Miniaturization of luminometric assays: Optimizing the performance of common luminescent assays in 384-, and 1536-well plate formats

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Abstract

Increasing use of small assay volumes with high density plates is a clear trend in today's bioscience research. Most of the commercial assay kits are still designed for 96 well plates and all instructions are given for that plate format. Therefore, the user needs most often to modify the assay procedure to be able to use low volume/high density microplates. There are no general rules how this modification is done making miniaturization of the assays sometimes complicated.

In this paper we have optimized the performance or common luminometric assays for 384, 384 low volume and 1536 well plates. The key parameters to be followed to evaluate the assay performance with the high density plate assays were the detection limit (LOD), CV%, and Z-prime.

Based on these results, it can be recommended that, considering all aspects (reagent and material consumption, performance etc.), the optimal assay setup would be 384-low volume plate with about 10 total assay volumes.

Materials & Methods

The first two luminometric assays to be studied were the very common luminometric ATP quantization assays representing both flash and glow type reaction chemistry. Third assay was luminescent dual reporter gene using both firefly and Renilla luciferase. All reagent kits, standards and different microplates used in these assays are listed in Table I. All measurements were performed with Thermo Scientific Varioskan Flash multitechnology reader equipped with Thermo Scientific LumiSens high-sensitivity luminometric module.

Flash ATP assay:

- ATP standard curves were measured between 0.2 amol – 50 pmol of ATP in the well
- Standards were added into different microplates in variable volumes using 8 replicates of each ATP standard and 24 replicates of blank wells
- Varioskan Flash was programmed to perform both luciferase/luciferin reagent dispensing and luminescence reading:
  - An equal volume of luciferase/luciferin reagent was dispensed (5-50 ul, depending on the plate format)
  - Three seconds were waited to let the luminescence reaction to be fully started
  - Luminescence signal was collected for four seconds

Glow ATP assay:

- ATP standard curves were measured between 2 amol – 50 pmol of ATP in the well
- Standards were added into different microplates in variable volumes using 8 replicates of each ATP standard and 24 replicates of blank wells
- Equal volume of luciferin/luciferase reagent from the kit was added using automatic dispenser (Thermo Scientific MultiDrop Combi)
- Plate was incubated in room temperature for two minutes to let luminescence signal to be generated
- The signal was read with Varioskan Flash using 1000 ms integration time

Luminometric cross talk assay:

- Luminometric cross talk was measured with 96, 384 and low volume 384 well plates using glow ATP reaction
- Single very high luminescence well was created in the middle of the plate in different assay volumes and the whole plate was measured with 1000 ms integration time.
- Cross talk value for each well wal calculated as percentage from the high signal well

Table I: Luminometric kits, assay standards and microplates.

	Flash ATP assay	Glow ATP assay	Dual luciferase assay
Kit/ chemistry	**Kikkoman:** Checklite HS Kit	**BioThema:** ATP Kit SL	**Promega:** Dual Luciferase (DLR) 1000 Assay System
Standards	**BioThema:** ATP Standard		**Promega:** QuantiLum Firefly luciferase **LUX Biotechnology:** Renilla Luciferase
Microplates	**96-well:** Thermo Scientific, Microlite 1+ **384-well:** Thermo Scientific, Microlite 384 **384-well low volume**: Corning, 384-low volume		**96-well:** Thermo Scientific, Microlite 1+ **384-well:** Thermo Scientific, Microlite 384 **384-well low volume:** Corning, 384-low volume **1536-well plate:** Corning, 10 ul, round bottom

Dual-Luciferase reporter gene assay:

- Both firefly and Renilla standard curves were measured between 0.001 – 100 000 amol/well
- Both standards were added into same well of different microplates in variable volumes using 8 replicates of each luciferase standard and 24 blanks
- Varioskan Flash was programmed to perform standard DLR assay:
  - LAR II firefly luciferase reagent was dispensed first and firefly signal was measured with 10 s integration time after two second delay
  - Stop & Glow reagent wad dispensed with another dispenser and similar measurement was performed for the Renilla signal

Results and Discussion

Flash ATP Assay:

Results of flash type ATP assays are shown in Table II and Figure 1 and following findings can be summarized:

- Assay sensitivity improves slightly when higher density plates are used and the lowest detection limit is achieved with low volume 384 well plates with highest assay volume
- All microplate formats with all volumes show sufficient assay performance (Z-prime > 0.6, CV% < 5%.)

