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The Protein Experts

MANUAL | CYTOSKELETON.COM

RalA G-LISA® GTPase Activation Assay Kit (Colorimetric Based): 96 Assays

CATALOG # BK129

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Introduction

Background

The Ras superfamily of small GTPases comprises more than 150 human proteins that regulate diverse cellular processes, including cell proliferation, vesicle trafficking, and cytoskeletal organization (1-3). The Ral subfamily consists of two closely related proteins, RalA and RalB, which share approximately 82% amino acid identity and function downstream of Ras and other signaling pathways (3). Ral signaling has important roles in exocytosis, endocytosis, cell migration, and tumorigenesis, with dysregulated Ral activity increasingly implicated in pathologies such as cancer progression, metastasis and diabetes (3-5).

Like other small GTPases, RalA functions as a molecular switch, cycling between an inactive GDP-bound state and an active GTP-bound state (1). In its GTP-bound form, RalA interacts with specific downstream effectors, including RalBP1 and components of the exocyst complex, to regulate cellular responses (3). Measurement of RalA-GTP therefore provides a direct assessment of RalA activation and pathway activity.

Keys to a successful G-LISA® assay

The G-LISA® assay is simple to perform, and a little preparation goes a long way toward achieving reliable, reproducible results.

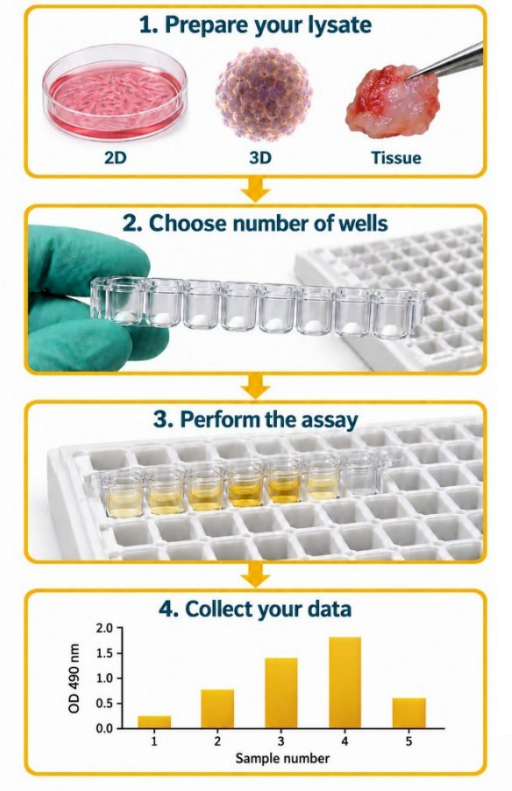
- **Prepare high-quality lysates**. Lysate preparation is the most critical part of the assay. Review the lysate preparation guidelines before beginning and follow them carefully to preserve the GTPase activation state and ensure reproducible data.
- **Have everything ready before you start.** Prepare all reagents, equipment and samples in advance so that the time-sensitive assay steps can be completed accurately and without interruption.

Introduction continued

Assay principle

The RalA G-LISA® kit contains a Ral-GTP-binding protein linked to the wells of a 96 well plate. Active, GTP-bound Ral in cell lysates will bind to the wells while inactive GDP-bound Ral is removed during washing steps. The bound active RalA is detected with a RalA specific antibody and colorimetric detection. A basic schematic diagram of the steps involved in the G-LISA® is shown in figure 1.

Figure 1: Simple workflow



Critical step: Lysate collection typically takes 10-15 minutes per sample. Guidelines for lysate preparation pages 13-25

Assay time: Approximately 3h. Protocol, pages 26-32

Data analysis: Analyze results using Microsoft Excel®, Google Sheets™, or other compatible statistical and graphing software, pages 33-34

Introduction continued

Typical RalA G-LISA® results

The G-LISA® assay provides information on relative levels of small G-protein activation, for example between treated and non-treated cells or diseased vs healthy tissue samples. Figure 2 shows data showing RalA signal from 12 µg of total cell lysate input per condition.

Figure 2: Typical RalA G-LISA® results

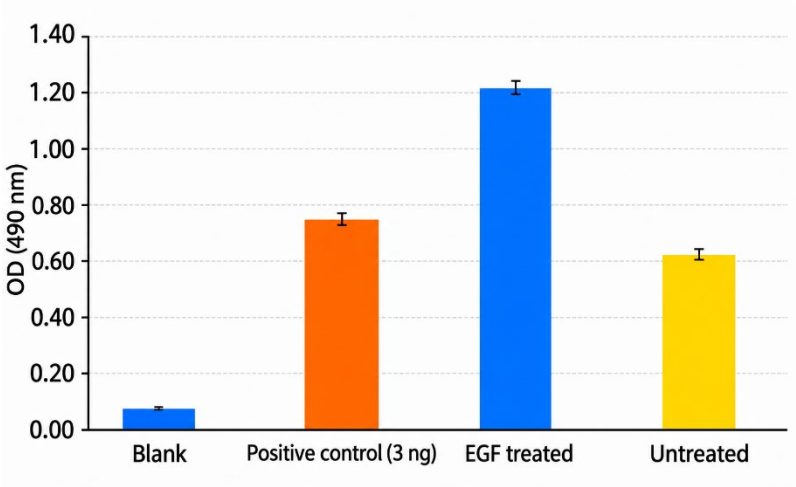


Figure 2 legend: Rat2 cells were grown to 70% confluence and serum starved for 48 hours. Cells were the either treated with EGF (100 ng/ml) for 2 minutes or left untreated. Lysates were harvested in cell G-LISA® Cell lysis buffer and snap frozen immediately in assay sized aliquots and stored at -80°C. The G-LISA® assay was performed as outlined in this manual; 12 µg of lysate was used per assay. A 3 ng sample of constitutively active RalA was included in the assay as a positive control (part # RLCA). All reagents except cell lysate samples are provided in this kit.

Introduction continued

The RalA G-LISA® advantage

Why Choose G-LISA® over a traditional RalA pull-down assay?

FEATURE	G-LISA® RAL ACTIVATION ASSAY	TRADITIONAL RAL PULL-DOWN ASSAY
Assay Time	<3 hours	Longer, multi-step workflow
Sample Required	5–25 µg total cellular protein	500–1,000 µg total cellular protein
Sample Efficiency	20–200× less sample Ideal for primary cells, rare cell populations, and precious tissue samples	High sample requirement Can limit experiments when biological material is scarce
Results	Quantitative measurement of Ral-GTP	Semi-quantitative Western blot-based measurement
Throughput	High Analyze many samples simultaneously	Limited Fewer samples can be processed efficiently
Workflow	Simple plate-based format No glutathione beads, affinity precipitation, or Western blotting	Complex, multi-step workflow Requires glutathione bead handling, affinity precipitation, and Western blotting
Overall Advantage	Faster, quantitative, sample-sparing, and scalable Ideal for limited material and large studies	Labor-intensive, sample-intensive, and semi-quantitative Less efficient for complex experimental designs

Kit contents

This kit contains sufficient reagents to perform **96 assays**. The flexible format allows **1–96 samples to be analyzed per run**, enabling efficient use of reagents based on your experimental needs. Kit components are listed below.

