Santander Travel Grant Report:

The travel grant was awarded to learn Cas9 protein production, with the outlook to expedite my research and utilise this techniques in a wild range of biomedical settings to the benefit of the School of Biological Sciences. Cas9 protein is a nuclease, a form of molecular scissor that can be utilised to cut DNA at specific locations. In the context of my work, it is planned to use these scissors to make a cut within the DMD gene. If a mutation is present or a change sequence within this gene, it results in Duchenne Muscular Dystrophy (DMD). Clinically, this presents in patients as muscle weakness which later progresses to affect respiratory and cardiac systems resulting in premature death. Current medical treatments do not address the current medical need. Genome surgery, the work proposed by my project, cutting the DNA within the mutated DMD gene and introducing the correct DNA sequence, provides promise for a permanent amelioration of clinical symptoms.

Due to Cas9 being a novel nuclease at the forefront of pioneering scientific research, a great deal of work has been on-going to establish this system at Royal Holloway for a range of biomedical applications. The aim was to learn the theoretical and practical elements of Cas9 protein production, with the outlook to implement an in-house protein production system. A brief description of the methodology I learnt to produce protein is as follows:

1) A plasmid coding the Cas9 protein and an appropriate HisTag (Figure 1), needs to be introduced into bacteria, by quickly heating and cooling bacteria in a process known as heat shock.
2) Once, the bacteria has taken up the plasmid, the bacteria continues to grow generating more copies of the plasmid of interest, much like a biological photocopier. Once enough bacteria is present in high enough quantities, the plasmid is chemically stimulated (induced) to produce the protein of interest from the plasmid introduced; once the protein is produced, bacteria is broken open (lysed) and all of the contents of bacteria are released (Figure 2).
3) At this stage, the Cas9 protein is present with a range of impurities, including non-specific bacterial proteins, nucleic acid and partial Cas9 protein fragments. Thus this mixture now needs to be put through a variety of columns to remove the impurities and concentrate the Cas9 protein. Three sequential columns are used to this purpose: Initially a histrap column which binds to the special tag on the Cas9 protein until a salt releases it from the binding (Figure 3), a sepharose ion exchange column where the positive protein binds to negatively charged beads, until once again a salt releases it (Figure 4), finally, this is put through a superdex column whereby protein of the size of interest will be eluted together (Figure 5).
4) After each purification process a gel is used to visualise the purity of the protein; finally the protein's ability to cut DNA is tested at the end of the process (Figure 6).

Learning Cas9 protein production has been hugely beneficial to my work at Royal Holloway. This protein can be used to produce cuts in challenging muscle cells lines and will expedite the route to DNA repair of the DMD gene. This is particularly important, as the delivery to certain cells lines was a limiting factor to my research. In addition, the experience was hugely enriching, providing me a unique insight into how other groups worked and the opportunity to work alongside experts to refine my understanding of gene editing. The techniques will also be introduced back into the Department of Biological Sciences as protein production has wide applicability beyond that of Cas9

alone. As such the protocols obtained will also be written and made freely available to the department from the Dickson group so others may benefit from this work as much as I have. I am currently working to set up an in-house protein production system with the skills I have learnt and am hugely grateful for the opportunities afforded to me and my research as a result of this funding.

