ZO-1 Sequence Analysis: A Paper and Computer-based Exercise in Cloning

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Molecular cloning is used to study the relationship between the structure of a protein and its function. However, the process can be time consuming and not necessarily practical in the constraints of a 3hr weekly lab. This "dry" lab walks students through key points of the process used to develop the plasmids needed to express altered ZO-1 proteins within epithelial cells in order to understand the role of each binding domain in the formation and function of the tight junction. Students practice finding and labeling protein domains and enzyme restriction sites within the ZO-1 DNA sequence and plasmid sequences. They also explore some methods used to put a sequence of interest into expression vectors. This lab can be used as a stand-alone unit or as the final exercise of your favorite cloning wet lab. This lab is recommended for a 300 or 400 level cell and molecular biology type course.

Introduction

Cloning is a topic frequently covered in advanced cell biology courses. In our labs, our students often transform competent cells with a pre-established plasmid to express a protein of interest. However, our students don't necessarily understand the process that underlies the creation of the plasmids. We also often discuss protein structure-function relationships in the cell biology lecture, but do not have a lab related to this topic. I developed this lab based on my post doctorate research project in order to provide students a glimpse into the process required to study the relationship between protein structure and its function through the use of molecular cloning. In this "dry" lab students walk through the process I used to create the plasmids needed to express the full tight junction scaffolding protein, Zonula Occludens (ZO-1), and truncated versions of the protein lacking different binding domains. I ultimately used the truncated proteins to study the role of each binding domain in the formation of tight junctions in epithelial cells. Ideally, this lab should be completed within a three-hour lab period, but it could be modified to be finished within a two-hour lab period or expanded into two lab periods. I personally have chosen to do this exercise during lab instead of during lecture because of the amount of time required for students to work through the entire process. You also may choose to use this lab in conjunction with a cloning based wet lab. I have used cloning lab kits from BioRad some years and use this lab as a follow up showing an example of how molecular cloning was used for a published study.

Prior to the lab, students use their textbook and any other resources available to them to research the function of tight junctions and ZO-1 in epithelial cells. I will also request that they download the free plasmid viewing program, SnapGene viewer, prior to arriving to class. At the start of the lab period I lead a short discussion about tight junctions, ZO-1, and the functional binding domains within ZO-1. I then explain the purpose of my post doctorate research (more details provided in instructor notes), outline the lab they will be completing, and then allow them to begin working through the assignment at their own pace. Throughout the lab, students will practice looking at DNA sequences, performing BLAST searches, aligning and comparing two different sequences, making predictions based on provided data, and discussing the limitations that may arise during protein structure-function studies.

This lab is not an "easy" lab for students to complete on their own, so it is important for the lab instructor to circulate the classroom and double check that students understand each step as they are working. I will provide details in the instructors notes regarding areas in

which students are more likely to be confused and thus require extra guidance.

I have two main goals for this lab. The first is for students to work through the thought process required to generate plasmids for a structure-function assay. The second is for students to see a real example of how "messy" science can be. The lab has several examples in which protocols did not work the first time and the results were not what was expected based on the current knowledge of the protein domain functions.

Student Outline
Sequence Analysis – An Exercise in Cloning

Student Pre-Lab Assignment

Use your textbook and any other resource (online is acceptable) to answer the following questions. If you use a source other than your textbook you will need to cite your source. This assignment will not be formally graded, but you will be given credit for completing the questions.

1. What is the function of the tight junction? What type of cells have tight junctions?
2. What proteins are found within the tight junction region of a cell?
3. What role do Zonula Occludens (ZO) proteins play in the structure and/or function of the tight junction?
4. Why might a researcher want to knock out ZO-1 from a mouse or epithelial cell line?

Software you need to download to your computer prior to lab:

Snapgene:
http://www.snapgene.com/products/file_compatibility/DNASTAR_Lasergene/

Note: At least one person per lab pair will need to have a computer in lab this week in order to complete the assignment!

For this exercise you will use a realistic cloning scenario in order to practice analyzing DNA sequences. This assignment is to be completed during lab and turned in before you leave. Write your answers on a separate piece of paper. You will find all of the required sequences in this week's lab folder online.

