Fall 2017 Molecular Techniques Project Descriptions.

MDH & CS FRET Constructs: FRET or Forster (aka Florescence) Resonance Energy Transfer is a method that allows us to determine molecular interactions using two fluorescent proteins. One compound is excited by a particular wavelength and transfers an electron to an acceptor compound rather than relaxing to ground state and emitting light. The second compound is excited to the higher state due to the transfer of energy and will eventually relax and emit light. Fluorescent proteins are often used to conduct FRET assays in living cells to identify protein-protein interactions. A protein FRET background can be found here (https://www.addgene.org/fluorescent-proteins/fret/). We will be using two fluorescent protein pairs: mTURQ-GFP and sYFP2. mTURQ-GFP (mTurquoise-GL also called Turquoise GFP) is a donor mutant of a classic CFP donor protein. Nat Commun. 2012 Mar 20; 3: 751. SYFP2 is an acceptor protein created from EYFP and is over 30 times brighter than the original clone (Cyan and Yellow Super Fluorescent Proteins with Improved Brightness, Protein Folding, and FRET Forster Radius. Biochem. 2006, 45: p. 6570-6580).

Scientists at RPI wish to test for the interactions between MDH and CS (Krebs cycle!) using FRET. Your job will be to insert CS into one plasmid and MDH into a second plasmid. Humans have two isoforms of MDH, MDH1 found in the cytoplasm of all cells and MDH2 which is located in the mitochondria. They want to investigate cytosolic vs mitochondrial MDH interactions with CS. We will also clone wgMDH (watermelon glyoxyl) MDH into the same plasmid as a negative control. For now, this is all without the transit peptide. That is the peptide that is recognized and causes the protein to be brought into a mitochondria. They wish to do this in the cytoplasm. Later, the will add this transit peptide to your constructs!

You will be using a unique construct that has both a FRET donor and acceptor protein cloned into the same plasmid. The vector was prepared by Dr. William A Barton at the Dept of Biochemistry and Molecular Biology at Virginia Commonwealth University Medical School. The paper describing the construction of this vector (he used two proteins not included in our version) is found here (Mol Cell. 2010 Mar 12; 37(5): 643–655. Tie1-Tie2 interactions mediate functional differences between angiopoietin ligands).

The base vector is PCDNA 3.1 (hygro+) with additional sections already cloned into it. From 5' to 3' there is an RE, start sequence – turquoise GFP – RE – linker amino acids – RE – SYFP2 - stop codon – RE.

NheI – ATG - Turquoise GFP - BamHI - linker(GSAAA) – AflII - sYFP2 – TAG - XbaI

Your job will be to clone MDH into one of the fluorescent proteins so that the remaining fluorescent protein is on the C terminus of MDH. We need to do this for wgMDH, MDH1 and MDH2. Also CS will be cloned into one of the sites (not the same as the MDH) so the fluorescent protein is on the N terminus of CS.

Turquoise constructs will use AflII and XbaI RE sites and must include STOP codon.
AflII RE site C'TTAAG XbaI RE site T'CTAGA

SYFP2 constructs will use NheI and BamHI and must include a start codon but no STOP codon.
NheI site G'CTAGC BamHI site G'GATCC

NOTES:

- MDH cannot have any amino acids, tags or fusion protein partners on the N terminus and MUST be on the C terminus of the protein. If you aren't clear – review and ensure you know where N and C terminus of a protein is AND how that aligns with the coding frame (5' or 3').
- The information on the base plasmid PCDNA 3.1 (hygro+) is made by Invitrogen and the instruction manual is linked on our webpage.
- Find the sequencing primers for the plasmid from the manual
- Pay attention to start and stop sites. You will need to add one or the other – see the info above
- Check the clone you are going to insert into PCDNA 3.1 to ensure the REs you are using are not already in the coding region. If they are… what will you do? The answer is easier than you think.
- The sequence for each MDH and CS is found on our web page. You will have to create your project using these sequences.

ADDITIONAL JOB!!!. There is no map for this plasmid. The FIRST thing each group will do is to make one! Have a full circular map, the complete sequence of the plasmid AND the sequence annotated of the Nhe1 to XbaI site.

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MDH & CS FRET Epitope Tag Constructs: An epitope tag is a series of additional amino acids cloned into a gene on either terminus of the protein. This set of amino acids adds a unique functionality to the protein. These tags are recognized by antibodies and can be used for detection or isolation using a technique called immunoprecipitation. A broader definition of an epitope tag includes affinity tags which can be used for the same purpose as an epitope tag but are also used for affinity chromatography where epitope tags are not commonly used for such techniques. We are going to use two epitope tags. First we are creating a FLAG tag protein (an 8 amino acid tag DYKDDDDK) which was patented by Sigma Chemical Company and is also called DDK tag by non-Sigma companies. We will also make a HA tag (human influenza hemagglutinin, a glycoprotein involved in the influenza virus) we use nine of the amino acids of the HA protein (YPYDVPDYA) to make the tag. There are very specific antibodies for both tags and few proteins have the amino acid sequences of either FLAG or HA tags making the antibody specific only for the tag.

