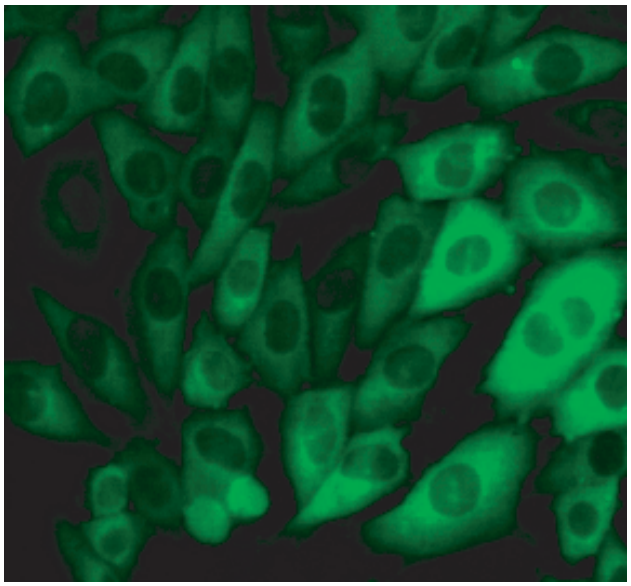
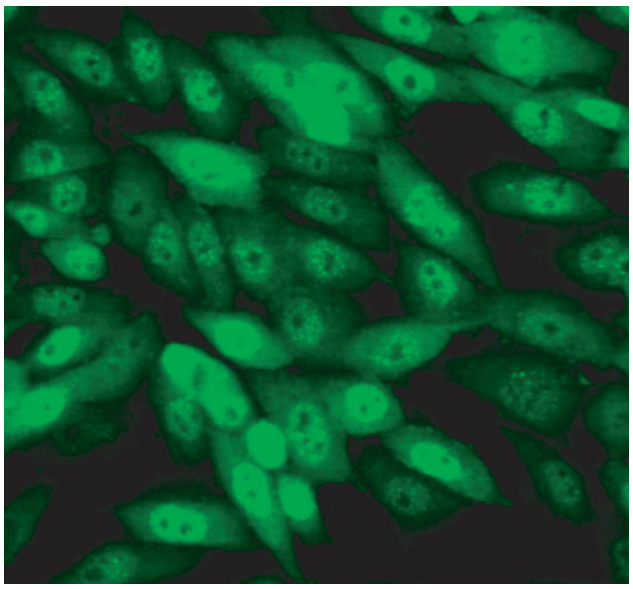
GE Healthcare

EGFP-SMAD2 Assay

Product User Manual

Codes: 25-8010-46
25-8010-47
25-8010-48
25-8010-49





Page finder

Front cover

Top image: CHO-hIR cells expressing the EGFP-SMAD2 fusion protein before the addition of agonist.

Bottom image: CHO-hIR cells expressing the EGFP-SMAD2 after stimulation with agonist (3 ng/ml TGF-β1).

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

1. Introduction

1.1. The SMAD protein family

SMAD proteins are a family of transcription factors ubiquitously expressed throughout development and in all adult tissues in vertebrates, insects and nematodes (6,8). SMAD gene family members are homologs of the *Drosophila* 'mothers against decapentaplegic' (Mad) gene and the related Sma genes in C. *elegans*. Hence, the name for the SMAD protein family derives from the longer name 'SMA- and MAD-related protein'. SMADs are currently categorized into three classes based on their structures and functions (9): **R-SMADs**, or receptor-activated SMADs, are phosphorylated by activated receptors and mediate specific response pathways. **Co-SMADs**, or common-mediator SMADs, associate with R-SMADs and are common to multiple response pathways. **I-SMADs**, or inhibitory SMADs, also associate with R-SMADs, but inhibit their ability to transduce signals. The R-SMAD class includes SMADs 1,2,3,5 and 8, while the most well-characterized Co-Smad is Smad 4. The I-SMAD class includes SMADs 6 and 7.

SMADs 2 and -3 mediate TGF-β/Activin signalling and are the only TGFB substrates with a demonstrated ability to propagate signals (10) Prior to activation, SMAD2 is anchored to the plasma membrane by SMAD Anchor for Receptor Activation (SARA)(1), this brings the SMAD into proximity with the TGF-β receptor kinases. The SMADs 2 and 3 are activated when phosphorylated at their c-termini by the TGF-β receptor complex. There then occurs a ligand-stimulated nuclear translocation of the receptor-regulated Smads. Activated R-SMAD, forms a tri-oligomeric configuration with SMAD4 (Co-SMAD), which then translocates from the cytosol to the nucleus and forms complexes that regulate transcription (2, 3, 6, 7). Co-SMADs are not required for nuclear translocation, but for the formation of functional transcriptional complexes. SMAD4 appears to play a crucial role in regulating the efficiency of transactivation of the SMAD complexes in the nucleus (4). This is done in several ways including: binding to DNA; interacting with other transcription factors; interaction with co-activators e.g. p300/CBP, which have intrinsic Histone Acetyltransferase (HAT) activity; Co-repressors such as SKI, SnoN and TGIF, which interact directly with Histone deacetylase (HDAC) containing complexes (5).

All SMADs affect transcriptional activity. R-SMADs and the Co-SMAD complexes participate in DNA binding and recruitment of transcriptional cofactors *in vivo*. I-SMADs (SMADs 6 and 7) repress signalling by other SMADs by binding tightly to the TGF-β receptor thus occluding the binding sites for the R-SMADs (2, 6).

1.2. SMADs and TGF-β Signalling

The transforming growth factor-β (TGF-β) superfamily are peptide growth factors known to exert a wide spectrum of biological responses on a large number of cell types in both vertebrates and invertebrates[1]. A striking feature of the TGF-β superfamily is the variety and potency of effects they can evoke, depending on the cellular context. Many of these effects have important roles in embryonic development and adult homeostasis, being involved in cell growth, proliferation, apoptosis, extracellular matrix secretion and adhesion and terminal differentiation and specification of developmental fate (1, 2, 3, 4, 5). Transcriptional control by the TGF-β family has become a subject of intense investigation in recent years, since imbalance or disruption of critical SMAD-mediated pathways has been implicated in many disease states.

There are two general branches of the TGF-β superfamily: The BMP/GDF pathways are mediated by SMADs 1,5 and 8, while the TGF-β/Activin/Nodal branch is mediated by SMADs 2 and 3, whose members have diverse, often complementary effects (2, 3). The first step in TGF-β signalling (Fig 1.1.) is the extracellular binding to a complex of transmembrane protein serine/threonine kinase receptors (6, 7). In general, TGF-β superfamily ligand binding brings together two distinct types of

receptors, designated type I and type II. Upon ligand binding, the type II receptor phosphorylates and activates the type I receptor. The activated receptor in turn phosphorylates the carboxy termini of the SMAD molecules (5, 6, 7, 8). This is followed by translocation of the SMAD complex and subsequent downstream transcriptional regulation.

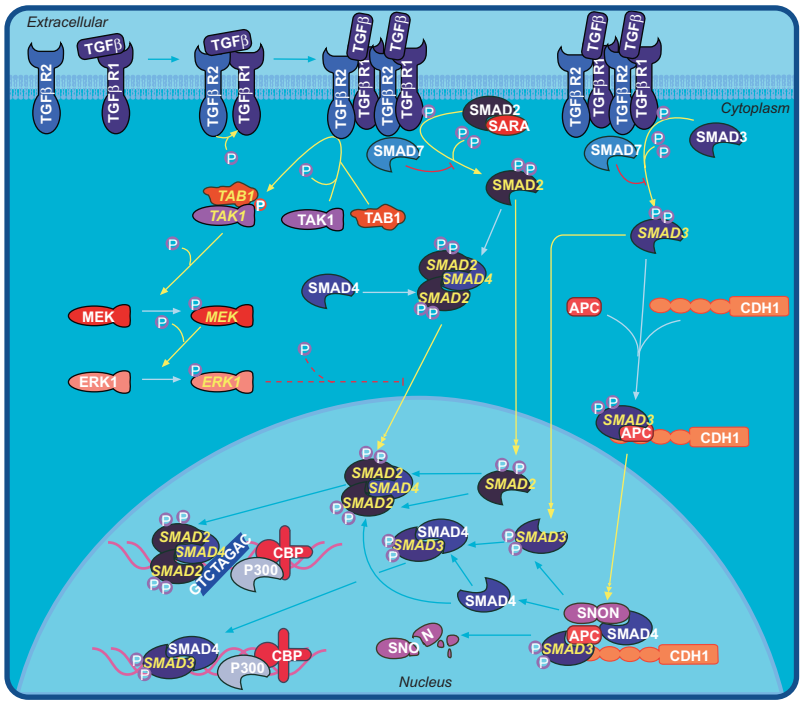


Fig 1.1. The role of the SMAD superfamily in the control of TGF-β transcriptional control. Provided with permission from BioCarta, www.biocarta.com

1.3. EGFP-SMAD2 assay

A cell-screening assay examining the response of the Transforming Growth Factor (TGF)-β signalling pathway has been developed. The assay is based on Redistribution™ technology to quantify the intracellular translocation of an EGFP-SMAD2 fusion protein in a stably transfected mammalian cell line. Following activation with TGF-β, EGFP-SMAD2 fusion proteins hetero-oligomerize and translocate from the cytosol to the nucleus. The SMAD2 Redistribution™ assay is designed to monitor the redistribution of activated SMAD2 from the cytoplasm to the nucleus.

This assay is optimized for image acquisition and analysis on the IN Cell Analysis System, using the Nuclear Trafficking Analysis Module, although the assay can also be imaged on other systems. The Nuclear Trafficking Analysis Module measures the degree of pathway response by quantifying the nucleus-to-cytoplasm intensity ratio of the EGFP-SMAD2 fusion protein in cells challenged with test compounds. When the pathway is stimulated, the EGFP-SMAD2 fusion protein moves to the nucleus (Fig 1.2), thus increasing the nucleus-to-cytoplasm ratio of green channel fluorescence. The assay is designed in an agonist format using TGF-β1 with a typical EC_{50} value of 3 pM.

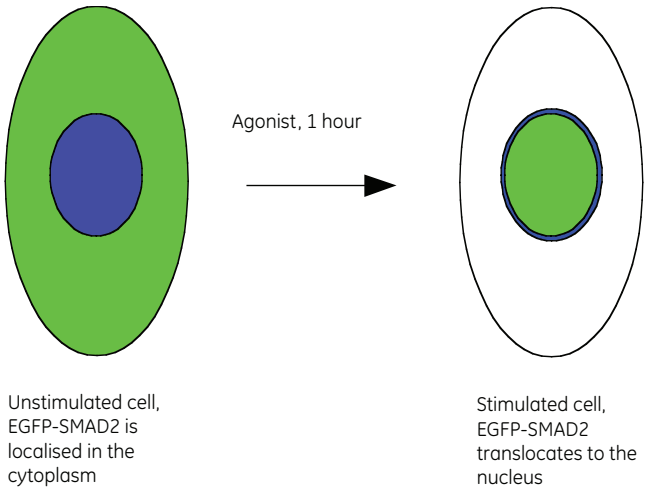


Fig 1.2. Agonist induced redistribution of EGFP-SMAD2 from the cytoplasm to the nucleus.