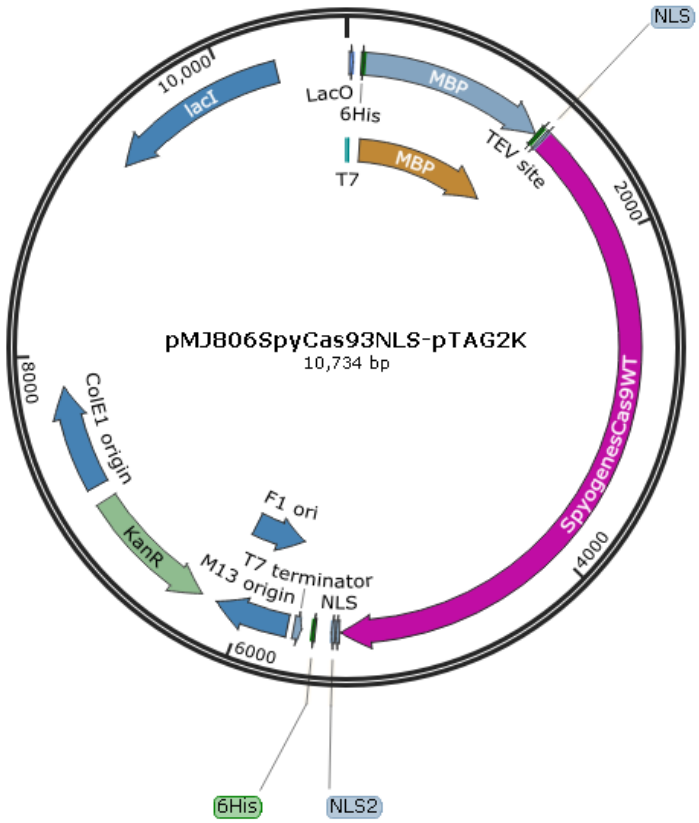

Figure 1: A plasmid map, showing the DNA that will be introduced to bacteria to the produce the Cas9 protein of interest. Notice the 5' MBP tag and the 3' His Tag that are adjacent to the Cas9 protein.

