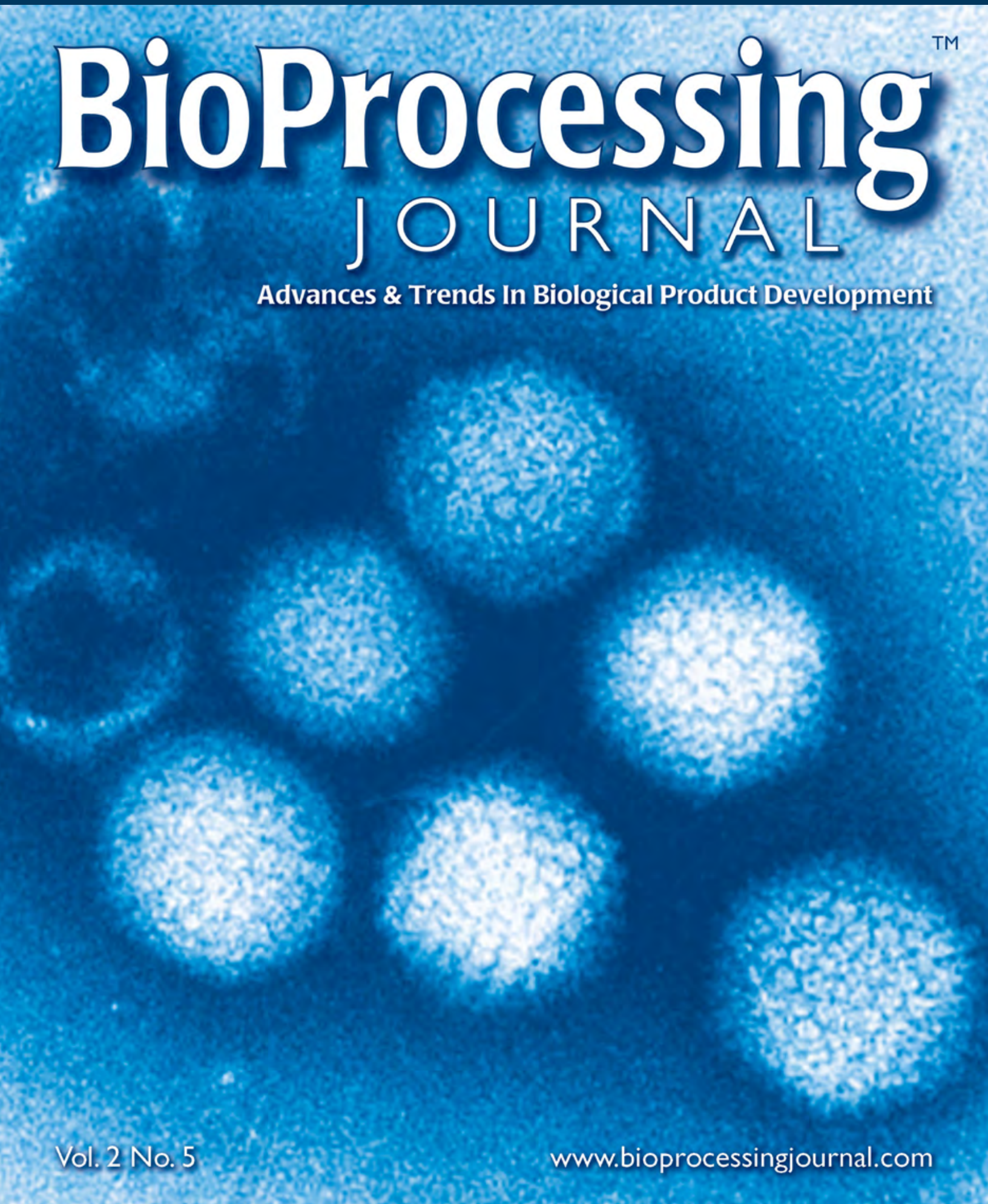
A publication of
The Williamsburg BioProcessing Foundation

September/October 2003

BioProcessing™ JOURNAL

Advances & Trends In Biological Product Development



Vol. 2 No. 5

www.bioprocessingjournal.com

The Study of GPCR Activity Using Insect Cell Membranes and AlphaKey Peptide Probes

