Hybrid Reporter Gene Assay for the evaluation of nuclear receptors as targets

Version 1.0

Version Date: April 2021

1. Rationale/Aim

The hybrid reporter gene assay allows to investigate the effect of compounds on nuclear receptor activity. It is a cellular assay system, which, unlike cell-free assay systems, ensures that the compound of interest reaches the cellular site of action. To minimize non-specific effects, chimeric receptors are used which consist of the DNA binding domain of the yeast receptor Gal4, a hinge region and the ligand binding domain of the human nuclear receptor in question. Additionally, a Gal4-responsive reporter (firefly luciferase) and a constitutively expressed (SV40 promoter) control gene (renilla luciferase) to monitor transfection efficiency and test compound toxicity are used. Human cells, e.g., human embryonic kidney cells (HEK293T) are transiently transfected with plasmids coding for these components.
The hybrid reporter gene assay allows a characterization of the modulation of a precisely defined nuclear receptor exhibited by different compounds in a cellular system [1].

2. Experimental conditions

2.1 Key Requirement

Preparation of hybrid receptor construct:

For each nuclear receptor in question, a hybrid receptor expression construct (pFA-CMV-NR-LBD) based on pFA-CMV (Agilent Technologies, Cat. No.: 219036) is needed. It codes for the Gal4 DNA binding domain fused to the ligand binding domain (LBD) of the nuclear receptor in question which must be inserted into the multiple cloning site (MCS). The reporter plasmid (pFR-Luc) and control gene plasmid (pRL-SV40) serve for all hybrid reporter gene assays.

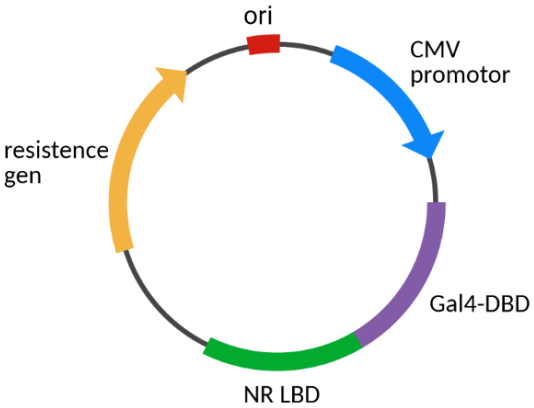


Fig. 1: Schematic representation of pFA-CMV-NR-LBD.

Required premises:

To perform the Hybrid Reporter Gene Assay, a genetic engineering laboratory is required according to §14 of the Genetic Engineering Safety Ordinance (Gen-TSV). This includes working under a class II safety workbench.

2.1.1 Instruments and Materials

Material	Description	Suppliers	Cat. No.
Counting Chamber BLAUBRAND®	Neubauer improved; 0.100 mm depth; 0.0025mm^2	Brand	717805
CB 260 CO_2 Incubator	Incubator	Binder	9040-0150
Tecan Spark® Microplate Reader	Plate Reader	Tecan	
Thermo Scientific™ Heraeus™ Labofuge™ 400 R	Centrifuge	Fischer Scientific	10638422
Julabo Pura 10	Waterbath	Julabo	9550510
RF3000 Pipette Controller	Pipette Controller	Heathrow Scientific	HEA300
HLC Safety Vacuum Suction Systems	Vacuum Suction System		
Thermo Scientific™ Herasafe™	Safety workbench	Fischer Scientific	
Microsoft Excel	Software for data analysis		
Brand Singlechannel Transferpette® S	0.5 – 10 µL	Brand	705870
Brand Singlechannel Transferpette® S	10 – 100 µL	Brand	705874
Brand Singlechannel Transferpette® S	2 – 20 µL	Brand	705872
Brand Singlechannel Transferpette® S	20 – 200 µL	Brand	705878
Brand Singlechannel Transferpette® S	100 – 1000 µL	Brand	705880
Brand Multichannel Transferpette® S	5 – 50 µL	Brand	705906
Eppendorf Multipette® M4	1 µL – 10 mL	Eppendorf	4982000012

