Manual

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IFTA AG
Certified QMS according to
DIN EN ISO 9001:2000-12
Reg. No. IC 03214 034

Jena Bioscience

Rap activation Kit

Detection of cellular Rap-GTP

Rap GTPases

Cat.-No.	Amount
PR-951	10 x 6 assay points

For *in vitro* use only
Quality guaranteed for 12 months
Kit shipped on dry ice
Attention: components must be stored at different temperatures upon arrival

Avoid freeze / thaw cycles

Kit contents
(store at -20°C unless otherwise stated)

5 x Lysis Buffer stock, 30 ml (*store at* ***4°C***)

100 x Protease inhibitor mix, 650µl

1.5 mg GST-RalGDS-RA, 2-5 µg/µl in PBS , *upon thawing, aliquot as convenient for you, snap-freeze in liquid nitrogen and store at* ***-80°C*** *to avoid repeated freeze / thaw cycles*

GDP, 10 mM in water, 100 µl

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GMPpNHp, 10 mM in water, 100 µl

Rap nucleotide loading solution (NLS), 2 ml

1 M $MgCl_2$, 100 µl

25 µg His-Rap1A protein, 1-5 µg/µl in 50 % glycerol containing storage solution

Glutathione-Sepharose slurry, 5 ml (*store at* ***4°C***)

Rap1 polyclonal antibody, 50µl (*store at* ***4°C***)

To be provided by you

- Secondary anti-rabbit IgG antibody and western-signal detection system.
- Standard reagents/instruments for SDS-PAGE and Western Blot analysis

Description

The Rap activation Kit provides a rapid, cost-effective and reliable tool for the detection and semi-quantitative analysis of the cellular activation state of Rap GTPases. The kit exploits the selective interaction of the Ras-interaction domain (RA) of the Ras/Rap-effector RalGDS with the active, Rap-GTP conformation. Recombinant, GST-tagged RalGDS-RA is added to cell extracts to pull out Rap-GTP, which is consequently detected by Western blotting. The kit detects active, GTP-bound Rap1A, Rap1B, Rap2A and Rap2B. However, the antibody included in the kit allows detection of Rap1 isoforms only. The kit also includes recombinant Rap1A protein that, once preloaded with GDP or GMPpNHp (all included in the kit), can be used to "spike" the cell extracts thus serving as an internal control of signal specificity.

Background

As all other members of the Ras superfamily of small guanine nucleotide binding proteins (GTPases), Rap GTPases cycle between an active GTP-bound state and an inactive GDP-bound conformation. Cellular Rap-GDP/GTP levels are tightly controlled by two groups of proteins: 1. guanine nucleotide exchange factors (GEFs) catalyze GTP uptake by Rap in response to multiple second messenger signals like Ca2+, diacylglycerol or cAMP, and 2. GTP hydrolase activating proteins (GAPs) which promote conversion of Rap-bound GTP to GDP. Members of the Rap subfamily of GTPases, prominently Rap1A, Rap1B, Rap2A and Rap2B were originally thought to act as antagonists of Ras signalling. However it is now evident that Rap proteins have a function of their own in numerous cell types. Recent findings illustrate that Rap proteins regulate cell-cell and cell-matrix adhesion via the regulation of integrin, cadherin and other cell surface adhesion receptor function.
Traditionally, GTPase activity measurements have involved metabolic labelling of cells with inorganic [^{32}P]-phosphate followed by isolation of the GTPase and chromatographic analysis of bound guanine nucleotides. This methodology does provide quantitative data for GDP and GTP levels on Rap but it is a tedious and time consuming procedure that requires overexpression of heterologous tagged

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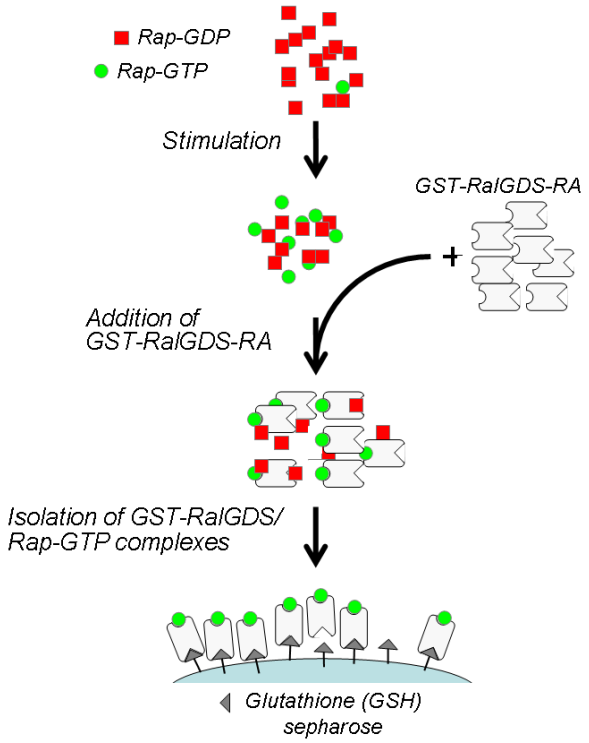
versions of Rap and working with large amounts of radioactivity.
An alternative non-radioactive technique exploits the selective interaction of GTPase binding domains with the active, GTP-bound cognate GTPase conformation. For Rap activity determinations recombinant, GST-tagged RalGDS-RA is added to cell extracts to pull out Rap-GTP, which is consequently detected by Western blotting.



