V 2.3

G-LISA® Arf1 Activation Assay

Biochem Kit

(Absorbance Based)

Cat. # BK132

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Manual Contents

Background

ADP ribosylation factor proteins 1-6 (Arfs) belong to the family of Ras small GTPases that are involved in various aspects of membrane trafficking events (1) including controlling of the assembly and disassembly of vesicle coat proteins (2, 3). Arf1 is the most abundant and best –characterized among the Arf GTPases and studies have shown that it regulates function of the Golgi, endoplasmic reticulum-to-Golgi transport, recruitment of transport vesicle coat proteins such as COPI, AP-1, and AP-3 (4-6).

The Arf switch operates by alternating between an active, GTP-bound state and an inactive, GDP-bound state. Understanding the mechanisms that regulate activation / inactivation of the GTPases is of obvious biological significance and is a subject of intense investigation. The fact that Arf family effector proteins preferentially recognize the GTP bound form of the protein (7) has been exploited experimentally to develop a powerful affinity purification assay that monitors Arf protein activation (8, 9).

Traditionally, the Arf1 activation assay has been performed using a pull-down method, wherein the Arf1-GTP binding domain of a Arf1 effector is coupled to agarose beads, allowing affinity based detection of the active Arf1 in biological samples (9). This method suffers from several drawbacks such as being time consuming, requiring large amounts of total cellular protein, being limited in the number of samples that can be handled simultaneously and yielding only semi-quantitative results.

The Arf1 G-LISA® Advantage

With the new G-LISA® kit (patent# 7,763,418B2) you can now measure Arf1 activation from cell and tissue samples in less than 3 h. G-LISA® requires only 1-5% of the material needed for a conventional pull-down assay. You will also be able to handle large sample numbers and generate quantitative results. The G-LISA® advantages are summarized in Table 1.

Table 1: The G-LISA® Advantage

	Traditional pull-down	G-LISA®
Assay Time	10-12 h (2 days)	<3 h
Cell material per assay	1-2 mg protein (100 mm plate)	10-50 µg protein (12-well plate)
*Lysate clarification needed**	Yes	No
Sample handling	Up to 10 samples	Up to 96 samples (or more)
*Quantitative Data***	Semi	Yes

* Clarification is still recommended for low sample numbers. HTS applications that omit clarification have been developed.

** Numerical readouts and fewer sample handling steps make this assay more quantitative.

Assay Principle

The Arf1 G-LISA® kit contains a Arf-GTP-binding protein linked to the wells of a 96 well plate. Active, GTP-bound Arf1 in cell lysates will bind to the wells while inactive GDP-bound Arf1 is removed during washing steps. The bound active Arf1 is detected with a Arf1 specific antibody. The degree of Arf1 activation is determined by comparing readings from activated cell lysates versus non-activated cell lysates. A basic schematic diagram of the steps involved in the G-LISA® is shown in Figure 1. Typical G-LISA® results are shown in Figure 2.

Figure 1: Simple and Quick Protocol

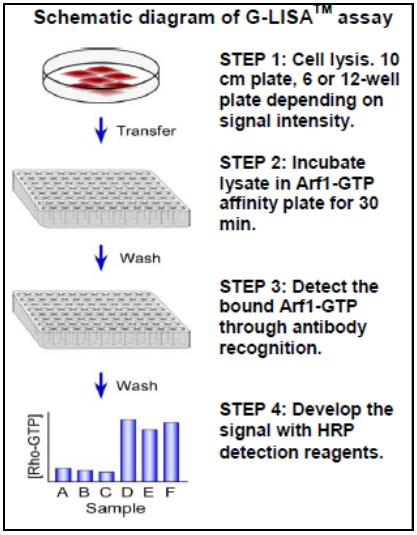
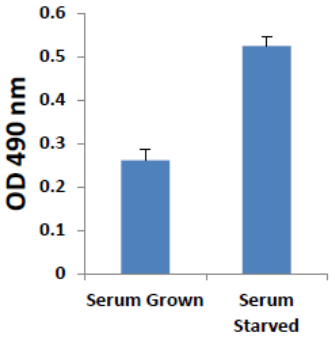


Figure 2: Typical G-LISA® Results



Arf1 activation by serum depletion measured by G-LISA®. C2C12 cells were grown in medium supplemented with 10% serum. Cells were then switched to serum free medium and incubated for an hour. 12.5 µg of cell lysates were subjected to the G-LISA®

II: Purchaser Notification

Limited Use Statement

The G-LISA® kits are based on patented technology developed at Cytoskeleton Inc. (Patent # 7,763,418B2). The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of product and components of product in research conducted by the buyer. The buyer cannot sell or otherwise transfer this product or any component thereof to a third party or otherwise use this product or its components for commercial purposes. Commercial purposes include, but are not limited to: use of the product or its components in manufacturing; use of the product or its components to provide a service; resale of the product or its components.

The terms of this Limited Use Statement apply to all buyers including academic and for-profit entities. If the purchaser is not willing to accept the conditions of this Limited Use Statement, Cytoskeleton Inc. is willing to accept return of the unused product with a full refund.

III: Kit Contents

This kit contains enough reagents for 96 assays. You can run anywhere from 2 to 96 samples at a time for your own convenience. Table 2 summarizes the kit contents.

