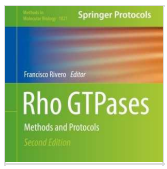


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# 3.5 Applications of G-Trap Assay

Book Chapter

In 1 collection

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ABSTRACT

Small, monomeric guanine triphosphate hydrolases (GTPases) are ubiquitous cellular integrators of signaling. A signal activates the GTPase, which then binds to an effector molecule to relay a signal inside the cell. The GTPase effector trap flow cytometry assay (G-Trap) utilizes bead-based protein immobilization and dual-color flow cytometry to rapidly and quantitatively measure GTPase activity status in cell or tissue lysates. Beginning with commercial cytoplex bead sets that are color-coded with graded fluorescence intensities of a red (700 nm) wavelength, the bead sets are derivatized to display glutathione on the surface through a detailed protocol described here. A different glutathione-*S*-transferase-effector protein (GST-effector protein) can then be attached to the surface of each set. For the assay, users can incubate bead sets individually or in a multiplex format with lysates for rapid, selective capture of active, GTP-bound GTPases from a single sample. After that, flow cytometry is used to identify the bead-borne GTPase based on red bead intensity, and the amount of active GTPase per bead is detected using monoclonal antibodies conjugated to a green fluorophore or via labeled secondary antibodies. Three examples are provided to illustrate the efficacy of the effector-functionalized beads for measuring the activation of at least five GTPases in a single lysate from fewer than 50,000 cells.

Section 3.5 'Applications of G-Trap Assay' from 'Small-Volume Flow Cytometry-Based Multiplex Analysis of the Activity of Small GTPases' https://www.protocols.io/view/small-volume-flow-cytometry-based-multiplex-analys-bpssmnee

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Small-Volume Flow Cytometry-Based Multiplex Analysis of the Activity of Small GTPases

ABSTRACT

Small, monomeric guanine triphosphate hydrolases (GTPases) are ubiquitous cellular integrators of signaling. A signal activates the GTPase, which then binds to an effector molecule to relay a signal inside the cell. The GTPase effector trap flow cytometry assay (G-Trap) utilizes bead-based protein immobilization and dual-color flow cytometry to rapidly and quantitatively measure GTPase activity status in cell or tissue lysates. Beginning with commercial cytoplex bead sets that are color-coded with graded fluorescence intensities of a red (700 nm) wavelength, the bead sets are derivatized to display glutathione on the surface through a detailed protocol described here. A different glutathione-*S*-transferase-effector protein (GST-effector protein) can then be attached to the surface of each set. For the assay, users can incubate bead sets individually or in a multiplex format with lysates for rapid, selective capture of active, GTP-bound GTPases from a single sample. After that, flow cytometry is used to identify the bead-borne GTPase based on red bead intensity, and the amount of active GTPase per bead is detected using monoclonal antibodies conjugated to a green fluorophore or via labeled secondary antibodies. Three examples are provided to illustrate the efficacy of the effector-functionalized beads for measuring the activation of at least five GTPases in a single lysate from fewer than 50,000 cells.

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## 3.5 Application of G-Trap Assay

### 3.5.1 Single-Target Format Measurement of GTP Loading of Rab 7 Associated with the Trafficking of EGF Receptors in EGF-Stimulated Cells

The single-target format of this assay recapitulates published data [16]. The example is shown here as a simplified example of the assay that shows quantification of the active GTPase-occupied sites on the beads using Quantum FITC™ MESF beads. In this



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experiment, HeLa cells were stimulated with EGF ligand for 10 min and then lysed [16]. Cell lysates of resting and EGF-activated cells were probed with RILP effector beads. One set of beads had no GST-effector protein, and was used to measure nonspecific binding; this is subtracted from the appropriate sample readings. The results are shown in Fig. 2.

