PathwayScan™

Multi-Pathway Reporter Kit

- Making new discoveries has never been easier

INSTRUCTION MANUAL

Catalog # GM8001

Revision A

For Research Use Only. Not for Use in Diagnostic Procedures

GM Biosciences

www.gmbiosciences.com

PathwayScan™ Multi-pathway Reporter Kit Catalog # GM8001

QUICK-REFERENCE PROTOCOL

1. **Pre-plate cells in 96-well plate, grow cultures to 60%~80% confluence.**

   Note *Calculate how many plates of cells are needed:*
   For example, in an experiment in triplicate with two conditions (stimulated vs non-stimulated), three 96-well plates are needed (48 wells x 3 x 2 conditions = 288 wells).

2. **Prepare transfection complexes of 48 reporter plasmids respectively, then add into cell cultures.**

   Note *If HEK 293 cells are transfected by Lipofectamine 2000, 0.1ug PathwayScan reporter plasmid is needed for each well. In an experiment in triplicate with two conditions, 0.6ug of each 48 reporter plasmids is needed.*
   If co-transfection is required, the amount of an interested plasmid should be same as that of PathwayScan reporter plasmid (1:1 ratio).

3. **Continue culturing cells for 16 ~ 24 hours, and stimulate the cells.**

   Note *If stimulation is required, add stimulant 16 hours after transfection and continue cultures for at least 5 hours before harvesting.*

4. **Lyse cells, test Firefly luciferase activity and Renilla luciferase activity for each well.**

PathwayScan™ Multi-Pathway Reporter Kit

CONTENTS

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KIT CONTENTS

	Catalog # GM8001
48 PathwayScan™ reporter plasmids [a,b,c,d,e] (including 46 transcription factor reporters, 1 negative control and 1 positive control)	100ng/ul x 10ul for each of 48 reporter plasmids

[a] Each of 48 plasmids contains a small amount of Renilla luciferase constitutive expression plasmid as internal transfection control.

[b] 48 plasmids are provided in six 8-well strips.

[c] PathwayScan is a trademark of GM Biosciences, Inc.

[d] All plasmids are not supposed to be transformed and replicated in E.coli.

[e] This protocol is not included with product shipments. Please go to www.gmbiosciences.com to download it.

STORAGE CONDITIONS

-20°C

MATERIALS NEEDED BUT NOT PROVIDED

- **Cell line**
- **Cell culture reagents** such as medium, Fetal calf serum (FCS), antibiotics
- **96-well cell culture plate**
- **Multi-channel pipettor**
- **Transfection reagent** such as Lipofectamine 2000
- **Reporter assay reagents for Firefly luciferase and Renilla luciferase**
- **Luminometer**

INTRODUCTION

Tens of signal transduction pathways have been identified, such as NFkB signaling pathway, GPCR signaling pathway, Erk signaling pathway. In general, signal transduction pathway ends up with transcription factors (TFs) activation and/or inhibition. TFs then regulate hundreds of, even thousands of genes' expressions. Therefore, in order to study signal transductions, it is advantageous to analyze TFs activities instead of downstream thousands of genes' expression level.

Human genome has approximately 20,000 genes. Studies on the functions of these genes in signaling pathways are promising ongoing projects and are intensively pursued world-widely. Guess and check strategy is a traditional way, but with less successful rate and time/labor consuming. Gene chip is an alternative way to find out what TFs/signaling pathways are influenced, but its drawback is obvious: (1) protein level does not have to be proportional to its RNA level; (2) protein level, especially TFs, does not correlate to its activity. In addition, it is a puzzle to infer a TF's activity based on expression level of hundreds of genes it regulated.

PathwayScan Multi-Pathway Reporters kit provides a comprehensive collection of proprietary TF luciferase reporters. It can be easily performed in a single experiment to scan 46 signaling pathways. Each of the TFs was carefully chosen to monitor its corresponding signaling pathway. The strongest DNA binding sequence of each TF was selected and proprietarily tandom-repeated as promoter of luciferase. The TFs activities are quantitatively reflected by luciferase activity. The luciferase gene was modified and optimized to achieve remarkable sensitivity.

