Module:
Techniques in Neuroscience

Week 5
Molecular biology: Going inside the cell

Topic 1
An introduction to molecular biology methods – Part 1 of 3

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In the previous week, we discussed how to visualise proteins in tissues using immunohistochemistry. Now, this approach is very useful because we can analyse protein distribution in the cells, we can determine whether pathological changes have occurred and we can analyse morphological features of stained cells. However, histochemistry on its own can't give you enough information to work out the complex signalling pathways that you can see on this image.

To discover and understand the function of detailed pathways like these, we need to be able to determine what protein cells and tissues contain and in what quantities, how these proteins are modified and which of these proteins interact with each other. So, this topic is in three parts and explains the methods that can be used to find the answers to these questions.

The first part is about recombinant DNA technologies, which gives us a means of producing the proteins that we're interested in, in quantities that we can study. Having successfully employed a means of producing the protein we're interested in is only half the challenge though. We still need a way of looking at the protein we've gone to such efforts to produce, so this means we need a way to separate the protein of interest from all the other proteins in the cell, and then we need a way of identifying the protein once it's been separated out.

So, in the second part of this topic, I'll describe SDS-PAGE which is a protein separation technique, and in the third part of this topic I'll describe western blotting which is a protein identification technique. As cell biologists, our aim is to often to understand the role of certain proteins in various cellular processes or signalling pathways. This could be very complex and can involve a lot of different proteins. To investigate the protein we're interested in, we need a way to identify or manipulate it. There are a variety of ways we can identify and manipulate proteins using recombinant DNA technologies. Recombinant DNA technologies involve artificially joining together DNA from different species. We can use recombinant DNA technology to artificially manipulate the protein we're interested in inside the cell. With these technologies, we can force the cells to make extra copies of our protein or we can remove the protein that the cell naturally makes. We can also make the cell create mutant versions of the protein or we can make the cell create tagged versions of the protein.

We can then look at the phenotype of the cell when it has none of the protein, normal levels or levels that are too high, and we can compare the mutant versions to wild-type versions of the protein. These can all give us clues about what the protein does functionally. For example, if a process stops happening when we remove the protein, then probably that protein is involved in that process. Mutant versions of a protein can be useful for working out what part of the protein is important for its function. For example, we can mutate what we think is the active site of an enzyme and then see if the enzyme is still catalytically active.

Tagging a protein is useful because it helps us find our protein amongst all the other proteins in the cell. For example, the tag could be something that fluoresces so we can literally see the location of the protein under a microscope, or it could be something which make it easy to purify our protein from the cells, or it could be something that binds antibodies very well, and you'll see why this might be important in the third section of this topic.

Before we go into detail of how we employ recombinant DNA technologies, let me first give you an overview of the steps we carry out. So, in the first instance, we're interested in investigating a particular protein in order to study its importance and function. To study it, we often, but not always, want to make it in significant amounts within working cells. So, we get hold of the gene that codes for the protein - we can either buy it or obtain it from a colleague. We also need something to express that gene and generate actual protein, something that we term the 'vector'. The vector we use is called a plasmid, a circle of DNA found in bacteria. The genes we obtain often already come in a plasmid, though we usually want to change that plasmid to one of our choice. So, we have to take the gene out of the original plasmid and insert it into the desired plasmid - a process we call cloning. To do this, we can use a technique called Polymerase Chain Reaction or PCR. This can make us millions of copies of a particular section of DNA, giving us plenty to work with.

Next, we insert the cloned plasmid into bacterial cells, which we call bacterial transformation. The transformed bacteria will make thousands of copies of the plasmid as they grow. We can then purify the plasmid from the bacteria so that we have a lot of DNA to work with. We can use the DNA to express the protein in cell types we're interested in, such as neurons, skin cells, etc. And finally, we extract the proteins from the cells and use special techniques to separate them from the other cell contents, in order to assay them. So, in the screens that follow, I will explain these steps in much more detail, starting with the use of plasmids.

A vector that we choose to express gene in cells is a plasmid, which is a circular piece of DNA. The plasmid contains the gene for the protein you are interested in, as well as promoter for that gene. We need to choose the promoter carefully - one that will be activated in the type of cells we want our protein to be expressed in, and also an antibiotic-resistance gene, so that once we've transformed bacteria with the plasmid, we can use the antibiotic resistance to selectively grow bacteria containing that plasmid. Often, the plasmid will also have the eukaryotic resistance marker as well so we can select the eukaryotic cells, such as nerve cells, into which we may have inserted the plasmid. The gene in the plasmid is the exact sequence of the gene, and does not contain any introns or upstream and downstream sequences.

