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bCUBE 2 validation

Comparative analysis

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Introduction

With the aim to fully validate bCUBE 2 on performing Real Time PCR and Loop mediated isothermal amplification (LAMP) analyses we set up comparative tests with competitor's instruments available on the market. As comparative machines for Real Time PCR and LAMP analyses we used CFX96™ produced by BIO-RAD and Genie® III produced by OptiGene, respectively.

Real Time PCR comparative analysis

We tested the ability of bCUBE 2 on performing Real Time PCR analyses with the most used biochemistries and reporter dyes:

- SYBR® Green I;
- TaqMan™ probe using FAM as reporter dye;
- TaqMan™ probe using VIC as reporter dye

For each biochemistry we performed amplification efficiency and limit of detection (LOD) tests using our internal developed kits, running the experiments simultaneously on the bCUBE2 and on the BIO-RAD CFX96 as comparative test. All the results showed in this report are the rough output of the two instruments, no setting adjustments were carried out.

Amplification efficiency analyses

Amplification efficiency indicates how the target DNA is amplified during the PCR reaction; it is usually evaluated in order to verify if the assay is working under efficient conditions (i.e. doubling the reaction product at each cycle). This is an essential parameter when performing quantitative PCR experiments, so we decided to verify the performances of bCUBE 2 with such tests. To this aim, we used 5 ten-fold serial dilutions of genomic DNA template starting from 5 ng/µl. Each serial dilution was run in triplicate and the obtained threshold cycle values (Ct) were plotted against the logarithm of the concentration. The slope of the straight line (regression line) that fitted the data allowed us to get the amplification efficiency as follow:

Amplification efficiency (E)= [10(-1/slope)] – 1

Limit of detection analyses (LOD)

LOD indicates the lowest amount of DNA template that can be detected by an assay. It is an essential test for publication of Real Time PCR experiments (Bustin, 2009), so we decided to investigate the behavior of bCUBE 2 with this kind of analyses. To this purpose we used 5 one-hundred-fold serial dilutions of genomic DNA template, starting from 0,05 ng/µl. For each test we recorded the lowest DNA template concentration that can be detected in a linear range (defined as discussed above for amplification efficiency analyses).

SYBR® Green I

As reference kit to evaluate the performances of bCUBE 2 with SYBR® Green I biochemistry we used our "demo kit Hyris SYBR® Green I" (DRHG) for the detection of Lambda phage (Lambda genomic DNA provided by Roche). Amplification efficiency and limit of detection analyses were performed in parallel on the bCUBE 2 and on the BIO-RAD CFX96.

Amplification efficiency analysis – SYBR® Green I

The following DNA concentrations and a No-Template-Control (NTC) were tested: 5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl. Each condition was tested in triplicate (except NTC). The runs were performed with 40 cycles of PCR amplification followed by a melting curve analysis (**Figure 1**, **Figure 2**). Cq mean, Cq standard deviation, efficiency and R^2 values relative to the runs on the two machines are summarized in **Table 1**.

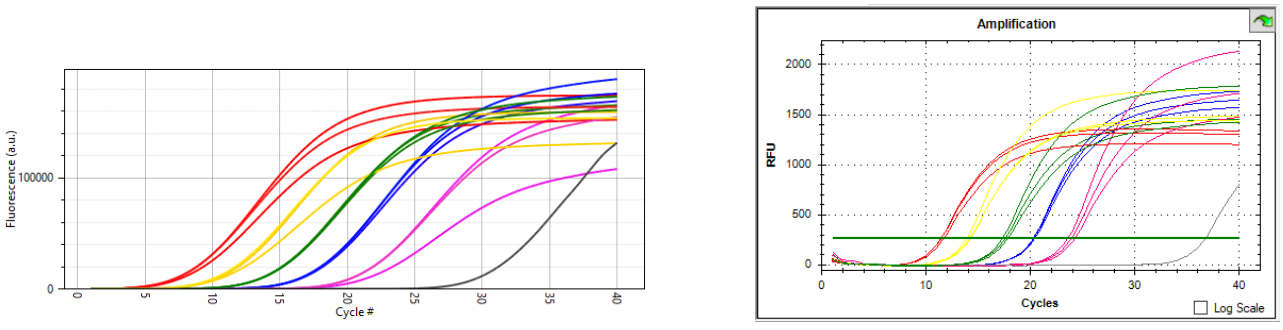

Figure 1: Amplification efficiency SYBR Green I.
The plots show the **a**mplification curves obtained for the kit DRHG using 5 different DNA template concentration (5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl and NTC) on the bCUBE 2 (left) and on the BIO-RAD CFX96 (right).