PathwayScan Multi-Pathway Reporters can be applied to a variety of scenarios. A single assay of PathwayScan easily identifies which signaling pathways are influenced by a gene/chemokine/cytokine/chemical/drug candidate. PathwayScan works best for (but not limited to):

- Screening for novel signaling pathways in which a gene may be involved
- Identifying which signaling pathways are influenced by a mutant gene, microRNA, siRNA, shRNA
- Identifying which signaling pathways are influenced by various stimuli (cytokines, chemokines, chemicals, hormones, drugs, etc.)
- Studying crosstalk of signal pathways
- Signaling pathway profiling in a variety of scenarios
- Screening which signaling pathways are influenced by home-made cells (transient or stable, overexpressed or knock-down, knock-out or knock-in)
- Identifying phenotype of transgenic, knock-out or knock-in mice
- Drug candidates screening and verification
- Identification of signaling pathways in which a protein kinase is involved

PathwayScan is easily performed as shown in Figure 1.

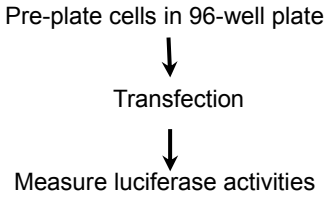


Figure 1. Flowchart of PathwayScan procedure

PathwayScan Multi-Pathway Reporter Kit dramatically promotes new discoveries. The following several examples show its unparalleled performances.

Cytokine EGF is known to activate TFs such as Erk[1,2], AP-1[3], STAT3[4] , EGR-1[5] and GATA[6]. In one single experiment of PathwayScan on EGF, the activations of Erk (66-fold), AP-1 (4-fold) , STAT3 (3.5-fold), EGR-1 (9.7-fold) and GATA (3.3-fold) were confirmed (see Fig 2). In addition, 4 transcription factors were found for the first time, whose activities were significantly changed by EGF stimulation (2.6-fold, 2.5-fold increase and 3.0-fold, 2.1-fold decrease respectively, as shown by red arrow and labeled as New discovery).

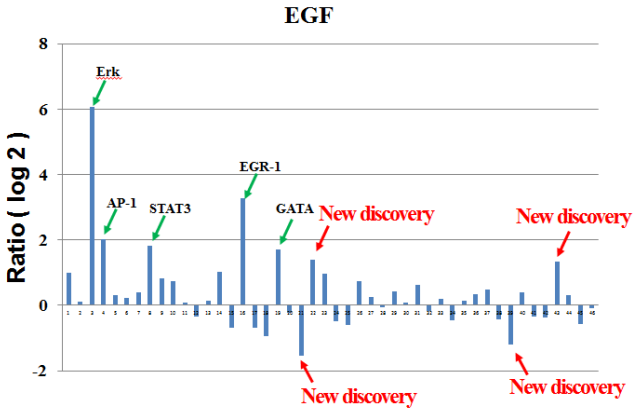


Figure 2. EGF stimulated HEK 293 cells were analyzed by PathwayScan Multi-Pathway Reporter Kit. The activity ratio was converted to logarithm with base 2.

Protein kinases are involved in most, if not all, of signal transduction pathways. It is known that IKK2 activates NFkB[7] and plays a critical role in NFkB signaling pathway. A single test of PathwayScan on IKK2 not only confirmed its activation on NFkB (25-fold) and Erk (2.8-fold), but also revealed 5 transcription factors for the first time whose activities were significantly changed by IKK2-CA overexpression (8.2-fold, 12.1-fold, 3.8-fold increase and 7.7-fold, 6.3-fold decrease respectively, as shown by red arrow and labeled as New discovery).

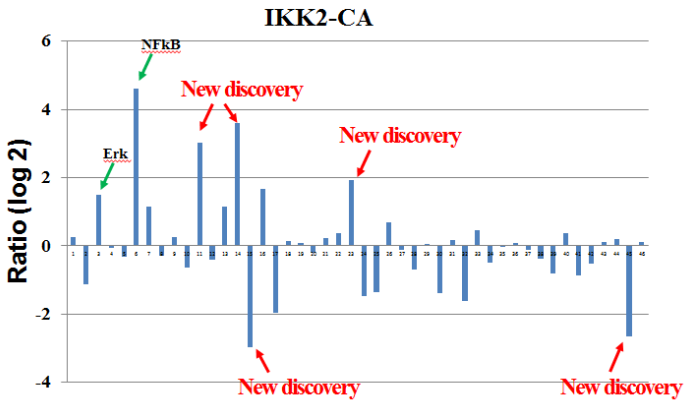


Figure 3. IKK2-CA overexpressed HEK 293 cells were analyzed by PathwayScan Multi-Pathway Reporter Kit. The activity was converted to logarithm with base 2.

