Bioluminescence Resonance Energy Transfer (BRET) as a means of monitoring dynamic receptor-protein interactions in living cells measured on LB 940 Mithras

K. M. Kroeger[1], K. A. Eidne[1], Bernd Hutter[2]

Introduction

Many aspects of cellular function involve protein-protein interactions, and bioluminescence resonance energy transfer (BRET) represents a newly emerging, powerful tool with which to investigate and monitor these interactions in real-time, in live cells. BRET is a cell-based, non-destructive technology based on the distance-dependent energy transfer between fusion proteins containing Renilla luciferase (Rluc) and variants of green fluorescent protein (either GFP or EYFP). Due to its strict proximity-dependence, BRET represents a powerful tool perfectly suited for applications in basic research and industry, in areas ranging from proteomics to drug discovery.

BRET Principle

BRET is a phenomenon observed naturally in several marine animals including the jellyfish, *Aequora Victoria*, and the sea pansy, *Renilla reniformis* and results from the transfer of energy between a bioluminescent donor and a fluorescent acceptor molecule. BRET assay technology exploits this principal by involving the non-radiative transfer of energy between the energy donor, Rluc to the energy acceptor, EYFP, when they are in close proximity (10-100Å) (1). This reaction occurs in the presence of the cell-permeable substrate coelenterazine. The critical distance dependence between donor and acceptor molecules makes BRET an ideal technique for monitoring protein-protein interactions in living cells.

[1] WA Institute for Medical Research, University of Western Australia, Perth, Australia, keidne@cylllene.uwa.edu.au

[2] BERTHOLD TECHNOLOGIES, Bad Wildbad, Germany, Bernd.Hutter@Berthold.com

Unlike, its derivative fluorescence resonance energy transfer (FRET), BRET avoids the need for excitation, thus circumventing the problems associated with autofluorescence, photobleaching and cell damage. Furthermore, the lower background fluorescence associated with BRET makes it a sensitive technique enabling the detection of low level or weak protein-protein interactions (2).

BRET can be usefully applied for the study of protein-protein interactions involving receptors and their related pathways. Before applying this system to the study of the proteins of interest, a requirement is for each protein and its partner protein to be genetically fused to either Rluc or to EYFP. Fusion proteins are co-expressed in cells and following the reaction of Rluc with its substrate, coelenterazine, if the two fusion molecules are in close enough proximity, then light is transferred from Rluc (peak emission 480nm) to EYFP, resulting in its excitation and emission of light at its characteristic wavelength of 530nm, with the degree of BRET quantified as a ratio of light emitted at 530nm over 480nm (Figure 1).