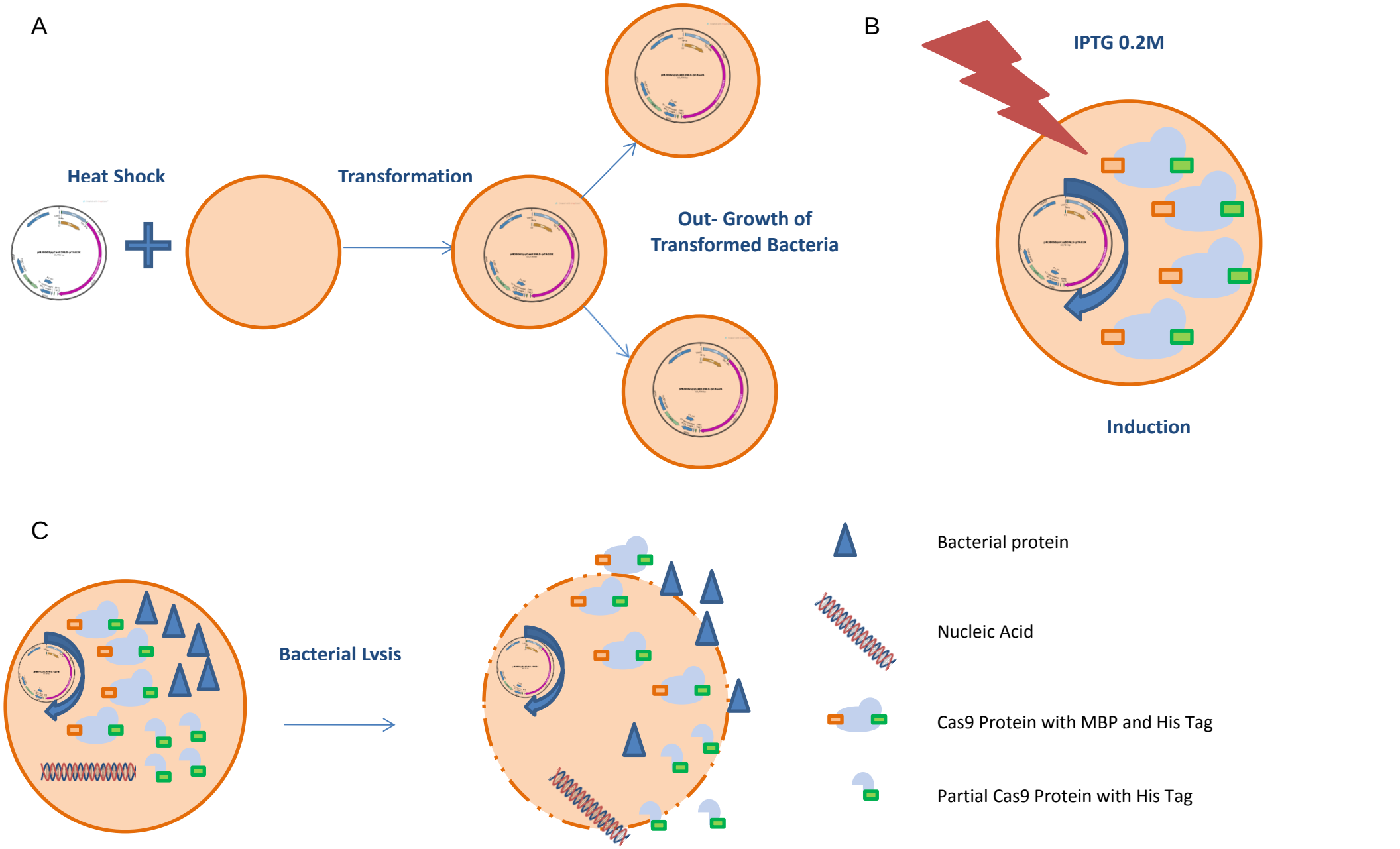

Figure 2: A schematic diagram showing the process of introducing the DNA hat codes for the Cas9 protein of interest and inducing its production. A. Heat shock of bacteria with plasmid a process where bacteria is heated and cooled quickly to allow DNA to be introduced into the bacteria, this is followed by outgrowth of bacteria that have taken up the plasmid. **B.** Induction, IPTG is added which prompts the bacteria to produce Cas9 protein. **C.** Bacterial Lysis, bacteria is lysed or broken up and its content is released including: Nucleic acid, bacterial proteins, partial Cas9 proteins and Cas9 protein.

