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**Adaptive Evolution of Escherichia coli:  
Fitness and Genetic Changes Beyond 100th Passage**

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## **Abstract**

*Escherichia coli* lives in the human intestine and any form of adaptation may affect the human body. The effects of food additives on *E. coli* has been less studied compared to antibiotics. Since food additives and preservatives are being consumed by humans so often nowadays, it is important to investigate this relationship. In this project, we continue to study the evolution of *E. coli* in different food additives (sodium chloride, benzoic acid, monosodium glutamate) in different concentrations, singly or in combination, for over 83 passages. Adaptability of the cells was estimated with generation time and cell density at the stationary phase. Polymerase Chain Reaction (PCR) / Restriction Fragments Length Polymorphism (RFLP) were used with 3 primers and 3 different restriction endonucleases to analyze the adaptation at genomic level. The PCR product and the digestion profiles using the 3 different restriction enzymes were analyzed using Nei-Li Dissimilarity Index. Our results showed that adaptation started to slow down and the gradients of generation time against passage are less steep compared to the first 70 passages of the experiment, suggesting that most adaptive mutations occurred within the first 500 generations. In the genomic level, ecological specialization was observed as we found that the cells adapted through a different mechanism and diverge from each other although the resulting effect of the medium is the same. It could further suggest that different concentrations of food additives cause different types of chemical stress, instead of different levels of chemical stress.

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## **Abbreviations**

BSA	Bovine Serum Albumin
CC	Correlation Coefficient
DI	Dissimilarity Index
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphates
EDTA	Ethylenedinitrilotetraacetic Acid
H BA	High Concentration of Benzoic acid
H MSG	High Concentration of Monosodium Glutamate Treatment
H SALT	High Concentration of Salt Treatment
H COMB	Low Concentration of Combination Treatment
L BA	Low Concentration of Benzoic acid Treatment
L MSG	Low Concentration of Monosodium Glutamate Treatment
L SALT	Low Concentration of Salt Treatment
L COMB	Low Concentration of Combination Treatment
NB	Nutrient Broth
STE	Sodium Chloride–Tris–EDTA

# **1      Introduction**

*Escherichia coli* is a Gram negative bacterium commonly found in the lower intestines of warm blooded organisms. Most *E. coli* strains are harmless, but others strains like O157:H7, can cause food poisoning in humans. As part of the normal flora of the gut, they can benefit the host by producing vitamin K and also preventing the establishment of pathogenic bacteria within the intestine. However, these harmless bacteria could adapt and evolve which could then cause harm to our human body.

*E. coli* evolutionary studies on antibiotics resistance have been widely studied but the mechanism on non-antibiotic agents, like food preservatives and food additives has not been well established yet. Food preservatives are used frequently to inhibit the growth of microbes and food additives can delay the spoilage of food. Since *E. coli* in our gut are constantly interacting with such chemicals, it is important to examine their relationship.

Experimental works had been done to study the effects of 4 different food preservatives of 2 different concentrations on bacterial cells for over 70 passages. In general, the cells started adapting after 25 passage and the cells have continued to adapt to their individual treatment until over 70 passages.

This project continues to observe the adaptation of *E. coli* cultured in different concentration of food additives, namely sodium chloride, benzoic acid and monosodium glutamate. *E. coli* cells are cultured in 8 different media over 83 passages and swapped at intervals among the treatments, making up a total of an estimated 992 generations through 153 passages including those of earlier studies. Adaptability over time is estimated by generation time and cell density of stationary phase. Polymerase Chain Reaction (PCR) and Restriction Fragments Length Polymorphism (RFLP) were used to characterize adaptation/evolution at genomic level.

## **2 Literature Review**

Evolution is the result of genetic changes within a population from one generation to the other. These changes refer to the modification to the DNA sequence resulting in genetic variation. The genetic traits in the individual are inherited down from one generation to the other. As these individual genetic changes accumulate over time, a population can be formed through the process of genetic divergence (Lenski et al., 1991). These traits may vary within population and show heritable difference of the organisms. Genetic changes originated in any generation are usually small and the difference accumulated in each successive generations can cause substantial changes in the population. Eventually, new species may be emerged from the ancestor (a speciation event).

### ***2.1 Sources and Effects of Genetic Variation***

Genetic variation arises from different sources such as from mutation, gene flow and independent assortment during sexual reproduction. A mutation is defined as a permanent change in the DNA sequence of the genome, which ranges from a single nucleotide change (point mutation) to one or more nucleotides being inserted or deleted (frame shift mutation). The result of accumulation of many mutations may result in evolutionary changes in the given population (Stanek et al., 2009) due to protein structures changing as an effect of nucleotides sequences changing after the mutations (You et al., 2007).

There are two types of mutation. The first is through heredity mutation, which is the mutation passed down from parents to the offspring. Successive generation will contain the inherited mutations. The second is mutation acquired during the organism's lifespan from exposure to the environmental, physical or chemical stress. The acquired mutation may be beneficial to improve survivability and adaptability (Travisano, 1997). An example will be in the case of sickle cell disease found inhabitants of Sub-Saharan Africa (Hanchard et al., 2007), where malaria rates are very high. The sickle cell mutation allows the survival rate of the individuals carrying the sickle cell allele to improve as it halts the infestation of *Plasmodium malariae* (Hanchard et al., 2007).

Gene flow, also known as gene migration, is defined as the transfer of alleles from one population to another (Gayden et al., 2007). Gene flow may result in an addition of new genetic variant to a pool of established gene population (Faure et al., 2009). For example,

technological advances allow humans to travel across the globe. Therefore, allowing individuals may search for mates in other geographical regions. If a child is successfully conceived, and subsequently delivered, the allele is considered to have been transferred from one population to the other. However, physical barriers exists in between the species, such as the Himalaya Mountains, can hinder the transfer of alleles as haplotype differences have been reported between populations on different side of the mountains (Gayden et al., 2007). Gene flow within or across population can have different effects in evolution. When gene flow occurs within the same population, it will increase the amount of genetic recombination and increase the variety of genetic variants. Gene flow allows distant population to be genetically similar to each other (Faure et al., 2009) which helps to reduce chances of speciation. Speciation is the evolutionary process by which a new biological species arises by diverging itself from the parental species (Schluter and Conte, 2009).

Sexual reproduction is the production of offspring by combining genetic materials from parental genome which allows the chance for gene recombination into population, resulting in genetic diversity. Sexual reproduction is important as it is a method to introduce new combination of genes by homologous recombination to every successive generation which increases the ability for the organism to adapt to new environments (Levin and Cornejo, 2009). An example will be one of the parents having brown eyes and curly hair, and the other having blue eyes and straight hair and the conceived child having brown eyes and straight hair through independent assortment during sexual reproduction, However, there is also a possibility that good combination of genes may be removed (Dawkins, 2006).

The two main mechanisms responsible for evolution (Koonin, 2009) are natural selection and genetic drift. The process whereby the heritable traits are passed on to successive generations to improve the survivability of organism is known as natural selection (Hurst, 2009). Each trait is linked to a gene; therefore, when traits are inherited, it will also mean that the gene that is linked to the trait has been passed down. For natural selection to occur, heritable variation for the particular traits must be present and able to exist within the population. In addition, there must be differential survival and reproduction associated with the possession of that trait. Through natural selection, the advantageous or traits are passed on to the next generation and more offspring will be able to survive and adapt better. On the other hand, a trait that does not confer an advantage is unlikely to be passed over to the next generation due to them not being favourable for survival. In other words, if a gene is lethal, it will tend to be

removed from the gene pool due to it causing the death of the organism before it is able to reproduce (Dawkins, 2006). Some lethal or disadvantageous genes do get passed down when they are late-acting, as the effect of the gene only shows when the organism is late into its life cycle, after living long enough to have successfully reproduced offspring (Dawkins, 2006). The offspring will have a chance of getting the late-acting gene from its parents. This will allow the lethal or disadvantageous gene to continue to reside in the gene pool, allowing them to be passed down through the generations. In the case of genetic disorders, unfavourable traits will be passed down to the next generation in the form of autosomal recessive disease such as autosomal recessive cataracts (Sajjad et al., 2008). Recessive diseases may or may not be expressed; therefore, the undesirable traits may get passed down to the next generation.

A classical example (Saccheri et al., 2008) would be peppered moth (*Biston betularia*) in England. The original peppered moths were light grey which blended in well with the light-coloured lichens and tree bark. The dark grey peppered moths were disadvantageous and were easily preyed on. However, during the industrial revolution, soot produced by coal factories cause many trees to darken, in turn the white peppered moth is unable to blend into the surroundings; thus, losing its camouflaging abilities. The once disadvantaged dark grey peppered moths are able to survive better than the light grey peppered moth due to their abilities to camouflage better in the soot covered trees. Over time, through natural selection and adaptation, the peppered moths eventually changed from light grey to dark grey to match the colour of the trees (Saccheri et al., 2008).

A more recent example (Calsbeek et al., 2009) would be on environment resulting in evolutionary changes in brown anole (*Anolis sagrei*). In natural conditions, the lizards will increase their use of vegetation in wet years resulting in an increase of limb size but not body size. During drought season, the dieback of vegetation will result in a selection on body size and relaxed selection on limb length. With the return of the rain, selection will revert to the previous pattern of selection. However, when vegetations are removed from an environment the anoles will only change in body size and not limb size due to the natural selection forces acting on it (Calsbeek et al., 2009). Without the vegetations, the use of legs during the year will be reduced and the selection on the legs will relax, with only the body size will change when the seasons change.

The central concept of natural selection is the evolutionary fitness of an organism (Orr, 2009). The fitness refers to the proportion of subsequent generations that contains the genes. This concept measures the organism survivability and reproducibility, determining the size of its genetic contributed to the next generation (Orr, 2009). For example, if an allele of one gene confers better fitness over the other allele in the population, this allele will be selected and passed over to the next generation (Lenz et al., 2009). Subsequently, this particular allele will become more common within the population after each successive generation due to the advantages that the gene gives to the organism; more of them carrying the gene are more likely to reproduce and pass on the gene, causing the particular gene to be more common within the population.

The second mechanism for evolution is genetic drift (Mank et al., 2009). Genetic drift, also known as allelic drift is the change in the frequency of a genetic variant (allele) occurring in the population due to random sampling. As compared to natural selection which determines the genetic variant due to successive generations, genetic drift randomly determines the variant and it is not affected by physical, chemical or environmental stress. The variant randomly selected may be beneficial such as Z-linked genes resulting in an increase in evolution (Mank et al., 2009); neutral, such as alteration of promiscuous proteins (Bloom et al., 2007); or even detrimental, such as lost of eye functions (Munguia-Vega et al., 2007); to the next generation.

An example of beneficial genetic drift will be the increase in evolution rate through Faster-Z evolution by using avian genetics to study the whole genome of Zebra Finch, *Taeniopygia guttata* to test for Faster-Z evolution. In a study (Mank et al., 2009), Z-linked genes shows a 50% increase in evolution rate as compared to autosomal genes. The Faster-Z Effect was not shown in genes expressed predominantly in females; therefore, the data indicates that the largest source of Faster-Z Evolution is the increased levels of genetic drift on the Z chromosome. This is likely a product of sexual selection acting on males, which reduces the effective population size of the Z relative to that of the autosomal genes (Mank et al., 2009). An increase evolution rate in Z-linked genes in males will result in an increase in genetic variation when mating (Mank et al., 2009); therefore, allowing evolution to happen at a faster rate and increase the chances of having beneficial genetic drift.

An example of neutral genetic drift will be the alteration of promiscuous protein functions, which can potentially aid in functional evolution (Bloom et al., 2007). Many of the mutations accumulated by naturally evolving proteins are neutral as they do not significantly alter a protein's ability to perform its primary biological function (Bloom et al., 2007). However, new protein functions evolve when selection begins to favour other, “promiscuous” functions that are incidental to a protein's original biological role. It means that different proteins coming together and interact with each other resulting in new protein function but at the same time does not change or impair the original biological function that those proteins. If mutations that are neutral with respect to a protein's primary biological function cause substantial changes in promiscuous functions, these mutations could enable future functional evolution (Bloom et al., 2007). Initially neutral genetic drift can lead to substantial changes in protein functions that are not currently under selection, effectively poised the proteins to more readily undergo functional evolution should selection favour new functions in the future (Bloom et al., 2007).

An example of detrimental genetic drift will be the lost of eye functions in the Mexican Cave Tetra *Astyanax mexicanus* (Protas et al., 2007). Many of the mapped quantitative trait loci (QTL) from the eyes, lens and the melanophore number of the cave tetra as compared to surface tetra shows a reduction in allele size (Protas et al., 2007). The lost of eye functions need not necessarily detrimental but the energy cost of maintaining the tissues that replaces the eyes is sufficiently high to be detrimental in the cave environment (Protas et al., 2007).

Genetic drift can have several important effects in evolution. The drift will eventually stop when an allele disappear from the population or replaced by other alleles entirely. Once a particular allele is fixed, the genetic variation in the population will be reduced and its ability to evolve in response to new stressed may be lowered owing to the fact that only a single variation of the gene exist (Otto and Whitlock, 1997); therefore, the chances of another allele replacing the fixed gene is unlikely. Another issue is that the effect of the genetic drift is larger in small population and smaller in large population (Otto and Whitlock, 1997). Genetic drift occurs faster and has more drastic impact in smaller population due to the relative chance of genetic drift occurring is higher, resulting in alleles being more likely to be lost from the population. Thus, rare and endangered species which exist in a smaller population will be affected most by the drift. Genetic drift can also contribute to the process of speciation (Devaux and Lande, 2008). Through the process of genetic drift, there is

possibility that a small isolated population will be diverged from the larger population also known as speciation. One example will be the variation in threespine stickleback (*Gasterosteus aculeatus*) which have undergone speciation since the last ice age (Schluter and Conte, 2009). These fishes shows structural differences including variation in fins, changes in the number or size of their bony plates, variable jaw structure, and colour differences (Schluter and Conte, 2009).

Evolution affects the behaviour and the organisms' characteristics in many aspects (Koonin, 2009). The outcomes of evolution can lead to adaptation, extinction or speciation. Adaption is process which an organism changes physiologically, structurally or changes their behaviour in order for them to survive better in the environment. For example, *Biston betularia* adapting to colour changes in the environment due to pollution (Saccheri et al., 2008), *Escherichia coli* evolved the ability to utilise citric acid as a nutrient source (Blount et al., 2008) and *Flavobacterium sp.* able to grow on the by-product of nylon manufacture through the two newly evolved enzymes (Okada et al., 1983). However, only the fitter species in the given environment will be able to survive and reproduce as they are able to survive in the environment, thus allowing higher chances of reproduction to pass on their genes. Although evolution can lead to beneficial advantages in organism, a certain proportion of species are able to survive and reproduce by out-competing the other proportion, which may cause extinction of a species (Kutschera and Niklas, 2004).

## **2.2 Experimental Advantages of *Escherichia coli***

Evolution occurs in every species. However, using higher organisms to study evolution is impossible due to them having a long reproduction cycle. Therefore, it is not feasible to study them under experimental setups. Whole animals or animal cells reproduce much slower than bacteria; however, long generation time constrain is removed by studying bacteria. This is due to rapid reproduction of bacteria under optimum conditions. By utilising bacteria in experiments, such as *Escherichia coli* in evolution experiments, one can expect several advantages to it.

Firstly, *E. coli* are able to grow rapidly on chemically defined environment (Lenski et al., 1991), allowing easy manipulation of media and also easy generation monitoring. For example, different mixture of chemicals can be added to the culture medium or by growing

them in different temperatures (Cooper et al., 2001). At the same time, the rapid generation time for *E. coli* allows mutation to occur at a higher chance (Travisano and Lenski, 1996) (Travisano, 1997). In addition, *E. coli* reproduces by binary fission, which means all the factors affecting the mutation will only solely be based on the environment and not DNA recombination.