Table 2: Kit Contents

Reagents	Cat. # or Part # *	Quantity	Storage
96 well Arf1-GTP binding plate	Part # GL54	12 strips of 8 wells each	Desiccated 4°C Stable for 6 months
Anti-Arf1 antibody	Part # GL12	2 tubes, lyophilized	Desiccated 4°C Stable for 3 months
Secondary antibody - horse radish peroxidase conjugate (HRP)	Part # GL02	1 tube, lyophilized	Desiccated 4°C Stable for 6 months
Arf1 control protein (constitutively active Arf1)	Part # A1CA	12 tubes, lyophilized	Desiccated 4°C Stable for 6 months
Cell Lysis Buffer	Part # GL36	1 bottle, lyophilized	Desiccated 4°C Stable for 1 year
Wash Buffer	Part # PE38	1 tablet	4°C Stable for 6 months
Binding Buffer	Part# GL37	1 bottle, lyophilized	Desiccated 4°C Stable for 6 months
Antigen Presenting Buffer	Part # GL39	1 bottle, 30 ml	Room temperature Stable for 6 months
Antibody Dilution Buffer	Part # GL40	1 bottle, lyophilized	Desiccated 4°C Stable for 6 months
HRP Detection Reagent A	Part # GL43	1 tablet, silver pack	4°C Stable for 6 months
HRP Detection Reagent B	Part # GL44	1 tablet, gold pack	4°C Stable for 6 months
HRP Stop Solution	Part # GL80	1 bottle, 8 ml	4°C Stable for 6 months
Precision Red™ Advanced Protein Assay	Part # GL50 (available in 500 ml size Cat. # ADV02)	1 bottle, 100 ml	Room temperature Stable for 6 months
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized	Desiccated 4°C Stable for 6 months

*** Items with Part numbers (Part #) are not sold separately and are available only in kit format. Items with Catalog numbers (Cat. #) are available separately.**

The 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).
- Concentrated sulfuric acid (need to add 1 ml to HRP Stop Buffer)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates
- Multi-channel or multi-dispensing pipettor for 25-200 µl range.
- Multi-channel pipettor solution basins (available from VWR Cat. # 21007-970). Used for liquid handling.
- Vortex for mixing cell lysate and Binding Buffer solutions.
- Two orbital microplate shakers (VWR, Cat. # 57019). Optimal shaker speed is 400 rpm (200 rpm is the minimal speed required). One at room temperature and one at 4°C.
- Microplate spectrophotometer (see Section V: Important Technical Notes for information on settings etc.)

IV: Reconstitution and Storage of Components

Many of the components of this kit have been provided in lyophilized form. Prior to beginning the assay you will need to reconstitute several components as shown in Table 3:

Table 3: Component Storage and Reconstitution

Kit Component	Reconstitution	Storage Conditions
Arf1-GTP binding 96 well plate	It is imperative to keep the plate in the sealed desiccant bag with desiccant at all times. Reconstitution is not necessary prior to the start of the assay. The protective white powder pellet in each well of the plate may become detached from the bottom of the well during shipping. This will not affect the assay performance. Pellets should be tapped down to the bottom of the well prior to resuspension.	Store desiccated at 4°C Stable for 6 months
Anti-Arf1 antibody	Centrifuge briefly to collect the pellet in the bottom of the tube. Resuspend each tube in 100 µl PBS.	Store at 4°C Stable for 3 months
Secondary antibody HRP	Centrifuge briefly to collect the pellet in the bottom of the tube. Dissolve the powder in 80 µl of PBS. Do not use sodium azide in combination with this antibody as it will inactivate the HRP.	Store at 4°C Stable for 6 months
Arf1 control protein (12 tubes)	Each tube is good for one experiment. Reconstitution is not necessary until starting the assay (see Table 4).	Store desiccated at 4°C Stable for 6 months
Cell Lysis Buffer	Reconstitute in 100 ml of sterile distilled water. This may take 5-10 min to resuspend. Use a 10 ml pipette to thoroughly resuspend the buffer.	Store at 4°C Stable for 1 year
Wash Buffer	Reconstitute in 1000 ml of distilled water. This will take 45-60 min to resuspend. A magnetic stir bar and stir plate can be used to help resuspension.	Store at room temperature Stable for 6 months
Antigen Presenting Buffer	No reconstitution necessary.	Store at room temperature Stable for 6 months
Binding Buffer	Reconstitute in 5 ml of sterile distilled water. This solution will take 5-10 min to resuspend. Use a 5 ml pipette to thoroughly resuspend the buffer.	Store at 4°C Stable for 6 months
Antibody Dilution Buffer	Reconstitute in 15 ml of sterile distilled water.	Store at 4°C Stable for 6 months
HRP Detection Reagent A	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes. Place in -70°C freezer for storage. NOTE -20°C is NOT good for storage	Store at -70°C Stable for 6 months
HRP Detection reagent B	Resuspend tablet in 10 ml sterile distilled water. Aliquot into 12 x 0.8 ml volumes. Place in -70°C freezer for storage. NOTE -20°C is NOT good for storage.	Store at -70°C Stable for 6 months
HRP Stop Solution	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 4°C.	Store at 4°C Stable for 6 months
Precision Red™ Advanced Protein Assay	No reconstitution necessary.	Store at room temperature Stable for 6 months
Protease Inhibitor Cocktail	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100 x stock.	Store at 4°C for 6 month stability. Note: the cocktail will freeze at 4°C. Store at -70°C for 1 year stability.

A. Updated Version Review

Changed part # GL32 to PE32 to reflect removal of 1L bottle from the product.

B. Growth and Treatment of Cell Lines

The ability to obtain reproducible activation of Arf1 in cells is dependent on the health and responsiveness of the cell type you are using. Experimental conditions described in the literature for Arf1 activation vary greatly, however, some basic principles for the treatment of cultured cells may prove beneficial if applied to your particular cell type and experimental design. The ideal culture conditions for Arf1 activation minimize the Arf1-GTP levels in the untreated "controlled state" and have a "responsive state" that maximizes the cellular response to the Arf1 activator being utilized.