Table II. Calculated detection limits and CV% of luminometric flash ATP assay with different microplate formats.

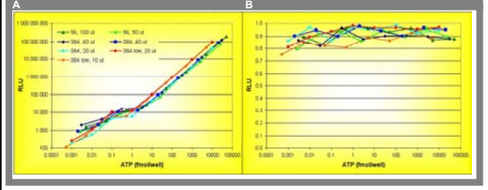
A

Sensitivity (amol ATP /well)			
96 well plate	**100 ul**	**50 ul**	
	3.0	6.0	
384 well plate	**60 ul**	**40 ul**	**20 ul**
	2.8	2.3	1.8
384 low volume plate	**20 ul**	**10 ul**	
	0.9	1.2	

B

CV% (1 fmol ATP /well)			
96 well plate	**100 ul**	**50 ul**	
	1.2	1.9	
384 well plate	**60 ul**	**40 ul**	**20 ul**
	1.2	0.6	1.0
384 low volume plate	**20 ul**	**10 ul**	
	0.6	3.6	

FIGURE 1. Calibration curves and calculated Z-prime values of luminometric flash ATP assay with different microplate formats. A) ATP calibration curve measured with Varioskan Flash. B) Calculated Z-prime values vs. amount of ATP in the well.



Glow ATP Assay:

Table III and Figure 2. show collected results of the tests measured with the glow type ATP assay. ATP calibration curves and Z-prime are shown in Figure 2. and calculated LOD values together with CV% are in Table III. Following conclusions can be made base on these results:

- Glow assay is less sensitive to changes in plate format and volume than flash assay
- Practically the same assay performance is obtained independent from the microplate format
- Typical glow assay vs. Flash assay sensitivity difference is seen in all plate formats. Good assay performance (Z-prime < 0.6) is achieved with about 1 fmol ATP when only few amol ATP is needed in flash ATP assay

Table III. Calculated detection limits and CV% of luminometric glow ATP assay with different microplate formats.

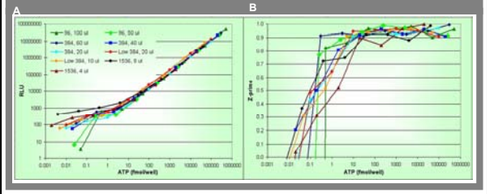
A

Sensitivity (fmol ATP /well)			
96 well plate	**100 ul**	**50 ul**	
	0.6	0.2	
384 well plate	**60 ul**	**40 ul**	**20 ul**
	0.2	0.3	0.4
384 low volume plate	**20 ul**	**10 ul**	
	0.4	0.2	
1536 well plate	**8 ul**	**4 ul**	
	0.2	0.4	

B

CV% (100 fmol ATP /well)			
96 well plate	**100 ul**	**50 ul**	
	0.7	1.7	
384 well plate	**60 ul**	**40 ul**	**20 ul**
	1.6	1.2	1.9
384 low volume plate	**20 ul**	**10 ul**	
	1.6	1.9	
1536 well plate	**8 ul**	**4 ul**	
	2.3	2.3	

FIGURE 2. Calibration curves and calculated Z-prime values of luminometric glow ATP assay with different microplate formats. A) ATP calibration curve measured with Varioskan Flash. B) Calculated Z-prime values vs. amount of ATP in the well.



Dual-Luciferase reporter gene assay:

All results from the luciferase reporter gene assays are collected into Table IV and Figures 3. and 4. These results can be concluded as follows:

- Both 384 and 384 low volume plate formats showed in DLR reporter gene assay somewhat better assay sensitivity than 96 well format without having any effect on other performance characteristics
- These luciferase assay show little bit lower performance with the 1536-well plates than other plate formats
- All these differences in the detection limit values are anyhow within rather small window (about 15 times difference between the best and lowest obtained sensitivity)

Table IV. Calculated detection limits (A) and CV% (B) of luminometric dual luciferase reporter gene assay with different microplate formats.