2.1.2 Reagents & experimental parameters

Protocol step	Reagent	Volume per Well [µL]	Stock concentration	Final concentration
Preparation of the HEK293T cells	HEK293T	100	~ $2.5x10^5$ – $3x10^5$ cells/ml	~ $2.5x10^5$ – $3x10^5$ cells/ml = $2.5x10^4$ – $3x10^4$ cells/well
Transfection	pFA-CMV-NR-LB	variable	variable	1 – 50 ng/well
	pFR-Luc	variable	variable	25 – 100 ng/well
	pRL-SV40	variable	variable	2 – 6 ng/well
	Opti-MEM	15.1		
	Plus reagent	0.12		
	LTX reagent	0.20		
Incubation	Test Compound	50	100 mM	0.001 – 100 µM
Lysis & Measurement	Dual Glo firefly substrate	0.026		
	Dual-Glo®Stop & Glo®Substrate mix	0.026		
	Tecan Spark® Microplate Reader	1000 ms integration time, no specific wavelength		

2.1.3 Additional information to used assay media

Medium name	Medium composition	Quantities
Cell culture medium	DMEM	500 mL
	FBS	0.1 mL/mL
	Sodium pyruvate	1 mM
	Penicillin	100 U/mL
	Streptomycin	100 µg/mL
Transfection medium	Opti-MEM	500 mL
Incubation medium	Opti-MEM	500 mL
	Penicillin	100 U/mL
	Streptomycin	100 µg/mL
Dilution medium	Opti-MEM	variable
	Penicillin	100 U/mL
	Streptomycin	100 µg/mL
	DMSO	1 µL/mL