PathwayScan has remarkable sensitivity. This feature enables both activation and inhibition to be detected in the meantime. Pathway-specific stimulus is routinely employed to manifest inhibitory effect. For example, in order to study optineurin(OPTN)'s inhibition on NFkB[8], TNF is necessary to boost NFkB signal so that OPTN's inhibition becomes detectable. In contrast, PathwayScan is sensitive enough and the inhibition is detectable even without TNF stimulation. As shown in Fig 4, NFkB was successfully inhibited for 9-fold by OPTN (green arrow). Moreover, 4 transcription factors were found to be dramatically inhibited or activated (labeled as "New discovery" in Fig 4).

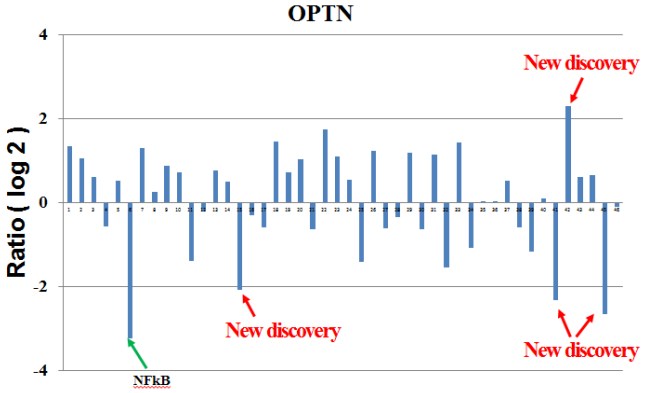


Figure 4. OPTN overexpressed HEK 293 cells were analyzed by PathwayScan Multi-Pathway Reporter Kit. The activity ratio was converted to logarithm with base 2.

Taken above together, PathwayScan has the following remarkable features:

- **Detect both activation and inhibition of TFs.**
- **Simultaneously screen 46 different signaling pathways**
- **One experiment leads to many new discoveries**

PathwayScan™ reporters list

48 PathwayScan reporter plasmids are included in the kit, which consists of 46 TF reporter plasmids, 1 negative control plasmid and 1 positive control plasmid. All the 48 reporter plasmids are ready for transfection.

The following table shows TF reporters that are included in PathwayScan and their corresponding signaling pathways.

Transcription Factor	Pathway	Transcription Factor	Pathway
AhR	Xenobiotic	MTF1	Heavy Metal Stress
Androgen Receptor	Androgen	Myb	Wnt-NLK
AP1(c-Jun/c-Fos)	MAPK/JNK	Myc/Max	Myc/Max
ATF2/3/4	Amino Acid Deprivation	Nanog	Nanog
C/EBP	C/EBP	NFAT	PKC/Ca++
CBF/NF-Y and YY1	ER Stress	NFE2	Oxidative Stress
CREB	cAMP/PKA	NFkB	NFκB
E2F/DP1	Cell Cycle	Oct4	Oct4
EGR1	EGR1	p53	p53/DNA Damage
Estrogen Receptor	Estrogen	Pax6	Pax6
ETS	ETS	PBX1	PBX1
FOXO	PI3K/AKT	PPAR	PPAR
GATA	GATA	PR	Progesterone
Gli	Hedgehog	RAR/RXR	Retinoic Acid/Retinoid X
GR	Glucocorticoid	RBP-J	Notch
HIF1	Hypoxia	SMAD	TGFβ
HNF4	Hepatocyte Nuclear Factor 4	Sox2	Sox2
HSF1	Heat Shock	SP1	SP1
IRF-1	Interferon Regulation	SRF	MAPK/ERK
KLF4	KLF4	STAT1	Interferon Gamma
LEF1/TCF1	Wnt	STAT2	Type I Interferon
LXR	Liver X	STAT3	IL-6
MEF2	MEF2	VDR	Vitamin D

Each of 46 reporter plasmids expresses Firefly luciferase under control of 46 TF's binding sequences. The negative control plasmid is Firefly luciferase gene without any promoter, and the positive control plasmid expresses Firefly luciferase driven by CMV promoter.

