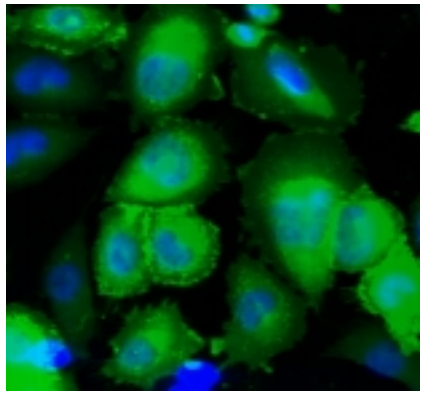
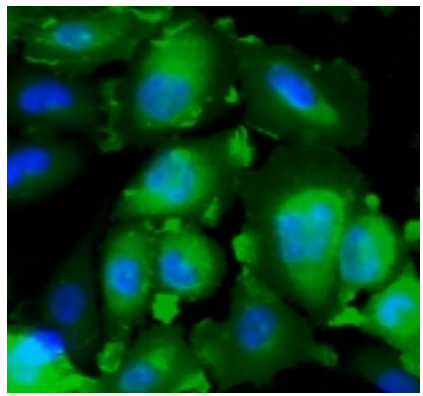
GFP-Rac1 Assay

user manual
GFP Assays
25-8007-27
25-8007-50
25-8010-15
25-8010-16

GFP-Rac1 Assay





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Page finder

Front cover:

Top image: CHO cells containing the GFP-Rac1 fusion protein before the addition of the agonist.

Bottom image: The same field of view, 6 min after stimulation with an agonist (30 nM Insulin).

Images show 1/18th of the full image acquired by the IN Cell Analyzer 3000.

BioImage is a Danish Biotech company specializing in developing drug candidates that exert their activity through modulation of protein translocation. For more information, visit their Web site at www.bioimage.dk

Chapter 6. Vector use details

Chapter 7. Quality control

Chapter 8. Troubleshooting guide

Chapter 9. References

Chapter 10. Related products

Chapter 11. Appendix

Chapter 1. Introduction

1.1. Rac1

Rac1 (ras-related C3 botulinum toxin substrate 1) is a member of the ras gene superfamily and Rho GTPase family of proteins (1). Rho GTPases regulate many essential processes, including actin dynamics, gene transcription, cell-cycle progression and cell adhesion. They function as molecular switches that cycle between an active GTP-bound and an inactive GDP-bound state (2). In the GTP-bound state, Rho family members are able to interface with effectors or target molecules to initiate downstream responses. An intrinsic GTPase activity returns the proteins to the GDP-bound state, completing the cycle and terminating the signal transduction (3). Currently there are three reported Rac isoforms (Rac1, 2 and 3), of which Rac1 is arguably the best characterized.

The nucleotide state of the Rho family proteins is controlled by three classes of regulatory proteins (4). Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP. GTPase–activating proteins (GAPs) promote the intrinsic GTP hydrolysis by Rho GTPases, leading to their rapid conversion to the GDP-bound state. Rho guanine nucleotide dissociation inhibitors (RhoGDIs) preferentially bind to the GDP-bound form of Rho proteins and prevent both spontaneous and GEF-catalyzed release of nucleotide. RhoGDIs therefore appear to maintain Rho proteins in the inactive state (5).

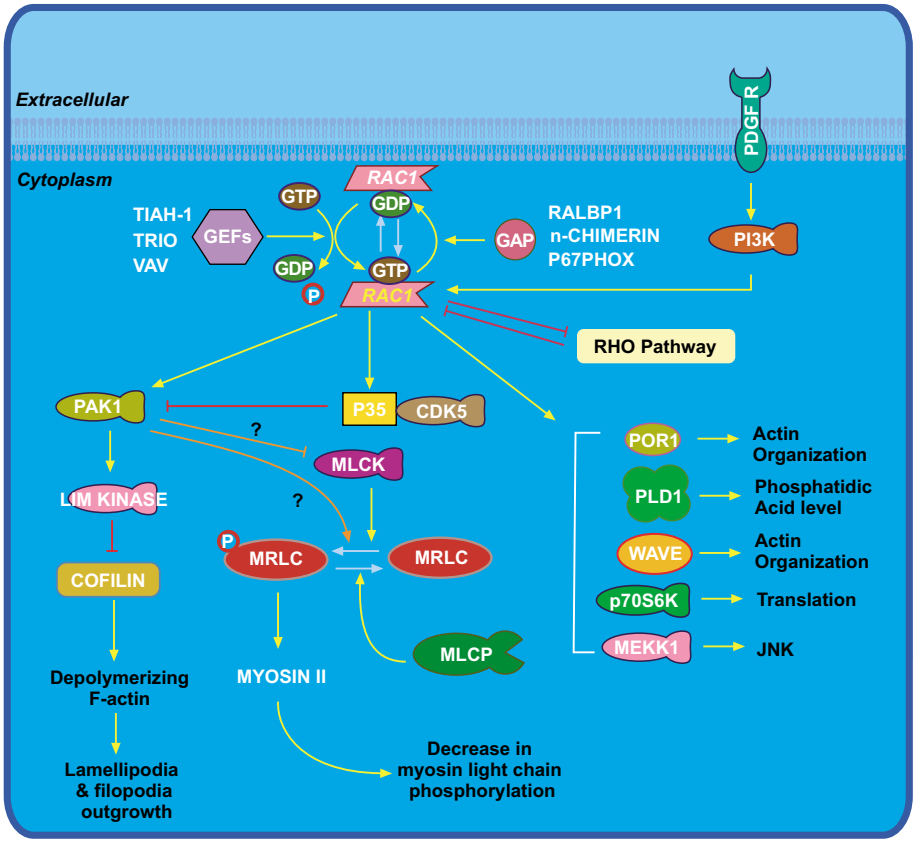
Rac1, Cdc42 and RhoA are the most extensively characterized Rho family members. The Rho/Rac/Cdc42 family is primarily devoted to controlling the status of the actin cytoskeleton, but each of the GTPases also has input into other signalling pathways such as the mitogen activated protein (MAP) kinase pathway and the c-Jun NH(2)-terminal kinase (JNK) pathway (in the case of Rac1) (3, 6). Activation of each of the Rho/Rac/Cdc42 proteins has fundamentally different outcomes on the state of the actin cytoskeleton. Rho activation generally leads to formation of actin stress fibers whereas Cdc42 activation generates filopodia. Activation of Rac1 favors the formation of lamellipodia and / or membrane ruffles (7, 8). There is little doubt that the co-ordinated activities of at least four small GTPases (Cdc42, Rac, Rho and Ras) are required for cell migration in a variety of cell types (6).

Rac homologs are found in a broad distribution of widely divergent eukaryotic species. The action of Rac on actin is strongly conserved across species and probably reflects a primordial function of this protein (7). In addition, Rac has important roles in gene transcription (via activation of MAPKinase pathways, and the transcription factor NF-κB), generation of reactive oxygen species, apoptosis, and cell-cycle progression. Rac may have at least two distinct roles in cell migration. Firstly, it regulates actin polymerization, leading to membrane protrusions, and secondly, it facilitates the activation of extra-cellular signal related kinase (ERK) MAP kinase. Rac is also involved in the phagocytosis of macrophages, again by controlling the actin skeleton (9, 10). Rac activation is restricted to the site of actin polymerization, independent of the overall distribution of Rac (8). Rac also stimulates cell proliferation (11, 12) and activates the JNK / stress-activated protein kinase (SAPK) cascade (13). Rac1 is known to regulate E-cadherin-

mediated cell-cell adhesion (14).

In mammalian cells, Rac is crucial for generating the actin-rich lamellipodial protrusions that are thought to be a major part of the driving force for forward movement (15). A number of assays have clearly demonstrated that the inhibition of Rac completely prevents movement (6). The generation of the lamellipodia comes about through the localized activation of multiple Rac effectors. Rac effectors include p21-Activated Kinase (PAK), Wiskott-Aldrich syndrome protein (WASP) / WASP family Verprolin-homologous protein (WAVE), formin, lipid-kinase, IQ motif-containing GTPase-activating protein (IQGAP) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (16). PAK is a serine / threonine kinase that is activated by an interaction with Rac1. PAK itself controls the activity of two key protein kinases, the Myosin Light Chain Kinase (MLCK) and LIM domain kinase (LIMK), thereby inhibiting actomyosin contraction while at the same time increasing actin filament stabilization (16, 17). Rac1 also stimulates actin filament nucleation and polymerization through the effector WAVE. It is the localized combination of the effector inputs to the actin cytoskeleton that eventually leads to the generation of lamellipodia and membrane ruffles (18). As a result of its role in lamellipodia production, activated Rac1 translocates from the cytoplasm to the membrane ruffles in response to stimulation with mitogenic factors (19). The activation state of the GFP-Rac1 fusion protein can be assessed by quantifying its agonist-induced translocation. It is possible to screen for compounds that have a positive or negative effect on the cascade that leads to the activation of Rac1.

Fig 1.1.: Rac1 activation mediates a range of cellular processes (provided with permission from BioCarta, www.biocarta.com)



1.2. GFP-Rac1 assay

The GFP-Rac1 assay provides a live-cell screening assay that measures the response of the Rac1 signalling pathway. The assay uses Redistribution™ technology to quantify the intracellular translocation of a GFP-Rac1 fusion protein in stably transfected mammalian cell lines. The GFP-Rac1 fusion protein behaves similarly to native Rac1 protein. The GFP-Rac1 Redistribution assay monitors the redistribution of activated Rac1 from the cytoplasm to the membrane ruffles that are formed upon the reorganization of the actin filaments in the plasma membrane.

This assay is optimized for image acquisition and analysis on the IN Cell Analyzer 3000, using the Plasma Membrane Spot analysis module , although the assay can also be imaged on other systems. The Plasma Membrane Spot analysis module measures the degree of Rac1 pathway response by quantifying the intensity of the GFP-Rac1 fusion protein at the cell surface, in cells challenged with test compounds. When the pathway is stimulated, the GFP-Rac1 fusion protein translocates to ruffle formations at the cell surface (Fig 1.2.). The Plasma Membrane Spot analysis module detects and quantifies these fluorescent cell-surface structures. Reference agonists, insulin or IGF-1, are used as controls (EC_{50} typically 0.57 nM and 0.12 nM, respectively). Wortmannin can be used as an inhibitor of the GFP-Rac1 fusion protein.

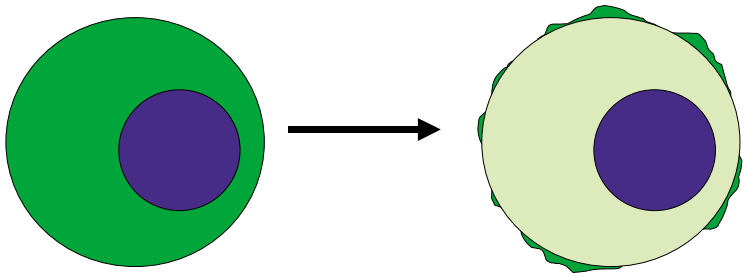


Fig 1.2.: Agonist induced redistribution of GFP-Rac1 from the cytoplasm to membrane ruffles

Chapter 2. Licensing considerations

2.1. Product right to use

Use of the GFP Rac1 assay is limited as stated in the terms and conditions of sale. These vary in accordance with the product code purchased.

Description	Product code
Non-commercial educational scientific	25-8010-15
Research for the discovery and development of human therapeutics	25-8007-50
Screening for the discovery and development of human therapeutics	25-8007-27
Assay Evaluation	
for 6 month period	25-8010-15
for 12 month period	25-8010-16

2.2. Legal

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This product was developed in collaboration with BioImage A/S and is the subject of patent application GB123856 in the name of Amersham Biosciences. The product is sold under license from: BioImage A/S under patents US 6 172 188, EP 851874 and EP 0896753 and other pending and foreign patent applications, and Vertex Pharmaceuticals (formerly Aurora Biosciences Corporation) under US patents: 5 625 048, 5 777 079, 5 804 387, 5 968 738, 5 994 077, 6 054 321, 6 066 476, 6 077 707, 6 090 919, 6 124 128, 6 172 188, European patent 1104769 and Japanese patent JP3283523, and other pending and foreign patent applications; and Iowa Research Foundation. The CMV promoter is covered under US patents 5 168 062 and 5 385 839 and its use is permitted for research purposes only. Any other use of the CMV promoter requires a license from the University of Iowa Research Foundation, 214 Technology Innovation Center Iowa City IA52242 USA; and Columbia University. This product is sold under license from Columbia University under US patent Nos. 5 491 084 and 6 146 826. Rights to use this product, as configured, are limited to internal use for screening, development and discovery of therapeutic products; NOT FOR

DIAGNOSTIC USE OR THERAPEUTIC USE IN HUMANS OR ANIMALS. No other rights are conveyed.