2.2 Key Resources Table

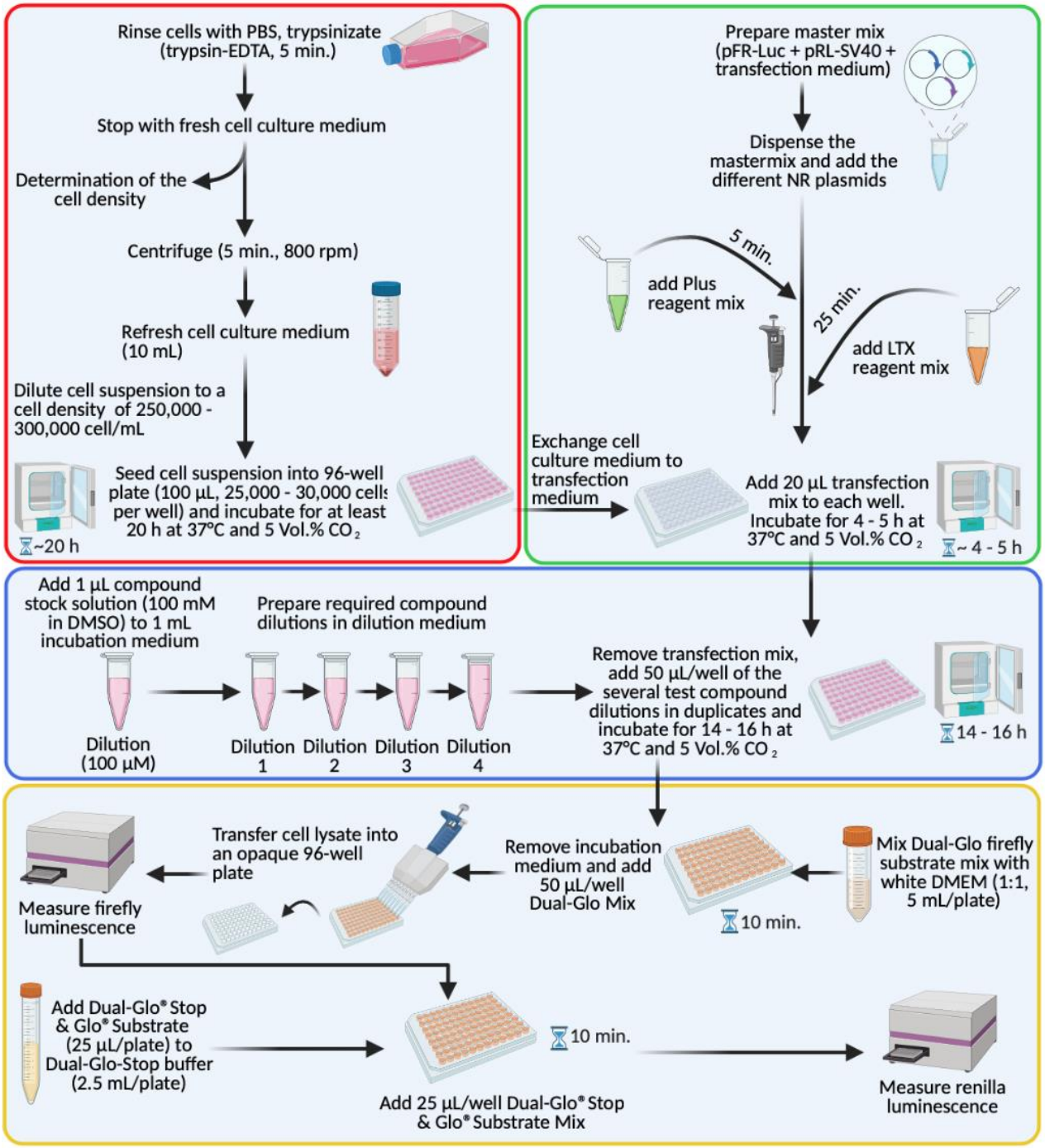
Reagents	Suppliers	Cat. No.
Cell Culture Flasks, 650 mL, 175 cm^2, PS, red standard screw cap, TC, clear, sterile	Greiner	661160
HEK293T cells	ATCC	CRL-3216
pFA-CMV-NR-LBD*	Agilent Technologies	219036
pFR-Luc	Agilent Technologies	219050
pRL-SV40	Promega	E2231
DMEM (1X)	Gibco, Life Technologies	41965-039
Pen Strep	Gibco, Life Technologies	15140-122
Sodium Pyruvate (100mM)	Gibco, Life Technologies	11360-070
Fetal Bovine Serum Premium Plus FBS	Gibco, Life Technologies	A4766801
0.5% Trypsin-EDTA (10X)	Gibco, Life Technologies	15400-054
DPBS (1X)	Gibco, Life Technologies	14190-094
Trypan blue solution 0.4%	In-house	
Opti-MEM® I (1X) + GlutaMAX™-I	Gibco, Life Technologies	51985-026
DMEM (1X)	Gibco, Life Technologies	31053-028
CELLSTAR® 96 Well Cell Culture Plate; clear	Greiner	655 180
CELLSTAR® 96 Well Cell Culture Plate; opaque	Greiner	655 083
Lipofectamine® LTX and Plus™ Reagent	Invitrogen, Life Technologies	15388100
Dual-Glo® Luciferase Assay System	Promega	E2980
Dimethyl sulfoxide**	Carl Roth	4720.1

* Expression plasmid with CMV promoter, Gal4-DBD fused to the ligand binding domain of the human nuclear receptor of interest (e.g. RXR, RAR, FXR etc.)

** ROTIPURAN® ≥ 99.8%, p.a.

3. Protocol

3.1 Workflow



3.2 Protocol

3.2.1 Detailed protocol

Preparation of the HEK293T cells:

HEK293T cells are grown in a 175 cm^2 flask in cell culture medium to 70-80% confluence. For seeding in 96-well plates, medium is removed and the cells are rinsed with 5 mL of DPBS (1X) (Cat.-No.:14190-094). 5 mL of 0.5% Trypsin-EDTA (Cat.-No.: 15400-054) is then added to the cells. After incubation for 3 – 5 minutes at 37°C and 5 Vol.% CO_2, the trypsinization is stopped by the addition of 5 mL cell culture medium and the resulting cell suspension is transferred to a 50 mL Falcon tube. 10 µL of the cell suspension are incubated with 40 µL of trypan blue solution for 1 minute and 10 µL of this mixture are applied to a counting chamber (Neubauer improved; 0.100 mm depth; 0.0025mm^2) for cell counting. The remaining cell suspension is centrifuged at 800 rpm for 5 minutes. After centrifugation, the supernatant medium is removed, and the cell pellet is immediately resuspended in 10 mL of fresh cell culture medium. Based on the cell number determined by counting, the cell suspension is diluted to a density of approx. 250,000 – 300,000 cells/mL. From this dilution, 100 µL per well are pipetted into a clear 96-well plate (Cat.-No.: 655 180). The cells are then kept in the incubator for at least 20 hours at 37°C and 5 Vol.% CO_2.