Table 1: Kit contents

Ral-GTP Binding plate	GL34B	12 strips/8 wells per strip/96 wells total	Desiccated 4°C Stable for 6 months
Anti-RalA antibody	GL08	1 tube lyophilized	Desiccated 4°C Stable for 6 months
Secondary antibody-HRP conjugated	GL02	1 tube lyophilized	Desiccated 4°C Stable for 6 months
RalA control protein Constitutively active	RLCA	12 tubes lyophilized	Desiccated 4°C Stable for 6 months
Ral Binding buffer	GL52	1 bottle lyophilized	Desiccated 4°C Stable for 6 months
G-LISA® Cell Lysis buffer	GL36	1 bottle Lyophilized	Desiccated 4°C Stable for 6 months
Wash buffer	PE38	1 tablet Lyophilized	Desiccated 4°C Stable for 6 months
Antigen presenting buffer	GL39	1 bottle 25 ml	Room temperature Stable for 6 months
Antibody dilution buffer	GL40	1 bottle Lyophilized	Desiccated 4°C Stable for 6 months
HRP detection reagent A	GL43	1 tablet silver pack	Desiccated 4°C Stable for 6 months
HRP detection reagent B	GL44	1 tablet gold pack	Desiccated 4°C Stable for 6 months
HRP stop solution	GL80	1 bottle 8 ml	Room temperature Stable for 6 months
Precision Red protein assay reagent	GL50	1 bottle 100 ml	Room temperature Stable for 6 months
Protease inhibitor cocktail	PIC02	1 tube Lyophilized	Desiccated -20°C Stable for 6 months

Kit contents continued

Reagents and equipment that are required but not supplied:

- Cold 4°C PBS buffer (10 mM phosphate buffer pH 7.4, 140 mM NaCl, 3 mM KCl).
- Cell scrapers
- Multi-channel or multi-dispensing pipettor for 25-200 µl range.
- Multi-channel pipettor solution basins. Used for liquid handling.

Two orbital microplate shakers. Optimal shaker speed is 400 rpm (200 rpm is the minimal speed required). One at room temperature and one at 4°C.

- Microplate reader: 96 well plate compatible, wavelength 490 nm ±20 nm, read type: endpoint, temperature: room temperature.

Component reconstitution & storage

Many of the components in this kit are supplied in lyophilized form. Reconstitution and storage of components is detailed below in Table 2.

Table 2: Component reconstitution & storage

Ral-GTP Binding plate	GL34B	• **Storage:** Keep the plate sealed in the desiccant bag with desiccant until use. • **Reconstitution:** No reconstitution is required before starting the assay. • **White pellets:** Pellets may detach during shipping; this does **not** affect assay performance.	Desiccated 4ºC Stable for 6 months
Anti-RalA antibody	GL08	• Centrifuge briefly to collect the pellet in the bottom of the tube. • Reconstitute in 200 µl of PBS (plus 0.02% sodium azide, optional).	4ºC Stable for 6 months
Secondary antibody-HRP conjugated	GL02	• Centrifuge briefly to collect the pellet in the bottom of the tube. • Reconstitute in 80 µl of PBS. **Do not use sodium azide** in combination with this antibody as it will inactivate the HRP.	4ºC Stable for 6 months
RalA control protein Constitutively active	RLCA	Do not reconstitute until immediately prior to the assay. Each tube is good for one experiment. **Do not** store reconstituted RLCA.	Desiccated 4ºC Stable for 6 months Reconstitute when instructed in assay
Ral Binding buffer	GL52	Resuspend in 10 ml of sterile, nanopure water.	4ºC Stable for 6 months
G-LISA® Cell Lysis buffer	GL36	• Reconstitute in 100 ml of sterile, nanopure water. • This may take 5-10 min to resuspend. Use a 10 ml pipette to thoroughly resuspend the buffer.	Desiccated 4ºC Stable for 6 months

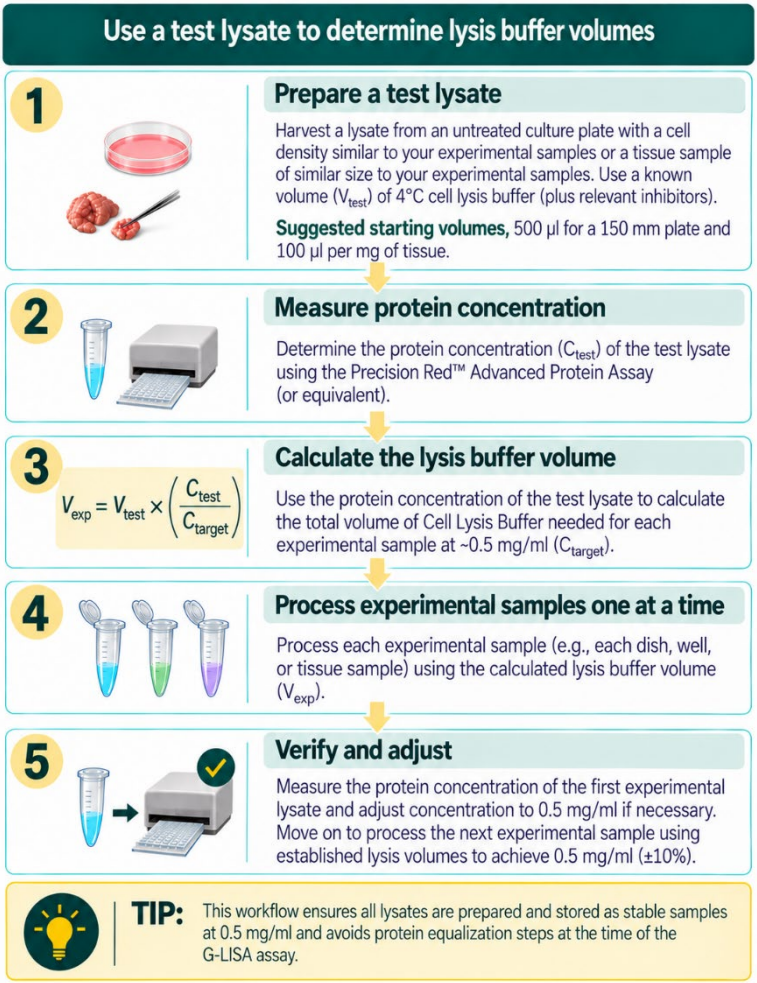
Wash buffer	PE38	• Reconstitute in 1000 ml of sterile, nanopure water. • Use a stirplate with magnetic stir-bar to aid reconstitution. This will take 45-60 min.	Room temperature Stable for 6 months
Antigen presenting buffer	GL39	No reconstitution necessary.	Room temperature Stable for 6 months
Antibody dilution buffer	GL40	Reconstitute in 15 ml of sterile, nanopure water.	4°C Stable for 6 months
HRP detection reagent A	GL43	• Resuspend tablet in 10 ml sterile, nanopure water. NOTE: **Light sensitive reagent** so cover tube with foil or similarly protect from light. • Place on a room temperature rotator for 10-15 minutes, until reagent dissolves completely. • Aliquot into 12 x 0.8 ml volumes. • Place in -70°C freezer for storage. • NOTE -20°C is **NOT** good for storage.	-70°C Stable for 6 months
HRP detection reagent B	GL44	• Resuspend tablet in 10 ml sterile, nanopure water. NOTE: **Light sensitive reagent** so cover tube with foil or similarly protect from light. • Place on a room temperature rotator for 10-15 minutes, until reagent dissolves completely. • Aliquot into 12 x 0.8 ml volumes. • Place in -70°C freezer for storage. • NOTE -20°C is **NOT** good for storage.	-70°C Stable for 6 months
HRP stop solution	GL80	Carefully add 1 ml of concentrated sulfuric acid (18 M) to HRP Stop Solution. • Check the box on the top of the bottle to indicate acid has been added. • Mix well and store at room temperature.	Room temperature Stable for 6 months
Precision Red protein assay reagent	GL50	No reconstitution necessary.	Room temperature Stable for 6 months
Protease inhibitor cocktail	PIC02	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100 x stock.	-20°C Stable for 6 months