Quite often the most critical element contributing to consistent and reproducible Arf1 activation is identifying the best "controlled state" for your particular cell type. The "controlled" state reflects conditions that favor a low basal level of Arf1 in the GTP-bound active state. For adherent cell types, two factors that can influence this state are the confluency of the cells in the culture vessel and the surface on which the cells are plated (e.g. tissue culture treated plastic or coated surfaces containing collagen, fibronectin etc.). These are parameters that can vary with each cell type being tested and each Arf1 activator being evaluated. Some cell types should not be allowed to grow to confluency (e.g Swiss 3T3 mouse fibroblasts), whereas other cell types may work best at confluency (e.g. endothelial cells). Engagement of the integrin receptors on the surface of cells by the matrix they are plated onto can also influence the basal level of Arf1-GTP and this will need to be evaluated for each cell type.

In addition to cell density and growth surface, the media the cells are growing in can have a profound effect on the basal level of Arf1-GTP.

Once the optimal "controlled state" of the cells is achieved, they should be checked for their responsiveness (i.e. their "responsive state") to a known stimulus. It should be noted that poor culturing technique can result in essentially non-responsive cells. This primarily occurs when cells are passaged too many times or are allowed to overgrow repeatedly.

If you are having difficulty determining a "controlled state" for your experiment then contact technical assistance at 303-322-2254 or e-mail tservice@cytoskeleton.com.

C. Assay Preparation For G-LISA®

It is critical to get the assay components ready before preparing cell lysates or thawing previously prepared lysates, as the GTP-bound form of Arf1 is very labile and should be assayed as soon as possible after preparation.

Table 4: Assay Preparation for G-LISA®

Reagent	Preparation
Arf1-GTP binding 96 well plate	1. Remove plate from 4°C and keep in its protective bag, place on your bench at room temperature for 30 min. 2. Do not remove the plate (or strips) from the bag until immediately prior to the experiment.
Distilled water	30 ml placed on ice.
Protease Inhibitor Cocktail	Keep at room temperature.
Lysis Buffer	1. Determine volume of Lysis Buffer needed per culture vessel by looking at Table 5. 2. Determine **total volume** of Lysis Buffer needed by multiplying the lysis volume per culture vessel (µl) by number of vessels x 1.3 (see Table 5 for guidelines). 3. Aliquot this volume of Lysis Buffer into a clearly labeled tube and place in ice. 4. Add 10 µl of protease inhibitor cocktail per ml of aliquoted Lysis Buffer. 5. Mix well and leave on ice. 6. Lysis Buffer needs to be **ice cold**.
Arf1 control protein	Dissolve one tube in 100 µl Lysis Buffer and leave on ice. This 1 ng/ml stock solution is good on ice for up to 8 h.
PBS pH 7.2	Phosphate buffered saline is not provided in the kit. It should be prepared prior to the assay and placed on ice for at least 30 min to ensure that it is ice cold.
Anti-Arf1 antibody	Have primary antibody stock ready on ice. For each 8-well strip, you will need to mix 12.5 µl antibody with 500 µl Antibody Dilution Buffer. This dilution step should be performed just prior to use as detailed in assay protocol.
Secondary antibody	Have secondary antibody stock ready on ice. For each 8-well strip, you will need to mix 2.5 µl antibody with 500 µl Antibody Dilution Buffer. This dilution step should be performed just prior to use as detailed in assay protocol.
Antibody Dilution Buffer	Place reconstituted buffer on the bench and use at room temperature.
Binding Buffer	Embed bottle in ice.
Wash Buffer	Place on the bench and use at room temperature.
Antigen Presenting Buffer	Place on the bench and use at room temperature.
HRP Detection Reagents A and B	The 0.8 ml aliquots of these reagents can remain at -70°C until secondary antibody addition as detailed in the assay protocol.
HRP Stop Solution	Make sure that the box on top of the bottle is checked, indicating sulfuric acid has been added to the solution. Place the bottle on your bench and allow to warm to room temperature.
Precision Red™ Advanced Protein Assay Reagent	Place on the bench and use at room temperature.

D. Timing and Intensity of Arf1 Activation

When using an Arf1 activator that hasn't been well characterized in the literature, it is best to consider a time course of Arf1 activation. Arf1 activation can occur rapidly and be transient in nature, which may be missed if a single time point is chosen for an experiment. Recommended time points for an Arf1 activator that is transient in nature are 0, 1, 3, 6, 12 and 30 minutes.

In practical terms the timed experiment should be performed sequentially. This allows rapid processing of each single time point. Once one time point lysate is collected, it should be snap frozen in "experiment sized" aliquots immediately and kept at -70°C. The G-LISA® kit uses 25 µl of lysate (0.5-1.0 mg/ml lysate protein concentration) per assay. We recommend duplicate or triplicate samples per time point or condition, therefore 80-120 µl aliquots are recommended for snap freezing.

E. Rapid processing of cells

GTP bound (active) Arf1 is a labile entity, the bound GTP is susceptible to hydrolysis by Arf-GAPs during and after cell lysis resulting in Arf1 inactivation. The following guidelines are useful for rapid washing of cells.

Washing

1. Retrieve culture dish from incubator, immediately aspirate out all of the media and place firmly on ice.

2. Immediately rinse cells with an appropriate volume of ice cold PBS to remove serum proteins (see Table 5 for recommended wash volumes).

3. Aspirate off all residual PBS buffer. This is essential so that the Lysis Buffer is not diluted. Correct aspiration requires that the culture dish be placed at an angle on ice for 1 min to allow excess PBS to collect in the vessel for complete removal.