Scientists at RPI wish to test for the interactions between MDH and CS (Krebs cycle!) using tagged proteins to investigate protein-protein interactions using a technique called co-immunoprecipitation. Your job will be to insert CS into one plasmid and each MDH isoform into a second plasmid. Humans have two isoforms of MDH, MDH1 found in the cytoplasm of all cells and MDH2 which is located in the mitochondria. They want to investigate cytosolic vs mitochondrial MDH interactions with CS. We will also clone wgMDH (watermelon glyoxyl) MDH into the same plasmid as a negative control. For now, this is all without the transit peptide. That is the peptide that is recognized and causes the protein to be brought into a mitochondria. They wish to do this in the cytoplasm. Later, the will add this transit peptide to your constructs!

You will clone **MDH** into the appropriate construct so that the tag is on the C terminus of MDH. MDH cannot have any amino acids, tags or fusion protein partners on the N terminus and MUST be on the C terminus of the protein. If you aren't clear – review and ensure you know where N and C terminus of a protein is AND how that aligns with the coding frame (5' or 3').

CS will be cloned into the other construct so that the tag is on the N terminus of CS.

Cloning plasmids. You will be using pCMV-HA from Clonetech, a 3.8 kb plasmid that has an MCS on the 3' side of the HA sequence. The map for Clontech is on our class website. You will also be using the plasmid pFLAG-CMV-5a from Sigma. This plasmid has THREE different reading frames. We have the "a" version. Pay attention to how to clone into this plasmid while maintaining the correct reading frame. The webpage has the manufactures information for each clone. Also search the Sigma website (***E3762 SIGMA***) for this product.

The sequence for each MDH and CS is found on our web page. You will have to create your project using these sequences.

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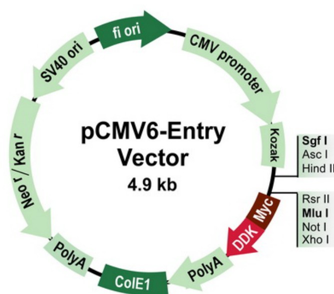
CHP 1 & 2 Hybrid Project: Calcineurin Homology Protein (CHP) is a protein whose sequence is homologous to calcineurin. CHP proteins bind calcium and proteins. Very little is known about what CHP does in the cell. There are three isoforms of CHP in the human genome, we focus on two, CHP1 and CHP2. CHP1 is expressed in nearly every cell in all human tissues while CHP2 has a much more limited expression profile. Interestingly CHP2 is expressed in most tumor cells examined, making it an interesting target in cancer cells. CHP1 and CHP2 have a short region that is distinct for each site (see the figure). The Provost group is very interested in seeing if this region is responsible for unique behaviors of the proteins and cells making each protein. Thus we would like to make a CHP1ΔCHP2-91-101 and a CHP2ΔCHP1-91-101.

```
CHP2 69 SFFPDGSQRVDFPGFVRVLAHFRPVEDEDTETQDPKKPEPLNSRRNKLHYAFQLYDLDRDGKISRHEM 136
CHP1 69 AFFPEGEDQVNFRGFMRTLAHFRPIED-NEKSKDVNGPEPLNSRSNKLHFAFRLYDLDKDEKISRDEL 135
```

You will start with two plasmids that already have CHP1 or CHP2 cloned into a plasmid called pCMV6-Entry vector. Your job will be to find a way using either site directed mutagenesis or Gibson cloning or another method to create a domain swap with your CHP. Find the info for the CHP sequence and make your plan!

<u>pCMV6-Entry vectors</u>: These are the plasmids which the CHP1 and CHP2 open reading frame (ORF) genes have been subcloned into the pCMV6-Entry vectors (entry vector [empty] is 4..9 kb and Kan^r). Once a gene has been cloned into the vector the plasmid is called pCMV6-CHP1 or so on. These plasmids are suitable for expression after transfection in mammalian cell culture (NOT in E. coli). Each pCMV6 clone has been purchased from Origene.com as TruORF Gold products:

- CHP1 Myc DDK tagged Human ORF clone (CHP, NM_0072360)
- CHP2 Myc DDK tagged Human ORF clone (CHP2, NM_022097)