BY MARGARET DEY, KEVIN RAMER, CARMEN ROQUES, RAINER BLAESIUS, WHITNEY NICHOLSON, ZOEY FREDERICKS, and HANNE GRON

G protein-coupled receptors (GPCRs) comprise a "superfamily" of cell surface receptors that play a prominent role in cell signalling and are classified into more than 100 subfamilies according to sequence, ligand structure, and receptor function.[1] They are cell surface receptor proteins with seven transmembrane domains which transduce extracellular signals to the interior of cells through heterotrimeric G proteins. GPCRs' exposure at the exterior cell surface and strong role in cell regulation has provided a rich target family for small compound therapeutics. Of the estimated 35,000 genes in the human genome, approximately 750 encode for GPCRs; half likely encoding sensory receptors, the remaining half representing potential drug targets. Only about 30 of these potential targets are currently modulated by existing pharmaceuticals with approximately 400 remaining potential pharmaceutical targets for validation.[2] The classic functional unit consists of the ligand-binding transmembrane protein (the receptor), and the associated intracellular heterotrimeric G protein (Gα, Gβ, and Gγ subunits) that is responsible for the signal transmission to effector molecules inside the cell. Upon binding of a select agonistic ligand (e.g. hormone or neurotransmitter) to the GPCR, a signal is transduced, causing the subunits within the G protein to undergo a conformational change. As a consequence the subunits are able to transmit signals to effector enzymes and cell membrane ion channels. As the β and γ subunits are considered conserved, the Gα subunit falls into one of four classes based on similarity of amino acid sequence and dependence on interaction with receptors, and effector molecules.[3] Therefore evaluation of a GPCR becomes dependent on specific activation of the associated Gα subunit. Because the membrane environments as well as correct protein folding and post-translational modifications are crucial for receptor function, the choice of an appropriate protein expression system is critical. Functional assays of this receptor class often rely on exogenous expression of one or more of the four essential components (receptor and three G-protein subunits) in a variety of recombinant mammalian cell systems. The use of these systems can be challenging as slower cell growth and the presence of naturally occurring GPCRs may lower measurable recombinant expression levels or offer inconsistent results. Traditional assays for receptor function are indirect, measuring the effect of ligand binding on levels of intracellular secondary messengers such as cyclic AMP or GMP, inositol phosphates, β-arrestin, or calcium. Radiolabeled ligand-binding assays are cell-free but require a known ligand and are not useful for the study of orphan receptors. The ligand-binding assay is also not a function-based assay and yields no information regarding the functionally of the receptor or the associated heterotrimeric G protein. Reporter assays that rely on the measurement of nuclear events triggered by GPCR activation by ligand binding are subject to interference from unrelated pathways. The AlphaKey™ assay we developed detects GPCR activation through the binding of a GTP conformation-specific peptide probe to the receptor-coupled Gα protein subunit.

The use of the baculovirus expression vector system (BEVS) in insect cells offers an alternative to traditional mammalian cell expression with numerous advantages. Insect cells combine high, consistent expression levels, expression of functional protein, and rapid cell growth with low background from endogenous GPCRs. Additionally, BEVS offers considerable flexibility permitting multiple protein products to be expressed. We have established an optimized co-infection protocol in Sf9 and Tni insect cells resulting in co-expression of the three intracellular G protein subunits

Margaret Dey *(peggy.dey@karobio.com) is a senior research investigator, Kevin Ramer, Ph.D. is a section head, Carmen Roques is a research investigator, and Hanne Gron, Ph.D. is a project manager, Karo Bio USA, Inc., Durham, NC; Rainer Blaesius, Ph.D. is a senior scientist and Whitney Nicholson is a scientist, TriPath Oncology, Durham, NC; and Zoey Fredericks, Ph.D. is a research scientist, Amgen, Seattle, WA.*

and the receptor. The system permits combining different G protein subunits and receptors and allows large-scale production of cell membranes which can be kept frozen for long-term storage.