Cell Lysis

To avoid making too dilute or too concentrated lysate samples (<0.25 or >2.0 mg/ml), it is recommended to adjust the amount of Lysis Buffer depending on your cell type and plate type. Table 5 gives guidelines for suitable lysis volumes for 3T3 cells which tend to give low protein yields. The exact lysis volumes for any given cell line will have to be determined empirically.

Table 5: Recommended Wash and Lysis Volumes for 3T3 Cell Cultures

Culture Vessel	Vessel surface area (cm^2)	Volume of PBS wash (ml)	Volume of Lysis Buffer (µl)
35 mm dish	8	2.0	100
60 mm dish	21	3.0	150
100 mm dish	56	10.0	400
150 mm dish	148	15.0	1200
6-well cluster plate	9.5 / well	3.0	100
12-well cluster plate	4 / well	1.5	60
T-25 Flask	25	4.0	160
T-75 Flask	75	10.0	800
T-150 Flask	150	15.0	1200

Cell Lysis, continued

The time period between cell lysis and addition of lysates to the wells is critically important. Take the following precautions:

1. Work quickly.

2. Keep solutions and lysates embedded in ice so that the temperature is below 4°C. This helps to minimize changes in signal over time. The Assay Protocol (Section VI) gives very specific instructions regarding temperature and must be strictly adhered to for successful results.

3. We strongly recommend cell lysates should be immediately frozen after harvest and clarification. A sample of at least 20 µl should be kept on ice for protein concentration measurement. The lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Under these conditions, frozen lysates should be stable for several months.

4. Thawing of cell lysates prior to the use in the G-LISA® assay should be in a room temperature water bath, followed by rapid transfer to ice and immediate use in the assay.

F. Protein Concentration

Equal protein concentration in all samples is a prerequisite for accurate comparison between samples in Arf1 activation assays. Cell extracts should be equalized with ice cold Lysis Buffer to give identical protein concentrations. For example, cell lysates of protein concentrations ranging from 0.5–1.3 mg/ml would all need to be diluted to 0.5 mg/ml. We highly recommend that the final concentration of equalized lysates is between 0.5 –1 mg/ml. However, due to the difference of Arf1-GTP levels in different cell lines, we highly recommend you titrate your lysate. Serially dilute your lysate from "controlled cell state" samples (*e.g.* serum starved) and perform a G-LISA® assay with them. Pick the lysate concentration that gives OD reading (after buffer blank subtraction) between 0.1-0.4. By doing this the OD readings of activated cell vs. controlled cell are more likely to stay in the linear range of the assay. Protein concentrations below 0.25 mg/ml often will not work with sufficient accuracy to warrant performing the assay. It is not necessary to equalize protein concentrations if the variation between them is less than 10%.

The Precision Red® Advanced Protein Assay Reagent (Part # GL50) is included with the kit in order to measure protein concentration with a rapid one-step procedure. It is ideal for this analysis because it is detergent compatible and it is rapid and simple to perform. The protein assay can be performed in a 1 ml cuvette format as described in the Assay Protocol section. Alternatively, a 96-well format can be used where 10 µl of sample is pipetted into a well followed by the addition of 300 µl of Protein Assay Reagent. In this case the absorbance reading at 600 nm is multiplied by 3.75 to obtain the protein concentration in mg/ml.

G. Assay Linearity

The assay is linear from 1 to 20 ng of bound activated Arf1. The positive control protein is at 16 ng, therefore any assay readings that are more than 1.25x the positive control will be out of the linear range of the assay. In such cases you should reduce the amount of total cell protein per assay. The lower level of detection of the assay is approximately 20% above the background reading of Lysis Buffer only. Readings lower than this will require increased cell protein per assay.

H. Use of a Multi-channel Pipettor

When processing more than 16 wells it is imperative to use a multi-channel or multi-dispensing pipettor with a range of 25 to 200 µl per dispense. Critical steps such as lysate addition, post-binding wash step and the Antigen Presenting Buffer step all have requirements for accurate and timely additions. Attempting to perform >16 assays with a single channel pipettor will also increase the likelihood of allowing wells to dry out before reagent addition can be completed, resulting in variable signals. Therefore, use a multi-channel or at least a multi-dispensing pipettor wherever possible. If neither of these pipettor options is available we highly recommend that you limit each experiment to a maximum of 16 wells.

I. Removal of Solutions from Wells

Removal of solutions from the wells is accomplished by turning the plate upside down and flicking out the well contents into a waste bin. This is followed by patting the plate several times on a paper towel to get rid of residual solution. It has been found that the complete removal of solutions from the well requires a vigorous flick of the plate and a vigorous series of pats onto paper towels (5-7 hard pats). The complete removal of solution from wells between steps of the G-LISA® is very important as it avoids high background readings in the buffer only wells. The buffer only wells should read between 0.1-0.25 at an absorbance of 490 nm. If background readings are higher than 0.1-0.25 then a more vigorous removal of solutions from the well should be practiced.

J. Plate Shaker Recommendations

It is recommended to use an orbital plate shaker at 400 rpm. As a back-up you can use a 200 rpm orbital shaking culture incubator or a normal orbital rotating platform. Signals will be lower with the 200 rpm option.

K. Spectrophotometer Settings

The majority of the work in the design of this assay has been based on the Molecular Devices SpectraMax 250, which is one of the most sensitive machines available. The parameters of a protocol file for the instrument are given below as a reference:

Table 6. Spectrophotometer settings

Parameters	Character	Contents
Wavelength	490 nm	Bandwidth 2 nm (can be ± 20 nm for filter based machines)
Protocol	End point	Standard end point assay
Shaking	Medium, orbital	5 s
Temperature	24°C	Room temperature is also fine for readings

Please inquire to Technical assistance for help is setting up other machines (call 303-322-2254 for immediate help, or e-mail tservice@cytoskeleton.com for assistance within 24 h).

