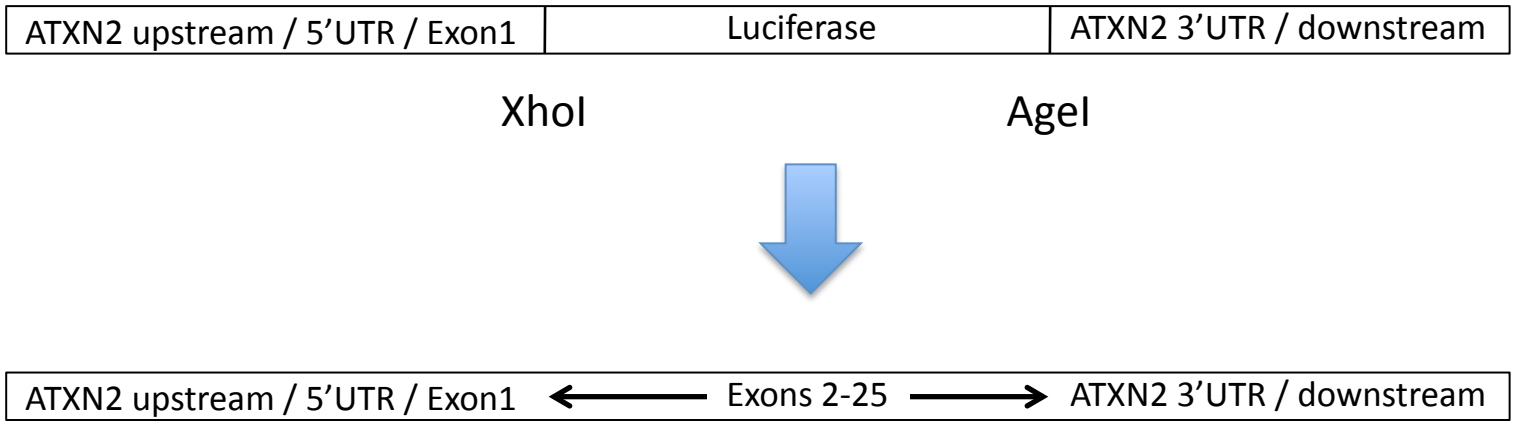
In-Fusion Cloning
&
Recent HTS Optimization Data

In-Fusion Cloning

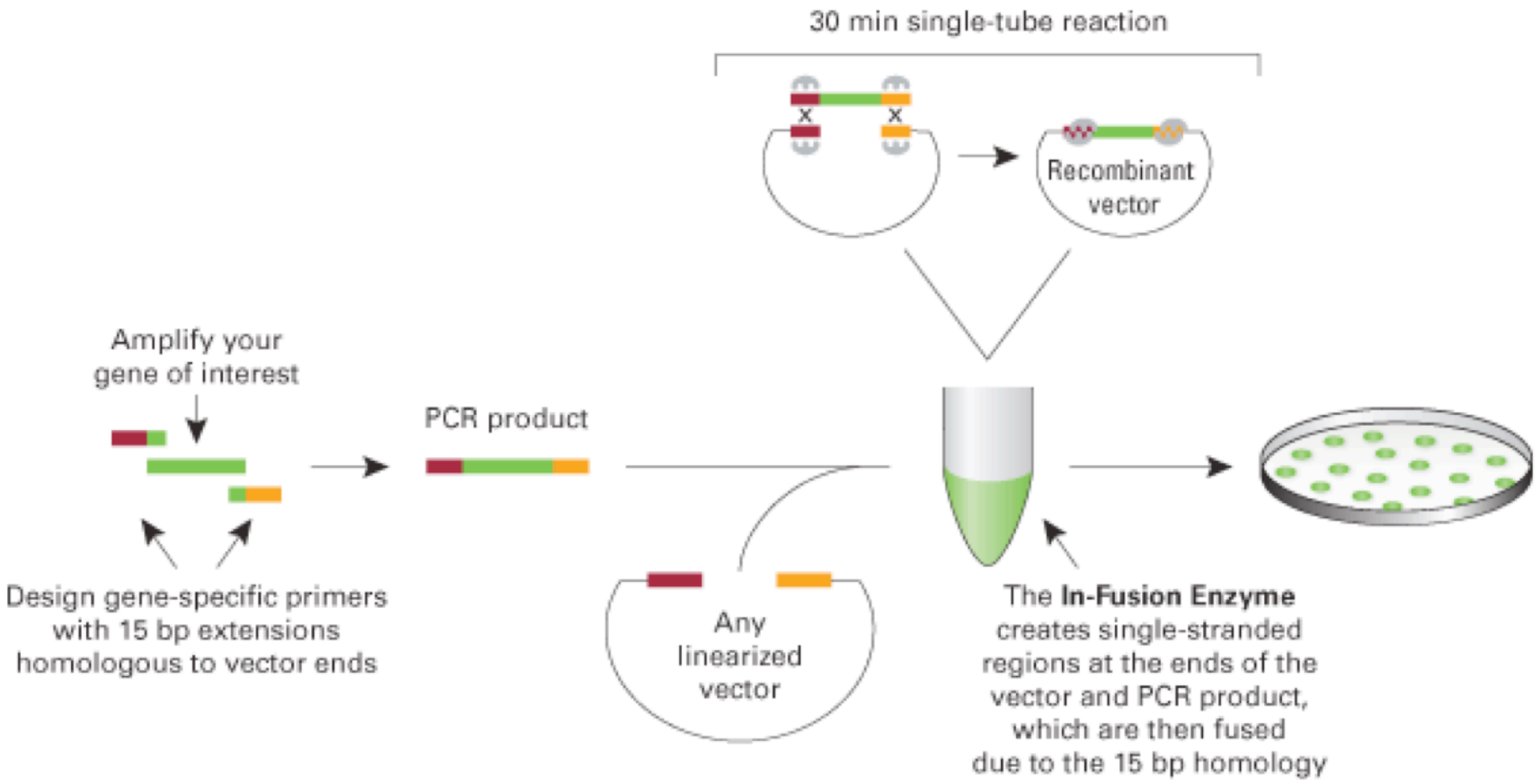
Objective is to use a recombination technique to swap the luciferase gene for the ATXN2 gene in pGL2c.5(B/C/D)3c in a single cloning step:



All native ATXN2 sequence with no parts of the XhoI or AgeI remaining

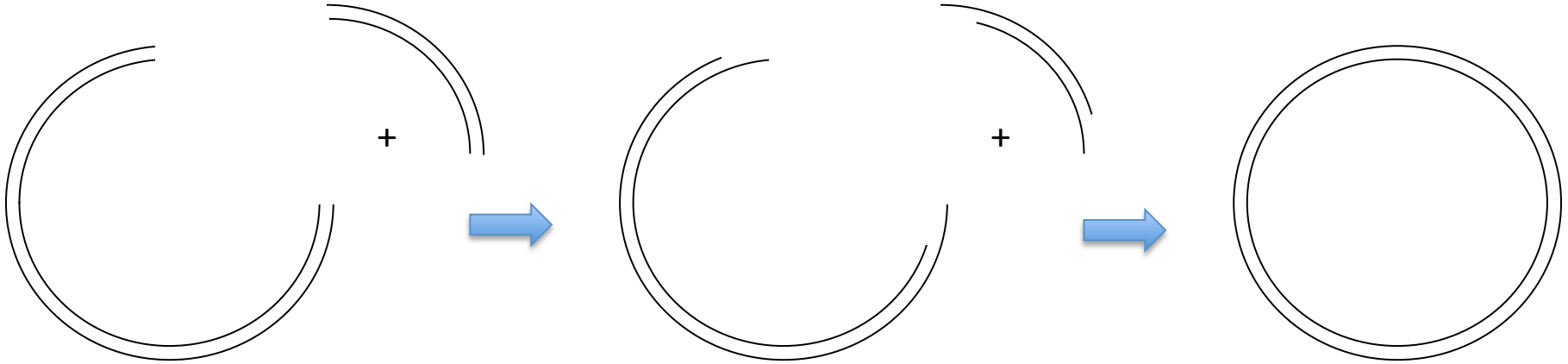
This approach was necessary so we could put ATXN2 downstream of the $(CAG)_n$ in different existing ATXN2-Luc clones with known CAG lengths. Without this kind of approach we would have to reclone the CAGs.

In-Fusion Concept



How In-Fusion actually works. This information was taken from web posts made by users of In-Fusion with intentions to understand the proprietary information of the product.