Scenario

You work in a lab that studies tight junctions (TJ). In order to understand the role of ZO-1 during the formation and stabilization of the TJ, you are tasked with creating ZO-1 constructs that lack individual binding domains and then expressing these constructs in cells that lack ZO-1. ZO-1 is a multi-domain scaffolding protein that binds to many different TJ proteins. The ability to bind to other TJ proteins is key to forming a functional TJ. Your boss hopes that this experiment will allow you to determine which individual binding domains within the ZO-1 proteins are required for normal TJ formation and function. Thankfully, you already know that the C-terminus half of the protein is not required for TJ formation and function, therefore you only need to focus on the N-terminus half of the protein.

The first half of this lab will help you better understand the ZO-1 structure and sequence. The second half of this lab will be focused on the steps required to create the expression constructs.

Problem Set A

Prior to constructing your vectors, let's first look more closely at the ZO-1 sequence. If researchers have a sequence for an unknown protein, or they want to know how similar their protein is to other proteins, they will perform a blast search. Open up your web browser and either Google "BLAST" or go to https://blast.ncbi.nlm.nih.gov/Blast.cgi

Each question is followed by instructions on what to do in order to figure out the answers.
1. **Which chromosome is ZO-1 found on?**
To find this answer, go the BLAST home page and click on "human." This will allow you to compare a portion of the ZO-1 sequence to the human genome. Open the ZO-1 sequence word document. Copy the DNA sequence for amino acids 1-876 (aa1-876) sequence and paste it into the large box labeled "enter accession number(s), gi(s), or FASTA sequence(s). Under "program selection" select "highly similar sequences." Click on the "BLAST" sequence. Once the search is complete, the software will list a description of the matches it has found, including the chromosome where your sequence is located. If you did not know what protein your sequence encoded, you would be able to scroll down and look at the "features" section to see what protein your sequence matched to.

2. **There are a total of three ZO proteins (-1, -2, and -3).**
a. **What other ZO protein is ZO-1 most similar to and what chromosome is this ZO protein found on?**
b. **How similar are the two proteins within the sequence region you searched?**

To find out, repeat your BLAST search, but this time use the DNA sequence for the aa115-317 portion of the ZO-1 sequence. You will also need to optimize for "somewhat similar sequences" instead of "highly similar sequences." Scroll through the results until you find a ZO protein.

3. **You have been provided with the human ZO-1 sequence. How similar is the human sequence to the chimpanzee, mouse, and dog ZO-1 sequence?**
   Return to the BLAST home page and click on "mouse" then perform a BLAST search just as you did for question 1. To complete a chimpanzee search you will have to return to the BLAST home page and type "Pan troglodytes" in the BLAST genome box. To complete a dog search, you will have to return to the BLAST home page and type "dog" or "9615" in the BLAST genome box. If you have trouble finding any of these organisms when doing a BLAST search, refresh the BLAST page and then try the search again.

Problem Set B

Next you need to create a map of the domains within the ZO-1 sequence. This map will help you later when you are constructing the plasmids you will use to express each of your modified ZO-1 proteins. To complete this problem set, you will need to use the ZO-1 domain deletion file (pdf) and the printed ZO-1 sequence provided with these instructions.

Each question is followed by instructions on what to do in order to figure out the answers.

1. The domain deletion file indicates the boundaries of each of the deletions you will need to create for your structure/function analysis of ZO-1 within epithelial cells.
   Label each of these boundaries on the ZO-1 sequence print out.
   For example, a line has been drawn through the sequence at the start of the PDZ1 domain and labeled "□P1 start," and a second line has been drawn through the sequence at the end of the PDZ1 deletion and labeled "□P1 end." Repeat this process yourself for the PDZ2, PDZ3, SH3, U5, GUK, and U6 domains. Note: The domain deletion file indicates the amino acid location, not the nucleotide location. You will need to convert the amino acid numbers to nucleotide locations.