VI: Assay Protocol

It is crucial to the success of this assay that the section entitled "Important Technical Notes" be read thoroughly and followed accurately. The ⚠ sign indicates steps that have particularly critical "Important Technical Notes". Have copies of Appendices 2 and 3 ready to fill out as you go through the assay. Filling these out will be a good reference both for you and of vital importance in case you need technical support.

To keep the assay in the linear range we highly recommend you titrate the "controlled cell" (serum starved in most cases) lysate concentration with a G-LISA® assay. Choose the lysate concentration that gives an OD reading (after buffer blank subtraction) between 0.1-0.4. By doing this the OD readings of "activated cell" vs. "controlled cell" are more likely to stay in linear range.

⚠ STEP 1: Assay Preparation

At least one hour prior to beginning the assay, prepare all G-LISA® assay components as described in Section IV and Section V: Important Technical Notes, Table 4. Use the check-off list below to confirm that the following reagents are ready;

- Arf1-GTP binding plate, at room temperature in the desiccant bag ☐
- Wash Buffer, resuspended at room temperature ☐
- Precision Red™ Advanced Protein Assay Reagent, room temperature ☐
- Water, 30 ml, ice cold ☐
- HRP Stop Solution, acid added and at room temperature ☐
- Lysis Buffer, ice cold with protease inhibitors (see Table 5) ☐
- PBS, ice cold (see Table 5) ☐
- 1.5 ml microfuge tubes, kept on ice ☐
- Arf1 Control Protein, resuspended in Lysis Buffer on ice ☐
- Antibody Dilution Buffer, room temperature ☐
- Ice bucket containing ice ☐
- Cell scraper for cell collection ☐
- Liquid nitrogen for snap freezing cell lysates ☐
- Vortex close to assay area ☐

STEP 2: Lysate Collection

We strongly recommend you snap freeze your cell lysate in liquid nitrogen right after you harvest and clarify. This is especially necessary if you have many samples. Save a small amount of lysate before you snap freeze them for protein quantitation.

1. Treat cells as your experiment requires.
2. After treatment, place culture vessel on ice, aspirate media, wash with ice cold PBS, see Table 5, Section V for recommended volumes.
3. Aspirate off PBS. Tilt plates on ice for an additional 1 min and re-aspirate all remnants of PBS. Residual PBS will adversely affect the assay.
4. Lyse cells in an appropriate volume of ice-cold Cell Lysis Buffer; see Table 5, Section V for recommended volumes.
5. Harvest cell lysates with a cell scraper. It is useful to incline the culture plate for this method because the Lysis Buffer is spread thinly on the surface.
6. Transfer lysates into pre-labeled sample tubes on ice.
7. Immediately clarify by centrifugation at 14,000 rpm, 4°C for 1 min. (For high throughput screens or well characterized experiments the clarification step can be omitted).
8. At this point each lysate volume should not exceed 130% of the original Lysis Buffer volume.
9. Save at least 20 µl lysate in a clean microcentrifuge tube for protein quantitation assay.
10. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Keep them in -70°C for future use. It is recommended to aliquot in 120 µl volume per condition (sufficient for triplicate G-LISA® assays).
11. Measure lysate protein concentration as follows.
    a. Add 20 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
    b. Add 1 ml of Protein Assay Reagent to each cuvette.
    c. Incubate for 1 min at room temperature.
    d. Blank spectrophotometer with the Lysis Buffer +Protein Assay Reagent cuvette at 600 nm.
    e. Read absorbance of samples.
    f. Multiply the absorbance by 5 to obtain the protein concentration in mg/ml.
12. Move on to next time point or condition and process the lysate as described above.
13. Calculate how to equalize the cell extracts with ice cold Lysis Buffer to give identical protein concentrations. It is essential to have equal protein concentration in each sample for a successful assay. It is also important that the equalized

protein concentration is not higher than 2.0 mg/ml or below 0.25 mg/ml. Specifically, we recommend the final concentration of 0.5 mg/ml for 3T3 cells and 1 mg/ml for HeLa cells. It is not necessary to equalize protein concentration if the sample variation is less than 10%.

The volume of cold cell Lysis Buffer to be added to the more concentrated samples can be calculated as follows:

$$\frac{A - B}{B} \times (\text{volume of } A) = \_\_\_\_\_ \ \mu l$$

Where A is the higher concentration lysates (mg/ml) and B is the concentration of the most dilute sample (mg/ml).

NOTE: If you have previously titrated the lysate and know the optimal protein concentration for your G-LISA™, you can calculate and may dilute all the samples to desired lysate concentration separately before snap freezing Be aware of the length of time cell lysates stay on ice; it should not exceed 10 min since the Arf1-GTP hydrolysis is very fast.

STEP 3. G-LISA® assay

1. Aliquot 60µl of Lysis Buffer into a labeled microfuge tube and dilute with 60 ul of ice cold Binding Buffer. Vortex for 3-5s on high and place on ice. This is your buffer blank control.

2. Aliquot 90µl of A1CA (Arf1 Control Protein) into a labeled microfuge tube and dilute with 50ul of Lysis Buffer. Add 140 µl of Binding Buffer. Vortex for 3-5s on high and place on ice. This is your positive control.

3. Take the Arf1-GTP binding plate out of its bag. Gently peel up the seal from the strips and pull out the number of strips required. Place strips in the extra strip holder provided, and place on ice. Immediately after removing the strips needed, put the rest of the plate back in the pouch with desiccant and place back in storage.