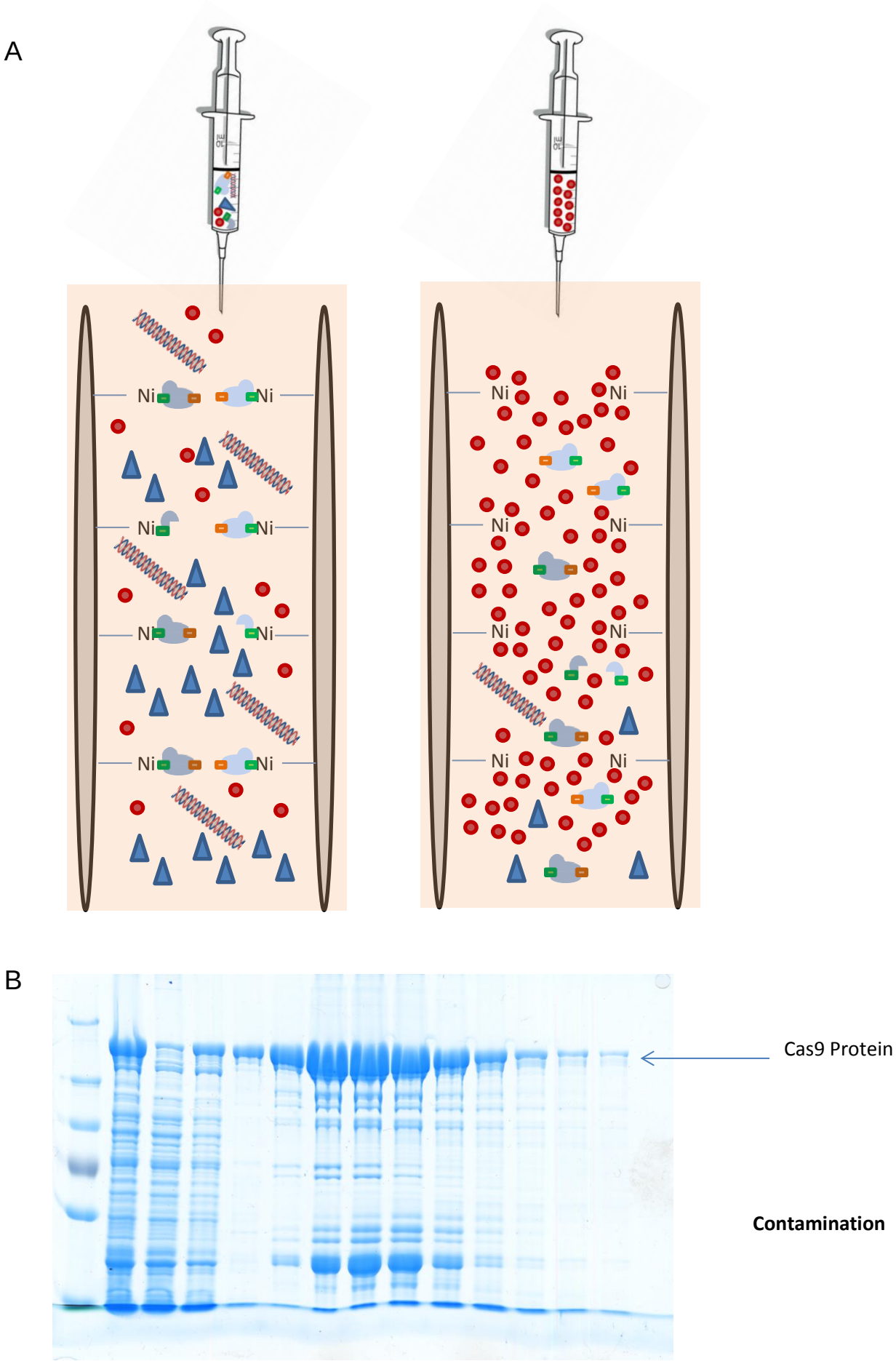

Figure 3: A schematic diagram showing the process of using the HisTrap column for the purification of Cas9 protein. A. Column on the left shows the loading of total bacterial protein in the presence of 20mM imidazole, this will allow the Cas9 protein to bind to the nickel column and will allow other proteins without the HisTag to flow through. Column on the right shows the elution of the Cas9 protein, where imidazole out competes the Cas9 protein and it is released from the nickel column. The resultant protein should have less contamination. **B.** A Gel Image to the stained protein fractions that were obtained from the column.