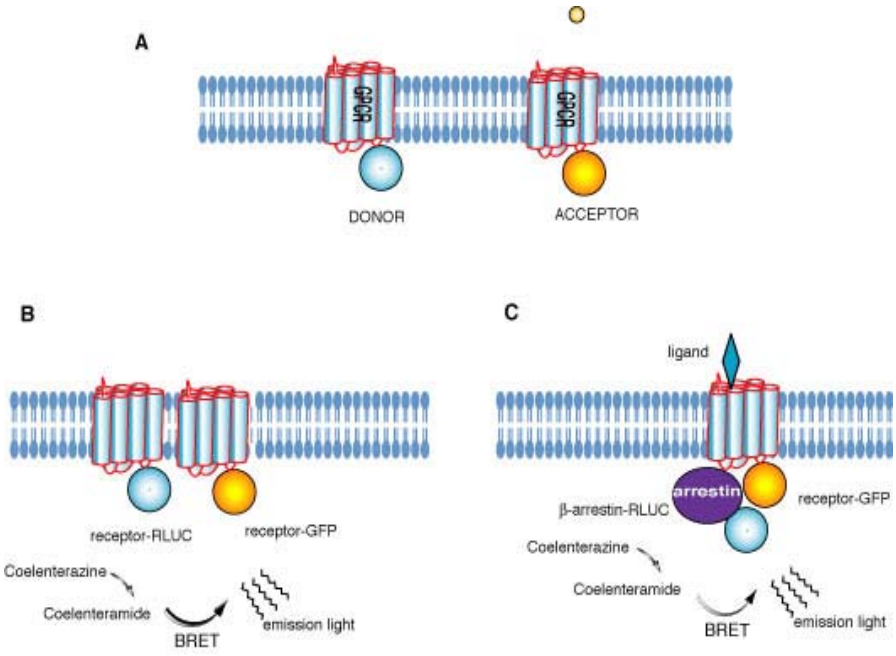

Figure 1: Schematic diagram illustrating the application of BRET to monitor G-protein coupled receptor (GPCR) interactions. BRET can be used to monitor receptor interactions in living cells in real time. A. GPCRs are genetically fused at their C-terminus to either donor, or acceptor molecule and co-expressed in live cells. B. BRET is used to demonstrate GPCR oligomerization and ligand-dependent interaction between GPCRs and β-arrestin. One molecule is labelled with Renilla luciferase (Rluc) and if it interacts with a second molecule (labelled with either GFP or EYFP) energy resulting from the degradation of the Rluc substrate, coelenterazine, will be transferred to the acceptor GFP, resulting in the emission of light at the wavelength characteristic of either GFP or EYFP.

Mithras LB 940

The Mithras is a multifunctional plate reader with multiple dedicated optical systems offering the sensitivity needed to measure BRET signals.

In the BRET mode, the LB 940 sequentially filters and measures light emission over the Rluc and EYFP wavelength ranges, and calculates the ratio between them.

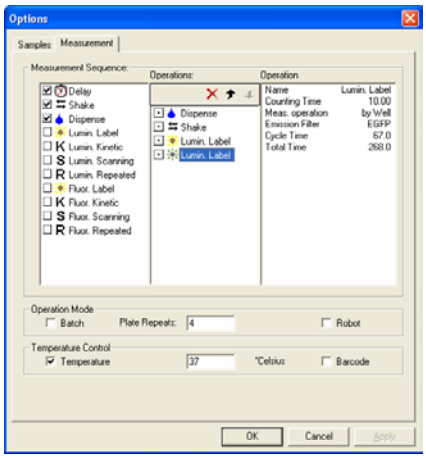

Figure 2: BRET mode requires two emission filters to be selected

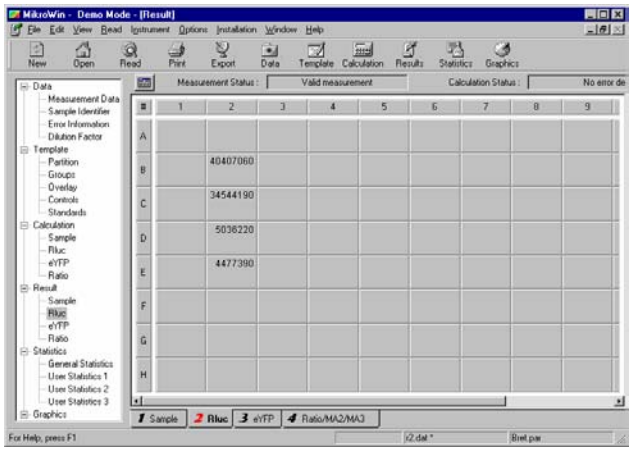

Figure 3A: Result window: the matrix showing the results counted through the first filter, for the Rluc emission peak. The matrix labelled '3 EYFP' similarly shows the results counted through the second filter, for the EYFP emission peak.

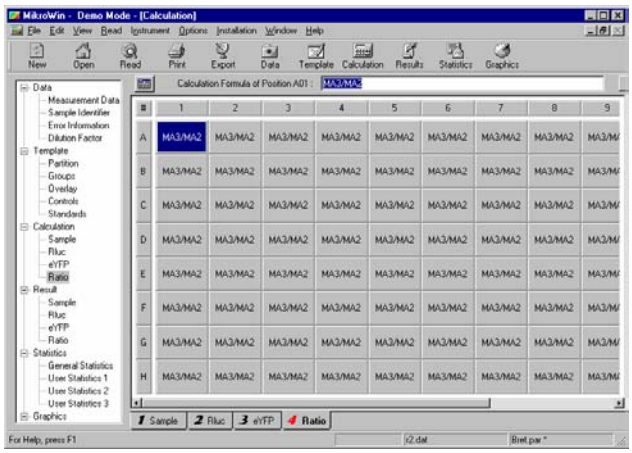

Figure 3B: Calculation window: The matrix showing the calculation to be performed in the fourth matrix; MA3/MA2 means that for each well, the result in matrix 3 is divided by the result in matrix 2, thus giving the ratio between EYFP and Rluc emission.

