Arf1 Activation Assay Biochem Kit™

Cat. # BK032-S

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Manual Contents

Background– Arf1 Activation Assay

The mammalian ADP-ribosylation factor (Arf) subfamily of Ras-related small G-proteins were originally named for their ability to stimulate cholera toxin mediated ADP-ribosylation of the $G_{s\alpha}$ subunit utilized by many GPCRs (1). The Arf GTPases have been grouped into three classes based on their size and amino acid similarity (2): class I (Arf1 and Arf3), class II (Arf4 and Arf5) and class III (Arf6). Arf1 functions in the anterograde (and retrograde) transport of proteins from the endoplasmic reticulum (ER) through the Golgi apparatus to the plasma membrane by activating lipid modifying enzymes and recruiting proteins needed to promote secretory vesicle development, scission and transport between secretory compartments (3, 4 and reviewed in 5). Arf1 has also been shown to function in the maintenance of structural integrity of the Golgi and ER (6).

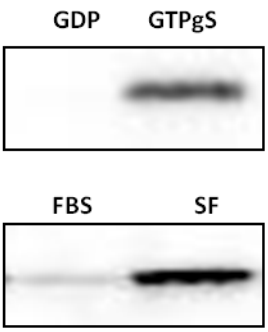
Arf1, like other small G-proteins, cycles between the inactive GDP-bound and the active GTP-bound states. The preferential association of effector proteins with the GTP-bound over the GDP-bound state of Arf1 provides the basis for Arf1's function in the cell. This highly specific association of effector proteins with Arf1-GTP has been exploited to develop affinity precipitation assays to monitor Arf1 activation (7).

Cytoskeleton's Arf1 Activation Assay Biochem Kit™ utilizes the Arf1 protein binding domain (PBD) of the effector protein GGA3 (*Golgi-localized γ-ear containing, Arf-binding protein 3*), which has been shown to specifically bind the GTP-bound form of Arf1 (7, 8). We have covalently conjugated purified GGA3-PBD (amino acids 1-316) expressed in *E. coli* to the colored sepharose beads provided in this kit. Using these beads, the researcher is able to "pull-down" Arf1-GTP and quantify the level of active Arf1 with a subsequent Western blotting step using the Arf1 specific antibody provided in this kit. This assay provides a simple means of analyzing cellular Arf1 activation levels in a variety of systems. A typical Arf1 pull-down assay is shown in Figure 1 using either GTPγS and GDP loaded MDCK cell extracts or extracts from C2C12 myoblasts that have been induced to differentiate by serum starvation.

Figure 1. Arf1 Activation Assay Biochem Kit™ Pull-down Assay Results.

A. MDCK cell lysates (500 μg) loaded with GTPγS or GDP using the method described in Section VI: Control Reactions.

B. C2C12 cell lysates (500 μg) from untreated cells (FBS) or cells that were serum starved for 1h (SF). All extracts were incubated with 20 μg of GGA3-PBD beads and processed as described in Section VI: Pull-down Assay. All bead samples were resuspended in 20 μl of 2x sample buffer and then separated on a 4-20% SDS-PAGE gel, transferred to PVDF, probed with a 1:250 dilution of anti-Arf1 antibody and processed for chemiluminescent detection as described in Section VI: STEP 4.



II: Purchaser Notification

Limited Use Statement

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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 approximately 20 pull-down assays. There is sufficient Arf1 antibody for 50 ml working strength primary antibody solution.

