7.016 Recitation 18 – Fall 2018

*(**Note:** The recitation summary should NOT be regarded as the substitute for lectures)*

Summary of Lectures 27 (11/19) & 28 (11/21):

GFP: a critical tool for biological science research: The story of GFP begins with Osamu Shimomura's research into the phenomenon of bioluminescence, in which chemical reactions within living organisms give off light. While studying a glowing jellyfish in the early 1960s, he isolated a bioluminescent protein that gave off blue light. However, the jellyfish glowed green. Further studies revealed that the protein's emitted blue light was absorbed by a second jellyfish protein, later called green fluorescent protein (GFP), which in turn emitted green light. The ability of GFP to absorb blue light and emit green (its fluorescence) was found to be inherent to its structure, occurring without the need for any accompanying factors.

In 1988, Martin Chalfie heard about GFP for the first time, and realized that its ability for independent fluorescence could perhaps make it an ideal cellular beacon for the model organisms he studied. Using molecular biological techniques, Chalfie succeeded in introducing the gene for GFP into the DNA of the small, almost transparent worm *C. elegans.* GFP production does not require the addition of any extra components, and without any indication of causing damage to the worms. Subsequent work showed that it was possible to fuse the gene for GFP to genes for other proteins, opening-up a world of possibilities for tracking the localization of specific proteins in living organisms.

The opportunities offered by GFP were immediately obvious to many, as was the desire of extending the range of available tags. Roger Tsien first studied precisely how GFP's structure produces the observed green fluorescence. He then used this knowledge to tweak the structure to produce molecules that emit light at slightly different wavelength, which gave tags of different colors. With time, his group added further fluorescent molecules from other natural sources to the tag collection, which continues to expand. Complex biological networks can now be labeled in an array of different colors, allowing visualization of a multitude of processes previously hidden from view.

Osamu Shimomura, Martin Chalfie and Rogen Y Tsein were awarded the Nobel Prize in Chemistry in 2008 for their discovery of GFP. Today GFP provides biologists with a wide and powerful array of tools with which they can visualize cell function, study intercellular communications, intracellular reactions, changes in cell shape and mobility and make fluorescent organisms. The effect of the discovery of GFP is widespread. For further details of this very fascinating molecule, please go to:

http://www.youtube.com/watch?v=cfg75ft7oOw

Luciferase assay: **Luciferase** is a generic term for the class of oxidative enzymes used in bioluminescence. It is distinct from fluorescent proteins like GFP. A variety of organisms regulate their light production using different luciferases in a variety of light-emitting reactions, the most famous being fireflies.

The protein structure of firefly luciferase consists of two compact domains: the N- terminal domain (composed of two beta sheets) and the C- terminal domain that are connected to each other by a flexible hinge, which can separate the two domains. The amino acid sequence on the surface of the two domains facing each other are conserved in bacterial and firefly luciferase, thereby strongly suggesting that the active site is located in the cleft between the domains. During a reaction, luciferase has a conformational change and goes into a "closed" form with the two domains coming together to enclose the substrate. This ensures that water is excluded from the reaction and does not hydrolyze ATP.

The chemical reaction catalyzed by firefly luciferase takes place in two steps.

- Luciferin + ATP → Luciferin Adenylate +PPi
- Luciferin adenylate + O2 → Oxyluciferin + AMP + Light

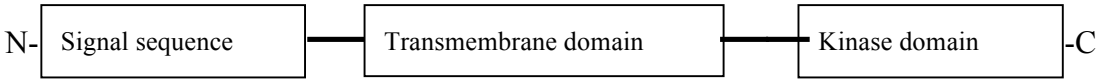
Light is emitted because the reaction forms oxyluciferin in an electronically excited state. The reaction releases a photon of light as oxyluciferin returns to the ground state. Photon emission can be detected by light sensitive apparatus such as a luminometer or modified optical microscopes. This allows observation of biological processes.

Luciferase can be produced in the lab through genetic engineering for a number of purposes. Luciferase genes can be synthesized and inserted into organisms or transfected into cells of many organisms such as mice, worms, plants etc. In biological research, luciferase is commonly used as a reporter to assess the transcriptional activity in cells that are transfected with a genetic construct containing the luciferase gene under the control of a promoter of interest. It can also be used to study enzyme activity or cell processes like programmed cell death (by measuring cytochrome C release from mitochondria). Luciferase can also be used to detect the level of cellular ATP in cell viability assay or for kinase activity assays. Luciferase is a heat sensitive protein that is used in studies on protein denaturation, testing the protective capacities of heat shock proteins. The opportunities for using luciferase continue to expand.