Secondly, several methods exist for the preservation of *E. coli* and other bacteria species and microorganisms. An effective and novel preservation method of *E. coli* by dehydrating them using pre-dried activated charcoal cloth based matrix contained within a re-sealable system (Hays et al., 2005) for long term storage and viability after revival of stored bacteria. Another method can be through the use of gelatin disks to store and transport a number of bacteria species which also includes *E. coli* (Obara et al., 1981). This gelatin disk drying method can be used to store and maintain viability of cells for a period of 1 to 5 years (Obara et al., 1981). *E. coli* can be cryopreserved for extended period of time at -79°C for couple of years (Proom and Hemmons, 1949). For shorter storage period, preservation can be done at -20°C to 4°C (Proom and Hemmons, 1949). Concerns about constantly changing test kits and instability factors can be removed due to the ability of preserved cells able to be taken out for competition against ancestor strain (Achá et al., 2005). Direct estimates of fitness, and fitness components, can be made in the selected environment as well as novel environments (Travisano, 1997). Should there be any incident that requires the experiment to be restarted, the preserved cells can be resurrected for the experiment again.

Lastly, being a model organism in genetics, genomics, molecular biology, biochemistry and cell physiology, it is well studied and several strains have been fully sequenced (Chaudhuri et al., 2010). This includes both pathogenic (*E. coli* O157:H7) and non-pathogenic strains (*E. coli* ATCC 8739 and *E. coli* S88). This not only provides information about *E. coli* that helps interpret results from studies but also enable researchers to determine the identity of the gene mutated, throughout the course of their experiments.

### **2.3 Examples of Evolution Experiments with Bacteria**

Considering the various advantages of utilizing bacteria for evolution experiments, there had been a number of studies using various bacteria strains. A few examples such as, *E. coli* MG1655 (Fong et al., 2005), *Escherichia fergusonii* (Touchon et al., 2008), *Streptococcus pneumoniae* (Levin and Cornejo, 2009), *Haemophilus influenzae* (Levin and Cornejo, 2009),

and *Bacillus subtilis* (Levin and Cornejo, 2009), *Caulobacter crescentus* (Ackermann et al., 2007), *Thermus thermophilus* (Akanuma et al., 1998), *Pseudomonas aeruginosa* (Prijambada et al., 1995), *E. coli* WATCC 9637 (Daumy et al., 1985), and *Proteus rettgeri* (Daumy et al., 1985).

Evolutionary experiments have also used *E. coli* in many of their experiments. An example where ara<sup>-</sup> and ara<sup>+</sup> *E. coli* strains to test on environmental constraints on adaptation and divergence (Travisano, 1997), target of selection and specificity on adaptation (Travisano and Lenski, 1997) where fitness have increase when bacteria are placed in novel environments with sugars that share the same uptake mechanism with glucose, but genetic variances increases more when bacteria are placed in novel environment containing sugars that do not share the same uptake mechanism with glucose.

A recent study (Ackermann et al., 2007) of evolution theory using the bacteria, *Caulobacter crescentus*, shows that there is a decline in reproduction and survival with increasing age, or in this case, increasing generations. According to evolutionary theory, aging evolves because selection late in life is weak and mutations exist whose deleterious effects manifest only late in life (Ackermann et al., 2007). The experiment data shows that a strong growth rate is present early in the experiment, but very weak as the generations continues. This is due to late acting deleterious mutations that have evolved in the bacteria genome.

Another recent study (Levin and Cornejo, 2009) looks at the population and evolution dynamics of homologous recombination in bacteria, using *Streptococcus pneumoniae*, *Escherichia coli*, *Haemophilus influenzae*, and *Bacillus subtilis*. The source of variation in this case will come from the external environment in such as horizontal gene transfer (HGT), HGT plays a central role in adaptive evolution (Levin and Cornejo, 2009). The contribution of homologous gene recombination (HGR) on the above bacteria are done and results shows that homologous gene recombination is happening at a predicted rate and the rate of adaption to the environment is much faster and also, once homologous gene recombination have occurred selection for higher rates of evolution will promote the maintenance of bacteria-encoded mechanisms for HGR (Levin and Cornejo, 2009).

A study (Daumy et al., 1985) demonstrating 2 different isoforms of Penicillin G acylase from *E. coli* and *Proteus rettgeri* with distinctly physical (isoelectric point and molecular weight)

and biochemical (regulation and chemical repressibility) activities can be made to evolve to similar physical and biochemical characteristics using selective media. Apart from altering biochemical properties of a protein, thermal stability has also been improved. In another study (Akanuma et al., 1998), the thermo stability of 3-Isopropylmalate dehydrogenase, an enzyme involved in leucine biosynthesis, from *Bacillus subtilis* was improved from 37°C to 70°C in *Thermus thermophiles* through sequential increase in culturing temperature. A sequence analysis of the mutant enzyme showed 3 amino acid substitutions as compared to the original enzyme in *Bacillus subtilis*.

## **2.4 Long-Term Experimental Evolution in *Escherichia coli***

*Escherichia coli* have often been used for experimental evolution studies (Luria and Delbrück 1943; Atwood et al., 1951) because of its short generation time which makes it possible to observe the effects of natural selection on mutation that have arisen during the course of the experiment (Lenski et al., 1991). *E. coli* has a relatively simple genome compared to eucaryotes, which can be manipulated easily and its natural occurrence at the lower gastrointestinal tract of mammals, including humans, were the reasons why it was so widely used in evolutionary experiments (Elena et al., 2005).

A well known evolution study initiated more than 20 years ago (Lenski, 1988) examines the adaptation in 12 populations of *E. coli* B in glucose-supplemented minimal medium. Adaptation is observed through competitive fitness relative to the ancestor (Lenski, 1988). The rate of increase in fitness relative to the ancestor in the first 1000 generations was higher compared to the next 1000 generations, demonstrating that the organism had adapted to the glucose environment (Lenski et al., 1991). This suggests that the bacterium adapted quickly during the first 1000 generations and the slower adaptation in the next 1000 generations suggests that the adapted bacterium tries to optimise its survival in the selected condition. Mutations had been found by RFLP screening (Schneider et al., 2000), some of which are beneficial. An example would be a rapid evolution of the loss of ribose catabolism, which suggests that this change was beneficial to the organism in adapting to the new environment (Cooper et al., 2001). There was a significant increase in fitness compared to strains that have the ribose catabolism activity.

Another common change was the loss of protein expression of the maltose regulon (Pelosi et al., 2006). To investigate the extent of genetic parallelism, the *malt* gene was sequenced in one evolved clone isolated at 2,000 generations from each of the other 10 long-term populations. Nine mutations were found in a spread of six populations, including the two focal populations, a total of eleven mutations were found in *malt* spreading among eight of the total of twelve populations. Mutations are constantly happening in evolution, most of them to increase its fitness in the selective condition. The others are deleterious and are harmful to the organism and most probably due to random drifts since the mutation is random and chances are the mutation is harmful to the organism.

Another adaptation is the change in the cell shape and size of *E. coli* (Philippe et al., 2009). This is due to a mutation in the gene coding for penicillin binding protein (PBP). PBPs catalyse the final stage of peptidoglycan synthesis and peptidoglycan is the major component of the bacterial cell wall. A mutation resulted in reduced cellular concentration of PBP (Philippe et al., 2009) was identified in the *pbpA* operon and this operon encodes for these penicillin binding proteins. As a result, spherical cells were generated. Rapid fitness gain was also an effect of this mutation as a 6-day competition assay was conducted to compete with the ancestor strain. Results demonstrated that the evolved strain which contained the mutated *pbpA* operon have significantly enhanced fitness (Philippe et al., 2009).

In another experiment, statistical analyses, including analyses of parallel substitution of mutation in *pbpA* in 6 of 12 populations, suggested that this mutation is beneficial (Woods et al., 2006), an inference which further supported their study which concluded that the evolved strain has a rapid fitness gain. However, this mutation has detrimental effects. The evolved strain has reduced resistance to osmotic stress and also it has reduced fitness during the stationary stage (Philippe et al., 2009) as lowered expression of PBP2 resulted in osmotic sensitivity due to the reduction in the cell wall. When the evolved *pbpA* allele was introduced in to the ancestral strain, the cells were more sensitive to osmotic stress; this phenotype was reversed by restoring the ancestral allele. This illustrated that the evolved strain is beneficial in the selective environment they arose from, but they are deleterious under other conditions like liquid Luria Bertani media containing salt; thus, indicating there is trade-off (Philippe et al., 2009). Trade-off mutation is an adaptation to the selective environment where an existing function is often lost and is traded for another function to allow optimal survival in that particular environment only. However, the function that was traded in is often not useful in

other different environments. This pattern of trade-off, implying that there is ecological specialisation.

Two processes, mutation accumulation and antagonistic pleiotropy, can produce this specialisation. Mutation accumulation (Cooper et al., 2000) includes random drift, hitchhiking, mutations that are not beneficial in the selective environment. Antagonistic pleiotropy refers to mutation that occurs to be beneficial in the selective environment but deleterious in any other environment (Cooper et al., 2000). Temperature is another factor that affects the metabolism rate of the bacteria (Achá et al., 2005).

A study (Cooper et al., 2001) where *E. coli* was cultured for 2000 generations in low and high temperature was conducted. Most of the populations were lost during the early phase of rapid adaptation to the environment but the surviving populations were believed to have undergone antagonistic pleiotropy. Antagonistic pleiotropy is driven by natural selection and the beneficial mutation is also the deleterious one in another environment, mediating a trade off (Cooper et al., 2000). The population adapted the fastest during the initial exposure to the environment and the rate of adaptation decreases over time. This was noticed to be similar in the rate of functional loss, which was fastest in the start and lower at the later phase. This differs from mutation accumulation because the rate of mutation which causes functional loss is constant. This type of mutation seems to be the best choice for the organism as it cannot predict which environment it is going to be placed in next and so, optimise survival in the current condition would be most appropriate.

Environmental conditions can affect the shape of the adaptive landscape and also the path of the evolving population (Cooper and Lenski, 2010). For example, a heterogenous environment might lead to a rugged adaptive landscape, and so it is more likely that the replicate population would evolve towards different and distinct adaptive peaks. Unlike a uniform environment, the replicate population would most likely move towards the same adaptive peak.

In another experiment (Cooper and Lenski, 2010), different environment were used to promote divergence of the bacteria, which caused the organism in the environment to evolve towards different adaptive peaks. Seven different environment were created; glucose, maltose, lactose, glucose and maltose, glucose and lactose, glucose/maltose and lastly,

glucose/lactose. In the environment with glucose and maltose, both sugars were presented to the bacteria simultaneously while in glucose/maltose environment, the two sugars were presented to the cells alternating daily. After 2000 generations, the results demonstrated that all treatment groups' fitness increased significantly in each of their respective environments which indicated that all populations have adapted to their selective environment. It was found that adaptive divergence was present in all environments but variation in fitness tends to be greater in the fluctuating environment; glucose/maltose, glucose/lactose, as compared to the constant environments (Cooper and Lenski, 2010). In addition, the divergence in the fluctuating environments was increasing over time (Barrick et al., 2009). This shows that the populations in these treatment groups were evolving toward different adaptive peaks (Cooper and Lenski, 2010).

However, it is less obvious whether the evolving populations that were moving towards different adaptive peaks were evolving as generalists, where the organism are adapted to both environment or coexisting specialists where the population have two types of specialists; one well adapted to lactose or maltose and the other well adapted to glucose. It could also be possible that both generalist and specialist both evolve in the replicate populations which could be the reason of the high level of variance in fitness.

Another possibility would be, if the evolved populations are all generalists, it is questionable whether they are similar or diverse generalists as diverse generalists with different adaptive peaks would give high variance in fitness. Compensatory evolution is a situation in which a second substitution compensates for the deleterious effects of an earlier substitution. Compensatory evolution of gene regulation in response to stress by *E. coli* lacking RpoS is another example of evolutionary study done on the bacteria (Stoebel et al., 2009). The RpoS sigma factor protein is a regulator of physiological responses to a variety of stresses, such as osmotic stress (Weber et al., 2005). This suggests that cells with non-functional RpoS will be not as fit as cells having the functional RpoS in stressful environment. The results showed that the non-functional cells evolved along the same mutation line were indeed, less fit than the functional RpoS cells (Stoebel et al., 2009). It was predicted that the less fit organism will have more possible mutations resulting in a higher rate of variation in fitness increase (Fisher, 1930).

However, it was found that the variation in the increase of fitness is low because the same mutation was fixed in all non-functional populations. One possible explanation is that the deletion of the *rpoS* causes the strain to move towards a smaller peak, rather than a higher peak which would give a higher rate of fitness increase. On this smaller peak, the impact of the adaptive mutations will be smaller, resulting in more parallel evolution. In other terms, this meant that trehalose synthesis is so important that upregulating of the *otsBA*, a requirement for osmoticprotectant trehalose synthesis, is much more favoured than any other adaptive mutation. It was also found that the mutation was mediated by insertion sequence 10 (IS10). While this is unusual (Cooper et al., 2001), IS elements have been frequently found as the causes of adaptation mutation in experimental evolution. This adaptive mutation is initiated by the insertion of an IS element into the promoter of the *otsBA* operon. OtsA and OtsB are required for the synthesis of osmoticprotectant trehalose, and the transcription of *otsBA* requires RpoS to be in the functional state. The evolved strain had IS10 inserted and rewrites expression of *otsBA* from RpoS-dependent to RpoS-independent phenotype; therefore, allowing for partial restoration of the wild-type response to osmotic stress.

By having this compensatory mechanism which requires a second mutation to partially reverse the deleterious effect of the first mutation; it suggests that abolished RpoS function may be unlikely or impossible to reverse. Therefore, needing this mechanism to increase the fitness of the bacterium once the RpoS function had been removed.

Changes like loss of protein expression of the maltose regulon, cell shape and loss of ribose catabolism are just mechanisms for one purpose - to increase the organism fitness in the selective environment. Both the loss of protein expression for maltose regulon (Pelosi et al., 2006) and ribose catabolism (Cooper et al., 2001) led to fitness gain, which suggests that the mutation is beneficial. The cell shape changes to be rounder and contain a higher volume (Philippe et al., 2008). This change increases the metabolism rate of the bacteria which then causes rapid growth rate. Whatever the change, the cell is moving towards an adaptive peak where it will be at its optimal fitness. When the cell cannot reverse the effects of a mutation, compensatory mechanism comes into the picture to reduce the effects of the deleterious mutation.

The environment also plays a role on how the cell is going to adapt to it. For example, in heterogenous environment where the cells would be placed in two different conditions

alternatively, the cells are going to adapt differently compared to cells placed in a uniform environment. When the cells adapt, rate of increase of fitness is usually high at the start of the adaptation and gets lower over time (Lenski et al., 1988), a reason for this could be because it had to quickly deal with the important adaptation which is necessary for survival in the selective condition. Although there is a low rate of increase of fitness after some time, adaptation is still occurring and these small adaptation could possibly be mutations that are not important but beneficial to the bacterium. Cells placed in a selective environment would move towards a certain adaptive peak and depending on the environment, the topology of the adaptive landscape changes. This adaptive peak represents the optimal condition of the cell in that selective condition and it would take a very long time if not, impossible to reach the peak. This can be observed from experiments where the fitness is continuously increasing although the rate maybe getting slower (Lenski et al., 1988).