Lysate preparation

Recommended lysate harvest workflow

Lysate preparation is critical for preserving rapid, transient GTPase activation. **Process each experimental condition individually through aliquoting and snap-freezing before starting the next.**

Before beginning, prepare a representative **Test Lysate**. Use its protein concentration to determine the Cell Lysis Buffer volume needed to prepare experimental lysates at approximately **0.5 mg/ml** (see schematic below).



2D Lysate preparation continued

2D Tissue culture lysates

G-LISA assays have been used extensively for analysis of small G-protein activation in 2D cultures (Table 3 and see cytoskeleton.com for most current citations).

Table 3: G-LISA® analysis in 2D cell culture models

2D culture model	G-LISA analysis	Application and sample processing	Ref.
RECENT hTERT-RPE1 and IMCD3 epithelial cells	**RhoA (BK124)**	Septin depletion and pathway perturbation were evaluated during primary-cilium assembly. Cells were lysed under cold conditions and active RhoA was quantified.	**a**
RECENT Primary mouse Schwann cells	**RhoA (BK124)**	Cultured Schwann cells from control and RhoA-deficient mice were analyzed during injury-associated dedifferentiation to connect RhoA activity with JNK signaling.	**b**
RECENT C3H10T1/2 cells and mouse embryonic fibroblasts	**Rac1 (BK128)**	Hedgehog signaling was activated or inhibited through Smoothened. Rapidly prepared cell lysates were used to compare Rac1 activation across treatments.	**c**
RECENT Human breast-cancer cell lines	**Rac1 (BK128)**	LDHA expression and catalytic activity were manipulated in adherent cultures. Rac1-GTP analysis linked noncanonical LDHA signaling to migration and tumor-cell behavior.	**d**
RECENT Primary rat dorsal-root-ganglion neurons	**Cdc42 (BK127)**	Dissociated neurons were cultured in compartmented chambers. Cdc42 activation was assessed after altering localization and translation of the prenyl-Cdc42 mRNA isoform.	**e**
FOUNDATIONAL MDA-MB-231 breast-cancer cells	**RhoA (BK124)**	Adherent cells underwent transcription-factor depletion or overexpression. RhoA activation analysis was paired with migration and metastasis assays.	**f**
FOUNDATIONAL HeLa cells	**Rac1 (BK128)**	Cells were synchronized through the cell cycle and separated into cytoplasmic and nuclear fractions. Rac1 activation was compared across cell-cycle stages.	**g**

a- O'Neill et al. 2023. Journal of Cell Biology 222:e201911062. doi:10.1083/jcb.201911062.
b- Liu et al. 2023. Glia 71:1715-1728. doi:10.1002/glia.24365.
c- Tang et al. 2022. Theranostics 12:1303-1320. doi:10.7150/thno.67702.
d- Zhang et al. 2023. Nature Metabolism 5:183-199. doi:10.1038/s42255-022-00719-9.
e- Lee et al. 2021. Journal of Cell Science 134:jcs251967. doi:10.1242/jcs.251967.
f- Zuo et al. 2010. Nature Cell Biology 12:188-195. doi:10.1038/ncb2047.
g- Michaelson et al. 2008. Journal of Cell Biology 181:485-496. doi:10.1083/jcb.200801047.

Detailed method for 2D tissue culture lysate preparation

A- 2D Cell Growth and Treatment

Plate cells and grow under the appropriate culture conditions to the desired confluency. Optimal conditions should be determined for each cell line.

NOTE: Remember to plate an extra culture for a “Test-Lysate” as discussed in the Recommeded Lysate Preparation section, page 13.

1- Perform experimental treatments, such as transfection, RNA interference, or serum starvation, as required by the experimental design.

2- When appropriate, serum-starve cells before stimulation to reduce basal RalA activity and establish a controlled baseline.

3- Stimulate cells using the appropriate experimental treatment. NOTE: RalA GTPase activation is typically rapid and transient; therefore, appropriate time points should be established experimentally. Activation may peak within 60 seconds to 2 minutes following stimulation and last for 15-30 minutes. RalA activation is highly cell type specific, an initial timepoint series of 0, 1, 3, 5 & 20 minutes is recommended.

B- Reagent preparation prior to 2D cell processing

NOTE: GTP-bound active RalA is labile and can undergo GTP hydrolysis during and following cell lysis. To preserve the activation state, keep cells, reagents, and lysates at ≤4°C and complete processing from cell lysis to snap-freezing as rapidly as possible (10-15 minutes per lysate).

To allow rapid lysate processing you should prepare all required reagents and materials before beginning cell lysis (see Table 4).