Samples	Cq mean bCUBE 2	Cq Std. Dev. bCUBE 2	Efficiency bCUBE 2	R^2 bCUBE 2	Cq mean BIO-RAD CFX96	Cq Std. Dev. BIO-RAD CFX96	Efficiency BIO-RAD CFX96	R^2 BIO-RAD CFX96
Lambda gDNA 5 ng/µl	8,55	0,12	102,60%	0,997	11,53	0,15	110,20%	0,996
Lambda gDNA 0,5 ng/µl	11,38	0,16			14,13	0,18		
Lambda gDNA 0,05 ng/µl	14,82	0,06			17,58	0,27		
Lambda gDNA 0,005 ng/µl	17,88	0,10			20,33	0,07		
Lambda gDNA 0,0005 ng/µl	21,61	0,20			23,93	0,35		
NTC	30,96	/			36,76	/		

Table 1: Amplification efficiency data Sybr Green I.
Cq mean, Cq standard deviation, amplification efficiency and R^2 values on the bCUBE 2 and on the BIO-RAD CFX96

The amplification efficiency and R^2 values are comparable between the two machines: 102,6% on the bCUBE 2 and 110,2% on the CFX96, with $R^2 \geq 0,99$ on both. NTC amplification indicates a partial contamination, however the great difference between targets and No-Template-Control Cq values suggests only a slight contribution on the analysis. After PCR amplification step, a melting curve analysis has been performed (**Figure 2**).

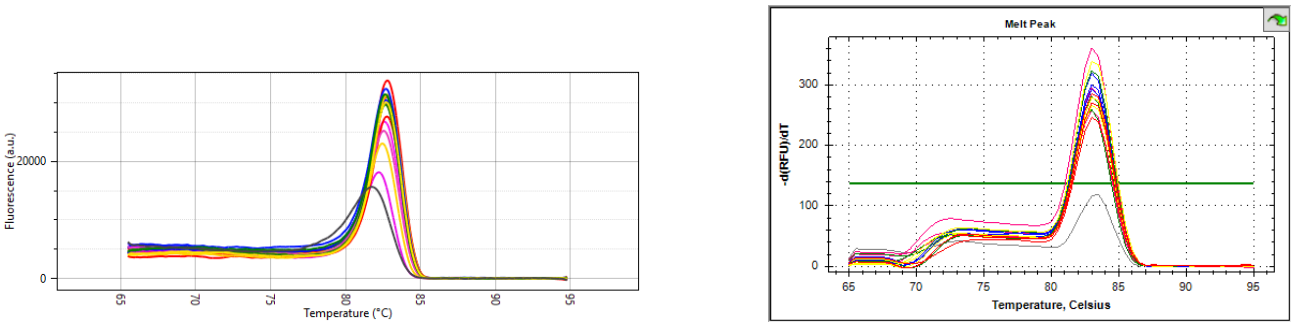

Figure 2: Melting curves SYBR Green I.
The plots show the melting curves for the kit DRHG using 5 different DNA template concentration (5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl and NTC) on the bCUBE 2 (left) and on the BIO-RAD CFX96 (right).

The melting curves are comparable between the two instruments; the peaks are between 82,4°C and 84,8°C on the bCUBE 2 and 83°C on CFX96 (data not shown). NTC peaks are slightly shifted on both the instruments: 81,6°C on bCUBE 2 and 83,5°C on CFX96, highlighting once again the low impact of the contamination in this analysis.