2. To create the ZO-1 construct lacking the PDZ1 domain you will need to use *Eco*R1 and *Nhe*I restriction enzymes to excise the PDZ1 domain. The *Eco*R1 site is not within the ZO-1 sequence, instead it is within the cloning vector we will be using (more about that later), but the *NheI* site is within ZO-1 gene.
   Label the location of the *Nhe I* cut site for these enzymes on your printed ZO-1 sequence.
   To locate the enzyme cut sites you will need to open the z1-876.seq file using the SnapGene software you downloaded prior to lab (or right now if you forgot). When prompted, be sure the "linear" is selected and not "circular." Click "view" and select "sequence." You should now see the linear sequence with a few enzyme sites listed. If the enzymes you are looking for are not listed, click "view" and select "enzymes." You should now see a list of enzymes and their location within the ZO-1 sequence. Next to "chosen enzymes" you can switch the setting to "New England Biolabs." Now you have a very long list of restriction enzymes and their location in the ZO-1 sequence. Scroll through and look for the enzymes listed above.

3. To create the ZO-1 construct lacking the GUK domain you will need to use the *Acc*1 restriction enzyme. **Label the location of the cut sites for this enzyme (there are three – we later engineered one of them out of the sequence) on your printed ZO-1 sequence.**

4. Now let's try to predict what we can learn by deleting individual binding domains. Open the file titled "ZO-1 binding partners." This document lists the proteins that are known to bind to each binding domain.
a. **Which domain would you need to delete from ZO-1 in order to study what happens in the absence of interactions with the following proteins: claudin, occludin, other ZO proteins, JAM, or ZONAB.**
b. **Why might it be hard to interpret the phenotype of a ZO-1 protein lacking PDZ1 or SH3?**

Problem Set C

Now it is time for you to work on assembling your ZNAdeltaPDZ2 (n-terminus half of ZO-1 lacking the PDZ2 domain) expression construct. In order to express an exogenous protein within a cell you must first insert the DNA sequence for the protein into an expression vector. This vector must be selectable – meaning it must contain an antibiotic resistance gene. Once we transform our cells with the vector, we can treat them with the appropriate antibiotic. Only the cells that contain the vector with the antibiotic resistance gene and the DNA sequence for our protein of interest will survive. For this project you will be inserting the n-terminus portion of the ZO-1 gene into a pTRE expression vector. First, you will need to

familiarize yourself with the pTRE vector, and then you will begin creating a ZNA (n-terminus half of ZO-1) DNA construct lacking the PDZ2 domain.

1. **a. What enzyme cut sites flank the multiple cloning site (MCS) within the pTRE vector?**
b. How will this vector be selected for once it is placed into cells (what antibiotic resistance is contained in this plasmid)?
c. What is the promoter driving the expression of your DNA sequence?
d. Why is a polyA region included after the MCS?
To answer these questions, you will need to open the pTRE sequence within SnapGene viewer. When prompted, be sure "circular" is selected. You should now see a map of the pTRE cloning vector.
Thankfully for you, you do not need to start from scratch when making your deletion construct. Last year your boss made all of the deletion constructs you need in a shorter version of ZO-1, a version that stops at the end of the GUK domain. However, these constructs are in a pCB6 plasmid, not a pTRE plasmid. You do have ZNA (no deletions) in a pTRE construct. All you need to do is replace the domain sequence in the pTRE vector with the modified sequences from the pCB6 vector. You will be able to accomplish this task by using the restriction enzyme sites you marked in problem set B.

2. After performing a restriction enzyme digest with *Eco*R1 and *Nhe*I you will need to run the digest products out on an agarose gel. This will allow you to isolate the open vector (pTRE vector) and insert (small region of ZO-1 lacking PDZ2). Figure 1 below represents the results (taped into your lab notebook) of your enzyme digest. The three columns containing two bands each on the left represent the enzyme digest of pTRE (vector containing your ZNA construct without deletions) and the three columns containing two bands each on the right represent the enzyme digest of pCB6 (vector containing the shorter version of ZO-1 with a deletion – provided by your boss).
a. Why are there two bands in each column?
b. Why did you circle the top bands of the pTRE digest and the bottom bands of the pCB6 digest as the correct ones to isolate?
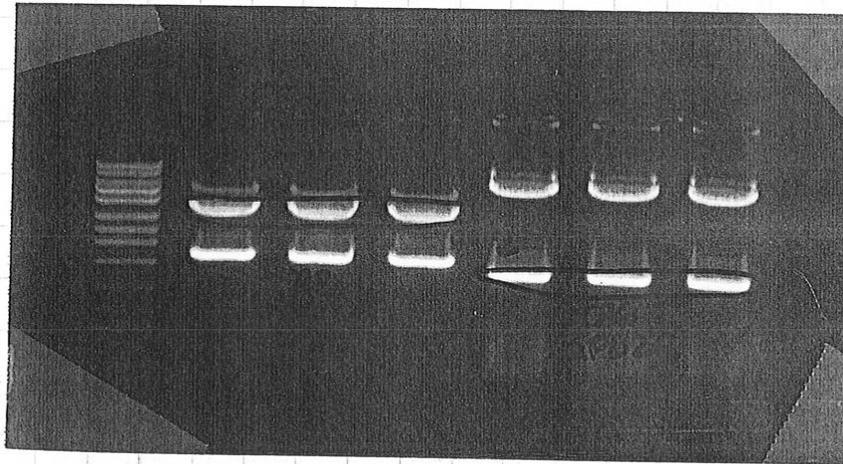