Table 4: Reagent preparation prior to processing lysate

Precision Red™ protein assay reagent	GL50	Used to quantitate lysate concentration. Reagent should be at room temperature. Protein concentrations are read in an absorbance spectrophotometer at 600 nm.
Microcentrifuge tubes (1.5 ml)	na	Label sufficient tubes to allow experiment sized aliquots of lysate to be made, and pre-chill on ice prior to cell lysis.
Ice buckets	na	Have sufficient ice to maintain cells, reagents and lysates at ≤4°C throughout processing.
G-LISA® Cell lysis buffer + Protease Inhibitor Cocktail	GL36 + PIC02	Add 10 µl Protease Inhibitor Cocktail per 1 ml G-LISA® Cell lysis buffer, mix thoroughly, and maintain on ice. The G-LISA® Cell lysis buffer must be ice-cold before use. A final lysate concentration of 0.5 – 0.6 mg/ml is recommended as a starting point, required G-LISA® Cell lysis buffer volumes can be calculated accordingly. **NOTE:** The protease inhibitor cocktail inhibits general serine, cysteine, and arginine proteases. Add experiment-specific inhibitors, such as kinase or phosphatase inhibitors, as needed.
PBS pH 7.2	na	PBS is not supplied with the kit. Prepare before beginning the procedure and chill on ice for at least 30 min before use.
Cell scrapers	na	Use to rapidly harvest adherent cells after adding G-LISA® Cell lysis buffer.
Liquid nitrogen	na	Use to immediately snap-freeze prepared lysate aliquots.

C- Wash 2D Cells

1- Remove the culture vessel from the incubator and immediately place it on ice.

2- Tilt the culture vessel and aspirate the culture medium.

3- Wash the cells with ice-cold PBS to remove residual serum proteins. See Table 5 for recommended wash volumes

4- Tilt the vessel to **completely** aspirate the PBS.

IMPORTANT: Residual PBS will dilute the G-LISA® Cell lysis buffer and may reduce the final protein concentration of the lysate. G-LISA® Cell lysis buffer volumes are usually very low (see Table 5) so small volumes of residual PBS will greatly reduce cell lysis efficiency.

TIP: Leave the vessel tilted for 30 seconds after initial PBS removal to collect residual PBS.

Table 5: Recommended wash & lysis volumes for 2D cell cultures

35 mm dish	9.6	1.5	33
60 mm dish	21	3	72
100 mm dish	55	8	190
150 mm dish	145	20	500
6 well plate	9.6	1.5	33
12 well plate	3.8	0.5	13
T25 flask	25	4	86
T75 flask	75	10	259

*G-LISA® Cell lysis buffer recommended volumes are given for Swiss 3T3 cells. A test plate should be used to determine lysis volumes for experimental samples. The volumes given above are starting points only.

D- Lyse and Clarify 2D Cells

1- Add an appropriate volume (determined from test lysate) of ice-cold G-LISA® Cell lysis buffer containing protease inhibitors directly to the cells.

Select the lysis volume to produce a final lysate protein concentration of approximately **0.5–0.6 mg/ml**. The optimal lysis volume should be determined empirically using a test lysate for each cell line and culture vessel.

2- Immediately harvest the lysate using a cell scraper.

3- Transfer the lysate to a pre-labeled, pre-chilled 1.5 ml microcentrifuge tube and keep on ice. Pipette cells 3-4 times to lyse cells completely.

4- Clarify the lysate by centrifugation at 10,000 × g for 1 min at 4°C.

5- Transfer the clarified lysate to a clean, pre-chilled tube.

6- Proceed immediately to protein concentration measurement.

E- Determine protein concentration of the lysate

Measure total protein concentration using Precision Red™ protein assay reagent (Part # GL50).

1- Add 20 µl of lysate to a separate disposable 1 ml cuvette.

2- Prepare a reference blank by adding 20 µl G-LISA® Cell lysis buffer to a separate cuvette.

3- Add 1 ml Precision Red™ protein assay reagent to each cuvette.

4- Mix and incubate for 1 min at room temperature.

5- Blank a spectrophotometer using the reference blank at 600 nm.

6- Measure the absorbance of each lysate at 600 nm.

7- Calculate the protein concentration:

[A_{600} sample – A_{600} blank] x 5 = protein concentration (mg/ml)

For additional information about the Precision Red™ Protein Assay Reagent and its 96-well assay format, refer to the ADV02 product page at www.cytoskeleton.com. The reagent is available as Part No. GL50 and Cat. No. ADV02.

F- Dilute lysate to a concentration of 0.5 mg/ml

1- Use G-LISA® Cell lysis buffer containing protease inhibitors to dilute the lysate to 0.5 mg/ml.

NOTE: 0.5 mg/ml is a recommended starting concentration for G-LISA assays. Optimal lysate concentrations will need to be determined empirically. Lysate concentrations> 2mg/ml are not recommended.

G- Aliquot and Snap-Freeze Lysate

1- Aliquot lysate into pre-labeled, pre-chilled microcentrifuge tubes.

Prepare aliquot volumes appropriate for the number of G-LISA® assays to be performed. Each G-LISA® requires approx. 30 µl of lysate per assay.

2- Immediately snap-freeze the aliquots in liquid nitrogen.

3- Store frozen lysate at -70℃. These should be stable for 3-6 months.

4- Move on to treat and process the next lysate until all conditions are complete and protein concentrations have been equalized.

H- Prepare the next lysate

1- Move on to treat and process the next lysate until all conditions are complete and protein concentrations have been normalized to 0.5 mg/ml.

Lysate preparation continued

3D Tissue culture lysates

G-LISA assays have been used extensively for analysis of small G-protein activation in 3D cultures (Table 6 and see cytoskeleton.com for most current citations).

Table 6: G-LISA® analysis in 3D cell culture models

3D culture model	G-LISA analysis	Application and sample processing	Ref.
RECENT HEK293, PANC-1 and MDA-MB-231 spheroids in Geltrex or type I collagen	**RhoA, Rac1 and Cdc42 (BK135)**	Spheroids recovered with Cell Recovery Solution after four days and lysed in kit Cell Lysis Buffer. Lysates: 2 mg/ml for RhoA; 1 mg/ml for Rac1 and Cdc42.	**a**
RECENT Mouse intestinal organoids	**Cdc42 (BK127) Rac1 (BK128)**	Organoids were dissociated and FACS-sorted. Cells were pelleted, lysed in ice-cold kit buffer, snap-frozen and equalized by total protein concentration.	**b**
RECENT Engineered blood–brain barrier microvessels in collagen–hyaluronan hydrogel	**RhoA (BK124) Rac1 (BK128)**	After flow or static exposure, hydrogels were washed briefly with ice-cold PBS. Microvessels were extracted with 50 µl ice-cold Cell Lysis Buffer plus protease inhibitors.	**c**
RECENT Mouse oviduct epithelial organoids	**Cdc42 (BK127)**	Cdc42-GTP was measured in organoid cultures following treatment with Cdc42 activity inhibitors.	**d**
FOUNDATIONAL Human glioblastoma cells in 3D collagen matrices	**Rac1**	G-LISA compared EGF-induced Rac1 activation in 2D and 3D cultures and related Rac signaling to migration speed and persistence.	**e**
FOUNDATIONAL Human corneal fibroblasts in fibrillar collagen matrices	**Rac1 (BK126)**	Four collagen matrices were pooled per treatment group. G-LISA quantified Rac activation after 0, 10 or 30 minutes of PDGF exposure.	**f**
FOUNDATIONAL Cells cultured in 3D extracellular-matrix systems	**Small-GTPase signaling methods**	Methods chapter for investigating integrin regulation and downstream signaling in 3D culture systems, including G-LISA-compatible analysis.	**g**