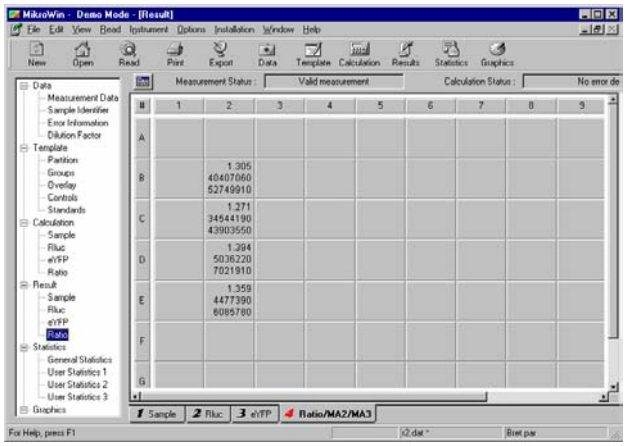

Figure 3C: Result window: the matrix showing the BRET ratio which has been calculated, and the counts from matrix 2 and 3 (MA2 and MA3).

Initially BRET was performed in E.coli to study the interaction of light-sensitive circadian clock proteins (1). BRET has also been used with success to study protein-protein interactions involving receptors belonging to different families. These receptors include (3-8):

β-adrenergic receptors
TRH receptors 1 & 2
GnRH receptors
Opioid receptors
Insulin receptors
Cholecystokinin A receptors

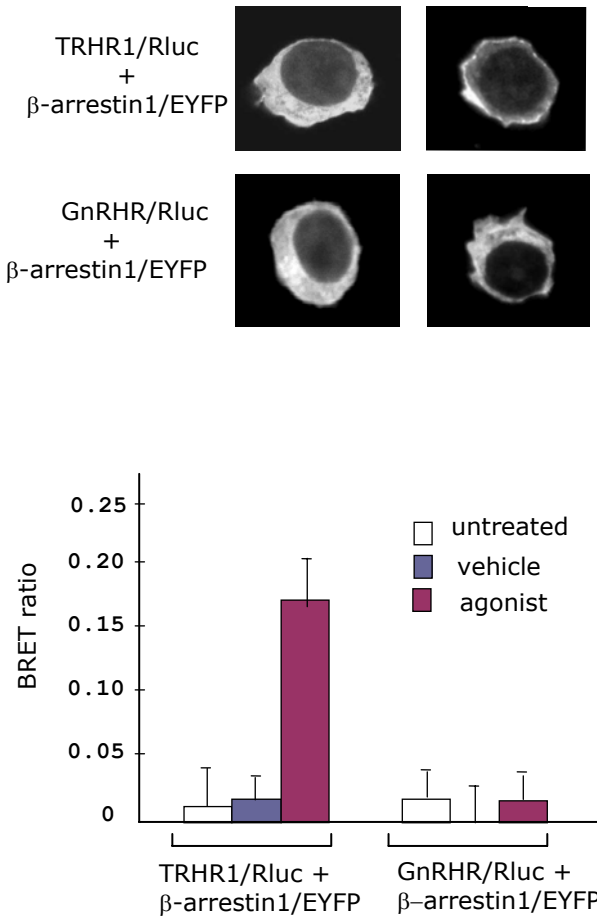

Figure 4: BRET can be used to monitor receptor-arrestin interactions in living cells in real time. (Upper panel) Cells co-expressing β-arrestin1/EYFP and either TRHR/Rluc or GnRHR/Rluc were visualized using confocal microscopy to monitor redistribution of arrestin following treatment with agonist (1μM). (Lower panel) BRET ratio was measured in COS 1 cells expressing TRHR/Rluc and β-arrestin1/EYFP, or GnRHR/Rluc and β-arrestin1/EYFP in the presence or absence of the appropriate ligand or vehicle and the data expressed relative to the BRET ratio for cells expressing either TRHR/Rluc or GnRHR/Rluc alone.

Interactions between GPCRs and intracellular proteins required for receptor function such as second messenger signalling, desensitisation, internalization and trafficking have also been monitored using BRET. For example, the interaction between the TRHR and β-arrestin (4) (Figure 4).

Thus, BRET represents a powerful tool with which to study the complex array of protein-protein interactions involved in cellular function.

Other applications on BRET

BRET can be used for a wide range of applications. It can theoretically be used to detect and monitor any protein-protein interaction and represents a powerful technique for both basic research and industry. Table 1 describes some of the many applications of BRET.