Transfection of Plasmid DNA:

For each nuclear receptor to be studied, an appropriate plasmid mixture is prepared according to the Excel template (Annex 1). This mixture contains 15.1 µL/well Transfection medium and the appropriate amounts of each plasmid (pFA-CMV-NR-LBD, pFR-Luc, pRL-SV40). It may be useful to prepare a master mix containing pFR-Luc and pRL-SV40 first and then splitting this master mix for the addition of the receptor plasmids.

For transient transfection with Lipofectamine LTX reagent, two reagent mixtures are prepared according to the Excel template (Annex 2):

- Plus™ reagent mix: 0.12 µL/well Plus™ reagent + 1.88 µL/well Transfection medium
- LTX™ reagent mix: 0.20 µL/well LTX reagent + 2.70 µL/well Transfection medium

Before transient transfection, the medium in the 96-well plate is exchanged to transfection medium (100 µL/well). Two rows should be aspirated and replaced at a time.

The transfection procedure proceeds as follows. Add 2.0 µL/well Plus™ reagent mix to the respective plasmid mixture. After 5 minutes incubation, add 2.9 µL/well LTX reagent mix to the plasmid + Plus mixture. After 25 minutes incubation, dispense the plasmid-lipofectamine mixture into the appropriate wells of the 96-well plate (20 µL/well). This procedure is performed for each plasmid mix. Incubate the cells for 4.5 – 5 hours at 37°C and 5 Vol.% CO_2 before addition of the test compounds.

Preparation of compound dilution series:

100 mM stock solutions of the test compounds are prepared in DMSO (Cat.-No.: 4720.1). From this DMSO stock, a 100 µM dilution is prepared by adding 1 µL of the 100 mM DMSO stock to 1 mL incubation medium. Further desired dilutions are prepared from the 100 µM dilution. Dilution medium is used for further dilutions.

Incubation with the test compounds:

After 4.5 – 5 hours incubation with the transfection mix, the transfection medium is aspirated from each well and replaced with 50 µL of the respective test compound solution. Each dilution is tested in duplicates. Dilution medium as negative control and a reference agonist as positive control are tested on each plate. The cells are then incubated with the test compound solutions for 14 – 16 hours at 37°C and 5 Vol.% CO_2.

Luminescence measurement:

For luminescence measurement, the Dual-Glo firefly substrate mix (2.5 mL per plate) is thawed and diluted with DMEM (Cat.-No.: 31053-028, 2.5 mL per plate). Additionally, Dual-Glo®Stop & Glo®Substrate (25 µL per plate) is added to Dual-Glo Stop buffer (2.5 mL per plate).

After 14 – 16 hours incubation with test compound solutions, the test compound solutions are aspirated from the cells and replaced with the Dual-Glo firefly substrate mix (50 µL/well, two rows at a time). After 10 minutes incubation, 50 µL/well of each cell lysate is transferred to an opaque 96-well plate (Cat.-No.: 655 083) using a multichannel pipette. The luminescence measurement should now be performed immediately (within 60 minutes max.) on the Tecan Spark® Microplate Reader (1000 ms integration time, no specific wavelength). After measuring firefly luminescence, 25 µL of Dual-Glo®Stop & Glo®Substrate mix is added to each well. After 10 minutes incubation, Renilla luminescence is measured on the Tecan Spark® Microplate Reader (1000 ms integration time, no specific wavelength).