Figure 1. Products of the *EcoR1* and *NheI* restriction enzyme digest of pTRE vector samples (columns 2-4) and pCB6 vector samples (columns 5-7). The top pTRE bands and the bottom pCB6 bands are circled in order to represent which bands will be purified from the gel and used for the next step in this project.

The next step is to ligate your insert (ZO-1 lacking PDZ2) into the open pTRE vector. The next step is to confirm that your ligation was successful. You accomplish this task by transforming competent bacteria cells with your new plasmid, selecting for colonies containing your plasmid with antibiotics (bacteria that live contain your plasmid and antibiotic resistance gene), growing a very large quantity of these colonies, re-isolating your plasmid, and then sending the plasmid off to be sequenced. In order to sequence, the plasmids sequencing primers that flank your region of interest in ZO-1 must be used. When your sequences are returned to you, you have to analyze them in order to determine which colonies have plasmids containing your ZNAdeltaPDZ2 construct.

3. Open the document titled "ZNAdeltaPDZ2 sequences." Within this word document are three sequences labeled ZNAdeltaPDZ2 seq1, ZNAdeltaPDZ2 seq2, and ZNAdeltaPDZ2 seq3. **Which plasmid(s) contain(s) your construct of interest?**

   To analyze the sequences, you will need to open a free online software called LALIGN. Either do a Google search for "LALIGN" and click on the link titled "LALIGN server" or go to the following link http://www.ch.embnet.org/software/LALIGN_form.html

   This software will allow you to compare the sequence of your plasmid to that of ZO-1. You are looking for plasmid sequences that lack the PDZ2 domain (check your original map to determine what region you are looking at). Copy and paste your ZO-1 sequence into the "1st query" box and past your sample sequence into the "2nd query" box. Click "run align" and analyze your results. You will need to look at the actual sequence alignment in order to compare the two sequences and confirm that the correct portion of your gene is missing. Simply looking at the percent similarity will not provide you with enough information to determine which plasmid you need to keep.

Problem Set D

   Now that you have successfully created a plasmid that will allow you to express ZNAdeltaP2 in your epithelial cells, you will next create a plasmid containing the sequence for ZNAdeltaGUK.

1. Just as above, your first step is to use an ACC1 restriction enzyme to digest both pTRE containing the full length ZNA sequence and the pCB6 plasmid containing a ZO-1 sequence lacking the GUK domain. Figure 2 shows the results below.

   a. What do your results tell you about the number of ACC1 cuts within the pCB6 vector itself?

   b. Did your ACC1 pTRE digest work? Explain your answer.

