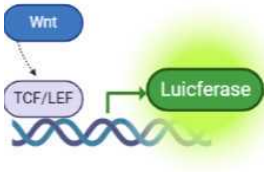
Oct 11, 2023 Version 2

Wnt-3a and R-spo1 conditioned media reporter assay V.2



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Natalia Martagón[1], Gurdrun Kliem[2]
[1]Charité Universitätsmedizin Berlin; [2]Robert Koch Institute

Natalia Martagón

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Protocol status: Working
We use this protocol and it's working

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Abstract

Protocol designed to measure the activity of Wnt-3a or R-spondin-1 (Rspo1) conditioned media.
A reporter HEK cell line expressing luciferase under Wnt-3a stimulation is cultured with conditioned media followed by cell lysis and a luciferase reporter assay. Activity is compared to previous media batches or references.

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Materials

Assay-reagent solution:

[M] 20 millimolar (mM) Tricine (MW 179,2)

[M] 1.07 millimolar (mM) Mg Carbonate x 5 H20 (MW 485,7)

[M] 2.67 millimolar (mM) Mg Sulfate x 7 H20 (MW 246,5)

[M] 0.1 millimolar (mM) EDTA (MW 372,2)

Preparation (warm up for preparation):

Dissolve:

3.584 g Tricine

0.519 g Mg-CO3

in ca. 850 mL water

Add 54 µL of the [M] 0.4 Molarity (M) MgSO4 pH 8 solution

adjust pH to pH 07.8

bring volume to 1 L Store at RT in glass bottles

Lysis solution

luciferase solution stocks preparation

Co Enzym A: .
Sigma C3019-100 mg
Dissolve 100 mg in 1,27 ml in water. Make aliquotes of100 µL

Luciferin
Dissolve 25 mg D-Luciferin-Na salt in 4,135 ml water. [Stock]=20 mM. Make aliquotes of 500 µL
0.5 M CDTA pH 8:

Dissolve 8,7 g in 50 ml. Buffer the solution first with NaOH-pearls and fine adjustment with liquid NaOH ca. 10 M
0.5 M MgS04-Stock, pH 8:
Dissolve 6,15 g in 50 ml in water

1M EDTA-Stock:
Dissolve 29,2 g in 100 ml water

0.5 M Tris-Stocksolution, pH 7,8:
Dissolve 15,14 g Tris-Base(MW 121,14) in 250 ml water. Adjust pH with phosphoric acid

1M DTT-Stock:
Dissolve 1,54 g (MW 154,3) in 10 ml water

Lysis solution stocks

125 mM Tris-Phosphate
10 mM DTT
10 mM CDTA
S0% Glycerin
5 % Triton X 100

5x Lysis-Solution preparation
12,5 ml Tris-Phosphat 0,5 M, pH 7,8
500 µL DTT 1 M
1 ml CDTA 0.5 M, pH 8
25 g Glycerin
2,5 g Triton X 100
Bring to a total vol. of 50 ml with water. Make10 ml aliquotes and store at- 20°C

HEK-medium
DMEM F12 HEPES (Gibco Cat. No. 11330),
20% FCS
200 µg/ml G418
material for cell counting

Microplatte for luminescence readers

Greiner Bio-one Cat. no. 655094

Before start

Be sure to have access to:

- Luminometer with or without injection system.
- 96 well plates appropriate for luminescence signal measurements
- Reagents for culturing reporter cell line. See reference (1)

*reagents might take a while to gather and prepare but once prepared they last for a long time

*time to produce conditioned media around 3 weeks. See reference protocols (2), (3).

Day 1: Seeding of Hek 293 STF cells

1 Culture HEK 293 STF CRL-3249™ (from now own referred as HEK-STF) according to the company specifications (1) until confluent and not too many passages old.