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Chapter 3. Product contents

3.1. Component summary

- CHO derived cells expressing the GFP-Rac1 fusion protein (two vials, each containing 1 ml of 1×10^6 cells) - NIF1955
- pCORON1000-GFP-Rac1 expression vector (one vial containing 10 µg DNA, at a concentration of 250 µg/ml, supplied in TE buffer: 10 mM Tris, 1 mM EDTA pH8.0) - NIF1992
- User manual

3.2. CHO derived cell line expressing GFP-Rac1 fusion protein - NIF1955

3.2.1. CHO derived parental cell line

The CHO-hIR cell line is of Chinese hamster ovary origin, derived from CHO-K1 (ATCC CCL-61) cells (20, 21, 22) that have been stably transfected with a non-mutated full-length human Insulin receptor (hIR) (23). The cells were transfected by a non-viral method and the hIR expression is under the control of the metallothionein promoter. The vector contains the DHFR gene that allows selection of expressing cells with methotrexate (MTX). The hIR expression appears to be extremely stable in CHO cells without selection pressure.

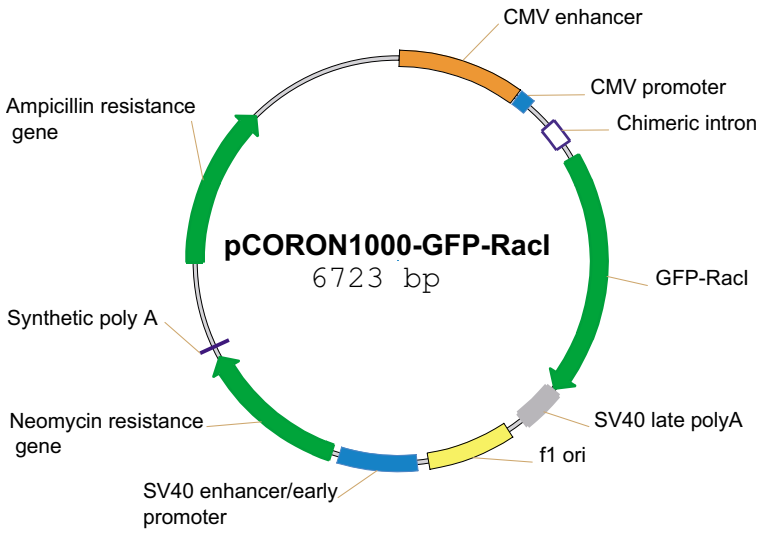
3.2.2. CHO derived GFP-Rac1 expressing cell line

CHO-hIR cells were transfected with the pCORON1000-GFP-Rac1 vector (supplied) using FuGENE 6. A stable clone expressing the recombinant fusion protein was selected using 500 µg/ml Geneticin. The isolated clone was grown for 18 passages before being sorted using a FACS machine. The cells were grown for a further passage before freezing. The cells tested negative for mycoplasma, bacterial and yeast contamination (testing details are available upon request). MTX selection pressure is not recommended with this particular cell line because CHO cells may develop MTX resistance by several reported mechanisms, including alterations to dihydrofolate reductase (DHFR) and decreased membrane transport (24). The present hIR expression levels in these cells are unknown. However, the cell line is responsive to both Insulin and insulin-like growth factor 1 (IGF-1) in the low nanomolar range, consistent with literature reports (26).

3.3. GFP-Rac1 expressing vector - NIF1992

The 6.7 kb plasmid, pCORON1000-GFP-Rac1, contains a bacterial ampicillin resistance gene and a mammalian neomycin resistance gene (see Fig 3.1.). The sequence of the construct is available, on a CD, upon request. Please e-mail incellanalyzer@uk.amershambiosciences.com . A detailed restriction map is shown in chapter 11, appendix A.

Fig 1.1.: Vector map of the supplied GFP-Rac1 expression vector



3.4. Materials and equipment required

The following materials and equipment are required, but not provided.

- Microplates. For analysis using the IN Cell Analyzer 3000, Packard Black 96 Well ViewPlates (Packard Cat # 6005182) should be used. For assays in 384 well format, please e-mail incellanalyzer@uk.amershambiosciences.com for recommendations.
- A CASY 1 Cell Counter and Analyzer System (Model TT) (Schärfe System GmbH) is recommended to ensure accurate cell counting prior to seeding. Alternatively a hemocytometer may be used.
- Environmentally controlled incubator (5% CO_2, 95% relative humidity, 37 °C)
- Imager / microscope (e.g. IN Cell Analyzer 3000)
- Laminar flow cell culture bench
- Tissue culture flasks (T-flasks) and pipettes
- Controlled freezing rate device providing a controlled freezing rate of 1 °C per min
- Standard tissue culture reagents and facilities (section 5.1.1.)

3.5. Software requirements

IN Cell Analysis System: The Plasma Membrane Spot analysis module is available from Amersham Biosciences for automated image analysis of the GFP-Rac1 assay. Analyzed data are exported as numerical files in ASCII format. ASCII format data can be imported into Microsoft™ Excel, Microsoft Access, or any similar package for further data analysis as desired.

Confocal or epifluorescence microscope: Suitable software will be required for analysis of images acquired on microscopes other than the IN Cell Analyzer 3000.

Chapter 4. Safety warnings, handling and precautions

4.1. Safety warnings

Warning: For research use only. Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

CAUTION! Contains genetically modified material

Genetically modified cells supplied in this package are for use in a suitably equipped laboratory environment. Users within the jurisdiction of the European Union are bound by the provisions of European Directive 98/81/EC which amends Directive 90/219/EEC on Contained Use of Genetically Modified Micro-Organisms. These requirements are translated into local law, which MUST be followed. In the case of the UK this is the GMO (Contained Use) Regulations 2000. Information to assist users in producing their own risk assessments is provided in sections 3.3.1 and 3.3.2 of '*The Genetically Modified Organisims (contained use) Regulations 2000*'. http://www.legislation.hmso.gov.uk/si/si2000/20002831.htm .

Risk assessments made under '*The Genetically Modified Organisims (Contained Use) Regulations 2000'* for our preparation and transport of these cells indicate that containment 1 is necessary to control risk. This risk is classified as GM Class 1 (lowest category) in the United Kingdom. For handling precautions within the United States, consult the National Institute of Health's Guidelines for Research Involving Recombinant DNA Molecules.

Instructions relating to the handling, use, storage and disposal of genetically modified materials:

1) These components are shipped in liquid nitrogen vapor. To avoid the risk of burns, extreme care should be taken when removing the samples from the vapor and transferring to a liquid Nitrogen storage unit. When removing the cells from liquid nitrogen storage and thawing there is the possibility of an increase in pressure within the vial due to residual liquid nitrogen being present. Appropriate care should be taken when opening the vial.

2) Genetically modified cells supplied in this package are for use in a suitably equipped laboratory environment and should be used only by responsible persons in authorized areas. Care should be taken to prevent ingestion or contact with skin or clothing. Protective clothing, such as laboratory overalls, safety glasses and gloves, should be worn whenever genetically modified materials are handled.

3) Avoid actions that could lead to the ingestion of these materials and NO smoking, drinking or eating should be allowed in areas where genetically modified materials are used.

4) Any spills of genetically modified material should be cleaned immediately with a suitable disinfectant.

5) Hands should be washed after using genetically modified materials.

6) Care should be taken to ensure that the cells are NOT warmed if they are NOT being used immediately. To maintain viability DO NOT centrifuge the cells upon thawing.

7) Most countries have legislation governing the handling, use, storage, disposal and transportation of genetically modified materials. The instructions set out above complement Local Regulations or Codes of Practice. Users of these products MUST make themselves aware of and observe relevant Local Regulations or Codes of Practice.

For further information, refer to the material safety data sheet(s) and / or safety statement(s).

4.2. Storage

The GFP-Rac1 expressing DNA construct (NIF1992) should be stored at -15 °C to -30 °C.

The CHO derived cells expressing the GFP-Rac1 fusion protein (NIF1955) should be stored at -196 °C in liquid Nitrogen.

4.3. Handling

Upon receipt, the cells should be removed from the cryo-porter and transferred to a gaseous phase liquid Nitrogen storage unit. Care should be taken to ensure that the cells are not warmed unless they are required immediately. The vector should be removed from the cryo-porter and stored at -15 °C to -30 °C until required.

4.3.1. Vector

After thawing the DNA sample, centrifuge briefly to recover the contents.

4.3.2. Cells

Do not centrifuge the cell samples upon thawing.

Chapter 5. Cell assay design

5.1. Culture and maintenance of CHO derived GFP-Rac1 expressing cell line

5.1.1. Tissue culture media and reagents required

The following media and buffers are required to culture, maintain and prepare the cells, and to perform the assay.

- GIBCO™ Nutrient Mixture F-12 Ham medium with Glutamax, Invitrogen™ life technologies 31765-027 or equivalent.
- GIBCO Fetal Bovine Serum (FBS), Invitrogen life technologies 10099-141 or equivalent.
- GIBCO Penicillin-Streptomycin (P/S), (5000 units/ml Penicillin G Sodium and 5000 µg/ml Streptomycin Sulfate), Invitrogen life technologies 15070-063 or equivalent.
- Geneticin (G418), Sigma G-7034 or equivalent.
- GIBCO Trypsin-EDTA in HBSS w/o Calcium or Magnesium, Invitrogen life technologies 25300-054 or equivalent.
- GIBCO Phosphate-Buffered Saline (PBS) Dulbecco's, w/o Calcium, Magnesium or Sodium Bicarbonate, Invitrogen life technologies 14190-094 or equivalent.
- Dimethylsulfoxide (DMSO), Sigma D-2650 or equivalent.
- Insulin, Sigma I-5500 or equivalent.
- Insulin-like Growth Factor 1, Sigma I-3769 or equivalent.
- Wortmannin, Sigma W-1628 or equivalent.
- Hoechst™ 33342, Molecular Probes H-21492.
- DRAQ5™, Biostatus.
- Bovine Serum Albumin (BSA), Sigma A-4503 or equivalent.
- 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Sigma H-3375 or equivalent.
- Cy5™ monocarboxyl dye, Amersham Biosciences PA05111.
- Fluorescein Isothiocyanate (FITC), Molecular Probes F-1300.
- Coumarin, Molecular Probes D-126.
- Formalin solution (10%), neutral-buffered, Sigma HT50-1-2.
- Standard tissue culture plastic-ware including tissue culture treated flasks (T-flasks), centrifuge tubes and cryo-vials.

5.1.2. Reagent preparation

NOTE - the following reagents are required, but not supplied.

- Growth-medium: Nutrient Mixture F-12 Ham medium with Glutamax supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin, and 0.5 mg/ml Geneticin.
- Freeze-medium: Nutrient Mixture F-12 Ham medium with Glutamax supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin, and 10% (v/v) DMSO.

- Assay-medium: Nutrient Mixture F-12 Ham medium with Glutamax supplemented with 10 mM HEPES, 0.2 % (v/v) BSA, and either 0.2 µM Hoechst nuclear stain or 1 µM DRAQ5 nuclear stain.
- Wortmannin: Wortmannin is light sensitive. Care must be taken in handling to prevent excessive degradation. Add 5 mg Wortmannin to 0.5 ml DMSO. Wortmannin is stable in DMSO at 4 °C for 2–3 weeks only. On the day of the assay, make up to 100 ml using PBS, to give a working stock of 117 µM. This should be kept in the dark at 4 ºC throughout the assay whenever possible. Prepare a 900 nM working dilution with Assay-medium (three fold of the final concentration, see section 5.2.6. for further details) less than an hour before it is required on the day of the assay. Keeping the working solution in the dark whenever practical. If a large number of assays is being performed over time during a day, we recommend preparing fresh working dilutions at regular intervals, with no working solution used for longer than 2 h.
- Flat field (FF) solution components:
  - Cy5 - 1 mM stock solution prepared in 10% (v/v) DMSO, 90% (v/v) PBS.
  - FITC - 1 mM stock solution prepared in 10% (v/v) DMSO, 90% (v/v) PBS.
  - Coumarin - 1 mM stock solution prepared in 10% (v/v) DMSO, 90% (v/v) PBS.

