Apr 12, 2024

# Assaying NLRP3-mediated LDH and IL-1β release

DOI
dx.doi.org/10.17504/protocols.io.dm6gpzr3plzp/v1

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**Protocol Citation:** Harper JW, Louis R R Hollingsworth 2024. Assaying NLRP3-mediated LDH and IL-1β release. **protocols.io https://dx.doi.org/10.17504/protocols.io.dm6gpzr3plzp/v1**



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

**Created:** April 11, 2024

**Last Modified:** May 31, 2024

**Protocol Integer ID:** 98085

**Keywords:** ASAPCRN, assaying nlrp3 activation, assaying nlrp3, nlrp3 activation through the downstream release, mediated ldh, nlrp3, ldh

**Funders Acknowledgements:**
**National Institutes of Health**
Grant ID: R01NS083524

## Abstract

A general protocol for assaying NLRP3 activation through the downstream release of LDH and IL-1β from cells. Included are protocol for iBMDMs and THP-1 macrophages.

## Materials

**Cell culture**

FBS (SH30910.03)
Sterile PBS (Thermo  J67802.AP)
DMEM (Thermo 1995-073)
DMEM, no phenol-red (Thermo 21063029)
RPMI (Thermo 11875119)
RPMI, no phenol-red (Thermo 11835030)
GlutaMAX™ Supplement (Thermo 35050061)
Trypsin Solution (VWR 45000-658)
Penicillin-Streptomycin (10,000 U/mL) (Thermo 15140163)

**Compounds**

CL097 (Sigma SML2566)
Imiquimod hydrochloride (MedChemExpress HY-B0180A)
Nigericin (Sigma N7143)
LPS (Invivogen tlrl-b5lps)
Phorbol 12-myristate 13-acetate (PMA) (Sigma P1585)

**Assay**

Multichannel pipettes

LDH release:
CyQUANT™ LDH Cytotoxicity Assay (Thermo C20300)
96 Well Flat Clear Bottom White Polystyrene Plates (Corning 3610)

IL-1B release:
Lumit® IL-1β Mouse Immunoassay (Promega  W7010)
Lumit® IL-1β Human Immunoassay (Promega  W6010)
384-well Low Flange White Flat Bottom Polystyrene Plates (Corning 3570)

## Troubleshooting

1 Optimize these protocol for your particular cells lines, treatment regimens, assay kits, etc. on the smaller scale with wild-type (and control NLRP3-KO, if on hand) cells prior to proceeding with large scale experiments. The sensitivity of the particular plate readers you have available is an important factor here, too.

STEP CASE

## Murine iBMDMs 15 steps

2 Grow iBMDMs (immortalized bone marrow derived macrophages) to full confluency in a 10-cm plate--one per genotype. Cells are maintained in complete DMEM (DMEM + GlutaMAX™ + Penicillin-Streptomycin + 10% FBS).

3 Trypsinize the cells, neutralize the trypsin with 5X medium, and resuspend cells thoroughly in a 50-mL falcon tube.

4 Count the resuspended cells, and then seed 200,000 cells in each well of a 96-well plate, in 200 µL of complete DMEM. To use a multichannel pipette, resuspend the cells at 1 M/mL and multichannel 200 µL of the suspension into wells.

Note

For each genotype, plate conditions in at least triplicate. In addition to any treatments, plate a triplicate of untreated cells and a triplicate used to calculate the maximum amount of LDH that can be released. Having these controls for each genotype is important because slight differences in growth rates or cell seeding will otherwise reflect in the amount of LDH or IL-1β released.

Note that this is a lot of cells to seed in 96-well plates. This is so that contact inhibition slightly slows the growth of these cells, since cells in mitosis have damped NLRP3 responses.

4.1 Here's an example of cell seeding (above) with the genotypes "WT", "FAPP1g1", and "FAPP1g2". The treatments are listed below (UT = untreated, Lysis = Lysis buffer total LDH release control). "Medium only" controls would go in row A, columns 10-12, on the day of the assay.

plate3

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | WT | WT | WT | FAPP1g1 | FAPP1g1 | FAPP1g1 | FAPP1g2 | FAPP1g2 | FAPP1g2 | | | |
| B | WT | WT | WT | FAPP1g1 | FAPP1g1 | FAPP1g1 | FAPP1g2 | FAPP1g2 | FAPP1g2 | | | |
| C | WT | WT | WT | FAPP1g1 | FAPP1g1 | FAPP1g1 | FAPP1g2 | FAPP1g2 | FAPP1g2 | | | |
| D | WT | WT | WT | FAPP1g1 | FAPP1g1 | FAPP1g1 | FAPP1g2 | FAPP1g2 | FAPP1g2 | | | |
| E | WT | WT | WT | FAPP1g1 | FAPP1g1 | FAPP1g1 | FAPP1g2 | FAPP1g2 | FAPP1g2 | | | |
| F | WT | WT | WT | FAPP1g1 | FAPP1g1 | FAPP1g1 | FAPP1g2 | FAPP1g2 | FAPP1g2 | | | |
| G | WT | WT | WT | FAPP1g1 | FAPP1g1 | FAPP1g1 | FAPP1g2 | FAPP1g2 | FAPP1g2 | | | |
| H | | | | | | | | | | | | |