## **2.5 Effects of Chemicals on Bacteria**

In terms of the effects of chemical treatments, bacterial resistance and tolerance to antibiotics are well established and the mechanisms widely studied (Cardonha et al., 2005, Karami et al., 2007, Langsrud et al., 2004, Odahara et al., 2006, Pezzotti et al., 2003). In contrast, mechanisms of insusceptibility to non-antibiotic agents, such as food preservatives and antiseptics which might include tolerance (cessation of growth without cell death) or resistance (adaptation to agents as a result of mutations to acquire unresponsiveness to agents), are less understood. Food additives are important means of limiting microbial growth (Salmond et al., 1984) and adding flavors to various types of food products. For example, citric acid inhibits the growth of proteolytic strains of *Clostridium botulinum* (Russell, 1991) because of its chelating properties which decrease the pH in the medium. Sodium chloride can inhibit the growth of many bacteria such as *Listeria monocytogenes* (Garner et al., 2006), *Ochrobactrum anthropi* (Kesseru et al., 2002) and *Lactobacillus plantarum* (Glaasker et al., 1998) by lowering the water activity (Verluyten et al., 2004). The water activity ( $a_w$ ) of a solution is the ratio of the vapour pressure of the solution to the vapour pressure of pure water at the same temperature (Lund et al., 2000). Water activity in solutions can be lowered through the addition of chemical species, such as crystalline salt (Karel et al., 2003). When a cell is placed from a medium of normal water activity (isotonic solution), into a medium of lower water activity (hypertonic solution), a difference in osmotic pressure would result, causing the transfer of water from inside the cell to the medium, thereby leading to cell

shrinkage and death of the organisms and cessation of microbial growth (Karel et al., 2003). However, some bacteria which are halophilic are able to grow well in high salt conditions (Kobayashi et al., 2000, Kushwaha and Kates, 1979, Chan et al., 1979). Fatty acids (Sheu and Freese, 1972, Carson and Daneo-Moore, 1980, Saito and Tomioka, 1988, Speert et al., 1979, Miller et al., 1977), such as formic (Dashper and Reynolds, 2000), acetic (Roe et al., 2002), propionic acid (Maruyama and Kitamura, 1985, Salmond et al., 1984), are also capable of inhibiting bacteria growth.

A recent study (Lee et al., 2010) on the effects of chemical treatments of bacteria with the use of monosodium glutamate (MSG), benzoic acid (BA), Salt (S), and the combination (COMB) of these 3 food additives, each at both high and low concentrations, were conducted. In their study (Lee et al., 2010), *Escherichia coli* ATCC 8739 (Reference Passage 4 from ATCC) inoculated into 8 different treatment supplementation in Nutrient Broth [0.025% (w/v) as high monosodium glutamate (H MSG), high benzoic acid (H BA); 0.0025% (w/v) as low monosodium glutamate (L MSG), low benzoic acid (L BA) 1% (w/v) NaCl as high salt (H SALT), Nutrient Broth as low salt (L SALT); H MSG, H BA, H SALT as high combination (H COMB); L MSG, L BA, L SALT as low combination (L COMB)]. Subculture was performed using 1% of the previous culture 3 times per week.

The results suggested that the *E. coli* cells from all the 8 treatments have evolved and adapted to their individual environments, but at different rates (Lee et al., 2010) Generally, it was noted that the higher the chemical stress, the higher the rate of adaptation, since higher levels of chemical stress force the cells to adapt quickly in order to survive, resulting in a faster rate of decrease in generation time. It is unlikely that the entire population will survive under higher chemical stress, but the surviving subpopulation will undergo mutation, leading to increased adaptation. However, past the threshold of chemical stress, cell death occurs in almost all populations, with no surviving cells that can adapt to the treatments. Higher chemical stress (below the threshold) will lead to more mutations which forms more variants in the surviving population that allow for increased adaptation (faster rate of decrease in generation time). Therefore, trends in rates of decline in generation time across the passages can indicate the level of chemical stress introduced to the *E. coli* cells.

Generation time trend had been employed to determine the adaptability of cells to different stress level because in an adaption study (Helling et al., 1987), *E. coli* was cultured in a

constant environment for 765 generations (Helling et al., 1987). Mutations occur mostly during the first half of the culture, indicating that adaption took place during the early half of the experiment. More variants were seen in subsequent passages suggesting that variants had a faster generation time. Therefore, a decrease in generation time would be indicative of variants.

Chemical stresses introduced to the *E. coli* were brought about by the 8 treatment media the bacterial cells were subjected to. Different types and levels of stress can be induced by different food additives at different concentrations (Lee et al., 2010). Different additives in a single treatment were also suggested to have interacting effects which alters the level of chemical stress (Lee et al., 2010).

Treatments exerting the highest level of stress (highest rate of decline in generation time) on the cells were H COMB, followed by L MSG, H BA, L BA, L COMB, L SALT, H SALT, and H MSG. H COMB was chemically most stressful, with the accumulation of stresses induced by H MSG, H BA, H SALT, together with their interacting effects. H BA induces more stress to L BA. MSG, at different concentrations, induces a difference type of stress to the cells. H MSG acts as a nutrient source for *E. coli* cells to aid their growth (lowering stress), while L MSG induced significantly more stress. L BA and L MSG were observed to have counteracting effects, with L MSG preventing the drop in pH caused by L BA, causing similar trends in L COMB and L SALT.

In addition, results from the PCR/RFLP (Lee et al., 2010) showed convergence in the Nei-Li dissimilarity index which was used to compare between bands produced from different treatments within the same passage. This could suggest similar mutation that may have evolved the same type of stress and DNA repair mechanisms (Global stress response). However, this convergence could only apply to areas of the genome amplified (0.37% of genome). Thus, there may still be other stress handling genes, not within the amplified regions that have played a part in adaptability through their mutation. Given spontaneous mutation, and a declining trend of adaptation, it was unlikely for genetic distance between cells in eight treatments to reach zero.

## **2.6 Aims and Hypothesis of Project**

Examination of the growth kinetics and genetic changes of *E. coli* growing in different concentration of food additives, namely sodium chloride, benzoic acid and monosodium glutamate, for 70 passages, over 465 generations were studied (Lee et al., 2010). This study aims to continue the long term study of evolution in *E. coli* under food additives to simulate a person consuming food additives for an extended period of time, observing growth kinetics and genetic changes in *E. coli* for a further 80 passages. Continuing the experiment over a further 80 passages allows for change in trends of generation time, or fitness in later parts of the adaptation to be detected, since some genetic changes may only occur in later passages. It is hypothesized that the rate of adaptation of the *E. coli* to the various treatments will slow down beyond the 150<sup>th</sup> passage, with the generation time stabilized by then, due to the deceleration in the rate of adaptation; thus, limiting the opportunity for further divergence in mean fitness, because any further divergence in fitness requires further adaptation (Lenski et al., 1991; Lenski et al., 1994). Most genetic variation and adaptation would have occurred in the early half of the experiment, with the rate declining as the selection progresses.

Since it was hypothesised that the rate of adaptation slows down beyond the 150<sup>th</sup> passage, with lesser genetic variation, RFLP/PCR profiles between treatment samples of the same passage are likely to be similar. RFLP/PCR profiles are also expected to be similar as a result of possible adaptation and activation of similar stress-handling mechanisms regardless of the type and level of stress subjected to the cells, leading to a convergence of the Nei-Li Dissimilarity Index (DI) from all treatments (Nei and Li, 1979).

In the first 70 passages studied (Lee et al., 2010), results from the swap experiments suggested that low and high treatments of the same food additive, although differing in just their concentrations, could pose a different type of chemical stress to the cells instead of just different levels of stress. To further prove this point, a new swap of L SALT (0.7% NaCl) and H SALT (1.7% NaCl) treatment cells into Higher salt (2.7% NaCl) media would be done. Since it was hypothesised that different concentrations of the same food additives will render different types of chemical stresses to cells, L SALT treatment cells would be expected to be able to adapt better (faster decline in generation time) to higher salt treatment as compared to H SALT treatment cells, with L SALT, H SALT and higher salt media each posing a different type of chemical stress to the cells. This would be unlike the case in which different

concentrations of the food additive just causes different levels of chemical stress, whereby H SALT cells would have adapted better to the higher salt media, since they have already gradually adapted to their own individual treatment before being swapped into higher salt media and are less chemically stressed when introduced into higher salt media as compared to the L SALT cells swapped into higher treatment media.

### **3 Materials and Methods**

#### ***3.1 Main Culture Experiment***

Lysophilised *Escherichia coli* ATCC 8739 strain (Reference Passage 4 from ATCC) were revived on nutrient agar plate and incubated at 37°C before inoculating into 8 different treatment supplementation in 10 mL Nutrient Broth [0.025% (w/v) as high monosodium glutamate (H MSG); 0.0025% (w/v) as low monosodium glutamate (L MSG); 0.025% (w/v) as high benzoic acid (H BA); 0.0025% (w/v) as low benzoic acid (L BA); 1% (w/v) NaCl as high salt (H SALT), Nutrient Broth as low salt (L SALT); 0.025% (w/v) monosodium glutamate, 0.025% (w/v) benzoic acid and 1% (w/v) NaCl as high combination (H COMB); 0.0025% (w/v) monosodium glutamate and 0.0025% (w/v) benzoic acid as low combination (L COMB)]. Subculturing was performed by transferring 1% (100 µL) of the previous culture on every Monday, Wednesday and Friday to the next passage. Optical density (OD) readings were taken before the next subculture at 600 nm wavelength to estimate the number of generations within the current passage and to also determine the number of cells that are being inoculated into the new passage. In addition, OD<sub>600</sub> readings were taken on day 5 and 7 of each Monday's, Wednesday's and Friday's culture to calculate the Day7/ Day 5 cell density ratio and the number of generations. Generation time was measured on every 3<sup>rd</sup> passage. Glycerol stocks for each treatment were made from 1% of the culture for every 12<sup>th</sup> passage after culturing on MacConkey agar. The main culture experiment was continued from passage 71 from the previous final year project in academic year 09/10, *Evolution Characterization of Escherichia coli using RFLP DNA fingerprinting* (Lee et al., 2010). This was continued and maintained until Passage 153.

Starting Passage: ATCC 8739, 4<sup>th</sup> Passage from ATCC reference

Continuation Passage: 70<sup>th</sup> Passage from Lee et al., 2010

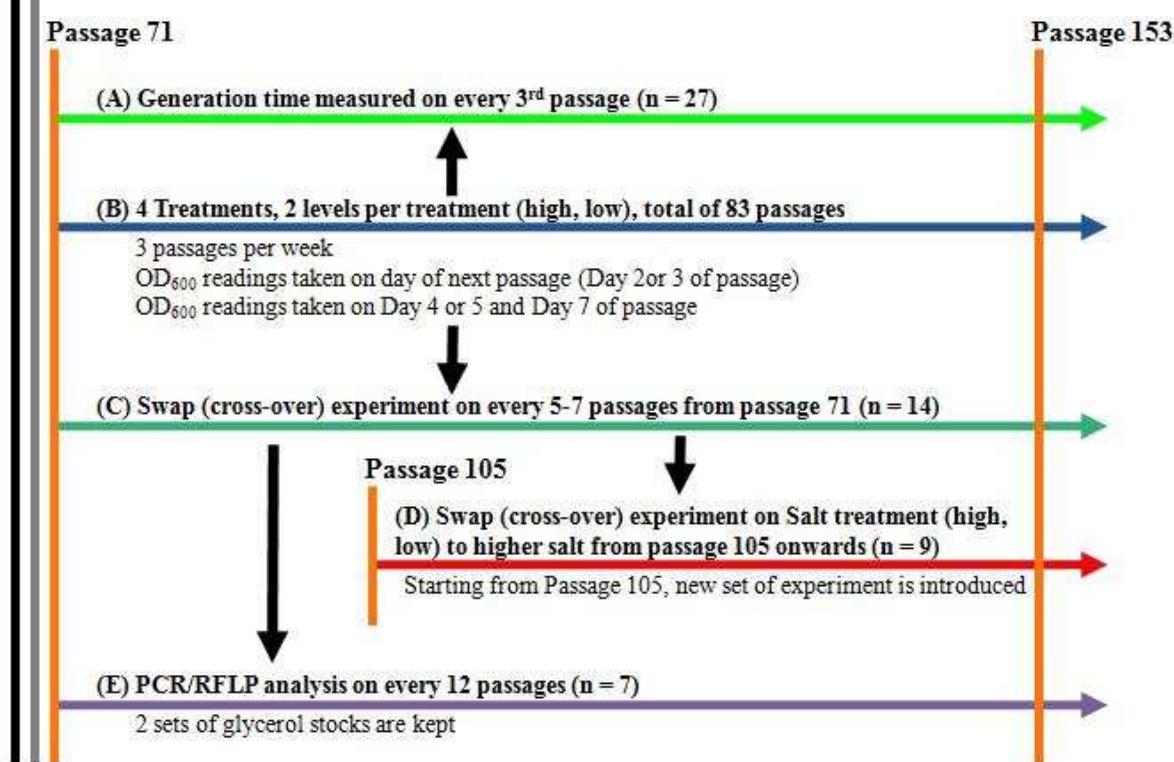


Figure 3.1: Complete Experimental Design.

### 3.2 Swap Experiment

The swap experiment was done fortnightly (6-7 passages interval), involving the transfer of *Escherichia coli* cells cultured in different treatments to other treatments for the measurement of generation time. Five types of swaps were carried out, whereby the cells were inoculated into the new treatment in a 100 times dilution. The first set of swap involves the inoculation of basal medium (L SALT) treated cells into the six non-salt treatments. An example would be inoculating cells grown in L SALT into H MSG treatment. For the second set, cells cultured in high and low concentrations of each treatment were swapped for all treatments. For example, cells growing in H MSG were inoculated into the L MSG media and vice versa. In the third set, cells of high concentration treatments (H MSG, H BA, H SALT) were each inoculated into the H COMB treatment. The next set is similar to the previous set except that cells of the low concentration were swapped. Cells from low concentration treatments (L MSG, L BA, L SALT) were each inoculated into L COMB media. The last set of swapping involved the inoculation of H SALT and L SALT treated cells into higher salt (2%) treatment.

media. OD600 readings were recorded down at intervals and generation times were calculated for each interval.

### ***3.3 Polymerase Chain Reaction / Restriction Fragments Length Polymorphism***

**Genomic DNA Extraction.** Treatment cultures from every 12<sup>th</sup> passage interval were used for Genomic DNA extraction. The extracted DNA was then subjected to Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP). The DNA extraction method employed (Cheng and Jiang, 2006) was based on the proposed method of DNA extraction for Gram- negative bacteria. The cultures were centrifuged at 4000 rpm for 15 minutes to retrieve cell pellets. These pellets were then washed twice with 400µl STE buffer (100mM NaCl, 10mM Tris/HCl, 1Mm EDTA, pH 8.0) used for each wash. The suspension was centrifuged at 13000rpm for 2 minutes. The pellets formed were then resuspended in 200µl Tris/HCl buffer. 200µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added into the cell suspension, and vortexed for 60 seconds to cause cell lysis and obtain a white suspension obtained. Centrifugation at 13000rpm for 5 minutes separated the aqueous phase from the organic phase. After removing the white interphase (cell debris) with the use of a sterile toothpick, the aqueous phase was further purified by repeated phenol/chloroform (two to three times) treatment until the white interphase was completely removed. The purified aqueous phase was then extracted, with 200µl of chloroform added to it and centrifuged at 13000rpm for 5 minutes, removing traces of phenol present in the aqueous phase. To precipitate the DNA in the aqueous phase, a volume of isopropanol (equal to the aqueous phase) was added to the aqueous phase and incubated at -20°C for at least 30 minutes. The precipitate was centrifuged at 13000rpm for 20 minutes. The pellet was air-dried and dissolved to 100ng/µL in pH 8.0 Tris/HCl buffer and stored at -20°C.