Methods and Materials

The recombinant baculovirus used for GPCR expression were screened and selected by a dual program based on preliminary screening of expression using a polyclonal virus followed by optimization of expression applied to confirmed monoclonal recombinant stock. Recombinant baculovirus for each Gα subunit was generated using BD Pharmingen's BaculoGold™ transfection method with Sf9 insect cells in TNM-FH media (HyClone) containing 10% fetal bovine serum (FBS) referred to as "complete media." Common GPCR Gβ and Gγ baculovirus were kind gifts.[4,5] Three days post transfection recombinant virus was harvested and plaque purification performed for generation and selection of individual recombinants. In parallel, the transfection stock underwent two rounds of virus amplification, the initial in a 60-mm dish, the secondary in shaker flask. Each was harvested at 72 hours post infection. Based on an estimated viral concentration of 10^8 plaque-forming units/ml (pfu), recombinant protein was expressed in Sf9 or Tni insect cells using a multiplicity of infection (MOI), the number of viral particles present per insect cell, of three. Western analysis by antibody detection was used to confirm potential recombinant protein expression. In parallel, several individual recombinant plaques (referred to as monoclonals) generated from a single viral particle were selected and underwent the initial 72-hour viral amplification, at which time an aliquot of the virus was processed for confirmation of the recombinant target DNA in the baculovirus by polymerase chain reaction (PCR). Amplified virus was precipitated from a polyethylene glycol (PEG) high salt buffer. The viral pellet was processed by Qiagen's QiaAmp™ protocol with the final DNA sample eluted in 50-ml sterile distilled water. Optimized PCR utilized baculovirus polyhedron primers and incorporated 5 ml of the purified DNA template in a 100-ml reaction volume. PCR products were analyzed on a 1% agarose TAE gel stained with ethidium bromide. Confirmed individual recombinant virus were selected for further amplification to high titer stock (HTS). Figure 1 indicates PCR amplification of template DNA purified from five monoclonal viral stocks. Four of the lanes confirm recombinant target baculovirus, and one of the lanes (sample #2) indicates a target product which is incomplete and potentially a defective infectious particle (DIP). Viral titers were determined by traditional plaque assay on the monoclonal HTS. The resulting time from transfer vector transfection and recombinant generation to protein confirmation of expression by Western analysis is approximately 2.5–3 weeks for polyclonal and 4–6 weeks for monoclonal baculovirus.

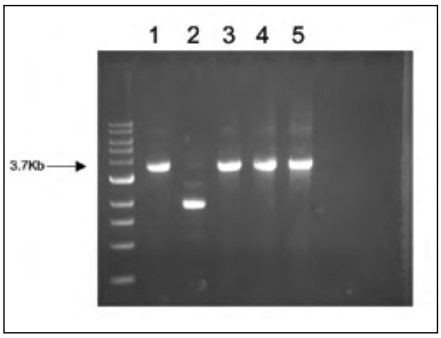


Figure 1. PCR Screen Using Monoclonal Baculovirus Stock
Using PCR amplification four monoclonal recombinant isolates with full target insert are confirmed. Also shown is one defective recombinant clone with partial target insert.

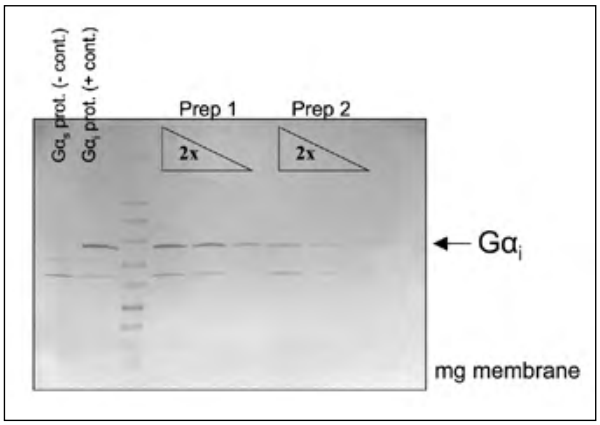


Figure 2: Confirmation of G-protein expression in insect cell membranes using Western Blot with anti-$G\alpha_i$ antibody. $G\alpha_s$ purified protein was used as a negative control for detection.