Limit of detection analysis (LOD) – SYBR® Green I

The following DNA concentrations and a No-Template-Control (NTC) were tested: $5 * 10^{-2}$ ng/µl, $5 * 10^{-4}$ ng/µl, $5 * 10^{-6}$ ng/µl, $5 * 10^{-8}$ ng/µl, $5 * 10^{-10}$ ng/µl. Each condition was tested in triplicate

(except NTC). The runs were performed with 40 cycles of PCR amplification (**Figure 3**) followed by a melting curve analysis. Cq mean, Cq standard deviation, efficiency and R^2 values relative to the runs on the two machines are summarized in **Table 2**.

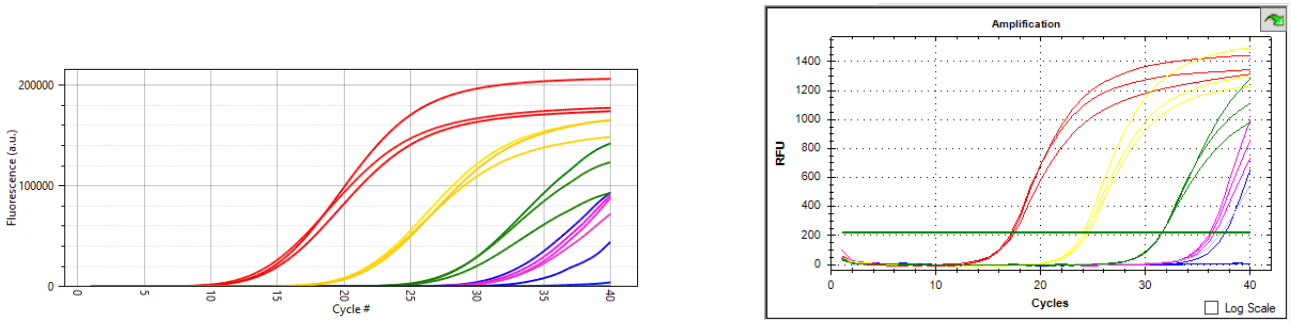

Figure 3: LOD test SYBR Green I.
The plots show the amplification curves for the kit DRHG using 5 different DNA template concentration (5 * 10 $^{-2}$ ng/µl, 5 * 10 $^{-4}$ ng/µl, 5 * 10 $^{-6}$ ng/µl, 5 * 10 $^{-8}$ ng/µl, 5 * 10 $^{-10}$ ng/µl and NTC) on the bCUBE 2 (left) and on the BIO-RAD CFX96 (right).

Samples	Cq mean bCUBE 2	Cq Std. Dev. bCUBE 2	Efficiency bCUBE 2	R^2 bCUBE 2	Cq mean BIO-RAD CFX96	Cq Std. Dev. BIO-RAD CFX96	Efficiency BIO-RAD CFX96	R^2 BIO-RAD CFX96
Lambda gDNA 5 * 10^{-2} ng/µl	14,58	0,48	94,7%	0,997	17,33	0,15	91,2%	0,999
Lambda gDNA 5 * 10^{-4} ng/µl	21,63	0,31			24,31	0,24		
Lambda gDNA 5 * 10^{-6} ng/µl	28,4	0,23			31,56	0,01		
Lambda gDNA 5 * 10^{-8} ng/µl	/	/			/	/		
Lambda gDNA 5 * 10^{-10} ng/µl	/	/			/	/		
NTC	/	/			37,14	/		

Table 2: LOD data SYBR Green I.
Cq mean, Cq standard deviation, amplification efficiency and R^2 values on the bCUBE 2 and on the BIO-RAD CFX96

The lowest DNA concentration that can be detected in a linear range is 5 * 10^{-6} with both the machines. Efficiency and R^2 values reported in table 2 refer to the conditions in the linear range (from 5 * 10^{-2} ng/µl to 5 * 10^{-6} ng/µl). Efficiency is comparable between the two instruments: 94,7% on the bCUBE 2 and 91,2% on the CFX96, with $R^2 \geq 0,99$ on both the instruments. The two more diluted conditions experienced (**Figure 3**) the "Monte-Carlo" effect (Bustin, 2004), an inherent limitation of PCR where small amount of DNA template show unpredictable amplification. The melting curves are reproducible (data not shown), too.