**Polymerase Chain Reaction.** Each reaction consisted of 50µl of mixture prepared using 200ng of DNA template in 10pmoles of DNTPs, 50pmoles of primer, 1 unit of Taq polymerase, and 1X standard buffer (with 1.5mM of MgCl<sub>2</sub>) provided by the supplier (New England Biolabs, Inc). Primer 5, CgCgCTggC; Primer 6, gCTggCggC and Primer 7, CAggCggCg were used separately. The PCR reaction was carried out (Hybaid Limited, PCR express) under the cycling condition of initial denaturation at 95°C for 10 minutes; 35 cycles of amplification at 95°C for 1 minute, 27°C for 1 minute, 72°C for 3 minutes; followed by a

final extension at 72°C for 10 minutes before gel electrophoresis in 2%(w/v) agarose gel with 1X GelRed.

**Restriction Fragments Length Polymorphism.** 11 $\mu$ L of PCR product was digested with 1 unit of restriction endonuclease (TaqI, Hinfl or MspI), in a reaction mixture consisting of 1X restriction digestion buffer and 100ng/ $\mu$ l acetylated BSA made to a total volume of 20 $\mu$ L with distilled water. Hinfl and MspI reaction mixtures were incubated at 37°C, while the TaqI reaction mixture was incubated at 65°C. All reaction mixtures were incubated for 16 hours before analysis on 2% (w/v) agarose gel with 1X GelRed.

### **3.4 Data Analysis**

**5 and 7 Day Cell density.** Cell density was calculated from OD600 readings using the cell size correction graph suggested by Sezonov et al. (2007), suggesting that the cell size remains constant up to OD600 reading of 0.3, which is equivalent to  $5 \times 10^7$  cells per millilitre. Any OD600 reading above OD600 reading 0.3, the relationship between the OD600 and the cell density changes due to the decrease in cell size. A correction graph shows a curve of Cell Density =  $52137400 \times \ln(\text{OD600 reading}) + 118718650$ .

A comparison was made between 5 day and 7 day OD600 reading, cell density was calculated and a quotient of cell density at 7 day over 5 day was made. A value of 100% would indicate no growth between fifth day and seventh day. A decrease of this value meant a lower cell density on the seventh day compared to fifth day and vice versa for an increase from the value.

**Generation Time.** OD600 readings were taken at intervals for up to 300 minutes after the inoculation of cells into fresh media. This experiment was done to analyze the generation time of each different treatment in the same passage for every 3 passage interval. Readings for the first interval was not used for generation time calculation because of the lag phase of bacteria cells growth. Geometric mean was calculated from the subsequent intervals, using cell density after cell size correction and obtained to represent the mean generation time for the treatment.

**Swap Experiment.** In the first swap experiment, low salt treatment to 6 other non-salt treatments, generation time across passage was measured and the results were used to compare the effects of nutrient broth (L SALT) on the adaptation of the cells growing in it. A gradient of 0 of the generation time graph would indicate that nutrient broth does not have any effect in causing adaptation that may promote better cell growth in other treatments. However, a positive or negative gradient would mean otherwise. The gradients of the generation time across passages were tested using t-test for regression coefficient (Gopal, 2006) to determine if they were statistically different from zero using the formula,

$$t - \text{statistic} = \frac{bS_x}{S_{y \cdot x}} (n - 1)^{-\frac{1}{2}},$$

where

$$\begin{aligned} S_x^2 &= \frac{\sum (x_i - \bar{x})^2}{n - 1} \\ b &= \frac{\sum x_i y_i - \frac{1}{n} \sum x_i \sum y_i}{\sum x_i^2 - \frac{1}{n} (\sum x_i)^2} \\ S_{y \cdot x}^2 &= \frac{\sum (y_i - \bar{y} - b(x_i - \bar{x}))^2}{n - 2} \end{aligned}$$

For the second experiment (high treatment cells to low treatment media and vice versa), the difference in stress level between the high and low concentration treatment, and the adaptability of the cells in both the high and low treatment can be determined. The generation time of each high and low treatment swap was compared across passage to analyse the effects of the concentration of the certain treatment.

For the swaps between low treatment cells into high treatment media, adaptability of the low treatment cells can be compared and analysed across passage. The adaptability of the low treatment cells increase if a trend of shorter generation time was observed. This could be likely due to the reduced effect of high concentration stress on the low treatment cells as adaptability improves.

Swaps between high treatment cells and the low treatment media can also be compared and analysed similarly across passages. Adaptability of the high treatment cells have increased if a trend of shorter generation time is observed. This may be the cause of the adaptation to the

high concentration treatment, therefore reducing the effects of the low treatment media. Another possible result would be observing a similar trend in both swapping of low treatment cells into high treatment media and vice versa. In this case, it is likely that the high concentration treatment and the low concentration treatment both cause different kinds of stresses. For example, if MSG is the only stress in the high and low MSG treatment, when low treatment cells are swapped into high concentration media, the generation time will increase because of the increased stress. On the other hand, shorter generation time will be observed if the high treatment cells are swapped into the low treatment media because of reduced stress. However, when a new kind of stress is present in one of the concentration, it would be equivalent to swapping MSG cells into BA treatment. In this case, the comparison will not be between high and low MSG only, but a number of factors will come into play.

Swap experiments 3 and 4 (high single treatment to high combination treatment, low single treatment to low combination treatment) were used to analyze if there are any similarities in the effect of different treatments on the cells. If a similar growth pattern among the treatments occurs, this may indicate that the different treatment has the same type of stress on the cells.

The last swap experiment (low/ high salt treatment cells to higher salt concentration media) was done to test the adaptability of salt treatment cells. It is expected that H SALT cells will grow better than L SALT cells because it has already partly adapted to the salt stress in the media.

**Polymerase Chain Reaction/ Restriction Fragment Length Polymorphism.** The migration distance of the bands of PCR and RFLP of different treatments within the same passage was tabulated and a Nei-Li dissimilarity index (DI) (Nei-Li, 1979) was obtained for each comparison (28 in total) between all treatments in the passage. Nei-Li DI is a way of measuring how different two organisms are according to the presence or absence of a band on the gel after the restriction endonuclease digestion or specific primer amplification. The bands are measured and the DI is measured using the following equation:

$$1 - \frac{2 \times \text{number of regions where both species are present}}{\left[ 2 \times (\text{number of regions where both species are present}) + \right. \left. \text{number of regions where only one species is present} \right]}$$

A maximum value of 1 is obtained when there are no common bands when comparing between the 2 treatments while a minimum of 0 will be obtained when the 2 treatments have exactly the same bands. Arithmetic mean of the DI is calculated for each comparison every passage, the average DI per passage was calculated also regardless of PCR or RFLP. The comparison can be analyzed by plotting the DI values for every passage on the graph. Another way of comparison can be the use of equations showing the effects of each treatment and by comparing 2 treatments, the difference can be seen more clearly. The effects of MSG, BA, and SALT are given the term MSG, BA and S respectively and the baseline effects from NB is termed NB.

All the single treatments effects would be the addition of effects from NB and the additive(s) which is shown as,

$$H \text{ MSG} = NB + 10 \text{ MSG}$$

$$H \text{ SALT} = NB + S$$

$$L \text{ MSG} = NB + MSG$$

$$L \text{ SALT} = NB$$

$$H \text{ BA} = NB + 10 \text{ BA}$$

$$H \text{ COMB} = NB + 10 \text{ MSG} + 10 \text{ BA} + S$$

$$L \text{ BA} = NB + BA$$

$$L \text{ COMB} = NB + MSG + BA$$

The difference of high and low concentration treatment is the multiplication of 10 in the high treatment with the exception of salt because no additional salt was added and the salt content in L SALT does not originate from NB. Therefore, the effects of comparisons between treatments would be,

$$H \text{ COMB} = NB + 10 \text{ MSG} + 10 \text{ BA} + S$$

$$L \text{ COMB} = NB + MSG + BA$$

Thus,  $H \text{ COMB} / L \text{ COMB} = 9 \text{ MSG} + 9 \text{ BA} + S$ , suggesting that the difference between the genome of cells from the treatments are due to the differing constituents between the treatments. The effect of all comparison is shown in Table 3.1.

Comparisons	Factors in Each Comparison						
	MSG	BA	Salt	9MSG	9BA	10MSG	10BA
LMSG / LSALT							
LBA / LCOMB							
LMSG / LCOMB							

LBA / LSALT							
HMSG / LSALT							
HBA / LSALT							
LMSG / HSALT							
LBA / H SALT							
LMSG / LBA							
LSALT / LCOMB							
HSALT / LCOMB							
HMSG / HSALT							
HBA / HCOMB							
HMSG / HCOMB							
HBA / HSALT							
HMSG / HBA							
HSALT / HCOMB							
LSALT / HCOMB							
HMSG / L BA							
LMSG / HBA							
HMSG / LCOMB							
HBA / LCOMB							
LSMG / HCOMB							
LBA / HCOMB							
HMSG / LMSG							
HBA / LBA							
HSALT / LSALT							
HCOMB / LCOMB							

Table 3.1: Effects of the 28 pair-wise comparisons among the 8 treatments. The shaded areas represent the individual effects of the comparisons.

By comparing the two comparisons, L MSG / L SALT and L BA / L COMB, the overall difference will be MSG. The DI of the two comparisons over passage was plotted against each other and the correlation coefficient (CC) value obtained. The CC value was then statistically tested against the CC value of 0.95 (~1) using the Z-test for two correlation coefficients (Gopal, 2006). A P-value of more than 0.05 would indicate that the null hypothesis (CC is equal to 0.95) is not rejected and the test is not statistically significant, whereas a P-value of lesser than 0.05 would indicate that the calculated CC is not equal to 0.95. The CC values were obtained and tested from comparisons having resulting effects of MSG, BA, BA + MSG, 10MSG + S, 10BA + S and 10MSG + 10BA.

**Resampling Statistics.** Jackknife resampling (Miller, 1974; Efron, 1979) was carried out to test the statistical difference between data obtained from this study, which comprises of Passage 71 to 153, to that of previous study (Lee et al., 2010). Using the jackknife resampling method, a distribution of the sample statistic could be generated from the original data set,

which in this case, would be the generation time means of the earlier passages (Lee et al., 2010).

For example, the generation time regression trend of the H BA cells from Passage 71 to 153 were compared against that of Passage 1 to 70, jackknife resampling was used to produce pseudoreplicates of the original data set where each pseudoreplicate contains one less data point (leaving out one generation time mean for each pseudoreplicate). Therefore, if the original data set has 10 generation time, 10 new data sets could be derived where an estimate of regression coefficient could be calculated. A distribution can be generated from the 10 estimates and is considered to be normal under central limit theorem. Generation time regression of H BA cells in Passage 71 to 153 can be tested against this distribution for statistical significance.

Jackknife resampling was used to test whether the generation time of Passage 71 to 153 is statistically significant from Passage 1 to 70 (Lee et al., 2010) in all experiments.

## 4 Results

### 4.1 Total Generations across 83 Passages

The 83 passages from the experiment were separated into 8 interval periods, each containing 10 passages with the last interval having 12 passages. The first interval started with an average of approximately 66 generations. Over the next 10 passages, the number of generations dropped till approximately 40, with H MSG, L BA and L COMB stopping at 46 generations (Figure 4.1). The number of generations increase across the next two intervals and stabilised at approximately 66 from the 4<sup>th</sup> till 7<sup>th</sup> interval. From the 7<sup>th</sup> passage interval onwards, the number of generations shows an increase due to the additional 2 passages that are being included in the average. The final two passages (Passage 152 and 153) were removed in order to obtain a more accurate comparison.

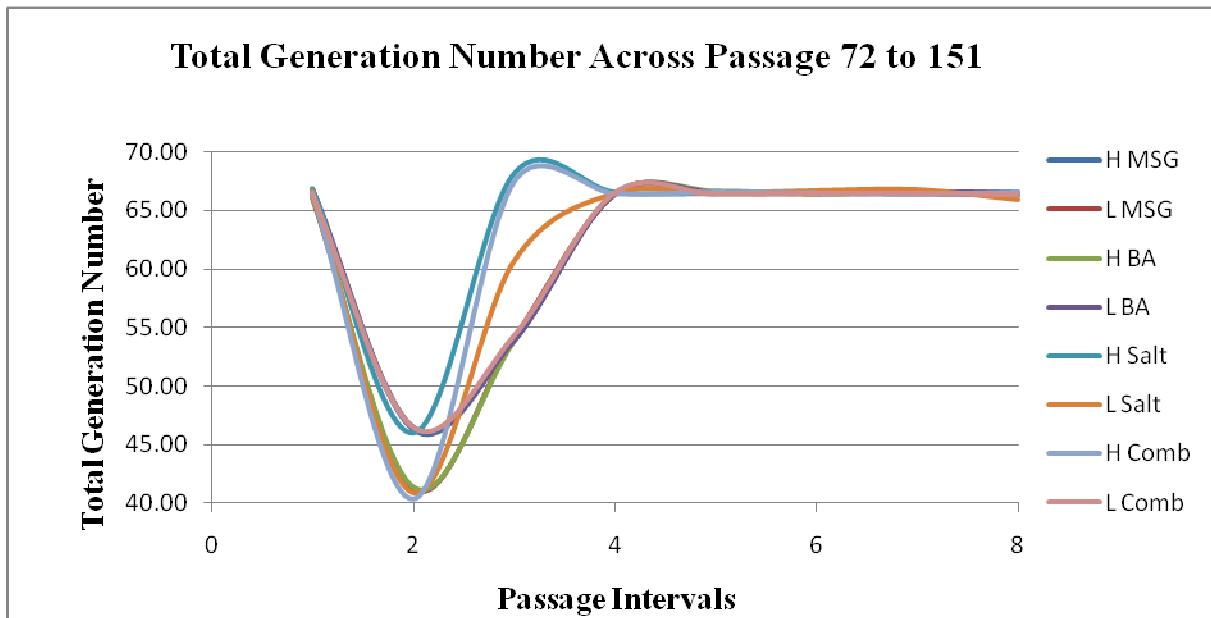


Figure 4.1: Total Generation Number across 8 Passages Intervals

### 4.2 Day 5 Day 7 Cell Density

At the start of experiment, all the treatments showed a similar trend with the exception of L COMB having a coefficient of variation (COV) at 12% (Table 4.1, Figure 4.2). The COV started to increase gradually with the exception of H MSG, H SALT and L COMB. This trend continued to increase until passage 113. The peak of most of the passages lies during

the interval of passage 99 to passage 113. The COV started to decline starting from passage 114, with L MSG and H COMB showing random peaks as shown in Figure 4.2.

