Protocol: Detection of antibodies to LASV using the Luciferase Immunoprecipitation Systems (LIPS)

Background

The Luciferase Immunoprecipitation Systems (LIPS) tests for interaction of antibody and antigen complexes. Serum from potentially infected humans or animals is incubated with cell lysate containing a specific viral protein fused to luciferase generated by overexpression from a transfected plasmid. If antibodies specific to the viral protein are present they will bind the antigen. A/G ultralink resin is then added. A/G resin is a durable beaded polyacrylamide with recombinant Protein A/G immobilized to it. Protein A/G specifically binds to IgG from several mammalian species enabling antibody capture from a variety of species. After allowing binding the beads are washed extensively. The luciferase enzyme will produce bioluminescence when the luciferase substrate is added and indicates presence of NP-antibody complexes. The amount of light is enumerated and detected using a luminometer. It is important to take into account the background bioluminescence readings and include the appropriate controls.

Constructs

Constructs: Constructs were cloned into the pcDNA3.1+ expression vector using BamHI and ApaI restriction sites. Two constructs were prepared, each with a Luciferase tag at the N terminus of the full length LASV nucleoprotein (lineage Josiah). The control construct had a stop codon introduced by site-directed mutagenesis after the Luciferase and before the linker. Constructs can be commercially cloned. For full sequences, including linkers please contact me.

Preparation of Plasmids

Once plasmids have been successfully cloned (commercially or in-house) they will need to be amplified in competent *E.coli. E.coli* can either be purchased as competent or treated with a combination of chemicals to induce this (let me know if a protocol is required to produce competent cells).

To transform the competent cells: 2μL plasmid is carefully added to the competent cells and incubated on ice for 20 minutes. Cells are then heat shocked at 42^0C for 45 seconds before immediately returning to ice. Add 500μL SOC media (you can use LB broth if you don't have any SOC). I usually recover the bacteria for 1 hour at 37^0C in a shaking incubator with 200rpm shaking. You will then need to select based on the antibiotic resistance specified by your plasmid – this will probably be ampicillin. Plate on LB amp plates O/N and select single colonies the following day. Grow up single colonies in 4mL of LB broth+ampicillin O/N and

then extract the plasmid using a commercially available plasmid miniprep clean-up kit. The plasmid is now ready to be transfected into your cell line.

Transfection of HEK293T cells and production of lysates

Lysates are prepared using plasmids transfected into HEK 293 T cells. HEK 293 T cells are a derivative human embryonic kidney cell line that expresses a mutant version of the SV40 large T antigen. These cells are very transfectable and we had much higher protein expression compared to Vero cells.

HEK 293T cells are grown in DMEM (Gibco, 41966029) with 10% foetal calf serum (ensure the FCS has been heat inactivated before use). Thaw cells and allow them to establish for at least a week before transfection. Transfection and harvesting is performed over a week, starting on the Friday before. Grow cells in 10cm^2 dishes with 10mL media.

Friday: Split confluent 10cm^2 dishes – depending on their growth you will want to split them 1:4 or 1:6 (you will require 1mL Trypsin-EDTA (0.05%) per dish). To split cells using trypsin you first carefully wash the cell layer with 10mL 1X sterile PBS. Add 1mL and the cells will rapidly slough off the plastic. To neutralise the trypsin add 1mL DMEM+10% FCS). If you want to split 1:4 take 0.5mL of this to a fresh 10cm^2 dish or 1:6 neutralise with 2mL media and add 0.5mL to the fresh dish. Always incubate in at least 10mL media with 5% CO_2 and at 37^{0}C.

Monday: Split the cells so that they will be about 80% confluent when you perform the transfection on Tuesday (if you aren't sure do a 1:4 and a 1:6 and look at the cells the next day to determine the optimal cell density).

Tuesday: Carefully change the media by aspirating the old media and replacing it with fresh (10mL).

Prepare the transfection mixture as follows (noting that Fugene should NOT touch the plastic tube – add directly to the opti-MEM).

In a 15mL tube add 200µL Opti-MEM (Gibco, 31985062), 10µL Fugene 6 (Promega, E2691) and 3.5µg plasmid.

Incubate at room temperature for 15 minutes

Whilst carefully swirling the dish add the transfection mixture dropwise. Incubate the cells at 37^{0}C.

