Protocol for expression and purification of a fluorescent binding proteins for a metabolite biosensor.

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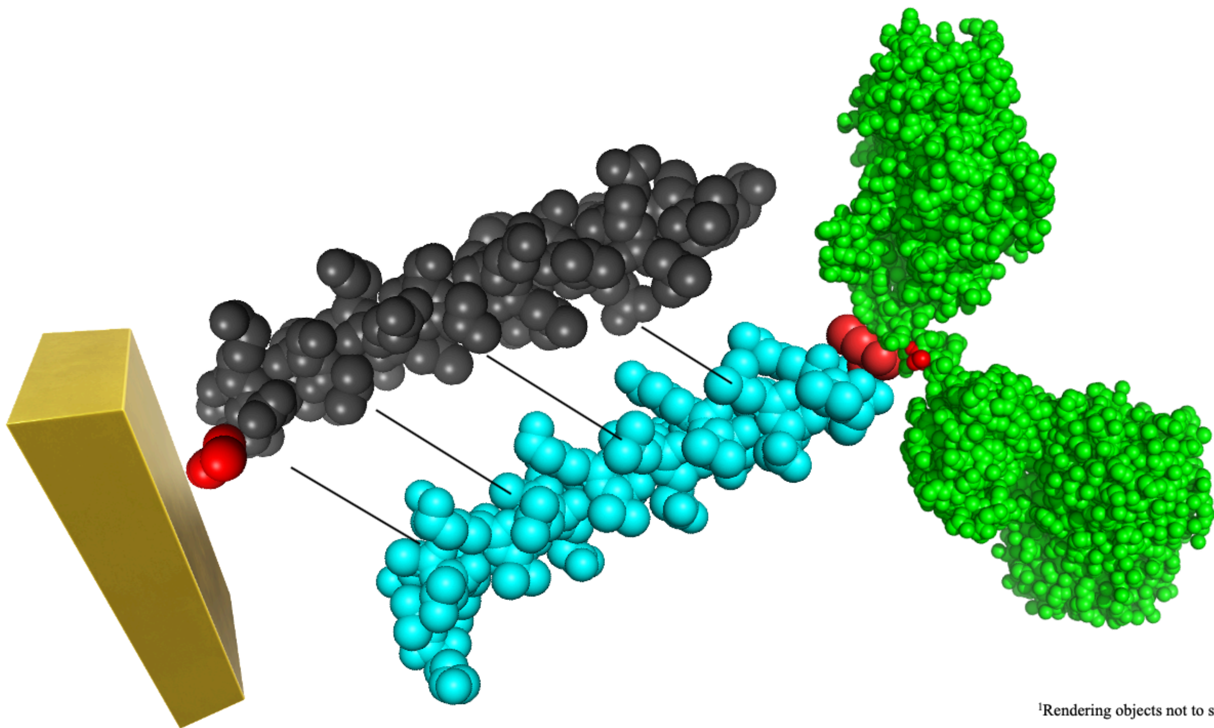
More project information can be found at queensigem.ca