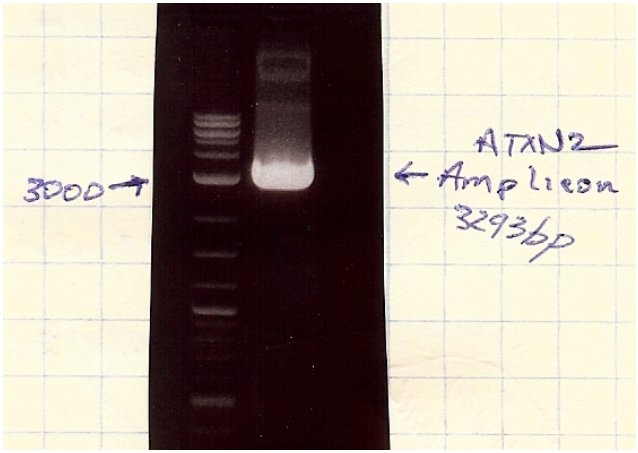
The In-Fusion Enzyme is a poxvirus DNA polymerase with 3' →5' exonuclease activity (Hamilton et al., 2007) from *vaccinia* virus. This enzyme promotes single stranded annealing reactions. It does this by first attacking the exposed ends of a broken double stranded DNA, removing bases on one strand in a 3'→5' direction, exposing ssDNA that can anneal with other homologous ssDNAs. A mixture of duplex molecules is created for about 8 min after the reaction started (Hamilton et al., 2007). Then, in the presence of dNTPs (also making duplexes resistant to exonuclease activity), the polymerase favors completion of duplex ends of DNA. The *E. coli* will fix the gaps and ligate the two pieces



In-Fusion Primers

tcctcgtcctcggccacggctccctcctcggtggt, "fusionA"
gggcagccttacaactgctgttggtggtgggcttgtac, "fusionB"

Amplification Conditions
Annealing T = 59C
Size=3263 bp without tags, 3293 bp with tags
Tm difference = 0.9 before tags added