a- Iwata W, *et al. Cell Reports.* 2025;44(12):116649.
b- Castillo-Azofeifa D, *et al. Cell Stem Cell.* 2023;30(2):188–206.e6.
c- DeOre BJ, *et al. FASEB Journal.* 2022;36(5):e22278.
d- Jiang R, *et al. Cell Death & Disease.* 2022;13:779.
e- Kim HD, *et al. Molecular Biology of the Cell.* 2008;19(10):4249–4259.
f- Petroll WM, *et al. Journal of Cellular Physiology.* 2008;217(1):162–171.
g- Keely PJ, *et al. Methods in Enzymology.* 2007;426:27–45.

General guidelines for processing cell lysates from 3D culture sample

Many 3D culture scaffolds are available. Consult the scaffold manufacturer's instructions and the references in Table 6. The following guidelines are recommended when preparing 3D-cultured cells for G-LISA® analysis:

1- Recover cells rapidly at 4°C by direct lysis of cells in the gel.

- Remove the gel from the culture well using forceps (volumes below are for a 35 mm^2 well).
- Briefly rinse the gel with ice-cold PBS.
- Gently blot the gel on blotting paper to remove residual PBS.
- Add the gel to 300–400 µl of ice-cold G-LISA® Cell lysis buffer (Part# GL36) supplemented with protease inhibitors (Part# PIC02) and necessary inhibitors not supplied in this kit e.g. kinase/phosphatase inhibitors as needed.
- Mechanically disrupt the scaffold using either: An 18-gauge needle to repeatedly shear the gel, or a micro homogenizer.
- Immediately centrifuge the homogenate to pellet insoluble scaffold material and cellular debris (10,000 x g, 4°C, 2 minutes).
- Transfer experiment sized aliquots of the clarified supernatant to labeled tube/s and snap-freeze for subsequent G-LISA® analysis.
- Do not use total protein concentration to normalize samples because most scaffolds contain high levels of intrinsic protein. Normalize samples according to cell number, as described below.

2- Use cell number when scaffold proteins interfere with protein measurement. Matrigel® and other protein-rich scaffolds can interfere with lysate protein quantification. When accurate protein measurement is not possible, normalize samples according cell number.

Determine the cell number using parallel gels prepared, cultured, treated, and harvested under identical conditions. Cell number may be determined by counting DAPI-stained nuclei or by another validated cell-counting method.

3- Avoid prolonged incubations and enzymatic digestion. Extended processing, particularly at elevated temperatures, or treatment with enzymes will alter the GTP-bound state of the target GTPase resulting in all samples having similar G-LISA® signal.

3D Lysate preparation continued

4-Avoid EDTA and unvalidated recovery reagents. Do not use EDTA to disrupt the scaffold. EDTA chelates Mg^{2+}, which is required to maintain guanine nucleotide binding by small GTPases.

If matrix recovery requires a chelating agent, EGTA may be less disruptive because it preferentially chelates Ca^{2+}. However, compatibility must be confirmed experimentally for the target GTPase, treatment concentration, and exposure time.

Avoid enzymatic or proprietary matrix-recovery reagents unless they have been validated for GTPase activation analysis.

Lysate preparation continued

Tissue lysates

G-LISA assays have been used extensively for analysis of small G-protein activation in tissue samples (Table 7 and see cytoskeleton.com for most current citations).

Table 7: G-LISA® analysis from tissue samples

Tissue extract	G-LISA analysis	Application and sample processing	Ref.
RECENT Mouse B16 tumors	**RhoA (BK124)**	Fresh tumor samples were homogenized directly in kit lysis buffer. Protein was measured immediately and lysates were equalized to approximately 0.5 mg/ml with less than 10% variation.	**a**
RECENT Mouse whole brain and cortical synaptosomes	**RhoA (BK124)** **Rac1 (BK128)**	Whole brains or crude synaptosome pellets were homogenized in kit lysis buffer with protease and phosphatase inhibitors. Protein was normalized with Precision Red before assay.	**b**
RECENT Live mouse kidney cortical slices	**Rac1 (BK128)**	Cortical kidney slices were treated ex vivo with a mitochondrial uncoupler, with or without an NCLX inhibitor. Rac1-GTP was then quantified from cortical proximal-tubule tissue.	**c**
RECENT Rat hippocampal tissue after cerebral ischemia	**RhoA (BK124)**	Hippocampi were homogenized in lysis buffer, total protein was measured by BCA, and absorbance at 450 nm was normalized to protein concentration.	**d**
FOUNDATIONAL Mouse left-ventricular tissue after ischemic injury	**RhoA (BK124)**	Left ventricles were homogenized in lysis buffer and protein was concentrated according to the assay protocol. RhoA activity was read by absorbance at 490 nm.	**e**
FOUNDATIONAL Glomeruli isolated from mouse kidneys	**RhoA (BK124)**	Kidneys were perfused and collagenase-digested, and glomeruli were magnetically isolated. Glomeruli were resuspended in lysis buffer with protease inhibitors and disrupted through a 22-gauge needle.	**f**

a- Wang et al. *Cell*. 2025;188(25):7155–7174.e25.
b- Ishchenko et al. *eLife*. 2025;13:RP103620.
c- Viquez et al. *Proc Natl Acad Sci USA*. 2025;122(47):e2504565122.
d- Chen et al. *ACS Omega*. 2024;9(11):13227–13238.
e- Jiao et al. *Exp Ther Med*. 2019;18(5):3783–3792.
f- Boucher et al. *Lab Invest*. 2012;92(5):662–675.

General guidelines for processing cell lysates from tissue samples

The end user is recommended to review the Recommended lysate preparation process, page 13 and the tissue preparation methods cited in Table 7 (see cytoskeleton.com for most current citations). The following guidelines are recommended when preparing tissue samples for G-LISA® analysis:

1- Freeze tissues immediately after harvest

- Process tissue immediately after collection. Remove blood, fat and unwanted tissue. Complete tissue handling as rapidly as possible and maintain the sample at approximately 4°C throughout.
- Cut the tissue into pieces approximately 3-5 mm in diameter, immediately snap-freeze them in liquid nitrogen, and store at –70°C or below. The frozen samples can then be processed individually and normalized to the same protein concentration, as described for 2D cell-culture lysates.