  As explained in the IN Cell Analyzer 3000 user manual, the flat field (FF) solution should be prepared to give a fluorescent signal in each channel between 700 and 3300 counts. Prepare an initial FF solution containing 20 µl 10 µM Cy5, 3 µl 10 µM FITC and 20 µl 1 mM Coumarin in 200 µl PBS, and adjust the solution as necessary to ensure fluorescent signal in the range of 700–3300 counts in each channel. Use 100 µl of FF solution for a 96 well microplate and 40 µl FF solution for a 384 well plate.

5.1.3. Cell thawing procedure

Two cryo-vials, each containing 1×10^6 cells in 1 ml of Freeze-medium are included with this assay kit. The vials are stored frozen in the vapor phase of liquid Nitrogen.

1. Remove a cryo-vial from storage.

2. Holding the cryo-vial, dip the bottom three-quarters of the cryo-vial into a 37 °C water bath, and swirl gently for 1–2 min until the contents are thawed. Do not thaw the cells for longer than 3 min as this decreases viability.

3. Remove the cryo-vial from the water bath and wipe it with 70% (v/v) Ethanol. Transfer the cells immediately to a T-25 flask and add 5 ml pre-warmed Growth-medium drop-wise to prevent cell damage. Add a further 2 ml Growth-medium and incubate at 37 °C.

NOTE: To ensure maximum cell viability, do not allow the cells to thaw at room temperature and do not thaw the cells by hand.

5.1.4. Cell subculturing procedure

Incubation: 5% CO_2, 95% humidity, 37 °C.
The cells should be passaged at a ratio of 1:10 when they are 70% confluent.

1. Warm all reagents to 37 °C.

2. Aspirate the medium from the cells and discard.

3. Wash the cells with PBS. Take care not to damage the cell layer while washing, but ensure that the entire cell surface is washed.

4. Aspirate the PBS from the cells and discard.

5. Add Trypsin-EDTA (2 ml for T-75 flasks and 4ml for T-162 flasks), ensuring that all cells are in contact with the solution. Wait for 3–10 min for the cells to round up / loosen. Check on an inverted microscope.

6. When the cells are loose, tap the flask gently to dislodge the cells. Add Growth-medium (8 ml for T-75 and 6 ml for T-162 flasks) and gently resuspend the cells with a 10 ml pipette until all the clumps have dispersed.

7. Aspirate the cell suspension and dispense 1 ml cells into a new culture vessel.

5.1.5. Cell seeding procedure

The following procedure is optimized for cells grown in standard T-75 and T-162 flasks to be seeded into 96 well microplates.

1. Warm all reagents to 37 °C.

2. Aspirate the medium from the cells and discard.

3. Wash the cells with PBS. Take care not to damage the cell layer while washing, but ensure that the entire cell surface is washed.

4. Aspirate the PBS from the cells and discard.

5. Add Trypsin-EDTA (2 ml for T-75 and 4 ml for T-162 flasks), ensuring that all cells are in contact with the solution. Wait for 3–10 min for the cells to round up / loosen. Check on an inverted microscope.

6. When the cells are loose, tap the flask gently to dislodge the cells. Add Growth-medium (3 ml for T-75 and 6 ml for T-162 flasks) and gently resuspend the cells with a 10 ml pipette until all the clumps have dispersed.

7. Count the cells using either a CASY1 Cell Counter and Analyzer System (Model TT) or a hemocytometer.

8. Using fresh Growth-medium, adjust the cell density to deliver the desired number of cells to each well. For example, to add 0.6×10^4 cells per well in a volume of 200 µl, adjust the suspension to 3×10^4 cells per ml. We recommend a concentration of $3–5 \times 10^4$ cells per ml.

9. Dispense 200 µl of the cells into each well of the microplate, except the well reserved for the flat field solution (see IN Cell Analyzer 3000 manual for further information).

10. Optionally incubate the plates undisturbed on a level surface for 1 h at room temperature (approximately 20 °C). This treatment may reduce edge effects.

11. Incubate the plated cells for 24 h at 37 °C before starting the assay.

N.B. If the cells are near confluence prior to trypsinization, they should be split into two T-flasks. They will then be ready for seeding the following day.

5.1.6. Cell freezing procedure

1. Harvest the cells as described in section 5.1.4 and resuspend the cells in a small volume of Growth-medium.

2. Count the cells as described in section 5.1.5.

3. Pellet the cells at approximately 300 g for 5 min. Aspirate the medium from the cells.

4. Gently resuspend the cells until no clumps remain in Freeze-medium at a concentration of 1×10^6 cells in 1 ml and transfer into cryo-vials. Each vial should contain 1×10^6 cells in 1 ml of Freeze-medium.

5. Transfer the vials to a cryo-freezing device and freeze at -80 °C for 16–24 h.

6. Transfer the vials to the vapor phase in a liquid Nitrogen storage device.

5.1.7. Growth characteristics

Under standard growth conditions, the cells should maintain an average size of 17.5 µm as measured using a CASY1 Cell Counter and Analyzer System (Model TT). The population doubling time of the cell line in exponential growth phase has been determined to be approximately 12 h under standard conditions.

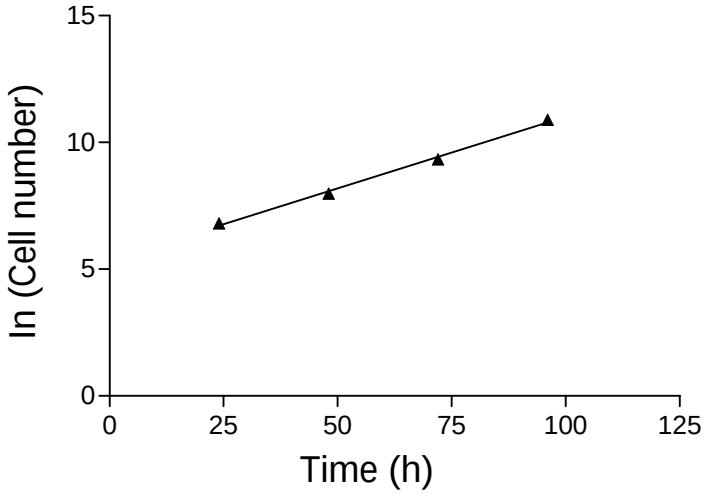


Fig 5.1. Growth curve of the CHO derived GFP-Rac1 expressing cell line. Only points on the linear portion of the curve are shown population doubling time = 12.2 h.

5.2. Assay set up

5.2.1. Live cell GFP-Rac1 assay using the IN Cell Analyzer 3000

This manual provides a suggested protocol to use the GFP-Rac1 assay for both agonist and antagonist screening on the IN Cell Analyzer 3000.

5.2.2. Microplate set up for 96 well format assays

The Rac1 assay can be run in both agonist and antagonist formats (see sections 5.2.4. and 5.2.6.). It is essential that the number of cells per well in the assay plates be consistent in order to minimize assay variability.

Insulin and IGF-1 can be used as reference agonists with typical EC_{50} values of 0.57 nM and 0.12 nM, respectively. Wortmannin is used as a reference antagonist. The GFP-Rac1 assay can be used with either Hoechst or DRAQ5 as the nuclear stain.

As explained in the IN Cell Analyzer 3000 user manual, each run must contain a flat field well to compensate for variations in fluorescence intensity

across each image. It is possible to prepare a plate solely for this purpose. Alternatively, a designated well on each plate can contain flat field solution. When seeding the plate, this well must not contain any cells if the auxiliary flat field correction tool is to be applied in the analysis module.

5.2.3. Schematic agonist assay protocol

Figure 5.2. shows a typical schematic of an agonist assay time-course. The cells should be seeded in the microplate the day before the experiment. Within 30 min to 3 h after the addition of the Assay-medium, the test compounds are added. Imaging occurs 6–8 min later.

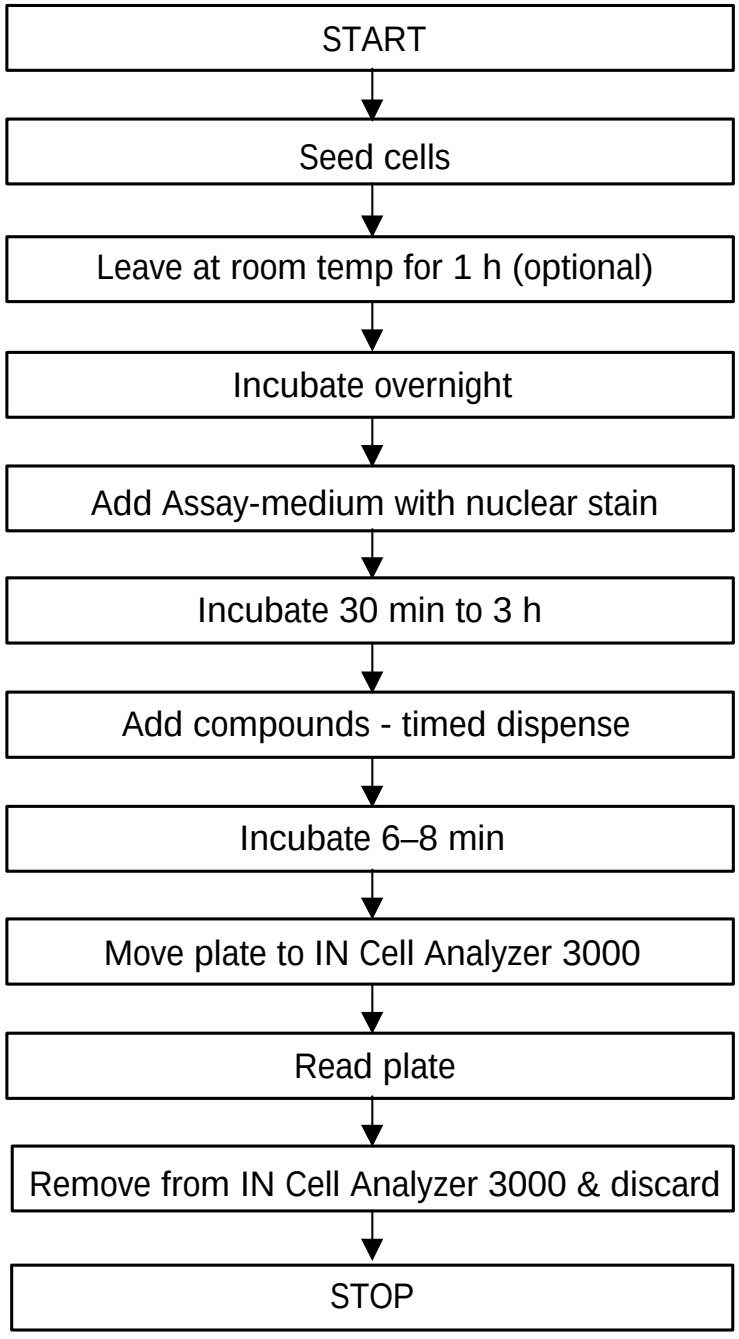


Fig 5.2. Flow diagram showing a basic protocol suitable for a GFP-Rac1 agonist screen. This is a minimal schematic showing how a GFP-Rac1 endpoint agonist screen could be performed with a 6–8 minute peak translocation time. All incubations are performed at 37 °C unless otherwise stated.

5.2.4. Agonist assay protocol (96 well format)

Note: whenever possible, keep the microplate at 37 °C, 5% CO_2, and 95% humidity.

1. The day before starting the assay, seed 0.6×10^4 cells per well in 200 µl of Growth-medium. Incubate at ambient temperature for 1 h (optional) before incubating for 24 h at 37 °C. If one of the wells on the cell plate is used for flat field correction, it should not contain cells.

2. On the day of the assay, prepare the test compounds, solvent controls (if used) and reference agonist controls (Insulin or IGF-1). These samples are typically prepared at four fold of the final concentration in Nutrient mixture F-12 Ham medium. For Insulin or IGF-1, final concentrations of 30 nM and 13 nM respectively, are suitable starting points. However, we recommend that users perform their own dose-response curves to establish optimal agonist concentrations.

3. Decant the Growth-medium from the cell plate, removing all excess liquid, and add 100 µl Assay-medium to wash the cells. Decant the wash.