2. Licensing considerations

2.1. Right to use

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

Description	Product Code
EGFP-SMAD2 Assay, Screening applications	25-8010-46
EGFP-SMAD2 Assay, Research applications	25-8010-47
EGFP-SMAD2 Assay, 6 month assay evaluation	25-8010-48
EGFP-SMAD2 Assay, 12 month assay evaluation	25-8010-49

The assay was developed in collaboration with BioImage A/S and sold under license from:

BioImage A/S under patents US 6172188, US 5958713, US 6518021, EP 851874, EP 815257, EP 0986753 and other pending and foreign patent applications; and

Invitrogen IP Holdings Inc (formerly Aurora Biosciences Corporation) under US patents: US 5625048, 5777079, 5804387, 5968738, 5994077, 6054321, 6066476, 6077707, 6090919, 6124128, 6319969, 6403374 European Patent 1104769, 0804457 and Japanese patent JP 3283523 and other pending and foreign patent applications; and

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University of Florida Research Foundation under patents US 5968750, 5874304, 5795737, 6020192 and other pending and foreign patent applications; and

Iowa Research Foundation. The CMV promoter is covered under US patents 5168062 and 5385839 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

The Research Institute of The Hospital for Sick Children, Toronto, Ontario, Canada under US patent 6,017,755 .

The exact terms of use for the product as configured are specified in the Terms and Conditions of Sale accompanying the product, but are limited to internal use for development and discovery of therapeutic products. No rights other than those expressly granted are conveyed.

2.2. Legal

http//www.gehealthcare.com/lifesciences

GE Healthcare UK Limited
Amersham Place Little Chalfont Buckinghamshire HP7 9NA UK

3. Product contents

3.1. Components summary

- CHO-hIR cells expressing the EGFP-SMAD2 fusion protein (two vials, each containing 1 ml and 1 x 10^6 cells)—NIF2031
- pCORON1000 EGFP- SMAD2 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 pH 8.0) - NIF2032
- User manual

3.2. CHO hIR derived cell line expressing EGFP-SMAD2 fusion protein NIF2031

3.2.1. CHO-hIR derived parental cell line

The parental cell line CHO-hIR cell line consists of CHO-K1 (ATCC CCL-61) cells that have been stably transfected with the human insulin receptor (12). The CHO-K1 cell line was derived as a subclone from the parental CHO cell line initiated from a biopsy of an ovary of an adult Chinese hamster by T.T. Puck in 1957 (13).

3.2.2. CHO hIR derived EGFP-SMAD 2 expressing cell line

CHO-hIR cells were transfected with pCORON1000 EGFP-SMAD2 vector (supplied) using the FuGENE 6 transfection method according to the manufacturer's instructions. A stable cell line was derived by applying a Geneticin selection pressure (0.5 mg/ml medium) for approximately two weeks. The isolated clone was grown for 2 passages before freezing. The cells tested negative for mycoplasma, bacterial and yeast contamination (testing details are available upon request).

3.3. EGFP-SMAD2 expression vector—NIF2032

The 6.7 kb plasmid, pCORON1000 EGFP-SMAD2, 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.amersham.com

A detailed restriction map is shown in chapter 11, appendix A.

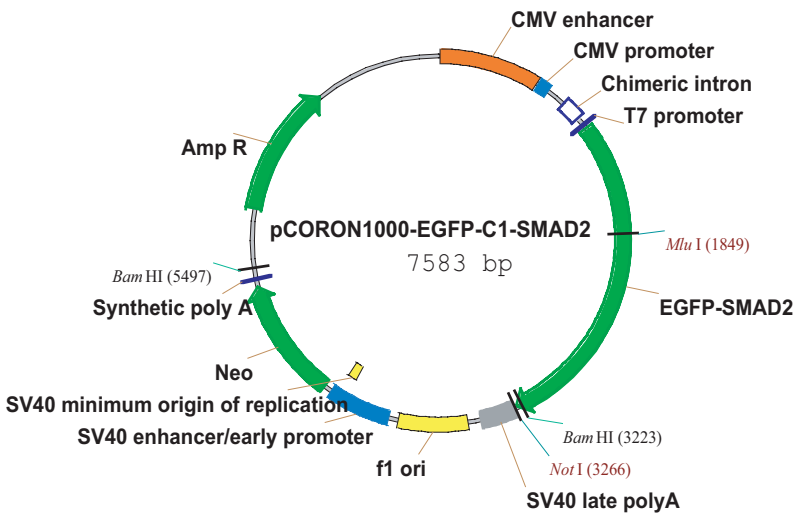


Fig 3.1. Vector map of the supplied EGFP-SMAD2 expression vector

3.4. Materials and equipment required

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

- Microplates. For analysis using the IN Cell Analysis System, Packard Black 96 Well ViewPlates (Packard Cat # 6005182) should be used. For assays in 384 well format, please email 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)
- Fluorescence/microscope (e.g. IN Cell Analyzer 3000/IN Cell Analyzer 1000
- 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 minute
- Standard tissue culture reagents and facilities (section 5.1.1.)

3.5 IN Cell Analysis System

The EGFP-SMAD2 assay has been developed and optimized for analysis using the IN Cell Analyzer 3000, in conjunction with the Nuclear Trafficking Analysis Module. Please refer to the instrument user manual for details on instrument set up and the analysis module manual for details on the algorithm settings. For further information on either of these products, please contact GE Healthcare.

3.5.1. 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 rapidly and at high resolution, enabling high-throughput and high-content testing of drug compounds

3.5.2. Nuclear Trafficking Analysis Module

The degree of translocation of the EGFP-SMAD2 fusion protein is determined using the Nuclear Trafficking Analysis Module. This image analysis algorithm relies upon the correct identification of the cell nucleus. It is therefore important to identify the fluorescently stained nuclei using the appropriate fluorescence channel of the IN Cell Analyzer 3000. The regions identified as nuclei are interrogated for green fluorescence enabling a measurement of nuclear EGFP. By dilating from the nucleus and measuring the GFP fluorescence in this surrounding area, cytoplasmic EGFP-SMAD2 fluorescence is sampled. A ratio of the green nuclear and cytoplasmic signal intensities is used to determine the level of translocation of the EGFP-SMAD2 fusion protein from the cytoplasm to the nucleus. Translocation data presented throughout this manual are expressed in terms of the Nuc/Cyt ratio.

3.5.3. IN Cell Analyzer 1000

The IN Cell Analyzer 1000 is a bench top automated microscope system designed for imaging sub-cellular end-point assays. The system's core components are a Nikon microscope, xenon lamp and high-resolution CCD camera. There are a number of analysis modules available with the system as well as the capability to export images and data into other commercial analysis packages. The Nuclear Trafficking Analysis Module for the IN Cell Analyzer 1000 measures nuclear translocation by comparing the fluorescence intensity of the labelled molecule in both the nuclear and cytoplasmic compartments following stimulation by a cellular activator.

3.6. EGFP-SMAD2 translocation assay on epifluorescence microscopes

For speed of screening and quality of the images obtained, we recommend performing the EGFP-SMAD2 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 x 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.

3.7. Software requirements

IN Cell Analysis System: The Trafficking Analysis Module is available from GE Healthcare for automated image analysis of the EGFP-SMAD2 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 Analysis Systems.

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.

Handle as a potentially biohazardous material.

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 that 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 the Regulations (in particular sections 3.3.1 and 3.3.2).

Risk assessments made under the *GMO (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 suitably equipped laboratory environment and should only be used by responsible persons in authorised 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 and users of these products MUST make themselves aware of and observe the Local Regulations or Codes of Practice, which relate to such matters.

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

4.2. Storage

The EGFP-SMAD2 expressing DNA construct (NIF2032) should be stored at -15°C to -30°C.

The CHO-hIR derived cells expressing the EGFP-SMAD2 fusion protein (NIF2031) 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.

5. Cell assay design

5.1. Culture and maintenance of CHO derived EGFP-SMAD2 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
- Fetal Bovine Serum (FBS), JRH Biosciences 12103 or equivalent. Heat inactivate the serum by incubation in a water bath at 56°C for 30 minutes.
- GIBCO Penicillin-Streptomycin (P/S), (5000 units/ml penicillin G sodium and 5000 µg/ml streptomycin sulfate), Invitrogen life technologies 15140-122 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 HEPES Buffer, 1 M solution, Invitrogen life technologies 15630-056 or equivalent
- Bovine serum albumin (BSA), Sigma A-7888 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-5879 or equivalent
- Transforming Growth Factor –β1 (TGF- β1) Human Natural Sigma T 1654
- Hoechst™ 33342, Molecular Probes H-21492
- DRAQ5™, Biostatus
- Cy5™ monocarboxyl dye, GE Healthcare PA05111
- Oregon Green (2', 7'-difluorofluorescein), Molecular Probes D-6145
- Alexa Fluor (carboxylic acid, succinimidyl ester), Molecular Probes A-10168
- 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.

- Ham's Nutrient Mixture F-12 medium supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-streptomycin, and 1% Geneticin (w/v; working concentration 0.5 mg/ml).
- Heat inactive Fetal Bovine Serum: Heat inactivate serum by incubation in a water bath at 56°C for 30 minutes.
- Freeze-medium: Ham's Nutrient Mixture F-12 medium supplemented with 10% (v/v) FBS, 1% (v/v) Penicillin-streptomycin, and 10% (v/v) DMSO.
- Assay Medium: Ham's Nutrient Mixture F 12 medium with Glutamax + HEPES 10 mM and 1.0 µM Hoechst Nuclear-stain.
- TGFβ1: Prepare a 10 µg/ml stock solution in 4 mM HCl, 1 mg/ml BSA. This can be stored at 2-8°C for up to 3 months. Prepare a 12 ng/ml working solution with Assay medium (four fold of final concentration). Suitable controls should also be prepared.

- Flat field (FF) solution components:
  - Cy5 - 1 mM stock solution prepared in 10% (v/v) DMSO, 90% (v/v) PBS
  - Oregon Green – 1 mM stock solution prepared in 10% (v/v) DMSO, 90% (v/v) PBS
  - Alexa Fluor- 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 FF solution should be prepared to give a fluorescent signal in each channel between 700-3300 counts. Prepare an initial FF solution containing 10 µl of 10 µM Cy5, 0.5 µl of 10 mM Oregon Green and 20 µl of 100 µM Alexa Fluor and make the volume up to 100 µl using PBS. Adjust these solutions if required. Use 100 µl of FF solution for a 96 well plate and 40 µl of FF solution for a 384 well plate.