Wednesday: Change the media on the cells

Thursday: Lyse and harvest the cells using 1-1.5mL of lysis buffer per dish. I have found the lysis buffer included with the Promega Luciferase Assay System kit to be good. Follow the lysis instructions in the kit. Cells can be scraped.

After lysis clear the lysate by centrifuging at 17,000g for 10 minutes.

Freeze vials of lysate (supernatant) at -70^{0}C in small aliquots (100-150µL). If you don't have a -70^{0}C you can store at -20^{0}C.

Western blotting

If there are antibodies commercially available then it is a good idea to run a western blot to test your lysates to check your protein of interest is present and the right size. You can definitely check with an anti-luciferase antibody. Run cells alone and include a loading control.

[NOTE: Lysates have been prepared and shipped to Sierra Leone for testing]

Testing RLU of lysates

Thaw an aliquot of lysate and transfer 20µL to a white plate in at least duplicate. Add 50µL of prepared Luciferase reagent to it (whichever system you plan to use for the main assay – either injector system or Glo System). Ascertain the relative light units per mL (RLU) according to the protocol. You cannot freeze-thaw aliquots more than once.

Luciferase Immunoprecipitation Systems (LIPS) for detection of LASV antibodies

In immunoprecipitation you must test different IP buffers in order to ascertain the optimal one for your antibody-protein interaction.

Materials and Reagents

Mini orbital shaker (capable of 100-150rpm per minute)
Vacuum pump
GloMax or other machine capable of measuring luminescence

Stock preparation
5M NaCl: Add 14.61g to 50mL dH_20
100mM MgCl2: Add 0.476g to 50mL {add slowly and wear goggles – exothermic reaction}
5g Dithiothreitol (DTT) add dH_20 to give a final volume 32.4mL for 1M stock (Freeze 1mL aliquots at -20^{0}C in 1.5mL tubes)

Prepare IP10 buffer as follows (you should be able to run several plates from 500mL):

For 500mL

10mM Tris-HCL pH 7.5 [Add 5mL 1M stock]
150mM NaCl [Add 15mL 5M stock]
10% glycerol [Add 50mL]
1% NP-40/IGEPAL [Add 5mL] – we are currently replacing NP-40 with 1% Triton-X
2mM $MgCl_2$ [Add 10mL 100mM stock]
2mM DTT [Add 1mL 1M stock]
Add distilled water to 500mL
Store IP-10 buffer in the fridge for up to 1 month

Start here for running plates if you have lysate stock and IP-10 buffer prepared

For a 96-well plate you need to prepare 20mL IP10 buffer with 2 protease inhibitor tablet dissolved in it (you only need just over 10mL and so if you are running multiple plates per day you don't 20mL for every plate)

Luc-NP from 7th July 2022 – measured at 20µL as containing $7.1x10^7/6.85x10^7$ RLU
This was re-tested in Bo on the 10th Feb 2023 and the following values obtained: **$5.75x10^7/5.81x10^7$ RLU in 20µL. Therefore 1µL lysate contains $2.89x10^6$ RLU**

For 10^7 RLU to be added per well = add 3.4µL lysate per well.

For the Luc control $1.52x10^8/1.47x10^8$ RLU in 20µL
For 10^7 RLU to be added per well = 1.3µL [N.B this should be re-tested in Bo before use. Take 20µL Luc control and add 50µL of prepared Renilla Glo substrate – measure the RLU and work out how many are in 1µL]

Prepare enough lysate per well in IP10 {each well will require 3.4µL LUC-NP lysate}

E.g for 96 well plate:

Each well needs a final volume of 100µL – adjust serum/blood/blood spot/control accordingly. If using the ELISA controls add 25µL for each well. For serum add 1µL. For whole blood add 2µL and for blood spots add 10-20µL. For plate setup remember to use the IP-10 + protease inhibitor tablet. For washes you don't add the protease inhibitor.

For the controls prepare these without the multi-channel since the buffer volume is quite different.

25µL control + 3.4µL lysate + 71.6µL IP-10 buffer per well [Run 2x positive controls and 2x negative controls per plate]

For the other wells the lysate + IP-10 buffer can be transferred into the plate with the multi-channel pipette.

3.4µL lysate + 94.6µL IP-10 buffer per well

In a reservoir: Prepare enough for 110 wells: **374µL lysate** (you will likely need 3-4 vials per plate) + **10.4mL IP-10 (For a full plate plus the controls and washing the A/G beads you will need to prepare 20mL + 2 protease inhibitor tablets)**. Mix well.

