In vivo zebrafish luciferase assays – a practical guide

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This is a detailed protocol for the *in vivo* zebrafish luciferase assay as previously described (Weger et al. 2013a) in the context of a chemical library screen. Different applications of the assay have been described in two publications (Weger et al. 2012; Weger et al. 2013b).

1. Materials

Buffer composition + reagents

D-Luciferin Firefly, potassium salt	Biosynth	L-8220
Methylene Blue	Sigma-Aldrich	M9140
E3	N/A	5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$

Plasticware + consumables

TopSeal-A	PerkinElmer	6005185	Adhesive transparent seals
OptiPlate-96	PerkinElmer	6005299	white opaque 96-well microplate

Animals

Tg(GRE:Luciferase) [GRE:Luc]	ZDB-TGCONSTRCT-120920-1	available at the European Zebrafish Resource Centre EZRC, Karlsruhe https://www.ezrc.kit.edu/catalog.php?text=GRIZLY
Tg(4xEBOX:Luciferase) [4xEbox:Luc]	ZDB-GENO-130516-2 ZDB-GENO-130516-3	available at the European Zebrafish Resource Centre EZRC, Karlsruhe https://www.ezrc.kit.edu/catalog.php?text=ka13

Potential Instruments

Instrument	Company	Special features	Remarks
EnVision XCite Multilabel Plate Reader	PerkinElmer	Equipped with enhanced luminescence detector	Tested - works
Topcount NXT counter	PerkinElmer		Tested - works
GLOMAX® 96 MICROPLATE LUMINOMETER	Promega		Tested – **low signal to noise ratio**
Infinite M200	Tecan		Sufficiently sensitive in a comparable assay (goo.gl/bvGjAV)

We would appreciate if you could share with us your experience when using other luminometers. If you feel your luminometer is not sensitive enough, check the "troubleshooting" section below.

2. Protocol

1. Breeding of Reporter Fish

Collect embryos from natural spawnings of group matings of GRE:Luc or 4xEbox:Luc transgenic fish. Raise embryos (not more than 60) in 9 cm Petri dishes containing E3 medium supplemented with 0.01 % (w/v) the fungicide methylene blue in an incubator at 28 °C. Change E3 medium regularly.

3. Preparation of Luciferase Medium (E3L)

Prepare an aqueous luciferin stock solution of 50 mM by adding distilled H_2O to the vial containing the luciferin powder (e.g. 1g D-Luciferin Firefly, potassium salt (Biosynth) in 62,8 ml dH_2O). This stock solution can be stored at -80 °C for several months. Dilute the luciferin stock into E3 medium to a final concentration of 0.5 mM to obtain E3L medium (e.g. add 500 µl 50 mM luciferin stock solution to 50 ml E3 Medium).

4. Distribute Larvae into 96 Well Plates

1. Prepare 1 ml pipette tips with wide bore by cutting off approximately 5 mm of the tip and briefly flaming the sharp edges.
2. Pool larvae from several crosses by carefully pouring them from the Petri dishes into a beaker. Harvest about 50 larvae at a time by gently pouring them onto a sieve (pore size of 0.25 mm diameter) and immediately place the sieve into a small Petri dish (diameter 5.5 cm) filled with E3L medium.
3. With a wide bore pipette tip, transfer e.g. 225 µl of medium containing one larva each to the wells of a white 96 well plate (OptiPlate, PerkinElmer).
4. Seal the plates with adhesive sealing sheets (*e.g.* TopSeal-A, PerkinElmer) and incubate them at 28 °C overnight. This preincubation prevents recording of transient changes in bioluminescence immediately after addition of luciferin, a phenomenon that occurs also in cultured cells (Hirota et al. 2008).