5.1.3. Cell thawing procedure

Two cryo-vials, each containing 1 x 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 minutes until the contents are thawed. Do not thaw the cells for longer than 3 minutes 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 sub-culturing procedure

Incubation: 5% CO_2, 95% humidity, 37°C.

The cells should be split in a ratio of 1:24 when they are 90% 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 4 ml for T-162 flasks), ensuring that all cells are in contact with the solution. Wait for 3–10 minutes 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 8 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 minutes 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 $2–5 \times 10^4$ 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. Incubate the plated cells for 24 hours at 37°C before starting the assay.

NOTE: 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 minutes. Aspirate the medium from the cells.

4. Gently resuspend the cells in Freeze medium until no clumps remain 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 hours.

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 14.6 µm as measured using a CASY1 Cell Counter and Analyzer System (Model TT). The doubling time of the cell line in exponential growth phase has been determined to be approximately 14.8 hours under standard conditions (Fig 5.1).

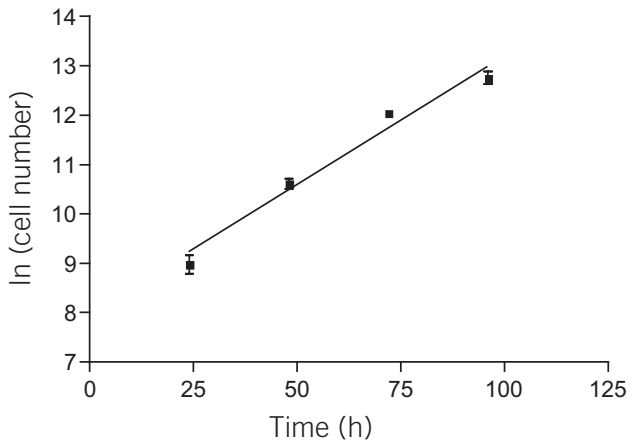


Fig 5.1. Growth curve of the CHO-hIR derived EGFP-SMAD2 expressing cell line points Doubling time = 14.8 hours.

5.2. Assay set up

5.2.1. Live cell EGFP-SMAD2 assay using the IN Cell Analyzer 3000

This manual provides a suggested protocol to use the EGFP-SMAD2 assay for agonist screening on the IN Cell Analyzer 3000.

5.2.2. Microplate set up for 96 well format assays

The SMAD2 assay is optimised for agonist format (see sections 5.2.3.). It is essential that the number of cells per well in the assay plates be consistent in order to minimise assay variability.

The assay protocol provided employs TGF-β1 as the reference agonist. TGFβ1 has an EC_{50} of approximately 3 pM in this assay when Hoechst 33342 is used as the nuclear counter-stain. The majority of data presented in this manual were obtained using Hoechst to stain the nuclei. Alternatively, the nuclei can be stained with the far-red emitting dye DRAQ5™ (see section 5.4.8).

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

Fig 5.2. shows a typical schematic of the agonist assay. The cells should be seeded in the appropriate microplate the day before the experiment. The Growth-medium is decanted, the cells washed and Assay-medium added to each well. Test compound and controls are added to required wells. After 1 hour incubation, the microplates are placed into the IN Cell Analyzer 3000. The Nuclear Trafficking Analysis Module is used to analyze each well.

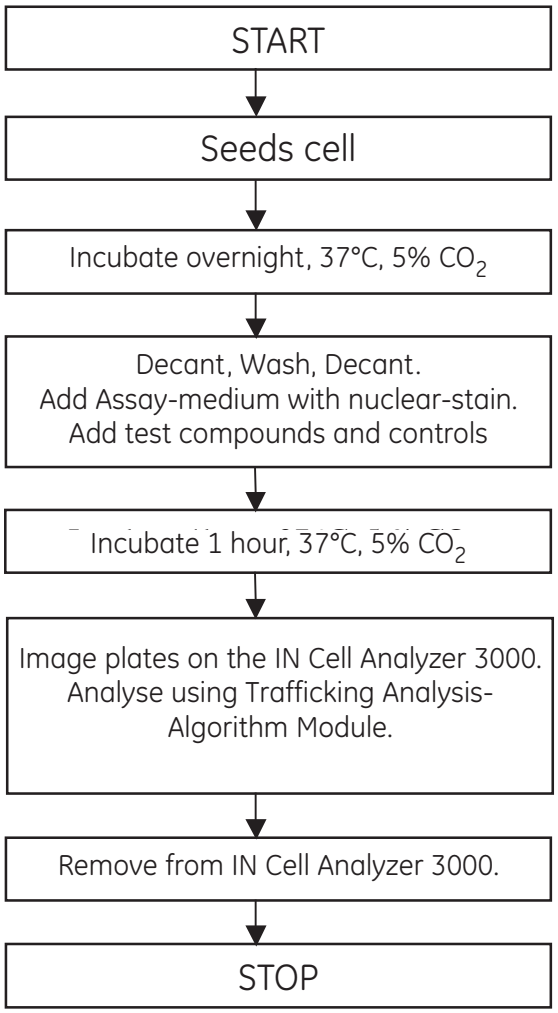


Fig 5.2. Flow diagram showing a basic protocol suitable for a EGFP-SMAD2 agonist screen. All incubations are performed at 37°C unless otherwise stated.

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 1.0 x 10^4 cells per well in 200 µl of Growth-medium. Incubate for 24 hours 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). These samples are typically prepared at four fold of the final concentration in Assay-medium. For TGFß1, a final concentration of 3 ng/ml is recommended. However, we recommend that users perform their own dose response curve to establish optimal agonist concentrations.
3. Decant the Growth-medium from the cell plate, removing all excess liquid and add 200 µl Assay-medium to wash the cells. Decant the wash.
4. Add 150 µl Assay-medium.
5. Add 50 µl of the prepared four fold dilution stocks of the test and control compounds to the appropriate wells. The total well volume is 200 µl.
6. After the first well has been incubated for 1 hour, read the assay plate using the IN Cell Analyzer 3000.
7. Perform the data analysis using the Trafficking - Analysis Module.

5.3. Results

5.3.1. Calculating the Z'-factor

Assay performance can be assessed by calculating the Z'-factor, a dimensionless value defined by Zhang *et al.* (14). 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

5.3.2. Example results obtained from IN Cell Analyzer 3000

The following figures (Fig 5.3. and Fig 5.4.) were acquired from a single experiment, and provide an example of the images and results that can be obtained with the EGFP-SMAD2 assay using the IN Cell Analysis System. Fig 5.3. shows images acquired on the IN Cell Analyzer 3000 of the supplied CHO-hIR derived EGFP-SMAD2 expressing cells with and without stimulation with TGF-β1.

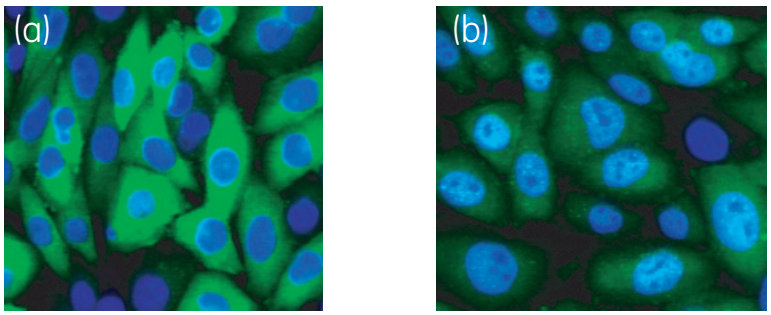


Fig 5.3. Cells expressing EGFP-SMAD2 in the absence (a) or presence (b) of 3 ng/ml TGF-β1 for one hour. (Only a fraction of the entire field of view is shown in each panel).

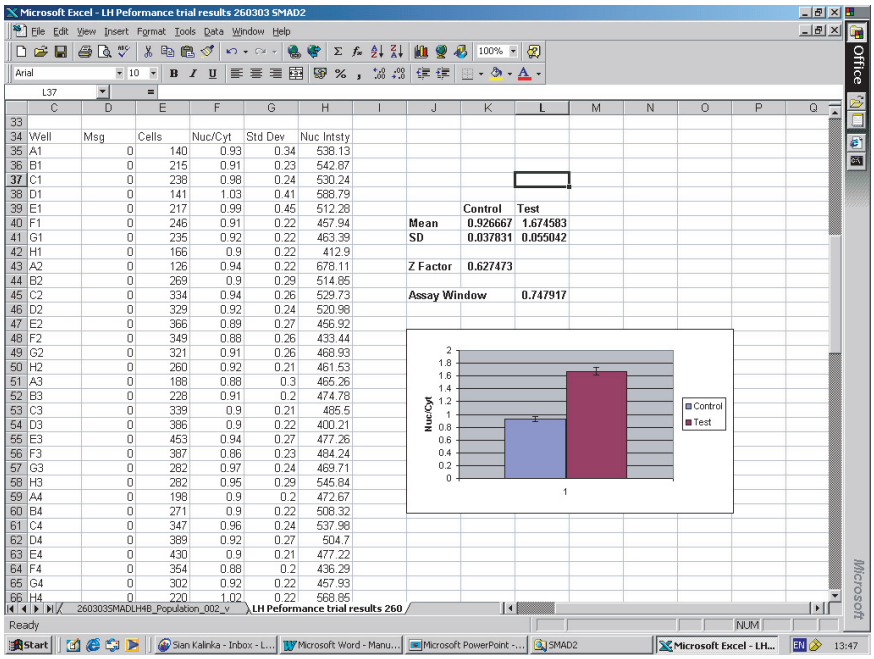


Fig 5.4. Data from the example experiment, generated by the Nuclear Trafficking Analysis Module, exported to and analyzed in Microsoft Excel.

5.4. Assay characterization

5.4.1. Translocation index

All validation assays for the EGFP-SMAD2 cell line were performed on the IN Cell Analyzer 3000 using the Nuclear Trafficking Analysis Module. The data generated by this module are in the format of Nuc/Cyt ratio. This is the average ratio of sampled nuclear and cytoplasmic intensities acquired in the signal channel.

5.4.2. Summary of quantitative assay parameters

A summary of typical assay data, using TGF-β1 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.

Parameter	Assay Data	#Assays	#Replicates
Signal to Noise	21.76	1	48
Z'-factor	0.65	1	48
Magnitude of Response	0.77	1	48
%CV			
Stimulated	3.25	1	48
Unstimulated	3.88	1	48

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

Signal to noise is (mean signal – mean background)/(background standard deviation) (14)

Magnitude of response is (mean signal – mean background)

%CV is (standard deviation x 100)/mean)

Z'-factor is a dimensionless characteristic useful for evaluation of assay quality (14).