Passage	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
71-83	6.61%	8.56%	5.70%	6.35%	4.95%	6.65%	4.02%	12.85%
84-98	27.04%	15.67%	14.95%	14.44%	33.71%	10.45%	18.54%	26.84%
99-113	24.20%	26.74%	19.01%	26.15%	25.45%	27.91%	26.84%	29.48%
114-128	3.60%	3.24%	3.57%	4.27%	4.54%	2.44%	4.47%	5.13%
129-143	2.11%	8.48%	1.08%	1.60%	1.34%	2.94%	12.03%	5.06%
144-153	2.16%	1.57%	3.51%	1.40%	1.68%	2.13%	3.58%	2.21%

Table 4.1: Tabulation of Coefficient of Variation of all Treatments for 83 Passages

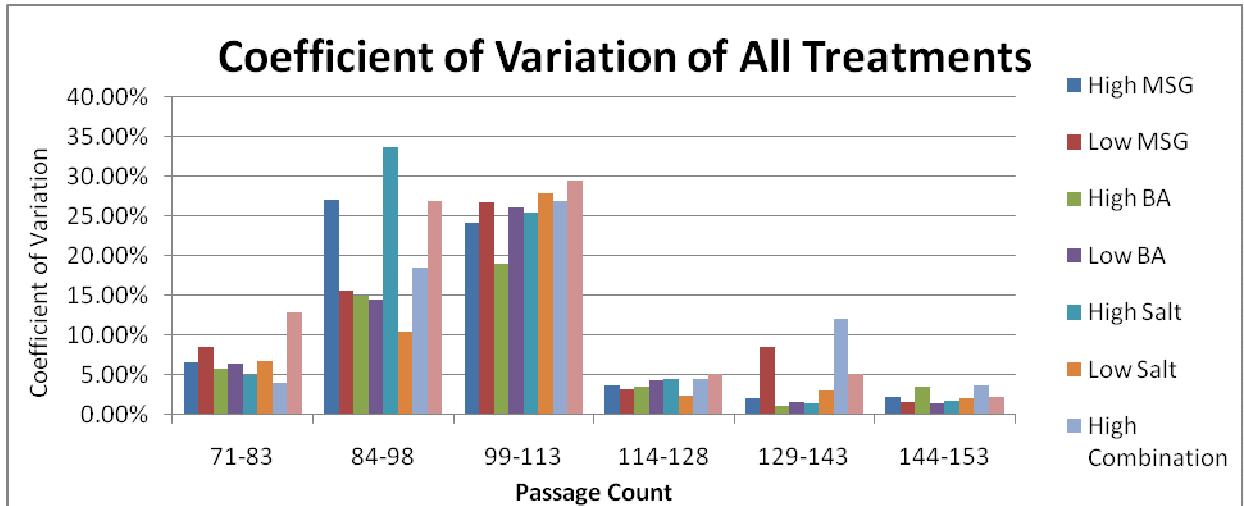


Figure 4.2: Coefficient of Variation of all Treatments for 83 Passages

The ratio of Day 7 to Day 5 cell density for MSG treatments shows a stabilised 100% (Figure 4.3). However, from the 84 to 98 passages, the variation doubles for H MSG (200%) and an increase in 50% for L MSG, before H MSG dropping to 150% which is the same as L MSG. The ratio stabilised again at 110% up till passage 104, before both MSG treatments peaks to 200%. After the 110<sup>th</sup> passage, both ratios stabilised at 110% until the end of the experiment (Passage 153).

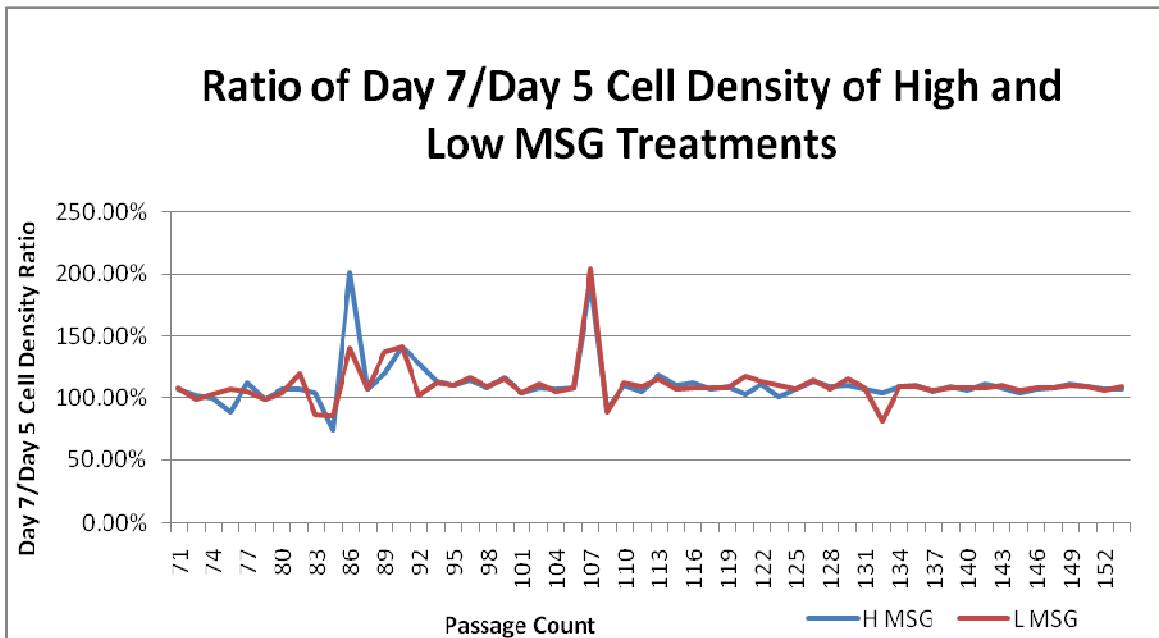


Figure 4.3: Ratio of Day 7 to Day 5 Cell Density of High and Low MSG Treatments over 83 Passages

From Figure 4.4, the cell density ratios for both BA treatments remained stabilised at 110% from passage 71 to 104 with the exception of a drop of 20% at passage 83 and a rise in 40% at passage 92. At passage 104, both treatments show a sharp increase of 70% to 100% (H BA – 110% to 200%, L BA – 110% to 170%). Both treatments returned to a stabilised ratio of 110% from passage 110 onwards and there is less fluctuations as compared to passage 71 to 104.

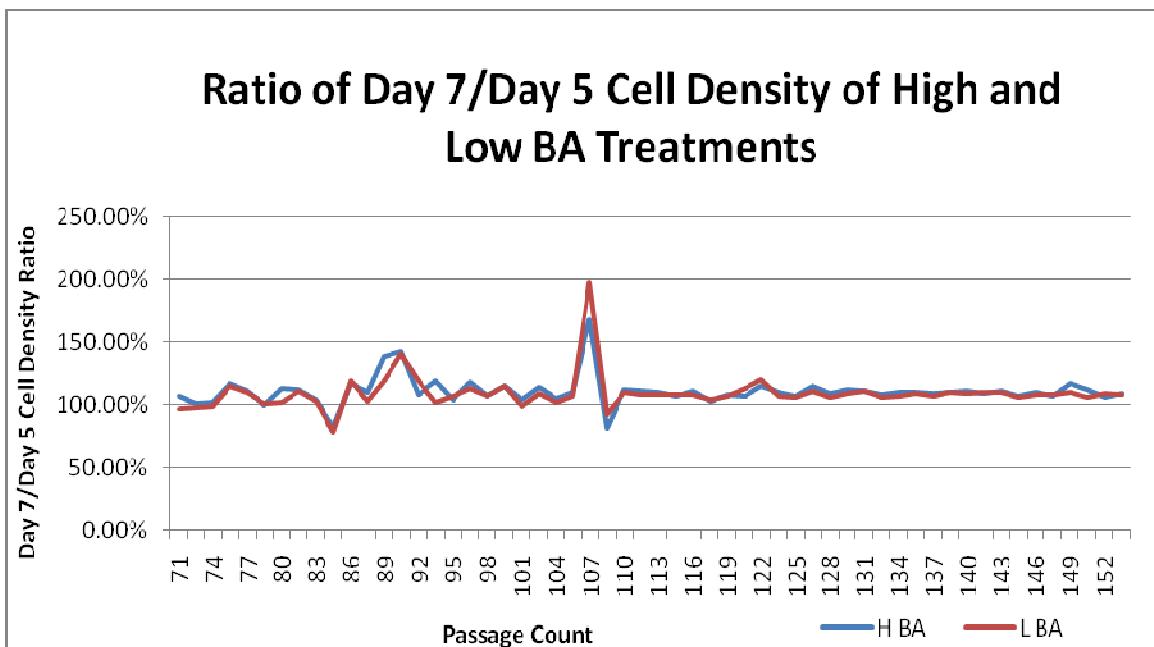


Figure 4.4: Ratio of Day 7 to Day 5 Cell Density of High and Low BA Treatments over 83 passages

The Day 7 to Day 5 cell density ratio for both salt treatments showed low fluctuations from passage 71 to 104 with the exception of H SALT having a sharp increase in ratio at passage 92 reaching 220% (Figure 4.5). From passage 95 to 104, the ratio returned to stabilised at 110%. At passage 107, both salt treatments rose to 200% before returning again to stabilised level at 110% until end of the experiment (Passage 153).

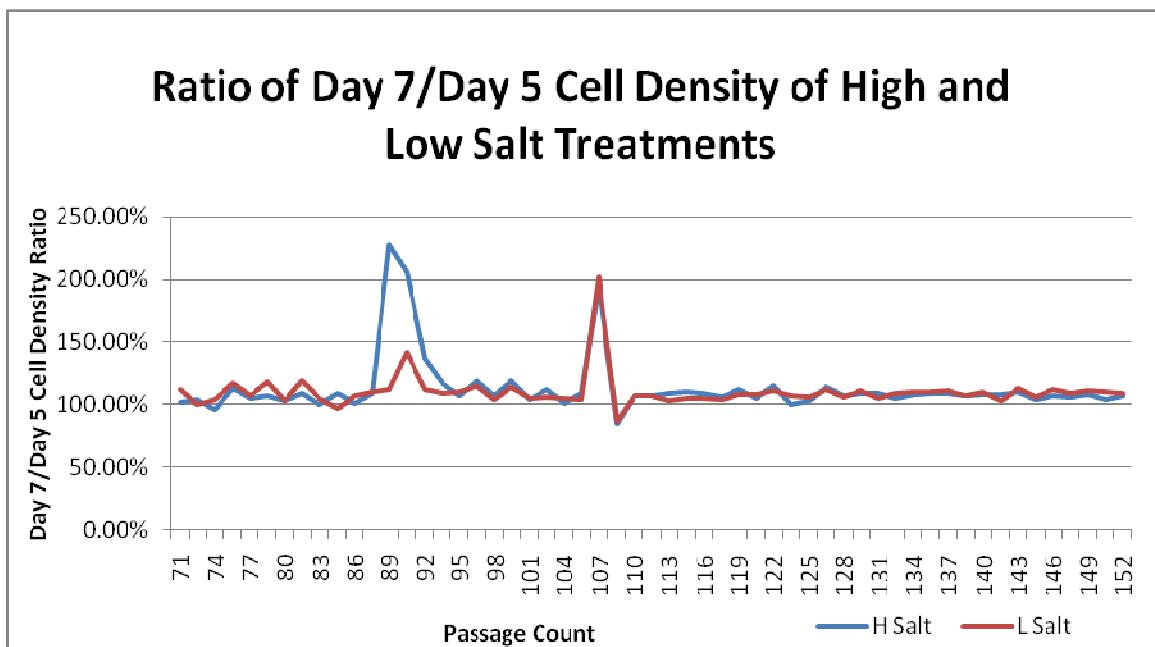


Figure 4.5: Ratio of Day 7 to Day 5 Cell Density of High and Low Salt Treatments over 83 Passages

The Day 7 to Day 5 cell density ratio for both combination treatments showed a fluctuation of 25% to 50% from passage 71 to passage 104 with H COMB peaking at 200% at passage 98 (Figure 4.6). At passage 107, both combination treatments showed a peak of 210% before subsequently returning to a stabilised level of 110%. From passage 110 to the end of the experiment (Passage 153), the fluctuations of the ratios are moderate, with occasional fluctuation of 50% (Passage 129, H COMB).