5. Compound Treatment (if applicable)

1. Remove adhesive sealing sheets from the larvae plates.
2. Pipette your treatment solution to the wells containing the larvae (e.g to stimulate GRE:Luc larvae with a final concentration of 10 µM, pipet 25 µl of 100 µM Dexamethasone [in E3/3% DMSO] into each well containing 225 µl E3L).
3. Seal the plates with the adhesive sealing sheets.

6. Luminescence Recording

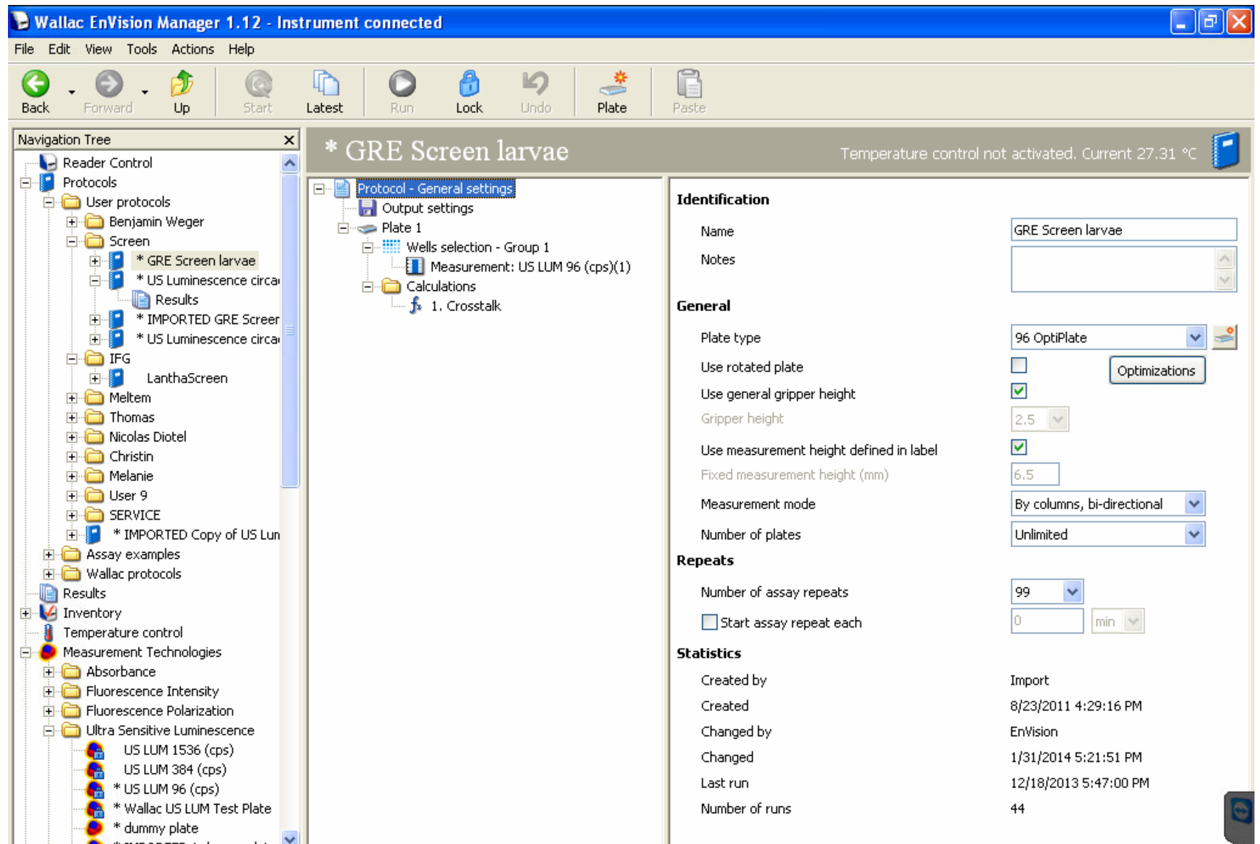
1. Put the plates containing the larvae into bioluminescence reader.

The following step by step instruction is for the EnVision bioluminescence reader with enhanced luminescence sensitivity (PerkinElmer).

