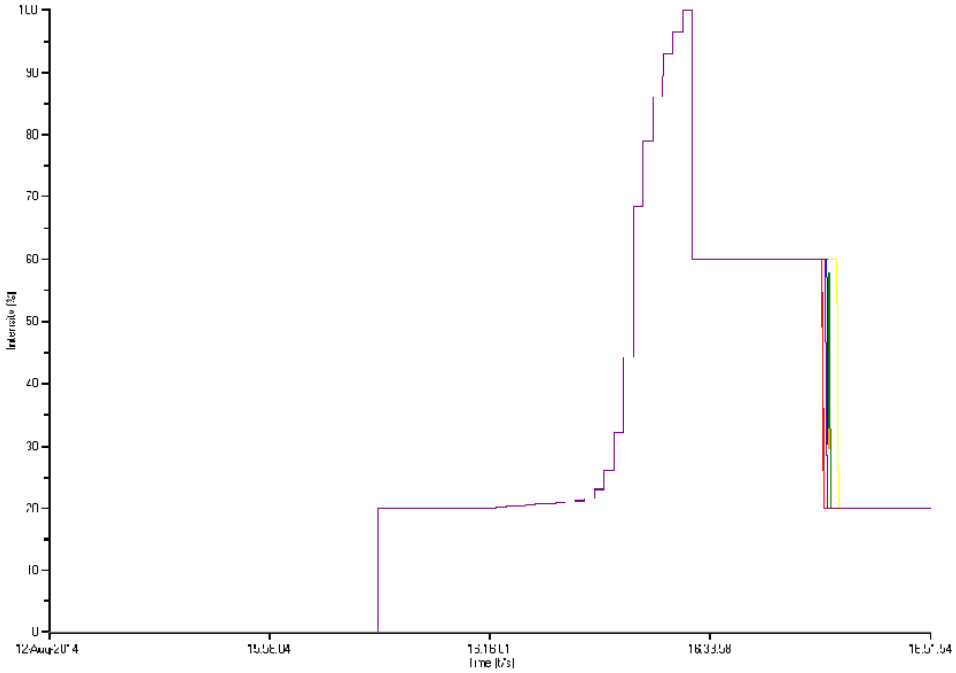
30-Oct-2014

Approved signatory

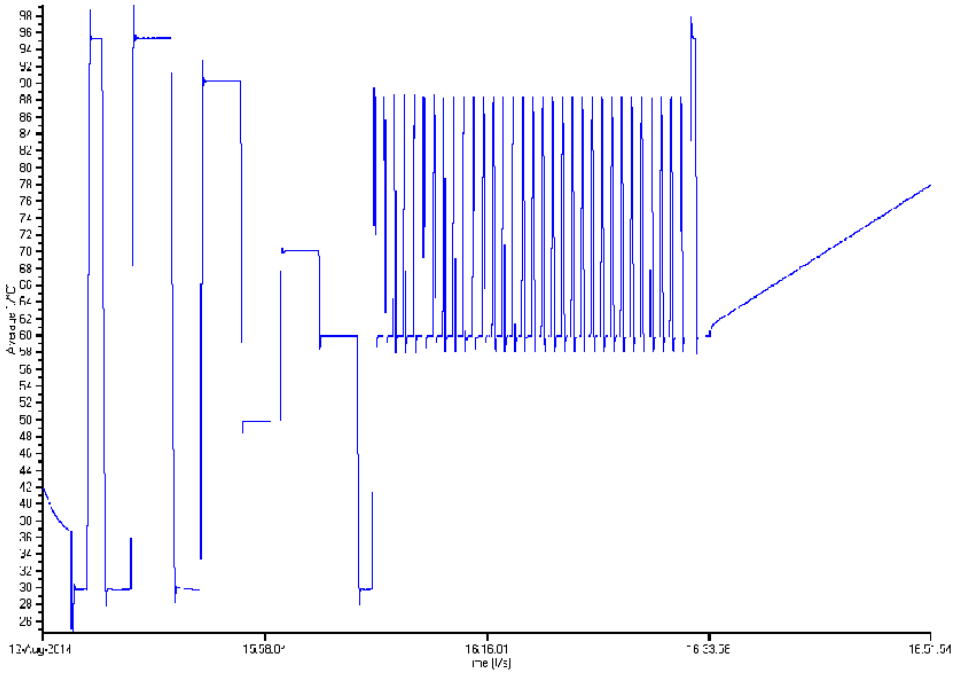
Default User (Debugging mode)
Operations co-ordinator