4. Add 150 µl Assay-medium. Incubate the plates at 37 °C, 5% CO_2 for 30 min to 3 h.

5. Add 50 µl of the prepared four-fold dilution stocks of the test and control compounds to the appropriate wells in a strictly time controlled manner, so that each well has been exposed to the compound / control solutions for 6–8 min by the time that well is imaged. The total well volume is 200 µl.

6. After the first well has been incubated for 6–8 min, read the assay plate using the IN Cell Analyzer 3000.

7. Perform the data analysis using the IN Cell Analyzer 3000 System Plasma Membrane Spot analysis module.

5.2.5. Schematic antagonist assay protocol

Figure 5.3. shows a typical schematic of an antagonist assay time-course. The cells should be seeded in the microplate the day before the experiment.

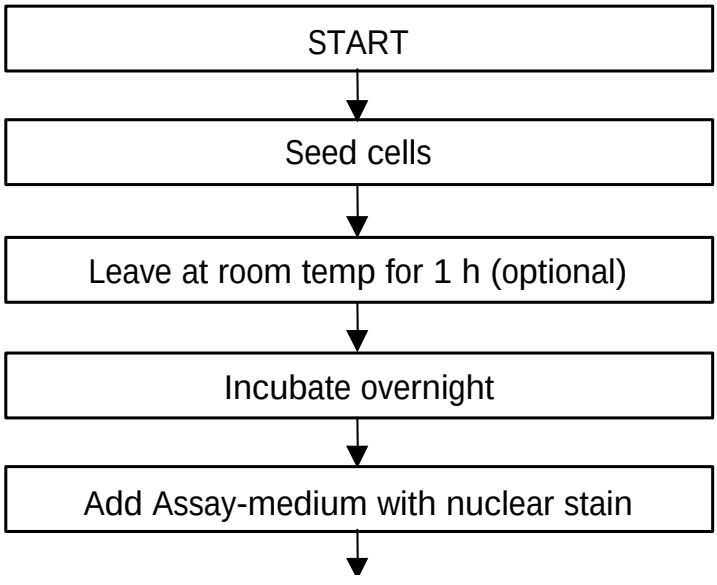
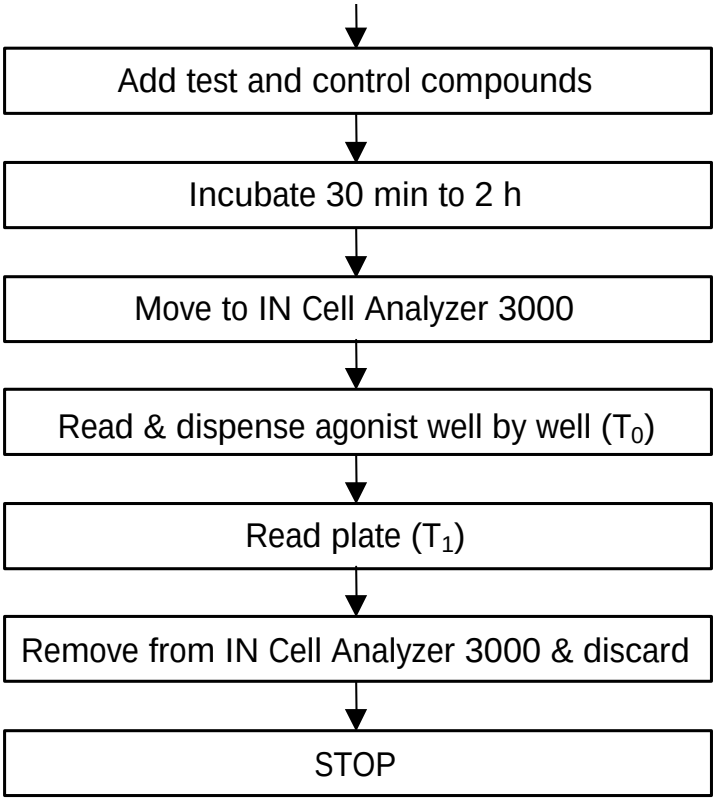


Fig 5.3. Flow diagram showing a basic protocol suitable for a GFP-Rac1 antagonist screen. This is a minimal schematic showing how a GFP-Rac1 endpoint antagonist screen could be performed with a 6–8 minute peak translocation time. All incubations are performed at 37 °C unless otherwise stated.



Within 30 min to 2 h after the addition of the Assay-medium and the test compounds, the agonist is added. Imaging occurs 6–8 min later.

5.2.6. Antagonist assay protocol (96 well format)

Note: whenever possible, keep the microplate at 37 °C, 5% CO_2, and 95% humidity.

1. The day before starting the assay, seed 0.6×10^4 cells in 200 µl of Growth-medium in each well of a 96 well microplate. Incubate for 24 h at 37 °C, 5% CO_2 following an optional 1 hour incubation at ambient temperature. If one of the wells on the cell plate is used for flat field correction, it should not contain cells.

2. On the day of the assay, prepare the solvent controls (if used), reference antagonist control (Wortmannin) and test compounds. These samples are typically three fold of the final concentration in Nutrient Mixture F-12 Ham medium. Also prepare a four-fold stock of the reference agonist. For Wortmannin, a final concentration of 300 nM is a suitable starting point. However, due to Wortmannin instability, we recommend that users perform their own inhibition curves to establish an optimal antagonist concentration.

3. Decant the Growth-medium from the cell plate and remove excess liquid. Add 100 µl Assay-medium to wash the cells. Decant the wash.

4. Add 100 µl of Assay-medium to the wells.

5. Add 50 µl of the prepared three fold dilution stocks of the test and control compounds to the appropriate wells. The total well volume is now 150 µl.

6. Incubate the plates at 37 °C, 5% CO_2 for 30 min to 2 h.

7. Read the assay plate before agonist addition, using the IN Cell Analyzer

3000. This is the T_0 read.

8. Immediately after each well is read, use the IN Cell Analyzer 3000 to dispense 50 µl of the four-fold reference agonist solution to that well. Recommended final concentrations of insulin and IGF-1 are 30 nM and 13 nM, respectively. The total well volume is now 200 µl.

9. After the first well has been incubated for 6–8 min, read the assay plate again, using the IN Cell Analyzer 3000. This is the T1 read.

10. Perform data analysis using the IN Cell Analyzer 3000 Plasma Membrane Spot analysis module.

5.2.7. Timing schedule

Because the maximal translocation of GFP-Rac1 occurs 6–8 min after the addition of, Insulin (or 8–12 min after the addition of IGF-1), timing is critical. Following the supplied protocols, scanning an entire 96 well microplate using a single pass (suitable when using DRAQ5 and GFP) will take approximately 6 min. Off-line addition of test agonists should be performed in a timed manner, either manually or automatically, to ensure that each well of the plate is incubated for the same amount of time.

Hoechst stained cells take longer to scan than DRAQ5 stained cells because the GFP and Hoechst signals must be imaged sequentially due to the spectral overlap of the emission profiles of these two probes. Consequently, the average time to image an entire 96 well microplate using Hoechst nuclear stain is 10 min. Therefore it would not be possible to screen a full plate without some of the wells incubating in the presence of the agonist (Insulin) for longer than the recommended 6–8 min. In this instance, it may be acceptable to perform a reduced assay (maximum 70 wells per plate) or use IGF-1 as the agonist. An alternative approach, which avoids the necessity of timed dispensing, is to configure the assay in a fixed-cell format (see section 5.2.9. for further details).

Similar considerations need to be taken for antagonist screens. Here, users have the option to use the IN Cell Analyzer 3000 dispenser to add agonist sequentially rather than using timed off-line dispensing.

5.2.8. Important considerations

When performing an antagonist screen, it is important to remember that the test compound added to the plates will be diluted by the addition of the agonist. It is recommended that the cells incubate in the target test compound concentration prior to the addition of the agonist. This means that the test compound concentration during the agonist-induced translocation will be 75% of the target concentration. Other options are available and can be determined by the user.

Although the assay is stable between 30 min and 3 h in Assay-medium prior to the addition of the agonist, we recommend that the incubation time be restricted to 2 h, in the case of the Wortmannin antagonist assays, due to the instability of the diluted Wortmannin solution.

5.2.9. Fixed cell assay format

In order to avoid the need for strictly time controlled dispensing, it is possible to complete the assay using live cells (as described in the agonist and

antagonist assay protocols) but then fix the cells prior to analysis:

1. Perform the assay as described.

2. After incubating with agonist for the optimal time, decant the Assay-medium and wash the cells in each well with 200 µl PBS.

3. Add 100 µl 10% (v/v) formalin and leave for 30 min at room temperature.

4. Decant the fixative and wash the cells in each well with 200 µl PBS.

5. Store the plate at 4 °C with 100 µl fresh PBS in each well.

6. Image and perform analysis as required.

If the cells are to be fixed, the assay can be performed with or without the nuclear stain in the Assay-medium for the duration of the assay. Nuclear staining, at the same concentrations as normal, can be performed after fixation. If no stain is used during the live-cell portion of the assay, the first PBS wash step can be omitted.

5.3. IN Cell Analysis System

The GFP-Rac1 assay has been developed and optimized for analysis using the IN Cell Analyzer 3000, in conjunction with the Plasma Membrane Spot analysis module. Please refer to the instrument user manual for details on instrument set up and the module manual for details on the algorithm settings. For further information on either of these products, please contact Amersham Biosciences.

5.3.1. IN Cell Analyzer 3000

The IN Cell Analyzer 3000 is a line-scanning, laser-based, confocal imaging system, with three high-speed CCD cameras. It has been developed specifically for performing information-rich cellular assays very rapidly and at high resolution, enabling high-throughput and high information-content testing of drug compounds.

5.3.2. Plasma Membrane Spot analysis module

The Plasma Membrane Spot analysis module enables the quantification of GFP-Rac1 translocation. It samples the level of GFP fluorescence (Rac1 expression) in the cell and then identifies and quantifies any bright fluorescent objects (in this case, ruffles) at the edge of the cell.

5.4. GFP-Rac1 translocation assay on epifluorescence microscopes

For speed of screening and quality of the images obtained, we recommend performing the GFP-Rac1 assay on the IN Cell Analyzer 3000. However, it is possible to adapt the assay to be read on alternative imaging platforms.

Laboratory grade inverted epifluorescence microscopes such as the Nikon Diaphot or Eclipse models or the Zeiss Axiovert model are suitable for image acquisition. A high-quality objective (Plan/Fluor 40 × 1.3 NA or similar) and epifluoresence filter sets compatible with GFP and the desired nuclear dye will be required. A motorized stage with multi-well plate holder and a heated stage enclosure are also recommended for assays performed on epifluorescence microscopes, and a suitable software package will be required

for image analysis.

