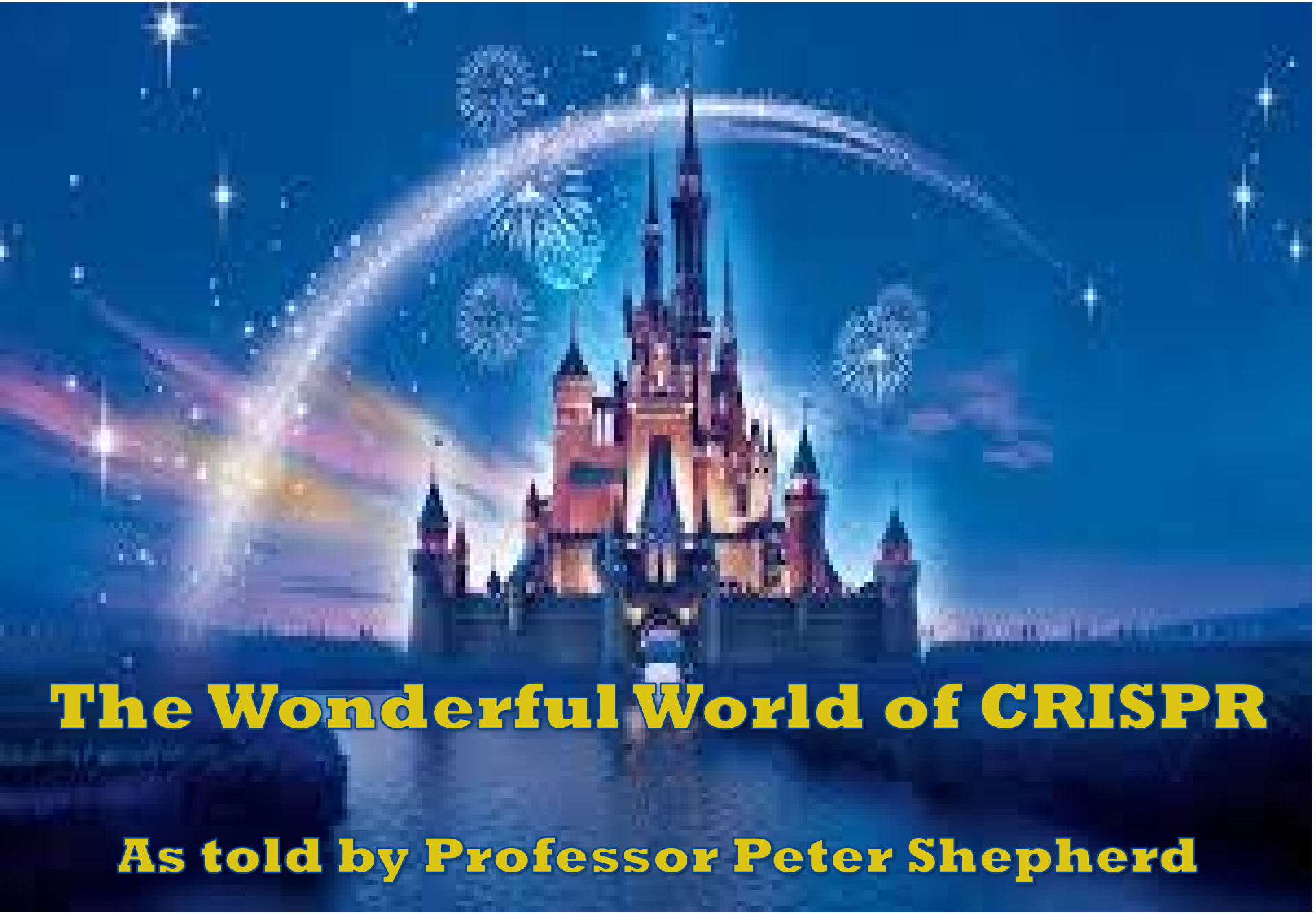
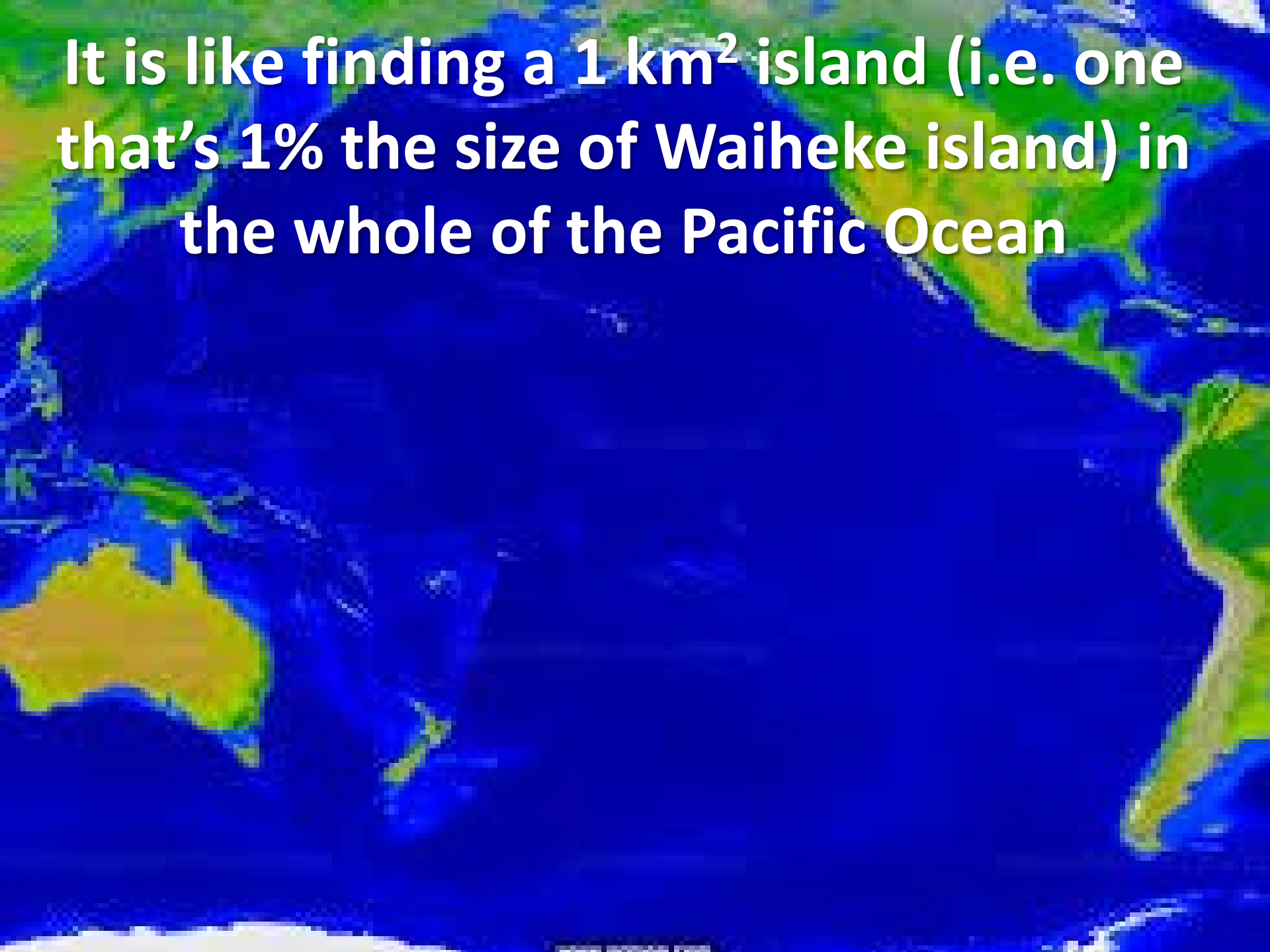

The Wonderful World of CRISPR
As told by Professor Peter Shepherd

To do precise genetic engineering we need to be able to find and specifically modify regions of DNA

But the human genome has 3,000,000,000 base pairs so how are we going to find a 20 base pair region in this huge sea of DNA ?


It is like finding a 1 km² island (i.e. one that's 1% the size of Waiheke island) in the whole of the Pacific Ocean

Its like finding a needle in a haystack

But we can find needles in haystacks if we use the right methods

Method 1 - Random

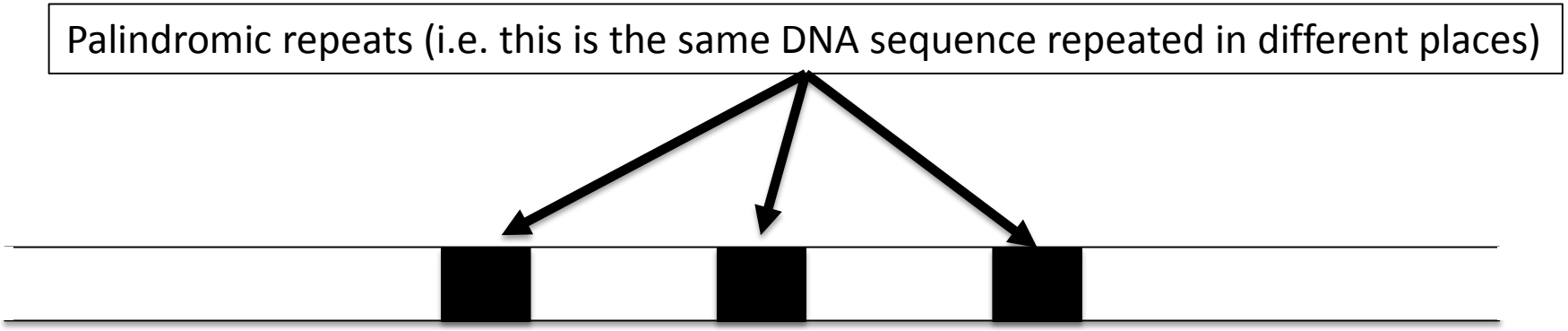
But we can find needles in haystacks if we use the right methods
Method 2 - Targeted

But we can find needles in haystacks if we use the right methods
Method 2 - Targeted

What is CRISPR ?