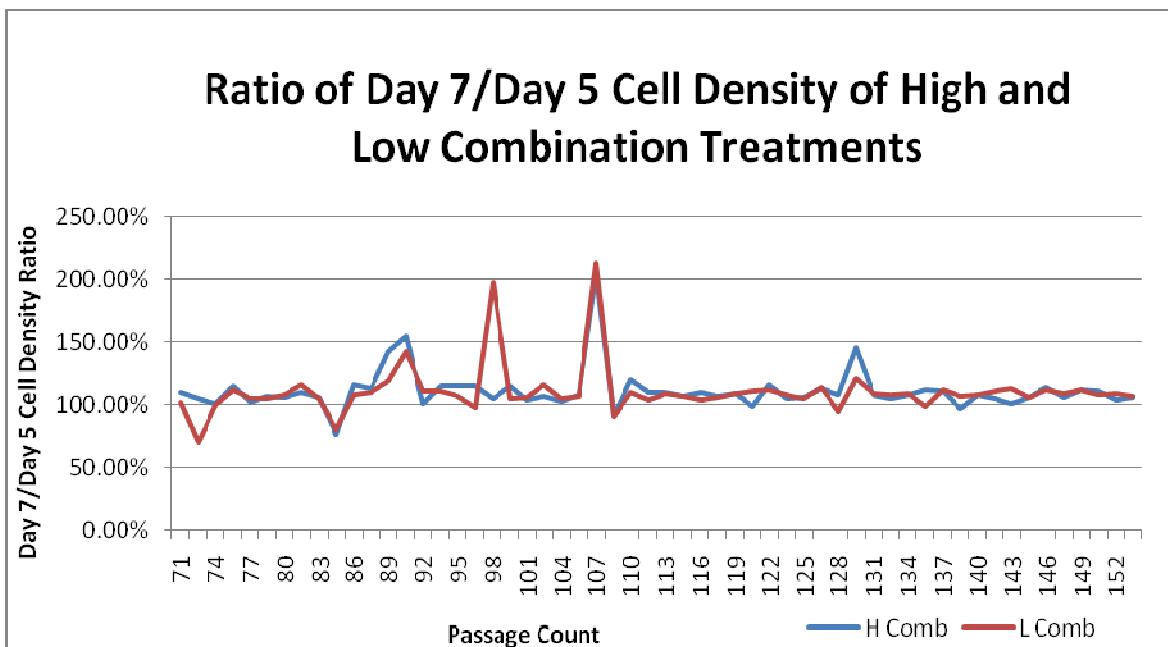
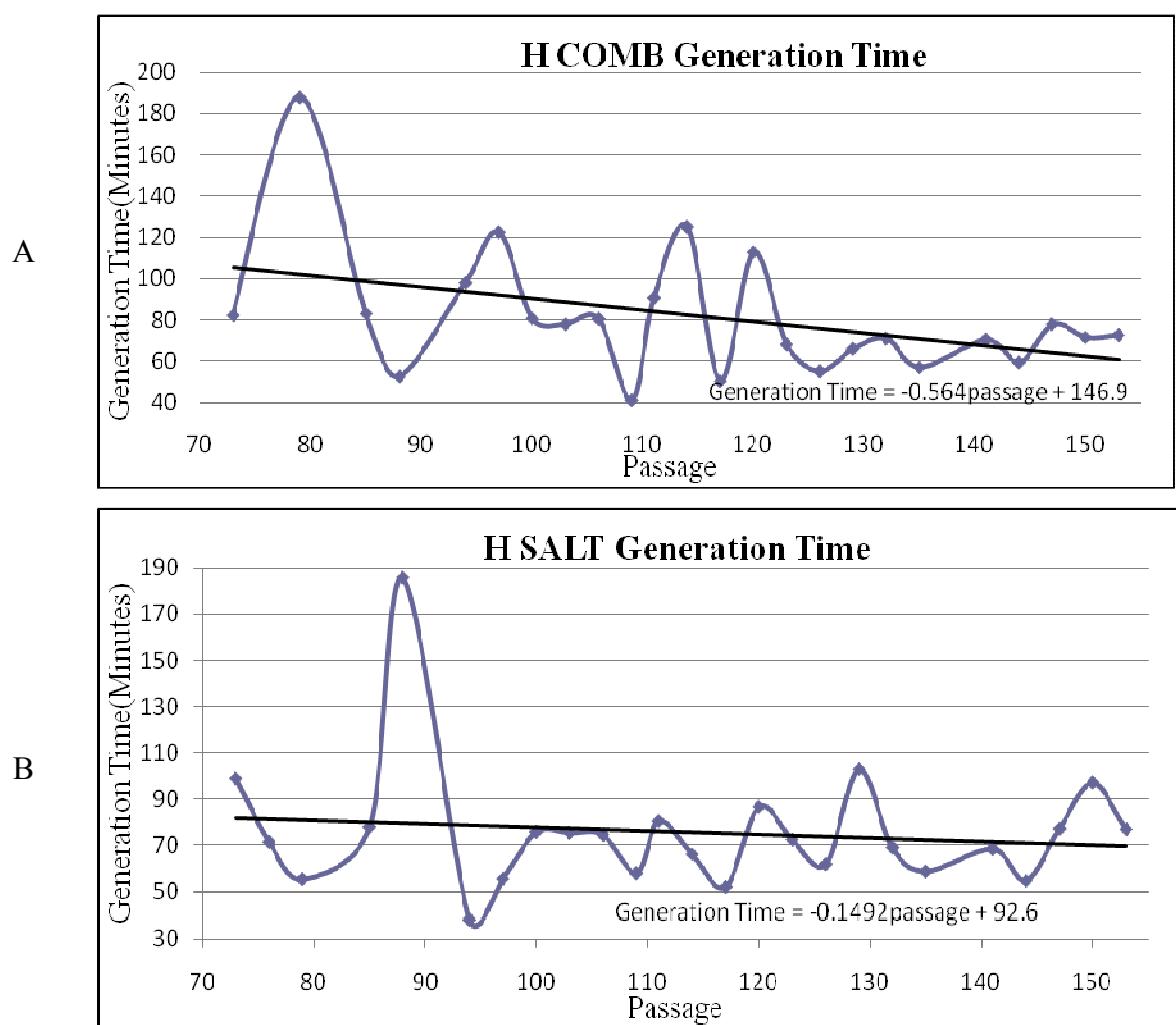
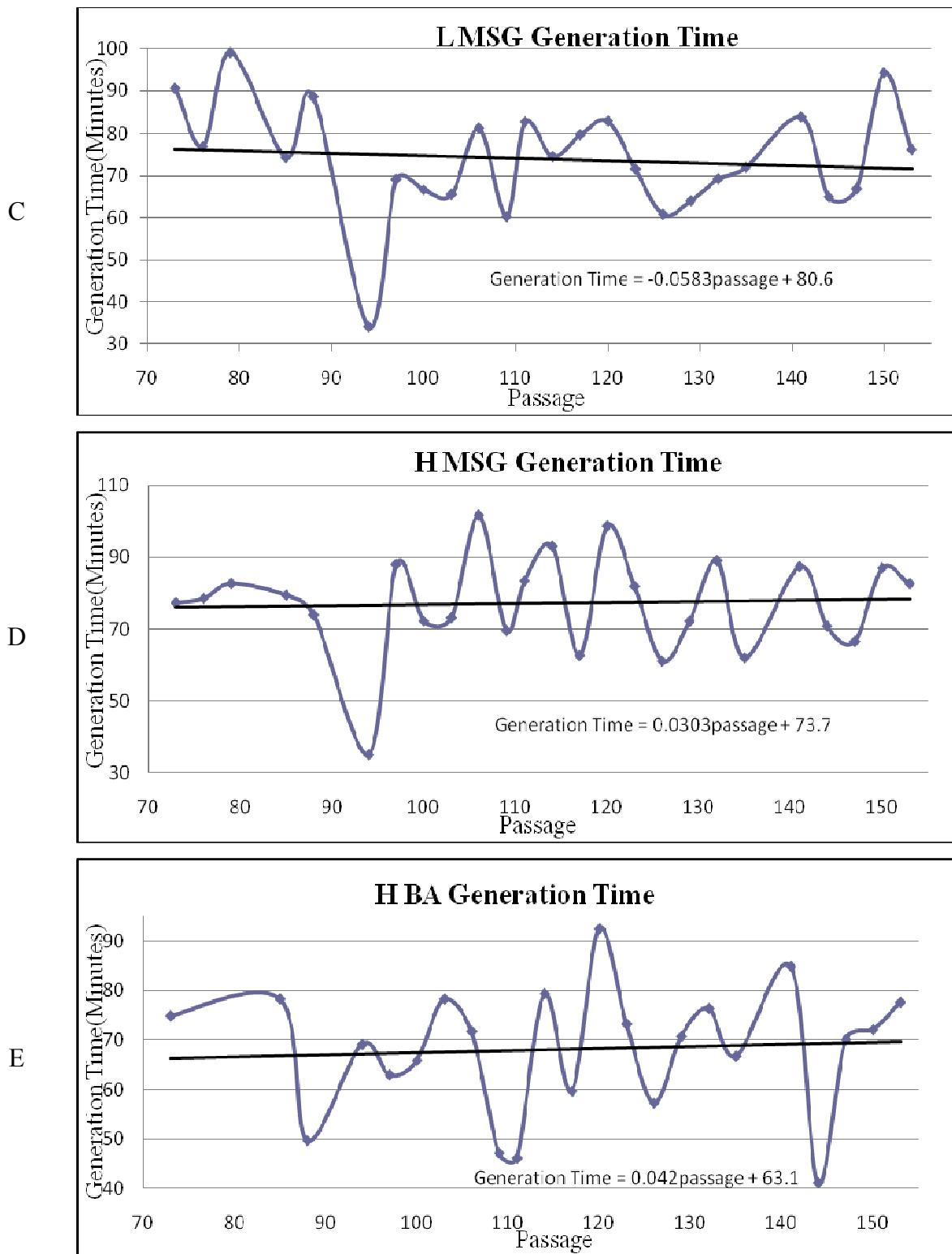


Figure 4.6: Ratio of Day 7 to Day 5 Cell Density of High and Low Combination Treatments over 83 passages

### 4.3 Generation Time

Analysis of the generation time of all 8 treatments showed different gradients over passages (Figure 4.7). H COMB (Figure 4.7A), H SALT (Figure 4.7B) and L MSG (Figure 4.7C) showed decreasing generation time and the rest of the 5 treatments have increasing generation time. The steepest decrease in generation time occurs in H COMB, which has an estimated of 0.56 minutes reduction in generation time per passage, followed by H SALT (0.15 minutes) and L MSG (0.06 minutes). L COMB (Figure 4.7H) has the steepest increasing gradient of 0.22 minutes increase in generation time per passage, followed by L SALT (0.18 minutes), L BA (0.11 minutes), H BA (0.04 minutes), H MSG (0.03 minutes).





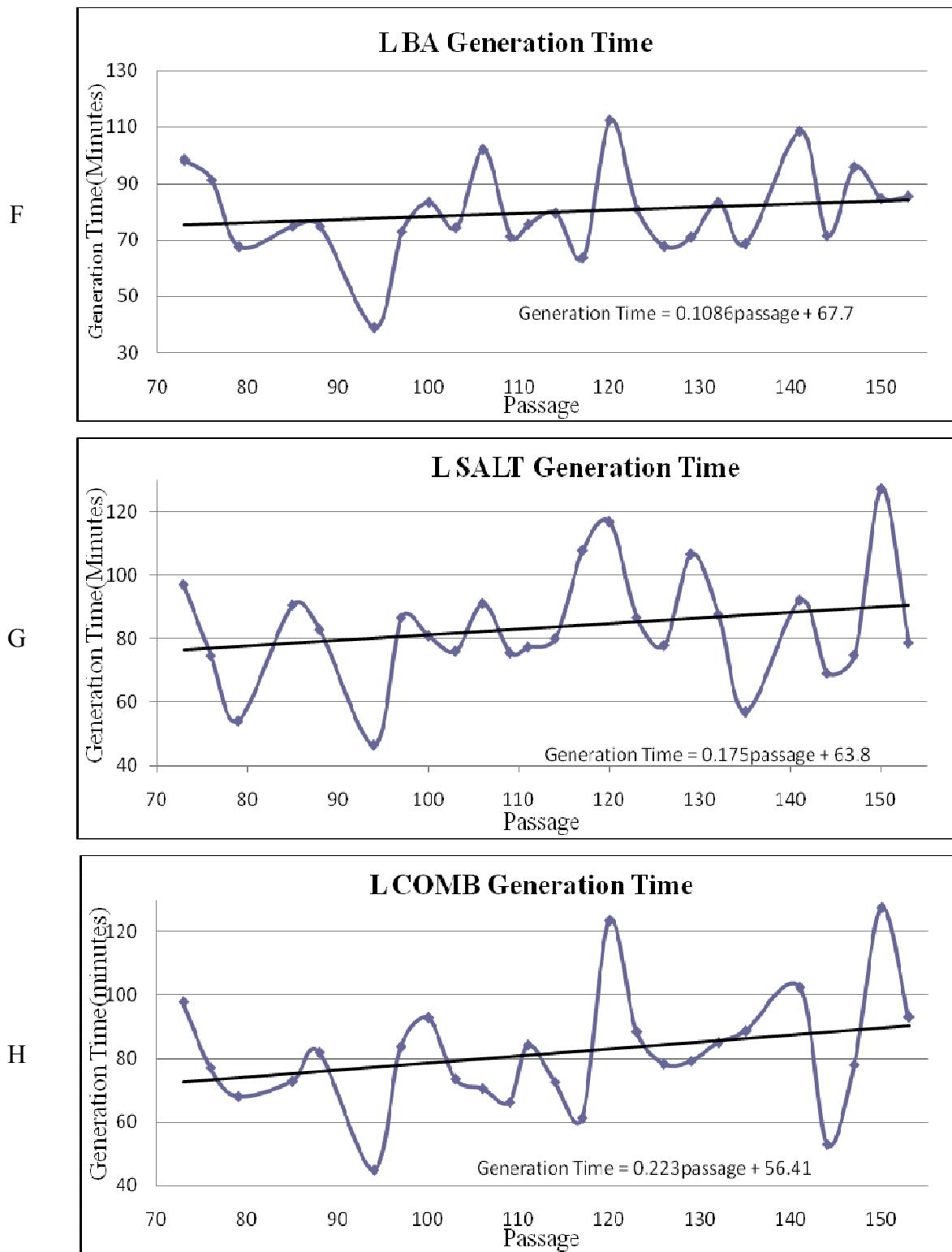


Figure 4.7: Generation Time of 8 Different Treatments Starting from H COMB on the Top, followed by H SALT, L MSG, H MSG, H BA, L BA, L SALT and L COMB Across a Total of 80 Passages Starting from 73 to 153.

To compare the difference between the first 70 and second 70 passages of the experiment, a table was constructed to observe the difference in generation time clearer (Table 4.2).

Gradient Of	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
Passage 0 - 70	-0.91	-1.87	-1.15	-1.39	-1.12	-1.24	-2.02	-1.22
Passage 73 - 153	0.03	-0.058	0.042	0.109	-0.149	0.175	-0.564	0.223

Table 4.2: Comparison of Generation Time gradient between Passage 0-70 and Passage 73-153.

Jackknife resampling was also done to compare the similarity of the generation time between Passage 2-68 and Passage 73-153. The results show that both phases of generation time are significantly different (Table 4.3). L MSG is the most different between all the treatments, followed by L COMB, H MSG, L BA, L SALT, H SALT, H BA and H COMB.

	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
Jackknife P-value	<3.46E-14	<2.33E-15	<1.13E-11	<1.69E-14	<1.9E-13	<1.6E-14	<1.51E-10	<7.8E-14

Table 4.3: P-values of the eight different treatments of the Jackknife resampling technique.

#### 4.4 Swap Experiment

Figure 4.8 shows the generation time trend of low salt treatment cells inoculated into 6 other non-salt treatment media over 14 swaps. All of the linear regression gradients of the equations are not equal to zero, indicating that the generation times are not constant through the 14 swaps.

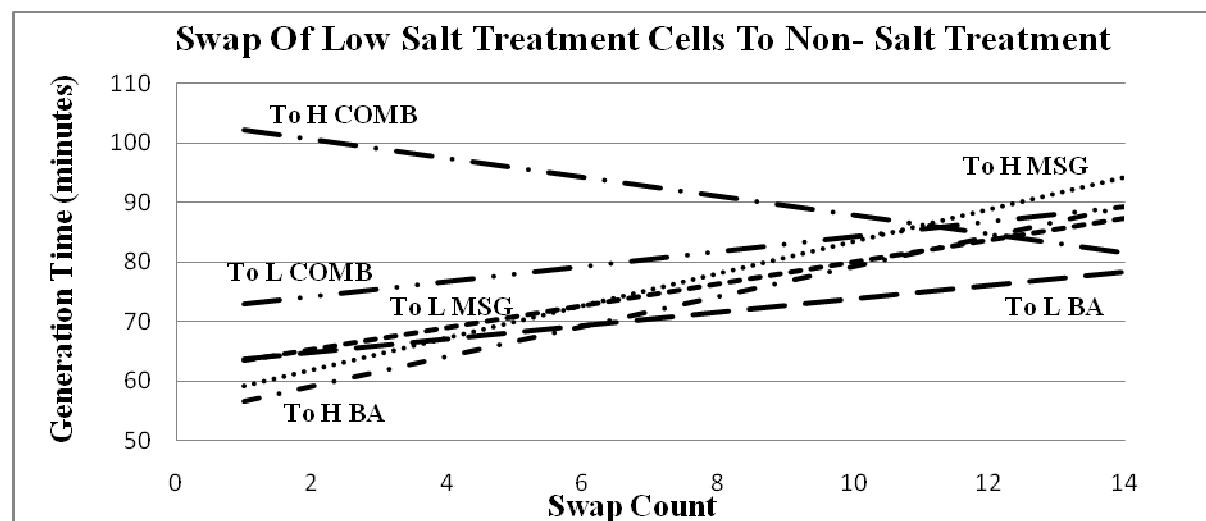


Figure 4.8: Generation Time Trend of Low Salt Treated Cells into Inoculated Non-Salt Treated Media (High MS, Low MSG, High BA, Low BA, High Combination and Low Combination) over 14 Swaps.

Swap Treatment (From L SALT to)	Linear Regression Equations
H MSG	Generation Time = 2.70 (Swap Count) + 56.5
L MSG	Generation Time = 1.83 (Swap Count) + 61.6
H BA	Generation Time = 2.52 (Swap Count) + 54.0
L BA	Generation Time = 1.12 (Swap Count) + 62.6
H COMB	Generation Time = -1.59 (Swap Count) + 104
L COMB	Generation Time = 1.25 (Swap Count) + 71.8

Table 4.4: Linear Regression Equations of Generation Time Trend of Low Salt Cells into Non-Salt Treatment Media (Passages 75 to 153).

The linear regression equations are shown in Table 4.4. A decreasing trend in generation time was observed when the low salt treatment cells were inoculated into the H COMB medium, while the rest of the swaps saw an increasing trend in generation time. The p-values of the 6 swaps were as follows: L SALT cells to H MSG media, 0.551; L SALT to L MSG media, 0.533; L SALT cells to H BA media, 0.535; L SALT cells to L BA media, 0.524; L SALT cells to H COMB media, 0.509; L SALT cells to L COMB, 0.519. Therefore, all the linear regression gradients of the 6 low salt to non-salt swaps were considered to be not significant from zero as in earlier passages (Lee et al., 2010).