5.5 Results

5.5.1. Calculating the Z'-factor

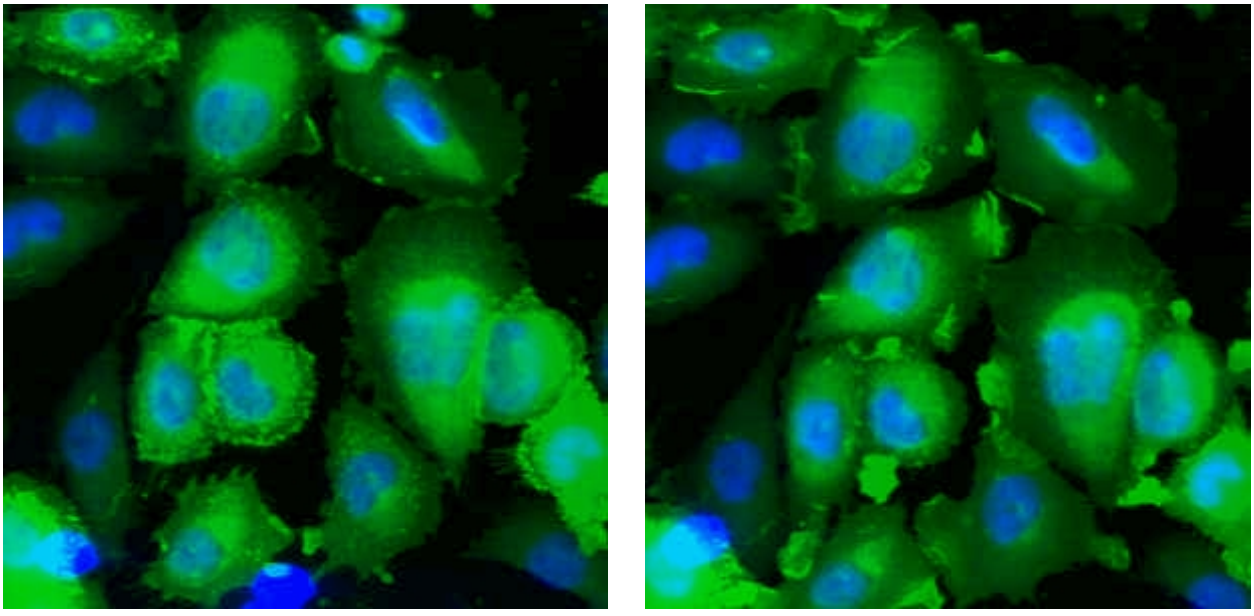
Assay performance can be assessed by calculating the Z' factor, a dimensionless value defined by Zhang *et al.* (27). Using the IN Cell Analyzer 3000, a Z'-factor of ≥ 0.3 should be obtained with the assay under standard conditions, if the experiment is performed as described in this manual.

$$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} + \mu_{c-}|}$$

where σ = standard deviation
μ = mean signal
$c+$ = positive control
$c-$ = negative control

5.5.2. Example results

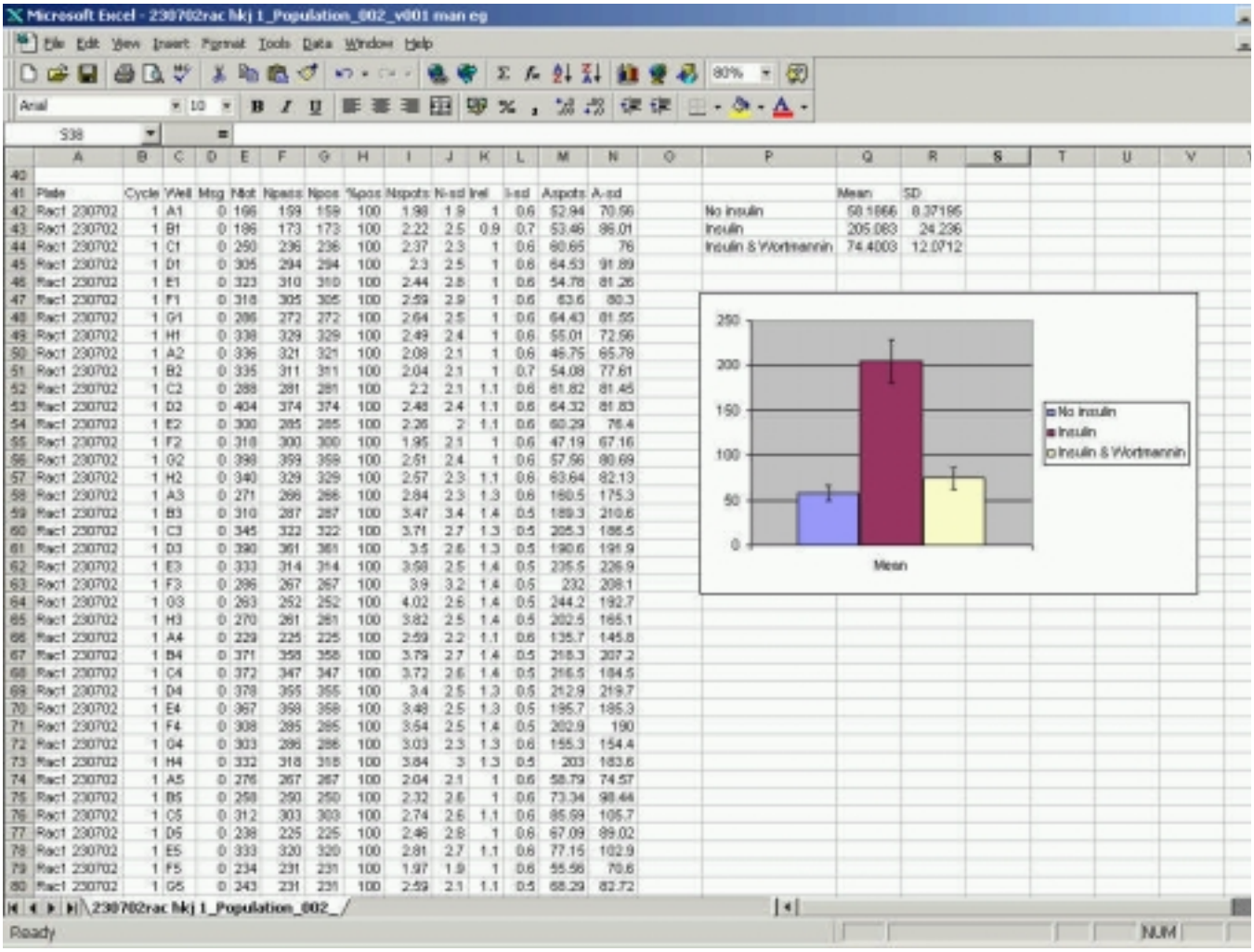
The following figures (5.4. and 5.5.) are taken from a single experiment, to give the user an overall view of the images and results that can be obtained with this GFP-Rac1 assay, using the IN Cell Analysis System. Figure 5.4. shows images captured on the IN Cell Analyzer 3000 of the supplied CHO derived GFP-Rac1 expressing cells before and after stimulation with 30 nM Insulin.



A) Before B) After

Fig 5.4. The same cells expressing GFP-Rac1 before and after stimulation with 30 nM Insulin. Only 1/18th of each image is shown in this figure.

Fig 5.5. Data from the example experiment, generated by the Plasma Membrane Spot analysis module module, exported to and analyzed in Microsoft Excel



Further analysis of these results indicated a Z'-factor of 0.33 for stimulated (30 nM Insulin) vs unstimulated (no Insulin) cells. The Wortmannin response is measured as a percentage decrease in response when compared to the Insulin stimulated cells, in this case, 89%.

5.6. Assay characterization

5.6.1. Translocation index

All of the characterizations for the GFP-Rac1 assay were performed on the IN Cell Analyzer 3000 using the Plasma Membrane Spot analysis module. The data generated by this module is in the format of A-spots. A-spots is a measure the area occupied by spots (ruffles) per cell, averaged for all identified cells in a well. This translocation index is used in all the following data.

5.6.2. Summary of quantitative assay parameters

A summary of typical assay data, using insulin as the agonist, is shown in Tables 5.1. and 5.2. In particular, Table 5.1. shows the results obtained from a single assay plate, indicating the level of well to well variation. Table 5.2. shows a summary of the results obtained from 19 assays, performed by different operators on different occasions, giving an indication of inter-assay variation.

Table 5.1. Results from a typical single assay, performed using the suggested protocol

Signal to noise (ref 27) is -

$$\frac{\text{mean signal - mean background}}{\text{background standard deviation}}$$

Magnitude of response is the mean signal - mean background

$$\%\ \text{CV is } \frac{\text{(standard deviation)}}{\text{mean}} \times 100$$

Z'-factor is a dimensionless characteristic useful for evaluation of assay quality (ref 27)

Parameter	Assay Data	# Assays	# Replicates
Signal to Noise	7.98	1	26
Z'-factor	0.30	1	26
Hill Slope			
Stimulated	0.92	1	6
Magnitude of Response	128.55	1	26
%CV			
Stimulated	7.69	1	26
Unstimulated	29.51	1	26

Table 5.2. Summary results from assays performed by different operators on different occasions, using the suggested protocol

∗ Standard deviation of the assays Signal to noise is -

$$\frac{\text{mean signal - mean background}}{\text{background standard deviation}}$$

Magnitude of response is the mean signal - mean background

$$\%\ \text{CV is } \frac{\text{(standard deviation)}}{\text{mean}} \times 100$$

Z'-factor is a dimensionless characteristic useful for evaluation of assay quality (ref 27)

Parameter	Assay Data (± SD∗)	# Assays	# Replicates
Signal to Noise	16.8 ± 7.5	19	32
Z'-factor	0.36 ± 0.09	19	32
Magnitude of Response	138.36 ± 30.89	19	32
%CV			
Stimulated	10.2 ± 1.97	19	32
Unstimulated	16.1 ± 5.62	19	32

5.6.3. Seeding density

Figure 5.6. shows the effect of varying seeding density in a 96 well microplate. The data were collected 7 min after the addition of 30 nM Insulin. Significant differences between stimulated and non-stimulated cells were seen at cell densities ranging from 4×10^3 to 1×10^4 cells per well.

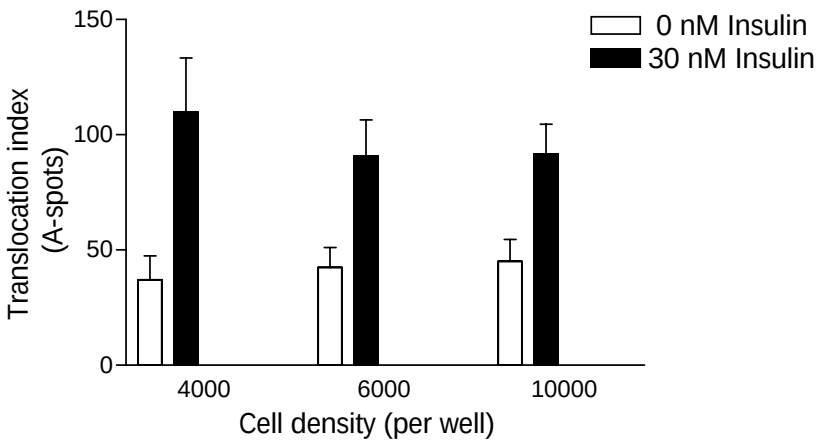


Fig 5.6. Insulin-induced GFP-Rac1 translocation as a function of seeding density. Stimulated cells were treated with 30 nM Insulin for 7 min prior to the scan. Error = ± SD, n = 4 replicates per data point.

5.6.4. Insulin dose response

Figure 5.7. shows an agonist dose-response curve for the supplied cells to Insulin. The data were collected 7 min after addition of the agonist, and demonstrate an EC_{50} of 0.6 nM.

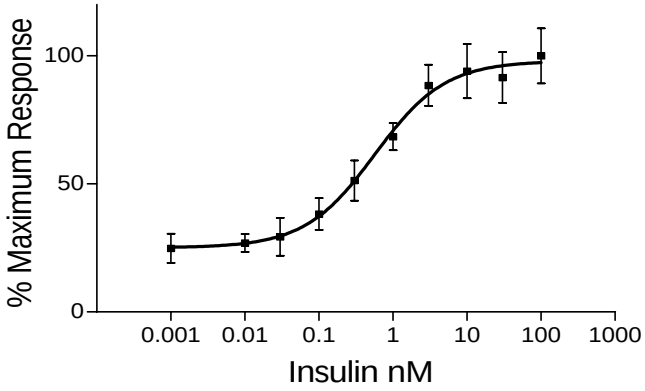


Fig 5.7. Insulin dose response curve using the supplied GFP-Rac1 cell line. Error = ± SD, n = 6 replicates per data point.

5.6.5. Wortmannin inhibition curve

Figure 5.8. shows a typical inhibition curve for Wortmannin. Data were collected 6–8 min after the addition of 100 nM Insulin and with varying concentrations of Wortmannin, and demonstrate an IC_{50} of 6.53 nM.

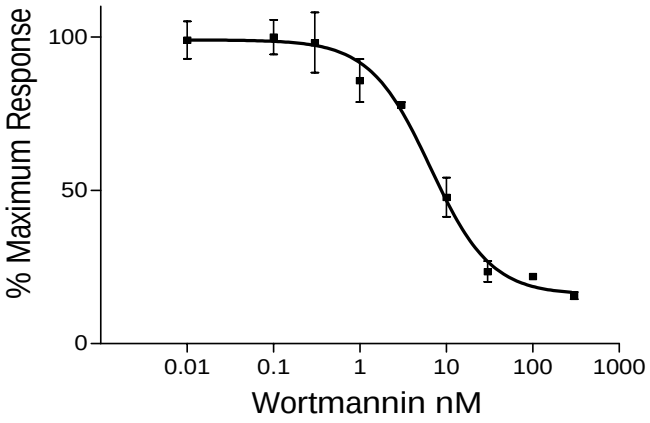


Fig 5.8. Wortmannin inhibition curve, in the presence of 100 nM Insulin. Error = ± SD, n = 3 replicates per data point.

