The Next-Generation Assay for Mammalian Protein Interactions: The CheckMate™/Flexi® Vector Mammalian Two-Hybrid System

ABSTRACT Analyses of protein:protein interactions are an integral part of better understanding protein functionality. Mammalian two-hybrid systems provide an excellent means to detect and validate protein interactions within mammalian cells. The next-generation CheckMate™/Flexi® Vector Mammalian Two-Hybrid System provides additional features for ease-of-use and expanded capabilities. The cloning vectors are now compatible with the Flexi® Vector System, allowing easy transfer of protein-coding regions to and from other Flexi® Vectors, which contain specialized functionalities. The reporter vector encodes firefly luciferase with rapid-response characteristics and a hygromycin-resistance marker for generating stable cell lines. In this article, we demonstrate the efficient transfer of protein-coding regions into the CheckMate™/Flexi® Vectors and the ease of screening for interacting partners. We also show how to use the mammalian two-hybrid system to generate stable cell lines and modulate protein interactions with small molecules in a stably transfected cell line.

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Mimic in vitro context
to examine the proteins within the context of mammalian cells where post-translational modifications and cellular compartmentalization may affect functionality.

INTRODUCTION

Prodigious sequencing data and genomic information have been generated for a variety of organisms. Studying the proteins, which are encoded by much of this genetic information, is at the top of the agenda for biologists. Advancements in molecular genetic tools and instrumentation enable researchers to bridge genomics and proteomics. Understanding structures, modifications and cellular and extracellular functions of proteins often requires knowledge about additional proteins and molecular entities with which the proteins of interest interact. Interactions between proteins play central roles in cellular regulation (e.g., in transcription, translation, signal transduction pathways and metabolism). Several strategies are used to identify and confirm protein interactions. The yeast two-hybrid system introduced by Fields and co-workers was developed to facilitate identification of pairs of interacting protein partners from cDNA clones using the power of yeast genetics for screening (1). Scale-up of this assay has provided information about interactions between multiple proteins within multicomponent complexes as well as the intricate web of interactions among hundreds of proteins in network displays (2,3).

The yeast two-hybrid system is now an established screening tool for initial identification of putative protein partners. Additional in vitro and in vivo methodologies are subsequently used to further confirm and examine interactions between the suspected protein partners. For mammalian proteins of interest, it is important to examine the proteins within the context of mammalian cells where post-translational modifications and cellular compartmentalization may affect functionality. Differences exist between yeast and mammalian cell patterns of post-translational modifications, such as glycosylation, phosphorylation and acylation (4). Currently, there are several confirmatory types of assays to study protein interactions within mammalian cells. These include immunohistochemical co-localization, as well as in vitro "pull-down" approaches that allow isolation and characterization of cellular proteins bound to an immobilized "bait", one of the interacting proteins of interest. An in vivo, mammalian cell-based assay such as the CheckMate™/Flexi® Vector Mammalian Two-Hybrid System[a–g] (Cat.# C9360) provides an additional powerful means to identify, confirm, validate and study protein:protein interactions.

MAMMALIAN TWO-HYBRID ASSAYS

The new CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System and the original CheckMate™ Mammalian Two-Hybrid System[a–d] (Cat.# E2440) are variations of the yeast two-hybrid system (1, 5–7). In these systems, one of the proteins of interest ("X") is fused to a DNA-binding domain of GAL4, and the other protein ("Y") is fused to a transcriptional activation domain of VP16. Interaction between proteins "X" and "Y" results in association of the GAL4 DNA binding domain with the VP16 transcriptional activation domain. When the GAL4 DNA binding domain binds to the GAL4 binding sites on a specially designed reporter vector, transcriptional activation of a firefly luciferase reporter gene occurs (Figure 1). Assay of firefly luciferase activity is easy, rapid and sensitive, and correlates with the interaction of proteins "X" and "Y".