Parameter	Assay Data (±SD*)	#Assays	#Replicates
Signal to Noise	21.0 ± 3.4	14	48
Z'-factor	0.60 ± 0.06	14	48
Magnitude of Response	0.76 ± 0.022	14	48
%CV			
Stimulated	3.7 ± 0.8	14	48
Unstimulated	4.0 ± .58	14	48

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

* SD shown is the standard deviation of the assays

Signal to noise is (mean signal – mean background)/(background standard deviation) (14)

Magnitude of response is (mean signal – mean background)

% CV is (standard deviation x 100/mean)

Z'-factor is a dimensionless characteristic useful for evaluation of assay quality (14)

5.4.3. Seeding density

Fig 5.5. shows the effect of varying seeding density in a 96 well microplate 18 hours prior to the assay. The data were collected 1 hour after the addition of 3 ng/ml final TGF-β1. Assay magnitude of response was found to be acceptable at cell densities ranging from 0.4 x 10^4 to 1.4 x 10^4 cells per well.

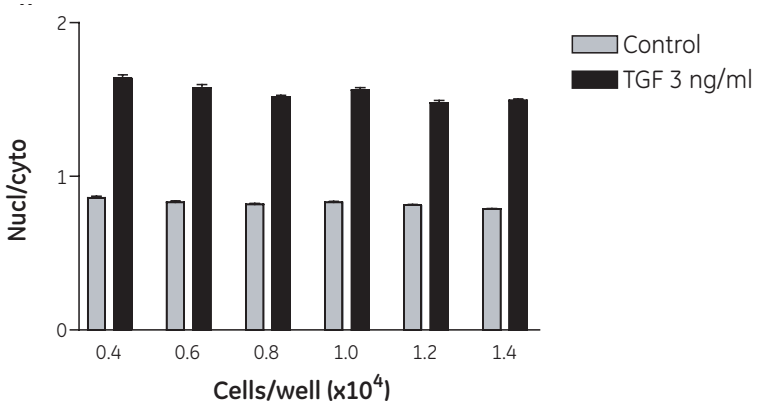


Fig 5.5. TGF β1 induced EGFP-SMAD2 translocation as a function of seeding density. Stimulated cells were treated with TGF β1 (3 ng/ml) 1 hour prior to imaging. Error = ± SD, n = 8 replicates per data point.

5.4.4. TGF β1 dose response

Fig 5.6. shows an agonist dose response curve for TGF-β1. The data were collected 1 hour after addition of agonist, and demonstrate an EC_{50} of 0.07 ng/ml (2.7 pM) .

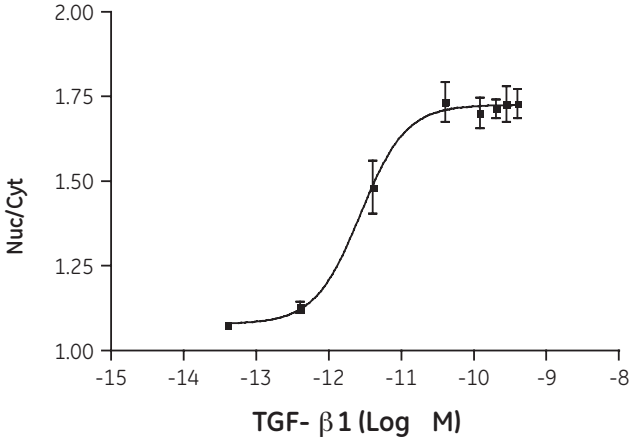


Fig 5.6. TGF -ß1 dose response curve using the supplied EGFP-SMAD2 cell line. Error = ± SD, n = 8 replicates per data point.

5.4.5. Time course

Fig 5.7. shows a typical time course of the translocation and indicates that the maximal translocation occurs 1 hour after the addition of TGF-β1.

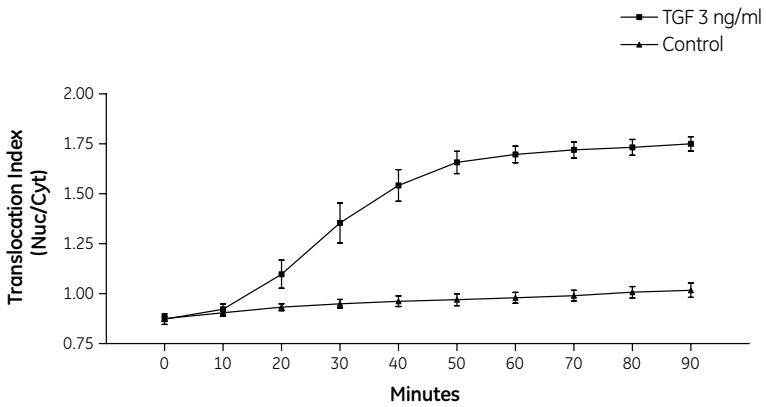


Fig 5.7. Time course of EGFP-SMAD2 translocation using TGF-β1 as agonist. Maximal response is seen after 1 hour. Error = ± SD, n = 32 replicates per data point .

Bone Morphogenic Protein (BMP) was titrated against the cell line with no effect.

5.4.6. Sensitivity of assay to DMSO, Ethanol and Methanol

The EGFP-SMAD2 translocation was measured in the presence of DMSO (≤ 2%), Ethanol (≤ 2%) or Methanol (≤ 2%). As can be seen in Fig 5.8. the TGF-ß1 -induced translocation can withstand at least 0.5% of each solvent.

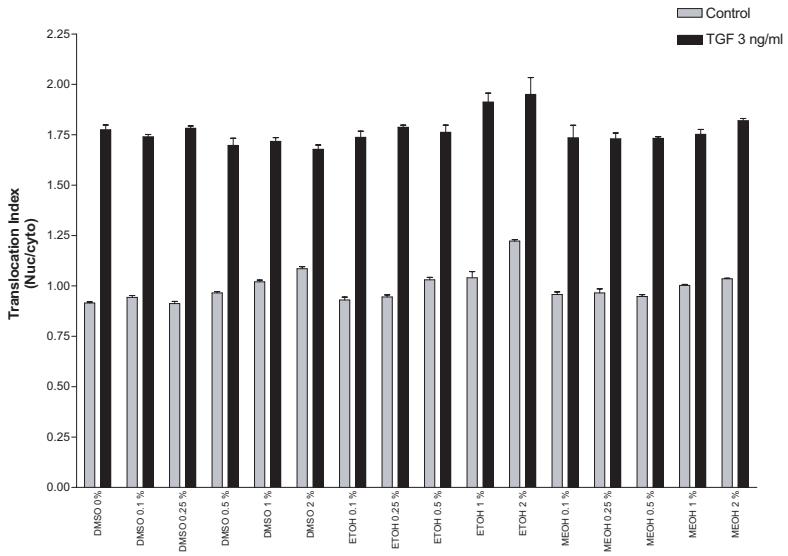


Fig 5.8. Effect of DMSO, ethanol or methanol on the EGFP-SMAD2 translocation. Error = ±SD, n = 4 replicates per data point.

5.4.7. Effect of different assay media

To determine the effect of varying the assay media on the TGF- β1 induced EGFP-SMAD2 fusion protein translocation, the stable CHO-hIR cells were assayed in either Nutrient Mixture F-12 Ham with Glutamax or KrebsRingerWollheim medium with a range of additives (10 mM HEPES, BSA and FBS). KrebsRingerWollheim medium consists of 140 mM NaCl, 3.6 mM KCl, 0.5 mM $NaH_2PO_4.H_2O$, 0.5 mM $MgSO_4.7H_2O$, 2 mM $NaHCO_3$, 1.5 mM $CaCl_2.2H_2O$, 6 mM D-Glucose and 10 mM HEPES. The results, shown in Fig. 5.9, demonstrate that the assay is tolerant to a range of assay media.

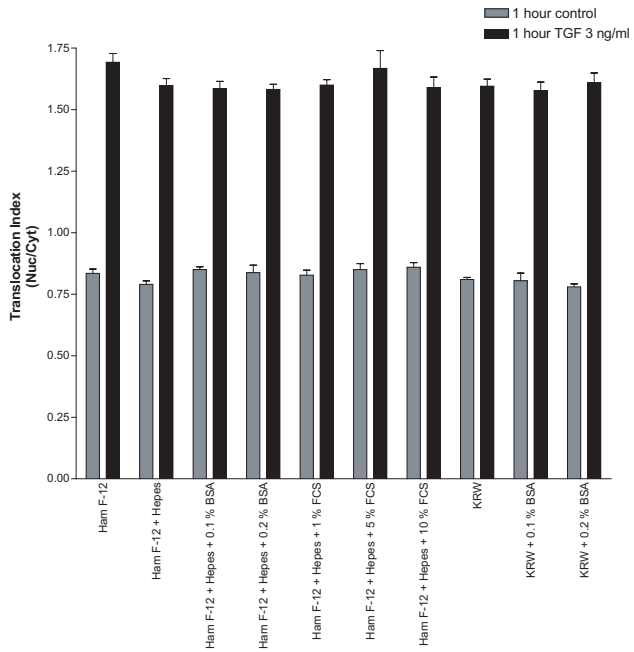
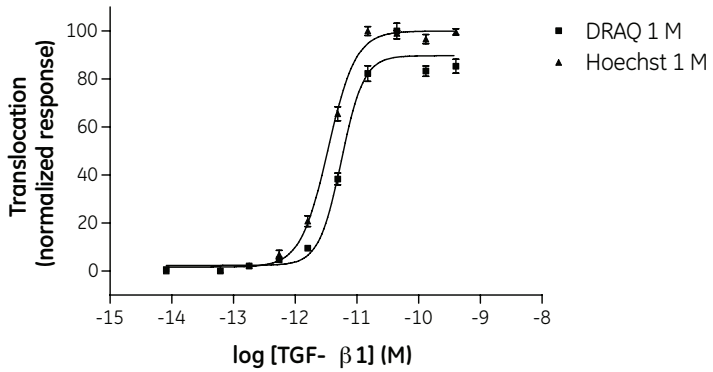


Fig 5.9. The effect of different assay media on the translocation of EGFP-SMAD2 Error = ± SD, n = 8 replicates per data point.

5.4.8. Effect of using the nuclear stain DRAQ5 in the translocation assay

GFP expressing cells which have been stained using Hoechst 33342 nuclear stain must be imaged sequentially due to overlap of the spectral profiles of these two probes. For speed of imaging, the far-red emitting nuclear stain DRAQ5 can be used instead of Hoechst. Since there is no spectral overlap between DRAQ5 and GFP, images can be acquired simultaneously.

Fig 5.10. shows a TGF-β1 dose response curve where the nuclear stain was 1 µM DRAQ5. A shift in the apparent EC_{50} of TGF-β1 was observed.



	DRAQ 1 M	Hoechst 1 M
EC50	5.5814e-012	3.4060e-012

Fig 5.10. TGF-β1 dose response curve using 1 µM DRAQ5 as the nuclear stain.
Error = ± SD, n = 8 replicates per data point.

5.4.9. Results obtained from the IN Cell Analyzer 1000

The following figures Fig 5.11. and Fig 5.12. show typical data for EGFP-SMAD2 translocation obtained using the IN Cell Analyzer 1000. Assays were set up as described in section 5.2. except that after the 1 hour incubation the cells were fixed using 2% formaldehyde. Also Hoechst 33342 nuclear stain is used at a higher concentration of 5 µM.