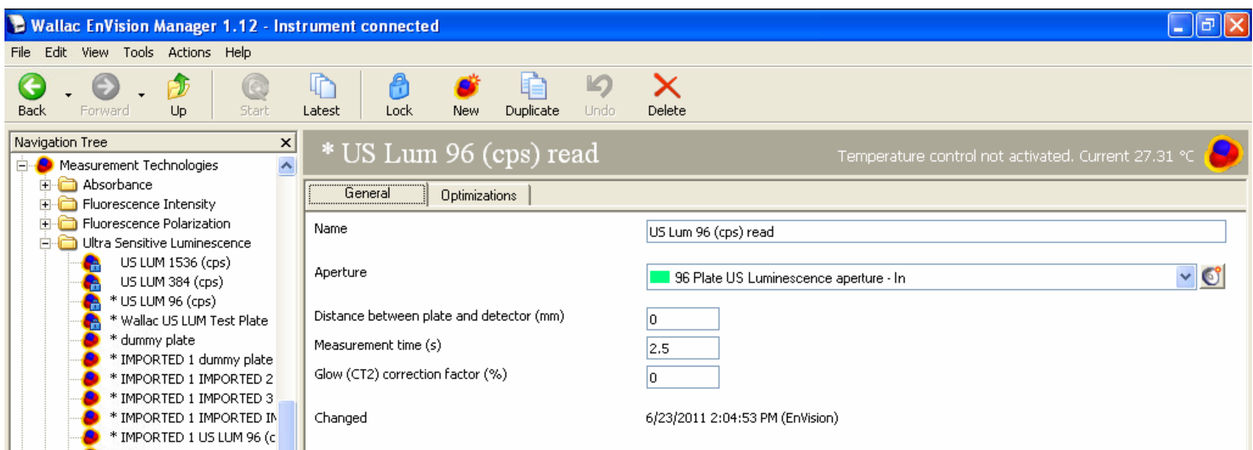
2. Record bioluminescence e.g. for two days using reader settings analogous to those described in table 1 and shown in the screenshots of the Wallac EnVision Manager. The measurement is significantly improved by measuring luminescence in each well for 2.5 s.

Table 1:

General settings:	
Number of assay repeats	*dependent on the length of the run*
Number of plates	unlimited
Temperature control	28 °C (if the room is not temperature adjusted)
Calculations	Crosstalk correction
Measurement Technology	US Lum 96 (cps) read
Measurement time	2.5 sec
Distance between plate and detector	0 mm
Glow (CT2) correction factor	0 %