Intensity chart from the CU (during calibration)

Tm_{set} CU = 70 ºC

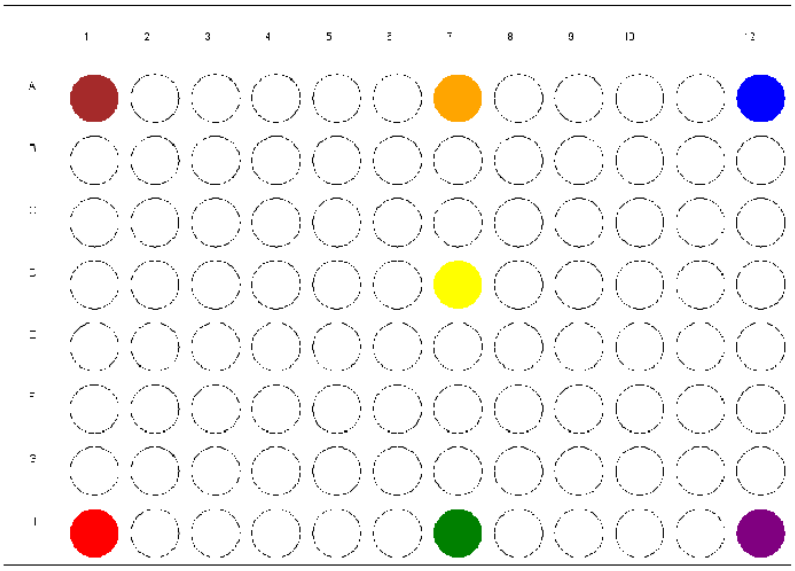


Temperature chart of the DUT, measured by the CU (during calibration)



Legend: temperature sensor and LGE positions

Sensor 1 (A1)
Sensor 2 (H1)
Sensor 3 (A7)
Sensor 4 (D7)
Sensor 5 (H7)
Sensor 6 (A12)
Sensor 7 (H12)



Cq detection

When the qPCR thermocycler (DUT) starts to collect Optical Signals, the Calibration Unit (CU) supplies an identical low light intensity to all channels for a number of thermal cycles (base line detection) after which the CU increases, in pre-defined steps, “light intensities packages” in defined portion and identical for each channel. By doing so the external calibration unit CU emulates a typical Absolute Quantification qPCR profile.
The DUT measures "light intensity" generated the by the CU and calculates Cq values for each channel.

Cq results (FAM CT_ST1)

Position	Value	Specifications	
A1 (1)	13.68	11.50 ± 2.50	✔
H1 (85)	12.64	11.50 ± 2.50	✔
A7 (7)	13.48	11.50 ± 2.50	✔
D7 (43)	13.22	11.50 ± 2.50	✔
H7 (91)	12.05	11.50 ± 2.50	✔
A12 (12)	11.68	11.50 ± 2.50	✔
H12 (96)	12.05	11.50 ± 2.50	✔
Average	12.69		
Uniformity	2.00		
Standard deviation	0.73		

Legend: ✔ = Pass, ✘ = Fail

Melting Point

While the qPCR thermocycler (DUT) is slowly increasing its block temperature, the optical unit of the DUT monitors changes in light intensity. The Calibration Unit (CU) which is positioned in the DUT dynamically measures the temperature of each individual channel. The measured temperature is processed and proportionally converted into a defined intensity of light. Defined thresholds of temperature readings are set as a threshold for the release of the designate amounts of light intensity.
For an ideal qPCR thermocycler, each well is identical in temperature and identical optical readings are detected at each position. Ideally there should be one peak, where all the signals of all channels overlay each other and the signal intensity detected by the qPCR thermocycler should be identical for all channels.

Peak Shifts

Since the calibration unit (CU) measures temperature for each channel and converts temperature to light, melt curve peak shifts will occur. In the case of ideal optics, light detection and analyzing software of the DUT, peak shifts will be identical to the temperature uniformity of the qPCR thermocycler as measured by the CU. In case of an ideal qPCR thermocycler with 0 °C temperature uniformity, ideal optics, light detection and analyzing software one would only see one peak where all channels overlay each other.