The linear regression equations for the 6 low salt to non-salt swaps in earlier passages of 10 to 74 (Lee et al., 2010) are tabulated in Table 4.5. In comparison between linear regression gradients of generation time trends from earlier (10-74) and later (75-153) passages, generation time trends which were initially decreasing were shown to be decreasing at a slower rate, or increase in swaps of the later passages.

Swap Treatment (From L SALT to)	Linear Regression Equations
H MSG	Generation Time = 0.90 (Swap Count) + 151
L MSG	Generation Time = -5.87 (Swap Count) + 230
H BA	Generation Time = -6.68 (Swap Count) + 385
L BA	Generation Time = 1.29 (Swap Count) + 151
H COMB	Generation Time = 2.34 (Swap Count) + 312
L COMB	Generation Time= 3.44 (Swap Count) + 138

Table 4.5: Linear Regression Equations of Generation Time Trend of Low Salt Cells into Non- Salt Treatment media (Passages 10 to 74)(Lee et al., 2010)

A test against swap experiment data from previous passages (Lee et al., 2010) showed that generation time linear regression line gradients of L SALT cells inoculated into L MSG and H BA media were significantly different from regression line gradients determined in the

similar swap of the earlier passages (Lee et al., 2010), with p-values of 0.000637 and 0.0377 respectively. The other p-values were as follows: L SALT cells to H MSG media, 0.112; L SALT cells to L BA media, 0.538; L SALT cells to H COMB media, 0.712; L SALT cells to L COMB, 0.941.

However, overall generation time trend lines for the total of 26 swaps including swaps from previous passages were plotted for L SALT cells inoculated into the 6 non-salt media. L SALT cells inoculated into the 6 non-salt media were determined to have generation time linear regression trend lines which were not- significant to zero. The p-values of the 6 swaps were as follows: L SALT cells to H MSG media, 0.552; L SALT to L MSG media, 0.528; L SALT cells to H BA media, 0.565; L SALT cells to L BA media, 0.522; L SALT cells to H COMB media, 0.546; L SALT cells to L COMB, 0.540.

From Figure 4.9, it was shown that there was a general increasing trend in all of the High treatment cells inoculated into their corresponding Low treatment media through the 14 swaps. This was in contrast to the generation time trends in the earlier passages (Lee et al., 2010), whereby all 4 high treatment cells (MSG, BA, SALT, COMB) inoculated into low treatment media had shown decreasing trends in generation time through the swaps. The linear regression equations are shown as in Table 4.6.

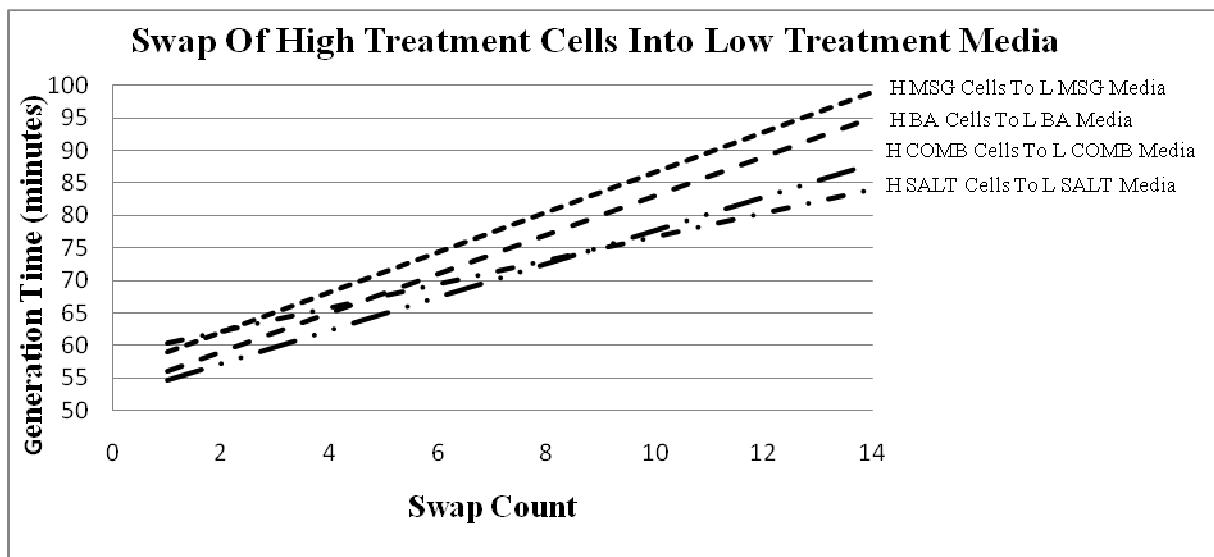


Figure 4.9: Generation Time Trend of High Treatment Cells Inoculated into Low Treatment Media over 14 Swaps (H MSG cells to L MSG Media, H BA cells to L BA Media, H SALT cells to L SALT Media, H COMB cells to L COMB Media).

Swap Treatment (From H Treatment to)	Linear Regression Equations
L MSG	Generation Time = 3.08 (Swap Count) + 55.9
L BA	Generation Time = 3.01 (Swap Count) + 53.0
L SALT	Generation Time = 1.82 (Swap Count) + 58.5
L COMB	Generation Time = 2.56 (Swap Count) + 52.2

Table 4.6: Linear Regression Equations of Generation Time Trend of High Treatment Cells Inoculated Into Low Treatment Cells

The linear regression equations for the 4 high treatment cells to low treatment media swaps in earlier passages of 10 to 74 (Lee et al., 2010) are tabulated in Table 4.7.

Swap Treatment (From H Treatment to)	Linear Regression Equations
L MSG	Generation Time = -6.91 (Swap Count) + 214
L BA	Generation Time = -5.25 Swap Count + 182
L SALT	Generation Time = -1.53 (Swap Count) + 193
L COMB	Generation Time = -4.20 Swap Count + 192

Table 4.7: Linear Regression Equations of Generation Time Trend of High Treatment Cells Inoculated into Low- Salt Treatment Media (Passages 10 to 74)(Lee et al., 2010)

A test against swap experiment data from previous passages (Lee et al., 2010) showed that generation time linear regression line gradients of all high treatment cells inoculated into low treatment cells, except for H SALT cells ( $p$ -value = 0.070) were significantly different from regression line gradients determined in the similar swap of the earlier passages (Lee et al., 2010), with  $p$ -values of lesser than 0.05. The rest of the  $p$ -values were as follows: H MSG cells to L MSG media, 0.00106; H BA cells to L BA media, 0.000144; H COMB cells to L COMB media, 0.0143.

Figure 4.10 shows the generation time trend through 14 swaps of low treatment cells inoculated into their corresponding high treatment media. L COMB and L MSG cells were observed to have an increase in generation time through the swaps when inoculated into H COMB and H MSG media respectively, while L BA and L SALT cells saw a decrease in generation time through the swaps when inoculated into H BA and H SALT media respectively. The linear regression equations are shown as in Table 4.8. In contrast, all generation time trends of the low treatment cells to high treatment media (including L MSG and L COMB) in earlier passages were shown to be decreasing.

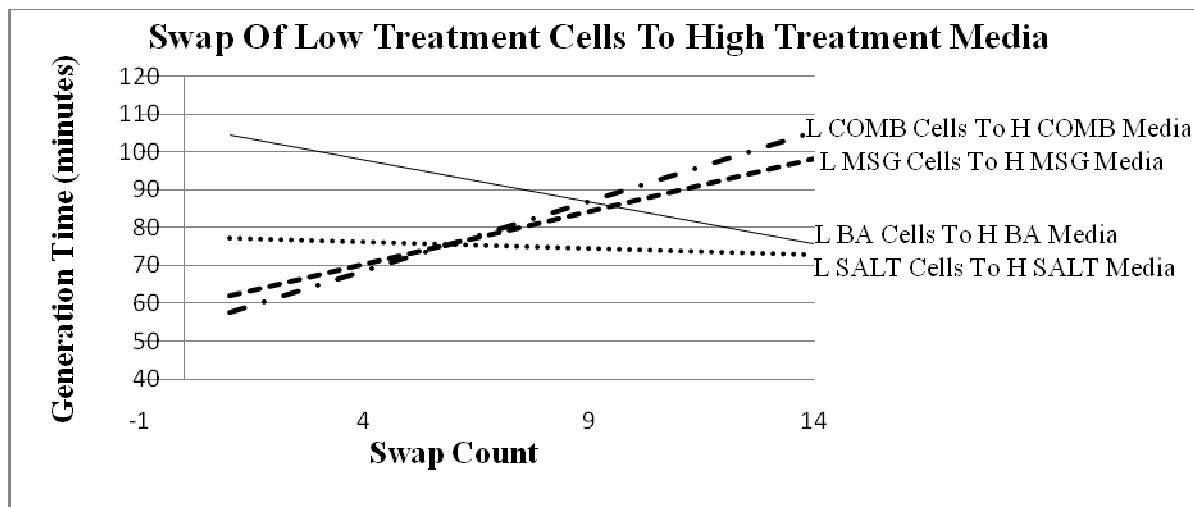


Figure 4.10: Generation Time Trend of Low Treatment Cells Inoculated into High Treatment Media over 14 Swaps (L MSG cells to H MSG Media, L BA cells to H BA Media, L SALT cells to H SALT Media, L COMB cells to H COMB Media)

Swap Treatment (From Low Treatment To)	Linear Regression Equations
H MSG	Generation Time = 2.78 (Swap Count) + 59.3
H BA	Generation Time = -2.19 (Swap Count) + 107.0
H SALT	Generation Time = -0.335 (Swap Count) + 77.5
H COMB	Generation Time = 3.66 (Swap Count) + 54.1

Table 4.8: Linear Regression Equations of Generation Time Trend of Low Treatment Cells Inoculated into High Treatment Media

The linear regression equations for the 4 Low treatment cells to High treatment media swaps in earlier passages of 10 to 74 (Lee et al., 2010) are tabulated in Table 4.9.

Swap Treatment (From Low Treatment To)	Linear Regression Equations
H MSG	Generation Time = -4.37 (Swap Count) + 237
H BA	Generation time = -15.0 (Swap Count) + 398
H SALT	Generation Time = -10.7 (Swap Count) + 290
H COMB	Generation Time = -17.2 (Swap Count) + 348

Table 4.9: Linear Regression Equations of Generation Time Trend of Low Treatment Cells into High Treatment media (Passages 10 to 74)(Lee et al., 2010)

A test against swap experiment data from previous passages (Lee et al., 2010) showed that generation time linear regression line gradients of all low treatment cells inoculated into high treatment media were significantly different from regression line gradients determined in the similar swap of the earlier passages (Lee et al., 2010), with p-values of lesser than 0.05. The p-values were as follows: L MSG cells to H MSG media, 0.00285; L BA cells to H BA

media, 0.000810; L SALT cells to H SALT media, 0.0132; L COMB cells to H COMB media, <2.78E-15.

With reference to Figure 4.11, it can be shown that all low treatment cells (L MSG, L BA, L SALT) inoculated into L COMB media had an increasing trend in generation time through the 14 swaps. This was similar to the generation time trend of the same swap in earlier passages. The linear regression equations of the 3 trend lines were tabulated in Table 4.10.

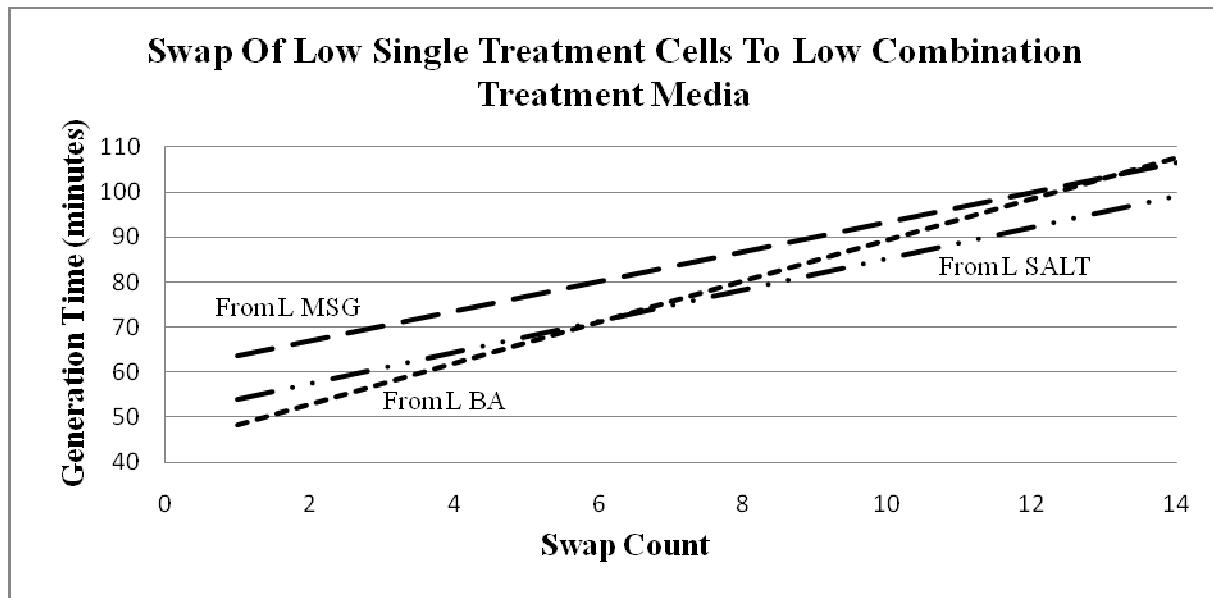


Figure 4.11: Generation Time Trend of Low Single Treatment Cells Inoculated into Low Combination Treatment Media over 14 Swaps (L MSG cells to L COMB Media, L BA cells to L COMB Media, L SALT cells to L COMB Media)

Swap Treatment (To L COMB)	Linear Regression Equations
L MSG	Generation Time = 4.58 (Swap Count) + 43.7
L BA	Generation Time = 3.30 (Swap Count) + 60.3
L SALT	Generation Time = 3.47 (Swap Count) + 50.5

Table 4.10: Linear Regression Equations of Generation Time Trend of Low Single Treatment Cells Inoculated into Low Combination Treatment Media

The linear regression equations for the 4 low treatment cells to high treatment media swaps in earlier passages of 10 to 74 (Lee et al., 2010) are tabulated in Table 4.11.

Swap Treatment (To L COMB)	Linear Regression Equations
L MSG	Generation Time = 4.55 (Swap Count) + 164
L BA	Generation Time = 9.18 (Swap Count) + 148
L SALT	Generation Time = 14.8 (Swap Count) + 109

Table 4.11: Linear Regression Equations of Generation Time Trend of Low Treatment Cells into Low Combination Media (Passages 10 to 74)(Lee et al., 2010)

A test against swap experiment data from previous passages (Lee et al., 2010) showed that generation time linear regression line gradients of all low treatment cells inoculated into L COMB media, except for L SALT cells to L COMB treatment media were not significantly different from regression line gradients determined in the similar swap of the earlier passages (Lee et al., 2010), with p-values of lesser than 0.05. The p-values were as follows: L MSG cells to L COMB media, 0.483; L BA cells to L COMB media, 0.100; L SALT cells to L COMB media, 0.00361.

With reference to Figure 4.12, it can be shown that all high treatment cells (H MSG, H BA, H SALT) inoculated into H COMB media had an increase in generation time through the 14 swaps. This was different in comparison to similar swaps done in previous passages, whereby all high treatment cells had shown decreasing trends in generation time when inoculated into high combination treatment. The linear regression gradients of the 3 generation time trend lines are tabulated in Table 4.12.