Microarrays: Not all cancers respond equally well to all treatments, so knowing the specific type or subtype of cancer is important for successful treatment. Some cancer subtypes can be identified by using histological tools, but other are best characterized by determining the gene expression profile. A DNA microarray is a multiplex technology that allows comparison of cells based upon the expression of many different genes. A DNA microarray consists of an arrayed series of thousands of microscopic spots of DNA, each spot representing a gene. When a DNA microarray is probed with mRNA isolated from cells, individual mRNA molecules will hybridize to the appropriate DNA spot. If a gene is highly expressed, more mRNA will be made from that gene, so more mRNA will hybridize to the corresponding DNA spot, and the signal from that spot will be greater.

Questions:

1. The following schematic represents the different domains that are the part of a protein kinase X (PKX).



Does the above schematic represent a…

a) Cytosolic/ Organelle specific/ Secreted or Membrane protein? **Explain** why you selected this option.

b) How does PKX activate its target protein? **Explain**.

c) Once the PKX has activated its target protein, how is this protein converted to its inactive state by a specific.…

i. Phosphatase? What are the amino acid residues that it can potentially act on?

ii. Protease? Briefly explain the mechanism.

2. You plan to clone a PKX gene. You start by fusing the DNA encoding GFP (green fluorescent protein) to the DNA encoding the C terminus of PKX gene. The following is the partial **cDNA sequence** encoding the C terminus of the PKX gene. The sequence encoding the stop codon is shown in bold. The bars above the sequence show restriction enzyme recognition sites.

```
          Z                    Y
5′-TCAAGAGGATCCCCGCGGTACCGAATTCCATGTTA TAG CAAGCTCGGAATTAACCCTCAC-3′
3′-AGTTCTCCTAGGGGCGCCATGGCTTAAGGTACAAT ATC GTTCGAGCCTTAATTGGGAGTG-5′
```

The following is the partial cDNA sequence encoding the N terminus of GFP. The codons are underlined. The bars above the sequence show the restriction enzyme recognition sites.

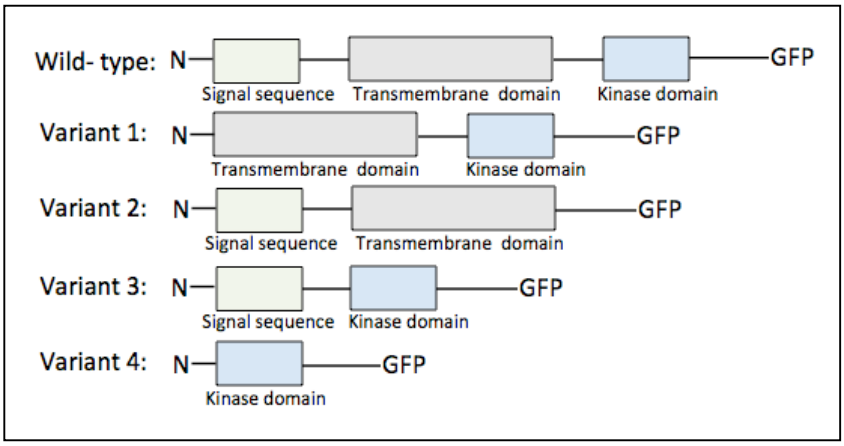
```
                Z          Y
5′-TCTAGAGGTACCGGGATCCGAATTCCC GTG CCA AGC GGC-3′
3′-AGATCTCCATGGCCCTAGGCTTAAGGG CAC GGT TCG CCG-5′
```

The recognition sequences and the cleavage sites (indicated by /) for each enzyme are given below.

Enzyme Z	*Enzyme Y*
5′ G/GATC C 3′	5′ G/AATT C 3′
3′ C CTAG/G 5′	3′ C TTAA/G 5′

Choose the restriction enzyme that you will use to cut the two genes before ligating them to make a fusion gene. **Explain** why you chose this restriction enzyme.

3. You create the following GFP –PKX variants.

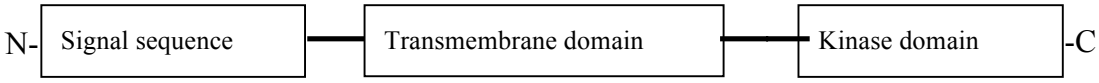


Complete the table for each of the variants provided.

Variant	Part of the cell that will fluoresce?	Is the PKX-GFP gene product functional protein? **Explain**
Wild- type		
1		
2		
3		
4		

Solution key:

1. The following schematic represents the different domains that are the part of a protein kinase X (PKX).



Does the above schematic represent a……

a) Cytosolic/ Organelle specific/ Secreted or Membrane protein? **Explain** why you selected this option.

It represents a membrane protein. The transmembrane domain that is present in this protein will stay in the ER membrane during translation leading to the protein in the cell membrane after vesicle fusion.

b) How does PKX activate its target protein? **Explain**.

As a kinase, it will activate its target proteins by phosphorylating them.

c) Once the PKX has activated its target protein, how is this protein converted to its inactive state by a specific….

iii. Phosphatase? What are the amino acid residues that it can potentially act on?