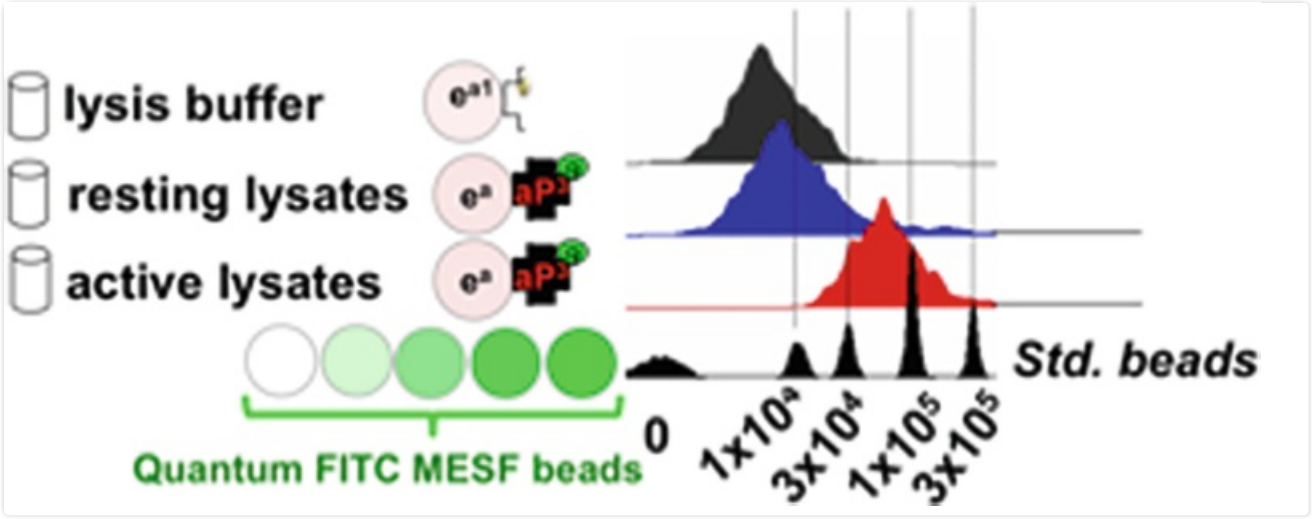


**Fig. 2**
Single-analyte assay for RILP: GTP·Rab7 captured on beads. Fluorescently labeled detection antibody added to lysis buffer is used to assess nonspecific binding of the antibody to beads. Flow cytometry histograms of RILP-RBD effector beads incubated at 4 °C with resting HeLa cell lysates or with EGF -stimulated HeLa cell lysates show increased Rab7-GTP bound, the levels of which can be quantified using commercial standard calibration beads (Quantum™ FITC MESF). Quantum™ FITC MESF beads comprise five sets of distinct bead populations. Each bead population is distinguished by a discrete number of doped fluorophores of known calibration. The average fluorophores/bead on each bead population is shown on the *x*-axis. The calibration beads are used to quantify the occupancy of Rab7-specific antibodies on RILP-effector beads. After correcting for nonspecific binding, $7.1 \pm 1.2 \times 10^3$ Rab7-GTP molecules/bead were recovered in resting cell lysates, and $6.7 \pm 0.3 \times 10^4$ Rab7-GTP molecules/bead were retrieved in EGF-stimulated cell lysates [16]

### 3.5.2 Sin Nombre Virus Induces Multiple GTPase Signaling Cascades

Integrins are cell adhesion receptors that signal bidirectionally ("inside-out" and "outside-in") across the plasma membrane [32]. Inside-out signaling stimulates increases in the ligand-binding affinity of integrins [33]. Outside-in signaling by integrins occupied by immobilized ligands [34, 35] induces cell spreading, retraction, migration, proliferation, and survival [32, 35]. Integrin signaling requires both heterotrimeric and monomeric small GTPases [37]. Many viruses engage cellular receptors such as integrins to transit the plasma membrane by hijacking the intrinsic endocytic pathways of desensitizing receptors [38, 39]. Recent studies from our lab have shown that SNV engages the $\beta_3$ integrin plexin-semaphorin-integrin (PSI) domain and initiates integrin outside-in signaling downstream of $G\alpha_{13}$ activation [17]. In the setting of SNV engagement, outside-in signaling stimulates cytoskeletal remodeling, receptor clustering, internalization, and trafficking [17]. The signaling events involved GTP loading of several GTPases associated with integrin activation (Rap1) [40], cytoskeletal remodeling (RhoA, Rac1) [41], and cargo trafficking (Rab7) [42]. Here we highlight the use of the glutathione bead sets (synthesized as detailed in Section 3.1, Protocol 'Synthesis of Glutathione Beads') in a multiplex assay of RhoA, Rap1, Rac1, and Rab7 (*see* Fig 3a) to determine the signaling outcome of SNV-induced outside-in signaling as previously established [17]. We also use a myristoylated peptide (Myr-FEEERA-OH) called mP6 [43] to inhibit $G\alpha_{13}$-dependent outside-in signaling caused by SNV [17]. In this setting there are three different experimental conditions (*see* Fig. 3b). 50,000 CHO-A24 cells in 48-well plates are treated with mP6 or with 0.1% DMSO (solvent for mP6) for 30 min. UV -inactivated particles of Sin Nombre virus (SNV) are added and the cells are incubated for 5 min at 37 °C. The cells are lysed and GTP loading of four GTPases is measured as described in Sections 3.2-3.4. Lysis buffer is used to determine the aggregate nonspecific binding of the four reporter antibodies used to detect GTPases associated with the beads. As shown in Fig. 3b, blocking the interaction between the $\beta_3$integrin cytoplasmic tail and $G\alpha_{13}$with mP6 inhibits GTP loading of all GTPases associated with integrin activation (Rap1), cytoskeletal remodeling (Rac1), and trafficking (Rab7). The reader is referred to ref. [17] for the rationale and further details. The top histograms are derived from lysates of cells treated with UV-inactivated SNV, whereas the bottom histograms are derived from beads incubated in RIPA buffer alone, and serve as a measure of nonspecific binding. The quantitative data are shown in Fig. 3c [17].