5.6.6. Time course

Figure 5.9. shows a typical time course of the translocation and indicates that the maximal translocation occurs 6–8 min after the addition of the Insulin.

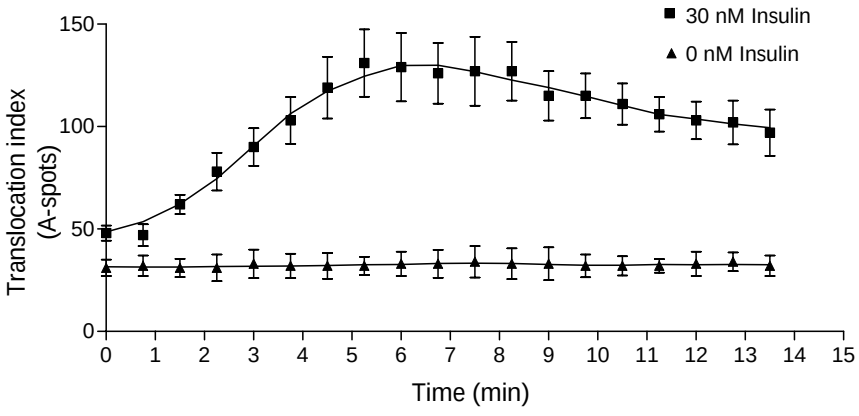


Fig 5.9. This graph shows the time course of GFP-Rac1 translocation using insulin as an agonist. Maximal response is seen after 6–8 min. Error = ± SD, n = 6 replicates per data point.

5.6.7. Sensitivity of assay to DMSO, Ethanol, and Methanol

The GFP-Rac1 translocation was measured in the presence of DMSO (≤ 2%), Ethanol (≤ 2%) or Methanol (≤ 2%).

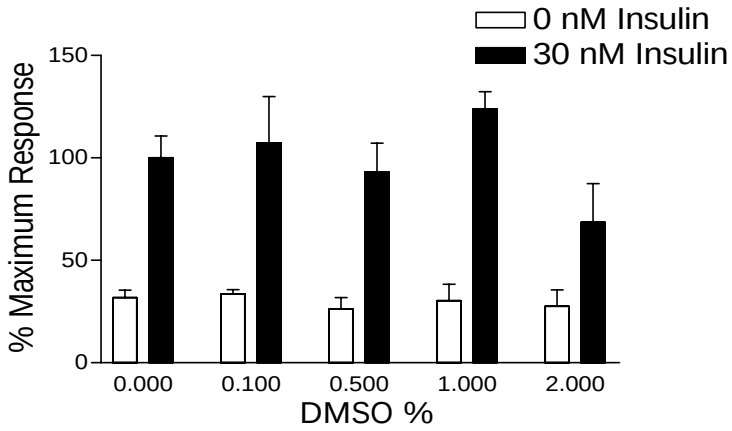


Fig 5.10a. The effects of DMSO on the fluorescent fusion protein translocation. Error = ± SD, n = 3 replicates per data point.

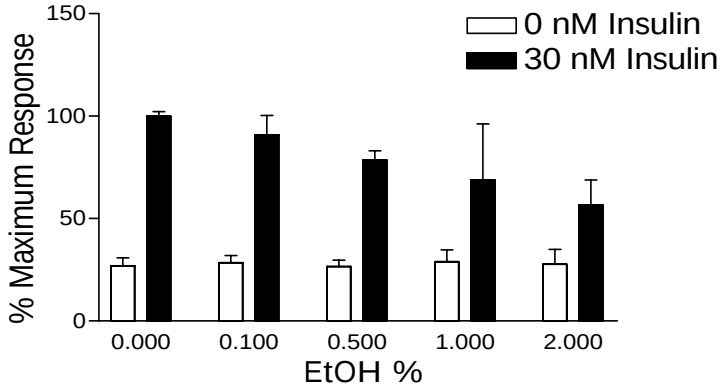


Fig 5.10b. The effects of Ethanol on the fluorescent fusion protein translocation. Error = ± SD, n = 3 replicates per data point.

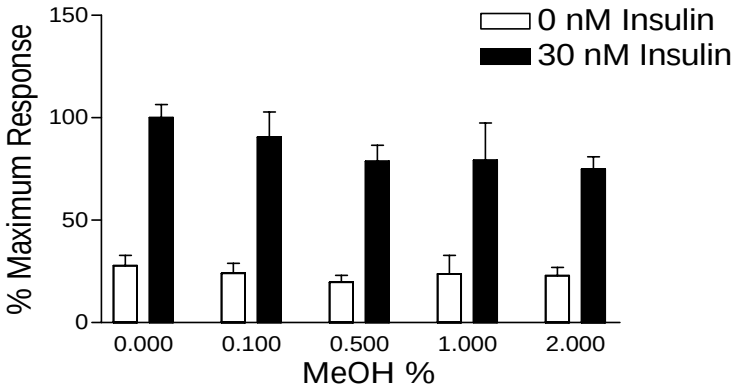


Fig 5.10c. The effects of Methanol on the fluorescent fusion protein translocation. Error = ± SD, n = 3 replicates per data point.

5.6.8. Effects of different culture conditions

To determine the effects of varying culture conditions on the Insulin induced GFP-Rac1 fusion protein translocation, the stable CHO hIR cells were cultured for 7 d in either the recommended Growth-medium or Dulbecco's modified eagle medium (DMEM) (Invitrogen life technologies, 32430-027), in either 10%, 5% or 1% (v/v) FCS. The results, shown in Figure 5.11., demonstrate that the assay is optimal in the recommended Growth-medium.

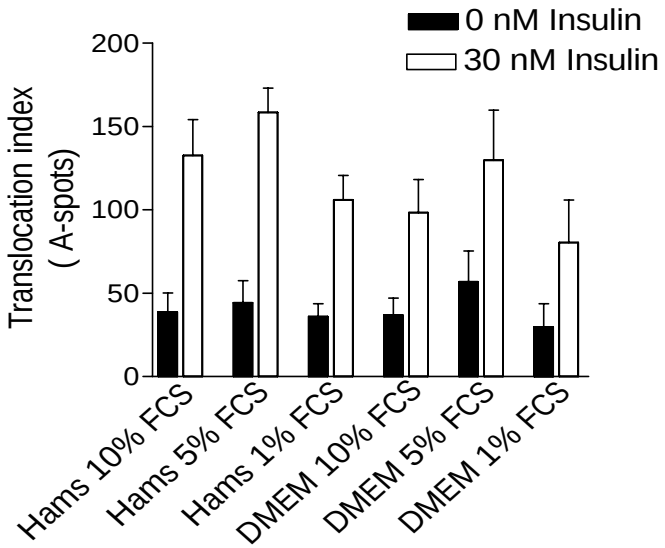


Fig 5.11. The effect of different culture conditions on the cytoplasmic to membrane ruffles translocation of GFP-Rac1. Error = ± SD, n = 32 replicates per data point.

5.6.9. IGF-1 agonist

IGF-1 can be used as an alternative to Insulin as an agonist. The following figures (5.12.–5.15.) demonstrate the optimal cell densities, IGF-1 concentration, assay time and Wortmannin concentration for use with this agonist.

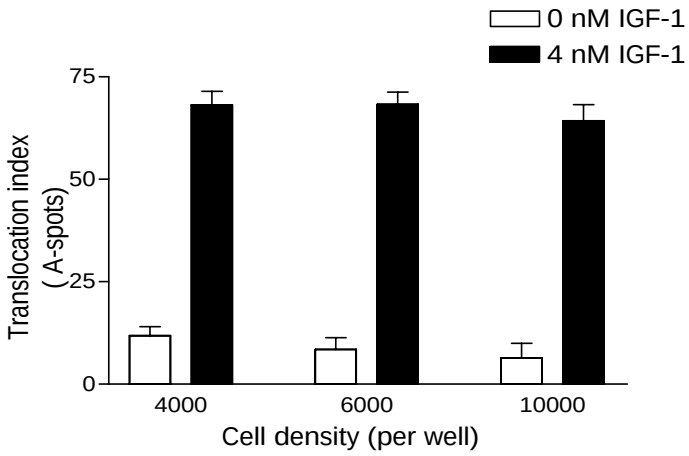


Fig 5.12. IGF-1-induced GFP-Rac1 translocation as a function of cell seeding density. Stimulated cells were treated with 4 nM IGF-1. Error = ± SD, n = replicates (0 nM IGF-1), n = 12 replicates (13 nM IGF-1).

Fig 5.13. IGF-1 dose response using the supplied GFP-Rac1 cell line. EC_{50} = 0.12 nM. Error = ± SD, n = 3 replicates per data point.

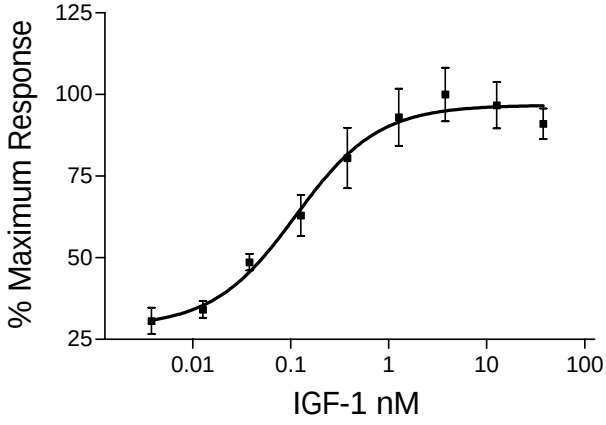


Fig 5.14. This graph shows the time course of the GFP-Rac1 translocation using IGF-1 as an agonist. Maximal response is seen 8–12 min. Error = ± SD, n = 6 replicates per data point.

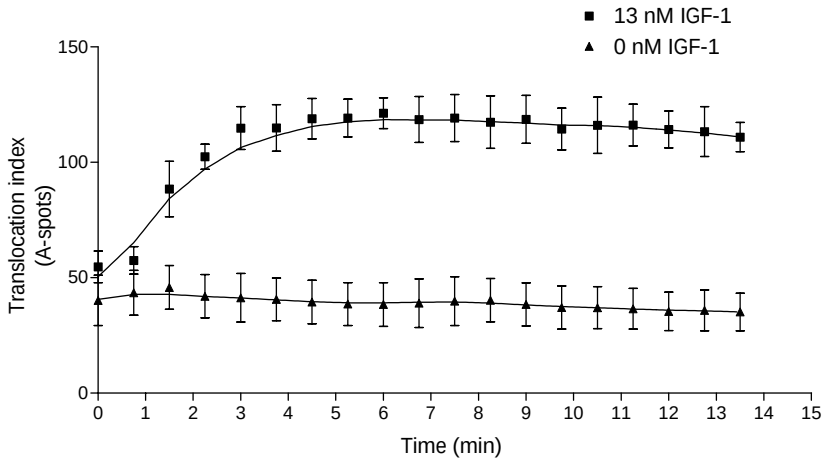
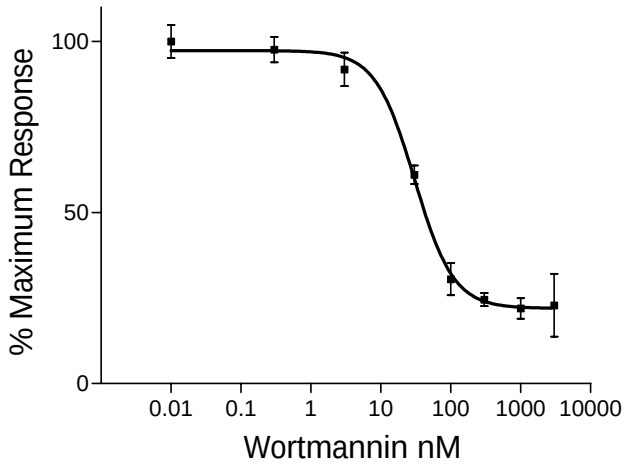


Fig 5.15. Wortmannin inhibition curve, in the presence of 13 nM IGF-1. IC_{50} = 26.6 nM. Error = ± SD, n = 3 replicates per data point.



Chapter 6. Vector use details

The plasmid vector pCORON1000-GFP-Rac1 (Fig 3.1.) can be used to transiently or stably express GFP-Rac1 fusion protein in the cell line of choice.