4. Dissolve the powder in the wells with 100 µl ice cold water.

5. Thaw the snap frozen cell lysates in ROOM TEMPERATURE water bath. Immediately place on ice after they are thawed.

6. Based on the calculation of equalization, add required amount of Lysis Buffer to respective tubes to equalize all lysate concentration.

NOTE: It is recommended to calculate the dilution factors required BEFORE thawing out lysates as this allows rapid sample processing.

7. Aliquot sufficient lysate for duplicate (60 µl) or triplicate (90 µl) assays into ice cold microcentrifuge tubes.

8. Add an equal volume of ice cold Binding Buffer to each tube. Vortex 3-5s on high. Keep on ice.

9. Completely remove the water from the microplate wells as follows:

   Complete removal of solutions from the well requires a vigorous flick of the plate and a vigorous series of pats onto paper towels (5-7 hard pats). The complete removal of solution from wells between steps of the G-LISA® is very important as it avoids high background readings in the buffer only wells. The buffer only wells should read between 0.1 – 0.25 at an absorbance of 490 nm. If background readings are higher than 0.25 then a more vigorous removal of solutions from the well should be practiced.

10. Put plate back on ice.

11. Immediately add 50 µl of lysate to respective wells.

12. Pipette 50 µl of buffer blank control to duplicate wells.

13. Pipette 50 µl of Arf1 positive control (16 ng) into duplicate wells.

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

NOTE: An ORBITAL microplate shaker set to a minimum of 200 rpm must be used. Slower shakers or rockers will not be sufficient.

15. During the incubation, dilute the anti-Arf1 primary antibody to 1/40 in Antibody Dilution Buffer by adding 12.5 µl of antibody to every 500 µl Antibody Dilution Buffer. Note: The final volume of 500 µl is adequate for one strip (8 wells).
16. After 30 min, flick out the solution from the wells and wash twice with 200 µl Wash Buffer at room temperature using a multi-channel pipettor. Do not leave this plate unattended at this time. Vigorously remove the Wash Buffer after each wash by flicking and patting the plate as detailed in step 9.
17. Place plate on the bench.
18. **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.**
19. Vigorously flick out the Antigen Presenting Buffer, pat inverted plate 5-7 times on a paper towel as outlined in step 9.
20. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer. Vigorously remove Wash Buffer after each wash as detailed in step 9.
21. Add 50 µl of diluted anti-Arf1 primary antibody to each well and leave the plate on the orbital microplate shaker (200-400 rpm) at room temperature for 45 min.
22. During the primary antibody incubation, dilute the secondary HRP labeled antibody to 1/200 in Antibody Dilution Buffer by adding 2.5 µl of antibody to every 500 µl Antibody Dilution Buffer. Note: The final volume of 500 µl is adequate for one strip (8 wells).
23. Vigorously flick out the anti-Arf1 primary antibody, pat inverted plate 5-7 times on a paper towel as outlined in step 9.
24. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer. Vigorously remove Wash Buffer after each wash as detailed in step 9.
25. 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.
26. During the secondary antibody incubation prepare the HRP detection reagent. The HRP reagent is in two parts (A & B) which should be mixed in equal volumes prior to adding 50 µl to each well. For each strip of 8 wells, mix 250 µl of Reagent A with 250 µl of Reagent B into a clean tube. Components A and B should be thawed in a room temperature water bath and removed as soon as they are thawed. The mixture must be protected from light and is stable at room temperature for 1 h. Unused mixed solution should be discarded. Unused, non-mixed, solutions should be re-frozen immediately for later use.
27. Vigorously flick out the secondary antibody, pat inverted plate 5-7 times on a paper towel as outlined in step 9.
28. Immediately wash the wells three times with 200 µl of room temperature Wash Buffer. Vigorously remove Wash Buffer after each wash as detailed in step 9.
29. Pipette 50 µl of HRP detection reagent into each well and incubate at room temperature for 10 min.
30. Add 50 µl of HRP Stop Buffer.
31. Immediately read the signal by measuring absorbance at 490 nm using a microplate spectrophotometer as described in Section V: Important Technical Notes. Designate Lysis Buffer only wells as the assay Blank.

VII: Data Analysis

1. It is recommended to use the Lysis Buffer wells as reference blanks in all studies with this kit. Based on the operator designating the appropriate wells, most machines have associated protocols that perform this operation automatically; call Technical Help for the company supplying the plate reader for information on how to perform this function. When the data are "Lysis Buffer subtracted" (Lysis Buffer only samples have been allocated as Blanks in the assay) then you can import them into a simple graph software like Excel or Sigma plot. Alternatively, the Lysis Buffer background can be subtracted manually or in the spreadsheet application.

2. Data should be arranged in columns where the headings are "Sample", "Mean", "Standard Deviation", "rep1", "rep2", "rep3" and "rep4" for the number of replicates performed on each sample.

3. List your samples under the "Sample" column in the same order that they were assayed in the plate.

4. Enter the following formula into the first sector under "Mean", "=average(Xn:Yn)" where X = the column designator for "rep1", Y = column designator for "rep4", and n= row designator of the row that you are working on. Repeat for each sector under the "Mean" header until there are sufficient rows to cover the number of samples in your experiment.

5. Enter the following formula into the first sector under "Standard deviation", "=stdev (Xn:Yn)" where X = the column designator for "rep1", Y = column designator for "rep4", and n= row designator of the row that you are working on. Repeat for each sector under the "Standard deviation" header until there are sufficient rows to cover the number of samples in your experiment.