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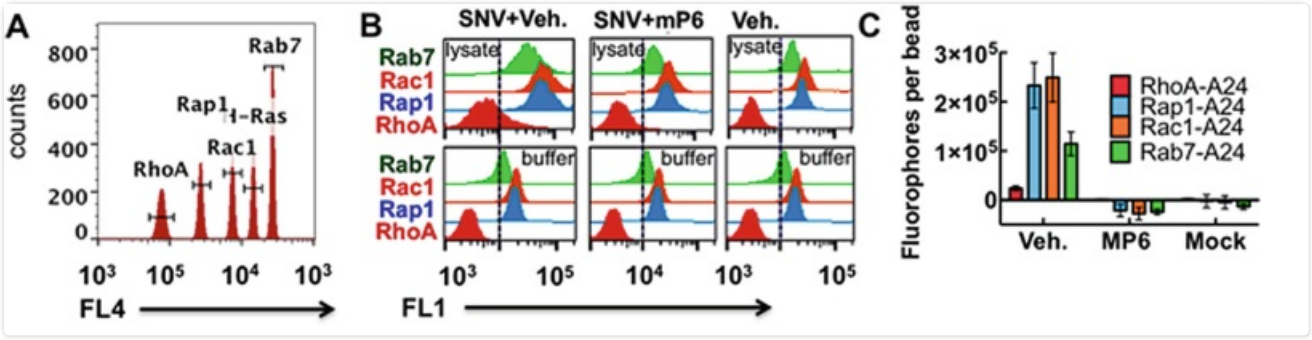


**Fig. 3**

Example output from G-Trap multiplex assay . (**a**) Histograms of a mixture of four Cyto-Plex™ bead populations, identified by their red fluorescence address. Each bead is functionalized with an effector molecule for Rac1 (PAK-1 RBD), H-Ras (RAF RBD), Rho A (Rhotekin-RBD), Rap1 (Ral GDS-RBD), and Rab 7 (RILP-RBD). Gates are used to select beads associated with effector proteins labeled with green fluorescent antibodies shown in panel **b**. (**b**) Top panels show histograms of beads incubated with CHO -A24 cell lysates derived from cells treated with SNV and vehicle (0.1% DMSO), 250 µM DMSO-solubilized mP6 and SNV, and resting cells. Bottom panels represent beads incubated in cell lysis buffer to determine nonspecific binding of anti-GTPase antibodies. (**c**) Respective plots of site occupancy/bead of active GTPases, established from Median Channel Fluorescence (MCF) of histograms shown in b after correction for nonspecific binding. Quantum™ FITC MESF were used to analyze the data shown in b. The error bars represent triplicate measurements for each target. Error bars represent standard deviation. Reproduced from ref. [17] with permission

### 3.5.3 GTPase Signaling Downstream of Protease-Activated Receptors

Here we use the G-Trap assay to measure in parallel RhoA·GTP, Rac1·GTP, and Rap1·GTP levels in endothelial cells exposed to plasma samples from *de-identified* subjects previously hospitalized for hantavirus cardiopulmonary syndrome (HCPS). Sample use was approved under UNM IRB#15-166. The bead sets were prepared as described in Subheading 3.3 (Protocol '[Molecular Assembly of GST Effector Proteins on Glutathione Beads]'). The assay required five sets of beads for the conditions of the experiment and was performed in a single afternoon (≤4 h).