   c. Did the digest work the second time you repeated the digest? Explain your answer.
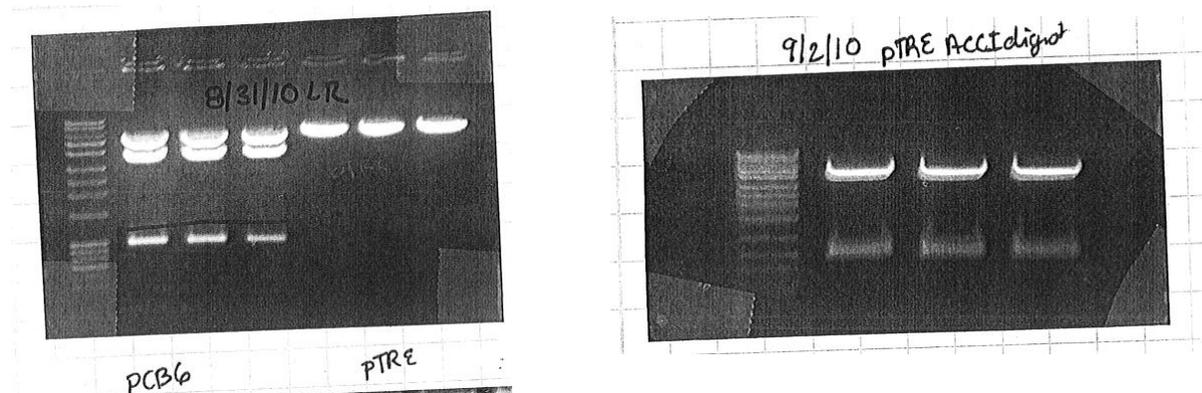

Figure 2. The gel on the left (8/31/10) shows the products of the *ACC1* restriction enzyme digest of pCB6 vector samples (columns 2-4) and pTRE vector samples (columns 5-7). The the bottom pCB6 bands are circled in order to represent which bands will be purified from the gel and used for the next step in this project. The gel on the right (9/2/10) shows the products of the second *ACC1* restriction enzyme digest of pTRE vector samples.

   2. After you ligate your insert (deltaGUK region) into your vector (pTRE) you will need to check your sequences. Open the file titled "ZNAdeltaGUK sequences." This word document contains three sequences you received from the sequencing facility. You will first need to determine whether you have "good" sequences. Sometimes a sequence comes back and it is very short or contains a lot of "N's," indicating that you do not have enough sequence information to analyze.

   a. Which sequencing reactions resulted in "good" sequences that can be analyzed?

   b. Of the reactions that worked and provided "good" sequences, which reactions contain the correct deletion and can thus be used in your epithelial cells?

Follow Up:

   The plasmids with a "good" sequence are used to transfect MDCK cells lacking ZO-1 and ZO-2. A different MDCK culture is created for each of the binding domain deletions. We then assessed the cells for tight junction formation and function. The results of this study can be found in the following reference.

Cited References

Rodgers LS, Beam MT, Anderson JM, Fanning AS. 2013. Epithelial barrier assembly requires coordinated activity of multiple domains of the tight junction protein ZO-1. J Cell Sci. 126(7):1565-1575.

Materials

Each student will need a computer with internet access and a downloaded trial of SnapGene.

Notes for the Instructor

Lab Set Up

Each pair of students will need a laptop with access to the internet. SnapGene (or a similar program) needs to be installed on all computers prior to the start of lab. SnapGene provides a one-month free trial, which is sufficient for the purposes of this lab. I do not have a site license for SnapGene. Instead, my students use the free trial to download the program onto their own computers just prior to or at the start of lab. Students will need a printed copy of this lab and a printed copy of the ZO-1 DNA sequence. While you could complete the lab using all electronic documents, I find that my students prefer to complete this exercise using printed copies. Your students will also need access to an electronic folder containing all of the sequencing files. Be sure you have worked through the lab yourself at least once before presenting it to students so that you can more easily explain each step.

Length of Lab

This lab can be completed in a 3hr time period if students are focused. However, if you have a shorter lab period, or your students tend to work slowly, the lab can be broken up into two pieces. The first lab period can be used to complete part A and B and the second lab period can be used to complete part C and D.

Starting the Lab

This lab is taught in my 400-level cell biology course. This course is usually taken by Juniors and Seniors, and thus I expect them to know basic cell structures prior to the course. I start the lab with a PowerPoint containing images of the tight junction (can be provided upon request), but I do not lecture to the students, instead I ask leading questions based on their pre-lab assignment in order to ensure that everyone understands what the tight junction is and how the proteins within the tight junction lead to its structure and function. The information I want my students to understand before we start the lab is outlined below.

What Is the Tight Junction?