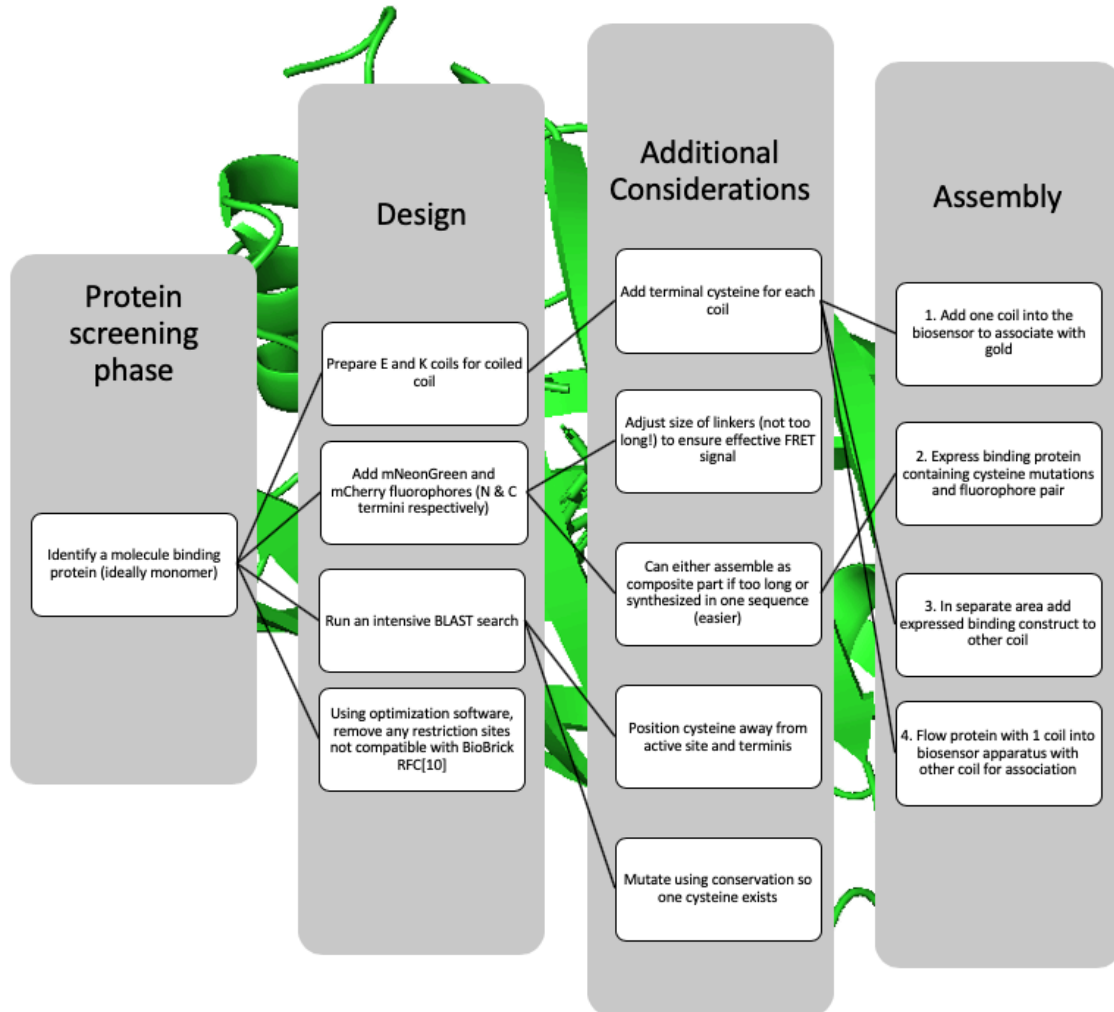
Project Background

This year's project goal is to produce a novel transdermal metabolite biosensor for point-of-care diagnostic quantification of phosphate, potassium, parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23) and glucose levels in sweat or interstitial fluid. We will generate a genetically encoded Förster resonance energy transfer (FRET) metabolite sensor by fusing existing binding proteins to fluorescent proteins (FPs). To quantify physiologically relevant levels of metabolites, highly optimized mNeonGreen and mCherry fluorophore pairs will be used as these are relatively stable and impose a large fluorescent linear range as they are quite intense (bright) (1). Upon conjugation of fluorophores, we will use site-directed mutagenesis to add a cysteine residue to the protein. This will serve as a critical first step to immobilizing the biosensor onto a microfluidic surface. Upon purification of the recombinant protein, we aim to integrate our metabolite biosensor into a microfluidics system that will exist in a handheld device of our own design. Through this, we will measure the fluorescence signal given off by the protein construct and correlate it to a metabolite concentration (1).

The scope of our project is immense in the Chronic Kidney Disease (CKD) landscape. Phosphate levels are associated with a slew of cardiovascular and renal complications, with hyperphosphatemia serving as a direct stimulus to vascular calcification – a leading cause of the morbidity and mortality associated with CKD (2). Currently, there is no point-of-care method for phosphate detection, with blood analysis being the main clinical diagnostic tool. This method is slow, and insufficient to measure the fluctuating levels of phosphate in a CKD patient (3).

This protocol will focus on the expression and purification of the fluorescent protein constructs used in our biosensor apparatus. Topics will include creating of cells that will translate the synthetic DNA into our proteins, plasmid ligation, protein purification techniques, and assembly techniques. Using our schematic overview, focusing on the 'Assembly' sub column, this protocol provides instructions for the building of our multi-part construct with both the coiled-coil and fluorescent binding protein.

A critical assumption being made in this protocol is that the entire fluorescent binding protein can be synthesized and ordered as one long DNA strand. This is not always the case for larger proteins. If this is the case, a scarless assembly method must be used. Most of our proteins are quite small, and with our two fluorophores attached, comfortably falls within the limit that may be ordered online.