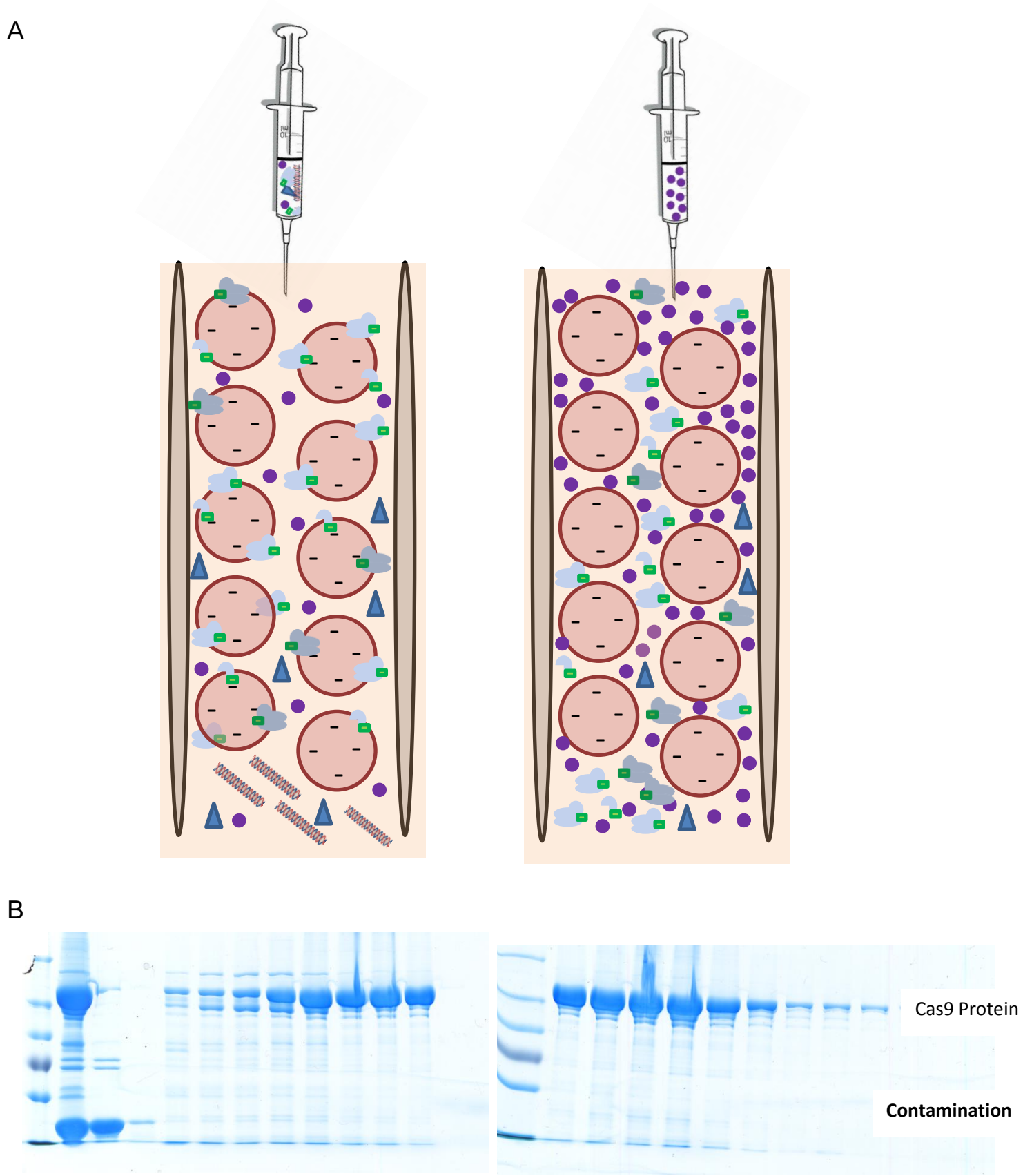

Figure 4: A schematic diagram showing the process of using the Sepharose Ion Exchange column for the purification of Cas9 protein. A. Column on the left shows the loading of His Trap purified Cas9 protein in the presence of 100mM KCl as this will allow the Cas9 protein to bind to the negatively charged beads. Column on the right shows the elution of the Cas9 protein, where KCl out competes the Cas9 protein and it is released from the negatively charged beads. The resultant protein should have less contamination. **B.** Two gel images showing elution series 1 and 2 of protein fractions obtained from the column.