Table 1: Kit Contents and Storage Upon Arrival

Reagents	Cat. # or Part # *	Quantity	Storage
GGA3-PBD beads	Cat # GGA07	1 tubes, lyophilized; 500 μg of protein per tube conjugated to colored sepharose beads	Desiccated 4°C
Anti-Arf1 monoclonal antibody	Cat # ARF01	1 tube, lyophilized, 50 μg protein	Desiccated 4°C
Arf1 control protein (His-tagged)	Part # A1CA	1 tube, lyophilized; 100 ng protein; (~28 kDa) as a Western Blot standard.	Desiccated 4°C
Cell Lysis Buffer	Part # A1LB	1 bottle, lyophilized; 50mM Tris pH 7.5, 10mM $MgCl_2$, 0.3M NaCl, 2% Igepal and 0.01% SDS when reconstituted	Desiccated 4°C
Wash Buffer	Part # WB01-S	1 bottle, lyophilized; 25 mM Tris pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl when reconstituted	Desiccated 4°C
Loading Buffer	Part # LB01	1 tube, 1 ml; 150 mM EDTA solution	4°C
STOP Buffer	Part # STP01	1 bottle, 1 ml; 600 mM $MgCl_2$ solution	4°C
GTPγS stock: (non-hydrolysable GTP analog)	Cat # BS01	1 tube, lyophilized; 20 mM solution when reconstituted	Desiccated 4°C
GDP stock	Part # GDP01	1 tube, lyophilized; 100 mM solution when reconstituted	Desiccated 4°C
Protease Inhibitor Cocktail	Cat. # PIC02	1 tube, lyophilized; 100X solution: 62 μg/ml Leupeptin, 62 μg/ml Pepstatin A, 14 mg/ml Benzamidine and 12 mg/ml tosyl arginine methyl ester when reconstituted	Desiccated 4°C
DMSO	Part # DMSO	1 tube, 1.5ml. Solvent for protease inhibitor cocktail	4° (will freeze at 4°C)

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

The reagents and equipment that you will require but are not supplied:

- Cell lysate (see Section V: B-D and Section VI: Step 2)
- 2X Laemmli sample buffer (125mM Tris pH6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% beta-mercaptoethanol)
- Polyacrylamide gels (12% or 4-20% gradient gels)
- SDS-PAGE buffers
- Western blot buffers (see Section VI: Step 4)
- Protein transfer membrane (PVDF or Nitrocellulose)
- Secondary antibody (e.g. Goat anti-mouse HRP conjugated IgG, Jackson Labs. Cat# 115-035-068)
- Chemiluminescence based detection system (e.g. SuperSignal West Dura Extended Duration Substrate; ThermoFisher)
- Cell scrapers
- Liquid nitrogen for snap freezing cell lysates

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 detailed in Table 2. When properly stored and reconstituted, components are guaranteed stable for 6 months.

Table 2: Component Storage and Reconstitution

Kit Component	**Reconstitution**	**Storage Conditions**
GGA3-PBD beads	Reconstitute each tube in 500 µl distilled water. Aliquot into 20 µl volumes (20 µl of beads = 20 µg of protein, under these conditions 500 µl is sufficient for 20 - 25 assays). **Snap freeze in liquid nitrogen.**	Store at –70°C.
Anti-Arf1 monoclonal antibody	Resuspend in 200 µl of PBS, pH 7.4. Use at 1:250 dilution.	Store at 4°C.
Arf1 control protein (His tagged)	Reconstitute in 100 µl of Cell Lysis Buffer (Part # A1LB). Aliquot into 10 x 10 µl sizes and snap freeze in liquid nitrogen.	Store at –70°C.
Cell Lysis Buffer	Reconstitute in 30 ml of sterile distilled water. This solution may take 5-10 min to resuspend. Use a 10 ml pipette to thoroughly resuspend the buffer.	Store at 4°C
Wash Buffer	Reconstitute in 30 ml of sterile distilled water.	Store at 4°C
Loading Buffer	No reconstitution necessary.	Store at 4°C
STOP Buffer	No reconstitution necessary	Store at 4°C
GTPγS stock (non-hydrolysable GTP analog)	Reconstitute in 50 µl of sterile distilled water. Aliquot into 4 x 12.5 µl volumes, snap freeze in liquid nitrogen.	Store at –70°C
GDP Stock	Reconstitute in 50 µl of sterile distilled water. Aliquot into 4 x 12.5 µl volumes, snap freeze in liquid nitrogen.	Store at –70°C
Protease Inhibitor Cocktail	Reconstitute in 1 ml of dimethyl sulfoxide (DMSO) for 100x stock.	Store at –20°C.