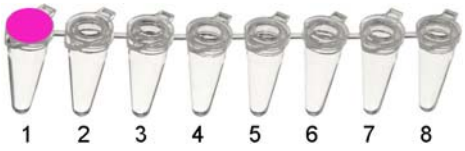
Each of the 48 PathwayScan plasmids is pre-mixed with Renilla plasmid which constitutively expresses Renilla luciferase. The expressed Renilla luciferase functions as an internal control to normalize variations of cells number and transfection efficiency among wells.

The 48 PathwayScan reporter plasmids are provided in six 8-tube strips which are labeled with 6 color tags on the cap: red, pink, yellow, green, blue and white. Only the first tube of each strip is color-tagged on its cap (as shown in pictures below).

The red tagged strip contains the following 8 plasmids from left to right. Only #1 tube is red color-tagged.

1	2	3	4	5	6	7	8
AhR	Androgen Receptor	AP1	ATF2/3/4	C/EBP	CBF/NF-Y and YY1	CREB	E2F/DP1

The pink tagged strip contains the following 8 plasmids from left to right. Only #1 tube is pink color-tagged.



1	2	3	4	5	6	7	8
EGR1	ER	ETS	FOXO	GATA	Gli	GR	HIF1

The yellow tagged strip contains the following 8 plasmids from left to right. Only #1 tube is yellow color-tagged.

1	2	3	4	5	6	7	8
HNF4	HSF1	IRF-1	KLF4	LEF1/TCF1	LXR	MEF2	MTF1

The green tagged strip contains the following 8 plasmids from left to right. Only #1 tube is green color-tagged.

1	2	3	4	5	6	7	8
Myb	Myc/Max	Nanog	NFAT	NFE2	NFkB	Oct4	p53

The blue tagged strip contains the following 8 plasmids from left to right. Only #1 tube is blue color-tagged.

1	2	3	4	5	6	7	8
Pax6	PBX1	PPAR	PR	RAR/RXR	RBP-J	SMAD	Sox2

The white tagged strip contains the following 8 plasmids from left to right. Only #1 tube is white color-tagged.

1	2	3	4	5	6	7	8
SP1	SRF	STAT1	STAT2	STAT3	VDR	Positive Control	Negative Control

Note *All of the PathwayScan plasmids are not supposed for transformation and replication in E.coli.*

PROTOCOL

1. **Pre-plate cells in 96-well cell culture plates 1 day prior to transfection, grow cultures to 60%~80% confluence.**

   Cell lines that are easily transfected are preferred. For example, adherent 293H, 293F, CHO-S, Cos-7L and HeLa cells can be easily transfected by Lipofectamine 2000.

   Calculate how many plates of cell are needed. In an experiment in triplicate with two conditions (stimulation vs non-stimulation), three 96-well plates are needed (48 wells x 3 x 2 conditions = 288 wells).

   Pre-plating is not required to transfect suspension cells and some adherent. For example, adherent cell line 293H, 293F, CHO-S and Cos-7L work very well without pre-plating for Lipofectamine 2000 transfection. It should be determined empirically whether pre-plating is needed for specific cell lines and transfection reagents.

   It is recommended to culture the cells in medium supplemented with 10% FCS during and after transfection if the cells are normally cultured in the presence of serum. High basal signals can be induced by FCS, which is good for detecting inhibitory effect. However, if only activation effects are desired, 0.5% FCS can be used after transfection, which may give lower basal signals.

   It should be cautious in using antibiotics during transfection. However, some cell lines such as HEK293 cells tolerate antibiotics very well. Whether to use antibiotics during transfection should be determined empirically according to cell lines and transfection reagents.

2. **Prepare transfection complexes of 48 reporter plasmids respectively.**

   Perform transfection when cells become 60%-80% confluence. Choose desired transfection reagent. Lipofectamine 2000 has been tested and is recommended.