Overview of protein construct design and assembly process. To develop the metabolite biosensor using an immobilized fluorescent binding protein, with cysteine modifications, and the coiled coil system – a schematic overview is created. Process should be followed Left-Right from Protein screening phase to Assembly, respectively. Branching indicates subsequent steps.

Creating competent *E.coli* K-12 cells

The protocol we use for creating competent *E. coli* K-12 cells is based off of the protocol used by iGEM, found in the Registry of Standard Biological Parts (4). Our bacteria that we grow is *E. coli,* K-12 strain. This bacterium is primarily used for safety and environmental reasons. It is not able to aggregate in the environment and is also non-toxic, should an accident occur, it will likely not have a dangerous effect.

Our *E. coli* K-12 will be stored in the -80C freezer for future work. To do this we need to grow our cells on a SOB plate and have them colonize. SOB stands for sugar optimal broth, otherwise known as Hanahan's Broth (5). This provides nutrition and the chance for our bacteria to grow.

Method (4):

1. Add cells onto a SOB plate and grow (room temperature)
    a. Protocol lists about 16 hours as the time for growth
2. Remove single colonies and place into 2 ml of SOB
    a. Shake overnight using an incubator for about 14-16 hours at room temperature
3. Add glycerol to about 15%
4. Add 1 ml samples into Nunc cryotubes
5. Add tubes to a sealed bag and place bag into a dry ice/ethanol bag for 5 minutes
6. Store in the -80C freezer until needed for use

When growing competent cells, it is important to 1) ensure quality of the cells and 2) ensure a sterile environment, which can be done by treating the lab area with ethanol. This production protocol uses SOB as previously discussed and a CCMB80 buffer.

CCMB80 buffer contains:

Item	**Amount**
KOAc	10 mM @ pH 7.0 10 ml of a 1M stock/L
CaCl2 x 2 H2O	80 mM @ 11.8 g/L
MnCl2 x 4H2O	10 mM @ 4.0 g/L
MgCl2 X 6H2O	10 mM @ 2.0 g/L
Glycerol	10 % @ 100 ml/L

It is important to make sure the pH is 6.4. To do this you may have to titrate down using HCl. Store the buffer at 4C in the fridge. Now off to growing the competent cells.

Method (4):

Add 250 mL of SOB to a 1 mL vial of cell stock, grow at 20C to an optical density of 0.3 at 600 nm.

1. Pre chill flat bottom centrifuge bottles in an ice bucket
2. Transfer the culture made into the centrifuge bottle
    a. Weigh and balance bottles
3. Centrifuge at 3000g at 4C for 10 minutes
4. Decant the supernatant from the centrifuge bottles into waste and keep pellet
5. Resuspend the pellet in 80 ml of ice chilled CCMB80 buffer
6. Incubate on the ice bucket for 20 minutes
7. Re-centrifuge at 3000g at 4C and decant the supernatant into waste
8. Again, resuspend cell pellet in 10 ml of ice cold CCMB80 buffer
9. Measure the OD of the mixture of 200 µL SOC (growth medium) and 50 µL of the resuspended cells
    a. The 200 µl SOC and 50 µl CCMB80 buffer is the blank
10. Add chilled CCMB80 to get a final OD of about 1.0-1.5.
11. Incubate on ice for 20 minutes and prep for aliquoting
    a. Prep dry ice
12. Aliquot into chilled 2 mL microcentrifuge tubes
13. Store at -80C

It is expected according to the iGEM protocol that cells should yield about 100-40 colonies. To calculate the competent cell efficiency, refer to our supplementary tests section in this document.

DNA ligation into plasmid

The DNA ordered for our fluorescent binding proteins or coiled-coil components must be inserted into a plasmid backbone. We use restriction digest enzymes recommended by iGEM since the DNA parts ordered are all RFC10 compatible. Our plasmid used is the pET24D vector. This ligation protocol is taken from the iGEM Registry of Standard Biological Parts (6).