This also means you could only express one isoform of the protein, so you have to choose which isoform of the protein you want. As I said earlier, when we get the DNA sequence for the protein we're interested in, we usually buy it from a company or ask for it from another scientist. However, it's not usually in the vector that we need for our studies and so we have to choose a plasmid vector that is suitable for our requirements - one with the right promoter or the right tag - and then clone the piece of DNA into the new vector. PCR is the tool we use for copying or amplifying the gene in the original plasmid so we can clone it into the desired plasmid. I'll explain how PCR works next.

This is the basics of how a PCR reaction works. To run a PCR reaction, we mix together template DNA (the DNA we want to amplify), DNA polymerase (an enzyme that synthesises DNA), primers (short strands of DNA that are complimentary to the start and end of the DNA we want to amplify) and the four nucleotides that make up DNA: C, G, A and T, the building blocks of DNA.

We then put this mixture through a serious of heating and cooling steps. An initial heating to a high temperature breaks the hydrogen bonds holding the two strands of the template DNA together so that the DNA double helix splits open, exposing the bases on the inside. Splitting the DNA open like this is called DNA denaturation. The denatured DNA is cooled, which allows the primers, shown here in green, to bind to the start and end of the DNA sequence we want to amplify - this is called primer annealing. The primers show the DNA polymerase which length of DNA to copy, and the enzyme cannot start the reaction without them. The mixture is then heated to the optimum temperature for the DNA polymerase to work on the exposed DNA strand. The DNA polymerase slides along the strand and links spaces together and so it synthesises a new strand of DNA. This step is called extension. This process doubles the amount of DNA. In order to amplify the gene we've just copied, we repeat that cycle of heating, cooling and warming the DNA. With each cycle, we double the number of copies of DNA we had. The process is exponential which means we can very quickly amplify our target piece of DNA.

So now that you know how we copy and amplify a gene, let's return to our plasmids and how to manipulate them. Remember that we usually buy the gene we're interested in from a company, and that this probably comes in a plasmid which isn't the actual vector we're going to use, so we also buy that plasmid as well. But then, we have the problem of getting the gene out of the original plasmid and inserting - or cloning - it into the target plasmid.

So first, we use PCR to make multiple copies of the gene from the original plasmid. To insert the gene into the target plasmid, we first need to linearise the plasmid - remember, they're circular - and open it up at the point we want to insert our gene. We can do this using PCR: we simply design primers that will bind to the point on the plasmid where we want to open it and that will amplify in opposite directions.

The PCR reaction will then generate millions of copies of polymerase plasmid. To insert the gene as I described earlier, we start by amplifying it using PCR. However, we have to make sure that we design primers where half the primer will bind to the gene we want to amplify, and half of the primer is a copy of the part of the plasmid we want to insert the gene into. When the gene is amplified by PCR in this way, the primers are incorporated into the resulting product, so we end up with a copy of the gene which also has the same DNA sequences as the plasmid we want to insert it into. So once the PCR reactions are complete, we have a linearised vector for the new gene to be inserted into and the gene we want to insert with sequences from the plasmid now added to the end.

We're now ready to move on to the cloning proper, inserting the gene into the new plasmid. To do this, we use an enzyme to remove base pairs from the ends of just one strand from the DNA double helix to create single-stranded lengths of DNA at the ends of both the linearised vector and the gene to be inserted. As the ends of the gene PCR products are the same as the plasmid we want to insert it into, we produce complimentary sticky ends that will line up by complimentary base-pairing, thus inserting the gene into the plasmid. We can then use a DNA ligase to close the phosphodiester bonds. This connects the inserted DNA to the plasmid, a step called the ligation reaction. This creates a complete plasmid which we can use to transform the bacteria.

Now, let's look at how we produce the cloned plasmid in usable quantities. As I mentioned earlier, what we do is transform bacteria with the cloned plasmid. We do this because this is an easy way to replicate the plasmid. Rather than doing it ourselves, we simply grow the bacteria. The bacteria replicate the plasmid for us, and we can then purify it from the bacterial cells. This is where the antibiotic-resistance gene comes in. If the plasmid included an antibiotic-resistance gene, the bacteria will contain it. We can then grow the bacteria on an agar plate

containing the antibiotic, and thus ensure that only bacteria containing the plasmid are present. The plasmid is mixed in with the bacteria. We heat them to 42° C for a few seconds then immediately cool them to 4° in a box of ice.

The thermal shock causes the cells to take up the plasmid from their surroundings. When the bacteria take up exogenous DNA, we call this process transformation. We then spread the bacteria on an agar plate and incubate them over night. The following day, a colony can be picked off the plate and grown in a culture. Once the bacterial culture has grown, you purify the plasmid from the culture. We then confirm the gene has been correctly inserted by sequencing the DNA in the plasmids. It's important to sequence the whole insert in case any unexpected mutations were introduced during the PCR. Once we have confirmed that the insertion has been successful, we're ready to use the plasmid in experiments to express the protein of interest in living cells.