Procedure
The following protocol is suited for a 6-well plate format experiment, i.e. 6 assay points, each on about 10^6 adherent cells. If working with more cells/point or with suspension cells, the assay may be scaled up or otherwise varied as applicable except for the indicated amount of GST-RalGDS-RA and Glutahione-sepharose beads per assay point, which should remain the same irrespective of the assay format.

Analysis of cellular Rap-GTP levels
Reagents provided in kit are highlighted in **green.**

1. Seed and cultivate cells in 6-well plates. Perform serum deprivation or other treatments as appropriate.
2. Prepare 6 ml Lysis solution on ice:

   1.2 ml **5 x lysis buffer** + 4.8 ml water

   + 60 µl **100 x protease inhibitors** as applicable

   If you prefer to supplement the lysis buffer with your own protease inhibitor mixture, all common inhibitors except NaF and EDTA may be used.

   + 6 µl **100 mM GDP**

   Inclusion of GDP at this point quenches post-lytic GTP-loading of Rap and thus avoids false-positive signals.

   + **150 µg GST-RalGDS-RA**

   Inclusion of GST-RalGDS-RA in the lysis solution quenches post-lytic GAP-promoted hydrolysis of Rap-bound GTP and thus minimizes the loss of signal. Thaw GST-RalGDS-RA aliquot quickly in a 37°C water bath. Thawed GST-RalGDS-RA protein is stable for up to 3 weeks kept at 4°C. Note: actual concentration of GST-RalGDS-RA varies from batch to batch.
3. After appropriate treatment/stimulation aspirate off medium from wells and lyse cells by addition of 1 ml ice-cold lysis solution. Scrape off cell debris with a rubber policeman and transfer extracts to vials on ice. Vortex.
4. Clarify lysates by centrifugation (15 min, 12000 g) in the cold. Transfer supernatants to new vials on ice.

Samples must be processed straightaway and may not be frozen or stored otherwise at this stage. Freeze-thaw cycles or too extensive delay in sample processing may cause significant loss of Rap-GTP levels by denaturation or GTP hydrolysis, respectively.

5. Take sample of lysate (commonly 30-50 l) and process for SDS-PAGE analysis, e.g. mix 30 l sample and 30 l of Laemmli SDS-PAGE loading solution containing 4 % SDS and boil the sample for 5-8 min. This represents the *total Rap load* samples.
6. Add 40 l of **(1:1) Glutathione-sepharose slurry** (pre-washed and equilibrated (1:1) in 1 x

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lysis buffer) to the clarified lysate. Incubate 30 min under constant mixing (e.g. on a rotating wheel) in the cold.

7. Briefly (about 10 sec) spin down beads in a microfuge. Discard supernatant.
8. Wash beads 3 times with 1 ml ice-cold 1 x **lysis buffer**.

The affinity purification of Rap-GTP is usually very reliable and suffers from only little false-positive signals owing to carry over or unspecific binding of Rap-GDP to the sepharose matrix. However, some cell types express high amounts of Rap proteins. We therefore recommend three washing steps for most applications, in order to avoid carry over of Rap-GDP.

9. Drain well beads and process for SDS-PAGE analysis, e.g. add 50 µl of Laemmli SDS-PAGE loading solution containing 2% SDS and boil the sample for 5-8 min. This represents the *Rap-GTP pulldown* samples.
10. Load and resolve *total Rap load* and *Rap-GTP pulldown* samples on 12% or higher concentrated polyacrylamide gels.

We recommend loading Rap-GTP pulldown and total load samples on separate gels. Moreover, the presence of GST-RalGDS-RA in large excess over the Rap proteins can seriously compromise the gel-to-membrane transfer efficiency of the latter and lead to marked signal loss. We strongly recommend cutting the gel containing the resolved Rap-GTP-pulldown samples horizontally at around the molecular weight marker size of 30 kD (use pre-stained molecular size markers!). This will yield two gel pieces: the lower one contains isolated Rap proteins and should be processed for western blotting as described below. The upper slice contains the GST-RalGDS polypeptide bands and may be stained with Coomassie or other protein dye solutions to ascertain the presence of the affinity probe (see Fig.1).