Stimulation3

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| A | UT | UT | UT | UT | UT | UT | UT | UT | UT | |
| B | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | LPS | |
| C | LPS+lysis | LPS+lysis | LPS+lysis | LPS+lysis | LPS+lysis | LPS+lysis | LPS+lysis | LPS+lysis | LPS+lysis | |
| D | Nig, 10 uM | Nig, 10 uM | Nig, 10 uM | Nig, 10 uM | Nig, 10 uM | Nig, 10 uM | Nig, 10 uM | Nig, 10 uM | Nig, 10 uM | |
| E | Nig, 20 uM | Nig, 20 uM | Nig, 20 uM | Nig, 20 uM | Nig, 20 uM | Nig, 20 uM | Nig, 20 uM | Nig, 20 uM | Nig, 20 uM | |
| F | CL, 50uM | CL, 50uM | CL, 50uM | CL, 50uM | CL, 50uM | CL, 50uM | CL, 50uM | CL, 50uM | CL, 50uM | |
| G | CL, 100uM | CL, 100uM | CL, 100uM | CL, 100uM | CL, 100uM | CL, 100uM | CL, 100uM | CL, 100uM | CL, 100uM | |
| H | | | | | | | | | | |

5 The next day, carefully exchange cells into phenol red-and serum-free DMEM ± LPS (1 µg/mL, 100 µL). 4h

Note

Phenol red can contribute to background absorbance in the assay. Serum can contain large amounts of LDH, contributing to assay background. Additionally, serum starvation might prevent or slow cells from entering mitosis, which could increase the population that can undergo an Nlrp3 inflammasome response (untested).

5.1 Add 100 µL of medium alone to 3 wells. Return plate to the incubator for 4 h.

6 Resuspend compounds or lysis buffer to 2X the desired final concentration in phenol red-and serum-free DMEM. For all wells that were treated with LPS, resuspend compounds in phenol red-and serum-free DMEM + LPS (1 µg/mL). Add 100 µL to each well with a multichannel pipette and tap to mix.

Note

There are many different NLRP3 treatment regimens described in the literature. For two of the compounds, CL097/imiquimod is usually added anywhere between 50 - 300 µM, whereas nigericin is often used at 5 - 20 µM. Inhibitors are often added during LPS priming, 1 h prior to the addition of activating compounds. Conditions should be optimized for the individual cell type, chemical compounds, and biological question. Additionally, different treatment times have been described for different compounds - for example, 1 - 2 h for nigericin, or 2 h for CL097. Stagger treatments so that the plates can be harvested at the same time. If there are timing differences, make a note of whether the LPS pre-treatment is extended or whether LPS pre-treatment was staggered (it should probably not make a difference, except in rare cases).

6.1 Add another 100 µL of medium alone to the set of 3 wells from step 5.1. Place the plate back into the cell culture incubator.

7 When the treatment(s) have run their course, tap the plate to mix, then centrifuge the plate (1000 g, 5 min) to pellet any cells that have lifted.

8 Carefully transfer 150 µL of the supernatant to a new 96-well plate. Excess supernatant can be kept on ice while performing assays for any technical repeats or technical optimization (e.g., if LDH/IL-1β assays are over or under saturated).

STEP CASE

## LDH release 5 steps

### LDH release from iBMDMs

9 Transfer supernatant (10 µL) and fresh cell culture medium (40 µL) to a 96-well plate assay plate; for example, 96 Well Flat Clear Bottom White Polystyrene Plates (Corning 3610).

Note

200,000 cells is far outside of the linear range of this assay, which is why this dilution, at a minimum, is necessary. Depending on the multichannel pipette(s) available, I would even recommend starting with 5 µL supernatant in 45 µL fresh cell culture medium. Being within the linear range is important because differences in cell seeding will be reflected in the data. For example, if the assay saturates when two genotypes are seeded differently, the condition with a larger amount of cells will have an artificially small total lysis control. This will compress the range of released LDH so that the relative %LDH of the more confluent condition always looks larger. Therefore, be wary of changes when there are meaningful differences in the total lysis controls, particularly if the more confluent cells show higher %LDH release in all treatment conditions (often on the order of up to 10%). Be prepared to further dilute the supernatant and repeat the assay to get more representative data.

10 Follow the manufacturer's instructions for the CyQUANT™ LDH Cytotoxicity Assay (Thermo C20300).

10.1 The incubation time can be reduced to 10 - 15 min if the wells are approaching saturation. Be sure to wrap the plate in foil during incubation and prior to reading the wells. The plate can be briefly spun without its lid to get rid of bubbles before reading absorbance values.

11 Correct all absorbance values (A490 nm - A680 nm).

Calculate % maximum LDH release for each replicate as follows: (Treatment – Average[medium])/(Average[Geneotype-matched maximum lysis control] – Average[medium]).

Where "Treatment" is the absorbance value for the individual treatment replicate; "Average[medium]" is the average absorbance value of the three medium control wells (same for all genotypes); "Average[Geneotype-matched maximum lysis control]" is the average value of the three lysis controls for the specific genotype.

Note

Some protocols include having a specific medium control for the lysis buffer. Since the volume difference from the lysis buffer is only about 10% (using a 10X stock), I have found that there isn't a quantifiable difference in serum-free medium with this kit.

11.1 This is an example excel file for calculating these values.

mock_TMEM165.xlsx

## Protocol references

https://link-springer-com.ezp-prod1.hul.harvard.edu/protocol/10.1007/978-1-62703-523-1_7