Method (6):

1. Add 2 µL of NcoI – XhoI digested plasmid backbone, about 25 ng
    a. Use the pET24D backbone
2. Add under 3 µL of NcoI—XhoI digested fragment
    a. Should be equimolar
3. Add 1 µL of the T4 DNA ligase buffer
4. Add 0.5 µL of DNA ligase
5. Add water so that the final volume of the tube is 10 µL
6. Cool tube to 16C for 30 min for ligation, followed by 80C for 20 min to kill restriction digests
7. 1-2 µL of plasmid product will be used for transformation

pET24D + vector with fluorescent synechococcus phosphate binding protein (PiBP) inserted. Plasmid sequence obtained from the Addgene vector database (#69752-3) and sequence was reversed for a forward orientation of the T7

promoter start site. Fluorescent PiBP was inserted into the plasmid to be transcribed by bacterial RNA pol after the ribosomal start site (cut site NcoI). XhoI cut site inserted in-frame at the C-terminus site of fluorescent PiBP, for expression of the protein with a 6-residue His-tag. Vector contains kanamycin resistance.

Transformation of DNA into cells

Transformation of DNA is required to enter our competent cells in order for those cells to grow our proteins of interest. Colonies can then be selected and purified. This protocol is from the iGEM Registry of Standard Biological Parts (7). Our group uses the single tube transformation protocol since the number of samples we have to transform is limited. This protocol requires a transformation control like a plasmid with a simple GFP, SOC media (for growth), and an antibiotic which will aid in colony selection (7).

Method (7):

1. Add resuspended DNA to selected wells with 10 μL of distilled water
    a. Sit for several minutes (should be red colour)
2. Label the 1.5 mL tubes and pre-chill tubes on ice
    a. Should be one tube per transformation (In our case we have seven parts needing to be expressed so seven tubes)
3. The competent cells created should be thawed over ice
4. Add 50 μL of competent cells via pipette to the 1.5 mL tubes followed by adding 1 μL of resuspended DNA to the 1.5mL tubes
5. Add 1 μL of the control DNA via pipette into a 2 ml tube
    a. Concentration of control DNA should be 10 pg/μL
6. Incubate the 1.5 mL tubes on ice for 30 min
7. Heat tubes for 45 seconds at 42C – water bath
8. Return the tubes to ice and incubate on ice bucket for 5 min
9. Pipette 950 μL of the SOC media into each transformation tube
10. Incubate the tubes at 37C for 1 hour, shaking at about 200-300 rpm
11. Add 100 μL of the transformation via pipette onto petri plates
    a. It is important to spread the plates with spreader or glass beads so single colonies can be selected
12. Spin down at 6800g for 3 mins, discarding 800 μL of the supernatant after centrifugation
    a. Suspend the cells in the 100 μL of remaining supernatant and pipette onto the petri plates
13. Incubate overnight for about 14-18 hours at 37C (agar side up)
14. Pick single colonies from the transformations, you can do a colony PCR to verify the part size, or make stocks, or grow up the cell cultures

To grow cell cultures up and produce protein, the following steps are used in our lab, adapted from a protocol by New England BioLabs (8):

Note: *E.coli* K-12 cells such as the Top 10 or DH5a strain of cells are great for cloning, however, lack transcriptional machinery and have no T7 polymerase (can’t recognize T7 promoter in pET24D vector). They are good for storage since cells with the T7 machinery can destroy the DNA

over time. To express the proteins, use BL21(DE3) cells, which have T7 RNA polymerase. You may miniprep the storage cells and transform the DNA into the BL21(DE3) cells before expression.

15. Resuspend a selected colony in a 10 mL liquid culture with antibiotic
16. Incubate at 37C until the colony reaches an optical density between 0.4 – 0.8 at 600 nm
17. Induce the cells with 40 µL of 100 mM stock of IPTG (sugar solution to overexpress proteins, removing the *lac* repressor)

Note: Optional to do a competent cell efficiency test after this protocol. For a large-scale protein expression, New England BioLabs recommends 1 L of liquid medium with antibiotic (8).

Cell ligation

To isolate the cellular contents and remove the cell membranes of our bacterium – eventually leading to a purified protein, the cell must undergo lysis. Our preferred method is sonication. Our lab has a Branson sonicator to conduct this, and the method described in this document is what a PhD candidate advisor of ours (Nolan Neville) typically employs.

Method:

1. Cells will be resuspended first in lysis buffer. To know the appropriate buffer pH we just have them either +/- 1 pH unit from our proteins theoretical pI. TRIS and HEPES biffers will be used for a pI less than or greater than 7.5, respectively. Also, add 150 mM NaCl and a reducing agent like DTT to keep cysteines reduced.