Seed 3 wells of HEK-STF cells per sample and following controls:
-negative control: conditioned media from L-cells (not transfected with luciferase construct) (4)
-positive control: previous batch with known activity, HEK-STF cells with recombinant Wnt-3a or agonist stimulation
-control lysates as blank for luminescence: HEK-SFT cells with HEK-medium only

1.1 Start with one almost confluent T75 culture bottle of HEK-STF cells

1.2 1x wash with DPBS: take out medium, add 5 mL DPBS, turn gently the bottle, take out DPBS

1.3 Detach cells with 1-2 mL Trypsin/EDTA (37 °C) and transfer to a conical tube with 8-9 mL HEK-medium 5m

1.4 Centrifuge at 100-200 g for 00:05:00 5m
count cells (Neubauer chamber or automatic cell counter)

1.5 count cells (Newbauer chamber or automatic cell counter) 5m

1.6 Seed cells in a 24 well plate. HEK-STF : 15m
24 well Platte (0.05×10^6 cells/well). $1{,}3\times10^6$ cells / 13ml for the whole plate
cover each well with 0.5 mL of HEK medium.

Day 2: cell stimulation 1d

2 Aspirate medium and discard. 30m

Add a total of 500 µL of HEK medium with the following amount of conditioned medium (CM) to test:

- For R-spondin CM: [M] 12.5 % volume Wnt-3a CM + [M] 2.5 % volume Rspo1 CM (3)
- For Wnt3a CM . [M] 50 % volume

2.1 Incubate at stadard culture conditions for aprox. 24:00:00 1d

Day3 : luicferasse reaction 20m

3 Luiferase solution
Prepare on the day luciferase solution. Keep in the fridge until ready to use
When using a plate reader with automatic injection, calculate the extra volume needed for it (ca 3 mL)
fill to final volume with **Assay reagent** solution

	A	B	C	D
	Reagent	units	[stock]	[working]
	DTT	M	1	0,033
	CoA	mM	10	0,266
	Luciferin	mM	20	0,467
	ATP	mM	100	0,633

4 Prepare 1x Lysis medium from the stock with destiled water 20m

5 Cell lysis
Aspirate medium from HEK-STF cells 2m

5.1 Add 150 µL /well of 1x Lysis buffer. 2m

6 Leave the plate for 00:20:00 at Room temperature . (Note: pippeting seems to make cell aggregates and bubbles) 20m

7 Check under the microscope that most cells are lysed

8 Shake gently for 00:05:00 on plate shaker 5m

9 Transfer 20 µL of each sample to a 96 well plate for luminescence read. 10m
Avoid bubbles or cell clumps. Recommended pipetting scheme as in the 24 well plate of HEK-STF culture-

10 If using a plate reader without automatic injection: 20m
Take to the plate reader with luci. solution, multichanel pipette, and reagend reservoir
Once the plate reader is set, add 100 µL of luciferse solution per well and read.
For accuracy, use a multichanel pipette and add the solution covering not one sample at a time but one of the triplcates of all samples at one time.

11
(signal decreases ca. 20% in the first 10 minutes)

12 Check that the relative luminescence units from negative controls are orders of magnitude lower that test samples. Average values from triplicates.
Values below more than 50-60% lower than a working batch of conditioned media are considered of poor quality.
Examples of equipment and methods: Berthold Tristar-GAS ISRE Luciferase Assay, SpectraMax i3x-SpectraMax Glo Steady-Luc Reporter Assay

Protocol references

(1) HEK 293 STF CRL-3249™
https://www.atcc.org/products/crl-3249

(2) Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation
https://www.nature.com/articles/nprot.2016.097

(3) Establishment and Culture of Human Intestinal Organoids Derived from Adult Stem Cells
https://currentprotocols.onlinelibrary.wiley.com/doi/10.1002/cpim.106

(4) Notes on the use of conditioned medium
https://web.stanford.edu/~rnusse/assays/actvwnt.html#:~:text=Comparing%20the%20Wnt%20conditioned%20medium,conditioned%20medium%20may%20therefore%20contain
"*Comparing the Wnt conditioned medium to medium from control L cells is a reasonable way of controlling for specificity but it should be realized that Wnt expression can lead to the induction of genes encoding other secreted factors (such as FGFs, see* ***target genes****) and that the conditioned medium may therefore contain such factors.*"

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