Optimization of expression for GPCR and G-protein subunits was performed by examining individual proteins by Western analysis as well as the effects of the co-expression environment. Factors examined in expression included the transfection method used to generate the recombinant baculovirus, MOI (.005 to 10), cell line (Sf9 vs. Tni), the presence or absence of serum during expression or generation of the baculovirus stock, the cell density at the time of infection, time to harvest, and the effects of co-expression itself. With consideration of the large number of parameters for study, expression conditions were evaluated in 100-mm dishes by immunoblotting with a dot blot apparatus for detection of the specific G protein of interest. Validation of enhanced expression was then performed in shaker flasks and bioreactor. Initial cell membrane expression studies were performed in Sf9 insect cells in media containing serum with a significant increase in total protein expression when expressed in later studies employing Tni insect cells in a serum–free environment. The final co-expression conditions for GPCR, Gα, Gβ, and Gγ cell membrane proteins included individual recombinant baculovirus at an MOI of 3 each, a cell density at the time of infection of 1.5 to 2.0 x 106 cells/ml, and a post-infection harvest time of ~48 hours.

GPCR assays were conducted on membranes isolated from baculovirus-infected cells lysed in a Parr bomb apparatus. We assayed factors such as whole cell vs. cell membranes, total membrane protein, membrane and probe preparation, storage condition of prepared membranes, and consistency between membrane preparations. Recombinant cell membranes were confirmed for G protein expression by Western analysis similar to the parameter screen, employing a specific antibody to the G

For proteins to come

a p a r t

so many things must come

together

Remember to visit us at ASME, at booth number 38

To avoid delays in your race to market, we offer you our Secure Solutions guarantee*. It's as simple as this. We **guarantee** that our chromatography columns will be delivered to you within three weeks of your order placement. We **guarantee** that our selected process systems will be delivered within six weeks. And we **guarantee** the performance of the hardware when used with our chromatography media. Never before has predictability been this exciting. Secure Solutions. You have our word on it. For a brochure, or to speak with your Amersham Biosciences sales representative, call 1-800-526-3593 X5764. Or simply go to:

www.amershambiosciences.com

*Please request a brochure for a detailed explanation of the guarantees offered in this program

Amersham Biosciences

Amersham Biosciences UK Limited,
Amersham Place, Little Chalfont,
Buckinghamshire, England HP7 9NA.

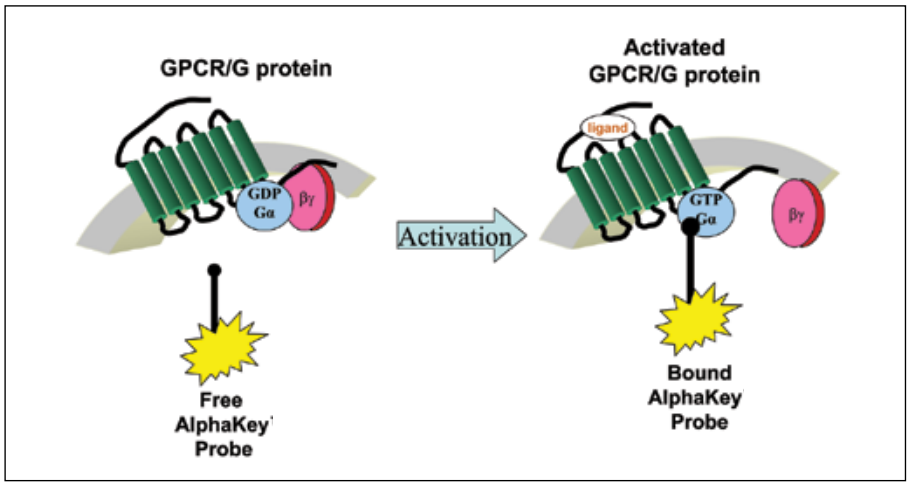


Figure 3: GTP-dependent AlphaKey probes as reporters of GPCR activity bind specifically to the Gα subunit protein only when the receptor has been activated.

protein of interest (Fig. 2). Varying amounts of cell membrane were examined for expression and consistency between membrane preparations. Total membrane protein concentration in each well of a 96-well format detection assay was standardized using the BCA protein assay from Pierce. Using radioactive ligand binding to well-characterized GPCRs and [^{35}S]–GTPγS binding to the associated Gα protein allowed detection of specific binding and activation and characterization of both agonist and antagonist ligands for use in the AlphaKey assay. The AlphaKey peptide probe, which binds to the Gα subunit in the activated or the GTP-bound conformation, is labeled with an enzyme tag. Monitoring the enzyme activity (β-galactosidase or alkaline phosphatase) associated with the AlphaKey probe, permitted detection of GTPγS exchange onto the Gα subunit. Thus, GPCR activation was assayed by indirect measurement of GTPγS exchange on Gα, increased signal over background indicating binding of the probe to the G protein, or, more significantly, a ligand-binding event activating the GPCR (Fig. 3).