TaqMan™ probe using FAM as reporter dye

As reference kit to evaluate the performances of bCUBE 2 with TaqMan™ probe biochemistry using FAM as reporter dye, we used our "demo kit Hyris Probe FAM" (DRHP_FAM) for the detection of Lambda phage (Lambda genomic DNA provided by Roche). Amplification efficiency and limit of detection analyses were performed in parallel with the bCUBE 2 and the BIO-RAD CFX96.

Amplification efficiency analysis – TaqMan™_FAM

The following DNA concentrations and a No-Template-Control (NTC) were tested: 5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl. Each condition was tested in triplicate (except NTC). The runs were performed with 40 cycles of PCR amplification (**Figure 4**). Cq mean, Cq standard deviation, efficiency and R^2 values relative to the runs on the two machines are summarized in **Table 3**.

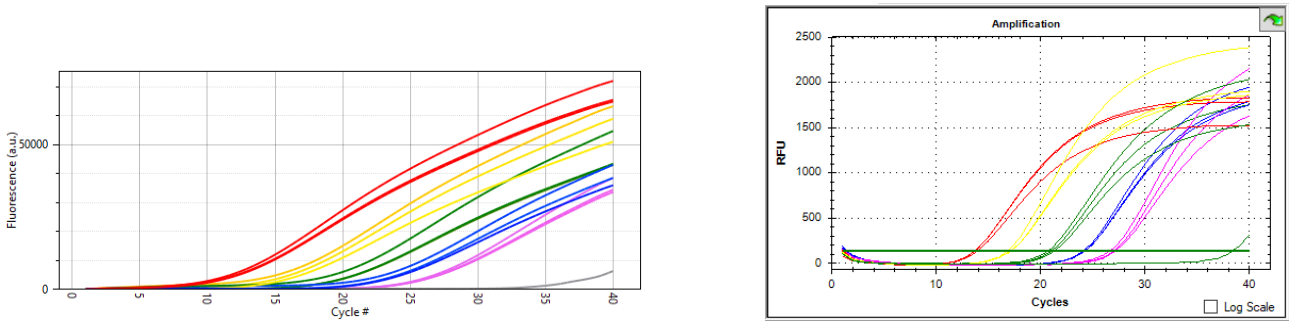

Figure 4: Amplification efficiency TaqMan_FAM.
The plots show the amplification curves for the kit DRHP_FAM using 5 different DNA template concentration (5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl and NTC) on the bCUBE 2 (left) and on the BIO-RAD CFX96 (right).

Samples	Cq mean bCUBE 2	Cq Std. Dev. bCUBE 2	Efficiency bCUBE 2	R^2 bCUBE 2	Cq mean BIO-RAD CFX96	Cq Std. Dev. BIO-RAD CFX96	Efficiency BIO-RAD CFX96	R^2 BIO-RAD CFX96
Lambda gDNA 5 ng/µl	12,84	0,12	94,0%	0,994	13,77	0,18	98,8%	0,995
Lambda gDNA 0,5 ng/µl	16,62	0,44			17,03	0,19		
Lambda gDNA 0,05 ng/µl	20,49	0,46			21,01	0,30		
Lambda gDNA 0,005 ng/µl	23,59	0,42			24,19	0,08		
Lambda gDNA 0,0005 ng/µl	26,73	0,03			26,95	0,28		
NTC	34,69	/			38,39	/		

Table 3: Amplification efficiency data TaqMan_FAM.
Cq mean, Cq standard deviation, amplification efficiency and R^2 values on the bCUBE 2 and on the BIO-RAD CFX96

The amplification efficiency is comparable between the two instruments: 94% on the bCUBE 2 and 98,8 % on the CFX96 with $R^2 \geq 0,99$ on both. Although present, NTC amplification signal seems negligible as it is very delayed compared to the target.

Limit of detection analysis (LOD) – TaqMan™_FAM

The following DNA concentrations and a No-Template-Control (NTC) were tested: 5 * 10^{-2} ng/µl, 5 * 10^{-4} ng/µl, 5 * 10^{-6} ng/µl, 5 * 10^{-8} ng/µl, 5 * 10^{-10} ng/µl. Each condition was tested in triplicate (except NTC). The runs were performed with 40 cycles of PCR amplification (**Figure 5**). Cq mean, Cq standard deviation, efficiency and R^2 values relative to the runs on the two machines are summarized in **Table 4**.