Critical: Do not allow the tissue or lysate to warm during processing. Delays or elevated temperatures may reduce the amount of GTP-bound GTPase detected.

2- Homogenize and Clarify the Tissue Lysate

- We recommend processing each tissue sample individually.
- Pulverize the frozen tissue in a micro-pestle. This will allow efficient protein extraction.
- Place the tissue in a pre-chilled microcentrifuge tube and add the appropriate volume of ice-cold G-LISA® Cell lysis buffer (Part# GL36) supplemented with protease inhibitors (Part# PIC02) and necessary inhibitors not supplied in this kit e.g. kinase/phosphatase inhibitors as needed. Suggested starting volume for tissue lysates is 100 µl buffer per mg tissue.
- Keep the tube on ice and homogenize the tissue thoroughly using a micro-homogenizer.
- Centrifuge the homogenate at 10,000 x g for 1 minute at 4°C to remove insoluble material.
- Transfer the clarified supernatant to a clean, pre-chilled microcentrifuge tube.

3- Determine the protein concentration and store the lysate

- Determine the protein concentration of the lysate as described for the 2D lysate preparation. Dilute the lysate to **0.5 mg/ml** with supplemented G-LISA® Cell lysis buffer.
- Divide it into single-experiment aliquots to avoid repeated freeze–thaw cycles.
- Snap-freeze the aliquots in liquid nitrogen and store them at −70°C or below until use.
- Move on to process the next tissue sample making sure to equalize the protein concentration with the first tissue sample.

G-LISA® assay protocol

Notes prior to assay

- **First-time users:**
  Read the Important technical notes section before performing the G-LISA® assay.

- **Lysate preparation:**
  Follow the cell and tissue processing guidelines provided in the Lysate preparation section of this manual.

  We strongly recommend snap-freezing lysates in experiment-sized aliquots and storing them at −70°C until use. Thaw each aliquot only once. **DO NOT refreeze lysates.** Discard any unused lysate remaining after thawing.

  The following instructions assume that frozen lysates are being used for the assay. Lysates should remain frozen until indicated in the assay protocol.

  If fresh lysates are to be used it is critical that they are processed rapidly after preparation to avoid non-physiological changes in the GTP state of your small G-protein target. This can occur due to intrinsic GTP hydrolysis or residual GEF/GAP activity in the lysate.

Assay Preparation

30 minutes prior to beginning the assay, prepare the following reagents and materials:

Table 8: Reagent preparation prior to assay

Ice supply for lysates and some buffers	na	4°C	The assay is temperature sensitive
Ral-GTP binding plate	GL34B	Room temp.	Equilibrate to room temperature inside the sealed desiccant bag
RalA control protein Constitutively active	RLCA	4°C	Reconstitute with 500 µl of G-LISA® Cell lysis buffer (GL36) and place on ice. Discard any unused RLCA.
Sterile, nanopure water	na	4°C	30 ml
G-LISA® Cell lysis buffer	GL36	4°C	
Wash buffer	PE38	Room temp.	
Ral binding buffer	GL52	4°C	
Antigen presenting buffer	GL39	Room temp.	
Antibody Dilution Buffer	GL40	Room temp.	
Precision Red® advanced protein assay reagent	GL50	Room temp.	Only required if lysate protein concentrations need to be equalized after thawing.
G-LISA® Cell lysis buffer containing 1x protease inhibitors	GL36 + PIC02	4°C	10 µl of 100x protease inhibitor stock per 1 ml of G-LISA® Cell lysis buffer. Only required if lysate protein concentrations need to be equalized prior to assay.

Prepare Controls and Plate

1- Prepare the buffer blank control.
Combine 60 µl G-LISA® Cell lysis buffer (GL36) with 60 µl ice-cold Binding Buffer (GL52) in a labeled microcentrifuge tube. Mix and keep on ice. This volume is sufficient for two wells.

2- Prepare the RalA positive control.
   a- Briefly centrifuge a tube of RalA positive control protein (Part# RLCA) to make sure the protein pellet is in the bottom of the tube.

   b- Reconstitute the protein in 500 µl of G-LISA® Cell lysis buffer (Part# GL36), mix well and place on ice.

   c- Combine 40 µl RalA Control Protein with 26 µl G-LISA® Cell lysis buffer.Add 66 µl ice-cold Binding Buffer (Part# GL52).

   d- Mix and keep on ice. This volume is sufficient for two wells and provides **3 ng positive control** per well.

   e- Briefly centrifuge a tube of RalA positive control protein (Part# RLCA) to make sure the protein pellet is in the bottom of the tube.

3- Prepare the Ral-GTP binding plate (Part# GL34B)
Remove the plate from its pouch and gently peel back the seal. Transfer the required number of strips to the extra strip holder and place on ice. Immediately reseal the remaining strips in the pouch with desiccant and return to storage.

   Add 100 µl ice-cold nanopure water to each assay well to dissolve the protective powder. Leave on ice until use. NOTE: wells should be used within 5-15 minutes of rehydration.

Thaw experimental lysates

4- Thaw snap-frozen cell lysates in a room-temperature water bath. As soon as the lysates are thawed, immediately place them on ice.

5- If necessary, add the calculated volume of G-LISA® Cell lysis buffer to each sample to equalize lysate concentrations.

NOTE: Calculate all required dilution volumes before thawing the lysates to minimize sample-processing time.

6- Transfer sufficient lysate to pre-chilled microcentrifuge tubes:

- 60 µl for duplicate wells
- 90 µl for triplicate wells

7- Add an equal volume of ice-cold Binding Buffer to each lysate. Mix thoroughly by pipetting and keep on ice.

Ral-GTP (active Ral) binding

8- Completely remove the water from the assay wells by vigorously flicking the plate, then patting the inverted plate firmly 5–7 times on paper towels.

NOTE: Complete liquid removal between assay steps is critical for minimizing background. Buffer blank wells should typically give an absorbance of 0.05–0.12 at 490 nm. Higher values may indicate insufficient liquid removal.

9- Return the plate to ice.

10- Immediately add 50 µl prepared lysate to the appropriate wells.

11- Add 50 µl buffer blank control to duplicate wells.

12- Add 50 µl RalA positive control to duplicate wells.

Immediately incubate the plate on **a cold orbital microplate shaker at 4°C for exactly 20 min.**

NOTE: An orbital microplate shaker operating at ≥200 rpm is required. Rockers and slower shakers are not suitable. Use 400 rpm if possible; 200 rpm minimum.

Primary antibody incubation

13- During the lysate incubation, dilute the **anti-RalA primary antibody 1:50** in Antibody Dilution Buffer:

Table 9: Primary antibody dilutions

1	1.2	58.8
2	2.4	117.6
3	3.6	176.4
4	4.8	235.2
5	6.0	294.0
6	7.2	352.8
7	8.4	411.6
8	9.6	470.4
>8 wells	1.2 x # wells	58.8 x # wells

*Volumes required have been multiplied by 1.2 to allow for pipetting error.