Results and Discussion

In the non-activated (unbound) state of the receptor protein, GDP is bound to the Gα subunit. Upon agonist binding to the receptor protein, or "activation," a conformational change is induced for the heterotrimeric G protein, and GDP is exchanged for GTP. The basis of the AlphaKey technology is selection of peptide probes which specifically bind to the Gα subunit in the receptor activated or GTP conformation. These probes have allowed us to build a GPCR assay using insect cell membranes that co-express the receptor and heterotrimeric G protein.

Using AlphaKey technology in combination with BEVS, we established a novel, highly flexible GPCR assay platform compatible with high-throughput screening in a 96 well format. As shown in Figure 4, binding of the AlphaKey probe is specific to the GTP conformation of the subunit protein. Addition of the labeled probe to purified Gα protein in the presence of increasing GTP or GDP shows increased signal and binding only in the GTP conformation. Binding of an antagonist to the GPCR will not activate the binding site and will not initiate the GDP to GTP heterotrimeric G-protein conformation change and the conformation-specific AlphaKey will not bind. With an increasing titration of known agonist, isoproterenol, to the β2-adrenergic receptor (β2AR) in the presence of insect cell membranes, increasing activation of the receptor is indicated by detection of the $G\alpha_s$ GTP conformation-specific probe. Under similar conditions with an initial agonist-activated receptor, addition of antagonist ICI118,551 results in reduced binding of the GTP conformation-specific probe. AlphaKey probe binding is specific only to an activated receptor event (Fig. 5). Further studies were performed to differentiate specific receptor coupling to the Gα protein. If GPCR activation is specifically coupled to the appropriate Gα protein, the activation would be agonist-specific. Recombinant cell membranes were exposed to two different ligands: one an agonist which, when bound to the GPCR, causes activation and nucleotide exchange on the coupled Gα protein, the other a ligand which should not induce GPCR activation. Recombinant cell membranes were prepared which co-expressed Gβ and Gγ subunit proteins as well as $G\alpha_{i2}$ and the coupled m2 acetylcholine receptor (m2AChR). In the presence of carbachol (an agonist for the m2AChR), or isoproterenol (an agonist for the β2AR), specific activation and conformational change was specifically detected only with the carbachol. This proved functional receptor specific coupling to the Gα protein (Fig. 6, left). In a second confirmation experiment, insect cell membranes which co-expressed the four signaling proteins incorporated $G\alpha_s$ and the specific coupled β2AR as the GPCR. Again the membranes were exposed to the same ligands, isoproterenol and cabachol (Fig. 6, right). Activation and conformational change was detected only with the agonist specific to the GPCR indicating receptor-

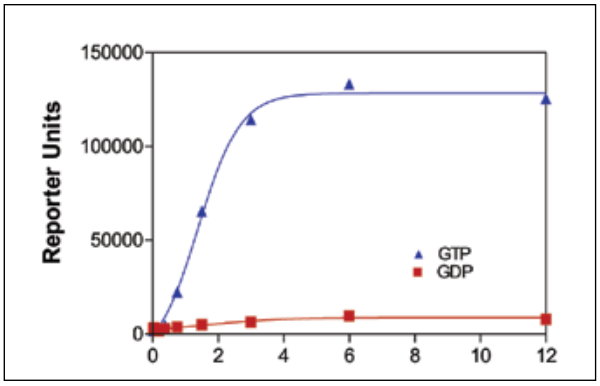


Figure 4: Binding of AlphaKey probe to purified G protein in the presence of GTP or GDP shows strong nucleotide selectivity.