Data analysis:

To normalize for transfection efficiency and cell growth, firefly luciferase data are divided by renilla luciferase data and multiplied by 1000 to obtain relative light units (RLU). Fold nuclear receptor activation is then obtained by dividing the mean RLU of a test compound at a respective concentration by the mean RLU of the negative control (DMSO). Relative activation can be obtained by dividing the fold nuclear receptor activation caused by a test compound at a respective concentration by the fold nuclear receptor activation of the respective reference agonist. EC_{50} or IC_{50} values can be calculated from dose-response curves based on RLU, fold activation or relative activation by a four-parameter logistic regression with variable slope.

3.2.2 Step-by-step Protocol

The Assay is performed as descripted in the literature. [1]

Preparation of the HEK293T cells:

1. Grow HEK293T cells in a 175 cm^2 flask in Cell culture medium to 70-80% confluence.
2. Aspirate cell culture medium, rinse with 5 mL PBS and aspirate it again
3. Incubate with 5 mL of 0,5% Trypsin-EDTA for 3 – 5 min at 37°C and 5 Vol.% CO_2
4. Add 5 mL of fresh cell culture medium to stop and mix by pipetting up and down
5. Incubate 10 µL of the cell suspension with 40 µL trypan blue solution for 1 min, add 10 µL of the suspension to counting chamber and determine cell density
6. Centrifuge remaining cell suspension (5 min, 800 rpm), remove supernatant medium
7. Resuspend cells in 10 mL of fresh cell culture medium
8. Dilute cell suspension to a cell density of approx. 250,000 – 300,000 cells/mL according to formula

$$V = \frac{MW \cdot 5}{300 \cdot 0.3}$$

V = total volume (mL)
MW = Mean value of the cell count from the 3 squares
5 = Dilution factor in trypan blue
300 = Chamber factor
0.3 = 300.000 cells/mL

E.g.: V = 8 mL → Dilute 1 mL cell suspension with 7 mL fresh cell medium

9. Seed cell suspension to 96-well plate (100 µL/well) and place in incubator for at least 20 h at 37°C and 5 Vol.% CO_2.

Transfection of Plasmid DNA:

1. Prepare plasmid master mix with appropriate amounts of pFR-Luc and pRL-SV40 in transfection medium (15.1 µL/well) according to template (Annex 1)
2. Split the master mix and add the respective pFA-CMV-NR-LBD according to the template (Annex 1).
3. Prepare Plus™ reagent mix and LTX reagent mix according to template (Annex 2)
4. Replace cell culture medium on 96-well plate with 100 µL/well transfection medium
5. For each plasmid mix, prepare transfection mixes by addition of Plus™ reaction mix (2.0 µL/well) - 5 min incubation - addition of LTX reaction mix (2.9 µL/well) - 25 min incubation
6. After the incubation time (total 30 minutes), add 20 µL/well of transfection mixes to each well of the 96-well plate
7. Incubate cells for 4.5 – 5 h at 37°C und 5 Vol.% CO_2

Preparation of compound dilution series:

1. Prepare 100 mM stocks of the test compounds in DMSO
2. Dilute the 100 mM 1:1000 to 100 µM dilution in incubation medium, from which further dilutions are prepared with dilution medium in a dilution series according to the template (Annex 3). All test compound solutions should contain 0.1% DMSO.

Incubation with the test compounds:

1. After 4.5 – 5 h incubation with the transfection mix, remove the transfection mix from the 96-well plate and add the appropriate test compound mixes (50 µL/well). Include negative control (incubation medium) and positive control (reference agonist) on each plate. Each dilution is tested at least in duplicate
2. Incubate at 37°C und 5 Vol.% CO_2 for 14 – 16 h.

Luminescence measurement:

1. Thaw Dual-Glo firefly substrate (2.5 mL/plate) and mix with 2.5 mL DMEM (light medium)
2. Add Dual-Glo®Stop&Glo®Substrate (25 µL/plate) to Dual-Glo Stop buffer (2.5 mL/plate)
3. Remove test compound mixes from 96-well plate, add 50 µL/well Dual-Glo firefly substrate mix and incubate for 10 min
4. After 10 min of incubation, transfer the cell lysate (50 µL/well) into an opaque 96-well plate using a multichannel pipette
5. Determine firefly luminescence within 60 min after lysis on a Tecan Spark® Microplate Reader (1000 ms integration time, no specific wavelength)
6. Add 25 µL Dual-Glo®Stop & Glo®Substrate mix to each well
7. After 10 min incubation, determine renilla luminescence on a Tecan Spark® Microplate Reader (1000 ms integration time, no specific wavelength)
8. Use the excel template (Annex 4) to calculate relative light units (RLU), fold-activation and relative activation. Calculate EC_{50}/IC_{50} from dose-response curves (RLU, fold-activation or relative activation) by a four-parameter logistic regression with variable slope using SigmaPlot or GraphPad Prism