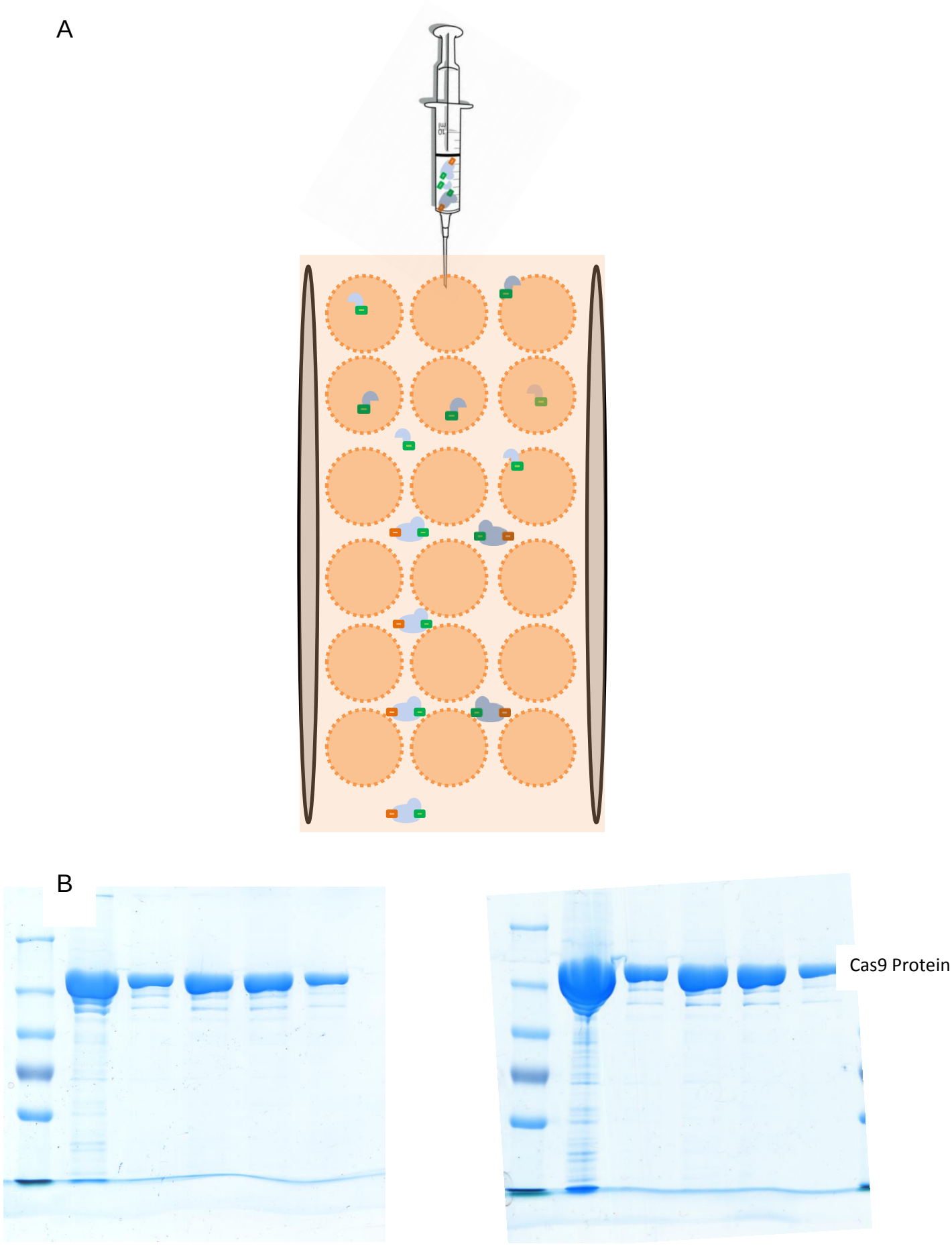

Figure 5: A schematic diagram showing the process of using the size exclusion supderdex column for the purification of Cas9 protein. A. The schematic shows the process of size exclusion chromatography, smaller partial protein can access the space inside and around the beads and thus is eluted later. Larger Cas9 protein cannot access the area inside the bead and thus is eluted sooner. **B.** Two gel fractions showing the stained protein fractions that were obtained from the column, from two sequential elutions this is the purest aliquots of protein and will be pooled.