V: Important Technical Notes

A) **Notes on Updated Manual Version**

1) Version 2.2 has been updated to version 3.0.
2) In version 3.0 the amount of Arf1 control protein has been optimized to 100 ng. The recommended amount of control protein to run on a western blot is 5ng. The control protein is therefore sufficient for 10-20 westerns. The control protein is constitutively active Arf1 and will be visible as a 28kDa band on the Arf1 western blot.

B) **Growth and Treatment of Cells**

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. Media requirements will need to be evaluated for each cell type.

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. A few examples of Arf1 modulators are given in Appendix 1. 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) **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 must be performed sequentially. This allows rapid processing of each single time point. Once one time point lysate is collected, is should be snap frozen in "experiment sized" aliquots immediately and kept in –70°C. The Activation Assay uses approximately 300-800 µg of total protein per assay; this translates to 300-800 µl of a 1 mg/ml cell lysate. We recommend duplicate samples per time-point or condition, therefore 0.6 – 1.6 ml aliquots are recommended for snap freezing.

D) **Rapid processing of cells**

GTP bound (active) Arf1 is a labile entity and the bound GTP is susceptible to hydrolysis by Arf1-GAPs during and after cell lysis, resulting in Arf1 inactivation. Rapid processing at 4°C is essential for accurate and reproducible results. The following guidelines are useful for rapid washing of cells.

Washing

a. Retrieve culture dish from incubator, immediately aspirate out all of the media and place firmly on ice.
b. Immediately rinse cells with an appropriate volume of ice cold PBS to remove serum proteins (see Table 3 for recommended wash volumes). Note: it may be necessary to wash the cells twice with PBS if the media the cells were in contained FBS. This ensures that the resulting protein quantification (see Appendix 2) is not influenced by the protein in the residual FBS.
c. Aspirate off all residual PBS buffer. This is essential so that the Cell Lysis Buffer is not diluted. Correct aspiration requires that the culture dish is placed at a steep 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 Cell Lysis Buffer depending on your cell type and plate type. Table 3 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. NOTE: Cell Lysis Buffer should contain 1X Protease Inhibitor Cocktail, and may benefit from being supplemented with phosphatase inhibitors (e.g. final concentrations indcated: 10mM sodium fluoride, 1mM sodium orthovanadate, 2mM β-glycerophosphate and 2mM sodium pyrophosphate)

Table 3: 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)
100 mm dish	56	10.0	250
150 mm dish	148	15.0	700
T-75 Flask	75	10.0	500
T-150 Flask	150	15.0	700

The time period between cell lysis and addition of lysates to the GGA3-PBD beads 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 that cell lysates be immediately frozen after harvest and clarification. A sample of at least 20 µl should be kept on ice for protein concentration measurement. A 10-20 µg sample should also be kept for Western blot quantitation of total Arf1 per sample. The lysates **must** be snap frozen in liquid nitrogen and stored at -70°C. Lysates can be stored at -70°C are stable for several months.

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

E) **Protein Concentration Equivalence**

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 Cell 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. It is not necessary to equalize protein concentrations if the variation between them is less than 10%. To quantitate the relative amounts of total Arf1 in each sample, we recommend including samples of total lysate from experimental samples in the Western blot. Samples of 20-50 µg total cell lysate per sample should be sufficient to detect total Arf1.

F) **Assay Linearity**

There are several factors to consider when performing the Arf1 activation assays:

1) **Bead Titration:** GGA3-PBD beads will bind to Arf1-GDP with a much lower affinity than Arf1-GTP. If too many GGA3-PBD beads are added to the pull-down assay there will be significant binding to inactive (GDP-bound) Arf1. The result of this will be an underestimate of Arf1 activation. For this reason we highly recommend performing a bead titration to determine optimal conditions for any given Arf1 activation or inactivation assay. Once optimal conditions have been established, bead titrations should no longer be necessary. We recommend 10, 20 and 40 µg bead titrations.

2) **Strictly Maintain Experimental Conditions:** Once assay conditions are established one should strictly maintain experimental conditions. For example, lysate concentrations should be consistent between experiments. Thus, if 20 µg of beads are used to assay 400 µg of lysate at 0.5 mg/ml protein concentration, it is recommended to keep subsequent assays at 0.5 mg/ml lysate rather than using half the volume of a 1 mg/ml lysate to give 400 µg total protein. As a further example, the growth and treatment of cell lines should be consistent between experiments; this point can not be over-emphasized and is discussed in detail in Section V: B.