Mammalian two-hybrid assays have been used to study protein interactions critical in many processes (Table 1). For example, interactions between MAPK11 and histone deacetylase-3 proteins, verified in the mammalian two-hybrid assay, provided a

link between the TNFα inflammatory signal cascade and histone modification of chromatin that modulates transcriptional activity (8). Transcriptional activation and repression functionalities of protein complexes implicated in developmental differentiation (9–11) and cancer (12) were also validated with this assay format. Homomultimerization and heteromultimerization propensities of cocksackieviral proteins and mutant proteins within mammalian cells were also studied using the mammalian two-hybrid assay (13).

ENHANCED FEATURES OF THE CHECKMATE™/ FLEXI® VECTOR MAMMALIAN TWO-HYBRID SYSTEM

Three vectors form the foundation for the CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System and need to be co-transfected into mammalian cells. They are a reporter vector (pGL4.31[*luc2P*/*GAL4*UAS/Hygro]), a vector designed for fusion of protein "X" with the GAL4 DNA-binding domain [pFN10A (ACT) Flexi® Vector] and a vector designed for fusion of protein "Y" with the VP16 activation domain [pFN11A (BIND) Flexi® Vector]. The new CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System differs from the original CheckMate™ System in the properties of these three vectors.

The pGL4.31[*luc2P*/*GAL4*UAS/Hygro] Vector, like the original reporter vector, contains five GAL4 binding sites (*GAL4*UAS) upstream of a minimal TATA box adenoviral promoter that drives expression of a luciferase gene reporter. This new reporter vector contains a synthetic firefly luciferase gene (*luc2P*) that has been engineered for increased mammalian expression as well as rapid-response characteristics due to incorporation of a protein degradation sequence, PEST. Another distinguishing feature of this reporter vector is the hygromycin resistance gene, which allows long-term selection of stably transfected cells.

The pFN10A (ACT) Flexi® and pFN11A (BIND) Flexi® Vectors share several functional similarities with the first-generation CheckMate™ System vectors. The pFN10A (ACT) Flexi® Vector is designed to express an N-terminal VP16 activation domain fusion protein with the protein of interest. This vector also contains the neomycin phosphotransferase gene driven by the SV40 early promoter, allowing long-term selection of stably transfected cells by adding the antibiotic G-418 (Geneticin®) to transfected cells. The pFN11A (BIND) Flexi® Vector is designed to express an N-terminal GAL4 DNA-binding domain fusion protein with the protein of interest. This vector is also designed to express *Renilla* luciferase, which may be used to normalize for transfection differences between samples within an experiment. The Dual-Luciferase® Reporter Assay System (Cat.# E1910) is a convenient and simple assay for both *Renilla* luciferase and firefly luciferase enzyme activities from the same lysate sample. As a check for fusion integrity, both Flexi® Vectors maintain a T7 RNA polymerase promoter upstream of the fusion protein