- It is a very efficient method of **genetic engineering** that allows precision cutting and rearranging DNA in pretty much any way we want i.e we are now truly in a new age of genetic engineering.
- Unlike transgenic techniques (which leave foreign DNA behind in the genome) the CRISPR method leaves no evidence in the genome that the engineering ever happened.

Way back scientists noticed that about 40% of bacteria species contain 29bp palindromic repeats sequences in them – what did they do ?



CRISPR stand for“**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats”

We now know that this area of the bacterial genome contains an adaptive immune system for bacteria, particularly against bacteriophages (Bacteriophages are DNA viruses)

Question: How do bacteria survive the onslaught of bacteriophages ?

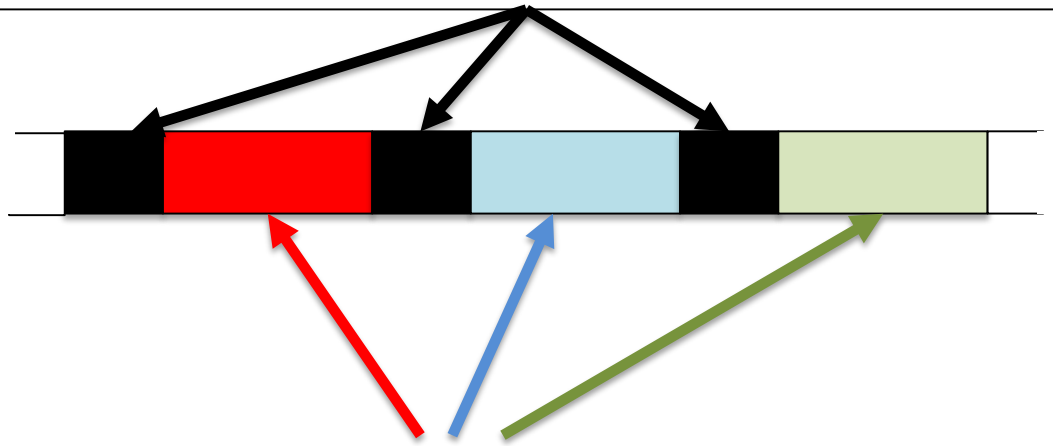
1. The classical defense most bacteria have is the restriction endonuclease system. This is a bit of a shotgun approach.

2. 40% of bacteria have a highly targeted ***adaptive immune system*** that uses mechanisms found in DNA in the CRISPR region of the genome to grab bits of the DNA of bacteriophages. These are used as a guidance system to take DNA cutting enzymes that the bacteria makes and target these specifically to the bacteriophages DNA and chop it up and so destroy the bacteriophage while leaving the bacteria's DNA intact.

What else is in the CRISPR locus ?

Shorts palindromic repeats (i.e. this is the same DNA sequence repeated in different places). These are part of the bacterial genome

Diagram of CRISPR locus in bacterial genome



These bits are derived from bacteriophage genome and each one is different and these provide the guidance system for the adaptive immune system

But wait
..... there's more

There are several other important regions of the bacterial DNA that are also always associated with the CRISPR locus and these provide the means for the palindromic repeat and the bacteriophage DNA sequences to actually destroy the bacteriophage.

These are called CRISPR Associated Sequences i.e. **Cas** genes .

How does this genetic material in CRISPR locus then manage to kill bacteria ?

The system can be slighty different in different types of bacteria but the best studies one is *Streptococcus pyogenes* so we will focus on that one

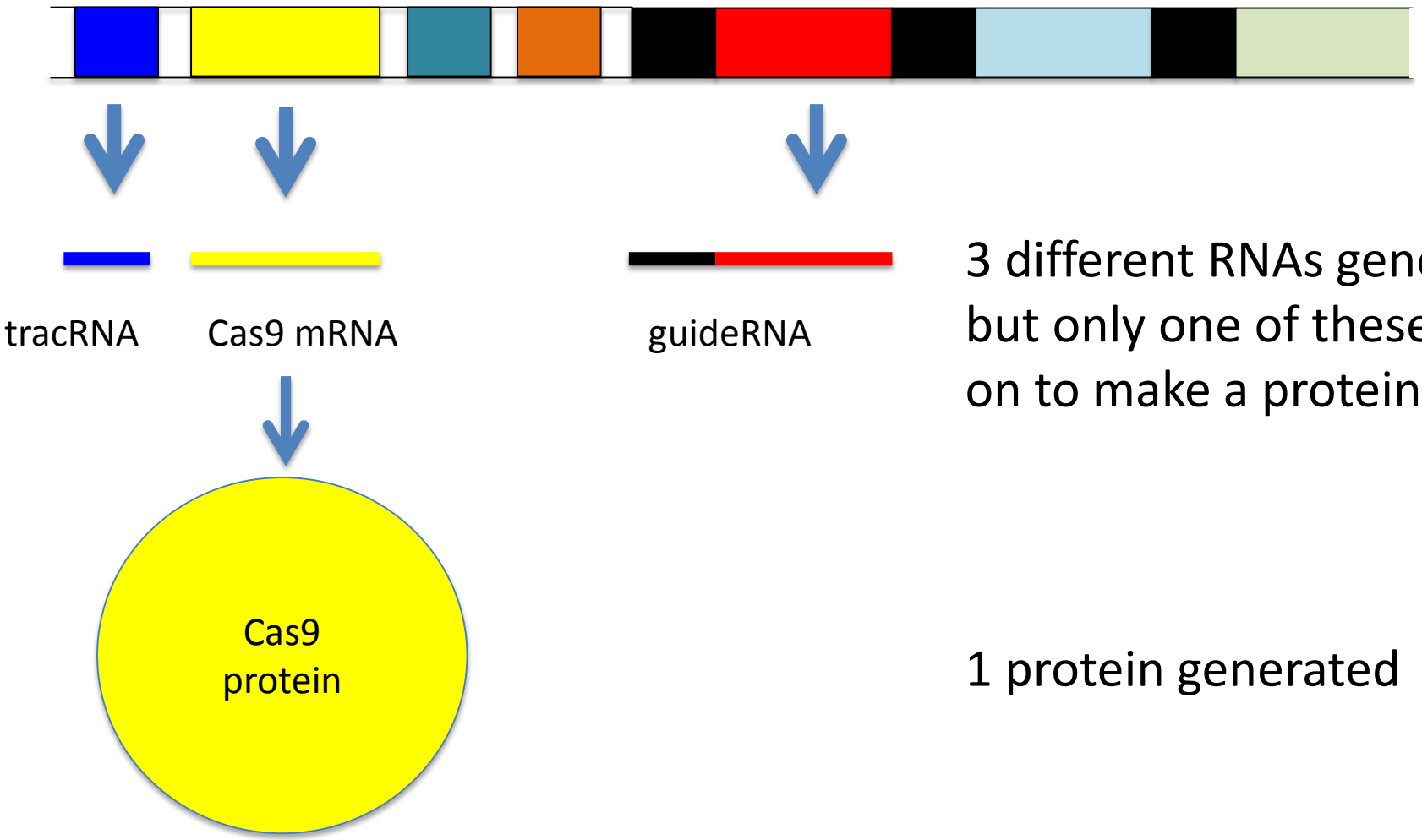
For the sake of simplicity lets focus on the 2 Cas genes most importantfor genetic engineering;

Codes for a protein that is a nuclease that cuts DNA but only if it is given a very specific set of signals to do so (otherwise it would potentially damage the bacteria's own DNA). The most common one used in genetic engineering approaches is called Cas9

Codes for a very specific piece of RNA that will help in the process of ensuring the whole process only cuts bacteriophage DNA

For now lets not worry about the other genes in the Cas locus

What is the *S. Pyogenes* CRISPR/Cas9 system

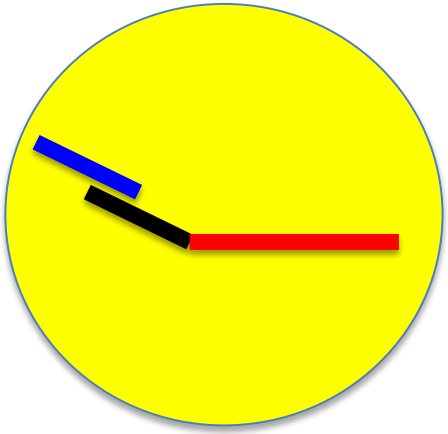


3 different RNAs generated but only one of these goes on to make a protein.