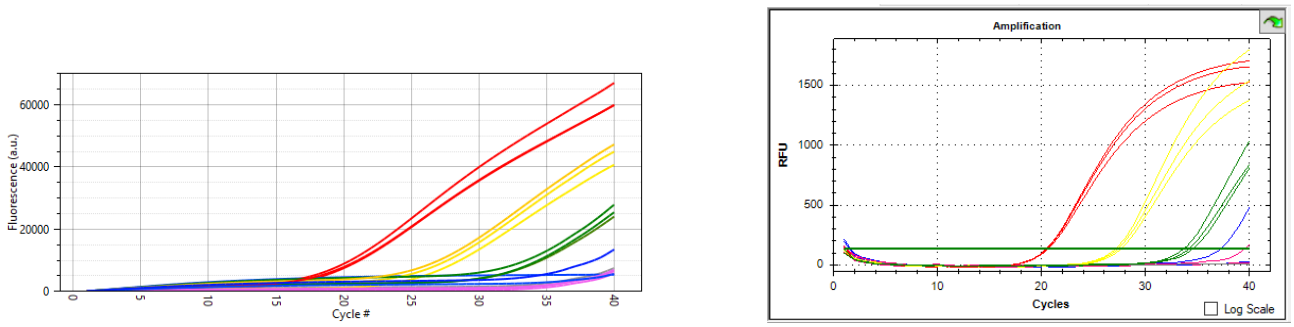

Figure 5: LOD test TaqMan_FAM.
The plots show the amplification curves for the kit DRHP_FAM using 5 different DNA template concentration (5 * 10^{-2} ng/µl, 5 * 10^{-4} ng/µl, 5 * 10^{-6} ng/µl, 5 * 10^{-8} ng/µl, 5 * 10^{-10} ng/µl and NTC) on the bCUBE 2 (left) and on the BIO-RAD CFX96 (right).

Samples	Cq mean bCUBE 2	Cq Std. Dev. bCUBE 2	Efficiency bCUBE 2	R^2 bCUBE 2	Cq mean BIO-RAD CFX96	Cq Std. Dev. BIO-RAD CFX96	Efficiency BIO-RAD CFX96	R^2 BIO-RAD CFX96
Lambda gDNA 5 * 10^{-2} ng/µl	19,23	0,16	101,8%	0,993	20,8	0,10	96,5%	0,998
Lambda gDNA 5 * 10^{-4} ng/µl	25,79	0,26			27,65	0,29		
Lambda gDNA 5 * 10^{-6} ng/µl	32,35	0,89			34,44	0,42		
Lambda gDNA 5 * 10^{-8} ng/µl	/	/			/	/		
Lambda gDNA 5 * 10^{-10} ng/µl	/	/			/	/		
NTC	39,76	/			/	/		

Table 4: LOD data TaqMan_FAM.
Cq mean, Cq standard deviation, amplification efficiency and R^2 values on the bCUBE 2 and on the BIO-RAD CFX96

As for LOD analysis with SYBR® Green I biochemistry, the lowest DNA concentration detectable in a linear range is 5 * 10^{-6} for both the instruments. The Efficiency and the R^2 values reported in table 4 refer to the conditions in the linear range (from 5 * 10^{-2} ng/µl to 5 * 10^{-6} ng/µl). The efficiency is:

101,8% on the bCUBE 2 and 96,5% on the CFX96, with R^2≥0,99 on both. Again, the two more diluted conditions (**Figure 5**) experienced the "Monte-Carlo" effect (Bustin, 2004).

TaqMan™ probe using VIC as reporter dye

With the aim to evaluate the performances of bCUBE 2 when performing TaqMan™ probe assays with VIC as reporter dye, we used our "demo kit Hyris Probe VIC" (DRHP_VIC) for the detection of Lambda phage (Lambda genomic DNA provided by Roche). Amplification efficiency and limit of detection analyses were performed in parallel with bCUBE 2 and BIO-RAD CFX96.