References

[1] J. Heering and D. Merk, “Hybrid Reporter Gene Assays: Versatile In Vitro Tools to Characterize Nuclear Receptor Modulators,” in *Nuclear Receptors: Methods and Experimental Protocols*, M. Z. Badr, Ed. New York, NY: Springer New York, 2019, pp. 175–192.

	c(stock)/µg/µl	ng/well	wells	µl 1:10 stock	**µl Stock**				**Mastermix µL**
Reporter	0,820	100	102	12,44	**12,44**	Reporter			
pRL-SV40	0,327	2	102	0,62	**0,62**	pRL-SV40			
Receptor A	0,058	1	35	0,60	**0,60**			Receptor A	538,95
Receptor B	0,012	6	30	14,63	**14,63**			Receptor B	461,96
Receptor C	0,789	25	20	0,63	**0,63**			Receptor C	307,97
Receptor D	0,555	50	17	1,53	**1,53**			Receptor D	261,78
Opti-MEM		15,1 µl/well	102		**1.540,20**	Opti-MEM			

Annex 1: Excel template for transfection. Calculation of the required volume of Transfection medium and the required volumes of Firefly plasmid (reporter) and Renilla plasmid (pRL-SV40) to create the mastermix (green). Distribution of the master mix among the different plasmid mixes (blue). Required volumes of receptor plasmids to be added to each receptor mix (orange).

			µl/well	wells		**µl**				**PLUS µL**
PLUS reagent mixture	PLUS		0,12	102		**12,24**	PLUS		Receptor A	70
	Opti-MEM		1,88	102		**191,76**	Opti-MEM	2 µl/well	Receptor B	60
								5 Min RT	Receptor C	40
									Receptor D	34
			µl/well	wells		**µl**				**LTX µL**
LTX reagent mixture	LTX		0,2	102		**20,40**	LTX	2,9 µl/well	Receptor A	101,5
	Opti-MEM		2,7	102		**275,40**	Opti-MEM	**25 Min RT**	Receptor B	87
									Receptor C	58
									Receptor D	49,3

Annex 2: Excel template for transfection. Calculation of the required volume of Transfection medium (green) and the required volumes of Plus™-Reagent and LTX-Reagent (orange). Indication of the volumes that must be added to the plasmid mix within the specified time schedule. Incubate Plus™-Reagent (red) for 5 minutes. Incubate LTX Reagent (purple) for 25 minutes.

Negative Control				
c in µM	Stock [µL]	Incubation medium [mL]	from previous	Dilution medium [µL]
0	---	---	---	1000
Reference Agonist [Stock = 10 mM]				
c in µM	Stock [µL]	Incubation medium [mL]	from previous	Dilution medium [µL]
10	1	1	---	---
1			100	900
Test Compound Nr. 1 [Stock = 100 mM]				
c in µM	Stock [µL]	Incubation medium [mL]	from previous	Dilution medium [µL]
100	1	1	---	---
30			300	700
10			300	600
Test Compound Nr. 2 [Stock = 100 mM]				
c in µM	Stock [µL]	Incubation medium [mL]	from previous	Dilution medium [µL]
100	1	1	---	---
50			500	500
30		from 100 µM	300	700
10			300	600
3			300	700
1			300	600
0.3			300	700

Annex 3: Template dilution series test compounds.