Peak Heights (CPHC)

The calibration unit (CU) converts temperature to traceable and pre-defined intensities of light. Melt curve peaks are detected by the thermocycler optics. In case of ideal optics, light detection and analyzing software of the DUT, peak heights (intensity of light) will be identical for each channel.
Differences in peak heights indicate differences in optical pathway, optics, optical detection and sensitivity of the DUT. Peak heights should be within pre-defined thresholds.
Individual peak heights are expressed as Channel Peak Height Consistency (CPHC).

Calculation of CPHC (Channel Peak Height Consistency):

[B-C] = Single Channel Raw data [(Tm -2°C) - (Tm +2ºC)]
[I] = Average Raw data (all channels) [(Tm -2ºC) - (Tm +2ºC)]
CPHC = [B-C] / [I]

Actual values
See table Tm results
847166

In case of an ideal qPCR thermocycler the CPHC will be 1.00

Tm Results (FAM)

Tm_{set} CU = 70 ºC Position	Tm [ºC]	Tm bias [ºC]	raw data Max A	raw data Tm -2°C B	raw data Tm +2°C C	Delta A-B	Delta B-C	CPHC	LSF
A1 (1)	69.30	-0.70	2740348	1647744	545897	1092605	1101847	1.30	0.99
H1 (85)	69.30	-0.70	2449756	1451075	476367	998681	974708	1.15	1.02
A7 (7)	69.50	-0.50	1685499	1008790	329099	676708	679691	0.80	1.00
D7 (43)	70.40	0.40	1718126	1032161	343455	685965	688706	0.81	1.00
H7 (91)	69.50	-0.50	1560627	936517	312586	624109	623931	0.74	1.00
A12 (12)	69.50	-0.50	2082147	1248244	409204	833904	839040	0.99	0.99
H12 (96)	69.30	-0.70	2544635	1541836	519594	1002799	1022241	1.21	0.98
Average	69.54	-0.46	2111591	1266624	419458	844967	847166		1.00

	Position	Value	Specification	
Ratio Tm	Average	0.98	1.00 ± 0.20	✔
TPSF	Δ*t* @Tm	1.13		
IFI	Average	0.03	≥ 0	✔
In this qPCR cycler identical amplicon will give a Tm spread of 1.13 max				✔ 1.13
$CPHC_{min}$	H7 (91)	0.74	1.00 ± 0.20	✘
$CPHC_{max}$	A1 (1)	1.30	1.00 ± 0.20	✘
LSF_{min}	H12 (96)	0.98	1.00 ± 0.20	✔
LSF_{max}	H1 (85)	1.02	1.00 ± 0.20	✔
LSF	Average	1.00	1.00 ± 0.20	✔

Legend: ✔ = Pass, ✔ = within expected values, ✘ = Fail

Validation of Tm (RTm or Ratio Tm)

Validation of Tm can be based on the absolute values, analyzed by the DUT, as well as by calculating the ratio between the delta Tm analyzed (ΔTm DUT) of all channels and the temperature uniformity during the Tm-set of the CU. (Δt Tm CU)

Calculation of Ratio Tm:	**Actual values**
[1] = $Tm_{max} - Tm_{min}$	**1.10**
[2] = $t_{max} - t_{min}$ @Tm	**1.13**
RTm = [1] / [2]	**0.98**

Whereas Tm is measured and calculated by the DUT and t_{max} and t_{min} @Tm is measured by the CU

Interpretation of Ratio Tm

Between 0.80 and 1.20	Tm deviations are based on temperature uniformity of the DUT
RTm > 1.20	Tm deviations are based on temperature uniformity, inaccuracies of optics and optical detection of the DUT
RTm < 0.80	Tm deviations are based on inaccuracies in analyzing software, optics and optical detection of the DUT

If RTm is within specifications: identical products will melt at different qPCR thermocycler block temperatures whereas the difference is only based on uniform temperature characteristics of DUT. Melt peak shifts, observed during melting are directly related to temperature inaccuracies of the qPCR thermocycler only.
If RTm is outside maximum specifications: identical products will melt at different qPCR thermocycler block temperatures whereas the difference in result is based on inaccuracies of temperature, optics and optical detection of the qPCR thermocycler.
If RTm is outside minimum specifications: identical products will melt at different qPCR thermocycler block temperatures whereas the difference in result is based on inaccuracies in analyzing software, optics and optical detection of the qPCR thermocycler.