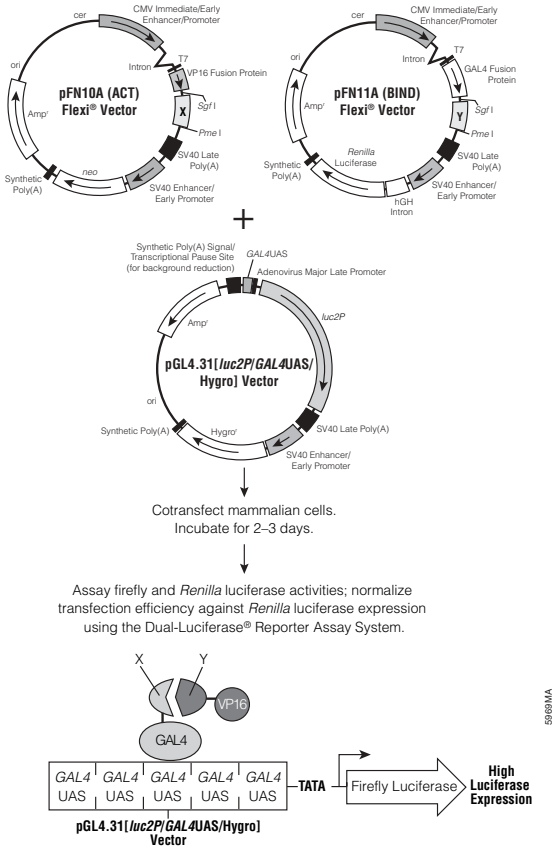

Figure 1. Schematic representation of protein:protein interactions using the CheckMate™/Flexi® Vector Mammalian Two-Hybrid System. The pFN11A (BIND)-X and pFN10A (ACT)-Y Flexi® Vectors are co-transfected into mammalian cells along with the pGL4.31[*luc2P*/*GAL4*UAS/Hygro] Vector. The pGL4.31 Vector contains five GAL4 binding sites upstream of a minimal TATA box that is upstream of the firefly luciferase gene. The cells are incubated for 2 to 3 days, lysed and then assayed for firefly and *Renilla* luciferase using the Dual-Luciferase® Reporter Assay System (Cat.# E1910). Interaction between the two proteins, "X" and "Y", in the GAL4-X and VP16-Y fusion proteins results in high levels of luciferase expression from the pGL4.31 Vector.

Unique features of the pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors provide compatibility with the Flexi® Vector System, which allows directional cloning and a rapid, efficient and high-fidelity transfer of protein-coding sequences between a variety of other Flexi® Vectors.

Table 1. Protein Interaction Processes Studied Using the Mammalian Two-Hybrid System.

Areas Studied	Citations
Signal transduction	Cho, Y.Y. *et al.* (2005) *Cancer Res.* **65**, 3596–603. Mahlknecht, U. *et al.* (2004) *J. Immunol.* **173**, 3979–90.
Transcriptional regulation	Suico, M. *et al.* (2004) *J. Biol. Chem.* **279**, 19091–8. Tsuzuki, S. *et al.* (2004) *Mol. Cell. Biol.* **24**, 6824–36. Dang, C.V. *et al.* (1991) *Mol. Cell. Biol.* **11**, 954–62.
Pathology and cancer	Deltour, S. *et al.* (2002) *Mol. Cell. Biol.* **22**, 4890–901. Deltour, S., Guerardel, C. and Leprince, D. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 14831–6.
Developmental biology	Zhou, R. *et al.* (2002) *Nucleic Acids Res.* **30**, 3245–52. Finkel, T. *et al.* (1993) *J. Biol. Chem.* **268**, 5–8.
Viral protein interactions and functions	Wessels, E. *et al.* (2005) *J. Virol.* **79**, 5163–73. de Jong, A.S. *et al.* (2002) *J. General Virol.* **83**, 783–9

Table 2
All desired constructs were successfully obtained after screening just three colonies for each construct.

constructs to allow the constructs to be transcribed and translated in vitro using the TNT® T7 Quick Coupled Transcription/Translation System (Cat.# L1170).

Unique features of the pFN10A (ACT) Flexi® and pFN11A (BIND) Flexi® Vectors provide compatibility with the Flexi® Vector System, which allows directional cloning and a rapid, efficient and high-fidelity transfer of protein-coding sequences between a variety of other Flexi® Vectors (14). These vectors incorporate two rare-cutting restriction enzymes sites, *Sgf* I and *Pme* I, flanking the protein-coding sequences. These cloning vectors also contain the lethal barnase gene for positive selection during the cloning step. Sequences for the protein coding sequences of interest may be transferred from another Flexi® Vector, preferably a vector coding for kanamycin resistance, because both of the CheckMate™/Flexi® Vectors code for ampicillin resistance in *E. coli*. Alternatively, protein-coding sequences may be captured by the vectors from amplification products generated with *Sgf* I and *Pme* I primers. Both vectors are designed such that the protein-coding regions are in-frame with the N-terminal fusion partner followed by a defined peptide linker sequence.

Controls included
Positive Control Vectors as well as Negative Control Vectors that can be directly transfected into cells are included in the CheckMate™/Flexi® Vector Mammalian Two-Hybrid System.