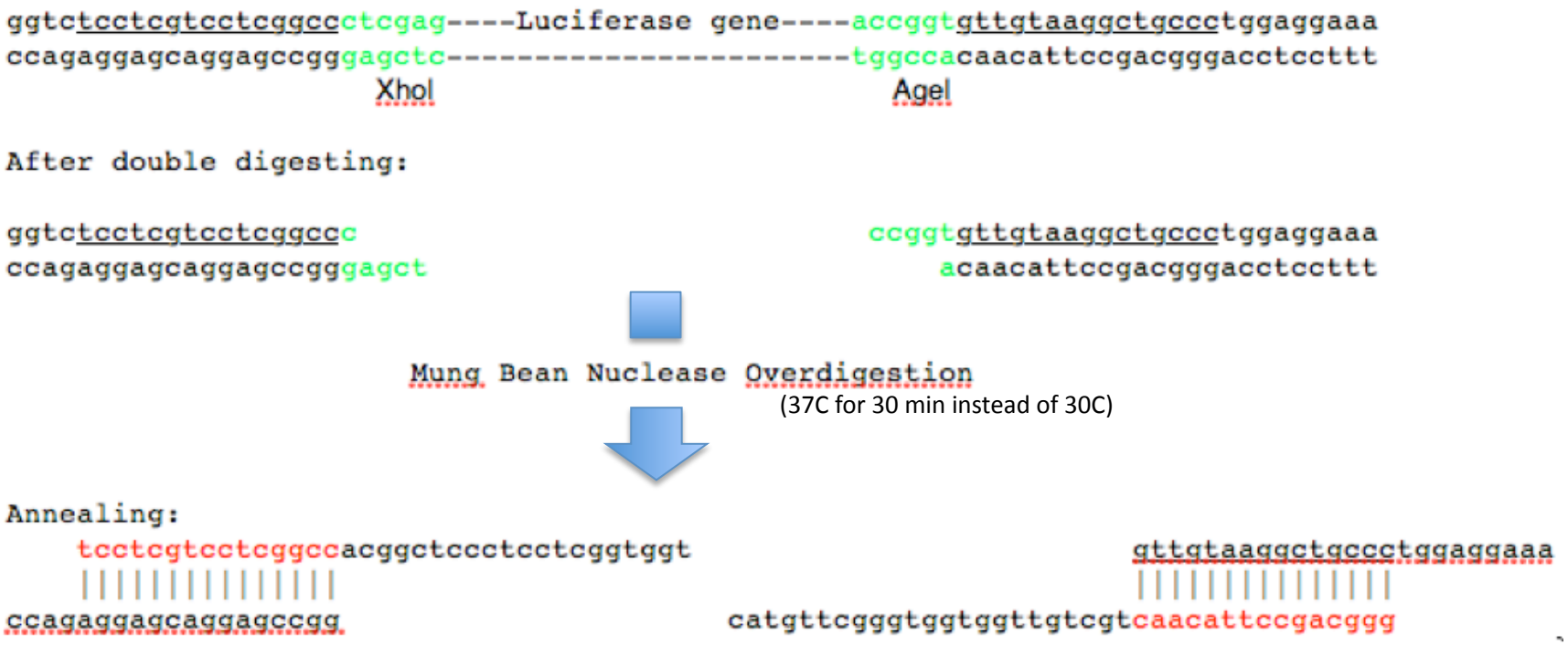


Double-strand sequence of vector and how oligos will anneal:

```
ggtctcctcgtcctcggccctcgag----Luciferase gene----accggtgttgtaaggctgccctggaggaaa
ccagaggagcaggagccgggagctc----------------------tggccacaacattccgacgggacctccttt
                        XhoI                          AgeI

After double digesting:

ggtctcctcgtcctcggccc                              ccggtgttgtaaggctgccctggaggaaa
ccagaggagcaggagccgggagct                              acaacattccgacgggacctccttt

Annealing:
    tcctcgtcctcggccacggctccctcctcggtggt                    ccggtgttgtaaggctgccctggaggaaa
    ||||||||||||||||                                        ||||||||||||||||
ccagaggagcaggagccgggagct                catgttcgggtggtggttgtcgtcaacattccgacggg
```

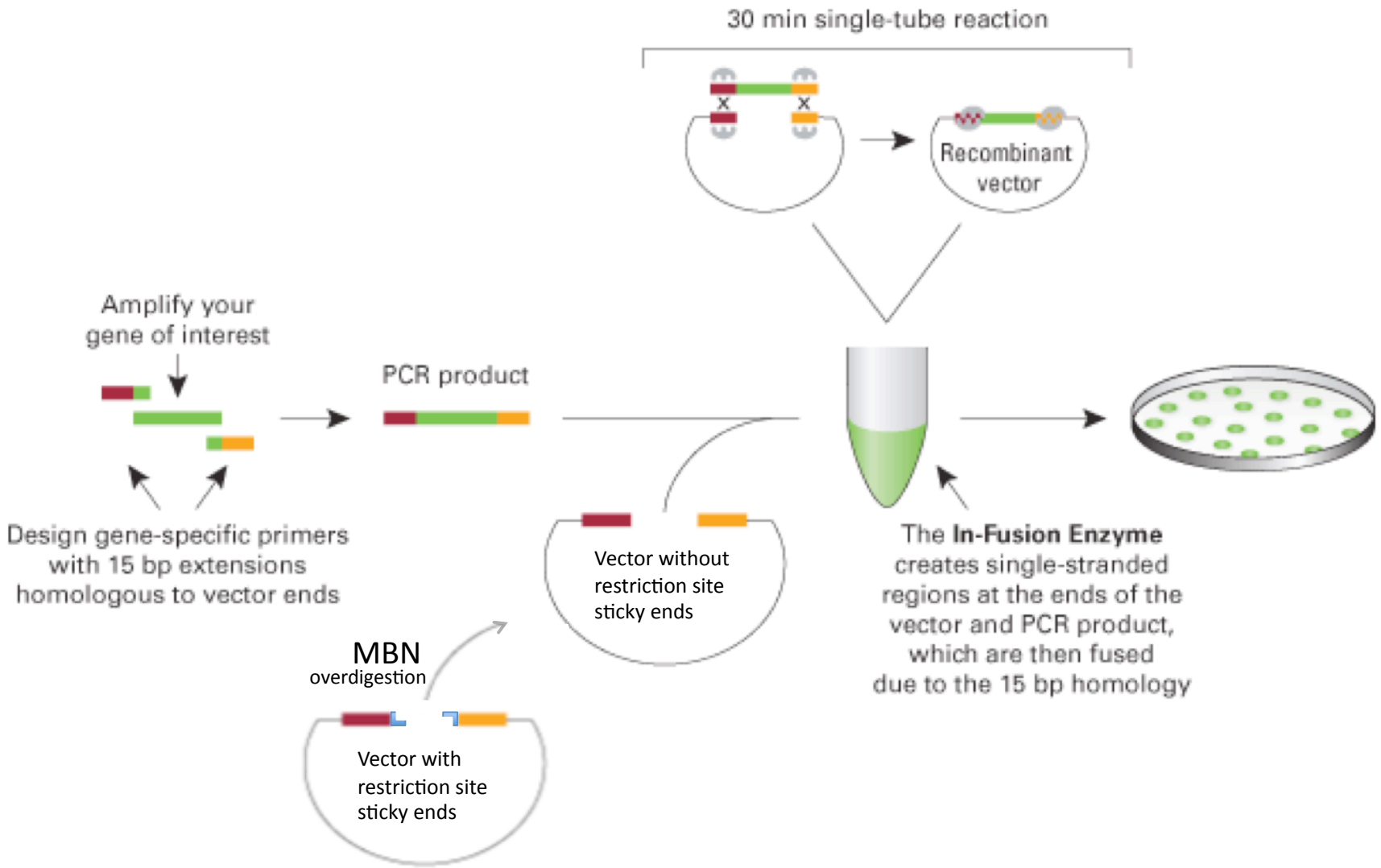
Because of the extra DNA from the restriction sites we predict this won't work.
The vendor told us there is no way around this problem but we found one…