Table 1 : Applications of BRET

I. Receptor-protein interactions
- Receptor homo- and heterodimerization
- Receptor activation
- Receptor signalling assays
- Orphan receptor screening
- HTS for GPCR analogs

II. Protein-protein interactions
- Light-sensitive (circadian clock proteins, retinal proteins, etc)
- Interactions in signalling cascades

III. Functional Proteomics
- Monitor protein-protein interactions (activation or inhibition)
- Validate and characterise yeast 2-hybrid results
- Mammalian cell screening assays based on BRET

IV. Drug screening

BRET assay protocol

Performing a BRET assay to investigate a potential protein-protein interaction involves several steps: (i) generation of the two proteins of interest genetically fused with either Rluc or EYFP at either the N- or C-terminus; (ii) confirmation that functionality of fusion proteins has not been compromised by presence of either Rluc or EYFP tags (iii) co-expression of the two BRET fusion proteins in mammalian cells and (iv) detection of the BRET signal.

1. Generation of BRET fusion constructs

Energy transfer is dependent on not only the distance between the energy donor, Rluc and the energy acceptor, EYFP, but also on the relative orientation of the donor and acceptor. Therefore, when performing a BRET assay to investigate the interaction between two proteins of interest it is best to generate all four combinations of fusion proteins for each protein being tested. This allows the optimal tag orientation (N- or C-terminal) and nature (Rluc or EYFP) to be determined. The potential for interference with interaction sites or incorrect protein folding and localization, may provide some justification for certain combinations of BRET fusions to be constructed and tested initially.

In addition, a BRET fusion positive control construct should also be used when performing BRET assays. This involves the direct fusion of the Rluc and EYFP proteins separated by an amino acid linker. Following addition of coelenterazine to cells expressing the Rluc-EYFP fusion, energy emitted from Rluc is directly transferred to EYFP resulting in a high BRET signal (1,9).

2. Testing functionality and expression levels of BRET fusion constructs

The functionality of BRET fusion proteins should be assessed and compared to the untagged protein prior to its use in a BRET assay. Addition of either the Rluc or EYFP to the receptor or protein of interest may disrupt the expression, folding, localization and/or function of the protein and in assessing the relevance of potential protein interactions it is important that function of tagged proteins is not compromised.

In addition, correct subcellular localization of fusion proteins can be monitored by confocal microscopy, either directly by visualization of the fluorescently tagged protein, or indirectly by antibody staining using antibodies directed against either the molecules themselves or to epitope tags engineered into the molecules. As expression can vary from one fusion protein to another and the levels of expression are important in BRET, it is recommended to carry out preliminary transfections to assess the relative expression of Rluc and EYFP fusion proteins. Relative expression levels of Rluc fusion protein can be determined by measuring luminescence following the addition of substrate coelenterazine h. Relative levels of EYFP fusion expression can be determined by measuring fluorescence intensity either by flow cytometry or by using the fluorescence mode of the Mithras LB 940 and the Fluorescein settings, with approximate excitation and emission wavelengths of 485 nm and 530nm.

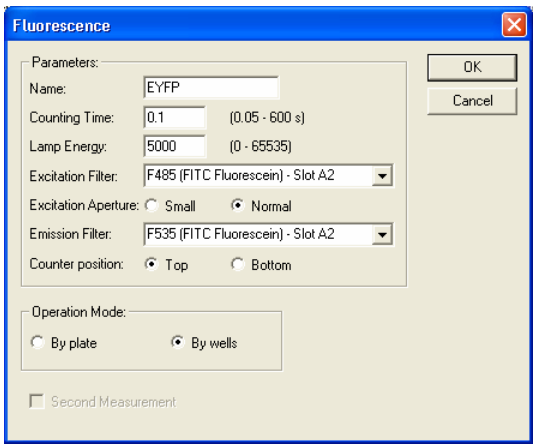

Figure 5: Settings for fluorescent mode, selected by double-clicking on 'fluor label' in the left pane of the 'Measurement' tab in the 'Read options' dialog box.

3. Transfection and co-expression of BRET fusion constructs

For each protein-protein interaction being tested, alongside those transfected with both the Rluc and EYFP fusion constructs, it is also necessary to transfect cells with the Rluc fusion construct alone. The amount of fusion constructs to be used in co-transfection studies, as well as the ratio of Rluc to EYFP fusion protein expressed, needs to be empirically determined in order to optimise the BRET signal. DNA concentrations for optimum expression levels will depend on several factors including the relative levels at which each construct is expressed, the amount of endogenous protein expression and the relative affinity of the two proteins. It is therefore recommended that transfections be carried out using various amounts of Rluc and EYFP fusion construct DNA. It is important to note that excessive Rluc fusion protein expression can mask small degrees of energy transfer leading to a reduced or non-detectable BRET signal and that generally it is better to overexpress the EYFP fusion construct relative to the Rluc fusion construct.