pCMV6-Entry

```
                       EcoR I           BamH I Kpn I   RBS        Kozak Consensus  Sgf I      Asc I
CTATAGGGCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGCCGCCGCGATCGCCGGCGCGCCAGATCT

Hind III      Nhe I   Rsr II    Mlu I       Not I     Xho I              Myc.Tag
CAAGCTTAACTAGCTAGCGGACCG ACG CGT ACG CGG CCG CTC GAG CAG AAA CTC ATC TCA GAA GAG
                          T   R   T   R   P   L   E   Q   K   L   I   S   E   E

                    EcoR V                   DDK.Tag                 Pme I    Fse I
GAT CTG GCA GCA AAT GAT ATC CTG GAT TAC AAG GAT GAC GAC GAT AAG GTT TAA ACGGCCGGCC
 D   L   A   A   N   D   I   L   D   Y   K   D   D   D   D   K   V  Stop
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Fall 2017 Molecular Techniques Project Descriptions.

CS – GST with TEV site Subclone Project: Citrate Synthase (CS) is a protein involved in the krebs cycle. A number of labs involved in a 12 university collaborative project to incorporate research into classrooms would like to use this construct to measure interactions between MDH and CS. We currently have human CS cloned into a pET28 vector but need to shuttle CS from this plasmid into a different plasmid that will generate a fusion protein with a protease cleavable site. The fusion protein is glutathione S-transferase (GST). As a fusion protein, GST leads to a higher expression level in bacterial systems and is easily purified by affinity chromatography. Including a protease cleavage side between the two proteins allows the researcher to express and purify the protein and then remove GST from CS by proteolysis. TEV is a cysteine protease (*Tobacco Etch Virus nuclear-inclusion-a endopeptidase*). It is a highly specific protease with a specific recognition and cleavage sequence.

Your job will be to subclone CS from its pET28 vector into a pGEX4T vector and at a TEV site between the two. Both sequences are located on our class website.

You must search the amino acid sequence of CS AND GST for any TEV sites. If they are, come up with a plan to still use the TEV cleavage site. Do this FIRST. Use any number of protease sequence prediction sites (use two to ensure there isn't any mistakes) and document the results in your laboratory notebook.

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cpMDH and TvMDH pET with TEV site Subclone Project: There are two interesting MDH isoforms that we would like to investigate in biochemistry labs here at USD and other universities.

cpMDH **–** MDH from Cryptosporidium parvum (a microbial parasite that causes diarrheal disease commonly called "Crypto"). The gene was purchased from Addgene (search for cpMDH or plasmid # 25584) but does not express well. Your job is to do two things. First, look up "rare codons" for bacterial expression. See your instructor for a simple background. Then analyze the gene for rare codons. Depending on the results you will conduct a site directed mutagenesis to correct for this issue ensuring the amino acid sequence remains unchanged. Second, you will shuttle (subclone) the coding region of cpMDH into a pET28 vector. Ensure that the histadine tag is on the c-terminus. Use the human MDH1 gene already in pET28 as your model for how to clone into the same backbone (pET28).

pTvMDH - LDH with an MDH activity. This MDH is currently in vector pQE31 with the His tag on the N terminus of MDH. Amp. Cloned from Trichomonas vaginalis, T. vaginalis (NIH-C1 strain ATCC 3001. The common sexual STD trichomoniasis, is caused by a protozoan parasite. Sequence in GenBank accession nos AF070994. Reference Proc Natl Acad Sci Vol 96 pp. 6285 1999. You will perform the same directions as for cpMDH. Check for rare codons and subclone into pET28.

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Taq – GFP Fusion Protein Project: This is an interesting project, Dr. Amy Parente from Mercyhurst has asked if you would make a clone for her student research project! She would like to clone Taq into an expression vector to make protein in bacteria that could easily be observed during expression and purification. But because of the temperatures involved with Taq the fusion protein must be removed from Taq after purification but before use. So we need to either 1) move a GFP gene with a His tag into her existing plasmid and ensure a TEV protease site lies between GFP and Taq or 2) move her Taq gene into another plasmid that will express in bacteria and has a His tag and a GFP in frame AND add or make sure there is an existing TEV site between Taq and GFP. We can leave the histadine tag on the protein. . TEV is a cysteine protease (*Tobacco Etch Virus nuclear-inclusion-a endopeptidase*). It is a highly specific protease with a specific recognition and cleavage sequence. You must search the amino acid sequence of Taq AND GFP for any TEV sites. If they are, come up with a plan to still use the TEV cleavage site. Do this FIRST. Use any number of protease sequence prediction sites (use two to ensure there isn't any mistakes) and document the results in your laboratory notebook.

Information for the Taq clone is in a paper linked from our website look for the pTTQ18 plasmid.

Clones to use may include the following:

- pEcoli-6xHN-GFPuv - Plasmid encoding GFPuv with N terminal 6XHis Tag. (alt His-Asn). T7 Promoter. Low copy number. Ori pBR322. 6460 bp. - Clontech – Cat 631417 & 631418. No TEV tag. Must be added if used.

- pET28a His-CHP2-GFP - Human CHP2 gene was synthesized after codon optimization for expression in E.coli. CHP2 was subcloned into pAcGFP1-N1 vector. CHP2-GFP was excised from pAcGFP and subcloned into pET28a. 5290 bp. There is no TEV site here. This would require you to cut out CHP2 and replace with Taq and add a TEV site.

- MGH – MDH – GFP – His. This is a fusion protein with GFP and His on the C-terminus of each other. There is no TEV site here. This is in a pQE60 vector.

Information for each vector is on the class website.