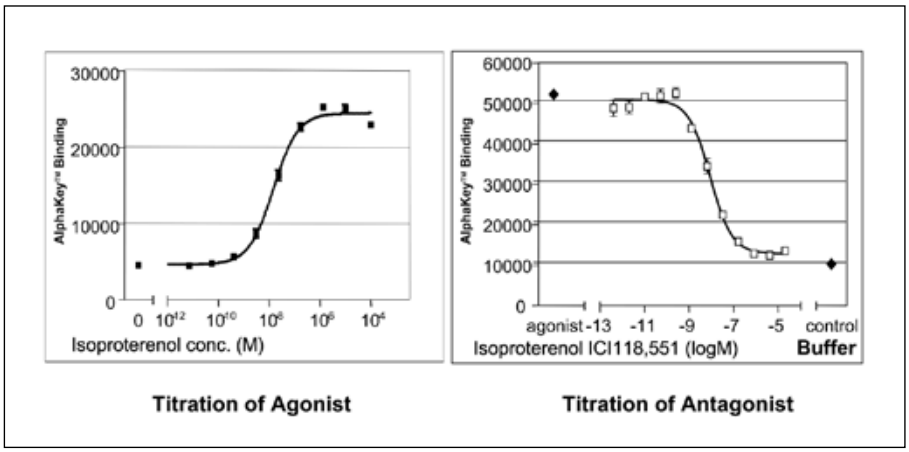


Figure 5: AlphaKey assay of purified β2-Adrenergic Receptor ($G\alpha_S$ coupled) protein with agonist titration, indicates increasing activation of the receptor by detection of the $G\alpha_S$ GTP conformation specific probe. Under similar conditions with an initial agonist saturated receptor addition of antagonist results in reduced binding of the GTP conformation specific probe.

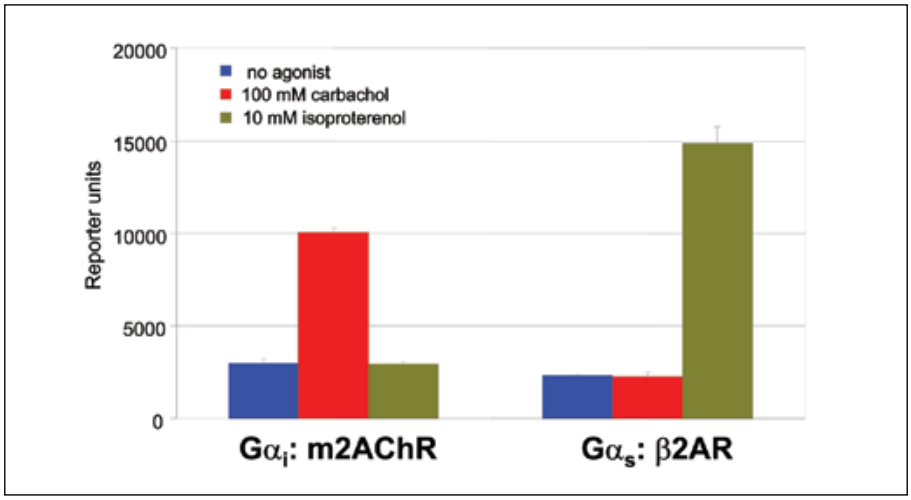


Figure 6: AlphaKey assay shows receptor specific coupling.

specific coupling. This method for generating functional custom cell membranes was used for screening a library of 640 known pharmacologically active compounds against the m2AChR. The library contained 12 known agonists to m2AchR; the AlphaKey assay detected 16 potential agonists. Upon confirmation of the assay results all 12 known agonists were detected. The remaining four confirmed "hits" comprised three of indeterminate chemical structure and one (TMB-8) allosteric activator. The ability to identify an allosteric activator is a significant advantage of a functional GPCR assay. In this way the AlphaKey probes provide for a cell-free, non-radioactive, functional assay of GPCRs.