Thrombin activates PARs and causes loss of cell barrier function [44-47]. $G_{12}/G_{13}$–RhoA·GTP–MLCK (myosin light chain kinase) and Gi-Rac·GTP (Gq-Rap1·GTP) signaling axes are cytoskeletal altering pathways that control cell contraction and spreading, respectively (*see* Fig. 4a). These signals ultimately combine to induce profound changes in vascular endothelial cells, including increased endothelial monolayer permeability [44, 47, 48]. High concentrations of thrombin expressed in the circulation of HCPS subjects significantly contribute to loss of cell barrier function in endothelial cells [18]. Argatroban, an orthosteric inhibitor of thrombin ($K_i$~ $10^{-8}$ M), can be used to block thrombin activity [49]. Because GTP loading of RhoA is associated with loss of cell barrier function, a set of beads were used to interrogate lysates of cells treated with an inhibitor of Rho kinase (Y27632) before exposing them to plasma samples. In a pilot study of a specific PAR4 antagonist, ML354 [50], we tested the G-Trap platform to determine the role of GTP loading of RhoA, Rap1, and Rac 1 on cell barrier function using telomerase-immortalized microvasculature endothelium (TIME) cells [17]. We first assayed the effects of plasma and PAR signaling inhibitors in terms of the status of cell-cell barrier integrity using electric cell-substrate impedance sensing (ECIS). We then correlate changes in cell barrier function to a time-course measurement of GTPase activity. As shown in Fig. 4b, ECIS measurements show that HCPS patient plasma caused loss of cell barrier function in TIME cells. The cell barrier function of Y27632-treated cells was conserved, consistent with normal activation of RhoA. ML354 treatment conferred only short-term barrier protection and argatroban supported long-term cell barrier integrity to cell monolayers (*see* Fig. 4b). We measured GTP loading of RhoA, Rap1, and Rac1 in a multiplex format. The G-Trap assays show that the short-time (15 min) exposure of cells to plasma increased RhoA·GTP 15-fold, Rap1·GTP 7-fold, and Rac1·GTP 4-fold relative to active GTPase levels in resting cells (*see* Fig. 4c). ML354 and argatroban limited GTP loading to ten-, five-, and threefold for RhoA, Rap 1, and Rac 1, respectively, in the short term. However, after 1-h exposure to plasma, the efficacy of ML354 at limiting GTP loading to the target GTPases is lost, while the activity of argatroban is conserved (*see* Fig. 4d). These assays demonstrate the utility of the G-Trap assay in easily connecting functional (ECIS cell barrier sensing) and mechanism (GTP loading primarily to RhoA).