Raw data Firefly

	1	2	3	4	5	6	7	8	9	10	11	12
A	12345	156641	5464165	252627	548623	46341	12345	156641	5464165	252627	548623	156641
B	56789	5646	151465	222324	213849	6541320	56789	5646	151465	222324	213849	5646
C	101112	465421	23100	192021	321894	651641	101112	465421	23100	192021	321894	465421
D	131415	65189	51612	161718	3106849	25416	131415	65189	51612	161718	3106849	65189
E	161718	213216	320050	131415	34984	65146	161718	213216	320050	131415	34984	213216
F	192021	21384	3021684	101112	315458	1326325	192021	21384	3021684	101112	315458	21384
G	222324	320684	3546	56789	458469	56541	222324	320684	3546	56789	458469	320684
H	252627	1651220	16516	12345	69841	65866	252627	1651220	16516	12345	69841	1651220

Raw data Renilla

	1	2	3	4	5	6	7	8	9	10	11	12
A	156641	548623	252627	5464165	156641	12345	46341	548623	252627	5464165	156641	12345
B	5646	213849	222324	151465	5646	56789	6541320	213849	222324	151465	5646	56789
C	465421	321894	192021	23100	465421	101112	651641	321894	192021	23100	465421	101112
D	65189	3106849	161718	51612	65189	131415	25416	3106849	161718	51612	65189	131415
E	213216	34984	131415	320050	213216	161718	65146	34984	131415	320050	213216	161718
F	21384	315458	101112	3021684	21384	192021	1326325	315458	101112	3021684	21384	192021
G	320684	458469	56789	3546	320684	222324	56541	458469	56789	3546	320684	222324
H	1651220	69841	12345	16516	1651220	252627	65866	69841	12345	16516	1651220	252627

RLU (Firefly/Renilla*1000)

	1	2	3	4	5	6	7	8	9	10	11	12
A	79	286	21629	46	3502	3754	266	286	21629	46	3502	12689
B	10058	26	681	1468	37876	115186	9	26	681	1468	37876	99
C	217	1446	120	8313	692	6445	155	1446	120	8313	692	4603
D	2016	21	319	3133	47659	193	5171	21	319	3133	47659	496
E	758	6095	2435	411	164	403	2482	6095	2435	411	164	1318
F	8980	68	29885	33	14752	6907	145	68	29885	33	14752	111
G	693	699	62	16015	1430	254	3932	699	62	16015	1430	1442
H	153	23643	1338	747	42	261	3835	23643	1338	747	42	6536