Amplification efficiency analysis – TaqMan™_VIC

The following DNA concentrations and a No-Template-Control (NTC) were tested: 5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl. Each condition was tested in triplicate (except NTC). The runs were performed with 40 cycles of PCR amplification (**Figure 6**). Cq mean, Cq standard deviation, efficiency and R^2 values are summarized in **Table 5**.

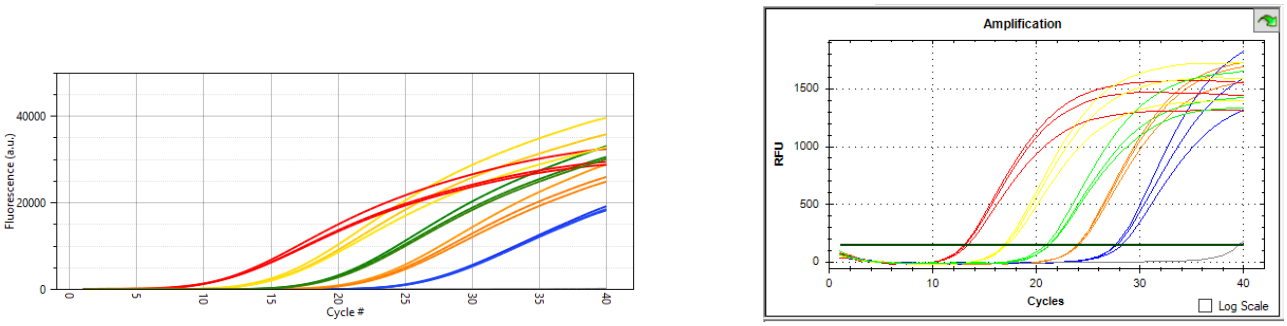

Figure 6: Amplification efficiency graph TaqMan_VIC.
The plots show the amplification curves for the kit DRHP_VIC using 5 different DNA template concentration (5 ng/µl, 0,5 ng/µl, 0,05 ng/µl, 0,005 ng/µl, 0,0005 ng/µl and NTC) on the bCUBE 2 (left) and on the BIO-RAD CFX96 (right).

Samples	Cq mean bCUBE 2	Cq Std. Dev. bCUBE 2	Efficiency bCUBE 2	R^2 bCUBE 2	Cq mean BIO-RAD CFX96	Cq Std. Dev. BIO-RAD CFX96	Efficiency BIO-RAD CFX96	R^2 BIO-RAD CFX96
Lambda gDNA 5 ng/µl	11,84	0,07	85,3%	0,996	13,14	0,16	88,5%	0,996
Lambda gDNA 0,5 ng/µl	16,19	0,07			16,95	0,14		
Lambda gDNA 0,05 ng/µl	20,22	0,10			21,1	0,19		
Lambda gDNA 0,005 ng/µl	23,21	0,13			23,97	0,09		
Lambda gDNA 0,0005 ng/µl	27,00	0,17			27,8	0,35		
NTC	/	/			39,5	/		

Table 5: Amplification efficiency data TaqMan_VIC.
Cq mean, Cq standard deviation, amplification efficiency and R^2 values on the bCUBE 2 and on the BIO-RAD CFX96

As for the biochemistries previously showed, the efficiency is comparable between the two instruments: 85,3% on the bCUBE 2 and 88,5% on the CFX96, with $R^2 \geq 0,99$ on both.

Limit of detection analysis (LOD) – TaqMan™_VIC

The following DNA concentrations and a No-Template-Control (NTC) were tested: 5 * 10^{-2} ng/µl, 5 * 10^{-4} ng/µl, 5 * 10^{-6} ng/µl, 5 * 10^{-8} ng/µl, 5 * 10^{-10} ng/µl. Each condition was tested in triplicate (except NTC). The runs were performed with 40 cycles of PCR amplification (**Figure 7**). Cq mean, Cq standard deviation, efficiency and R^2 values are summarized on **Table 6**.