It is also important when performing BRET assays to include negative controls to exclude the possibility that the BRET signal is merely generated from the non-specific, random interaction of the expressed Rluc and EYFP fusion proteins. These include the expression of each Rluc-tagged protein with EYFP alone and each EYFP-tagged protein with Rluc alone. Further negative controls using other tagged proteins should also be performed, with control proteins expressed at similar levels to the proteins being tested, in order to assess the specificity of the receptor/protein BRET signal. To further assess the specificity of the BRET signal, untagged proteins can be co-transfected along with the donor and acceptor BRET fusions. The untagged proteins should compete for binding sites with the tagged proteins, thus selectively disrupting the BRET interaction and thereby reducing the BRET signal (4).

By performing BRET at lower levels of protein expression (at or below physiological levels) may help to prevent non-specific BRET signals. However, each protein-protein interaction is unique with contributing factors affecting binding such as affinity, stability and kinetics. Therefore, different levels of protein expression and different ratios of donor and acceptor molecules may be required in order to detect a BRET signal indicative of an interaction. By titrating the quantity of EYFP fusion protein expression, whilst keeping the expression of the Rluc fusion constant, the $BRET_{50}$ (the EYFP fusion protein expression as measured by degree of fluorescence, at which the BRET value is half maximal) can be determined as a relative approximate measure of interaction affinities (10).

4. Detection of BRET signals

In order to measure BRET suitable instrumentation is required that can sequentially filter, then measure, light emission over two separate wavelength ranges. As extreme over-expression of proteins will result in non-specific interactions occurring, it is important that BRET can be performed and signals detected using low levels of protein expression ie. near/or below physiological levels. Therefore, it is important the application of an instrument capable of detecting low BRET signals will help reduce the detection of non-specific BRET signals.

Generally, BRET measurements are performed on suspensions of the transfected cells. Upon addition of coelenterazine to the cell suspension, light emission should be immediately measured as the luciferase reaction displays rapid decay kinetics. This can be performed manually by adding coelenterazine to each sample, one at a time, reading immediately after the addition or preferably by injection of substrate with the on-board reagent injectors. Repeated reads are taken on all samples, as the BRET signal takes several seconds to equilibrate following the addition of coelenterazine. Also, by performing repeated reads following the addition of substrate, the stability of the interaction can be monitored over time. This may be particularly important when monitoring the effect of a certain modulator on a protein-protein interaction, for example, the effect various agonists or antagonists may have on receptor-protein interactions. By taking repeated reads, a BRET kinetic analysis is being performed essentially allowing an interaction to be monitored in real-time.

A basic example of how a BRET assay can be performed is described below. Assays can be adjusted based on the nature of the interaction being investigated. For example alterations can be made to cell number assayed, treatments tested, number of reads taken.

Basic BRET assay protocol

1. At 48 h post-transfection, cells are detached using phosphate buffered saline (PBS)/0.05% trypsin and washed twice in PBS and resuspended in 500µl PBS (based on 2 x 10^5 cells transfected in a well of 6 well plate). For each transfection approximately 100,000 cells are analysed by flow cytometry for EYFP expression, or by using the fluorescent mode on the LB 940 Mithras. For BRET, 40µl cell suspension (approximately 20,000 cells) is distributed into each well of a 96-well plate.

2. Add 10µl of coelenterazine h (freshly diluted to 25µM) to each well either manually or preferably by injection using on-board reagent injectors, to obtain a final concentration of 5µM and immediately read using the Mithras LB 940 BRET plate reader. (see parameter settings in figures 2 and 6)

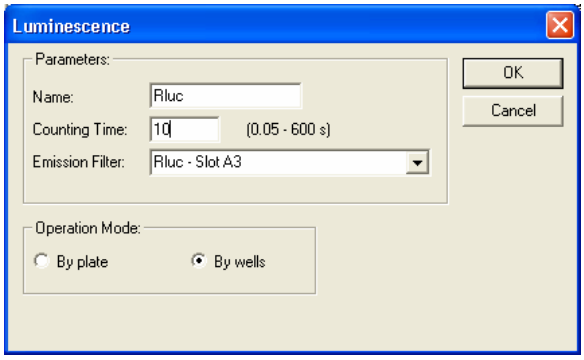

Figure 6: Properties for 'lumin label' in the 'Read options' dialog box

3. To test the effect of an agonist or antagonist (or another reagent/chemical/treatment) on the interaction and hence on the BRET signal the reagents can be added prior to the addition of coelenterazine to perform a pre-incubation. Alternatively, coelenterazine can be injected, a reading taken, then the reagent can be injected and the effect on the BRET ratio assessed over time. The Mithras is fitted with four injectors allowing easy, reliable and reproducible addition of either substrate and/or treatment.