		Firefly		Renilla					
Testcompound	Conc. [µM]	Mittelwert	Std.	Mittelwert	Std.	**Ø-RLU**	Std.-RLU	**fold-activ.**	**rel.Aktivi.**
#1	10	84493,0	102032,7	352632,0	277173,1	182,2	146,2	**0,0**	**0,07**
	30	2858396,0	3685113,9	2858396,0	3685113,9	10837,8	15261,6	**0,5**	**4,18**
#2	10	297482,0	355167,0	84493,0	102032,7	3628,1	177,8	**0,2**	**1,40**
	30	84493,0	102032,7	297482,0	355167,0	276,0	13,5	**0,0**	**0,11**
#3	0.3	2858396,0	3685113,9	2858396,0	3685113,9	10837,8	15261,6	**0,5**	**4,18**
	1	352632,0	277173,1	84493,0	102032,7	8095,5	6495,6	**0,3**	**3,12**
	3	31217,5	36163,6	109747,5	147221,8	5042,3	7093,6	**0,2**	**1,94**
	10	186894,5	50104,9	186894,5	50104,9	1074,6	556,2	**0,0**	**0,41**
	30	3377584,5	4474197,7	31217,5	36163,6	76531,3	54666,6	**3,2**	**29,48**
	50	31217,5	36163,6	3377584,5	4474197,7	17,5	12,5	**0,0**	**0,01**
#4	10	186894,5	50104,9	186894,5	50104,9	1074,6	556,2	**0,0**	**0,41**
	30	109747,5	147221,8	31217,5	36163,6	18987,8	26712,2	**0,8**	**7,31**
#5	10	283266,5	257605,4	393657,5	101488,9	831,6	868,8	**0,0**	**0,32**
	30	107560,5	119445,2	107560,5	119445,2	4216,4	5792,8	**0,2**	**1,62**
#6	10	486767,5	233166,3	283266,5	257605,4	3568,2	4068,1	**0,1**	**1,37**
	30	283266,5	257605,4	486767,5	233166,3	800,5	912,7	**0,0**	**0,31**
#7	10	107560,5	119445,2	107560,5	119445,2	4216,4	5792,8	**0,2**	**1,62**
	30	393657,5	101488,9	283266,5	257605,4	2647,3	2765,8	**0,1**	**1,02**
#8	10	98302,0	46828,9	1586019,0	2150778,4	1018,4	1410,6	**0,0**	**0,39**
	30	106665,0	77856,7	106665,0	77856,7	1726,2	1989,9	**0,1**	**0,67**
Control	DMSO	1566132,5	2178902,2	98302,0	46828,9	23926,3	33563,3	**1,0**	**9,22**
	Reference	98302,0	46828,9	1566132,5	2178902,2	2595,8	3641,3	**0,1**	**1,00**
#9	0.3	106665,0	77856,7	106665,0	77856,7	1726,2	1989,9	**0,1**	**0,67**
	1	1586019,0	2150778,4	98302,0	46828,9	24077,6	33349,3	**1,0**	**9,28**
	3	187467,0	36414,6	124100,0	126029,1	3426,6	3773,3	**0,1**	**1,32**
	10	225732,5	133385,1	225732,5	133385,1	1423,0	1431,8	**0,1**	**0,55**
	30	50065,0	21327,8	187467,0	36414,6	283,5	168,8	**0,0**	**0,11**
	50	187467,0	36414,6	50065,0	21327,8	4288,5	2554,3	**0,2**	**1,65**
#10	10	225732,5	133385,1	225732,5	133385,1	1423,0	1431,8	**0,1**	**0,55**
	30	124100,0	126029,1	187467,0	36414,6	741,3	816,3	**0,0**	**0,29**
#11	10	106702,5	120658,6	168421,0	207941,7	4523,7	6301,6	**0,2**	**1,74**
	30	1561398,0	2065156,3	1561398,0	2065156,3	14959,0	21107,9	**0,6**	**5,76**
#12	0.3	820891,5	714790,9	106702,5	120658,6	10829,6	5547,2	**0,5**	**4,17**
	1	106702,5	120658,6	820891,5	714790,9	106,3	54,4	**0,0**	**0,04**
	3	1561398,0	2065156,3	1561398,0	2065156,3	14959,0	21107,9	**0,6**	**5,76**
	10	168421,0	207941,7	106702,5	120658,6	7431,7	10352,5	**0,3**	**2,86**
	30	271504,0	69551,0	389576,5	97428,7	696,4	4,4	**0,0**	**0,27**
	50	30167,5	37648,5	30167,5	37648,5	8038,7	11280,1	**0,3**	**3,10**
#13	10	257505,0	284206,0	271504,0	69551,0	842,0	831,1	**0,0**	**0,32**
	20	271504,0	69551,0	257505,0	284206,0	2315,8	2285,8	**0,1**	**0,89**
	30	30167,5	37648,5	30167,5	37648,5	8038,7	11280,1	**0,3**	**3,10**
#14	10	389576,5	97428,7	271504,0	69551,0	1436,0	9,0	**1,4**	**9,48**
	30	951923,5	988954,6	860530,5	1118203,8	11897,8	16609,6	**11,4**	**78,53**
#15	10	14430,5	2949,3	14430,5	2949,3	1042,7	417,5	**1,0**	**6,88**
	30	67853,5	2810,7	951923,5	988954,6	151,5	154,5	**0,1**	**1,00**
#16	10	951923,5	988954,6	67853,5	2810,7	13739,0	14005,7	**1,0**	**13,18**
	30	14430,5	2949,3	14430,5	2949,3	1042,7	417,5	**0,1**	**1,00**
	50	860530,5	1118203,8	951923,5	988954,6	3289,2	4591,9	**0,2**	**3,15**

Annex 4: Excel template for data analysis. Raw data is inserted into tables (above). Calculations of RLU, fold-activation, relative activation etc. with corresponding standard deviation are done automatically. (note: random values were used as example)