Tm Peak Shift Fact (TPSF) and Identical Fragment Indicator (IFI)

A DUT with ideal optics, light detection and analyzing software will generate Tm peak shifts identical to the temperature uniformity of the qPCR thermocycler as measured by the CU. This Tm Peak Shift Fact (TPSF) is expressed in ºC and represents the temperature uniformity of the qPCR cycler at Tm (average). The Identical Fragment Indicator (IFI) is a precise calculation to determine if the DUT detected Tm range is solely temperature related or not.

IFI (Identical Fragment Indicator) is calculated as:

IFI = TPSF – (Tm_{max} - Tm_{min})

Interpretation IFI

IFI ≥ 0	amplicon is likely identical
IFI < 0	amplicon is not identical

Linear Sensitivity Factor (LSF)

During the Optical Calibration, the CU supplies defined intensity amounts of light with a known spectrum to the DUT (qPCR thermocycler). The CU provides a maximum intensity of 100% (saturation phase) whereas the decrease to 60% of intensity from saturation to "prior melt" is equal (∆40%) to the instant quench of intensity occurring during melt and after the melt to 20% intensity (∆40%) offered by the CU.

Calculating the ratio between a range of provided signal intensities of the CU and the detected intensities of the DUT enables the calculation of a linear sensitivity factor for each channel.

Calculation of LSF for each channel:

[1] = (DUT Raw data,max) - (DUT Raw data@Tm -2°C)
[2] = (DUT Raw data@Tm -2°C) - (DUT Raw data@Tm +2°C)
LSF = [1] / [2]

An ideal qPCR Thermocycler should have a Linear Sensitivity Factor of 1.00

Interpretation LSF	
Between 0.80 and 1.20	linear
LSF < 0.80	less linear at high intensities, alert for signal saturation
LSF > 1.20	less linear at low intensities, alert for minimum signal detection and sensitivity

For individual channel LSF values see the Tm results table given above

Terminology

CU	Calibration Unit. (device which is used to calibrate the qPCR thermocycler)
DUT	Device Under Test (calibrated instrument) qPCR thermocycler.
average [°C]	$t90_{avg}$, average value of all active sensors at a specific sample position.
minimum [°C]	minimum value of all active sensors at a specific sample position.
maximum [°C]	maximum value of all active sensors at a specific sample position.
uniformity [°C]	maximum temperature -minimum temperature at a specific sample position. Other commonly used terms for uniformity are temperature spread or homogeneity.
deviation [°C]	average measured temperature (t90) minus set temperature (t_{set}).
ramp rate [°C/s]	dT/dt, where dT= between 10% and 90% of the ramp. dt= interval t(T90%) -t(T10%).
average overshoot [°C]	average value of all active sensors at the sample position of the maximum overshoot.
maximum overshoot [°C]	maximum of all active sensors towards a static temperature hold.
overshoot duration [°C]	the time it takes for the plateau to reach the set temperature again.
set temperature [°C]	t_{set}, set or target temperature is the value which is programmed to be reached.
uncertainty [°C]	parameter, associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the quantity intended to be measured.
protocol run time [mm:ss]	the time it takes for the protocol to complete (pre-heat steps are not included).
hold time [s]	the measured time it takes for a plateau to complete.
ramp time [s]	the measured time it takes for an instrument to ramp from one temperature to another.
hertz [Hz]	the number of samples taken per second.
CMC	Calibration and Measurement Capabilities.
Light and Intensity	Radiant power of a source emitted in a certain direction (Radiant Intensity [W/sr] Watt per steradian (squared radian))
Cq	Quantification Cycle, represents the number of cycles needed to reach a set threshold fluorescence signal level
Tm	Melting temperature of the amplicon (PCR product), the temperature at which 50% of the helices are dissociated
CPHC	Channel Peak Height Consistency
LSF	Linear Sensitivity Factor
Ratio Tm	$(Tm_{max} - Tm_{min}) / (t_{max} - t_{min}$ @Tm)
TPSF	Tm Peak Shift Fact
IFI	Identical Fragment Indicator

Calibration information

Calibration number

Calibration engineer

Certificate generated

Equipment information

Calibration Unit (CU)

Acquisition software version	Optical 1.1.7.5
Definitions	25-Jul-2014
Data filename	14012503-00062.ulf
Sample interval	250 ms
Probe serial	140125-03
Probe type	Optical 7 x 0.2ml N/A
Probe thermal calibration date	N/A
Probe optical calibration date	
Probe software version	070211

Device Under Test (DUT)

Instrument software version	SDS v1.3.1
Runned cycler program	-

Disclaimer

Software and hardware are designed and manufactured in the Netherlands