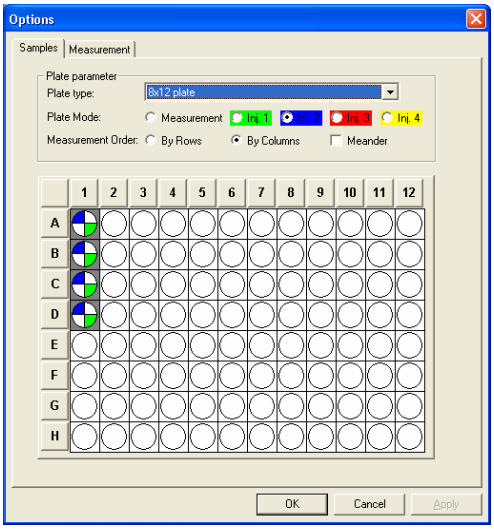

Figure 7A: Selection of wells for injections with substrate and treatment. The 4 injectors allow more than one treatment to be added to different wells as desired.

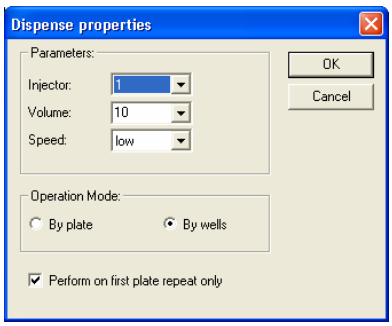

Figure 7B: After selecting wells for injectors, click on the measurement tab, double click on 'dispense' in the left pane and set the properties.

4. Integrated readings are taken for 10 secs through each of two filters, each with a different wavelength range. 480nm and 530nm interference filters are used on the Mithras. The BRET ratio is then calculated from these two reads according to the following calculation :

$$\text{BRET ratio} = \frac{\text{emission at 530nm}}{\text{emission at 480nm}}$$

5. Data is then represented as a normalized BRET ratio, which is defined as the BRET ratio for the co-expression of the Rluc and EYFP constructs normalized against the BRET ratio for the Rluc expression construct alone in the same experiment. The BRET signal is a ratiometric measurement, thus reducing or eliminating data variability due to differences in assay volume, cell number and/or signal decay over time.

Conclusion

BRET is a novel resonance energy transfer technique that represents a powerful tool for the detection and analysis of a wide range of protein-protein interactions and provides significant advantages over currently used methodologies. It represents a robust, homogeneous live-cell assay system that has been successfully applied to the study of receptor interactions, which can be easily adapted for the study on any protein-protein interaction.

Materials

- Black and White Isoplate 96-well (25/pack) - Perkin Elmer Cat #1450-581
- BRET buffer (PBS) - PBS Powder 10 x 1L - Gibco (Invitrogen) Cat # 21600-010
- Coelenterazine (h form) 250µg - Molecular Probes Cat # C-6780
- EYFP vectors – Clontech
  pEYFP-C1 Cat # 6005-1
  pEYFP-N1 Cat # 6006-1
- Rluc vector – Promega
  PRL-CMV Cat # E2261

References

1. Xu, Y *et al*. (1999) *Proc. Natl. Acad. Sci. USA*. 96: 151-156.
2. Zacharias, DA *et al*. (2000) *Curr. Opin. Neurobiol.* 10: 416-421.
3. Angers, S *et al*. (2000) *Proc. Natl. Acad. Sci. USA*. 97: 3684-3689.
4. Kroeger, KM *et al*. (2001) *J. Biol. Chem.* 276: 12736-12743.
5. Hanyaloglu, AC *et al*. (2002) *J. Biol. Chem*. 277: 50422-50430.
6. McVey, M *et al*. (2001) *J. Biol. Chem*. 276: 14092-14099.
7. Boute, N *et al*. (2001) *Mol. Pharmacol*. 60: 640-645.
8. Cheng, ZJ and Miller, LJ (2001) *J. Biol. Chem*. 276: 48040-48047.
9. Eidne KA *et al*. (2002) *Trends. Endocrinol. Metab*. 13: 415-421.
10. Mercier, JF *et al*. (2002) *J. Biol. Chem*. 277: 44925-44931.