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Protocol for Initiating New GPCR Target Collaborations with GPCR Network

1. **Collaborator nominates** the protein as a PSI:Biology community target (http://sbkb.org/cnt)

2. **Collaborative Agreement is established** between **GPCR Network (TSRI)** and **Collaborator (Collaborator's Institute).**
   Note: GPCR Network is required to deposit clones into NIH Materials Repository, and so must have freedom to operate with materials and DNA under the collaborative agreement.

3. **GPCR Network supplies** information on vectors, fusion protein clones and where to obtain materials from Materials Repository.

4. **Collaborator clones (or outsources cloning of)** proposed receptor into our defined vector, wild-type as well as a 2 previously validated fusion proteins T4 lysozyme (T4L) and apocytochrome b_{562}RIL (BRIL) inserted into the third intracellular loop (ICL3) or N-terminus, as well as 2 N- and 2 C-terminal truncations (and any mutations known that may help with expression). **(See MB Resource Plans attached)**.

5. **Collaborator sends** bacmids and pFastBac1 samples to GPCR Network along with sequencing (*e.g.* SCF, ABI, AB1) files.

6. If able, **Collaborator conducts** small scale expression studies and binding-activity assays.

7. If not able, **GPCR Network performs** small scale (5ml) *Sf*9 expression of the above expressed protein and sends frozen cells to collaborator for assays.

8. **Collaborator performs** ligand binding assays and activity assays.

9. **Collaborator supplies** 2-5 mgs of at least 5 different ***transferable*** ligands that have reasonable affinity and solubility, along with affinity and solubility data.
   Note: Collaborator is responsible for legal "transferability" of the ligands provided.

10. **GPCR Network performs** medium scale expression, thermal stability, and SEC profiles.

11. If materials achieve benchmarks for success in medium-scale studies, **GPCR Network performs** large-scale expression, FRAP and crystallization trials. Collaborators will receive regular updates and be kept involved in all steps of the structure determination process.

Molecular Biology Resource Plan: Outsourcing

1. The insertion point of the T4L and BRIL fusion within the ICL3 region will be based on protein sequence alignments of published GPCR structures of β_2AR, ADORA2A, CXCR4, and D3 and/or consultation. On the N-terminus, T4L or BRIL will be fused directly to the first residue of the wild type or truncated protein.
2. Obtain necessary material from PSI: Biology-Material Repository.
    a. Plasmids for subcloning (PSI:MR will designate unique identifiers for each below)
        i. XX N-terminal FLAG (we will use D3 plasmid)
        ii. XX C-terminal FLAG (we will use D3 plasmid)
3. Designated restriction sites for use in subcloning into plasmids obtained from PSI: Biology-Material Repository.
    a. N-terminus
        i. AscI
    b. C-terminus
        i. FseI
4. Synthesize wild-type GPCR of choice along with T4L and BRIL fusion partners and designated restriction sites (GenScript).
5. Subsequently, two truncations at both the N and C terminal junctions of the fusion partner boundaries should be considered along with any putative beneficial mutations.
6. Request commercial cloning into pFastBac1 vector with designated restriction sites.
7. Upon plasmid delivery, transform DH10Bac *E. coli* according to protocol.
8. Mail white colony agar stabs or glycerol stocks and DNA samples to the GPCR Network for bacmid preparation and transfection of insect cells.
9. Email DNA sequencing chromatogram files (*e.g.* SCF, ABI, AB1) to jeffvel@scripps.edu.

Molecular Biology Resource Plan:
In-house

1. The insertion point of T4L and BRIL fusion within the ICL3 region will be based on protein sequence alignments of published GPCR structures of β_2AR, ADORA2A, CXCR4, and D3 and/or consultation.
2. Obtain Wild-type GPCR template DNA.
3. Obtain necessary materials from PSI: Biology-Material Repository.
    a. Fusion template DNA
        i. GPCR Network T4 lysozyme (CCR5 version to be used)
        ii. GPCR Network BRIL (A2AAR version to be used)
    b. Plasmids for subcloning (PSI:MR will designate unique identifiers for each below)
        i. XX N-terminal FLAG(we will use D3 plasmid)
        ii. XX C-terminal FLAG(we will use D3 plasmid)
4. Design appropriate primers for PCR-driven splicing by overlap extension (SOE-PCR) to insert the T4L and BRIL partners into the IL3 region.
5. Perform SOE-PCR to insert the T4L and BRIL fusion partners into the ICL3 region without the use of restriction sites.
6. Analyze agarose gel for shifted SOE-PCR band sizes to ensure successful splicing of the T4L and BRIL fusion partners into the ICL3 region of the GPCR.
7. Purify amplified SOE-PCR product.
8. Design appropriate PCR primers encoding restriction sites of choice for ligation of the ORF with pFastBac1 vector.
9. Perform standard PCR to amplify the ORF.
10. Purify amplified ORF product.
11. Digest pFastBac1 vector and the amplified ORF with appropriate restriction enzymes.
12. Ligate pFastBac1 vector with the amplified ORF.
13. Transform *E. coli* and screen positive clones via DNA sequencing.
14. Sequence positive DNA should be used as templates for future site-directed mutagenesis to create at least two truncations at both the N and C terminal junctions of the T4L and BRIL fusion partner boundaries as well as any putative beneficial mutations.
15. Upon successful cloning, transform DH10Bac *E. coli* according to protocol.
16. Inspect agar plates for blue/white colonies.
17. Mail white colony agar stabs or glycerol stocks and DNA samples to the GPCR Network for bacmid preparation and transfection of insect cells.

Email DNA sequencing chromatogram files (*e.g.* SCF, ABI, AB1) to jeffvel@scripps.edu.