A

	Limit of Detection (amol luciferase /well)					
	Firefly luciferase			Renilla luciferase		
	220 ul	**110 ul**	**60 ul**	**220 ul**	**110 ul**	**60 ul**
96 well	0.013	0.007	0.027	0.063	0.031	0.021
	100 ul	**60 ul**	**40 ul**	**100 ul**	**60 ul**	**40 ul**
384 well	0.004	0.009	0.004	0.035	0.005	0.021
	20 ul	**10 ul**		**20 ul**	**10 ul**	
384 low volume	0.021	0.009		0.015	0.004	
	8 ul			**8 ul**		
1536 well	0.067			0.036		

B

	CV % (1 amol luciferase /well)					
	Firefly luciferase			Renilla luciferase		
	220 ul	**110 ul**	**60 ul**	**220 ul**	**110 ul**	**60 ul**
96 well	2.2	1.1	3.7	0.1	1.2	2.6
	100 ul	**60 ul**	**40 ul**	**100 ul**	**60 ul**	**40 ul**
384 well	3.2	1.1	2.5	2.5	3.0	1.7
	20 ul	**10 ul**		**20 ul**	**10 ul**	
384 low volume	2.7	3.2		3.3	3.5	
	8 ul			**8 ul**		
1536 well	5.1			2.8		

FIGURE 3. Calibration curves of firefly and Renilla luciferase assay with different microplate formats. A) Firefly luciferase assay. B) Renilla luciferase assay.

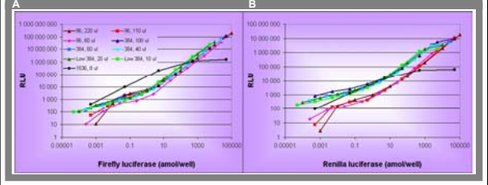
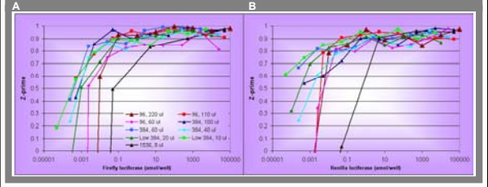


FIGURE 4. Z-prime values over luciferase concentration of firefly and Renilla luciferase assay with different microplate formats. A) Firefly luciferase assay. B) Renilla luciferase assay.



Luminometric cross talk assay:

Results of the luminometric cross talk assays are shown in Figure 5. and Tables V and VI.

- Luminometric cross talk is practically independent on the assay volume. The light is emitted similarly from the well with all volumes (emission forms always the similar spherical surface at the top of the well
- Remarkable cross talk effect can be seen only in the wells adjacent to the high signal wells and only with 348 or higher density plates

FIGURE 5. Crosstalk behavior of the luminescence signal with 384-well plate.

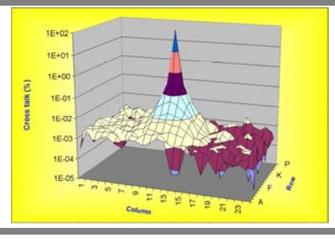


Table V. Crosstalk of the luminescence signal with 384-well plate. Crosstalk values are calculates as percentage from the high signal sample in well H12.

Table VI. Average crosstalk of the surrounding well loops with different distance from the high signal well.

	Crosstalk %						
	96-well plate		384-well plate			384-well low volume plate	
	100 ul	**50 ul**	**60 ul**	**40 ul**	**20 ul**	**20 ul**	**10 ul**
Cross talk Loop 1	0.033	0.030	0.272	0.248	0.209	0.321	0.312
Cross talk Loop 2	0.003	0.003	0.020	0.018	0.016	0.070	0.053
Cross talk Loop 3	0.002	0.001	0.006	0.005	0.005	0.010	0.008
Cross talk Loop 4			0.003	0.002	0.003	0.004	0.004
Cross talk Loop 5			0.002	0.001	0.002	0.002	0.002
Cross talk Loop 6			0.002	0.001	0.001	0.001	0.001
Cross talk Loop 7			0.002	0.001	0.001	0.001	0.001
Empty plate	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**	**0.001**

Conclusions

- **Assay performance in practically independent on the plate format.**
- **Very similar sensitivities, CV% and Z-prime values are achieved with 96, 384 and 384 low volume plates.**
- **Only 1536 well plate differs somewhat, little lower performance is some times observed.**
- **Decreasing assay volume does not reduce assay performance within usable volume range of the microplate.**
- **Glow ATP assays can be easily miniaturized up to 1536-well plate format using 4 ul total volumes without any remarkable effect on any followed performance characteristics.**
- **Considering all aspects (reagent costs, other material costs, performance etc.), the optimal assay setup can be considered to be 384-low volume plate with 10 - 20 ul total assay volume.**

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