3) **Densitometric Quantitation:** The linear range of X-ray film is very narrow. Multiple exposures of the western blot may be required to analyze data in the linear range of the film. As a general guideline, protein bands that appear grey rather than black will be within the linear range of the film.

STEP 1: Control Reactions

The correct control reactions are key components of the Arf1 Activation Assay. The following control assays should be performed as an integral part of each experiment:

1. **Whole Cell Lysate Protein:**

   Total Arf1 present in each sample should be determined by quantitative Western blot analysis. Usually 10 – 20 µg of cell lysate will result in a good signal.

2. **Positive Cellular Protein Control**:

   Total cell lysate (300 – 800 ug) should be loaded with GTPγS as a positive control for the pull-down assay. The following reaction details how to load endogenous Arf1 with the non-hydrolysable GTP analog (GTPγS), this is an excellent substrate for GGA3-PBD beads and should result in a strong positive signal in a pull-down assay.

   a. Perform GTP loading on 300 – 800 µg of cell lysate by adding 1/15th volume of Loading Buffer (67 µl Loading Buffer per 933 µl of lysate).

   b. Immediately add 1/100th volume of GTPγS (10 µl GTPγS per 990 µl of lysate) to give a 200 µM final GTPγS concentration. Under these conditions 5 - 10% of the Arf1 protein will load with non-hydrolysable GTPγS and will be "pulled-down" with the GGA3-PBD beads in the assay (see Figure 1).

   c. Incubate the control sample at 37°C for 20 min with occasional gentle mixing.

   d. Stop the reaction by transferring the tube to 4°C and adding 1/10th volume of STOP Buffer (100 µl STOP Buffer per 900 µl of lysate) .

   e. Use this sample immediately in a pull-down assay as detailed in STEP 3.

3. **Negative Cellular Protein Control:**

   This reaction should be performed in an identical manner to the Positive Control reaction except that 1/100th volume of GDP (1 mM final concentration) should be added to the reaction in place of the GTPγS. Loading endogenous Arf1 with GDP will inactivate Arf1 and this will bind very poorly to GGA3-PBD beads.

4. **His-Arf1 Protein Control:**

   The Arf1 family proteins have a molecular weight of between 20-25 kDa; the His-tagged control protein has a molecular weight of approximately 28 kDa. We recommend that 5 ng of His-Arf1 control protein be run on the gel as a positive control and as a quantitation estimate for endogenous Arf1 (see STEP 4).

STEP 2: Lysate Collection

We strongly recommend that you snap freeze your cell lysates in liquid nitrogen right after you harvest and clarify. This is especially necessary if you have many samples. It is recommended to freeze lysates in 0.6-1.6 ml aliquots and to save a small amount of each lysate (approximately 20 – 30 µl) for protein quantitation. Details of lysates processing are given below:

Cells Grown in Tissue Culture Vessels as Monolayers

1. Grow cells in appropriate culture conditions. It is important to keep cells in a "controlled state" prior to Arf1 activation. See Section V (B): Important Technical Notes.
2. Treat cells with Arf1 activator (or inactivator) as your experiment requires.
3. After treatment, place culture vessel on ice, aspirate media, wash twice with ice cold PBS. See Table 3, Section V: D for recommended volumes.
4. Aspirate off PBS. Tilt plates on ice for an additional 1 min to remove all remnants of PBS. Residual PBS will adversely affect the assay.
5. Lyse cells in an appropriate volume of ice-cold Cell Lysis Buffer (Lysis Buffer should be supplemented with 1X Protease Inhibitor Cocktail). See Table 3, Section V: D for recommended volumes.
6. 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.
7. Transfer lysates into the pre-labeled sample tubes on ice.
8. Immediately clarify by centrifugation at 10,000 x g, 4°C for 1 min.
9. At this point each lysate volume should not exceed 130% of the original Cell Lysis Buffer volume.
10. Save at least 20 µl of lysate for protein quantitation and 10-20 µg of lysate for Western blot or ELISA quantitation of total Arf1.
11. Aliquot and snap freeze the remaining cell lysates in liquid nitrogen. Store at -70°C for future use. It is recommended to aliquot into 0.6-1.6 ml of lysate per tube (This should be sufficient for duplicate assays of 300-800 µg per assay).
12. Measure lysate protein concentrations. We recommend using Precision Red Advanced Protein Assay (Cat. # ADV02) for quantitations (see Appendix 2):
    - Add 10 µl of each lysate or Lysis Buffer into disposable 1 ml cuvettes.
    - Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Cat # ADV02) to each cuvette.
    - Incubate for 1 min at room temperature.
    - Blank spectrophotometer with the Cell Lysis Buffer at 600 nm.
    - Read absorbance of lysates samples.
    - Multiply the absorbance by 10 to obtain the protein concentration in mg/ml.
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. 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:

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

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

NOTE: You can dilute the lysates to a given concentration (e.g. 0.5 mg/ml) prior to snap freezing aliquots. This makes subsequent pull-down assays simpler. Be aware of the length of time cell lysates stay on ice (should not exceed 10 min), since Arf1 GTP hydrolysis will occur.

STEP 3: Pull-down Assay

1. If using freshly prepared cell lysates, use as soon as possible after lysis and protein equalization and always maintain samples at 4°C. If using frozen lysates (recommended), thaw in a room temperature water bath and remove immediately to ice upon thawing. Use immediately.

2. Add equivalent protein amounts of lysate (300 – 800 µg total cell protein) to a pre-determined amount of GGA3-PBD beads from your bead titration test (see Section V.F.1).

NOTE: In general, a 20 µg (20 µl) bead pull-down will yield optimal results. Under these conditions the 500 µg of GGA3-PBD beads supplied in the kit are sufficient for 20-25 assays. We do however recommend a bead titration (10, 20 & 40 µg) to determine optimal pull-down conditions.

3. Incubate at 4°C on a rotator or rocker for 1 h.

4. Pellet the GGA3-PBD beads by centrifugation at 3-5,000 x g at 4°C for 2 min.

5. Very carefully remove 90% of the supernatant. Do not disturb the bead pellet. If you do disturb the pellet simply re-centrifuge the sample as in step 4.

6. Wash the beads twice with 600 µl each of Wash Buffer. **NOTE**: Add the buffer to the bead pellet in a manner that completely resuspends the beads. **DO NOT invert the tube as the beads will disperse over the surface of the tube and protein will be lost. This step should take less than 1 min to perform.**

7. Pellet the GGA3-PBD beads by centrifugation at 3-5,000 x g at 4°C for 2 min.

8. Very carefully remove the supernatant. Do not disturb the bead pellet. If you do disturb the pellet simply re-centrifuge the sample as in step 7.

9. Add 20 µl of 2x Laemmli sample buffer to each tube and thoroughly resuspend the beads by gently tapping the bottom of the tube. Boil the bead samples for 2 min.

10. Spin down the beads at 10,000 x g for 2 min.

11. The samples are now ready to be analyzed by SDS-PAGE and Western blot analysis (see STEP 4).

NOTE: The whole sample including the beads can be loaded onto the SDS PAGE gel. It is recommended that the necessary control samples be run on each gel.

