G-LISA RhoA Activation Assay Biochem Kit

Cytoskeleton, cat number : BK124

Quantify protein

1. Spin down embryo and remove excess liquid
2. Add 120ul of lysis buffer, keep on ice
3. Add 10ul of sample lysate to a well in a 96 well plate
4. Add 290ul of Precision red to each well
5. Blank is 10ul of lysis buffer in 290ul of precision red.
6. Incubate for 1 minute
7. Read at 600nm, multiply Abs by 3.75 to get mg/ml
   (Concentration = Absorbance reading/ pathlength x extinction coefficient x dilution factor)
   Concentration = Abs/ 0.8 cm x 10 mg/ml x 30

All samples should be the same protein concentration, between 0.4- 2mg/ml

Assay

It is important to completely remove all solutions between steps to avoid high background readings

1. Mix 60 µl Lysis Buffer with 60 µl ice-cold Binding Buffer. Place on ice. This is the buffer blank.

2. Mix 12 µl Rho Control Protein with 48 ul Cell Lysis Buffer and 60 µl Binding Buffer. Place on ice. This is the positive control sample. Do not re-use.

3. Remove the number of strips required from Rho plate, place in strip holder, and place on ice. Return remaining strips to storage.

4. Keep the plate on ice and dissolve the powder in the wells with 100 µl ice-cold water. Detachment of the white powder pellet will not affect assay performance. Tap pellets to the bottom of the wells prior to resuspension.

5. If not already equalized, add required amount of ice-cold lysis buffer to equalize all lysate concentrations. Calculate dilution factors required BEFORE thawing lysates.

6. Immediately aliquot sufficient lysate for duplicate (60 µl) or triplicate (90 µl) assays into fresh ice-cold microcentrifuge tubes.

8. Add an equal volume of ice-cold Binding Buffer to each tube. Vortex each tube for 3-5 s on a high setting and return tubes to ice.
9. Completely remove the water from the microplate wells as follows:

Vigorously flick the plate to remove solution from each well, followed by a series of 5-7 vigorous pats onto paper towels. The complete removal of solution from wells between steps of the G-LISA is very important to avoid high background readings. At an absorbance of 490 nm, buffer-only wells should read between 0.10 – 0.40 and positive control wells should read between 0.7 – 1.0 (after subtraction of blank).

10. Return plate to ice. Immediately add 50 µl of equalized cell lysate to wells.

11. Pipette 50 µl of buffer blank control into duplicate wells.

12. Pipette 50 µl of RhoA positive control into duplicate wells.

13. Immediately place the plate on a cold orbital microplate shaker (400 rpm recommended, 200 rpm minimum) at 4°C for exactly 30 min.

14. During the incubation, dilute anti-RhoA primary antibody to 1/250 in Antibody Dilution Buffer (add 2 µl of antibody to every 500 µl Antibody Dilution Buffer). Note: The final volume of 500 µl is adequate for one strip (8 wells).

15. After 30 min, remove the solution from the wells and wash twice with 200 µl Wash Buffer at room temperature using a multi-channel pipettor. Do not leave plate unattended at this time. Vigorously remove the Wash Buffer after each wash as described in step 9.

16. Place plate on the bench.

17. **Immediately pipette 200 µl of room temperature Antigen Presenting Buffer into each well using a multi-channel pipettor and incubate at room temperature for exactly 2 min.**

18. Vigorously flick out the Antigen Presenting Buffer as described in step 9.

19. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 9.

20. Add 50 µl of diluted anti-RhoA primary antibody to each well and leave the plate on the orbital microplate shaker (200-400 rpm) at room temperature for 45 min.

21. During primary antibody incubation, dilute the secondary HRP labeled antibody to 1/62.5 in Antibody Dilution Buffer (add 8 µl of antibody to every 500 µl Antibody Dilution Buffer). Note: The final volume of 500 µl is adequate for one strip (8 wells).

22. Vigorously flick out the anti-RhoA primary antibody as described in step 9.

23. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 9.

24. Add 50 µl of diluted secondary antibody to each well and leave the plate on a microplate shaker (200–400 rpm) at room temperature for 45 min.

25. During secondary antibody incubation, thaw an aliquot of HRP detection reagents A and B in a room temperature water bath and remove as soon as they are thawed. Do not mix.

26. Immediately prior to the end of the secondary antibody incubation, mix HRP detection reagents A and B in equal volumes (50 l of A/B mixture per well is needed). Protect mixture from light. Discard unused solution.

27. Vigorously flick out the secondary antibody as described in step 9.

28. Wash the wells three times with 200 µl of room temperature Wash Buffer as described in step 9.

29. Pipette 50 µl of the mixed HRP detection reagent into each well and incubate at 37°C for 10-15 min.

30. Add 50 µl of HRP Stop Buffer to each well.

31. Check that the wells are free of bubbles; if not, remove before continuing.

32. Read the signal by measuring absorbance at 490 nm using a microplate spectrophotometer. Designate Lysis Buffer only wells as the assay Blank.