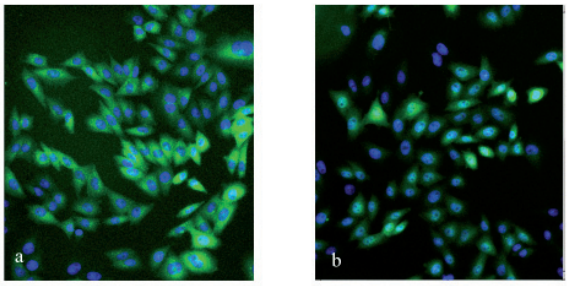


Fig 5.11. Cells expressing EGFP-SMAD2
Images obtained using IN Cell Analyzer 1000 with 20 x air objective lens. Cells were fixed 1 hour after addition of a) assay buffer (contol wells) or b) TGF-β1 (3 ng/ml) (Stimulated cells)

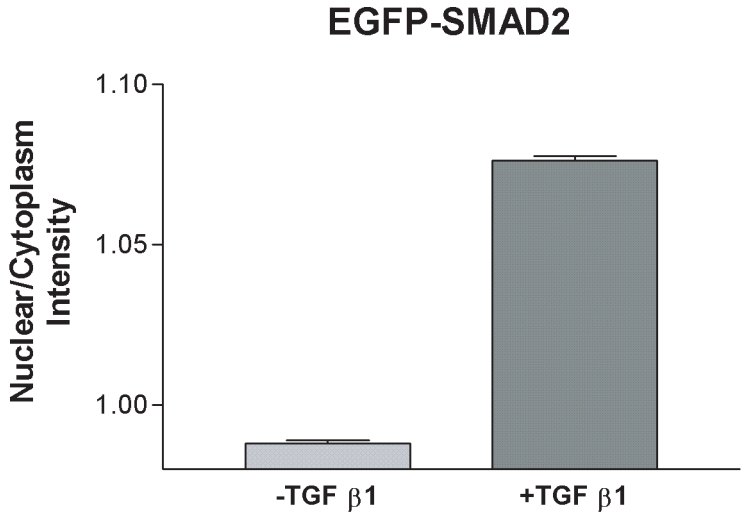


Fig 5.12. Results obtained using the Nuclear Trafficking Analysis Module for the IN Cell Analyzer 1000 Response in the absence or presence of 3 ng/ml TGF-β1 for 1 hour.
Error = ± SD, n = 12

6. Vector use details

The plasmid vector pCORON1000 EGFP-SMAD2 (Fig 3.1.) can be used to transiently or stably express the EGFP-SMAD2 fusion protein in the cell line of choice.

6.1. General guidelines for vector use

pCORON1000 EGFP-SMAD2 has been used successfully to express the EGFP-SMAD2 fusion protein both transiently and stably in the CHO-hIR derived cell line. Expression levels, translocation responses and other assay parameters may vary depending on the cell type and the transfection procedure.

6.2. Transient transfection with pCORON1000 EGFP-SMAD2

Transfection protocols must be optimised for the cell type of choice. Choice of transfection reagent and cell type will affect efficiency of transfection. FuGENE 6 Transfection Reagent (Roche) produced successful results when transfecting pCORON1000 EGFP-SMAD2 into CHO-hIR. For more information, refer to manufacturer's guidelines for the desired transfection reagent.

6.3. Stable cell line generation with pCORON1000 EGFP-SMAD2

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 (15).

pCORON1000 EGFP-SMAD2 has been used to generate stably transfected cell populations. The magnitude of the response with different cell lines are unknown, and may deviate considerably from the values specificed in this manual.

7. Quality control

7.1. EGFP-SMAD2 cell line

The EGFP-SMAD2 cell line is supplied at a concentration of 1 x 10^6 cells per ml in fetal calf serum containing 10% (v/v) DMSO.

The cell line has the characteristics detailed in Table 7.1.

Table 7.1. Quality control information for EGFP-SMAD2 cell line

Property	Value	Measurement method
Assay stability	Magnitude of response ≥ 0.4 for 20 passages after dispatch Z' factor ≥ 0.3	Quality Control Assay
Viability from frozen	> 80%	CASY1 - model TT cell counter
Cell diameter (mm)	14–16	CASY1 - model TT cell counter
Fluorescence at 5 x 10^4 cells per ml (RFU)	> 40 000 for 20 passages after dispatch	FARCyte (Gain 65)

7.2. EGFP-SMAD2 expression vector

The SMAD2 vector is supplied in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.9) at 250 µg/ml. The vector has the characteristics outlined in Table 7.2.

Table 7.2. Quality control information for the EGFP-SMAD2 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 @ 260 nm 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. Restriction digest map of pCORON1000 EGFP-SMAD2 expression vector

Enzyme(s)	Fragment(s) size (bp)
BamHI	2274, 5309
BsrGI	1720, 5863
Hind III	3768, 3851
NotI	7583
PvuI	871, 1938, 2019, 2755

8. Troubleshooting guide

Problem	Possible cause	Remedy
8.1. Low assay response. (positive vs. negative controls)	**8.1.1.** Passage number too high.	**8.1.1.** 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.
	8.1.2. Cell density too low or too high.	**8.1.2.** Verify density of cell plating; adjust plating density to values that yield optimal assay response.
	8.1.3. Incorrect selection of analysis parameters.	**8.1.3.** Check that the primary parameters are correct and suitable for the cells currently in use.
	8.1.4. Incorrect assay/incubation conditions.	**8.1.4.** 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 is added to the medium to maintain proper pH.
	8.1.5. Reagents were not stored properly or they are out of date.	**8.1.5.** Repeat assay with fresh reagents.
	8.1.6. Cells have been stressed during assay.	**8.1.6.** Use actively growing cells maintained at 37°C. Pre-warm reagents to 37°C.
8.2. Low nuclear intensity.	**8.2.1.** Nuclear stain concentration too low.	**8.2.1.** Adjust Nuclear stain concentration to recommended level.
	8.2.2. Nuclear stain incubation time too short.	**8.2.2.** Adjust Nuclear stain incubation time to recommended length.
8.3. Image is out of focus.	**8.3.1.** Autofocus Offset is chosen incorrectly or the system may need to be realigned.	**8.3.1.** Alignment and calibration of instrument. Perform Z-stack on cells. Change Autofocus Offset.
8.4. Cells do not adhere to well bottom in plate.	**8.4.1.** Plating density too high.	**8.4.1.** Reduce plating density.
8.5. Shading across image field.	**8.5.1.** Flat field correction not applied or flat field solution too weak.	**8.5.1.** Apply Flat field correction or adjust flat field solution.

9. References

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15. Freshney, R. I. *Cloning and Selection of Specific Cell Types in Culture of Animal Cells,* 3rd Edition, Wiley-Liss Inc, Chapter 11, pp. 161–178, 1994.

10. Related products

Product Name:	Code:
GFP Assays	
GFP-PLCδ -PH domain assay	See below*
GFP-Rac1 assay	See below*
GFP-MAPKAP-k2 assay	See below*
AKT-1-EGFP assay	See below*
EGFP-2xFYVE assay	See below*
CypHer	
pCORON 1000 VSV-G Expression Vector	25-8008-51
pCORON 1000 SP VSV-G Expression Vector	25-8009-92
CypHer5 Labelled Anti VSV-G Antibody	PA45407
CypHer5 NHS Ester (1 mg pack)	PA15401
CypHer5 NHS Ester (5 mg pack)	PA15405

*Use of the GFP assays is limited as stated in the terms and conditions of sale. The product codes vary accordingly. Please contact your local representative for details.

IN Cell Analysis System	
IN Cell Analyzer 3000	25-8010-11
Nuclear Trafficking Analysis Module for IN Cell Analyzer 3000	63-0048-94
IN Cell Analyzer 1000	25-8010-26
Nuclear Trafficking Analysis Module for IN Cell Analyzer 1000	25-8010-31

11. Appendix

11.1. Appendix A: Restriction map of pCORON1000-EGFP-SMAD2

The following enzymes do not cut the vector: *Aar*I, *Acc*I, *Apa*I, *Asc*I, *Bae*I, *Bbr*PI, *Bbv*CI, *Bcl*I, *Blp*I, *Bmg*BI, *Bpu*1102I, *Bsi*WI, *Bsp*120I, *Bst*1107I, *Bst*EII, *Bst*Z17I, *Bsu*36I, *Btr*I, *Cel*II, *Eco*NI, *Eco*RV, *Esp*I, *Fse*I, *Fsp*AI, *Ksp*I, *Nru*I, *Pac*I, *Pma*CI, *Pme*I, *Pml*I, *Ppu*MI, *Psh*AI, *Psp*OMI, *Psr*I, *Sac*II, *Sal*I, *San*DI, *Sbf*I, *Sgr*AI, *Sse*232I, *Sse*8647I, *Swa*I, *Xba*I, *Xcm*I

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Aat*I	1	2153 4508
*Aat*II	5	279 332 415 601 5760
*Acc*65I	1	4159
*Acc*I	1	1825
*Ace*II	1	1090
*Ace*III	9	1058 1114(c) 1147(c) 1468(c) 1651 1801(c) 3981(c) 5633(c) 6375(c)
*Aci*I	80	129 212 240 252 266 399 433 524(c) 557(c) 669 690(c) 767(c) 1326 1367 1434 1473 1611 1724 1784 1787 1879 1940(c) 2425 3265(c) 3269 3546 3607(c) 3621(c) 3624(c) 3652 3679 4057(c) 4083(c) 4096 4104(c) 4172(c) 4357 4369 4378 4390 4400 4411 4457 4612 4675 4769(c) 4833(c) 4934(c) 4937(c) 5177 5217(c) 5222 5272(c) 5288 5314 5370(c) 5429 5501 5539 5565 5575 5614 5788(c) 5835 5934(c) 6043(c) 6120(c) 6164 6285(c) 6331 6522(c) 6613(c) 6975 6984(c) 7119 7229(c) 7350(c) 7369(c) 7496(c) 7524(c)
Ac/I	2	6077 6450
*Acs*I	8	1843 2102 2772 2978 3019 3354 4008 4019
*Acy*I	8	276 329 412 598 4703 5405 5757 6139
*Afe*I	1	1091
*Afl*II	4	829 848 1051 4557
*Afl*III	2	1849 2226
*Age*I	1	1095
*Ahd*I	1	6679
*Ale*I	4	1108 1138 1288 1465
*Alo*I	1	3864(c)
*Alu*I	35	728 759 834 1048 1128 1161 1233 1266 1482 1530 1641 1815 1918 2007 2034 2162 2633 3075 3393 3738 3995 4185 4473 4527 4809 5267 5628 5647 6326 6389 6489 7010 7267 7313 7403
*Alw*44I	4	3087 5510 6007 7253
*Alw*I	21	1602(c) 1801 2403(c) 3218(c) 3231 3511(c) 3520 4114 4882 4947(c) 5128 5492(c) 5505 6040 6044(c) 6361 6824(c) 6825 6921(c) 6923 7009
*Alw*NI	2	2665 7158
*Aos*I	4	3574 4113 4805 6456
*Apa*BI	3	804 4255 4327
*Apa*LI	4	3087 5510 6007 7253
*Apo*I	8	1843 2102 2772 2978 3019 3354 4008 4019
*Ase*I	2	161 6504
*Asi*SI	1	665
*Asn*I	2	161 6504
*Asp*700	2	3004 6079
*Asp*718	1	4159
*Asp*EI	1	6679
*Asp*HI	9	730 1717 3091 4816 5006 5514 6011 6096 7257
*Aps*I	1	4821
*Asu*II	1	5385
*Ava*I	2	1838 3260