STEP 4: Western Blot Protocol

1. Run the test protein samples and controls on a 4-20% or 12% SDS gel until the dye front reaches the bottom of the gel.
2. We recommend running a lane containing 5 ng of His-Arf1 control protein as a positive control. To do this the protein should be diluted as follows;
   a) Thaw one of the 10 µl aliquots of His-Arf1 control protein (see Table 2).
   b) Dilute to 0.5ng/µl by adding 10 µl of 2X Laemmli sample buffer (125mM Tris pH6.8, 20% glycerol, 4% SDS, 0.005% Bromophenol blue, 5% beta-mercaptoethanol).
   c) Load 10 µl (5ng).
   d) Discard any unused control protein as it will "crash out" during storage at 4°C or frozen.
3. Equilibrate the gel in Western blot buffer (See recipe below) for 15 min at room temperature prior to electro-blotting.
4. Transfer the protein to a PVDF membrane for 45 minutes at 75V.
5. Wash the membrane once with TBS (10 mM Tris-HCl pH 8.0, 150 mM NaCl).
6. Allow the membrane to air dry completely (typically this takes 20-30 minutes)
7. Re-wet the blot with TBST (blot should be uniformly wet in appearance).
8. Block the membrane surface with 5% nonfat-dry milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature with constant agitation.
9. Incubate the membrane with a 1:250 dilution of anti-Arf1 antibody (Cat. # ARF01, provided with kit) diluted in TBST (no blocking agent) for 2 h at room temperature or overnight at 4°C with constant agitation.
10. Wash the membrane 3 times for 10 min. with TBST.
11. Incubate the membrane with an appropriate dilution (e.g. 1:20,000) of anti-mouse secondary antibody (e.g. goat anti-mouse HRP conjugated IgG from Jackson Labs., Cat. # 115-035-068) in TBST (no blocking agent) for 30 min-1 h at room temperature with constant agitation.
12. Wash the membrane 5 times in TBST for 10 min each.

13. Use an enhanced chemiluminescence detection method to detect the Arf1 signal (e.g. SuperSignal West Dura Extended Duration Substrate; ThermoFisher)

Recipe for Western Blot Buffer (1 L)

1 M Tris pH 8.3	25 ml	(25 mM final)
Glycine	14.4 g	(192 mM final)
Methanol	150 ml	(15% final)
Distilled water to 1 L		

VII: Troubleshooting

Observation	Possible cause	Possible Remedy
No signal from the His-tagged Arf1 control protein.	1. Repeated freeze/thaw cycles of the reconstituted positive control stock protein 2. Attempts to store the diluted stock at 4°C or frozen for future use.	1. The stock protein must be aliquoted as described in Table 2. Repeated freeze thaws of the stock will result in denaturation and precipitation. 2. We recommend loading 5 ng of the positive control on the gel as a positive control and quantitation estimate for endogenous Arf1. The diluted protein is unstable and will precipitate. Unused protein must be discarded. The Arf1 family proteins have a molecular weight of between 20-25 kDa; the His-tagged control protein has a molecular weight of approximately 28 kDa.
No difference in signal between GTPγS positive control and GDP negative control assay	1. Protein lysate concentrations were not equalized. 2. Titration of GGA3-PBD beads not performed. 3. GDP requirements are higher for your cell line. 4. Loading buffer and/or STOP buffer were not added to the reactions	1. The absolute amount of protein in lysates can have a dramatic effect upon Arf1 signal. It is therefore very important to have equal amounts of cell lysate protein in each reaction. See section V (E). 2. Perform bead titration per section V (F). In cases where there is a high signal in both GTPγS and GDP lanes, using half the amount of GGA3-PBD beads will often result in a better differential signal. 3. Some cell lines have very high levels of endogenous GTP and exchange of GDP requires addition of greater than the 1 mM GDP outlined in this manual. We recommend trying 10 mM GDP in these cases. 4. Make sure you use the Loading and STOP buffers in the amounts recommended. Load the lysates immediately prior to use.
No detectable Arf1 activation in the positive control (GTPγS) assay	1. STOP buffer not added to the reactions. 2. Leaving the lysates for >10 minutes before use.	1. Follow the instructions carefully, for example, STOP buffer must be added to the reaction or you will not get an Arf1-GTPγS signal. 2. GTPγS AND GDP loaded lysates should be used within 2-3 minutes after STOP buffer has been added.

VII: Troubleshooting (cont.)