It acts by dephosphorylating the target protein. The amino acids with an –OH group in their side- chains are the ones that get phosphorylated (Serine, Threonine and Tyrosine), so these will be targeted by phosphatase.

iv. Protease? Briefly explain the mechanism.

They act by hydrolyzing the peptide bonds and breaking the target protein into individual amino acids.

2. You plan to clone a PKX gene. You start by fusing the DNA encoding GFP (green fluorescent protein) to the DNA encoding the C terminus of PKX gene. The following is the partial **cDNA sequence** encoding the C terminus of the PKX gene. The sequence encoding the stop codon is shown in bold. The bars above the sequence show restriction enzyme recognition sites.

```
          Z                    Y
5'-TCAAGAGGATCCCCGCGGTACCGAATTCCATGTTATAGCAAGCTCGGAATTAACCCTCAC-3'
3'-AGTTCTCCTAGGGGCGCCATGGCTTAAGGTACAATATCGTTCGAGCCTTAATTGGGAGTG-5'
```

The following is the partial cDNA sequence encoding the N terminus of GFP. The codons are underlined. The bars above the sequence show the restriction enzyme recognition sites.

```
                        Z         Y
5'-TCTAGAGGTACCGGGATCCGAATTCCC GTG CCA AGC GGC-3'
3'-AGATCTCCATGGCCTAGGCTTAAGGG CAC GGT TCG CCG-5'
```

The recognition sequences and the cleavage sites (indicated by /) for each enzyme are given below.

Enzyme Z	*Enzyme Y*
5' G/GATC C 3'	5' G/AATT C 3'
3' C CTAG/G 5'	3' C TTAA/G 5'

Choose the restriction enzyme that you will use to cut the two genes before ligating them to make a fusion gene. **Explain** why you chose this restriction enzyme.

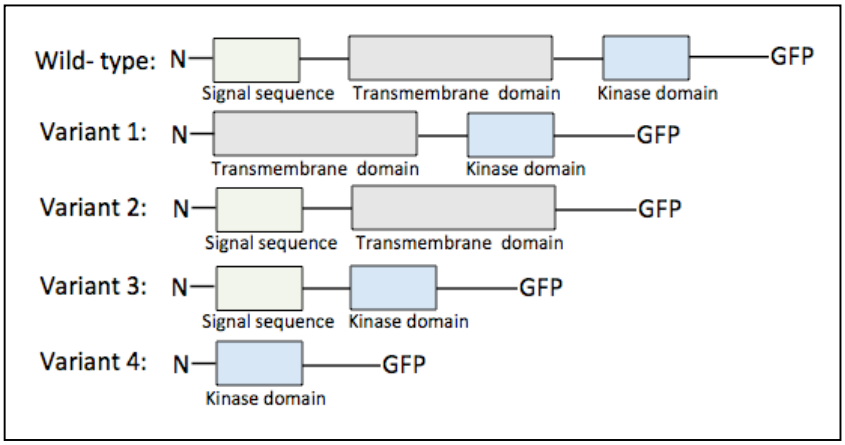
You CAN use restriction enzyme Z since it keeps the codons of the fusion gene in frame.

```
5'-TC AAG AGG ATC CGA ATT CCC GTG CCA AGC GGC-3'
3'-AG TTC TCC TAG GCT TAA GGG CAC GGT TCG CCG-5'
```

You cannot use the restriction enzyme Y since it puts the GFP cDNA out of frame.

```
5'-TC AAG AGG ATC CCC GCG GTA CCG AAT TC CCG TGC CAA GCG GC-3'
3'-AG TTC TCC TAG GGG CGC CAT GGC TTA AG GGC ACG GTT CGC CG-5'
```

3. You create the following GFP –PKX variants.



Complete the table for each of the variants provided.

Variant	Part of the cell that will fluoresce?	Is the PKX-GFP gene product functional protein? **Explain**
Wild- type	*Plasma membrane*	*Yes, this fusion protein has all the domains (its your wild- type!) needed for it to be localized at the right location and function. As shown in the schematic, GFP will be close to the cytoplasmic side of the plasma membrane.*
1	*Cytosol*	*No, In the absence of signal sequence it will be made as a cytosolic protein and will most likely be degraded by proteasome complex.*
2	*Plasma membrane*	*No, In the absence of its Kinase domain it will not be able to phosphorylate its target protein and hence will be non-functional*
3	*Outside of the cell in the extracellular environment*	*No, In the absence of its transmembrane domain it will be made a secretory protein that will be released by the cell into the extracellular space. It will again be non-functional since it is not at the correct location.*
4	*Cytosol*	*No, In the absence of signal sequence and transmembrane domain it will be made as a cytosolic protein and will most likely be degraded by proteasome complex.*

7.016 Introductory Biology
Fall 2018