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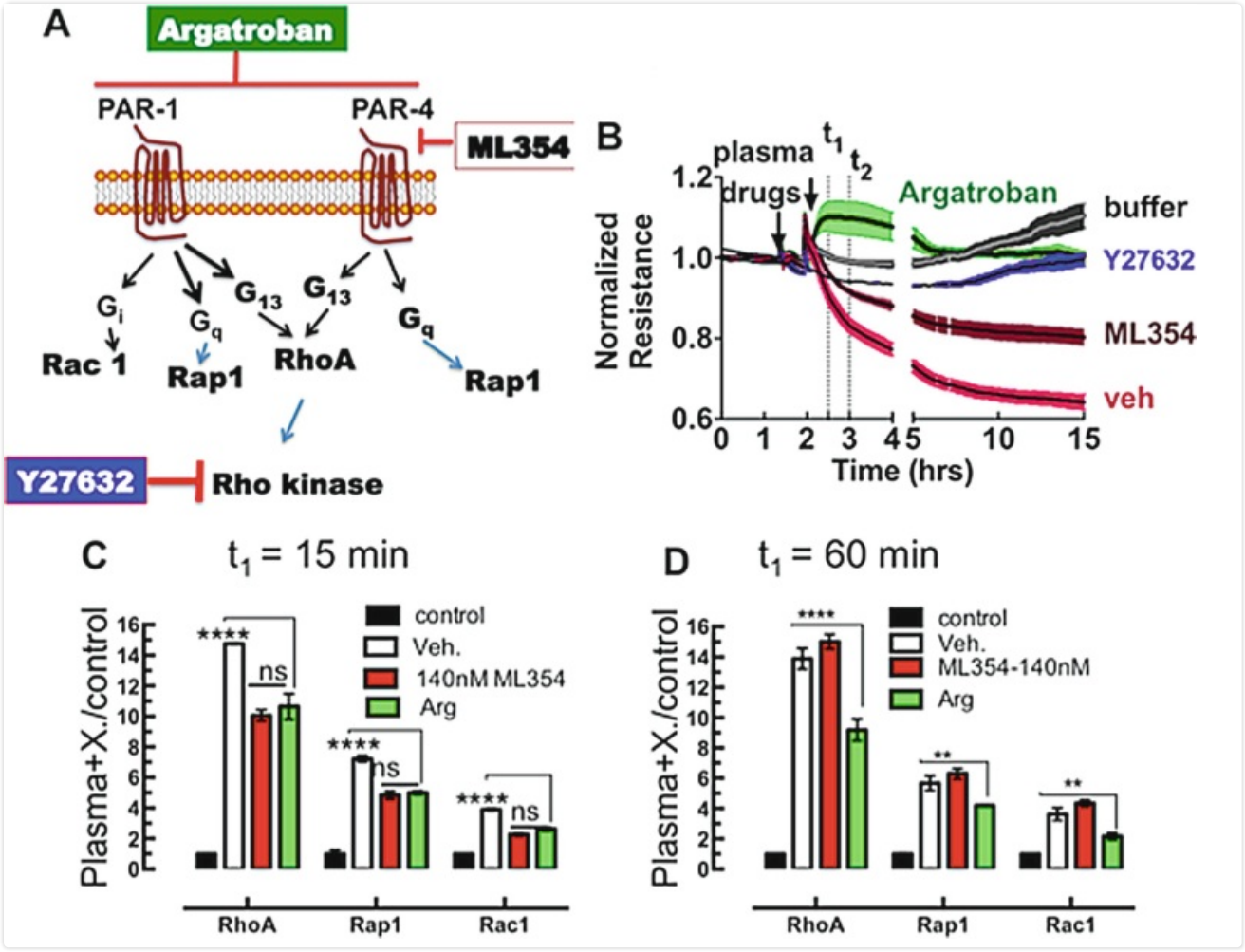


**Fig. 4**

RhoA activation stimulates loss of cell barrier function. (**a**) Model of protease-activated receptor-1 and -4 (PARs) coupling to multiple G proteins ($G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$), upstream of small GTPase activation. Activation of PARs results in cell barrier disruption (RhoA) whereas Rac1 and Rap1 signaling are believed to be barrier protective [57, 58]. Argatroban is an orthosteric inhibitor of thrombin interaction with PARs and is barrier protective. ML354 is a specific inhibitor of PAR4 activation. (**b**) Electric cell-substrate impedance sensing (ECIS) measurement of the effects of plasma from a patient with hantavirus cardiopulmonary syndrome (HCPS) on the cell barrier function of telomerase-immortalized microvascular endothelial (TIME) cell monolayers. Inhibitors are added to cell monolayers 30 min before the plasma is added as indicated by arrows and barrier function is measured. (**c**) GTPase activity measured in TIME cells after $t_1$ = 15-min exposure to HCPS plasma. The results are corrected for nonspecific binding and normalized to resting cells. GTP loading increases in plasma treated cells. Drugs limit the increase in GTP loading compared to untreated cells. GTP loading in cells treated with ML354 is comparable to argatroban treatment. Cell barrier disruption is consistent with a significant increase in GTP loading in RhoA. Each multiplex data point was measured using effector beads prepared as described in Subheading 3.3 (Protocol '[Molecular Assembly of GST Effector Proteins on Glutathione Beads]'). (**d**) After 1-h exposure to plasma, RhoA activity is observed in ML354 but not argatroban-treated samples. GTP loading is consistent with cell barrier disruption shown in the ECIS time course. The multiplex beads used for this dataset were similar to panel **c**, for cells lysed after 1-h exposure. Error bars are standard deviation. *$P$ < 0.05; **$P$ < 0.01, ****$P$ < 0.0001 by Dunnett's $t$-test

### 3.5.4 Testing Septic Patient Plasma for GTPase Activity

Bacteria overcome host defenses, by hijacking Rho GTPases that regulate the actin cytoskeleton [11, 12, 51]. During initiation of infection, bacterial adhesins favor tissue colonization, whereas, at later stages, exotoxins promote bacterial spread and blockage of immune cell responses [52]. By downregulating Rho GTPases, bacterial pathogens can block crucial immune cell functions such as chemotaxis, phagocytosis, and antigen presentation [12, 53]. Bacteria produce various toxins and virulence factors that activate or inactivate Rho GTPases by different mechanisms. The processes include (a) posttranslational modification of the GTPases; (b) bacterial protein mimics of GTPase regulatory factors, including guanine nucleotide exchange factors (GEFs), GTPase-activating



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proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs); and (c) modification of upstream regulators of Rho GTPases [1, 12].