14- After the Ral GTP binding step is complete, vigorously flick out the well contents. Wash the wells **twice** as rapidly as possible with **200 µl** room-temperature Wash Buffer per well using a multichannel pipettor. After each wash, completely remove the buffer by vigorous flicking and patting as described in Step 8.

Do not leave the plate unattended during this stage.

15- Place the plate on the bench at room temperature.

16- Immediately add **200 µl** room-temperature **Antigen Presenting Buffer** to each well. Incubate at room temperature for exactly 2 min.

17- Vigorously flick out the Antigen Presenting Buffer and pat the inverted plate 5–7 times on paper towels.

18- Immediately wash the wells three times with 200 µl room-temperature Wash Buffer. Completely remove the buffer after each wash.

19- Add **50 µl** diluted **anti-RalA primary antibody** (Part# GL08) to each well. Incubate on an orbital microplate shaker at 200–400 rpm for **45 min at room temperature**.

G-LISA® assay protocol continued

Secondary Antibody Incubation

20- During the primary antibody incubation, dilute the HRP-conjugated **secondary antibody 1:100** in Antibody Dilution Buffer:

Table 10: Secondary antibody dilutions

1	0.6	59.4
2	1.2	118.8
3	1.8	178.2
4	2.4	237.6
5	3.0	297
6	3.6	356.4
7	4.2	415.8
8	4.8	475.2
>8 wells	0.6 x # wells	59.4 x # wells

*Volumes required have been multiplied by 1.2 to allow for pipetting error. Dilutions are shown for up to one plate strip of wells.

21- After the primary antibody incubation, vigorously flick out well contents and pat the inverted plate 5–7 times on paper towels.

22- Immediately wash the wells three times with 200 µl room-temperature Wash Buffer, completely removing the buffer after each wash.

23- Add **50 µl** diluted **secondary antibody** to each well. Incubate on an orbital microplate shaker at 200–400 rpm for **45 min at room temperature**.

HRP detection

During the secondary antibody incubation, prepare the HRP Detection Reagent by mixing equal volumes of Reagents A and B. For each 8-well strip, combine:

- 250 µl Reagent A
- 250 µl Reagent B

  Thaw Reagents A and B in a room-temperature water bath and remove them as soon as they thaw. Protect the mixed reagent from light. The mixture is stable for 1 h at room temperature. Discard unused mixed reagent and immediately refreeze unused unmixed reagents.

24- After the secondary antibody incubation, vigorously flick out the well contents and pat the inverted plate 5–7 times on paper towels.

25- Immediately wash the wells three times with 200 µl room-temperature Wash Buffer, completely removing the buffer after each wash.

26- Add **50 µl HRP Detection Reagent** to each well and incubate for **15 min** at **room temperature.**

27- Add **50 µl** HRP Stop Buffer to each well.

28- Immediately measure absorbance at 490 nm using a microplate spectrophotometer. Designate the buffer blank control wells as the assay blank.

Data analysis

Data can be plotted as a simple excel bar chart;

Subtract the Lysis Buffer Background

Use the Lysis buffer-only wells as assay blanks. Subtract the mean Lysis Buffer absorbance from all sample and control readings before analyzing the data. Most plate reader software can perform blank subtraction automatically when the appropriate wells are designated as blanks. Refer to your plate reader software instructions for details. Alternatively, blank subtraction can be performed manually in Excel or other data-analysis software.

Organize the Data

Transfer the blank-corrected data into a spreadsheet using the following columns:

Sample | Mean | Standard Deviation | Rep 1 | Rep 2 | Rep 3 | Rep 4
Enter samples in the same order in which they were assayed on the plate. Enter the individual replicate values under Rep 1–Rep 4. If fewer than four replicates were performed, leave the unused cells blank.

Calculate the Mean

Calculate the mean absorbance for each sample from its replicate values.

In Excel, use:

=AVERAGE(Xn)
where X and Y are the first and last replicate columns and n is the sample row.

Calculate the Standard Deviation

Calculate the standard deviation (SD) for each set of replicates.

In Excel, use:

=STDEV.S(Xn)

where X and Y are the first and last replicate columns and n is the sample row.

Copy the Mean and SD formulas down the spreadsheet for all samples.

Plot the Results

Create a column chart using the sample names and mean values:

- X-axis: Sample
- Y-axis: Blank-corrected absorbance
- Error bars: Standard deviation

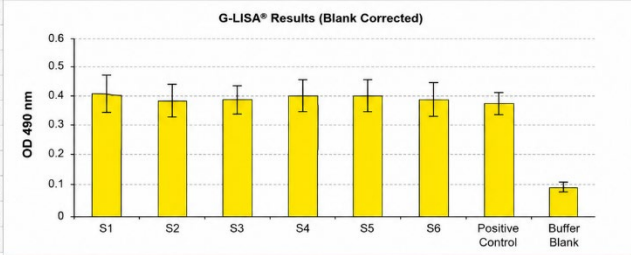
Add custom error bars using the values in the Standard Deviation column.

A representative spreadsheet layout and data plot are shown in Figure 3.

Figure 3: Typical excel layout

Template: G-LISA® Data Analysis

	A	B	C	D	E	F	G
1	Date:	mm/dd/yyyy					
2	Name:						
3	Animal/experiment:						
4	IC used:	(Insert IC/protein description)					
5							
6	Sample name	Rep 1	Rep 2	Rep 3	Rep 4	Mean (Blank Corrected)	St Dev (±)
7	S1	0.40	0.39	0.41	0.40	**0.400**	0.008165
8	S2	0.40	0.36	0.39	0.41	**0.390**	0.020548
9	S3	0.39	0.40	0.38	0.39	**0.390**	0.008165
10	S4	0.41	0.39	0.40	0.40	**0.400**	0.008165
11	S5	0.40	0.41	0.38	0.40	**0.398**	0.012910
12	S6	0.40	0.36	0.39	0.40	**0.388**	0.020548
13	Positive Control	0.40	0.09	0.36	0.36	**0.370**	0.020000
14	Buffer Blank	0.10	0.09	—	—	**0.095**	0.007071



Formulas used (example shown for row 7):

- Mean (F7): =AVERAGE(B7:E7)
- Standard Deviation (G7): =STDEV.S(B7:E7)

Copy the formulas down for all samples.

Instructions:

1. Use Lysis Buffer wells as blanks and subtract the mean blank from all sample and control readings.
2. Enter replicate values in columns B–E.
3. Excel ignores blank cells in calculations.
4. Create a column chart using Sample name and Mean. Add error bars using the St Dev column (custom error bars).