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Ava*II	5	1764 3184 5219 6315 6537
*Avi*II	4	3574 4113 4805 6456
*Avr*II	1	4509
*Bam*HI	2	3223 5497
*Ban*I	9	619 977 1143 2957 3784 4159 4702 4737 6726
*Ban*II	4	730 2932 3754 5068
*Bbe*I	1	4706
*Bbr*7I	1	967
*Bbs*I	1	962
*Bbv*I	25	821(c) 1253(c) 1359 1643 1650 1676(c) 1679(c) 3012(c) 3380(c) 3587 3655 4126 4650(c) 4776 4818 4834(c) 4927(c) 5339 5634(c) 6245(c) 6636 6939(c) 7145(c) 7148(c) 7238
*Bce*83I	8	2056 2266(c) 3228(c) 5079(c) 6075 6943 7184(c) 7482
*Bce*AI	14	1182 1194 1242 1323 1434 1524 1602 1644 1695 3049(c) 3798 4662(c) 5129 7067(c)
*Bce*fI	14	1182 1194 1242 1323 1434 1524 1602 1644 1695 3050(c) 3798 4663(c) 5129 7068(c)
*Bcg*I	2	1232 6141(c)
*Bci*VI	5	2136(c) 2214 4922 5842 7369
*Bfa*I	12	154 753 1058 1087 3080 3321 3672 4510 4564 6486 6821 7074
*Bfr*BI	3	2728 4257 4329
*Bfr*I	4	829 848 1051 4557
*Bgl*I	8	137 244 366 437 3265 3584 4462 6561
*Bgl*II	3	1834 3002 7579
*Bln*I	1	4509
*Blo*HII	2	839 4756
*Bme*1580I	10	1148 1277 1526 2396 3091 4649 4742 5514 6011 7257
*Bmr*I	8	443(c) 892 945 1028(c) 1713(c) 4388(c) 4652 6639
*Bmt*I	1	1090
*Bmy*I	18	730 1148 1277 1526 1717 2396 2932 3091 3754 4649 4742 4816 5006 5068 5514 6011 6096 7257
*Bpl*I	2	4476 5391
*Bpm*I	7	1552 1792 1949 2182(c) 2366(c) 2573(c) 6610
*Bpu*10I	3	1711 1729 2790(c)
*Bpu*AI	1	962
*Bpu*EI	8	2056 2266(c) 3228(c) 5079(c) 6075 6943 7184(c) 7482
*Bsa*AI	3	494 3825 5007
*Bsa*BI	2	3511 5496
*Bsa*HI	8	276 329 412 598 4703 5405 5757 6139
*Bsa*I	3	916(c) 2186 6613
*Bsa*JI	19	514 1106 1136 1276 1439 1463 1518 2822 3260 4120 4221 4293 4416 4451 4460 4509 4866 5135 7407
*Bsa*WI	6	1095 1825 4734 6383 7214 7361
*Bsa*XI	4	2532 2601 3168 3864(c)
*Bsc*AI	23	514(c) 1209(c) 1483 1498 1597 2736 3355(c) 4047(c) 4083 4265 4337 4664(c) 4919(c) 5001 5065 5135(c) 5340 5528(c) 5618 5981(c) 6226 6421(c) 7473(c)
*Bse*AI	1	1825
*Bse*MII	16	1702(c) 1720(c) 1845 2104(c) 2672 2885(c) 3052 3229 4158(c) 4460(c) 5357(c) 5531 6192 6709(c) 6875(c) 7284(c)
*Bse*RI	5	1138 1946 1958 2870 4505
*Bse*YI	6	1899(c) 2472 4230 4302 5207(c) 7263
*Bsg*I	4	1236(c) 1333 1657 2944
*Bsi*EI	9	665 1100 2684 3269 3555 4612 6161 6310 7233

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Bsi*HKAI	9	730 1717 3091 4816 5006 5514 6011 6096 7257
*Bsi*YI	15	203 1277 1440 1790 2521 3606 3932 4417 4684 5228 5641 7089 7368 7534 7552
*Bsl*I	15	203 1277 1440 1790 2521 3606 3932 4417 4684 5228 5641 7089 7368 7534 7552
*Bsm*AI	13	588 826 916(c) 941(c) 1887(c) 2186 2200 2359(c) 4554 5642 5684(c) 5837(c) 6613
*Bsm*BI	3	2200 5642 5684(c)
*Bsm*FI	9	329 480 648 2123 4203(c) 4275(c) 4339(c) 4854 5386
*Bsm*I	2	3330 3423(c)
*Bsp*1286I	18	730 1148 1277 1526 1717 2396 2932 3091 3754 4649 4742 4816 5006 5068 5514 6011 6096 7257
*Bsp*24I	3	443 5779 6873(c)
*Bsp*CNI	16	1703(c) 1721(c) 1844 2105(c) 2671 2886(c) 3051 3228 4159(c) 4461(c) 5358(c) 5530 6191 6710(c) 6876(c) 7285(c)
*Bsp*DI	2	3515 5484
*Bsp*EI	1	1825
*Bsp*HI	4	2274 5734 5839 6847
*Bsp*MI	5	878(c) 2673 4590(c) 4971 5421
*Bsp*WI	45	137 244 366 398 437 530 554 803 1054 1199 1259 1272 1316 1325 1875 1956 2159 3034 3265 3554 3584 3616 3618 3660 3687 3717 4254 4326 4377 4456 4462 4694 4778 4801 4940 4946 5063 5099 5146 5413 5509 6561 6949 7521 7569
*Bsr*BI	4	3681(c) 5316(c) 5370 5837(c)
*Bsr*DI	4	66(c) 4936 6445 6619(c)
*Bsr*FI	6	1095 1258 3720 5022 5203 6594
*Bsr*GI	2	97 1817
*Bsr*I	21	449(c) 887 940 1034(c) 1719(c) 1882(c) 2371(c) 2383(c) 2647(c) 3914 4394(c) 4647 4848 6034 6204(c) 6473 6516 6634 7040 7152(c) 7165(c)
*Bss*HII	1	5100
*Bss*KI	31	242 435 1135 1151 1276 1388 1463 1517 1789 2176 2198 2520 2985 3014 3259 3260 3519 4221 4276 4293 4705 4865 5088 5605 5640 6141 6492 7188 7406 7419 7540
*Bss*SI	5	1288(c) 5295(c) 5703(c) 6010 7394
*Bst*API	3	803 4254 4326
*Bst*BI	1	5385
*Bst*F5I	13	997(c) 1148(c) 1514(c) 1909 2331 2439 4373(c) 5020 5045 5610(c) 6233 6520 6701
*Bst*KTI	36	665 750 1610 1758 1796 1837 2136 2262 2411 2684 3005 3226 3515 3519 3555 4109 4877 4955 5036 5045 5123 5500 5999 6035 6052 6310 6356 6374 6715 6820 6832 6910 6918 6929 7004 7582
*Bst*NI	19	244 437 1153 1278 1390 1465 1519 2178 2200 2522 2987 3016 4223 4278 4295 5090 7408 7421 7542
*Bst*UI	21	214 1436 1754 1851 3597 3621 3641 4017 4104 4769 5070 5102 5503 5583 5686 5688 5788 6120 6613 6943 7524
*Bst*XI	1	5424
*Bst*YI	16	1607 1834 2408 3002 3223 4106 4874 5120 5497 6032 6049 6817 6829 6915 6926 7579
*Btg*I	6	514 1106 2822 4120 4416 5135
*Bth*CI	48	837 1269 1328 1350 1634 1641 1692 1695 1789 1881 3028 3268 3271 3396 3578 3610 3624 3646 4117 4459 4614 4666 4677 4767 4772 4809 4850 4937 4940 4943 5179 5275 5316 5330 5431 5541 5650 5937 6166 6261 6288 6627 6955 7161 7164 7229 7372 7527
*Bts*I	6	844 1321 1991 3335 6260 6280(c)
*Cac*8I	36	240 433 669 1088 1231 1264 1312 1636 1643 1938 2254 2956 3526 3546 3665 3679 3722 4236 4255 4308 4327 4597 4783 5002 5068 5074 5102 5106 5147 5151 5205 5553 6566 6957 7517 7554
*Cfo*I	33	1092 1397 1438 1754 2252 2864 3575 3599 3612 3621 3643 3669 3677 4114 4697 4705 4769 4806 5072 5102 5104 5332 5585 5688 5788 6120 6457 6550 6943 7052 7226 7326 7393
*Cfr*10I	6	1095 1258 3720 5022 5203 6594