1 protein generated

What is Cas9 ?

- Cas9 is an endonuclease that can cut double stranded DNA
- Cas 9 is only activated when the tracRNA and the guide RNA are associated with it (i.e it is a nucleoprotein). Imagine this a bit like the fail safe mechanism they use to prevent accidental launch of nuclear missiles where 2 people have to insert keys at exactly the same times
- In fact the tracRNA and the guide RNA have a short overlapping sequence that means they actually have to bind to each other in this complex for this to work properly



Active Cas9

How is Cas9 activated ?

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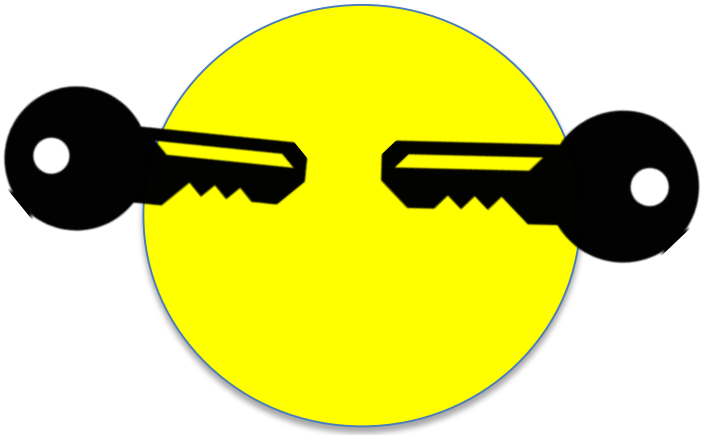
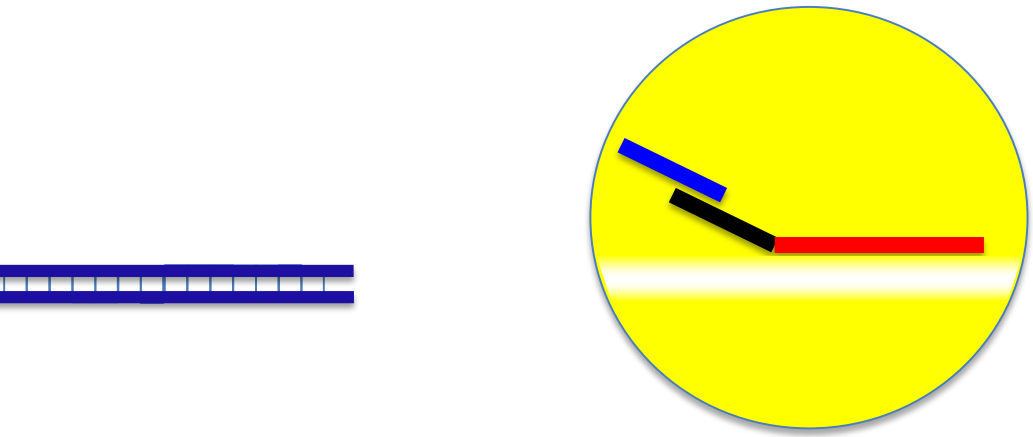


Active Cas9

How does Cas9 work ?

- Cas9 has a channel that DNA can fit into.
- It scans the DNA looking for sequence that match the guide sequence



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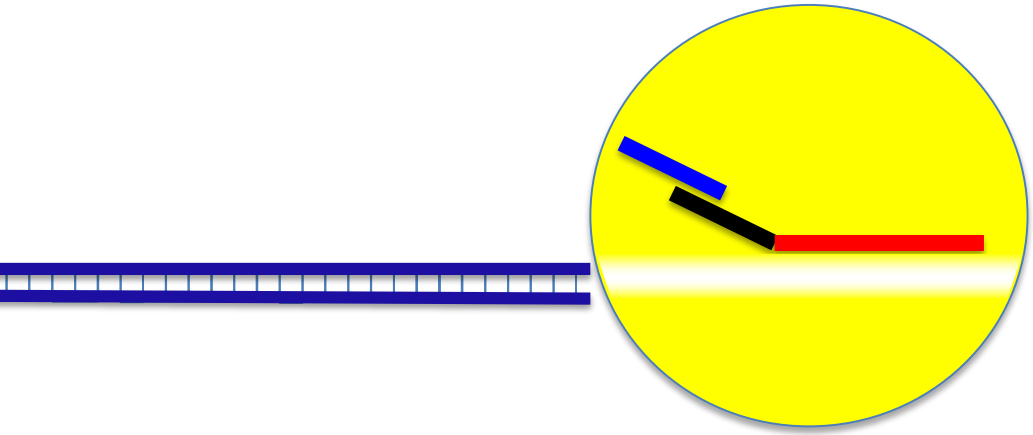

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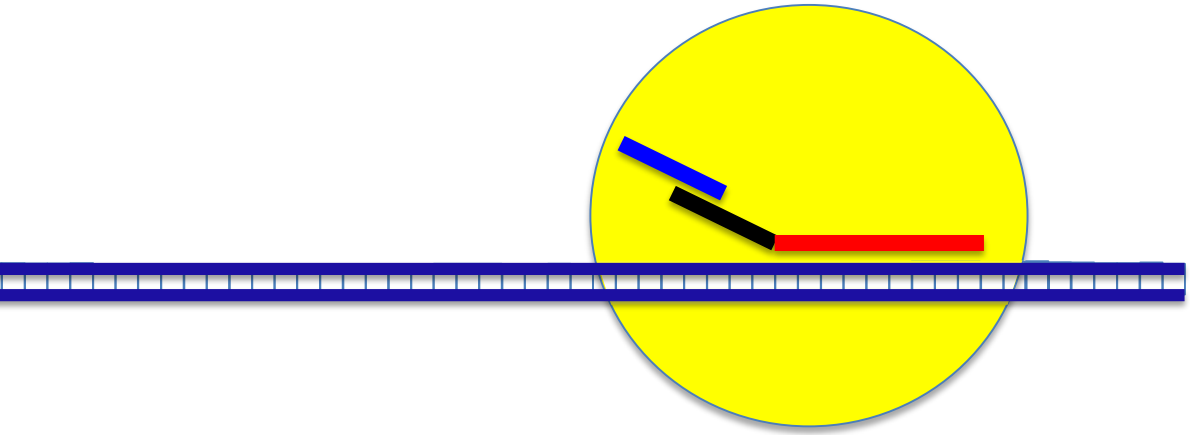
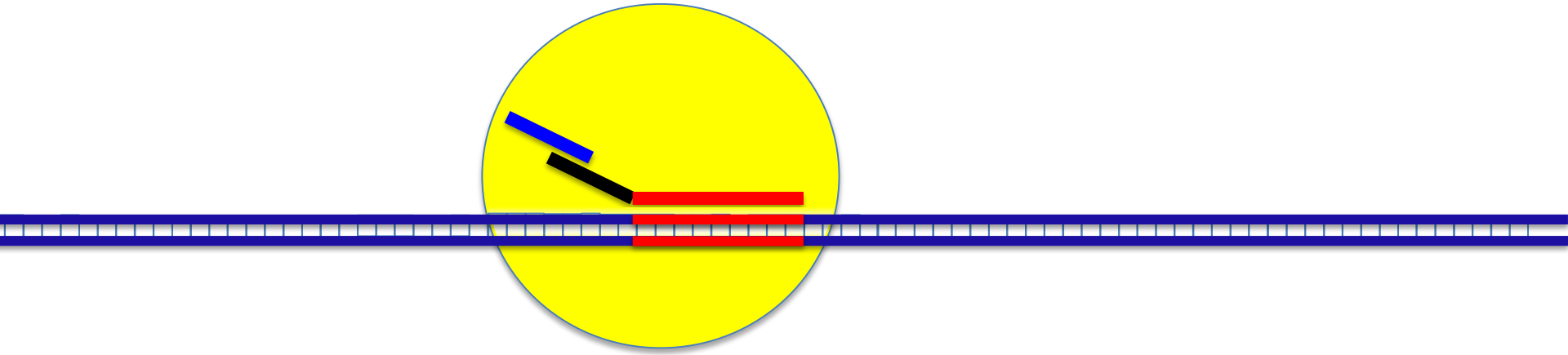


Active Cas9

How does Cas9 work ?