Protein	**pI**	**Buffer Choice**
Phosphate binding protein with FRET	5.47	TRIS @ pH ~ 6.47
Potassium binding protein with FRET	6.16	TRIS @ pH ~ 7.16
Glucose binding protein with FRET	5.83	TRIS @ pH ~ 6.83
PTH receptor with mNeonGreen	6.46	TRIS @ pH ~ 7.46
PTH with mCherry	6.65	TRIS @ pH ~ 7.65
Alpha klotho with mNeonGreen	7.12	TRIS @ pH ~ 8.12
FGF23 with mCherry	6.66	TRIS @ pH ~ 7.66
E coil with TEV cut site and GFP	5.18	TRIS @ pH ~ 6.18
K coil with TEV cut site and GFP	6.22	TRIS @ pH ~ 7.22

2. Pre-treat the cells with 0.2 mg/ml lysozyme
    a. Lysozyme is used to weaken the cellular membranes in our cells
3. Sonicate the sample with the Branson Sonicator Probe for 5 mins
    a. Alternate, 5 seconds sonicating, 15 seconds off. The sonicator generates lots of heat. Since proteins can denature at high temperatures it is important to rest the sample on ice during the '15 seconds off'.

Protein purification steps

Nickel Chromatography:

For our larger protein constructs that contain fluorophores, adding a 6 residue histidine tag to either fluorophore tail may bind to nickel – using principles of affinity chromatography – will allow us to capture our proteins quickly and without disturbing native function (9). Histidine is an amino acid that will most readily associate with nickel, which is a transition metal. Nickel is immobilized on the chromatography column. The protocol used here is adopted from Bornhorst et al. in the journal Methods of Enzymology (9). Many of the materials and buffers used may be purchased from a supplier.

Method (9):

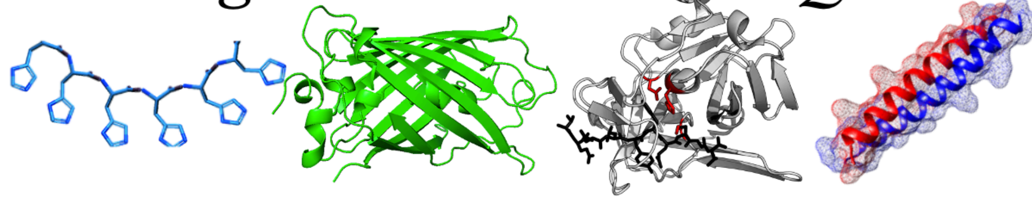
1. Centrifuge the cell lysate at 30,000 g for 30 mins at 4C
2. Discard the pellet
3. Add QIAgen 50% Ni^{2+}-NTA (nickel-nitrilotriacetic acid) which should be mixed with ice chilled loading buffer, this will bind the protein that is His-tagged
    a. Add about 5-10 mg/ml of the 50% Ni^{2+}-NTA
    b. Stir for 1 hour at 4C
4. Load this resin into a column, washing with loading buffer at 4C (loading buffer volume should be 20 times the column volume)
5. Wash the resin now with wash buffer, 20 times the column volume
    a. This is the same as the loading buffer, however, it contains 10 mM imidazole, pH set to 8.0 (1 unit from physiological pH which our proteins operate at)
6. Elute the protein with 20 column volumes of 10 to 250 mM imidazole in loading buffer
    a. Collect 1 ml fractions of protein

Note: Fractions may be assayed using SDS-PAGE ensuring that only the protein of interest is present. SDS-PAGE should be run with a MW marker and at 300 volts. Imidazole is also used to compete with histidine-nickel binding, therefore, allowing the purified protein to elute in fractions.

TEV-protease (tobacco etch virus) technique:

The TEV protease-technique is necessary when expressing our very small proteins – the coiled-coils for example that at only about 3kDA would be very difficult for *E.coli* to express on their own. TEV-protease targets a sequence most commonly ‘ENLYFQS’ with its catalytic triad (10). Expressing the coiled-coil with a His tagged GFP will allow us to isolate and then cleave it. Therefore, our construct would be; N – His-tag – GFP – ENLYFGQ- coil – C. Using 50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, and 1mM DTT will function as a reaction buffer for the isolated protein after nickel chromatography (10). Typical reaction duration is overnight (10). A rule of thumb regarding how much TEV-protease to use is 1 OD at 280 nm of protease per 100 OD at 280 nm of substrate protein (10).