Observation	Possible cause	Remedy
No detectable signal in the experimental samples	1. Insufficient cell lysate used	1. Titrate the protein amount used in the assay. We recommend 300-800 µg lysate, however, in some cases more lysate may be required.
	2. Lysates not processed rapidly at 4°C	2. Arf1 is still able to hydrolyze GTP during lysate preparation; hydrolysis is stopped only when the GGA3-PBD beads are bound to Arf1-GTP. The temperature and speed of lysate preparation are therefore very important parameters in this assay .
	3. Control reaction not performed for GTPγS. His-Arf1 control protein not used during Western blot.	3. Always run a GTPγS control to make sure the GGA3-PBD beads are working and always run the recombinant His-Arf1 control protein to make sure that the Western blot / Arf1 antibody is working correctly. Once these controls are working you can go on to determine the likely cause of a lack of signal or a lack of activation in the experimental samples.
Arf1-GTP signal does not change with the modulator that is being used.	1. Titration of GGA3-PBD Beads not performed.	1. Make sure that your control GDP and GTPγS lanes give a clear positive and negative response; this indicates that the bead and cell lysate levels are in the correct linear range to detect differential Arf1 activation states. This may require titrating bead and / or lysate levels.
	2. Culture conditions have caused cells to become unresponsive to Arf1 activators.	2. Continuous overgrowth of a cell line can result in unresponsive cells. Additionally, cells that have been excessively passaged can also lose their ability to respond to stimuli. In either case, the best solution is to start a fresh culture from a liquid nitrogen stock with as low a passage number as possible.
	3. Selected Arf1-GTP modulator may not work with your cell line.	3. Use a known Arf1-GTP modulator (e.g. QS11) to check the responsiveness of your cell line (see Appendix 1). Note that the response to any given modulator can vary considerably between cell lines.
	4. The dose of modulator and/or time of treatment of the cells is not optimal	4. Whenever possible, the dose of the modulator and the duration of cell exposure to the modulator should be varied to determine the optimal conditions for each parameter.
	5. Western blot is overexposed leading to inaccurate readings.	5. As a general guideline, you should expose the film so that the Arf1 signal gives a grey

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Appendix 1: Modulators of Arf1-GTP levels in cells

Non-transfection based Arf1-GTP (i.e. active Arf1) modulation

Modulator	Treatment	Cell type used	Response	Type of Assay Used	Ref.
Formyl methionyl-lysyl-phenylalanine (fMLF)	5 ng/ml	Polymorphonuclear neutophils (PMNs), PLB-985 cells	Treatment for 2 minutes with fMLF resulted in ~5-fold activation of Arf1 in PMNs.	GGA3 pull-down	10
QS11 (ARFGAP1 inhibitor)	1-2.5 μM	NIH 3T3 cells	Treatment for 36 hr resulting in dose-dependent activation of Arf1 and Arf6	GGA3 pull-down	9
Poliovirus	10 PFU/ cell	HeLa cells	Time-dependent increase in Arf1-GTP with a maximal activation of ~3.75-fold	GGA3 pull-down	11
Ephrin-B1	2 μg/ml EphB2-Fc	SUIT-4 cells	Treatment for 1.5 hr with EphB2-Fc resulted in elevated Arf1-GTP levels	GGA3 pull-down	12

Gene transfected	Treatment	Cell type used	Response	Type of Assay Used	Ref.
Arf1-HA + GBF1	5-25 μg/ml BFA	Cos7 cells	GBF1 increased Arf1-GTP levels 2-3 fold and this activation was not inhibited by BFA.	GGA3 pull-down	13
HCV NS5A	N/A	Huh7 cells	Hepatitus C virus NS5A protein expression reduced Arf1-GTP levels	GGA3 pull-down	14
Ephrin-B1	2 μg/ml EphB2-Fc	Capan-1 cells Panc-1 cells	Treatment for 1.5 hr with EphB2-Fc resulted in elevated Arf1-GTP levels	GGA3 pull-down	12
Enterovirus 3A protein	N/A	Cos7 cells	Expression of enterovirus 3A protein decreased total Arf1-GTP levels by 60%	GGA3 pull-down	15

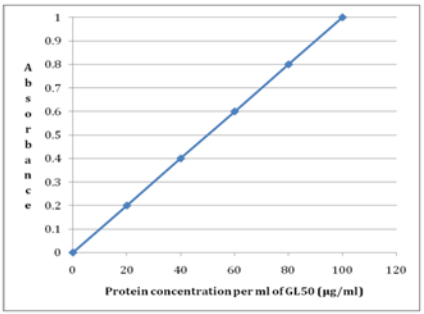
Transfection based Arf1-GTP (i.e. active Arf1) modulation