- When a DNA sequence complementary to the guide RNA is found the scanning stops



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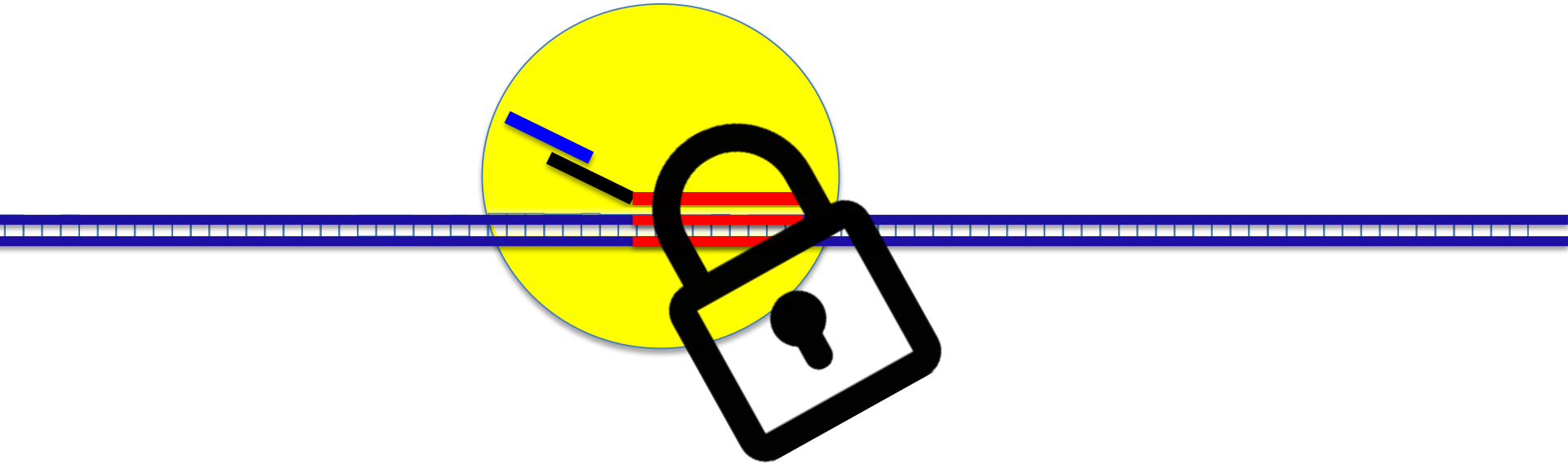
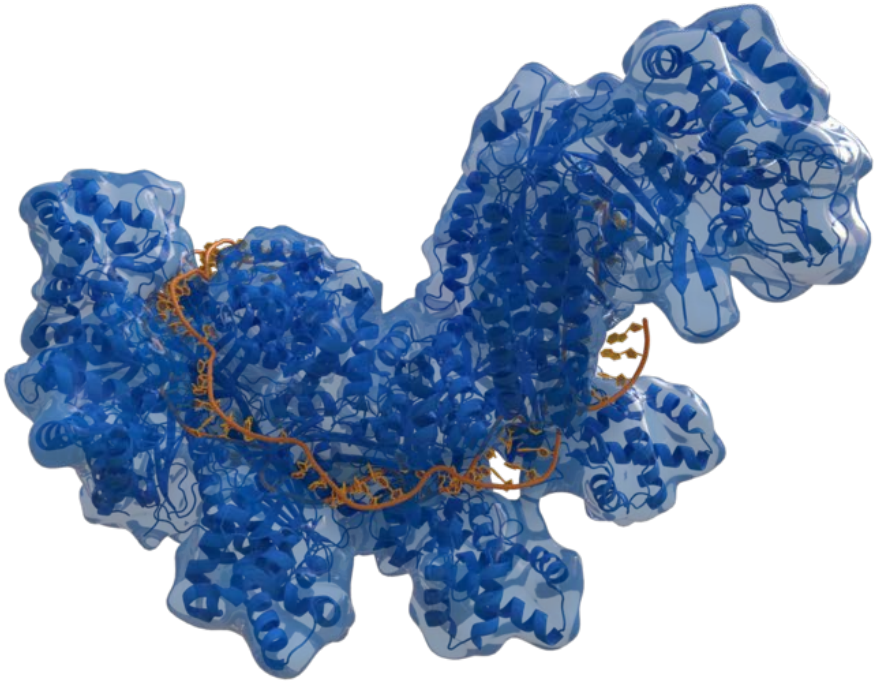


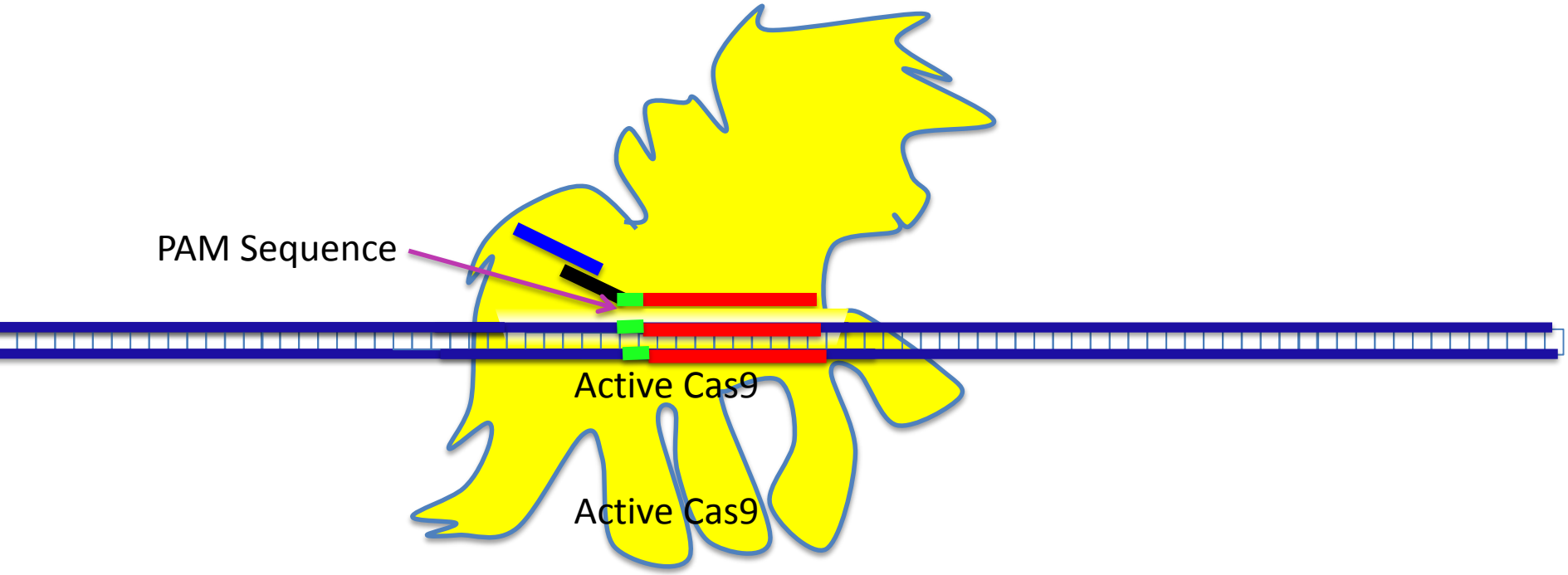
Structure of DNA bound to a Cas enzyme

Completely irrelevant aside



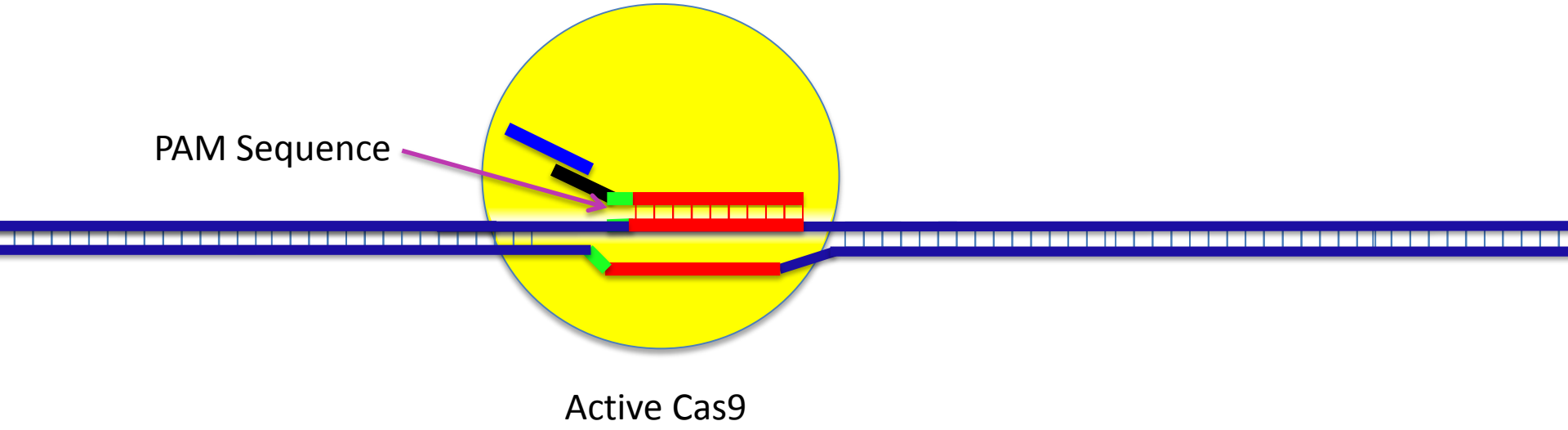
How does Cas9 work ?

- There is one additional check
- In this check the part of the RNA that came from the palindromic repeats of the bacteria has to also have a a very short piece of RNA that is complementary to bit of the bacteriophage DNA. This is called the PAM sequence (**P**rotospacer **A**djacent **M**otif)
- For *Staph Pyogenes* this needs a GG sequence
- Only when all this happens and we have the guide RNA bound do we have a fully active enzyme.