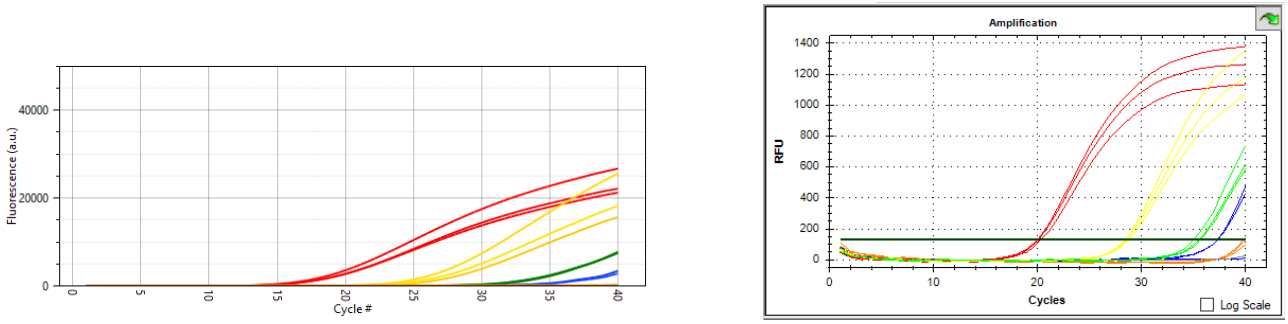

Figure 7: LOD test TaqMan_VIC.
The plots show the amplification curves for the kit DRHP_VIC using 5 different DNA template concentration (5 * 10^{-2} ng/µl, 5 * 10^{-4} ng/µl, 5 * 10^{-6} ng/µl, 5 * 10^{-8} ng/µl, 5 * 10^{-10} ng/µl and NTC) on the bCUBE 2 (left) and on the BIO-RAD CFX96 (right).

Samples	Cq mean bCUBE 2	Cq Std. Dev. bCUBE 2	Efficiency bCUBE 2	R^2 bCUBE 2	Cq mean BIO-RAD CFX96	Cq Std. Dev. BIO-RAD CFX96	Efficiency BIO-RAD CFX96	R^2 BIO-RAD CFX96
Lambda gDNA 5 * 10^{-2} ng/µl	19,29	0,12	94,6 %	0,989	20,13	0,18	82,8%	0,995
Lambda gDNA 5 * 10^{-4} ng/µl	27,39	0,41			28,54	0,17		
Lambda gDNA 5 * 10^{-6} ng/µl	33,12	0,26			35,38	0,32		
Lambda gDNA 5 * 10^{-8} ng/µl	/	/			/	/		
Lambda gDNA 5 * 10^{-10} ng/µl	/	/			/	/		
NTC	/	/			/	/		

Table 6: LOD data TaqMan_VIC.
Cq mean, Cq standard deviation, amplification efficiency and R^2 values on the bCUBE 2 and on the BIO-RAD CFX96

As for the previous LOD tests, the lowest DNA concentration that can be detected in a linear range is 5 * 10^{-6} with both the instruments. Efficiency and R^2 values reported in table 6 refer to the conditions in the linear range (from 5 * 10^{-2} ng/µl to 5 * 10^{-6} ng/µl). Again, the two more diluted conditions (**Figure 7**) experienced the "Monte-Carlo" effect (Bustin, 2004). The amplification

efficiency differs between the two instruments: 94,6% on the bCUBE 2 and 82,8% on the CFX96, with $R^2 \geq 0,99$ on both.

LAMP comparative analysis

Loop-mediated isothermal amplification (LAMP) is a DNA based technique first proposed by Notomi et al. in 2000 and widely used in the diagnostic field for its rapidity (Nagamine et al.). The reaction is based on the strand displacement activity of the *Bst* DNA polymerase. This enzyme, together with a specific set of six primers, allows amplification of target DNA at 65°C in less than 30 minutes. Successively a melting curve analysis is performed to assess the specificity of the reaction. To monitor the analysis in real time an intercalating dye as SYBR® Green I is added to the reaction mix.

The ability of bCUBE 2 on performing LAMP assays was compared with the competitor device Genie® III developed by OptiGene Limited. As reference kit, we used our internal demo kit Hyris LAMP (DLH). Here we show the amplification curves and the melting curves (**Figure 8**, **Figure 9**, and **Table 7**) obtained with the two devices using 1 ng of Lambda genomic DNA (gDNA) as template (in triplicate). A No-Template-Control (NTC) was added to the reaction, too.