11. Transfer proteins from acrylamide gel to PVDF or alternative blotting membrane. Block the membrane in conventional western blot wash solution containing 1-2% BSA.
12. Incubate membrane overnight in the same BSA-containing wash-solution as above supplemented with **anti-Rap1 antibody** (1:1000 dilution) on an orbital shaker or similar at 4°C.

Rap1 antibody solutions can be supplemented with 0.001% azide to avoid microbial growth and re-used several times.
Signals detected in the *Rap-GTP pulldown* represent active Rap-GTP complexes whereas the *total Rap load* samples include the totality of Rap, i.e. Rap-GDP and Rap-GTP. *Total Rap load* signals should therefore ideally be of equal intensity in all points (see Fig.1)

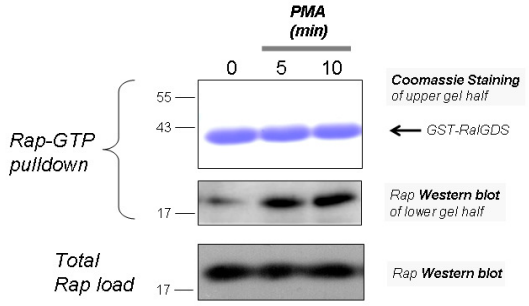

Fig. 1 *COS-7 fibroblasts were stimulated with phorbol ester (PMA) for the indicated time periods and subjected to a Rap-GTP pulldown.*

Preparation of Rap-GDP and Rap-GMPpNHp for spiking of cell extracts

Spiking of cell extracts (either at the level of step 3 or step 4 in the procedure described above) with Rap-GDP and Rap-GMPpNHp (GMPpNHp is a non-hydrolysable GTP analogue) serves as a control for the specificity of the affinity purification. We recommend performing this control experiment for each new application (e.g. when performing the Rap activation assay on a novel cell type).

Owing to the low intrinsic nucleotide exchange rate of Rap, preparation of Rap-nucleotide complexes takes several hours and, for convenience, should be started the day before the Rap activity determination. If kept on ice, Rap-GDP and Rap-GMPpNHp complexes are stable for several hours.

1. To load Rap separately with GDP and GMPpNHp dilute 2 µg **His-tagged Rap1A** in 50 µl **NLS**. Mix well and split à 2 x 25 µl in two fresh vials.

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The concentration of His-Rap1A protein provided in the kit varies depending on the preparation batch.

2. Add 2 µl **10 mM GDP** or **10 mM GMPpNHp** separately to each of the samples. Mix well and briefly spin to collect the liquid on the bottom of the tubes. Incubate overnight or a minimum of 12 h on the bench at room temperature.
3. Add 2 µl **1 M $MgCl_2$** and mix well. Briefly spin down the liquid. Let stand 5 min on the bench and place on ice.
4. Add 2-10 µl each of the prepared Rap-GDP or Rap-GMPpNHp complexes separately to cell extracts being processed in a Rap activation assay either before (step 3 above) or after the centrifugation step (step 4).

Use extracts from unstimulated, serum-starved or otherwise treated cell points that are expected to yield little endogenous Rap-GTP.

5. Proceed with standard Rap activation assay as described above.

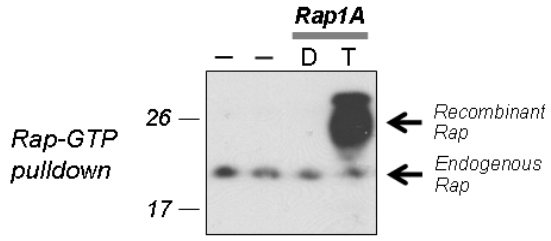

Fig. 2 *HeLa extracts were spiked with 5 µl of His-Rap1A preloaded with GDP (D) or GMPpNHp (T) as described above, and subjected to a Rap-GTP pulldown. Note that the presence of the His-tag allows discrimination of recombinant and native Rap proteins by the difference in size*

Troubleshooting

Problem	Measure
No Rap-GTP signal whatsoever is detectable, not even from control cells.	1. Spike cell extracts with Rap-GDP and Rap-GTP- -S to ascertain the functionality of all assay components 2. Increase the number of cells/assay point 3. Use a new Rap-antibody solution
No increase in Rap-GTP signal in stimulated cells as compared to control unstimulated point.	1. Use a different batch of agonist/stimulus or check otherwise for its functionality 2. To avoid Rap-GDP-derived false-positive signal in unstimulated points, increase the number of washing steps for the glutathione-sepharose precipitates

Selected references

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