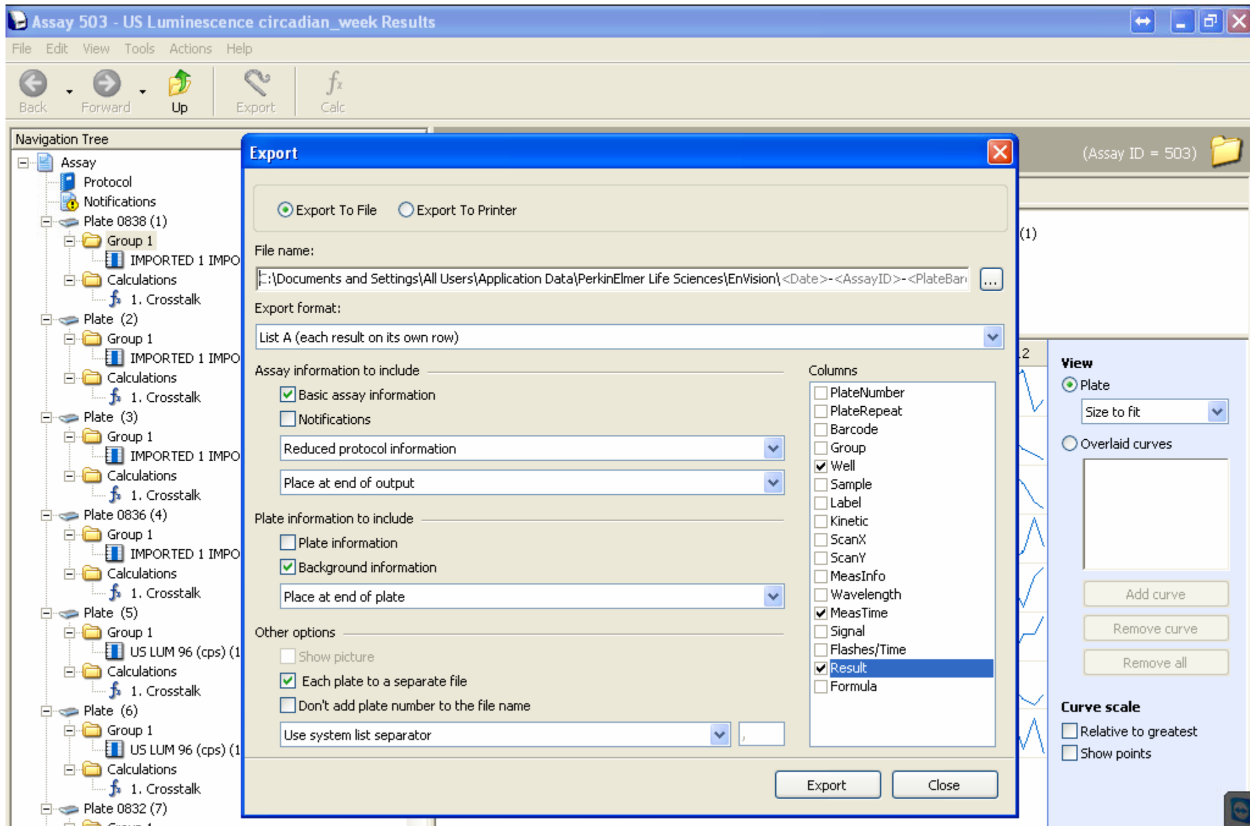


Screenshot 1: General settings menu



Screenshot 2 Measurement technologies menu

3. Export your data e.g. according to the parameters show in screenshot 3 and perform a basic analysis e.g. in Excel.



Screenshot 3 Suitable parameters for the export of the data

	A	B	C
1	Well	MeasTime	Result
2	A01	00:00.0	682
3	A01	42:15.7	683
4	A01	24:31.3	621
5	A01	06:47.0	525
6	A01	49:01.4	477
7	A01	31:17.0	464
8	A01	13:32.6	427
9	A01	55:48.2	422
10	A01	38:03.9	403
11	A01	20:20.1	413
12	A01	02:36.4	406
13	A01	44:52.2	403
14	A01	27:07.9	349
15	A01	09:23.5	362
16	A01	51:39.0	365
17	A01	33:55.2	398
18	A01	16:10.6	392

Screenshot 4 Example of an exported file

3. Hints, Tips and Troubleshooting

Embryos do not look good/are dead after the measurements: Embryos survive best when they are kept as shortly as possible in 96-well plates. For long-term measurements, keep embryos as long as possible in Petri dishes with fresh fish water and methylene blue in order to provide optimal development conditions before they are transferred into well plates.

Signal too low or not detectable: We have some preliminary experience in pooling 20 larvae and measuring the reporter activity in an *in vitro* luciferase assay to increase the signal-to-noise ratio. This method may be suitable for endpoint measurements in cases where live measurements are not sensitive enough.

Homogenize 20 embryos/larvae in Reporter Lysis Buffer (# E3971, Promega) using micropistilles (#0030120.973, Eppendorf) and by passing the solution four times through a syringe (0.45×25 mm, Braun sterican).

Record reporter activity following the protocol of the Luciferase Assay System (#E1500, Promega).

4. References

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