Positive Control Vectors as well as Negative Control Vectors that can be directly transfected into cells are included in the CheckMate™/Flexi® Vector Mammalian Two-Hybrid System and are also available separately. The Positive Control Vectors constitute pBIND-Id and pACT-MyoD and, when co-transfected with a CheckMate™ reporter vector, result in firefly luciferase expression. The mouse proteins involved in muscle cell differentiation, Id and MyoD, interact strongly and provide a positive luciferase signal for reference purposes (15–17). In the absence of a protein-coding insert between the *Sgf* I and *Pme* I sites, the CheckMate™/Flexi® Vectors, when directly transfected, are toxic to mammalian cells due to the presence of the barnase gene in these vectors. Therefore, the negative controls, pACT and pBIND Vectors are substituted for the pFN10A (ACT) Flexi® and pFN11A (BIND) Flexi® Vectors to assess background levels of interactions in the presence of the reporter vector.

SCREENING FOR PROTEIN INTERACTION PARTNERS

To demonstrate that a specific interaction partner can be identified among a random collection of proteins using the CheckMate™/Flexi® Vector Mammalian Two-Hybrid System, we conducted an experiment in which we fixed the "bait" protein (i.e., MyoD) and screened for the "prey" protein (i.e., Id) in a panel of 57 other random proteins. The panel contained receptor, transcription factor, kinase and protease representatives. The protein-coding sequences were obtained from several sources, including Origene (MD, USA) and Kazusa DNA Research Center (Chiba, Japan), which were already in a Flexi® Vector backbone, and the IMAGE consortium and MGC (via ATCC, VA, USA), which were amplified and cloned in a Flexi® Vector. Each of the 57 donor Flexi® Vector clones was digested

Table 2. Efficient Transfer of Protein-Coding Regions Between Flexi® Vectors.

Acceptor Vector	3 of 3 Correct Clones	Correct Clones Transferred
pFN10A (ACT) Flexi® Vector	50/57 (88%)	163/171 (95%)
pFN11A (BIND) Flexi® Vector	51/57 (90%)	163/171 (95%)

with *Sgf* I and *Pme* I enzymes together with the acceptor vector, either pFN10A (ACT) Flexi® Vector or pFN11A (BIND) Flexi® Vector, according to the protocols provided in the *Flexi® Vector Systems Technical Manual* #TM254. Following ligation, transformation and plating, three colonies were selected for each of the constructs and tested for expected insert size by *Sgf* I and *Pme* I restriction digestion. All desired constructs were successfully obtained after screening just three colonies for each construct (see Table 2). Overall, approximately 95% of the total number of selected colonies contained plasmids with the correct clone, demonstrating the efficient transfer using the Flexi® Vector System. Protein-coding sequences for MyoD or Id were amplified and cloned into the *Sgf* I and *Pme* I sites of the pFN10A (ACT) Flexi® or pFN11A (BIND) Flexi® Vectors, respectively. One representative clone for each construct was chosen, and plasmid DNA was purified for transfection experiments.

In one experiment, NIH/3T3 cells grown in 96-well culture dishes were co-transfected with three plasmids: pFN10A (ACT)-MyoD, one of the 57 pFN11A (BIND) Flexi® Vector clones or the Id positive control, and the pGL4.31 Vector. Luciferase activities were assayed, and results are shown in Figure 2. None of the 57 random proteins interacted to a significant extent with the MyoD

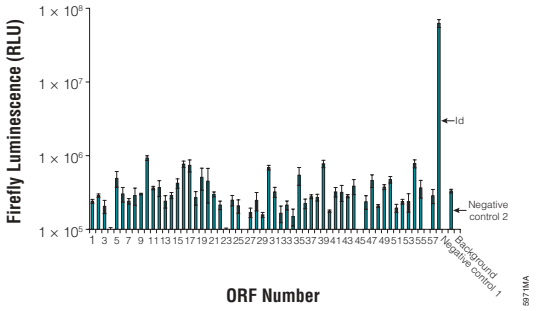

Figure 2. Screening for specific protein interaction partners using MyoD as bait protien. NIH/3T3 cells were plated at a density of 10,000 cells per well in a 96-well plate and allowed to adhere overnight. Transfections were performed by complexing 200ng DNA (equal amounts of pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector, pFN10A (ACT)-MyoD and pFN11A (BIND) Flexi® Vectors) and 0.6µl TransFast™ Reagent in 40µl serum-free media per well. After 1 hour, the wells were overlaid with 160µl complete medium with serum and allowed to incubate for 24–48 hours. Firefly and *Renilla* luciferase activities were measured with the Dual-Glo™ Luciferase Reagent. Normalized firefly luciferase data were generated by dividing the firefly RLU (relative light unit) signal by the *Renilla* luciferase RLU signal for each well. Samples 1–57 represent the panel of protein-coding sequences cloned into pFN11A (BIND) Flexi® Vector. The next sample is pFN11A-Id. Control samples are pACT and pBIND Vectors (background).