6.1. General guidelines for vector use

pCORON1000-GFP-Rac1 has been used successfully to express GFP-Rac1 fusion protein both transiently and stably in the CHO derived cell line. Expression levels, translocation responses and other assay parameters may vary if a different cell type or transfection procedure is chosen.

6.2. Transient transfection with pCORON1000-GFP-Rac1

Transient transfection protocols must be optimized for the cell type of choice. Choice of transfection reagent and cell type will affect efficiency of transfection. To ensure the best quality reproducible results are obtained, define optimal DNA concentration and cell seeding density. For more information, refer to manufacturer's guidelines for the desired transfection reagent.

6.3. Stable cell line generation with pCORON1000-GFP-Rac1

The process of establishing stable cell lines involves a large number of variables, many of which are cell-line dependent. Standard methods and guidelines for the generation of stable cell lines are widely available in the public domain (28).

pCORON1000-GFP-Rac1 has been used to generate stably transfected cell populations. The magnitude of the response and the kinetics of the translocation event achievable with different cell lines are unknown, and may deviate from the values specified in this manual.

Chapter 7. Quality control

7.1. GFP-Rac1 cell line

The GFP-Rac1 cell line is supplied at a concentration of 1×10^6 cells per ml in fetal calf serum containing 10% (v/v) DMSO. The cell line should have the characteristics detailed in Table 7.1.

Table 7.1.: Quality control information for GFP-Rac1 cell line

Property	Value	Measurement method
Assay stability	Z'-factor ≥ 0.3	Quality Control Assay
Viability from frozen	> 80%	CASY1 Cell Counter and Analyzer System (Model TT)
Cell diameter (mm)	15–18	CASY1 Cell Counter and Analyzer System (Model TT)
Fluorescence at 5×10^4 cells per ml (RFU)	> 15 000 for 20 passages after dispatch	FARCyte (Gain 62)

7.2. GFP-Rac1 expression vector

The GFP-Rac1 expression vector is supplied in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 250 µg/ml. The vector should have the characteristics outlined in Table 7.2.

Table 7.2.: Quality control information for the GFP-Rac1 expression vector

Property	Value	Limits	Measurement method
Concentration	250 µg/ml		UV Absorbance @ 260 nm in water
Purity - Minimal contamination of the DNA construct by RNA or protein	A_{260}/A_{280} ratio	Between 1.8–2.2	UV/Vis Absorbance@ 260nm and 280 nm
Expected restriction pattern	The restriction digests should give fragments of the sizes shown in Table 7.3.		Agarose gel electrophoresis

Table 7.3.: Expected restriction pattern for the GFP-Rac1 expression vector

Enzyme(s)	# of cuts	Fragment(s) size (bp)
*Hpa*I	2	955, 5723
*Nco*I	6	296, 719, 757, 985, 1004, 2962
*Nhe*I	2	734, 5989
*Not*I	1	6723
*Pvu*I	4	493, 1537, 1938, 2755

Chapter 8. Troubleshooting guide

8.1 Troubleshooting guide

Problem	Possible causes and remedies
❶ Low assay response (positive vs negative controls).	**Possible cause** **1.1.** Passage number too high. **1.2.** Cell density too low or too high. **1.3.** Incorrect selection of analysis parameters. **1.4.** Incorrect assay / incubation conditions. **1.5.** Reagents were not stored properly or they are out of date. **Remedy** **1.1.** Start a fresh batch of cells from an earlier passage number. Cells should be expanded, and additional vials should be frozen down from the vials delivered with the kit. **1.2.** Verify density of cell plating; adjust plating density to values that yield optimal assay response. **1.3.** Check that the primary parameters are correct and suitable for the cells currently in use. **1.4.** Ensure that proper incubation is maintained as consistently as possible during the assay. When plates are out of the CO_2 incubator for extended periods, it is essential that HEPES buffer be added to the medium to maintain the correct pH. **1.5.** Repeat assay with fresh reagents.
❷ Low nuclear intensity.	**Possible cause** **2.1.** Nuclear stain concentration too low. **2.2.** Nuclear stain incubation time too short. **Remedy** **2.1.** Adjust nuclear stain concentration to recommended level. **2.2.** Adjust nuclear stain incubation time to recommended length.
❸ Image is out of focus.	**Possible cause** **3.1.** Autofocus Offset is chosen incorrectly or the system may need to be realigned. **Remedy** **3.1.** Alignment and calibration of instrument. Perform Z-stack on cells. Change Autofocus Offset.
❹ Cells do not adhere to well bottom in plate.	**Possible cause** **4.1.** Plating density too high. **Remedy** **4.1.** Reduce plating density.
❺ Shading across image field.	**Possible cause** **5.1.** Flat field correction not applied or flat field solution too weak. **Remedy** **5.1.** Apply flat field correction or adjust flat field solution.

Chapter 9. References

9.1. References

1. Didsbury, J. *et al*. Rac, a novel ras-related family of proteins that are botulinum toxin substrates. *J. Biol. Che*m. **264**, 16378–16382 (1989).

2. Kjoller, L. and Hall, A. Signaling to Rho GTPases. *Exp Cell Res*. **253**, 166–179 (1999).

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Chapter 10. Related products

10.1. Related products

Product Name	Code:
GFP Assays	
GFP-PLCδ-PH domain Assay	25-8007-26
GFP-MAPKAP-k2 Assay	25-8008-82
EGFP - 2x FYVE Assay	25-8010-21
AKT-1 EGFP Assay	25-8010-17
EGFP SMAD2 Assay	25-8010-46
CypHer	
CypHer5 Labelled Anti VSV-G Antibody	PA45407
CypHer5 NHS Ester (1 mg pack)	PA15401
CypHer5 NHS Ester (5 mg pack)	PA15405
pCORON 1000 VSV-G Expression Vector	25-8008-51
pCORON 1000 SP VSV-G Expression Vector	25-8009-92
IN Cell Analysis System	
IN Cell Analyzer 3000	25-8010-11
Plasma Membrane Spot Analysis Module	63-0048-96

Chapter 11. Appendix

11.1. Appendix A: Restriction map of pCORON1000-GFP-Rac1

The following enzymes do not cut the vector: *Acc*III, *Apa*I, *Asc*I, *Ava*I, *Bpu*1102I, *Bse*AI, *Bsg*I, *Bsi*WI, *Bsp*120I, *Bsp*EI, *Bst*EII, *Cel*II, *Eco*47III, *Eco*NI, *Eco*RI, *Eco*RV, *Esp*I, *Ksp*I, *Mlu*I, *Mro*I, *Nru*I, *Pac*I, *Pae*R7I, *Pfl*MI, *Pme*I, *Ppu*MI, *Sac*II, *Sal*I, *Sbf*I, *Sgr*AI, *Sma*I, *Swa*I, *Van*91I, *Xba*I, *Xho*I, *Xma*I.

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Aat*I	2	2316 3648
*Aat*II	5	279 332 415 601 4900
*Acc*65I	1	3299
*Acc*I	1	1554
*Aci*I	73	129 212 240 252 266 399 433 524(c) 557(c) 669 690(c) 767(c) 2042 2128(c) 2243 2360 2405(c) 2409 2686 2747(c) 2761(c) 2764(c) 2792 2819 3197(c) 3223(c) 3236 3244(c) 3312(c) 3497 3509 3518 3530 3540 3551 3597 3752 3815 3909(c) 3973(c) 4074(c) 4077(c) 4317 4357(c) 4362 4412(c) 4428 4454 4510(c) 4569 4641 4679 4705 4715 4754 4928(c) 4975 5074(c) 5183(c) 5260(c) 5304 5425(c) 5471 5662(c) 5753(c) 6115 6124(c) 6259 6369(c) 6490(c) 6509(c) 6636(c) 6664(c)
*Acs*I	5	1181 1238 2494 3148 3159
*Acy*I	8	276 329 412 598 3843 4545 4897 5279
*Afl*II	4	829 848 1051 3697
*Afl*III	1	1427
*Age*I	1	1972
*Alu*I	26	728 759 834 1048 1781 1820 1862 2000 2224 2533 2878 3135 3325 3613 3667 3949 4407 4768 4787 5466 5529 5629 6150 6407 6453 6543
*Alw*44I	3	4650 5147 6393
*Alw*I	18	1727(c) 2335(c) 2651(c) 2660 3254 4022 4087(c) 4268 4632(c) 4645 5180 5184(c) 5501 5964(c) 5965 6061(c) 6063 6149
*Alw*NI	2	1784 6298
*Aos*I	4	2714 3253 3945 5596
*Apa*LI	3	4650 5147 6393
*Apo*I	5	1181 1238 2494 3148 3159
*Ase*I	2	161 5644
*Asn*I	2	161 5644
*Asp*700	1	5219
*Asp*718	1	3299
*Asp*EI	1	5819

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Asp*HI	8	730 2297 3956 4146 4654 5151 5236 6397
*Asp*I	1	3961
*Asu*II	2	1727 4525
*Ava*II	4	1759 4359 5455 5677
*Avi*II	4	2714 3253 3945 5596
*Avr*II	1	3649
*Bam*HI	1	4637
*Ban*I	8	619 977 2130 2924 3299 3842 3877 5866
*Ban*II	3	730 2894 4208
*Bbr*PI	1	1430
*Bbs*I	1	962
*Bbv*I	19	821(c) 1768(c) 2520(c) 2727 2795 3266 3790(c) 3916 3958 3974(c) 4067(c) 4479 4774(c) 5385(c) 5776 6079(c) 6285(c) 6288(c) 6378
*Bcg*I	1	5281(c)
*Bcl*I	1	1884
*Bfa*I	15	154 753 1058 1087 1637 1821 2159 2252 2461 2812 3650 3704 5626 5961 6214
*Bfr*I	4	829 848 1051 3697
*Bgl*I	7	137 244 366 437 2724 3602 5701
*Bgl*II	1	6719
*Bln*I	1	3649
*Bmy*I	13	730 1179 2297 2894 3789 3882 3956 4146 4208 4654 5151 5236 6397
*Bpm*I	4	1152 1932 2309 5750
*Bpu*AI	1	962
*Bsa*AI	4	494 1430 2965 4147
*Bsa*BI	2	2651 4636
*Bsa*HI	8	276 329 412 598 3843 4545 4897 5279
*Bsa*I	3	916(c) 1743(c) 5753
*Bsa*JI	13	514 1271 2256 3260 3361 3433 3556 3591 3600 3649 4006 4275 6547
*Bsa*WI	5	1972 3874 5523 6354 6501
*Bsi*EI	8	665 2202 2409 2695 3752 5301 5450 6373
*Bsi*HKAI	8	730 2297 3956 4146 4654 5151 5236 6397
*Bsi*YI	11	203 2746 3072 3557 3824 4368 4781 6229 6508 6674 6692
*Bsl*I	11	203 2746 3072 3557 3824 4368 4781 6229 6508 6674 6692
*Bsm*AI	11	588 826 916(c) 941(c) 1743(c) 1848(c) 3694 4782 4824(c) 4977(c) 5753

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Bsm*FI	10	329 480 648 1124(c) 2124(c) 3343(c) 3415(c) 3479(c) 3994 4526
*Bsm*I	2	2470 2563(c)
*Bsp*1286I	13	730 1179 2297 2894 3789 3882 3956 4146 4208 4654 5151 5236 6397
*Bsp*DI	2	2655 4624
*Bsp*HI	3	4874 4979 5987
*Bsp*MI	4	878(c) 3730(c) 4111 4561
*Bsp*WI	37	137 244 366 398 437 530 554 803 1054 2308 2345 2694 2724 2756 2758 2800 2827 2857 3394 3466 3517 3596 3602 3834 3918 3941 4080 4086 4203 4239 4286 4553 4649 5701 6089 6661 6709
*Bsr*BI	4	2821(c) 4456(c) 4510 4977(c)
*Bsr*DI	4	66(c) 4076 5585 5759(c)
*Bsr*FI	5	1972 2860 4162 4343 5734
*Bsr*GI	2	97 1381
*Bsr*I	18	449(c) 887 940 1034(c) 1135 1258 3054 3534(c) 3787 3988 5174 5344(c) 5613 5656 5774 6180 6292(c) 6305(c)
*Bss*HII	1	4240
*Bst*1107I	1	1555
*Bst*BI	2	1727 4525
*Bst*NI	11	244 437 1911 2288 3363 3418 3435 4230 6548 6561 6682
*Bst*UI	18	214 2737 2761 2781 3157 3244 3909 4210 4242 4643 4723 4826 4828 4928 5260 5753 6083 6664
*Bst*XI	2	1957 4564
*Bst*YI	12	1732 3246 4014 4260 4637 5172 5189 5957 5969 6055 6066 6719
*Bsu*36I	1	2121
*Cfo*I	28	2302 2715 2739 2752 2761 2783 2809 2817 3254 3837 3845 3909 3946 4212 4242 4244 4472 4725 4828 4928 5260 5597 5690 6083 6192 6366 6466 6533
*Cfr*10I	5	1972 2860 4162 4343 5734
*Cla*I	2	2655 4624
*Csp*45I	2	1727 4525
*Csp*6I	12	98 372 452 485 536 701 1063 1382 3300 4148 4661 5337
*Dde*I	12	2121 2180 2262 3307 3609 4506 4657 4892 5318 5858 6024 6433
*Dpn*I	32	664 749 1332 1734 1886 2178 2201 2342 2654 2658 2694 3248 4016 4094 4175 4184 4262 4639 5138 5174 5191 5449 5495 5513 5854 5959 5971 6049 6057 6068 6143 6721
*Dpn*II	32	662 747 1330 1732 1884 2176 2199 2340 2652 2656 2692 3246 4014 4092 4173 4182 4260 4637 5136 5172 5189 5447 5493 5511 5852 5957 5969 6047 6055 6066 6141 6719
*Dra*I	5	1495 2610 5241 5933 5952
*Dra*II	1	4839