6. Enter your replicate data into rep1, rep2 etc. The program will calculate the Mean and Standard deviation of your replicates.

7. When the data has been entered select the Sample, Mean and Standard deviation data sectors by the click and drag method. Then select the chart making function in Excel; this looks like a clickable square with a mini-bar chart in. This will guide you through the chart making process with the data you have selected. Choose "column chart" initially, designate the Mean numbers for input values. The Standard deviation column for the y-axis error bars needs to be designated after the Mean numbers chart is made. This is achieved by double clicking on the graph bars, and selecting the "Y-axis error" tab, then entering the location of the standard deviation data by clicking the "Custom" option and selecting the area in the worksheet. E-mail tservice@cytoskeleton.com for a free Excel Template. An example of a typical Excel layout and data plot is shown in Figures 4 and 5.

Figure 4: Typical Excel Layout

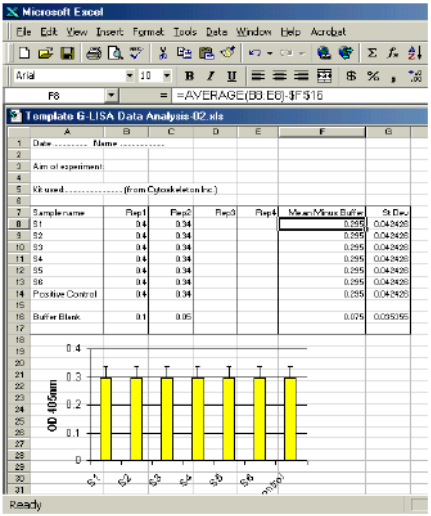
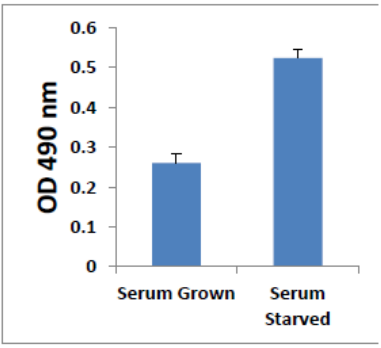


Figure 5: Typical G-LISA® Results



Arf1 activation by serum depletion measured by G-LISA™. C2C12 cells were grown in medium supplemented with 10% serum. Cells were then switched to serum free medium and incubated for an hour. 12.5 µg of cell lysates were subjected to the G-LISA™ assay. Absorbance was read at 490 nm.

VIII: Troubleshooting Guide

Observation	Possible cause	Remedy
Weak signal or no signal in all wells	1. Slow processing of samples or processing at above 4°C. 2. The wells were allowed to dry out during the experiment. 3. The plate was allowed to get damp during storage. 4. A step or component of the assay was omitted. 5. The HRP reaction was not developed for long enough.	1. Process samples quickly on ice. 2. Do not remove the solution in the wells unless the solution of next step is ready. 3. Store the plate in the Zip-top bag with desiccant. Keep the protective cover on the plate. 4. Read instructions carefully. 5. The manual guidelines state that HRP reaction should be allowed to develop for 10 min at room temp. In cases where Arf1 signal is very low, allowing longer times (up to 30 minutes) will give a stronger signal. HRP Stop Solution should be added prior to reading at 490 nm.
High signal in all wells	1. Concentration of antibodies is too high. 2. Insufficient washes were performed	1. Follow the recommended dilution of antibodies in the manual, if still too high, an antibody titration is necessary to optimize your results. 2. Follow the instructions for the washing in the manual.
Background readings are high (>0.25)	1. Inefficient removal of solutions from G-LISA® wells.	1. Background should read between 0.05-0.25. Complete removal of solutions from the wells is required to produce a clean assay. Vigorous flicking and patting of the inverted plate is required to completely remove solutions from the wells after each step is complete. See Important Technical Notes and the G-LISA® method for details.
Induced sample does not give significant signal increase	1. Poor inducer activity. 2. Technique not rapid or cold enough. 3. Too much extract in the wells or the concentration of extract is too high. 4. The endogenous GTP-Arf1 level is too high. 5. Tissue culture cells not correctly serum starved. 6. Temperature of lysis and incubation is not 4°C. 7. The basal level of Arf1 is too high.	1. Purchase a new vial of inducer. 2. Read instructions carefully and compare with your Experiment Record Sheet. 3. The linear range of the assay is 1-10 ng of active Arf1. 4. Titrate down the amount of extract to be added. 5. Details of how to serum starve cells is given in Appendix 1. Lyse cells on ice, keep Cell Lysis Buffer and distilled water on ice. 6. Titrate controlled cell states (e.g. serum starved) as indicated in the protocol. If basal level reading is over 0.5 (after buffer blank subtraction) it is too high to detect correct activation ratio.
Significant variation between duplicate/triplicate samples.	1. Incorrect volume of solutions for each step added in the wells. 2. Inaccurate pipetting.	1. Follow the instruction for recommended volume in the manual. 2. A multi-channel pipettor is recommended.
Positive control not working	1. Positive control protein was re-stored after reconstitution.	1. Use a new tube each time, there are 12 per kit.

Note: Refer to our website for timely updates on technical tips for G-LISA® assays.