Baculovirus expression in insect cells and generation of the membranes with up to four stoichiometric proteins allow custom expression and flexibility with little to no background from endogenous cell transmembrane proteins. Incorporating PCR confirmation of target recombinant baculovirus allows quality high titer stock generation and, if needed, sequence confirmation. Screening for monoclonal baculovirus in this manner reduces the likelihood of selection of a DIP plaque with ultimate reduced or unsuccessful recombinant protein expression. The dual method of recombinant protein expression uses polyclonal baculovirus for rapid expression confirmation followed by optimization of expression using a high-quality monoclonal baculovirus. The employment of both polyclonal and monoclonal recombinant baculovirus allows rapid screening in expression systems in parallel with optimization of robust quality high titer stocks and timely troubleshooting. This method is most beneficial because it allows specific target protein expression confirmation within days instead of weeks. Insect cell expression for GPCRs is multi-format compatible, adaptable to expression in 100-mm plates, shaker flasks, or bioreactors on a scale from 10 mls to 100 liters and can be employed with both Sf9 and Tni insect cells. In addition, BEVS allows any GPCR to be co-expressed with the Gα, β, and γ subunit proteins with a great deal of adaptability in specific expression. The AlphaKey assay provides a novel and general screening platform for any GPCR in a flexible, high-throughput format. The assay demonstrates appropriate ligand receptor specificity and can be successfully applied to screen thousands of ligands for GPCR agonists for proven therapeutic targets and could potentially be applied for evaluation of additional targets.

ACKNOWLEDGEMENTS

We would like to thank Kellie Duke, Karin Calles, Erica Zeim, Salman Ashraf, David Duffin, Erin Anderson, Brett Antonio, Nancy Hyde-DeRuyscher, and Robin Hyde-DeRuyscher for their contributions to this manuscript.

REFERENCES

1. Ji TH, Grossmann M, Ji I. G protein-coupled Receptors minireview. *J Biological Chemistry* 1998:273; 17299–17302.
2. Wise A, Gearing K, Rees S. Target Validation of G-protein coupled receptors. *Drug Discovery Today* 2002:7; 235–246.
3. Vaughan M. Signaling by heterotrimeric G proteins — minireview series. *J Biological Chemistry* 1998:273; 667–668.
4. Kozasa T, Gilman AG. Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of alpha 12 and inhibition of adenylyl cyclase by alpha z. *J Biochemistry* 1995:270;1734.
5. Kobilka BK. Amino and carboxyl terminal modifications to facilitate the production and purification of a G protein-coupled receptor. *Analytical Biochemistry* 1995:231;269.

YOUR PARTNER

FROM START.....

DISCOVERY

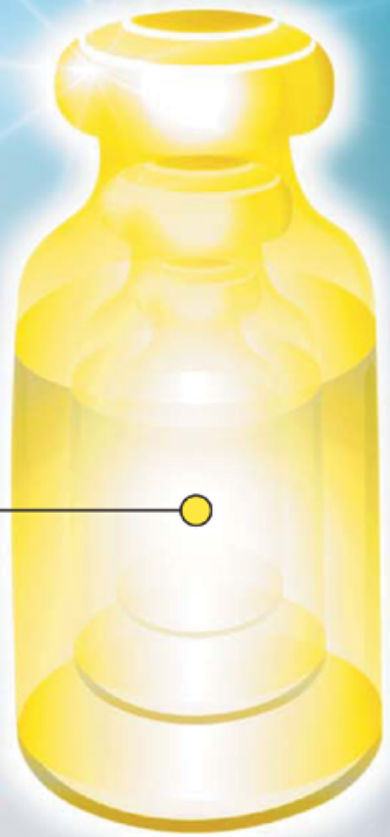
Target Validation
Lead Compound Selection
Molecular Toxicology Profiling

PRE-CLINICAL

Adventitious Virus Testing
Biodistribution Studies
Integration Studies
Plasmid DNA Production

CLINICAL

Clinical Sample Analysis
Host Cell DNA Contamination Assay
Adventitious Virus Testing
GMP Plasmid DNA Production
Aseptic Vial and Syringe Filling



.....TO FINISH.

ALTHEA TECHNOLOGIES accelerates gene-based drug development by providing a comprehensive portfolio of outsourcing solutions. We combine an unsurpassed commitment to quality with a high level of scientific excellence to consistently achieve project success.

ALTHEA technologies, inc.

FOR MORE INFORMATION, PLEASE VISIT WWW.ALTHEATECH.COM OR CALL 1.888.4ALTHEA