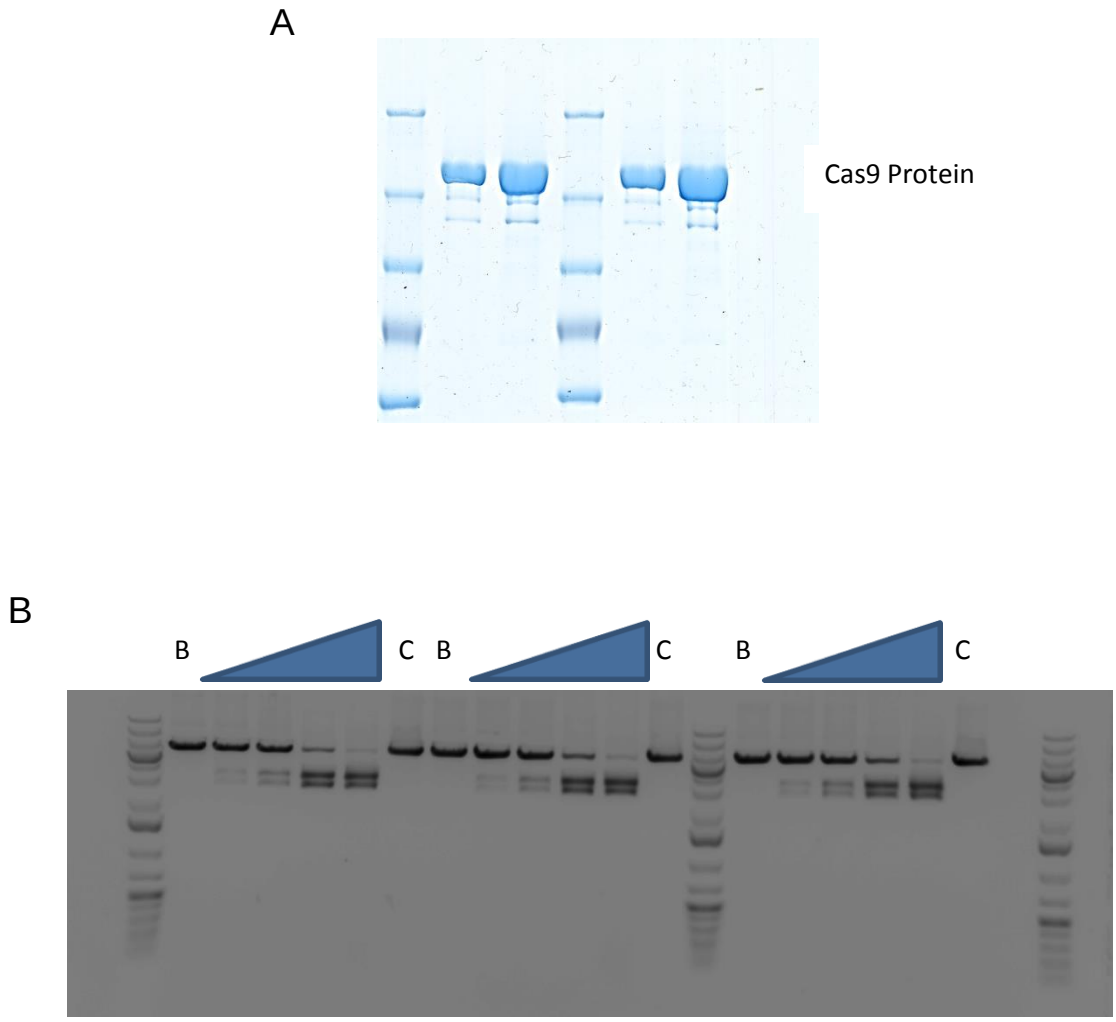

Figure 5: Purified Cas9 protein and Cutting Assay. **A**. A gel showing the stained purified two aliquots Cas9 protein. **B.** A cutting assay on a piece of DNA, B is a buffer control, C is a Cas9 alone (without guide control), the other samples are Cas9 complexes of guide and Cas9 protein at increasing concentrations 5nM, 15nM, 50nM and 15nM of complex to 5nM of DNA. If the Cas9 protein is functional 3 bands should be seen to be becoming more prominent as the concentration increases. This indicates that the single band of DNA has been cut. The far left is a reference sample of protein, the middle and right cutting assays are the protein aliquots produced.