IX: References

1. Takatsu H., et al. GGA proteins associate with Golgi membranes through interaction between their GGAH domains and ADP-ribosylatiion factors. *Biochem. J*. **365**: 369-378 (2002)

2. Donaldson, J.G. and Klausner, R.D. ARF: a key regulatory switch in membrane traffic and organelle structure. *Curr. Opin. Cell. Biol*. **6**, 527–532 (1994)

3. Moss, J., Vaughan, M. Molecules in the ARF Orbit. *J. Biol. Chem*. **273**: 21431-21434 (1998)

4. Donaldson JG, et al. ADP-ribosylation factor, a small GTP-binding protein, is required for binding of the coatomer protein beta-COP to Golgi membranes. *Proc Natl Acad Sci U S A.* **89**(14):6408–6412 (1992)

5. Donaldson JG, et al. Binding of ARF and beta-COP to Golgi membranes: possible regulation by a trimeric G protein. *Science.* **254**(5035):1197–1199. (1991)

6. Palmer DJ., et al. Binding of coatomer to Golgi membranes requires ADP-ribosylation factor. *J. Biol. Chem.* 268: 12083-12089 (1993)

7. Black MW and Pelham H. Membrane traffic: How do GGAs fit in with the adaptors? *Current Biology* **11**:R460–R462 (2001)

8. Zhang Q. et al, Small-molecule synergist of the Wnt/beta-catenin signaling pathway. *Proc Natl Acad Sci U S A.* ***104****(18): 7444-7448 (2007)*

9. Boulay, PL., et al. Arf1 controls the activation of the PI3K pathway to regulate EGF dependent growth and migration of breast cancer cells. *J. Biol. Chem*. **283**(52): 36425–36434. (2008)

Appendix 1: Experiment Record Sheet

Scientist Name …….

Contact Tel. # …….

e-mail …….

Kit Cat. # / Lot # …….

STEP		Comments or Changes
1	Type of cells or tissue ……..	
2	How were the cells prepared prior to lysis?	……. days in culture
		……...% confluency
		…… inducer
		……...mg/ml of protein in lysate
3	How long were the ice cold solutions on ice before lysis?	…….. Min
4	Time that cultures were removed from incubator?	……. am or pm
5	Was Binding Buffer added?	…….. Y or N
6	Time that binding reactions were placed on the shaker?	…….. am or pm
7	Did you add 50 µl of extract per well?	…….. Y or N
8	What locations are the 50 µl Lysis Buffer controls?	……. Wells
9	What speed and time was the shaking for the binding reaction? …… rpm ……min	
10	How long did you wait after the post-binding wash step?	…….. s or min
11	What was the time when the anti-Rho primary antibody reaction was started? .am or pm	
12	What was the time when the Secondary antibody reaction was started?..... am or pm	
13	What was the time when detection reagent was added?	……..… am or pm
14	What was the time when the plate was read?	…….. am or pm

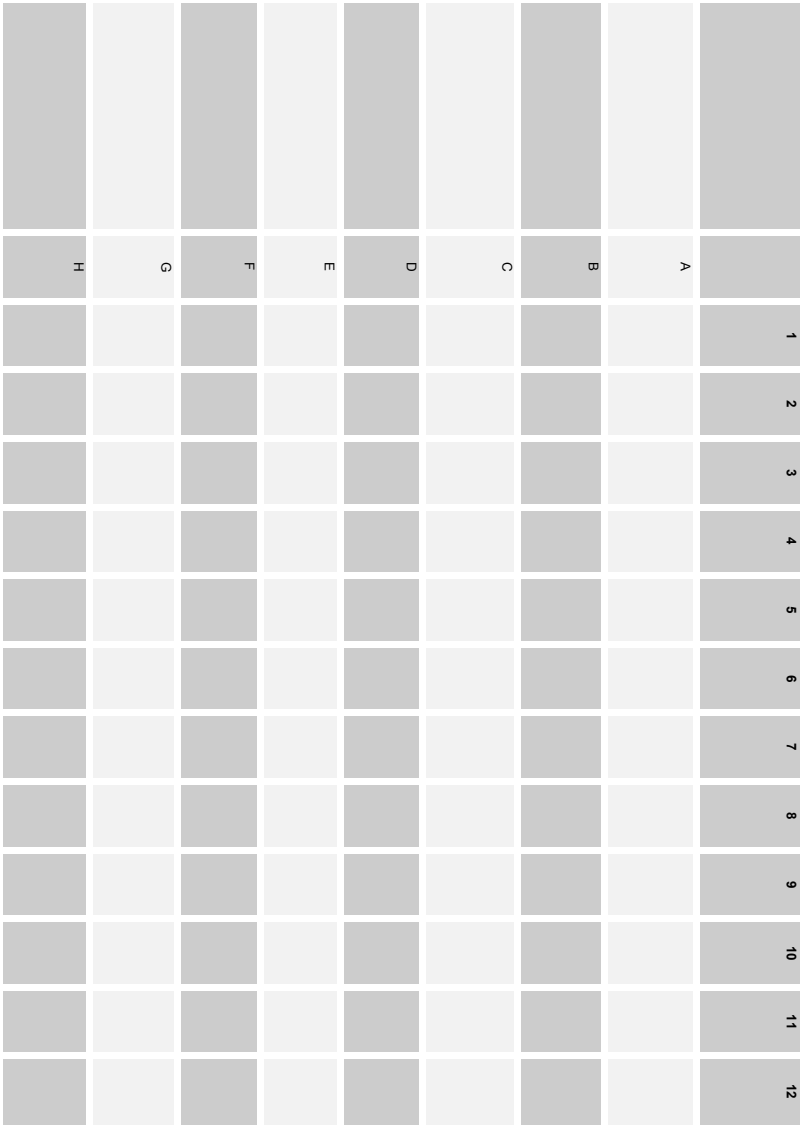
Technical Assistance: call either 303-322-2254 or e-mail tservice@cytoskeleton.com.

Appendix 2: Plate Record Template

Name of experiment: ……..

Date of experiment: ……

Technical Assistance: call either 303-322-2254 or e-mail tservice@cytoskeleton.com



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