N – His-tag – GFP – ENLYFGQ- coil – C



Tobacco etch virus (TEV) protease system for small protein expression. Protein structures were obtained from the RCSB Protein Data Bank. Coiled-coil image generated using PyMOL. Images from L-R: 6 residue Histidine tag, green fluorescent protein, TEV-protease (grey) with ENLYFGQ (black), coiled-coil.TEV-protease system used to express small proteins such as a 35-residue coil where otherwise bacterial expression may prove difficult. His-tag and GFP are attached to a cut site 'ENLYFGQ' for the TEV-protease. His-tag and GFP to be cleaved by TEV-protease after purification by nickel chromatography leaving a functional coil protein on its own.

Supplementary tests: For proof of concept and quality assurance

Calculating competent cell efficiency:

This test is to ensure that the cells used in the ligation and transformation of the synthetic DNA is efficient and has been grown correctly. This is important – having efficient cells means that our synthetic DNA will be translated into protein at the highest possible rate. This test uses plasmid controls and SOC, growth medium. This protocol is used by iGEM and is in the Registry of Standard Biological Parts (11). Ensure the area is sterile with ethanol.

Method (11):

1. Thaw the competent cells on an ice bath. Label a 1.5 mL microcentrifuge tube for each transformation and chill these tubes on ice. (Triplicates of each concentration has been recommended)
2. Spin the DNA tubes with the controls to collect the DNA
    a. Spin for 20-30 seconds between 8000-10000 rpm.
3. Pipet 1 µL of DNA into each of the microcentrifuge tubes
4. Pipet 50 µL of compotent cells into each tube and flick
5. Shock the cells (heat-shock) and place in water bath for 45 seconds
6. Transfer tubes back on ice for 5 minutes
7. Add 950 µL SOC into each tube
    a. Incubate at 37C for 1 hour while shaking at about 200-300 rpm
8. Pipet 100 µL from each tube onto the plate, spreading the mix evenly
    a. Incubate at 37C for about 16 hours, positioning the agar side at the top and lid at the bottom
9. Count the number of colonies on the plate

Using this equation, you can calculate the competent cell efficiency (11):

$$Efficiency\ \left(\frac{cfu}{\text{mg}}\right) = \frac{[colonies\ on\ plate]\ x\ 1000\ ng/\text{mg}}{Amount\ of\ DNA\ plated\ (ng)}$$

Where the amount of DNA plated is:

$$Amount\ of\ DNA\ plated\ (ng) = Volume\ DNA\ added\ (1\ \text{mL})\ x\ concentration\ of\ DNA\ in \frac{ng}{\text{mL}}\ x\ \frac{volume\ plated\ (100\ \text{mL})}{total\ reaction\ volume\ (1000\ \text{mL})}$$

A competent cell should have an efficiency between 1.5e8 and 6.0e8 cfu/µg of DNA.

Restriction Digest:

This test is used to ensure that the DNA inserted into the plasmid backbone is the correct length – therefore of quality. Our DNA is optimized to be RFC10 compatible, so we adopt the restriction digest protocol used by iGEM in the Registry of Standard Biological Parts (12).

Method (12):

Note: Keep all components on ice.

1. Add 250 ng of DNA to be digested
    a. Add water to reach a total volume of 16 µL
2. Add 2.5 µL of NEBuffer2 (for optimal color activity)
3. Add 0.5 µL of BSA
4. Add 0.5 µL of both restriction digests EcoRI and PstI
    a. Mix and spin the tube
5. Incubate at 37C for 30 min, followed by heat incubation at 80C for 20 min to ensure the restriction digests enzymes are denatured
6. Run 8 µL of the mixture on a gel
    a. Based on the size of the band you can ensure the part length and plasmid are both accurate, it is a good idea to have a standard MW band

Miniprep:

Miniprep is used to identify the cloned DNA in the competent cell. This is a quality control technique for our application. The DNA elution's from the cell may be run on an electrophoretic gel and the bands will provide insight regarding if our transformation was successful. iGEM recommends the use of a Qiagen Miniprep Kit – after a brief internet search this seems to be a popular choice (13). This miniprep method adopts the protocol described by iGEM in the Registry of Standard Biological Parts (13). Note that 'TE' used is a buffer solution which includes Tris, a pH buffer, and EDTA.