   The amount of DNA to use in transfection should be determined according to manufacture manual of transfection reagent. For example, if Lipofectamine 2000 is used to transfect HEK 293 cells, 0.1ug reporter plasmid is needed for each well; 0.6ug of each 48 reporter plasmids is needed for an experiment in triplicate with two conditions.

   A small amount of GFP (0.001~0.005ug) may be mixed with each of 48 plasmids to visually confirm transfection using fluorescent microscope. However, it should be

cautious to co-transfect GFP because GFP is reported to have potential toxic to cells in some cases and it can induce oxidative stress.

If co-transfection is required, the amount of an interested plasmid should be same as that of PathwayScan reporter plasmid (1:1 ratio). For example, if Lipofectamine 2000 is used to transfect HEK 293 cells, 0.1ug interested plasmid and 0.1ug reporter plasmid should be co-transfected in each well.

Co-transfection of miRNA/siRNA/shRNA with PathwayScan reporter plasmid is feasible. Please refer to manufacture protocol of transfection regent.

Co-transfection of peptide/protein with PathwayScan reporter plasmid is feasible. Please refer to manufacture protocol of transfection regent.

3. **Transfection by adding 48 transfection complexes into cell cultures respectively.**

   For a two-condition experiment in triplicate, each of transfection complexes prepared above should be added into 6 wells in equal amount.

   Generally, it is not necessary to remove complex or change/add medium after transfection. Please see manufacture protocol of transfection reagent for details. If Lipofectamine 2000 is used, changing/adding medium post transfection is not needed, but transfection complex may be removed after 4-6 hours.

   It is not recommended to change medium for cell line, such as HEK 293, which is loosely bound to culture plate.

4. **Continue culturing cells for 16 ~ 24 hours, then stimulate the cells.**

   If stimulation is required, directly adding stimulant into culture medium at 16 hours post transfection and continue cultures for at least 5 hours before harvesting.

   Changing medium is generally not necessary for stimulation. However, whether to change medium for stimulation should be determined empirically.

   It is not recommended to change medium for cell line, such as HEK 293, which loosely bound to culture plate.

5. **Lyse cells, test Firefly luciferase activity and Renilla luciferase activity for each well.**

   Remove culture medium to harvest cells. Add 100ul of lysis buffer (see PREPARATION OF SOLUTIONS for recipe) into each well. Slightly rock the plate several times from

side to side, and stand at room temperature for 5~10min to lyse the cells.

In order to increase pipetting accuracy, it is recommended to remove genome DNA (sticky materials) out of lysate. Insert a tip into lysate and swirl the tip 5-10 times. A small mess of sticky transparent materials will stick to the end of the tip. Pull out the tip and discard it. The remaining lysate is ready for testing luciferase activity.

Luciferase reagents (including luciferin and co-elenterazine) can be either commercial or home-made. Luciferin is the substrate for firefly luciferase's bioluminescence reaction, while co-elenterazine is the substrate for renilla luciferase activity. Promega's Dual-Luciferase system (either E1910 or E2920) uses identical lysate (in a single vial) to test activity of Firefly luciferase and activity of Renilla luciferase sequentially, which reduces the pipetting variations. E1910 is compatible with luminometer equipped with auto-injector. E2920 generates luminescence in a very stable manner, and is compatible with any luminometer.

For using home-made luciferase reagents, prepare luciferin reaction solution and co-elenterazine reaction solution respectively according to traditional recipe (not provided). However, home-made reagents are much less sensitive than Promega's Dual-Luciferase system, and are thus not recommended.

6. Data analysis

Firefly luciferase activity represents TFs activity, whereas Renilla luciferase functions as internal control to normalize variations of cell-seeding and transfection among wells. It is recommended to normalize the activity of Firefly luciferase to that of Renilla luciferase.

PREPARATION OF SOLUTIONS

Lysis buffer

10mM TrisHCl pH7.5
2mM DTT
1% TritonX100
2mM EDTA

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MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information is available on the web at *http://www.gmbiosciences.com*. MSDS documents are not included with product shipments.

LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, express or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by GM Biosciences. GM Biosciences shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

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