The tight junction is a structure found above the adherens junction within epithelial cells. Its function is to seal the space between two epithelial cells and regulate what is allowed to pass through that space. Many students do not realize that the tight junction will, in fact, allow ions to pass through the space between cells – but the type of claudin present in the tight junction controls the type of ion that can pass through this paracellular space (Anderson and

Van Itallie 2009). For example, the movement of sodium ions through the paracellular space in-between epithelial cells of the intestine is controlled by the tight junction and absolutely necessary for the absorption of glucose from food within the intestine (Wada et al 2013). In the kidneys, the type of claudin present in the tight junctions of the kidney tubules changes as you move through the tubule system – matching the changes in cell function in different regions of the kidney (Elkouby-Naor and Ben-Yosef 2010).

What Proteins Make Up the Tight Junction?

There are three transmembrane proteins within the tight junction – claudins, occludins, and the JAM proteins. The claudins of one cell will bind to the claudins on another cell – closing the space between two cells and creating a pore for claudin-specific ions to pass through the space. Occludins provide additional stability to the tight junction and assist in maintaining the barrier function of the tight junction. The inner cellular portion of the tight junction is very complex and contains the scaffolding proteins ZO-1, ZO-2, and ZO-3, the cytoskeleton protein actin, and other proteins that do not play a role in the function of the tight junction such as transcription factors. ZO-1 and ZO-2 are both required for the formation and maintenance of a functional tight junction. ZO-1and ZO-2 are both large proteins with multiple binding domains. They serve as scaffolding proteins by binding to claudins, occludins, cytoskeletal linker proteins such as catenins, and each other (Van Itallie and Anderson 2014). Our understanding of the tight junction proteins is constantly improving. I highly recommend that instructors do a quick literature search before teaching this lab in order provide students with the most up to date information.

Background on this Project

The results of this complete project are published in the Journal of Cell Science (Rodgers et al 2013). You may choose to have your students read the paper before or after this exercise, however it is not an easy paper to read. I usually explain the project to my students, and then show them the figures from the paper after the exercise. The purpose of this project was to determine the role of each major binding domain of ZO-1 in the formation and function of tight junctions. We knew from a previous study that the c-terminus half of ZO-1 is not necessary to form a functional tight junction, so I focused on deleting domains from the N-terminal half of the protein. Once plasmids were created that could express the N-terminal half of ZO-1 minus a binding domain, we transfected MDCK cells that lacked both ZO-1 and ZO-2. ZO-1 and ZO-2 have overlapping function, so you cannot eliminate tight junction formation without removing both from a cell. Once the constructs were expressed within MDCK cells, I used immunofluorescence microscopy to look at tight junction formation and cytoskeleton organization. I also

used fluorescein-dextrans and TER (transepithelial electrical resistance) to measure paracellular permeability, and thus whether the tight junction that did form was functional.

Areas where Students Struggle

I have found that every group of students I work with finds a different area of the case study to be difficult. In general, I introduce students to the lab and then turn them loose and will circulate to answer questions. Make sure you are familiar with SnapGene and the lab itself before running the case study with your students. The most frequent questions are in regards to using SnapGene because inevitably someone will click where they are not supposed to in SnapGene and then their screen no longer matches what they are reading in the text. The other area that I have to help some students is when they are mapping out the domains in the beginning. They will often miss the instructions to convert the amino acid sequence numbers to nucleotide sequence numbers. Finally, be prepared to draw the process of moving the insert from the pCB6 plasmid and putting it into the pTRE plasmid. A drawing on the board is often the only way some of my students can visualize what is being accomplished experimentally.

Supplemental Materials

The sequence files can be found on the ABLE website or by contacting Laurel Rodgers at lrodgers2@su.edu. You can find the ZO-1 domain deletion file needed for part A in Figure 2 of the original paper (Rodgers 2013).

Lab Follow-up

At the end of the lab I conclude by showing my students several figures from the original paper. I particularly like to point out the results that were "weird." For example, you would expect occludin to NOT be recruited into the tight junction if the GUK domain, which binds to occludin, is removed. However, occludin is still found at the tight junction in the absence of the GUK domain. In fact, the P1 and the P2 binding domains appear to be more important for recruiting and maintaining occludin at the tight junction. You can also point out in figure 7 that science is not always "pretty" and exactly the same from one trial to another. In this figure, we show the results of epithelial barrier function in two different clones created for each domain deletion. While the results are frequently similar, they are never exactly the same. The greatest difference in results can be seen between the two clones with the P2 domain deletion (Rodgers 2013).