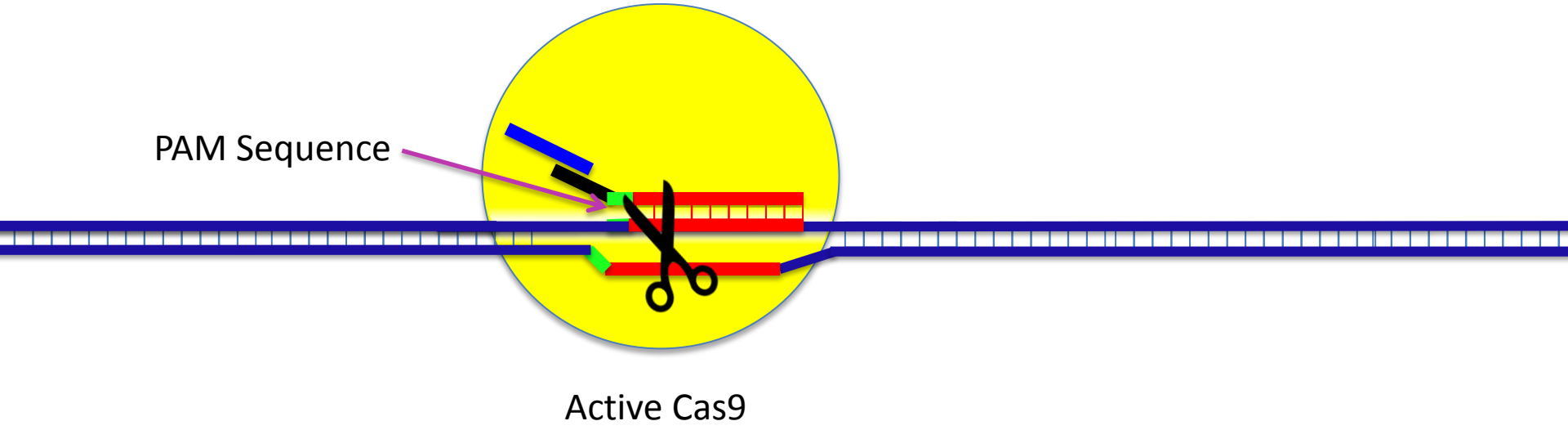
How does Cas9 work ?

- Now the RNA binds to the complementary strand of the DNA and opens up the DNA helix



How does Cas9 work ?

- Now the bacteriophages DNA gets cut very close to the PAM site



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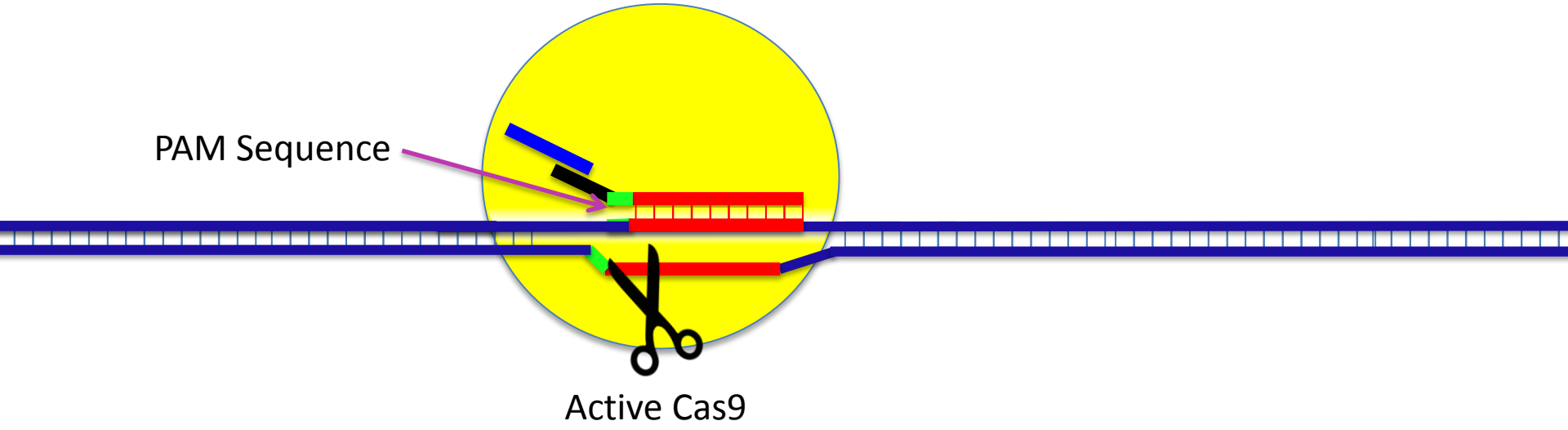
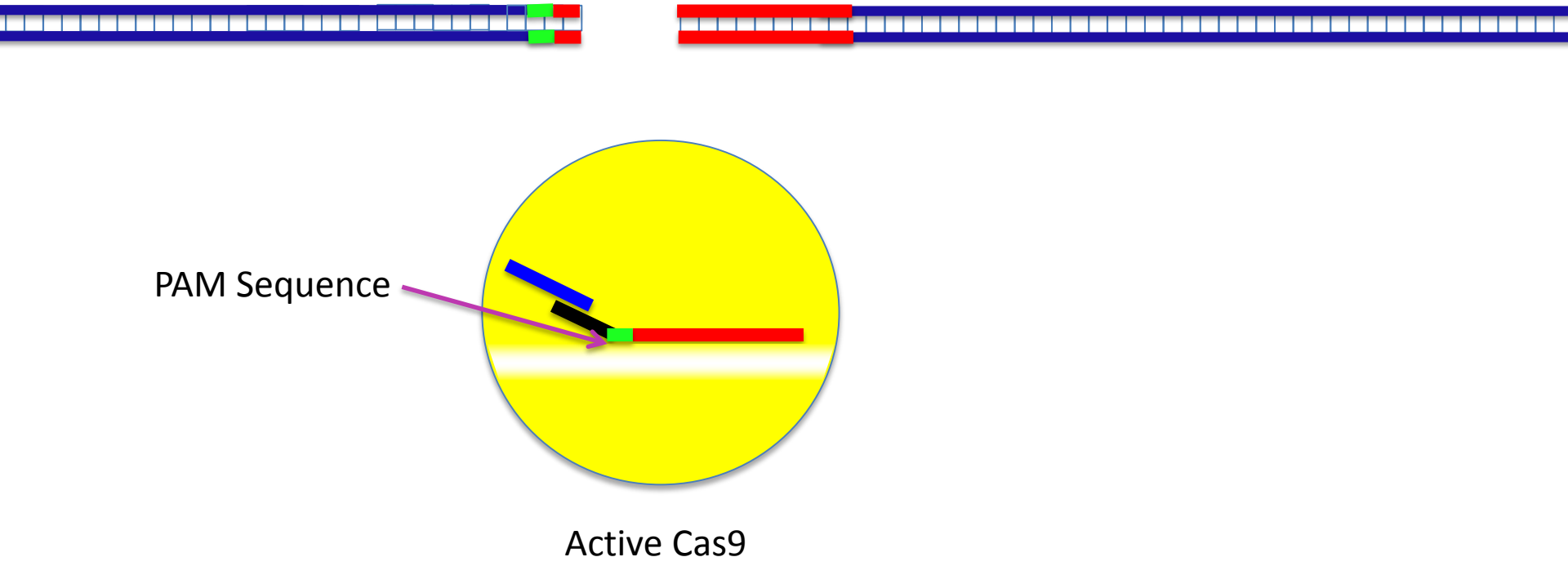


- Now the bacteriophages DNA gets cut very close to the PAM site so now it looks like this and the bacteriophage is essentially dead



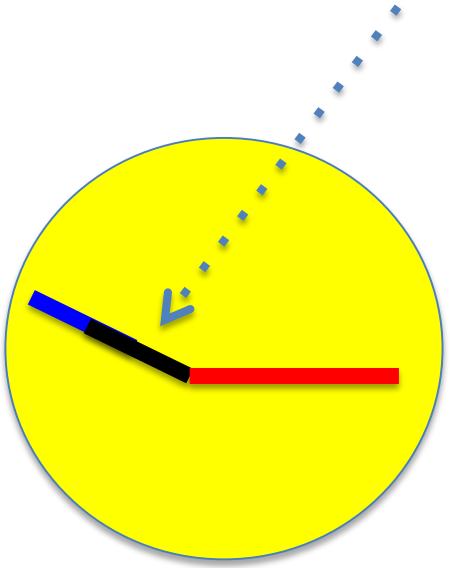
Features of the CRISPR/Cas9 system

- Its highly specific
- Tightly regulated
- Highly efficient

i.e. ALL THE THINGS YOU WANT IN A GENETIC ENGINEERING TOOL

How can we use CRISPR/Cas9 for genetic engineering?