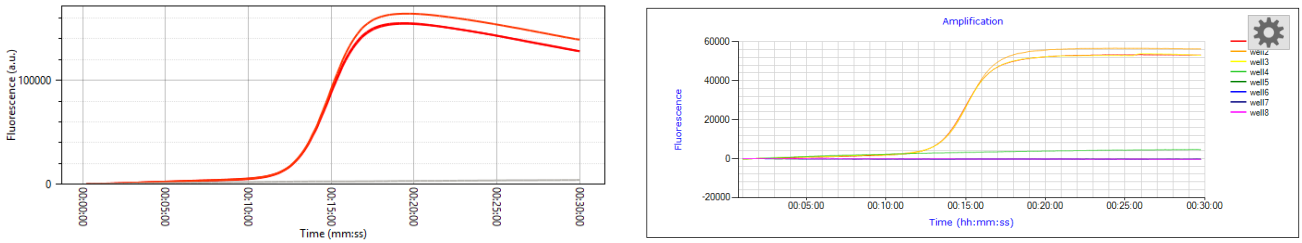

Figure 8: Amplification curves LAMP.
The plots show the amplification curves for the kit DLH on the bCUBE 2 (left) and on the Genie® III (right)

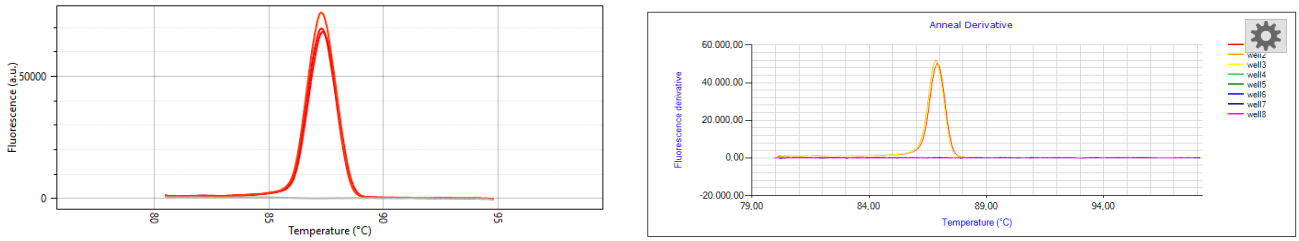

Figure 9: Melting curves LAMP.
The plots show the melting curve peaks for the kit DLH on the bCUBE 2 (left) and on the Genie® III (right)

Samples	TTS bCUBE 2 (hh:mm:ss)	Melting peaks bCUBE 2 (°C)	TTS Genie® III (hh:mm:ss)	Melting peaks Genie® III (°C)
Lambda gDNA 1 ng	00:12:40	87,3	00:14:45	86,9
Lambda gDNA 1 ng	00:12:43	87,3	00:14:45	86,8
Lambda gDNA 1 ng	00:12:41	87,3	00:14:45	86,9
NTC	/	/	/	/

Table 7: LAMP data.
TTS and melting peaks data on the bCUBE 2 (left) and on the Genie® III (right)

The amplification curves are comparable between the two machines. Time-To-Start (TTS) values repeatable on both the instruments. No differences are present for melting curve peaks.

Conclusion

With the aim to validate the ability of bCUBE 2 on performing Real Time PCR and LAMP experiments, we set up a series of tests using a comparative approach with competitor's machines. To this purpose we performed typical Real Time PCR and LAMP experiments with the most common biochemistries and reporter dyes. Referring to bCUBE 2 intraassay repeatability, it is positive and comparable to other instruments available on the market for all the experiments; in fact, Cq standard deviation never exceed 0,5 (except one triplicate in "TaqMan_FAM" LOD analysis). The reproducibility of the comparative analyses confirms this robustness, in fact all the runs carried out are comparable between the bCUBE 2 and the competitor's machines (only the amplification efficiency values in "TaqMan_VIC" LOD analysis show a significant and inexplicable difference). However, some open issues remain. In fact, TaqMan_FAM analyses on bCUBE 2 showed an initial increase in fluorescence that hampers Cq determination. Moreover, as final step to fully validate the bCUBE 2 performances with the most common biochemistries a "reproducibility study" followed by tests with other commercial kits are required.

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