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Dra*III	1	2968
*Drd*I	7	818 1757 3012 3686 3870 4736 6605
*Dsa*I	6	514 1271 2256 3260 3556 4275
*Dsa*V	19	242 435 1909 2286 2659 3361 3416 3433 3845 4005 4228 4745 4780 5281 5632 6328 6546 6559 6680
*Eae*I	10	9 63 1274 2406 3749 3923 4314 4341 4566 5426
*Eag*I	2	2406 3749
*Eam*1105I	1	5819
*Ear*I	6	2369(c) 2375(c) 2673(c) 4187(c) 4397(c) 5020(c)
*Ecl*136II	1	728
*Ecl*XI	2	2406 3749
*Eco*57I	6	1455 2231 3989 4421 5153 6165(c)
*Eco*O109I	1	4839
*Eco*RII	11	242 435 1909 2286 3361 3416 3433 4228 6546 6559 6680
*Esp*3I	3	1848(c) 4782 4824(c)
*Fnu*4HI	40	835 1782 2129 2406 2409 2534 2716 2748 2762 2784 3255 3597 3752 3804 3815 3905 3910 3947 3988 4075 4078 4081 4317 4413 4454 4468 4569 4679 4788 5075 5304 5399 5426 5765 6093 6299 6302 6367 6510 6665
*Fnu*DII	18	214 2737 2761 2781 3157 3244 3909 4210 4242 4643 4723 4826 4828 4928 5260 5753 6083 6664
*Fok*I	11	984(c) 1817 2141(c) 2198 3500(c) 4167 4192 4737(c) 5380 5667 5848
*Fsp*I	4	2714 3253 3945 5596
*Hae*II	5	2303 2810 2818 3846 6467
*Hae*III	30	11 65 238 431 1276 1677 1832 2316 2408 2684 2973 3115 3265 3590 3596 3605 3648 3751 3925 4316 4343 4568 4841 5428 5695 5775 6233 6667 6685 6696
*Hga*I	7	688 2743 4553 4729 5287 6017(c) 6595(c)
*Hgi*AI	8	730 2297 3956 4146 4654 5151 5236 6397
*Hha*I	28	2302 2715 2739 2752 2761 2783 2809 2817 3254 3837 3845 3909 3946 4212 4242 4244 4472 4725 4828 4928 5260 5597 5690 6083 6192 6366 6466 6533
*Hin*P1I	28	2300 2713 2737 2750 2759 2781 2807 2815 3252 3835 3843 3907 3944 4210 4240 4242 4470 4723 4826 4926 5258 5595 5688 6081 6190 6364 6464 6531
*Hinc*II	3	678 1594 2549
*Hind*II	3	678 1594 2549
*Hind*III	2	757 3665
*Hin*fI	19	564 842 958 1074 1095 1470 1584 1978 2080 2227 3013 3035 3671 4328 4462 4514 4621 5820 6337
*Hpa*I	2	1594 2549
*Hpa*II	22	1973 2660 2861 3748 3825 3847 3875 4006 4096 4163 4344 4747 4781 5282 5524 5634 5701 5735

Enzyme	# of cuts	Positions (c) indicates the complementary strand
		6139 6329 6355 6502
*Hph*I	16	530 1175 1214 1220 1463 1987 2227(c) 2965 4021(c) 4799(c) 4808(c) 5092(c) 5127 5333(c) 5749 5976
*Ita*I	40	835 1782 2129 2406 2409 2534 2716 2748 2762 2784 3255 3597 3752 3804 3815 3905 3910 3947 3988 4075 4078 4081 4317 4413 4454 4468 4569 4679 4788 5075 5304 5399 5426 5765 6093 6299 6302 6367 6510 6665
*Kas*I	1	3842
*Kpn*I	1	3303
*Ksp*632I	6	2369(c) 2375(c) 2673(c) 4187(c) 4397(c) 5020(c)
*Mae*I	15	154 753 1058 1087 1637 1821 2159 2252 2461 2812 3650 3704 5626 5961 6214
*Mae*II	18	75 276 288 329 412 493 598 1429 2854 2964 3007 3019 3959 4146 4897 5217 5590 6006
*Mae*III	22	215 302 651 839 902 1285 1774 1889 2519 2775 2787 3963 4269 4770 5158 5346 5499 5557 5888 6171 6287 6350
*Mam*I	2	2651 4636
*Mbo*I	32	662 747 1330 1732 1884 2176 2199 2340 2652 2656 2692 3246 4014 4092 4173 4182 4260 4637 5136 5172 5189 5447 5493 5511 5852 5957 5969 6047 6055 6066 6141 6719
*Mbo*II	22	967 1130 1511 1631 2021 2230 2383 2386 2392 2690 2826(c) 3666(c) 4204 4414 4494(c) 5037 5146 5224 5979 6050(c) 6202(c) 6714(c)
*Mcr*I	8	665 2202 2409 2695 3752 5301 5450 6373
*Mfe*I	2	1665 2558
*Mlu*NI	4	11 65 1276 3925
*Mnl*I	37	703(c) 870(c) 1101 1192(c) 1619(c) 2116(c) 2306(c) 2327 2363 2370(c) 2594(c) 2634 2674(c) 2938 3278(c) 3286 3302(c) 3580(c) 3586(c) 3610 3616 3623(c) 3626(c) 3638(c) 3758(c) 3894(c) 4251(c) 4444 4793(c) 4852 5446(c) 5652(c) 5799 5880 6280 6530(c) 6604
*Msc*I	4	11 65 1276 3925
*Mse*I	34	161 784 830 849 917 1052 1067 1170 1236 1464 1478 1494 1593 2060 2548 2609 2755 3026 3124 3141 3152 3164 3175 3698 4687 4868 5240 5605 5644 5879 5932 5946 5951 6003
*Msl*I	8	519 2159 4280 4562 4601 5048 5407 5566
*Msp*A1I	8	1781 2000 3325 3949 4717 5183 6124 6369
*Msp*I	22	1973 2660 2861 3748 3825 3847 3875 4006 4096 4163 4344 4747 4781 5282 5524 5634 5701 5735 6139 6329 6355 6502
*Mun*I	2	1665 2558
*Mva*I	11	244 437 1911 2288 3363 3418 3435 4230 6548 6561 6682
*Mvn*I	18	214 2737 2761 2781 3157 3244 3909 4210 4242 4643 4723 4826 4828 4928 5260 5753 6083 6664
*Mwo*I	37	137 244 366 398 437 530 554 803 1054 2308 2345 2694 2724 2756 2758 2800 2827 2857 3394 3466 3517 3596 3602 3834 3918 3941 4080 4086 4203 4239 4286 4553 4649 5701 6089 6661 6709
*Nae*I	2	2862 4345
*Nar*I	1	3843

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Nci*I	8	2661 3847 4007 4747 4782 5283 5634 6330
*Nco*I	6	514 1271 2256 3260 3556 4275
*Nde*I	2	388 1335
*Nde*II	32	662 747 1330 1732 1884 2176 2199 2340 2652 2656 2692 3246 4014 4092 4173 4182 4260 4637 5136 5172 5189 5447 5493 5511 5852 5957 5969 6047 6055 6066 6141 6719
*Ngo*MI	2	2860 4343
*Nhe*I	2	1086 1820
*Nla*III	33	118 136 458 518 1108 1275 1349 1369 1564 1759 1799 1804 1828 2260 2426 3264 3397 3469 3560 3717 4062 4248 4279 4305 4794 4878 4983 5376 5412 5490 5500 5991 6711
*Nla*IV	19	621 979 2132 2893 2905 2926 3301 3367 3439 3844 3879 4639 4932 5522 5733 5774 5868 6640 6679
*Not*I	1	2406
*Nsi*I	3	1906 3399 3471
*Nsp*I	5	1828 3397 3469 4248 4794
*Nsp*V	2	1727 4525
*Pin*AI	1	1972
*Ple*I	11	558(c) 836(c) 952(c) 1068(c) 2088 2221(c) 3021 3029(c) 4508(c) 5828 6331(c)
*Pma*CI	1	1430
*Pml*I	1	1430
*Ppu*10I	3	1902 3395 3467
*Psp*1406I	2	5217 5590
*Pst*I	2	839 3896
*Pvu*I	4	665 2202 2695 5450
*Pvu*II	4	1781 2000 3325 3949
*Rca*I	3	4874 4979 5987
*Rsa*I	12	99 373 453 486 537 702 1064 1383 3301 4149 4662 5338
*Rsr*II	1	4359
*Sac*I	1	730
*Sap*I	2	4187(c) 4397(c)
*Sau*3AI	32	662 747 1330 1732 1884 2176 2199 2340 2652 2656 2692 3246 4014 4092 4173 4182 4260 4637 5136 5172 5189 5447 5493 5511 5852 5957 5969 6047 6055 6066 6141 6719
*Sau*96I	12	237 430 1676 1759 2683 2971 4359 4839 5455 5677 5694 5773
*Sca*I	2	1064 5338
*Scr*FI	19	244 437 1911 2288 2661 3363 3418 3435 3847 4007 4230 4747 4782 5283 5634 6330 6548 6561 6682

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Sex*AI	1	3416
*Sfa*NI	20	511(c) 1201(c) 2096 2492(c) 3184(c) 3224 3406 3478 3801(c) 4056(c) 4142 4206 4272(c) 4481 4665(c) 4759 5118(c) 5367 5558(c) 6610(c)
*Sfc*I	8	835 1080 1863 2742 3892 5573 6251 6442
*Sfi*I	1	3602
*Sfu*I	2	1727 4525
*Sna*BI	1	494
*Sno*I	3	4650 5147 6393
*Spe*I	1	153
*Sph*I	4	1828 3397 3469 4248
*Ssp*BI	2	97 1381
*Ssp*I	4	6 53 3173 5014
*Stu*I	2	2316 3648
*Sty*I	7	514 1271 2256 3260 3556 3649 4275
*Taq*I	20	824 945 1093 1473 1727 2202 2414 2655 2930 3692 3956 4112 4136 4172 4334 4525 4624 5165 6609 6714
*Tfi*I	8	1095 1470 1584 1978 3671 4328 4462 4621
*Tha*I	18	214 2737 2761 2781 3157 3244 3909 4210 4242 4643 4723 4826 4828 4928 5260 5753 6083 6664
*Tru*9I	34	161 784 830 849 917 1052 1067 1170 1236 1464 1478 1494 1593 2060 2548 2609 2755 3026 3124 3141 3152 3164 3175 3698 4687 4868 5240 5605 5644 5879 5932 5946 5951 6003
*Tsp*509I	23	172 786 1040 1143 1154 1181 1238 1523 1602 1665 1939 2061 2494 2558 3148 3159 3185 3403 3475 3567 5386 5641 5947
*Tth*111I	1	3961
*Xcm*I	2	1840 2158
*Xho*II	12	1732 3246 4014 4260 4637 5172 5189 5957 5969 6055 6066 6719
*Xma*III	2	2406 3749
*Xmn*I	1	5219