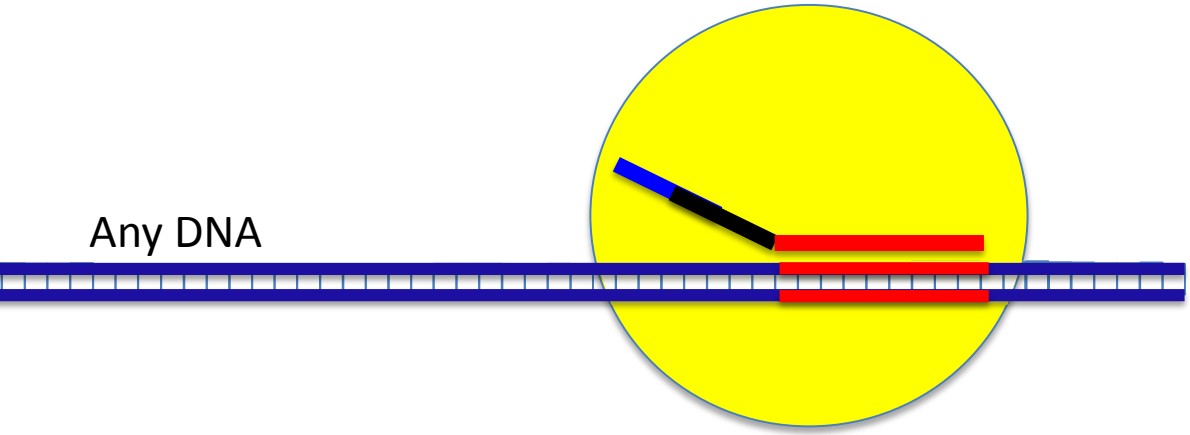
- Some clever people found you could combine the guide RNA and the tracRNA together into one artificial RNA called a **single guide RNA (sgRNA).**



Active Cas9

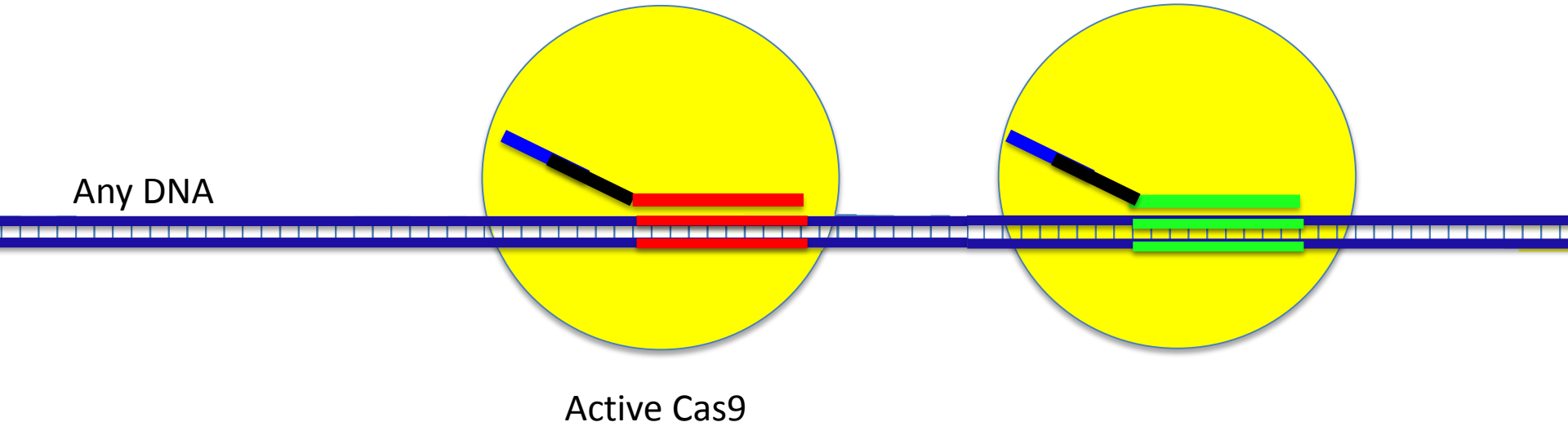
How can we use CRISPR/Cas9 for genetic engineering?

- This means we can artificially make a sgRNA that can be designed to target any part of the genome (as long as it has an appropriate PAM sequence nearby)
- All we have to do is artificially express the Cas9 and the sgRNA together and hey presto you can cut DNA anywhere you want pretty much

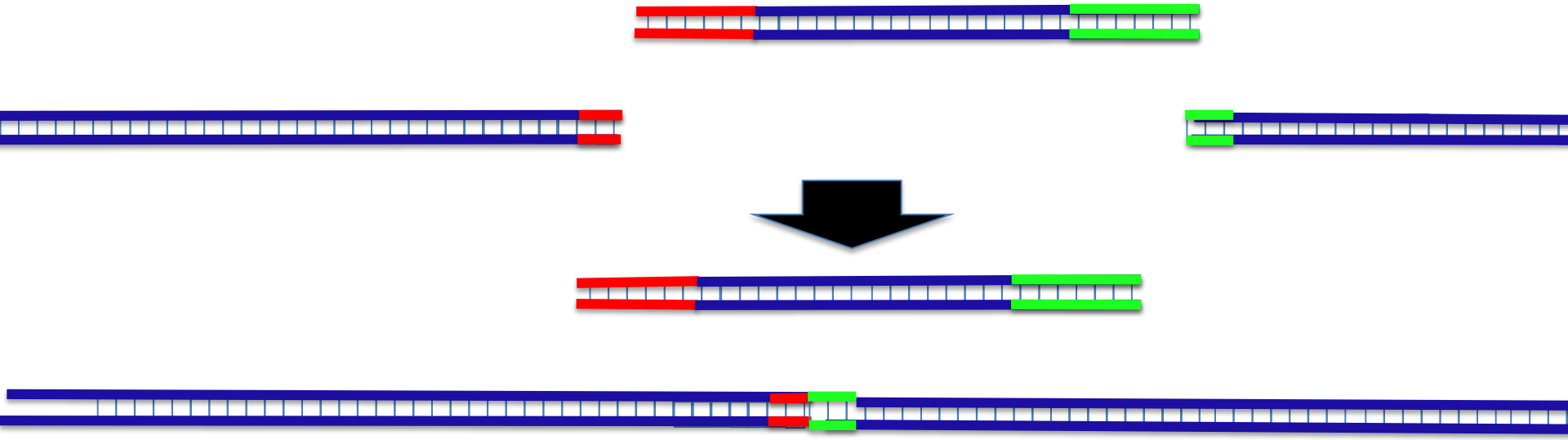


How can we use CRISPR/Cas9 for genetic engineering?

- We can put two different sgRNA into the same protein and cut at 2 places in the genome we can cut out large regions of DNA



This allows us to selectively “knock out” regions of the genome



Recipe for knocking out VEGFA gene

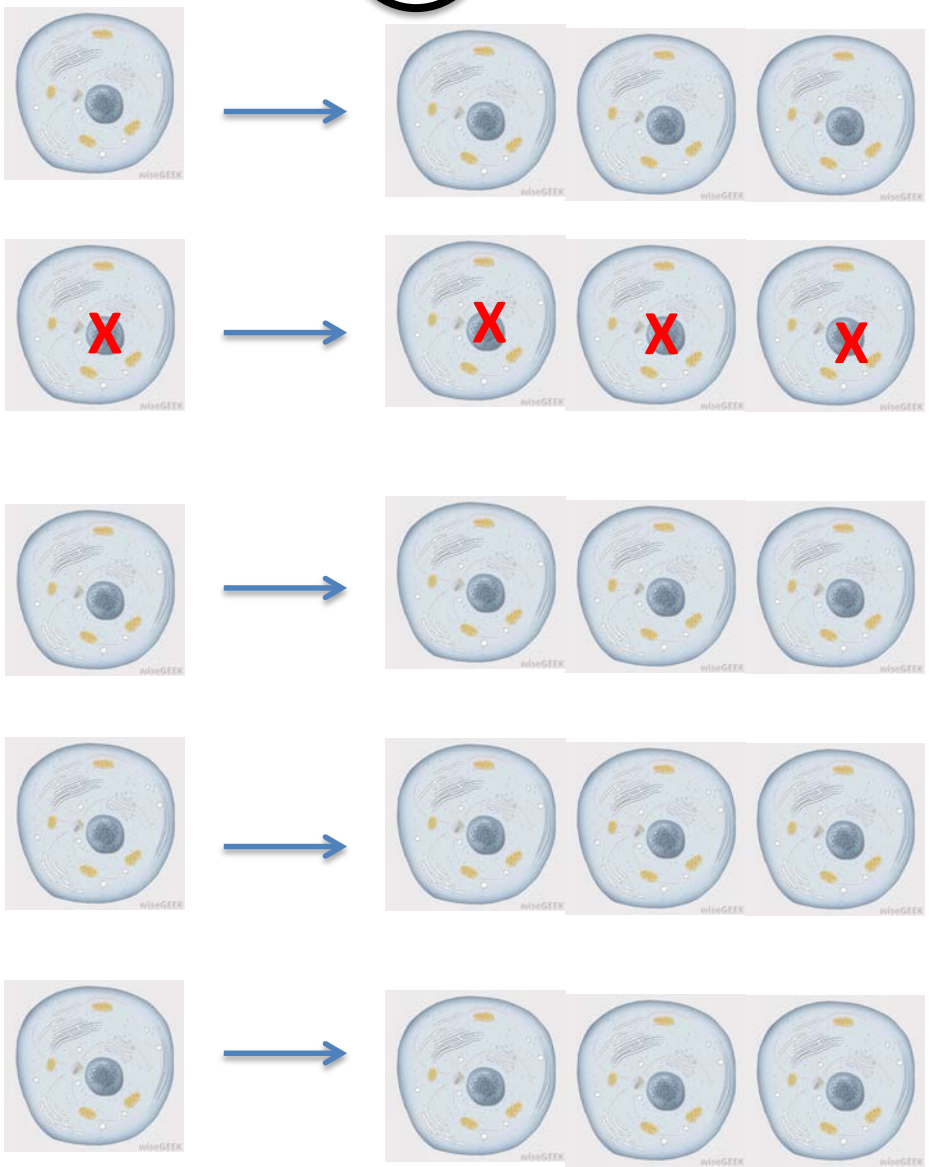
1

Take lots of cells and add the Cas9 protein plus 2 sgRNA that specifically bind to VEGFA gene