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Cha*I	36	666 751 1611 1759 1797 1838 2137 2263 2412 2685 3006 3227 3516 3520 3556 4110 4878 4956 5037 5046 5124 5501 6000 6036 6053 6311 6357 6375 6716 6821 6833 6911 6919 6930 7005 7583
*Cje*I	16	218(c) 411(c) 566 1053 2408 2736(c) 3182(c) 4171(c) 4266(c) 4504 5747(c) 6906 6970(c) 7006 7084 7525(c)
*Cje*PI	11	411(c) 1902(c) 1954(c) 2070 2958 3508(c) 5747(c) 6314 6786 6906 7019(c)
*Cla*I	2	3515 5484
*Csp*45I	1	5385
*Csp*6I	15	98 372 452 485 536 701 1063 1536 1818 2117 3152 4160 5008 5521 6197
*Cvi*AII	33	115 133 455 515 1107 1341 1371 1566 1761 1806 2227 2275 2725 3249 3283 4121 4254 4326 4417 4574 4919 5105 5136 5162 5651 5735 5840 6233 6269 6347 6357 6848 7568
*Cvi*JI	134	11 18 58 65 112 196 238 247 431 728 759 834 857 940 1048 1057 1086 1128 1161 1181 1233 1266 1281 1325 1382 1482 1530 1550 1570 1641 1682 1815 1918 2007 2034 2044 2153 2162 2256 2487 2585 2633 2646 2736 2756 2930 2954 2990 3037 3064 3075 3259 3268 3393 3524 3544 3578 3724 3738 3752 3833 3975 3995 4125 4185 4226 4298 4421 4450 4456 4465 4473 4496 4508 4514 4527 4563 4569 4611 4628 4636 4663 4688 4772 4781 4785 4809 4847 4923 4940 4959 5022 5066 5076 5093 5176 5203 5207 5244 5267 5283 5428 5551 5555 5595 5628 5647 5701 6288 6326 6389 6399 6489 6555 6564 6568 6594 6635 6647 7010 7039 7082 7093 7158 7237 7262 7267 7313 7403 7501 7527 7545 7556 7572
*Dde*I	19	1711 1729 1831 2113 2658 2790 2894 2902 3038 3215 4167 4469 5366 5517 5752 6178 6718 6884 7293
*Dpn*I	36	664 749 1609 1757 1795 1836 2135 2261 2410 2683 3004 3225 3514 3518 3554 4108 4876 4954 5035 5044 5122 5499 5998 6034 6051 6309 6355 6373 6714 6819 6831 6909 6917 6928 7003 7581
*Dpn*II	36	662 747 1607 1755 1793 1834 2133 2259 2408 2681 3002 3223 3512 3516 3552 4106 4874 4952 5033 5042 5120 5497 5996 6032 6049 6307 6353 6371 6712 6817 6829 6907 6915 6926 7001 7579
*Dra*I	4	3470 6101 6793 6812
*Dra*II	1	5699
*Dra*III	1	3828
*Drd*I	6	818 3872 4546 4730 5596 7465
*Dsa*I	6	514 1106 2822 4120 4416 5135
*Dsa*V	31	242 435 1135 1151 1276 1388 1463 1517 1789 2176 2198 2520 2985 3014 3259 3260 3519 4221 4276 4293 4705 4865 5088 5605 5640 6141 6492 7188 7406 7419 7540
*Eae*I	11	9 63 1179 1568 3266 4609 4783 5174 5201 5426 6286
*Eag*I	2	3266 4609
*Eam*1105I	1	6679
*Ear*I	7	1885(c) 2847(c) 3035 3533(c) 5047(c) 5257(c) 5880(c)
*Eci*I	15	388(c) 1356(c) 1600(c) 1713(c) 1955 2414(c) 4187 4358(c) 4379(c) 4389(c) 4400(c) 5232 6537 7365 7511
*Ecl*136II	1	728
*Ecl*XI	2	3266 4609
*Eco*47III	1	1091
*Eco*57I	9	1261 1305(c) 1504 3019 3215(c) 4849 5281 6013 7025(c)
*Eco*57MI	16	1261 1305(c) 1504 1552 1792 1949 2182(c) 2366(c) 2573(c) 3019 3215(c) 4849 5281 6013 6610 7025(c)
*Eco*HI	12	1135 1789 3259 3260 3519 4705 4865 5605 5640 6141 6492 7188
*Eco*ICRI	1	728
*Eco*O109I	1	5699
*Eco*RI	1	1843
*Eco*RII	19	242 435 1151 1276 1388 1463 1517 2176 2198 2520 2985 3014 4221 4276 4293 5088 7406 7419 7540
*Esa*BC3I	24	825 946 1158 1452 1479 1494 1623 1840 2212 2681 3275 3516 3791 4553 4817 4973 4997 5033 5195 5386 5485 6026 7470 7575

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Esp*3I	3	2200 5642 5684(c)
*Fal*I	5	874 1617 3173 4166 5043
*Fat*I	33	114 132 454 514 1106 1340 1370 1565 1760 1805 2226 2274 2724 3248 3282 4120 4253 4325 4416 4573 4918 5104 5135 5161 5650 5734 5839 6232 6268 6346 6356 6847 7567
*Fau*I	14	247 273 440 676 1441 3553 3617(c) 3686 4364 4385 4826(c) 5363(c) 5572 5582
*Fmu*I	14	240 433 1283 1684 1767 3187 3546 3834 5222 5702 6318 6540 6557 6636
*Fnu*4HI	48	835 1267 1326 1348 1632 1639 1690 1693 1787 1879 3026 3266 3269 3394 3576 3608 3622 3644 4115 4457 4612 4664 4675 4765 4770 4807 4848 4935 4938 4941 5177 5273 5314 5328 5429 5539 5648 5935 6164 6259 6286 6625 6953 7159 7162 7227 7370 7525
*Fnu*DII	21	214 1436 1754 1851 3597 3621 3641 4017 4104 4769 5070 5102 5503 5583 5686 5688 5788 6120 6613 6943 7524
*Fok*I	13	984(c) 1135(c) 1501(c) 1916 2338 2446 4360(c) 5027 5052 5597(c) 6240 6527 6708
*Fsp*I	4	3574 4113 4805 6456
*Gdi*II	10	1179 1568(c) 3266 3266(c) 4609(c) 4609 5174(c) 5201(c) 5426(c) 6286
*Hae*I	10	11 65 2153 2256 4125 4508 4785 7093 7545 7556
*Hae*II	6	1093 2253 3670 3678 4706 7327
*Hae*III	32	11 65 238 431 1181 1281 1570 1682 2153 2256 3268 3544 3833 3975 4125 4450 4456 4465 4508 4611 4785 5176 5203 5428 5701 6288 6555 6635 7093 7527 7545 7556
*Hae*IV	2	4899 6698
*Hga*I	7	688 3603 5413 5589 6147 6877(c) 7455(c)
*Hgi*AI	9	730 1717 3091 4816 5006 5514 6011 6096 7257
*Hha*I	33	1092 1397 1438 1754 2252 2864 3575 3599 3612 3621 3643 3669 3677 4114 4697 4705 4769 4806 5072 5102 5104 5332 5585 5688 5788 6120 6457 6550 6943 7052 7226 7326 7393
*Hin*4I	7	2601 4866(c) 4896(c) 4898 6591(c) 6665(c) 6697
*Hin*P1I	33	1090 1395 1436 1752 2250 2862 3573 3597 3610 3619 3641 3667 3675 4112 4695 4703 4767 4804 5070 5100 5102 5330 5583 5686 5786 6118 6455 6548 6941 7050 7224 7324 7391
*Hinc*II	3	678 2811 3409
*Hind*II	3	678 2811 3409
*Hind*III	2	757 4525
*Hinf*I	15	564 842 958 1074 1829 2565 3873 3895 4531 5188 5322 5374 5481 6680 7197
*Hpa*I	2	2811 3409
*Hpa*II	28	1096 1136 1199 1259 1790 1826 3261 3520 3721 4608 4685 4707 4735 4866 4956 5023 5204 5607 5641 6142 6384 6494 6561 6595 6999 7189 7215 7362
*Hph*I	17	530 1122 1125(c) 1455 1479 1608 2010 2040(c) 3825 4881(c) 5659(c) 5668(c) 5952(c) 5987 6193(c) 6609 6836
*Hpy*188I	33	747 811 972 1834 2100 2169 2357 2661 2705 2778 2945 2999 3179 3218 3506 3768 4140 4168 4470 4539 4670 5014 5367 5534 5659 6181 6301 6312 6758 6893 7027 7380 7458
*Hpy*188III	37	823 874 946 1290 1605 1779 1826 1838 1840 1928 2185 2275 2431 2455 2506 4051 4479 4551 4721 5037 5046 5129 5185 5229 5319 5352 5421 5705 5735 5840 6589 6848 6922 7005 7103 7201 7335
*Hpy*8I	23	355 538 678 701 739 1028 1133 1176 1470 1599 2119 2340 2797 2811 3089 3409 3822 3877 3894 5512 6009 6767 7255
*Hpy*99I	11	1174 1420 1681 4793 4821 5115 5130 5407 6414 6677 7471
*Hpy*AV	17	1372(c) 1479(c) 2730 2834(c) 3128(c) 3239 3569 3709 4173 4831(c) 5348 5358 5955 6314(c) 6567 7022(c) 7332(c)
*Hpy*CH4I	33	117 135 457 517 1109 1343 1373 1568 1763 1808 2229 2277 2727 3251 3285 4123 4256 4328 4419 4576 4921 5107 5138 5164 5653 5737 5842 6235 6271 6349 6359 6850 7570
*Hpy*CH4III	27	230 352 423 705 743 782 820 2062 2269 2561 2748 2826 2970 3144 3194 3569 3851 4548 5083 5199 5307 5636 5671 6239 6754 7067 7537
*Hpy*CH4IV	20	75 276 288 329 412 493 598 1172 1385 1556 3714 3824 3867 3879 4819 5006 5757 6077 6450 6866

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Hpy*CH4V	34	71 837 862 1253 1314 1638 2668 2728 2914 2925 2965 3089 3328 3396 3423 4248 4257 4320 4329 4444 4520 4595 4754 4929 4943 5512 5650 6009 6253 6341 6534 6624 6959 7255
*Hpy*F10VI	45	138 245 367 399 438 531 555 804 1055 1200 1260 1273 1317 1326 1876 1957 2160 3035 3266 3555 3585 3617 3619 3661 3688 3718 4255 4327 4378 4457 4463 4695 4779 4802 4941 4947 5064 5100 5147 5414 5510 6562 6950 7522 7570
*Ita*I	48	835 1267 1326 1348 1632 1639 1690 1693 1787 1879 3026 3266 3269 3394 3576 3608 3622 3644 4115 4457 4612 4664 4675 4765 4770 4807 4848 4935 4938 4941 5177 5273 5314 5328 5429 5539 5648 5935 6164 6259 6286 6625 6953 7159 7162 7227 7370 7525
*Kas*I	1	4702
*Kpn*I	1	4163
*Ksp*632I	7	1885(c) 2847(c) 3035 3533(c) 5047(c) 5257(c) 5880(c)
*Lpn*I	6	1091 2251 3668 3676 4704 7325
*Mae*I	12	154 753 1058 1087 3080 3321 3672 4510 4564 6486 6821 7074
*Mae*II	20	75 276 288 329 412 493 598 1172 1385 1556 3714 3824 3867 3879 4819 5006 5757 6077 6450 6866
*Mae*III	25	215 302 651 839 902 1290 1779 2076 2551 2649 3144 3379 3635 3647 4823 5129 5630 6018 6206 6359 6417 6748 7031 7147 7210
*Mam*I	2	3511 5496
*Mbo*I	36	662 747 1607 1755 1793 1834 2133 2259 2408 2681 3002 3223 3512 3516 3552 4106 4874 4952 5033 5042 5120 5497 5996 6032 6049 6307 6353 6371 6712 6817 6829 6907 6915 6926 7001 7579
*Mbo*II	28	967 1350(c) 1395(c) 1398(c) 1593 1902 1920 1975 2013 2254(c) 2548 2864 2997(c) 3012 3022(c) 3550 3686(c) 4526(c) 5064 5274 5354(c) 5897 6006 6084 6839 6910(c) 7062(c) 7574(c)
*Mcr*I	9	665 1100 2684 3269 3555 4612 6161 6310 7233
*Mfe*I	3	2288 2934 3418
*Mlu*I	1	1849
*Mlu*NI	3	11 65 4785
*Mly*I	10	558(c) 836(c) 952(c) 1068(c) 1823(c) 3882 3889(c) 5368(c) 6689 7191(c)
*Mme*I	5	2691(c) 3179(c) 3850(c) 7174(c) 7358(c)
*Mnl*I	47	703(c) 870(c) 1116(c) 1197(c) 1203(c) 1297 1434(c) 1446(c) 1497(c) 1617(c) 1924(c) 1936(c) 2390 2438 2528 2699(c) 2748 2774(c) 2848(c) 3197 3454(c) 3494 3534(c) 3798 4138(c) 4146 4162(c) 4440(c) 4446(c) 4470 4476 4483(c) 4486(c) 4498(c) 4618(c) 4754(c) 5111(c) 5304 5653(c) 5712 6306(c) 6512(c) 6659 6740 7140 7390(c) 7464
*Mro*I	1	1825
*Msc*I	3	11 65 4785
*Mse*I	34	161 784 830 849 917 1052 1067 2312 2318 2624 2698 2810 3047 3211 3408 3469 3615 3886 3984 4001 4012 4024 4035 4558 5547 5728 6100 6465 6504 6739 6792 6806 6811 6863
*Msl*I	16	519 1108 1138 1288 1465 1594 1852 2673 3097 3179 5140 5422 5461 5908 6267 6426
*Msp*A1I	7	1918 4185 4809 5577 6043 6984 7229
*Msp*I	28	1096 1136 1199 1259 1790 1826 3261 3520 3721 4608 4685 4707 4735 4866 4956 5023 5204 5607 5641 6142 6384 6494 6561 6595 6999 7189 7215 7362
*Mun*I	3	2288 2934 3418
*Mva*I	19	244 437 1153 1278 1390 1465 1519 2178 2200 2522 2987 3016 4223 4278 4295 5090 7408 7421 7542
*Mvn*I	21	214 1436 1754 1851 3597 3621 3641 4017 4104 4769 5070 5102 5503 5583 5686 5688 5788 6120 6613 6943 7524
*Mwo*I	45	137 244 366 398 437 530 554 803 1054 1199 1259 1272 1316 1325 1875 1956 2159 3034 3265 3554 3584 3616 3618 3660 3687 3717 4254 4326 4377 4456 4462 4694 4778 4801 4940 4946 5063 5099 5146 5413 5509 6561 6949 7521 7569
*Nae*I	2	3722 5205
*Nar*I	1	4703
*Nci*I	12	1137 1791 3261 3262 3521 4707 4867 5607 5642 6143 6494 7190