protein since the luminescent signals were comparable to those of negative controls. In contrast, the pFN10A (ACT)-MyoD and pFN11A (BIND)-Id pair showed a much higher luminescent signal, 100-fold over background, indicating interactions between these proteins.

In another experiment, pFN11A (BIND)-Id was tested with each of the 57 pFN10A (ACT) clones or the MyoD clone. Again, only MyoD was identified as the interacting partner for the bait protein Id (data not shown).

MODULATING PROTEIN INTERACTIONS WITH SMALL MOLECULES

Drug discovery efforts target protein interactions because of the importance of protein:protein interactions to cellular function (18). To demonstrate that the CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System can be used to monitor modulation of protein:protein interaction by small molecules, we employed a model system of rapamycin-mediated FKBP-FRB interaction. Rapamycin, a cell-permeable low-molecular-weight organic, immunosuppressant compound, binds to FKBP (FK506 Binding Protein 12) and FRB (FKBP-rapamycin binding domain of FKBP-rapamycin-associated protein), forming a ternary complex (19). When FKBP and FRB are co-expressed in mammalian cells, minimal interaction occurs between these proteins. However, in the presence of rapamycin, FKBP and FRB are brought into close proximity via the binding to rapamycin. Interactions between these two proteins, and fusion constructs of these proteins, can be modulated by the concentration of rapamycin (20).

We used the FKBP-rapamycin-FRB model system to illustrate several features of the enhanced CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System (Figure 3). First, the pGL4.31 Vector in the CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System can be used to generate stable cells that can be subsequently transfected with pFN10A (ACT) Flexi® and the pFN11A (BIND) Flexi® Vector clones to test directly for protein interactions. Second, the CheckMate™/Flexi® Vector System can be used to monitor small-molecule modulation of protein interactions. In this example, there was no significant interaction between the FKBP and FRB proteins in the absence of rapamycin, as indicated by the low level of luciferase activity in cell lysates, comparable to background levels from the transfected negative control samples (pBIND Vector with either pFN10A-FKBP, pFN10A-FRB, or pACT Vector). However, the presence of rapamycin, which provides a bridge for FKBP and FRB protein interaction, results in a significant increase of luminescent signal. This signal correlates with the expected interaction between FKBP and FRB fusion proteins in this assay. In addition, rapamycin mediates the binding interaction between FKBP and FRB in a dose-dependent manner (data not shown).

Switching the (ACT) and (BIND) fusion partners had no significant effect, and similar fold increases in the presence of rapamycin were measured for the FKBP and FRB pair. In addition, the high levels of luciferase expression from the MyoD:Id transfection positive control samples were not significantly altered by addition of rapamycin, indicating the rapamycin effect was specific for the FKBP:FRB protein interaction. This experiment demonstrates that the CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System can be used to monitor and assay specific protein interactions modulated by small molecules.

Protein interactions
The CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System can be used to generate stable cells to test for protein interactions and can also be used to monitor small-molecule modulation of protein interactions.

CONCLUSION

The CheckMate™/ Flexi® Vector Mammalian Two-Hybrid System is an excellent in vivo approach to monitor and characterize protein:protein interactions within mammalian cells. This second-generation system includes all the advantages of the original CheckMate™ Mammalian Two-Hybrid System while incorporating the additional features of the Flexi® Vector System. These features include cloning your protein-coding regions of interest with ease, and transfering your constructs to other Flexi® Vectors with different expression and purification options easily and conveniently. The ease and efficiency of transfer of protein-coding regions between Flexi® Vectors allows large sets of clones to be transferred, scored with minimal effort and used for larger scale experiments with mammalian cells designed to screen a panel of clones against one or more "bait" proteins of interest for putative interacting protein partners. The reporter vector maintains the