2

Isolate single cells (i.e select clones)

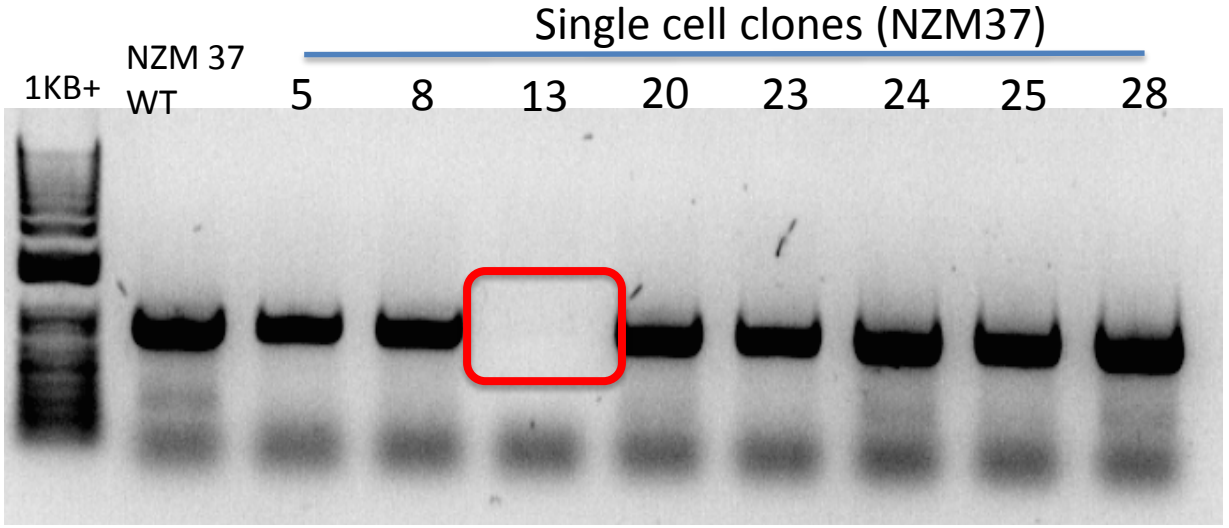
3 Grow cells



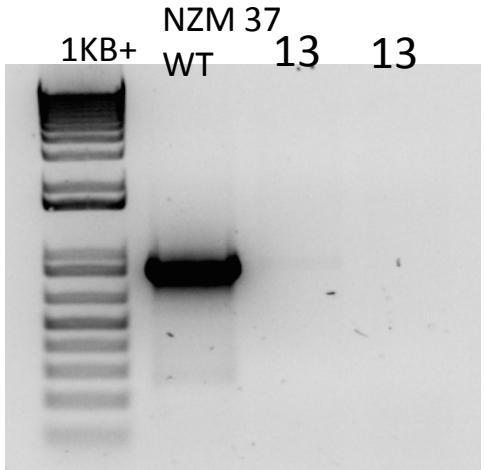
4

Isolate DNA from cells and find cells that have the gene knocked out

Here is an example of PCR of the VEGFA gene of melanoma cells where we have tried to use CRISPR to "knockout the VEGFA gene (achieved in clone 13)

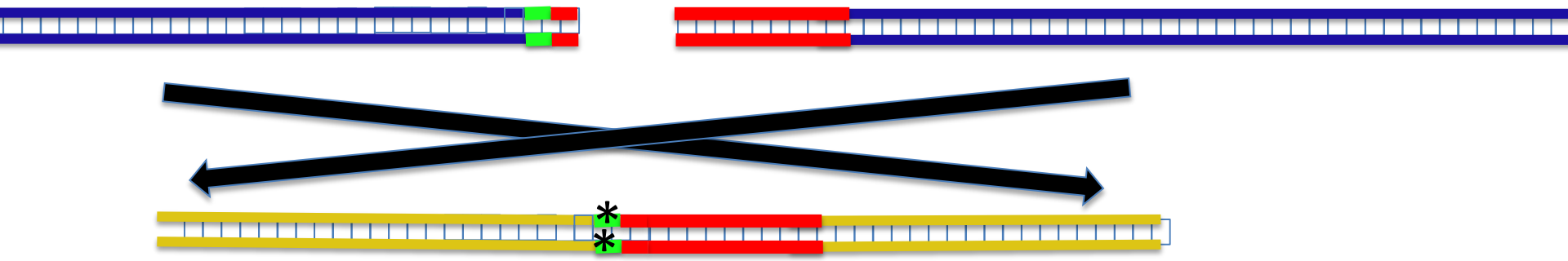


Repeat PCR



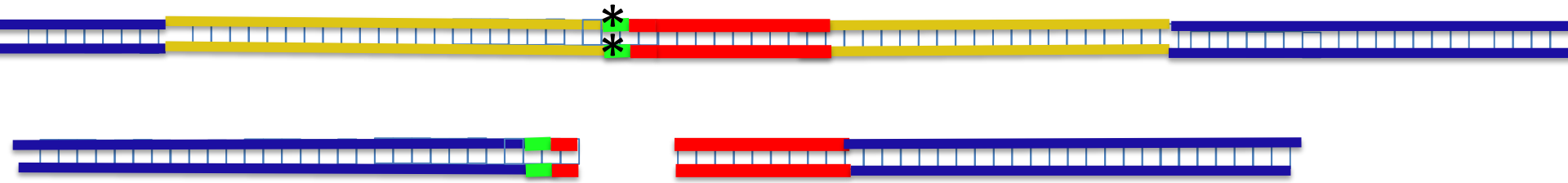
We can also use CRISPR/Cas9 to "knockin" bits of DNA

- If we make an artificial piece of DNA that is identical to the cleaved region of DNA then when the cell tries to repair its own chromasomal DNA it will sometimes accidentally incorporate this into its own DNA by homologous recombination