Here we test the applicability of the G-Trap assay for detecting bacterially induced GTPase activity in serial plasma samples from a de-identified septic patient (UNM IRB #13-312). The patient was diagnosed with community-acquired pneumonia *(S. pneumoniae* on hospital admission) and treated with antibiotics. Vero E6 cells from a cell culture were treated with 10 µL of plasma samples/test. Following a 30-min incubation, Vero cell lysates were prepared as described in Subheading 3.2 (Protocol 'Production of a Cleared Cell Lysate') and GTP loading of Rho A, Rac1, and Rap1 were simultaneously measured in each lysate using a mixture of PAK-1 RBD, Rhotekin-RBD, and Ral GDS-RBD beads. As shown in Fig. 5, the plasma samples added to Vero E6 cells in culture elicit GTP loading principally of Rac1 during the first 4 days after hospital admission. After that, plasma levels of the Rac1-activating factor decreased in response to antibiotic treatment and Rac1 GTP levels reverted to basal levels similar to RhoA and Rap1, which were unchanged across the entire time course of patient hospitalization. The selective activation of Rac1 by blood plasma collected during the infectious phase is consistent with the fact that *S. pneumoniae* produces a toxin, pneumolysin, which activates Rac1 GTPases [54, 55]. Serial samples from patients with sterile inflammations indicated basal GTPase activity only (not shown). It is also interesting to note that on day 5 the patient underwent stomach surgery, which did not elicit overt GTPase activity. These results illustrate the potential utility of the G-Trap assay for diagnostic purposes using serial samples.

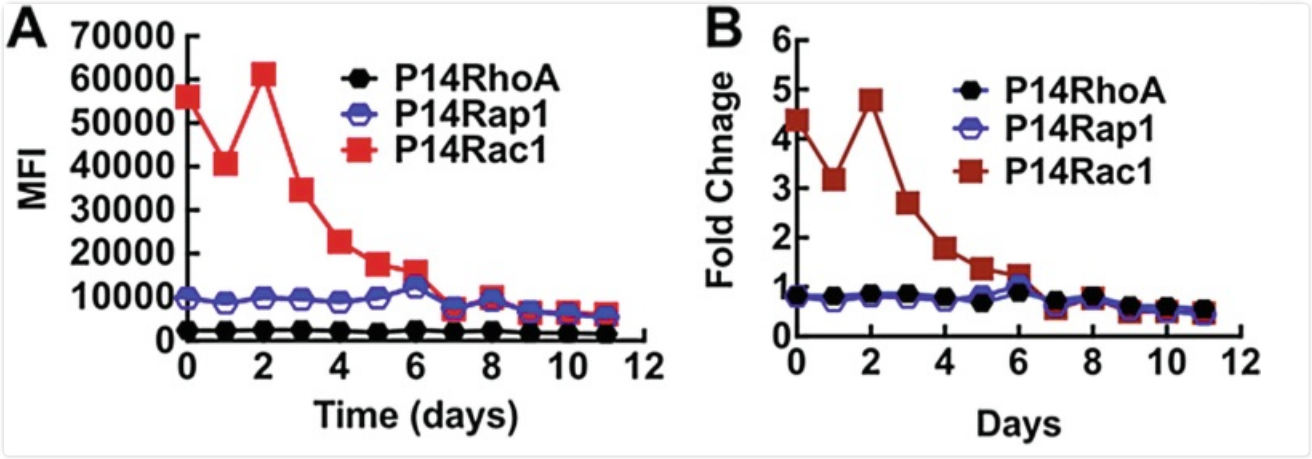


**Fig. 5**
GTP loading of RhoA, Rap1, and Rac1 measured in TIME cell lysates after 30-min exposure to serial plasma samples drawn from a septic patient (P14) 0–12 days after hospital admission for sepsis. The patient underwent gastric surgery after day 5 when the bacterial infection was brought under control. The sterile inflammation resulting postsurgery did not elicit any further spike in GTPase activity. (**a**) Raw G-Trap data for a patient who tested positive for bacterial infection (*S. pneumoniae*) on day 0, and was treated with antibiotics. (**b**) Data normalized to GTP loading of each GTPase as measured on day 11



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