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Nco*I	5	514 1106 4120 4416 5135
*Nde*I	1	388
*Nde*II	36	662 747 1607 1755 1793 1834 2133 2259 2408 2681 3002 3223 3512 3516 3552 4106 4874 4952 5033 5042 5120 5497 5996 6032 6049 6307 6353 6371 6712 6817 6829 6907 6915 6926 7001 7579
*Ngo*MI	2	3720 5203
*Ngo*MIV	2	3720 5203
*Nhe*I	1	1086
*Nla*III	33	118 136 458 518 1110 1344 1374 1569 1764 1809 2230 2278 2728 3252 3286 4124 4257 4329 4420 4577 4922 5108 5139 5165 5654 5738 5843 6236 6272 6350 6360 6851 7571
*Nla*IV	22	621 979 1145 1683 2959 3225 3753 3765 3786 4161 4227 4299 4704 4739 5499 5792 6382 6593 6634 6728 7500 7539
*Nli*3877I	2	1842 3264
*Not*I	1	3266
*Nsi*I	3	2730 4259 4331
*Nsp*I	6	2230 3252 4257 4329 5108 5654
*Nsp*V	1	5385
*Oli*I	4	1108 1138 1288 1465
*Pae*R7I	1	1838
*Pci*I	1	2226
*Pfl*MI	1	2521
*Pfo*I	3	1388 2520 5640
*Pin*AI	1	1095
*Ple*I	10	558(c) 836(c) 952(c) 1068(c) 1823(c) 3881 3889(c) 5368(c) 6688 7191(c)
*Ppi*I	5	1114(c) 1432(c) 3864(c) 6051 6861(c)
*Ppu*10I	3	2726 4255 4327
*Psi*I	2	3389 3953
*Psp*03I	5	1767 3187 5222 6318 6540
*Psp*1406I	2	6077 6450
*Psp*GI	19	242 435 1151 1276 1388 1463 1517 2176 2198 2520 2985 3014 4221 4276 4293 5088 7406 7419 7540
*Pss*I	1	5702
*Pst*I	2	839 4756
*Pvu*I	4	665 2684 3555 6310
*Pvu*II	3	1918 4185 4809
*Rca*I	4	2274 5734 5839 6847
*Rle*AI	1	3446(c)
*Rsa*I	15	99 373 453 486 537 702 1064 1537 1819 2118 3153 4161 5009 5522 6198
*Rsr*II	1	5219
*Sac*I	1	730
*Sap*I	3	3035 5047(c) 5257(c)
*Sau*3AI	36	662 747 1607 1755 1793 1834 2133 2259 2408 2681 3002 3223 3512 3516 3552 4106 4874 4952 5033 5042 5120 5497 5996 6032 6049 6307 6353 6371 6712 6817 6829 6907 6915 6926 7001 7579
*Sau*96I	14	237 430 1280 1681 1764 3184 3543 3831 5219 5699 6315 6537 6554 6633
*Sca*I	3	1064 3153 6198
*Sci*I	1	1840
*Scr*FI	31	244 437 1137 1153 1278 1390 1465 1519 1791 2178 2200 2522 2987 3016 3261 3262 3521 4223 4278 4295 4707 4867 5090 5607 5642 6143 6494 7190 7408 7421 7542
*Sel*I	21	212 1434 1752 1849 3595 3619 3639 4015 4102 4767 5068 5100 5501 5581 5684 5686 5786 6118 6611 6941 7522

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Sex*AI	2	2176 4276
*Sfa*NI	23	511(c) 1206(c) 1484 1499 1598 2737 3352(c) 4044(c) 4084 4266 4338 4661(c) 4916(c) 5002 5066 5132(c) 5341 5525(c) 5619 5978(c) 6227 6418(c) 7470(c)
*Sfc*I	9	835 1080 2747 3190 3602 4752 6433 7111 7302
*Sfi*I	1	4462
*Sfo*I	1	4704
*Sfu*I	1	5385
*Sgf*I	1	665
*Sim*I	12	181 260(c) 1235(c) 1430(c) 1737(c) 1925 2763(c) 3791(c) 5131(c) 6607 6893(c) 7376
*Sma*I	1	3262
*Sml*I	13	829 848 1051 1838 2035 2281 3243 4557 5094 6054 6922 7199 7461
*Sna*BI	1	494
*Sno*I	4	3087 5510 6007 7253
*Spe*I	1	153
*Sph*I	3	4257 4329 5108
*Srf*I	1	3262
*Ssp*BI	2	97 1817
*Ssp*D5I	17	530 1122 1124(c) 1455 1479 1608 2010 2039(c) 3825 4880(c) 5658(c) 5667(c) 5951(c) 5987 6192(c) 6609 6836
*Ssp*I	4	6 53 4033 5874
*Sth*132I	55	169(c) 246 272 439 456(c) 561(c) 624(c) 675 680 1128(c) 1277 1340 1382 1440 1691 1703 1782(c) 2403 3253(c) 3267 3512(c) 3552 3616(c) 3685 3737 3738(c) 4363 4384 4713 4783(c) 4825(c) 4858(c) 5116 5255 5326 5362(c) 5564 5571 5581 5583(c) 5613 5633(c) 5766(c) 6076 6134(c) 6137 6500 6659 6667(c) 6904 7181(c) 7225(c) 7234(c) 7342 7433(c)
*Sts*I	13	983(c) 1134(c) 1500(c) 1917 2339 2447 4359(c) 5028 5053 5596(c) 6241 6528 6709
*Stu*I	2	2153 4508
*Sty*I	6	514 1106 4120 4416 4509 5135
*Tai*I	20	78 279 291 332 415 496 601 1175 1388 1559 3717 3827 3870 3882 4822 5009 5760 6080 6453 6869
*Taq*I	24	824 945 1157 1451 1478 1493 1622 1839 2211 2680 3274 3515 3790 4552 4816 4972 4996 5032 5194 5385 5484 6025 7469 7574
*Taq*II	5	3923(c) 4740 5408 6147(c) 6332
*Tat*I	12	97 371 451 484 535 1062 1535 1817 2116 3151 5520 6196
*Tau*I	23	1328 1789 1881 3268 3271 3610 3624 4459 4614 4677 4772 4937 4940 5179 5275 5316 5431 5541 5937 6166 6288 7372 7527
*Tfi*I	5	2565 4531 5188 5322 5481
*Tha*I	21	214 1436 1754 1851 3597 3621 3641 4017 4104 4769 5070 5102 5503 5583 5686 5688 5788 6120 6613 6943 7524
*Tru*9I	34	161 784 830 849 917 1052 1067 2312 2318 2624 2698 2810 3047 3211 3408 3469 3615 3886 3984 4001 4012 4024 4035 4558 5547 5728 6100 6465 6504 6739 6792 6806 6811 6863
*Tsc*I	20	78 279 291 332 415 496 601 1175 1388 1559 3717 3827 3870 3882 4822 5009 5760 6080 6453 6869
*Tse*I	25	834 1266 1347 1631 1638 1689 1692 3025 3393 3575 3643 4114 4663 4764 4806 4847 4940 5327 5647 6258 6624 6952 7158 7161 7226
*Tsp*45I	10	839 1290 1779 2551 3647 4823 5129 5630 6206 6417
*Tsp*509I	24	172 786 1040 1843 2102 2288 2480 2496 2695 2772 2934 2978 3019 3354 3418 4008 4019 4045 4263 4335 4427 6246 6501 6807
*Tsp*DTI	15	17(c) 180(c) 1236(c) 1357 2291 2342 2708 3163(c) 3416(c) 4762 5180(c) 5440(c) 6409 6712 6789(c)
*Tsp*GWI	2	5898(c) 6240

Enzyme	# of cuts	Positions (c) indicates the complementary strand
*Tsp*RI	20	808 844 887 1321 1991 2143 2538 2751 2975 3199 3239 3335 4832 6260 6287 6634 6739 6888 7159 7172
*Tth*111I	1	4821
*Tth*111II	7	1591 2103 3261 5138(c) 6944(c) 6976 6983(c)
*Unb*I	14	236 429 1279 1680 1763 3183 3542 3830 5218 5698 6314 6536 6553 6632
*Van*91I	1	2521
*Vpa*K11AI	5	1763 3183 5218 6314 6536
*Xho*I	1	1838
*Xho*II	16	1607 1834 2408 3002 3223 4106 4874 5120 5497 6032 6049 6817 6829 6915 6926 7579
*Xma*I	1	3260
*Xma*III	2	3266 4609
*Xmn*I	2	3004 6079
*Zra*I	5	277 330 413 599 5758

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25-8010-46UM Rev B, 2006