…we will digest away the restriction sites by mung bean nuclease overdigestion



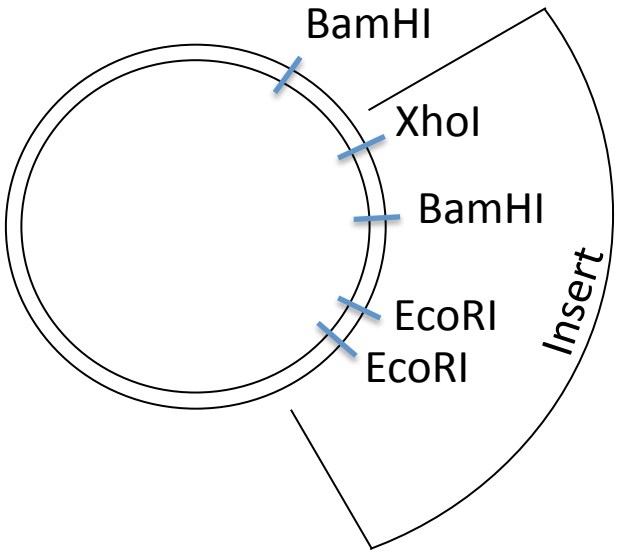
We predict this approach will work.

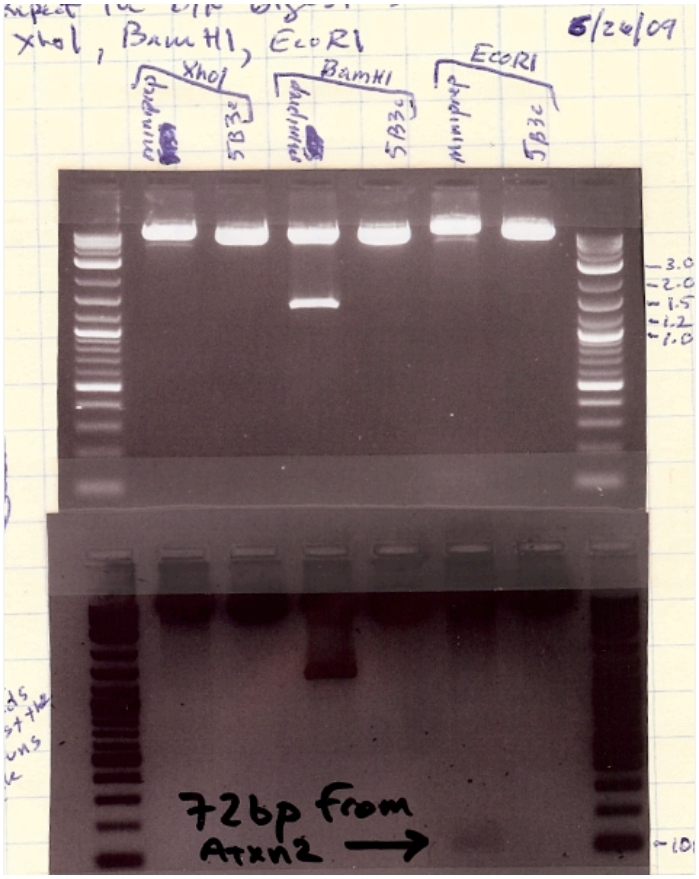
In-Fusion Concept w/ MBN



Result

Plated 2 ul of the reaction, got one colony.
Replated another 2 ul and got another colony.
Both were confirmed to possess the ATXN2 $(CAG)_{22}$ insert by sequencing, exactly as predicted.





We continued retrying transformations and eventually got colonies possessing all desired clones, containing ATXN2 genes with $(CAG)_{22}$, $(CAG)_{58}$, $(CAG)_{102}$ repeats. This was all accomplished upon the first use of the In-Fusion kit with the modified mung bean nuclease protocol.