Post-Lab Assignment

At this time, I do not have a post lab assignment for this lab. However, I have thought of two possible options if you want a post lab assignment. The first would be for you to have your students analyze the results of the original paper themselves and point out areas in which the data appears "odd" or does not match up with their predictions. For the second option, it may be interesting for students to complete a literature search to find out what has been learned more recently about the structure and function of the ZO-1 protein or the Tight Junction itself.

Cited References

Anderson JM, Van Itallie CM. 2009. Physiology and function of the tight junction. Cold Spring Harb Perspect Biol. 1(2): Doi: 10.1101/cshperspect.a2002584

Elkouby-Naor L, Ben-Yosef T. 2010. Functions of claudin tight junction proteins and their complex interactions in various physiological systems.Int Rev Cell Mol Biol. 279:1-32.

Rodgers LS, Beam MT, Anderson JM, Fanning AS. 2013. Epithelial barrier assembly requires coordinated activity of multiple domains of the tight junction protein ZO-1. J Cell Sci. 126(7):1565-1575.

Van Itallie CM,Anderson JM. 2014. Architecture of tight junctions and principles of molecular composition. Semin Cell Dev Biol. 36:157-165.

Wada M, Tamura A, Takahashi N, Tsukita S. 2013. Loss of claudins 2 and 15 from mice causes defects in paracellular Na+ flow and nutrient transport in gut and leads to death from malnutrition. Gastroenterology. 144:369 – 380.

Acknowledgments

About the Authors

Dr. Laurel Rodgers is an Associate Professor at Shenandoah University, where she teachers General Biology, Cell Biology, Developmental Biology, and Biology in Society.

Appendix A
Student Laboratory Hand Out – Answers

Problem Set A
1. Which chromosome is ZO-1 found on? Chromosome 15
2. a. There are at total of three ZO proteins (-1, -2, and -3). What other ZO protein is ZO-1 most similar to and what chromosome is this ZO protein found on? ZO-2, chromosome 9
   b. How similar are the two proteins within the sequence region you searched? 75% similar
3. You have been provided with the human ZO-1 sequence. How similar is the human sequence to the chimpanzee, mouse, and dog ZO-1 sequence? mouse-97%, Chimp-99%, dog-99%

Problem Set B
1. Label each of these boundaries on the ZO-1 sequence print out. Answers provided on labeled sequence file.
2. Label the location of the *Nhe*I cut site for these enzymes on your printed ZO-1 sequence.
   Answers provided on labeled sequence file.
   Label the location of the cut sights for this enzyme (there are two) on your printed ZO-1 sequence. Answers provided on labeled sequence file.
3. a. Which domain would you need to delete from ZO-1 in order to study what happens in the absence of interactions with the following proteins: claudin, occludin, other ZO proteins, JAM, or ZONAB? Claudin-delete PDZ1, Occludin-delete GUK, ZOs-delete PDZ2, JAM-delete PDZ3, ZONAB-delete SH3
   b. Why might it be hard to interpret the results of a ZO-1 protein lacking PDZ1 or SH3?
   PSZ1 and SH3 domains have many binding partners

Problem Set C
1. a. What enzyme cut sites flank the multiple cloning site (MCS) within the pTRE vector? ECOR1 and XbaI
   b. How will this vector be selected for once it is placed into cells? AMP resistance
   c. What is the promoter driving the expression of your DNA sequence? TET responsive region
   d. Why is a polyA region included after the MCS? To protect the mRNA from degradation
2. a. Why are there two bands in each column? When the plasmid is cut in two locations,
   b. Why did you circle the top bands of the pTRE digest and the bottom bands of the pCB6 digest as the correct ones to isolate? You want the pTRE vector (larger fragment) and the pCB6 insert (ZO-1 fragment)
3. Which plasmid(s) contain(s) your construct of interest? Seq 1 and 2

Problem Set D
1. a. What do your results tell you about the number of ACC1 cuts within the pCB6 vector itself? There are three cut sites
   b. Did your ACC1 pTRE digest work? Explain your answer. NO, because you only have one band
   c. Did the digest work the second time you repeated the digest? YES, because you have two bands Explain your answer.
2. a. Which sequencing reactions resulted in "good" sequences that can be analyzed? Seq 1 and 2
   b. Which reactions contain the correct deletion and can thus be used in your epithelial cells? Seq 2

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