Method (13):

1. Centrifuge cell culture to pellet cells – supernatant should be discarded
2. Suspend the pellet in 250 µL Buffer P1 and transfer to microcentrifuge tube (fully dissolve)
    a. Note Buffer P1 has RNase A and is from the Qiagen Miniprep Kit
3. Add 250 µL Buffer P2, invert tube several times until viscous and clear

   a. Limit reaction to 5 mins and do not vortex
4. Add 350 µL Buffer N3, invert tube several times – solution should be cloudy
5. Centrifuge at 13,000 rpm for 10 mins
6. Collect supernatant from centrifugation and pipette it into the QIAprep spin column
7. Centrifuge for 30 – 60 seconds, discarding the flow-through
8. (Optional step) Add 0.5 mL of Buffer PB to the spin column and wash it by centrifuging for 30 – 60 seconds, discarding the flow-through. This will remove nuclease activity.
9. Wash the QIAprep spin column adding 0.75 mL Buffer PE, centrifuge for 30 – 60 seconds
10. Discard flow-through, centrifuge for 1 min to remove wash buffer residue
11. Place QIAprep column in new 1.5 mL microcentrifuge tube
    a. Elute the DNA by adding 50 µL of Buffer EB (10 mM Tris Cl, pH 8.5) or water to the center of the QIAprep column
       i. Stand for 1 min, then centrifuge for 1 min

Standard curve – quantifying concentration of analytes:

A standard curve should be generated in order to quantify the concentration of analytes. This is a crucial test that must be done – it will directly affect how the fluorometer reads the interstitial fluid. The most basic method would be to input analytes of known concentration into the fluorometer device (that includes the protein constructs) and record the intensity.

The concentration of an analyte based off of the intensity (absorbance), can be determined using the Beer-Lambert law.

$$A = \varepsilon c l$$

Where A is the absorbance, ε is the molar extinction coefficient, c is the concentration in moles, and l is the distance between the light source and detector.

However, since we do not readily have available the molar extinction coefficient, we can generate a standard curve. That way using a linear regression, at any given intensity we can accurately estimate the concentration of metabolite. Although a non-linear regression may be better suited depending on the data – our proteins are all monomers and so we use a linear model for explanations sake. A non-linear fit may be required should the initial sample data appear that way.

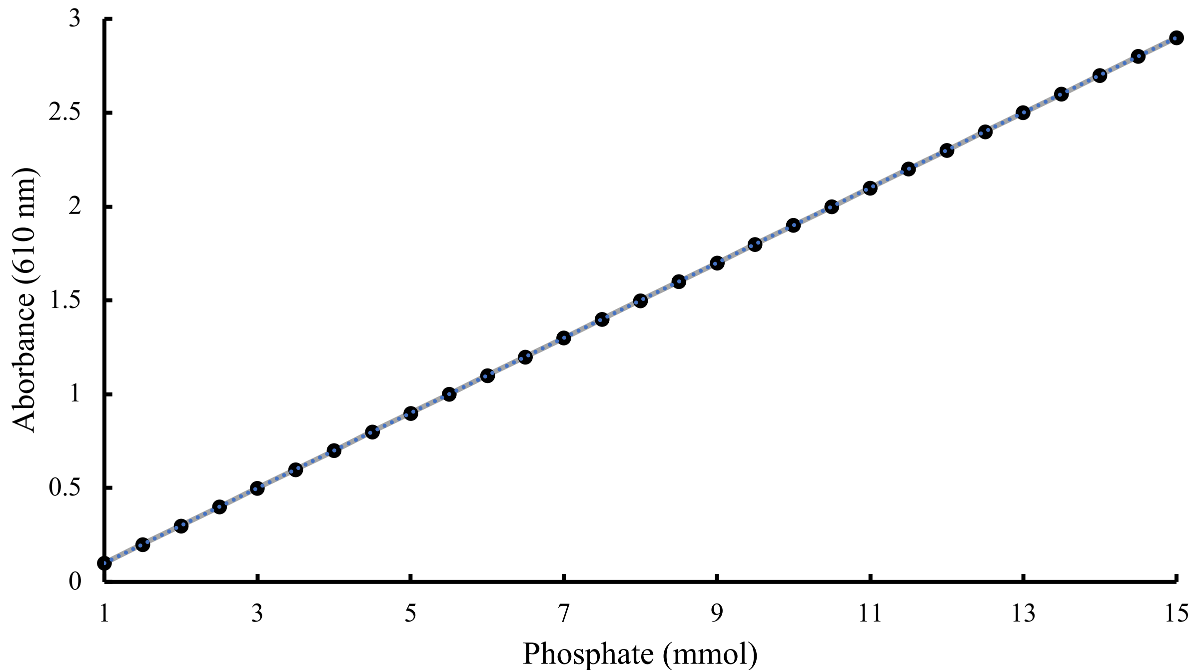
It is also important to note that when conducting this test – repeat the experiment multiple times. Since this data significantly effects how our device reports concentrations do more data collection than the ‘average value of 3 trials’ standard rule. The method described here is for phosphate but may be used with other analytes with small differences.