Transfer 98µL to each sample well as required.

For samples that are potentially infected with LASV – transfer 2µL blood into the appropriate wells using the Glovebox for safety. Controls can be aliquoted on the bench, since they don't present a hazard.

Incubate on a rotary shaker for 1 hour at room temperature (25 degrees) @ 180rpm

For a 96-well plate: transfer enough A/G resin into a 1.5mL Eppendorf tube for 120 wells (600µL). Mark where the volume is. Wash the A/G resin 3X with IP10 to remove preservative by adding 0.5mL IP-10 and centrifuging at 10,000rpm for 3 minutes each. You can do this during the incubator.

Add 5µL A/G Ultrabeads to Multiscreen plate (per well) [Cut off the end of a p20 pipette tip]

Transfer 100µL from plate on shaking incubator to each well.

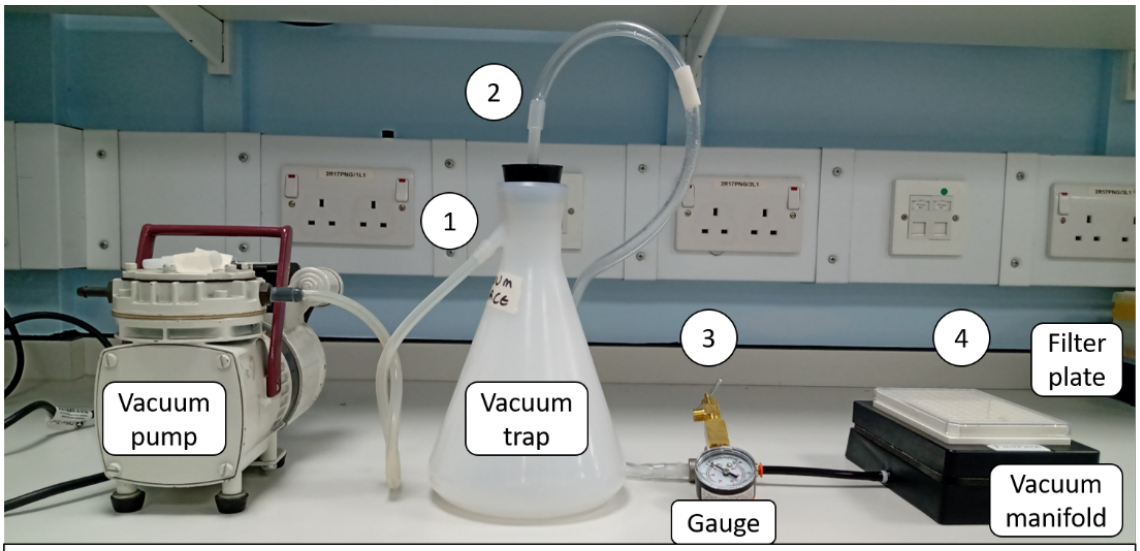
Incubate on a rotary shaker for 1 hour at room temperature (25 degrees) @180rpm

Wash the plate 8X using 100µL/well IP10 (doesn't need protease inhibitors for this stage but you can use up any that is left or if you are running more than 1 plate in a day you would only need 30mL + 3 tablets total)
Wash the plate 2X using 100µL/well 1X PBS

Blot the plate dry on paper towel.

Prepare Renilla Glo Luciferase assay reagent by adding 52µL substrate per 5.2mL buffer (5.2mL is required for 1x96 well plate if you add 50µL per well) – **prepare immediately before use**

Add 50µL per well of prepared luciferase substrate per well.
Incubate at room temp (25 degrees) for at least 10 minutes.
Read on the GloMax using the protocol for your kit – here we are using Renilla Luciferase Glo



1 – Connect the vacuum pump to the arm of the vacuum trap
2 – Connect the gauge pipe to the top of the vacuum trap
3 – Connect the vacuum manifold to the gauge: connect the black pipe and push the orange ring to lock it. Make sure the gauge knob is closed. Turn on the vacuum pump and check for vacuum pressure at the vacuum manifold.
4 – Place the filter plate on top of the vacuum manifold (frame on top of the base). Turn on the vacuum pump pressing the plate down while applying the vacuum