Appendix 2: Protein Quantitation (with Precision Red Reagent)

Background
The Precision Red Advanced Protein Assay Reagent is a simple one step procedure that results in a red to purple/blue color change characterized by an increase in absorbance at 600 nm. The reagent is not supplied in this kit, it is sold separately as Cat. # ADV02. Precision Red Advanced Protein Assay Reagent is supplied in the G-LISA activation assays (Part# GL50).
The assay exhibits low variance in readings between different proteins of the same concentration and high reproducibility of the colorimetric response. This allows one to utilize a generally applicable standard curve (Fig. 1) for protein quantitation. The assay can also be performed in approximately 1-2 minutes. These properties are particularly valuable when applied to the labile lysates required for activation assays.

Quick Protein Concentration Method for 1 ml Cuvette (recommended)

- Add 20 µl of each lysate or Cell Lysis Buffer into disposable 1 ml cuvettes.
- Add 1 ml of Precision Red™ Advanced Protein Assay Reagent (Cat# ADV02) to each cuvette.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 1 ml of ADV02 plus 20 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 5 to obtain the protein concentration in mg/ml



Legend: The standard curve shown in Fig. 1 represents the average absorbance reading of several common proteins (e.g., actin, BSA, casein) measured in a 1 ml cuvette format using 1 ml of ADV02 reagent. The protein reading pathlength for a cuvette is 1 cm. Linear range of this assay is 0.05 - 0.6.

Fig. 1: Standard Curve for Protein Quantitation in a 1ml Cuvette

Example Calculation
Assume a 20 µl sample of cell lysate added to 1 ml of ADV02 gives an absorbance reading of 0.1.

$$C = \frac{A}{\varepsilon \times l} = \frac{0.1}{10 \times 1} \times 50 = 0.5 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[mg/ml]^{-1}$ cm^{-1}) and the multiplier of 50 is the dilution factor for the lysate in ADV02 (20 µl lysate in 1 ml ADV02).

Thus for a 20 µl sample in 1 ml ADV02, the equation is C = A x 5
10 µl sample in 1 ml ADV02, the equation becomes C = A x 10

Quick Protein Concentration Method for 96 Well Plate

- Add 10 µl of each lysate or Lysis Buffer into the well of a 96 well plate.
- Add 290 µl of Precision Red™ Advanced Protein Assay Reagent to each well.
- Incubate for 1 min at room temperature.
- Blank spectrophotometer with 290 µl of ADV02 plus 10 µl of Lysis Buffer at 600 nm.
- Read absorbance of lysate samples.
- Multiply the absorbance by 3.75 to obtain the protein concentration in mg/ml

96 Well Plate Method
The linear range of this assay is 0.05 - 0.4 and is recommended when lysates are below the linear range of the 1 ml cuvette method. The pathlength for 96 well plate readings is 0.8 cm, hence the equation is modified as shown in the example below:

Example Calculation for 96 Well Plate Measurement
Assume a 10 µl sample of cell lysate added to 290 µl of ADV02 gives an absorbance reading of 0.1

$$C = \frac{A}{\varepsilon l} = \frac{0.1}{10 \times 0.8} \times 30 = 0.375 \text{ mg/ml}$$

Where c = protein concentration (mg/ml), A = absorbance reading, l = pathlength (cm), ε = extinction coefficient ($[mg/ml]^{-1}$ cm^{-1}) and the multiplier of 30 is the dilution factor for the lysate in ADV02 (10 µl lysate in 290 µl ADV02).

Thus, for a 10 µl sample in 290 µl ADV02, the equation becomes C = A x 3.75

For a 5 µl sample in 295 µl ADV02, the equation becomes C = A x 7.5

NOTE: The protein concentrations generated by using the standardized protein curve (Fig.1) will generate approximate lysate concentrations. Data will be highly reproducible from lysate to lysate and will generate excellent values for relative concentrations of a series of lysates. It should be noted for activation assays, the relative protein concentration between experimental extracts is far more important than the absolute protein quantitation. However, if desired, one can create a standard curve using BSA or IgG protein standards for each experiment. The standard curve should be performed prior to lysate preparations due to the labile nature of the lysates.

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