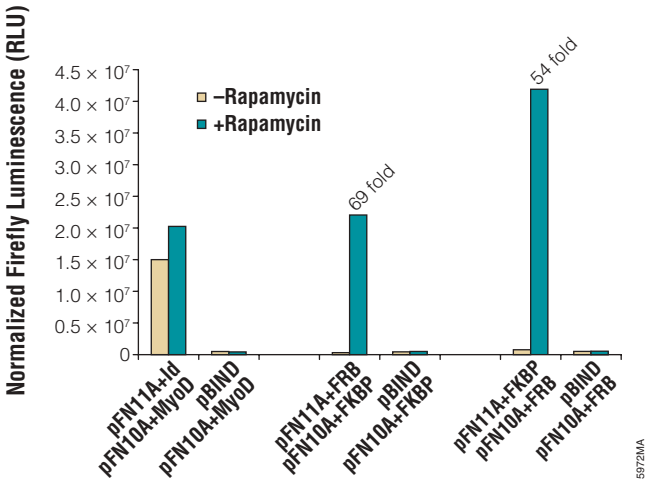

Figure 3. Specific small-molecule modulation of protein interactions can be assayed in a cell line generated with the reporter vector. A stable HeLa pGL4.31 cell line was generated previously by transfecting HeLa cells with pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector and selecting with G-418 (500µg/ml), followed by limited dilution cloning. The stable HeLa-pGL4.31 cells were plated at a density of 10,000 cells per well in a 96-well plate and allowed to adhere overnight. Transfections were performed by complexing 200ng DNA (equal amounts of pFN10A (ACT) Flexi® and pFN11A (BIND) Flexi® Vectors) and 0.6µl TransFast™ Reagent (Cat.# E2431) in 40µl serum-free medium per well. After 1 hour, the wells were overlaid with 160µl complete media with serum +/– 200nM rapamycin (Biomol Cat.# A-275) and allowed to incubate for 24–48 hours. Firefly and *Renilla* luciferase activities were measured using the Dual-Glo™ Luciferase Reagent. Normalized firefly data were generated by dividing firefly luciferase RLU (relative light units) signal by the *Renilla* luciferase RLU signal for each well. Positive control samples were MyoD and Id cloned into pFN10A (ACT) and pFN11A (BIND) Flexi® Vectors, respectively. Negative control samples were pACT and pBIND Vectors. Test samples were FKBP and FRB cloned either into pFN10A (ACT) Flexi® or pFN11A (BIND) Flexi® Vectors. Both orientations were tested to determine differences when binding partners are switched.

Ease and efficiency of transfer of protein-coding regions between Flexi® Vectors allows large sets of clones to be transferred, scored with minimal effort and used for larger scale experiments.

advantage of sensitive luciferase detection but now encodes an engineered form of the enzyme for increased expression in mammalian cells and rapid-response capabilities. Finally, a new feature confers hygromycin resistance to transfected cells, which allows stable cell lines to be isolated and used for future transfection experiments with the ACT and BIND fusion vectors of interest.

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PROTOCOL

- *CheckMate™/Flexi® Vector Mammalian Two-Hybrid System Technical Manual* #TM283, Promega Corporation. (www.promega.com/tbs/tm283/tm283.html)

ORDERING INFORMATION

Product	Size	Cat.#
CheckMate™/Flexi® Vector Mammalian Two-Hybrid System	each	C9360
pGL4.31[*luc2P/GAL4*UAS/Hygro] Vector	20µg	C9351
CheckMate™ Positive Control Vectors	1 set	C9370
CheckMate™ Negative Control Vectors	1 set	C9380
pFN10A(ACT) Flexi® Vector	20µg	C9331
pFN11A(BIND) Flexi® Vector	20µg	C9341

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[d]U.S. Pat. No. 5,670,356.

[e] Patents for the foundational PCR process, European Pat. Nos. 201,184 and 200,362, expired on March 28, 2006. In the U.S., the patents covering the foundational PCR process expired on March 29, 2005.

[f]Patent Pending.

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