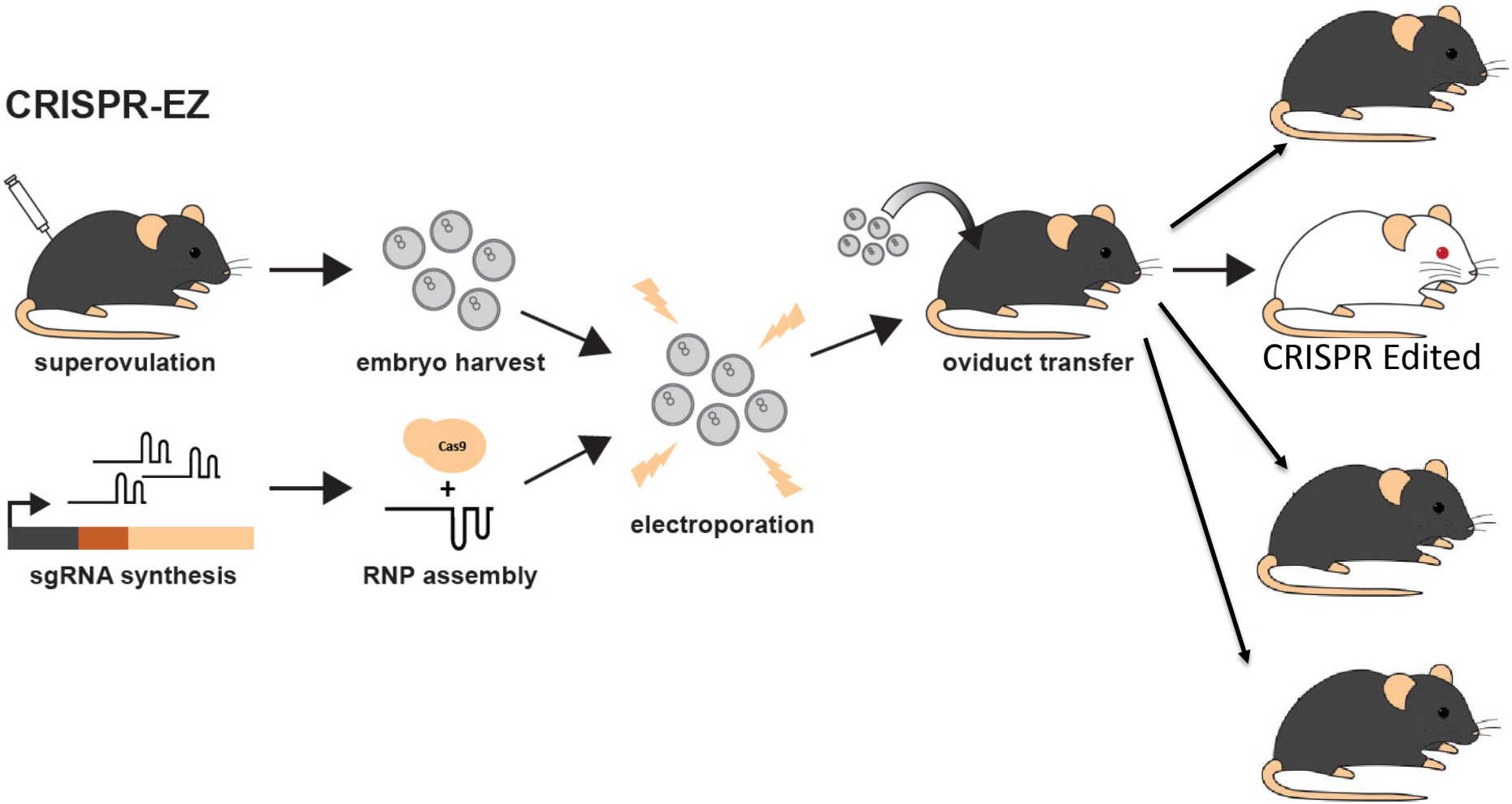


How can we use CRISPR/Cas9 for genetic engineering?

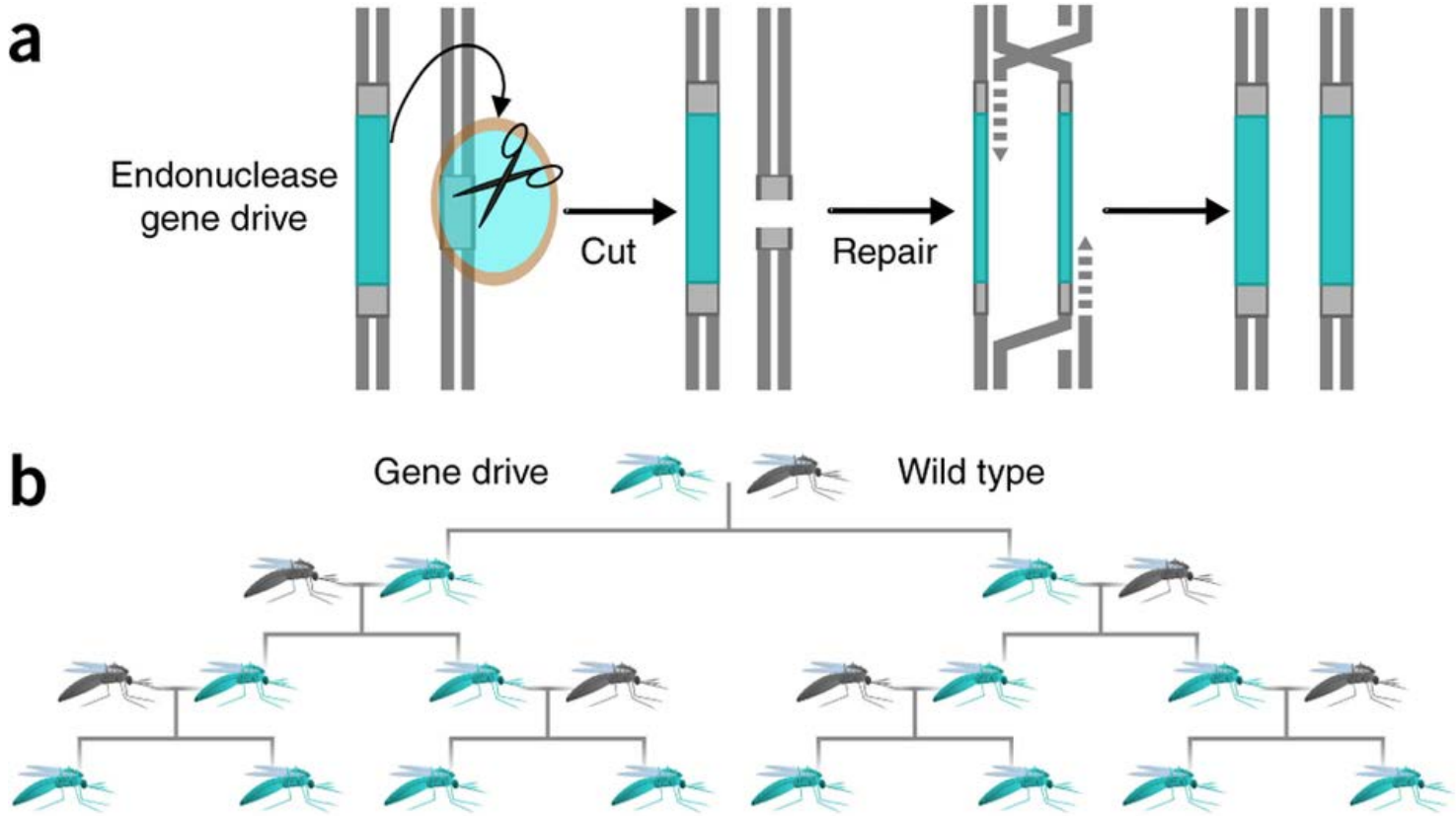
- Now the artificially produced piece of DNA is “knocked in” to the genome



Making mice where genes are knocked out is now super easy and cheap

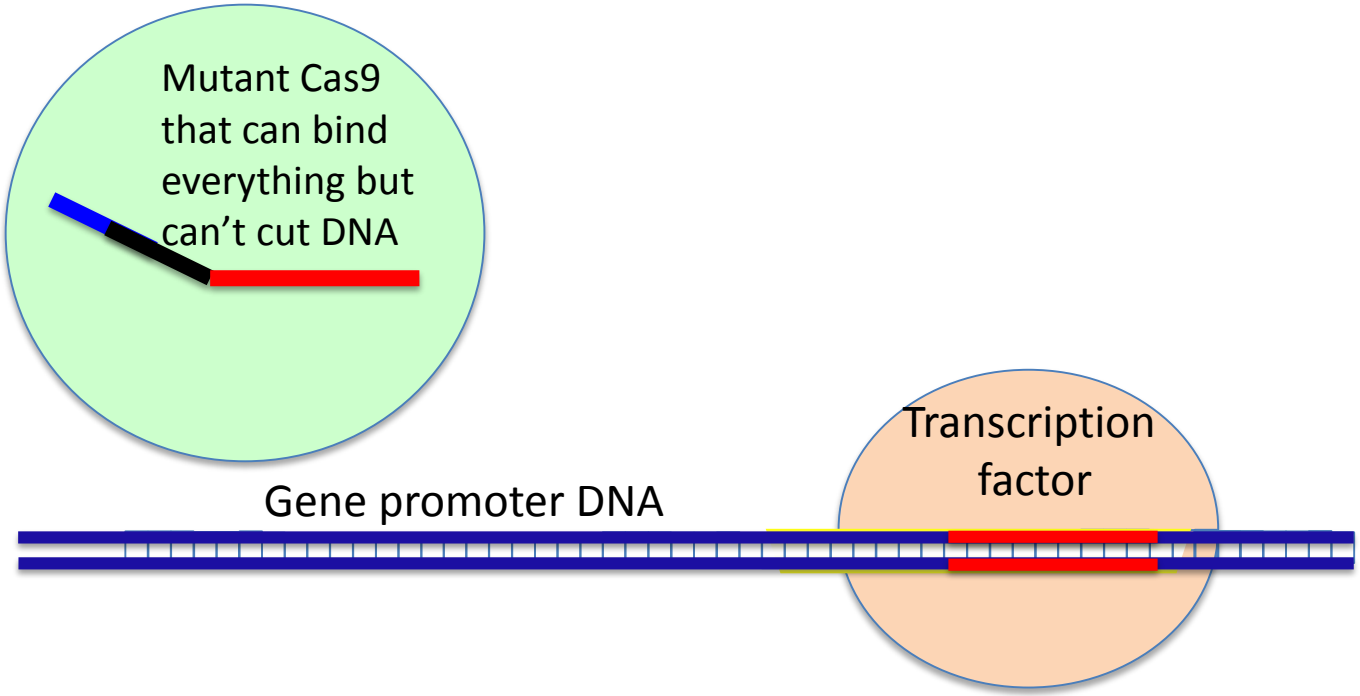


Using CRISPR a weapon to wipe out mosquitos



CRISPR/Cas9 can also be used to switch on or off genes

- Uses a mutant Cas9 that can bind everything but can't cut DNA
- This means it locks on tightly to the DNA that matches the guide sequence
- An example of how this can be used is by having a big Cas9 protein sitting at say a transcription factor binding site we can block the transcription factor from coming into the gene promoter so switch off the expression of that specific gene in a highly targeted way.



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