Need an Excel template? Contact Cytoskeleton Technical Support at tservice@cytoskeleton.com.

Important technical notes

Manual updates from previous version

The following updates have been incorporated into BK129 v19.0

- The Ral-binding plate has been redeveloped to provide a more robust signal from cell lysates. The updated plate, Part # GL34B, replaces Part # GL34 and provides a similar RalA activation window (see Figure 4).
- The updated assay requires fivefold less GL08 anti-RalA primary antibody than the original GL34 plate. Therefore, GL08 Lot 061 and onward contain fivefold less anti-RalA antibody. The antibody reconstitution and working dilution remain unchanged.

Figure 4: Active RalA measurements in GL34 plates vs GL34B plates

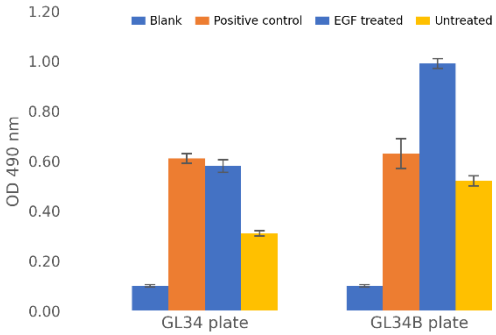


*Figure 4 legend: Rat2 cells were grown to 70% confluence and serum starved for 48 hours. Cells were the either treated with EGF (100 ng/ml) for 2 minutes or left untreated. Lysates were harvested in cell G-LISA® Cell lysis buffer and snap frozen immediately in assay sized aliquots and stored at -80°C. The G-LISA® assay was performed with plate GL34 and GL34B as outlined in this manual except that plate GL34 used 5X the amount of GL08 antibody; 12 µg of lysate was used per assay. A 3 ng sample of constitutively active RalA was included in the assay as a positive control (part # RLCA). The background subtracted activation level of EGF treated vs serum starved cell lysates was determined to be **2.24x** for plate **GL34** and **2.28x** for plate **GL34B**.*

Important technical notes continued

Use of a multichannel pipette

A single-channel pipette can be used to add the initial lysates and controls. For all subsequent steps, use a multichannel pipette capable of dispensing 25–200 µl. This is essential for completing time-sensitive steps, particularly washing, Antigen Presenting Buffer and developer solution addition, rapidly and consistently across all wells.

Consider experiment specific inhibitors

The protease inhibitor cocktail supplied in the kit contains pepstatin A (aspartic protease inhibitor), leupeptin (serine, cysteine & threonine protease inhibitor), benzamidine (serine protease inhibitor) and TAME (arginine protease inhibitor).

If your experimental system requires additional inhibitors, such as **kinase or phosphatase inhibitors**, these are not included with the kit and should be supplied by the end user.

Guidelines for control lysates

It is often beneficial to prepare control lysates, designed to give a known activation/inhibition profile. Some general guidelines are given below;

2D cultures

- Serum starvation vs a known RalA activator (see Table 11). Treatment with growth factors like epidermal growth factor (EGF) compared to untreated serum starved cell lysates has been shown to activate the RalA pathway. As responses are cell line specific, it is recommended to use a known activator for your given cell line.

3D cultures

- Consider using known RalA inhibitors such as BQU57.

Tissues

- When a positive tissue lysate control is required, use a tissue known to express the GTPase of interest and select an experimentally validated treatment or physiological condition that increases activation of the target GTPase.

Table 11: RalA activators

Activator / stimulus	RalA activation pathway	Cell line / cell type	Ref.
RECENT **Quinine**	Bitter taste GPCR signaling → Ras p21/MAPK activation; RalA inhibition or no effect	MCF-10A and MCF-7 mammary epithelial cells	**a**
RECENT **RalA overexpression**	CRISPR/Cas9 Rosa26 hRalA knock-in → increased RalA-GTP → RAC1-dependent HSC/LSC expansion and leukemogenesis	Mouse bone-marrow HSCs/HSPCs and BCR-ABL1-transformed LSCs	**b**
FOUNDATIONAL **De novo RALA variants**	GTP/GDP-binding-region variants → altered GTP hydrolysis and effector binding; S157A increased effector binding	Purified recombinant RALA proteins; variants identified in 11 affected individuals	**c**
FOUNDATIONAL **EGF**	EGFR → PLC/Ca^{2+} and Ras/RalGEF signaling → RalA-GTP	Rat-2 and 3Y1 fibroblasts; T24/T24T bladder carcinoma cells	**d–e**
FOUNDATIONAL **LPA**	LPA receptor → PLC → increased intracellular Ca^{2+} → Ral activation	Rat-2 fibroblasts	**e**
FOUNDATIONAL **PMA**	PKC-dependent signaling → increased RalA-GTP	T24/T24T bladder carcinoma cells	**d**

a- Bhatia et al. *Mol Cell Biochem.* 2024;479:567–577.
b- Yin et al. *Int J Biol Sci.* 2023;19(4):1211–1227.
c- Hiatt et al. *PLoS Genet.* 2018;14(11):e1007671.
d- Gildea et al. *Cancer Res.* 2002;62(4):982–985.
e- Hofer et al. *Curr Biol.* 1998;8(14):839–842.

Do not lyse samples at high initial protein concentrations

G-LISA® lysis buffers are formulated to minimize GEF and GAP activity and help preserve the GTPase activation state present at the time of harvest. However, residual enzymatic activity may still occur after lysis.

To minimize this risk, prepare lysates rapidly using ice-cold buffers and target an initial protein concentration of 0.5–2.0 mg/ml.

Do not prepare a highly concentrated lysate (e.g., 5 mg/ml) and then dilute it to 0.5 mg/ml. At high initial protein concentrations, residual GEF or GAP activity may alter the amount of GTP-bound GTPase before the sample is diluted. Dilution afterward cannot reverse these changes. Best practice for lysate harvest is given in Recommended lysate harvest workflow (page 13).

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2- Colicelli J. Human RAS superfamily proteins and related GTPases. *Sci STKE.* 2004;2004(250):RE13.

3- Richardson DS, Spehar JM, Han DT, Chakravarthy PA, Sizemore ST. The RAL enigma: distinct roles of RALA and RALB in cancer. *Cells.* 2022;11(10):1645. doi:10.3390/cells11101645

4- Zago G, Biondini M, et al. A family affair : A Ral-exocyst-centered network links Ras, Rac, Rho signaling to control migration. Small GTPases. 2017;10(5):323-330.doi:10.1080/21541248.2017.1310649

5- Thulasi N, Harithpriya K. et al. Targeting the Ras-Ral signaling axis in tupe 2 diabetes mellitus: A dual-modulation approach to correcting insulin resistance and beta-cell dysfunction. Pharmaceuticals. 2026;19(4):648. Doi:10.3390/ph19040648

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NOTES

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