Method:

1. Clean the gold chamber of the biosensor apparatus which contains the binding protein with a TRIS buffer solution (used for solvation)
   a. The buffer
2. Pipette 1 mmol of phosphate solution into the biosensor apparatus

a. Pipette directly into the gold chamber
3. Close the device and turn on the biosensor to activate the fluorometer
   a. Wait 5 seconds to ensure an accurate reading
4. Record the intensity value given by the fluorometer in the biosensor apparatus
   a. The biosensor apparatus will add a protein buffer solution to wash out the existing phosphate and free the binding protein of any analyte. Wait at least 30 min before repeating the experiment with a different concentration.
   b. TRIS buffer should be used for this generation step

Note: Use concentrations at both very low and very high physiological ranges. This is so if a patient using the sensor has an abnormal analyte value, a clinician may use that data accurately for diagnosis and treatment. E.g. Phosphate values between 1-15 mmol. Increments should be 0.5 mmol or smaller for the most accurate standard curve regression.



Hypothetical phosphate standard curve. mCherry intensity at 610 nm was observed at phosphate concentrations ranging from 1-15 mmol in a biosensor apparatus. Using the standard curve, a linear regression may be used to determine phosphate concentrations based on intensity alone. Relationship between absorbance and phosphate concentration can be expressed as Abs = 0.2(phosphate conc. in mmol) - 0.1.

References:

1. Bajar BT, Wang ES, Zhang S, Lin MZ, Chu J. A Guide to Fluorescent Protein FRET Pairs. Sensors [Internet]. 2016 Sep 14 [cited 2020 Jun 2];16(9). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5038762/

2. Webster AC, Nagler EV, Morton RL, Masson P. Chronic Kidney Disease. Lancet Lond Engl. 2017 25;389(10075):1238–52.

3. Chen TK, Knicely DH, Grams ME. Chronic Kidney Disease Diagnosis and Management: A Review. JAMA. 2019 01;322(13):1294–304.

4. Help:Protocols/Competent Cells - parts.igem.org [Internet]. [cited 2020 Jul 6]. Available from: http://parts.igem.org/Help:Protocols/Competent_Cells

5. Chan W-T, Verma CS, Lane DP, Gan SK-E. A comparison and optimization of methods and factors affecting the transformation of Escherichia coli. Biosci Rep. 2013 Dec 12;33(6).

6. Help:Protocols/Ligation - parts.igem.org [Internet]. [cited 2020 Jul 7]. Available from: http://parts.igem.org/Help:Protocols/Ligation

7. Help:Protocols/Transformation - parts.igem.org [Internet]. [cited 2020 Jul 8]. Available from: http://parts.igem.org/Help:Protocols/Transformation

8. Protein Expression Using BL21(DE3) (C2527) | NEB [Internet]. [cited 2020 Jul 10]. Available from: https://international.neb.com/protocols/0001/01/01/protein-expression-using-bl21de3-c2527

9. Bornhorst JA, Falke JJ. [16] Purification of Proteins Using Polyhistidine Affinity Tags. Methods Enzymol. 2000;326:245–54.

10. tev.pdf [Internet]. [cited 2020 Jul 10]. Available from: https://mcl1.ncifcrf.gov/waugh_tech/faq/tev.pdf

11. Help:Competent Cell Test Kit - parts.igem.org [Internet]. [cited 2020 Jul 6]. Available from: http://parts.igem.org/Help:Competent_Cell_Test_Kit

12. Help:Protocols/Restriction Digest - parts.igem.org [Internet]. [cited 2020 Jul 7]. Available from: http://parts.igem.org/Help:Protocols/Restriction_Digest

13. Help:Protocols/Miniprep - parts.igem.org [Internet]. [cited 2020 Jul 9]. Available from: http://parts.igem.org/Help:Protocols/Miniprep