### Swap Treatment Of High Single Treatment Cells To H Combination Treatment Media

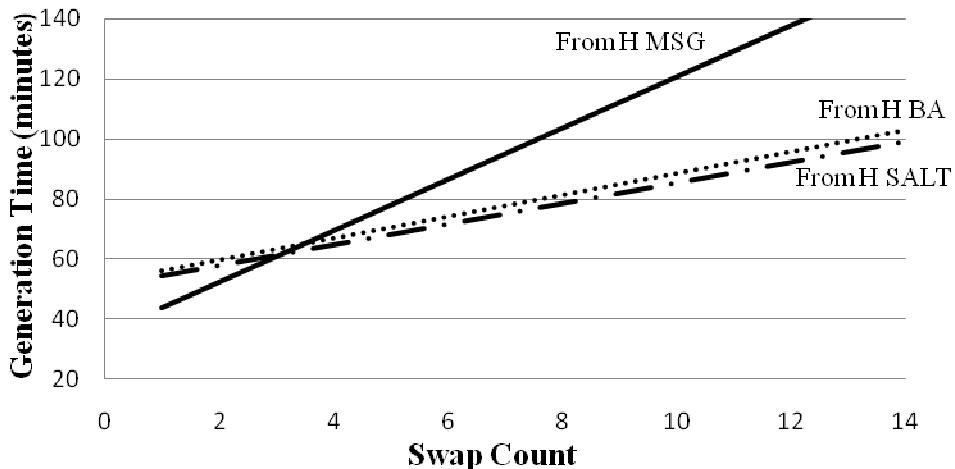


Figure 4.12: Generation Time Trend of High Single Treatment Cells Inoculated into High Combination Treatment Media over 14 Swaps (H MSG cells to H COMB Media, H BA cells to H COMB Media, H SALT cells to H COMB Media)

Swap Treatment (To H COMB)	Linear Regression Equations
H MSG	Generation Time = 8.53 (Swap Count) + 35.4
H BA	Generation Time = 3.58 (Swap Count) + 52.7
H SALT	Generation Time = 3.45 (Swap Count) + 51.1

Table 4.12: Linear Regression Equations of Generation Time Trend of High Single Treatment Cells Inoculated into High Combination Treatment Media

The linear regression equations for the 4 low treatment cells to high treatment media swaps in earlier passages of 10 to 74 (Lee et al., 2010) are tabulated in Table 4.13.

Swap Treatment (To H COMB)	Linear Regression Equations
H MSG	Generation Time = -63.6 (Swap Count) + 814
H BA	Generation Time = -37.1 (Swap Count) + 849
H SALT	Generation Time = -29.3 (Swap Count) + 480

Table 4.13: Linear Regression Equations of Generation Time Trend of High Treatment Cells into High Combination Media (Passages 10 to 74)(Lee et al., 2010)

A test against swap experiment data from previous passages (Lee et al., 2010) showed that generation time linear regression line gradients of all high treatment cells inoculated into H COMB media, were significantly different from regression line gradients determined in the similar swap of the earlier passages (Lee et al., 2010), with p-values of lesser than 0.05. The p-values were as follows: H MSG cells to H COMB media, 0.000138; H BA cells to H COMB media, 0.00583; H SALT cells to H COMB media, 3.95E-05.

With reference to Figure 4.13, it can be shown that both L SALT and H SALT cells have a declining trend in generation time through the 9 swaps when inoculated into higher salt treatment. However, it could be observed that the rate of decline of generation time was higher in L SALT cells than H SALT cells, as determined from the gradients of the linear regression trend lines tabulated in Table 4.14.

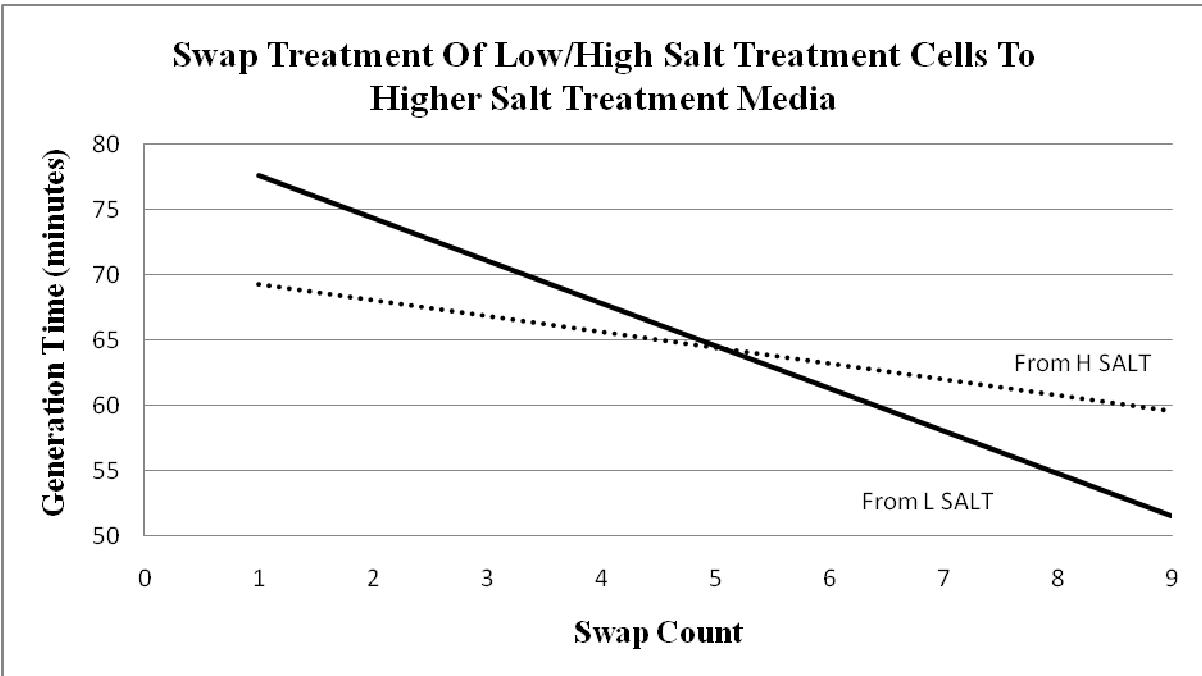


Figure 4.13: Generation Time Trend of H SALT and L SALT cells inoculated into Higher Salt Treatment Media over 9 Swaps (H SALT cells to Higher Salt media, L SALT to Higher Salt media).

Swap Treatment (To Higher SALT)	Linear Regression Equations
L SALT	Generation Time = -3.26 (Swap Count) + 80.9
H SALT	Generation Time = -1.22 (Swap Count) + 70.6

Table 4.14: Linear Regression Equations of Generation Time Trend of High/Low Salt Treatment Cells inoculated into Higher Salt Treatment Media

## 4.5 Polymerase Chain Reaction / Restriction Fragment Length Polymorphism

The PCR and RFLP products of all 8 treatments after agarose gel electrophoresis were used to study differences between the genome of the *E. coli* cells of the treatments across 83 passages. Nei-Li Dissimilarity Index (DI) was utilised to mathematically calculate the dissimilarity between pair-wise comparisons of the treatments. The dissimilarity index of the 28 comparisons showed a trend of slight divergence from PCR/RFLP #4 (Passage 117) onwards (Figure 4.14).

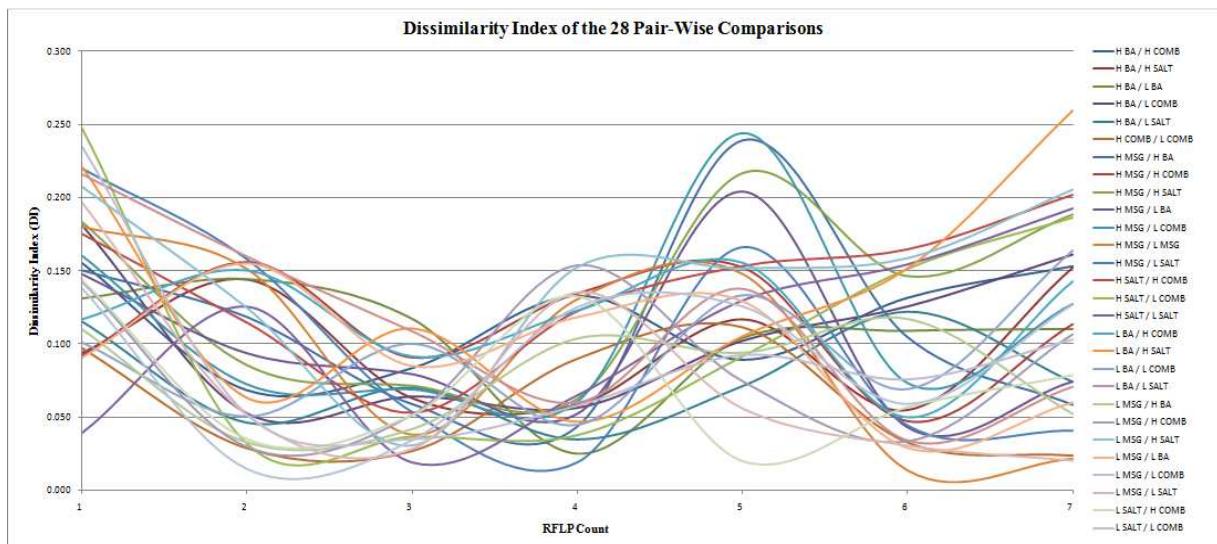


Figure 4.14: Dissimilarity Index of the 28 Pair-wise Comparisons for the 7 PCR/RFLP.

The trend is further analysed with the maximum (2SD) and minimum (-2SD) mean values (Figure 4.15) which shows slight diverging linear regression across the 7 PCR/RFLP.

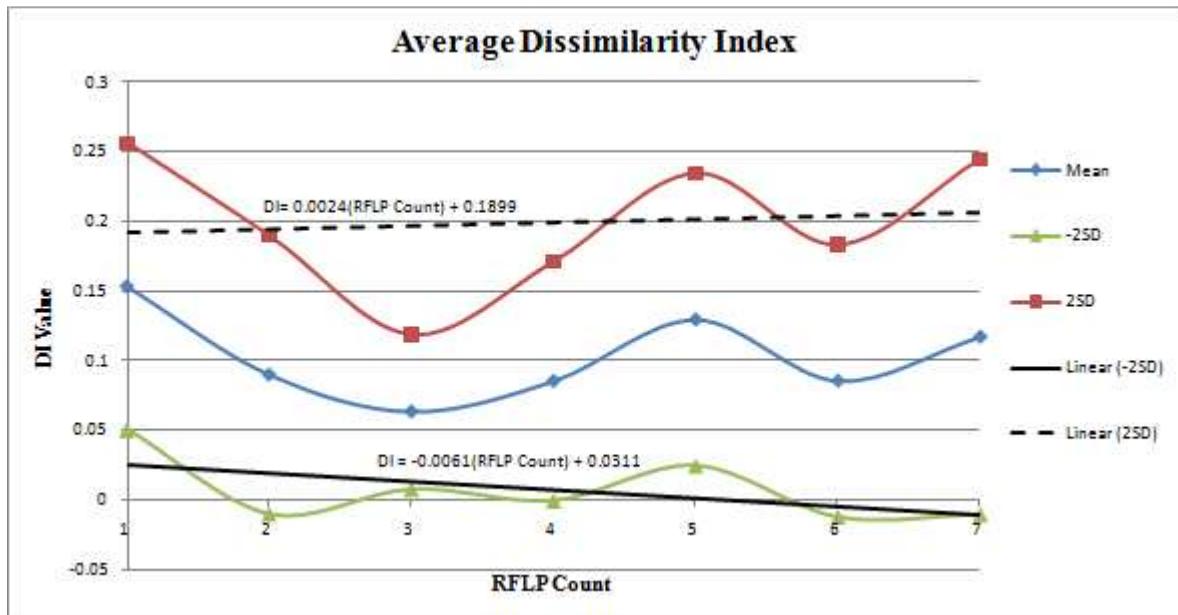


Figure 4.15: Estimation of the Average Maximum and Minimum Mean Values of the DI for each of the 7 PCR/RFLP Counts.

From the comparison of the DI values, six resulting effects were obtained and analysed. The similarity among the six resulting effects is that each type of effects has 2 originating comparisons. Therefore, by plotting the 2 comparisons against each other and testing for significance, we can deduce whether the genomic differences in each of the 2 comparisons are actually due to a consequent effect from the resulting effects. All resulting effects are statistically significant as tabulated in Table 4.15.

PCR/RFLP Comparison	Resulting Effect	Correlation Coefficient	Z-Statistic	P-Value	Significant
LMSG/LS, LBA/LC	MSG	0.078	-3.508	$2.26 \times 10^{-4}$	Yes
LMSG/LC, LBA/LS	BA	0.006	-3.651	$1.31 \times 10^{-4}$	Yes
LMSG/LBA, LS/LC	BA + MSG	0.022	-3.619	$1.48 \times 10^{-4}$	Yes
HMSG/HS, HBA/HC	10MSG + S	0.144	-3.373	$3.71 \times 10^{-4}$	Yes
HMSG/HC, HBA/HS	10BA + S	0.341	-2.954	$1.57 \times 10^{-3}$	Yes
HMSG/H BA, HS/HC	10MSG + 10BA	0.067	-3.529	$2.08 \times 10^{-4}$	Yes

Table 4.15: Tabulation of P-value for the resulting effects.

## **5 Discussion**

### ***5.1 Nutrient Broth Does Not Prime Cells for Adaptation in Other Treatments through 153 Passages***

The *E. coli* cells were grown in NB with various supplementations of treatments. Thus, it was important that the adaptations of the cells were resulting from the treatments rather than from the NB. The generation time trends for passages 71-153 of the L SALT (NB) cells inoculated into 6 non-salt treatments were shown in Fig 4.8. Although all of the 6 generation time trends were observed to be varying, they were statistically insignificant. This was similar to that of generation time trends of L SALT (NB) cells inoculated into 6 non-salt treatments in passages 0-70 (Lee et al., 2010). This suggests that NB does not assist the cells to grow better in the various treatment media; thus, not affecting the adaptability of cells in the various treatments through the 153 passages.

When comparing between the generation time trends of earlier passages (0-70) to later passages (71-153), it was observed that generation time trends of L SALT cells inoculated into L MSG and H BA media were significantly different (the rest being statistically insignificant). This may suggest that L SALT could prime cells for growth in L MSG and H BA treatment.

However, the overall generation time trends of passages 0- 153 for L SALT cells inoculated into the 6 non-salt media showed that all generation time trends were not statistically different from zero. This suggests that the significant difference in generation time trends between earlier and later passages of L SALT cells inoculated into L MSG and H BA could be due to random variations of generation times which are not caused by the ability of NB to prime cells for growth in the individual treatment.

### ***5.2 Decreased Adaptation Rates of Cells***

Comparing the generation time trends of cells in the 8 treatments (H MSG, L MSG, H BA, L BA, H SALT, L SALT, H COMB, L COMB) of earlier passages (0-74) (Lee et al., 2010) to that of later passages (71-153) in Table 4.2, there was either a slower decrease (as in L MSG, H SALT, H COMB), or slight increases (H MSG, H BA, L BA, L SALT, L COMB) in generation time trends, observed as gentler gradients the generation time linear regression.

All 8 generation time trends of passages 71-153 were significantly different from trends in passages 0-70. This suggested that there is a deceleration of the rate of adaptation of the cells to their individual treatments, and a stabilisation and optimization of relative fitness of the cells in the various treatments, as observed in previous long term experimental evolution experiments with *E. coli* (Korona, 1996, Travisano, 1997), whereby more than half of the improvements in fitness through adaptive mutation occurred in the first 500-700 generations of cells. This may suggest that most selection and beneficial mutations with the highest adaptive effects have already occurred in the earlier generations to render a faster rate of adaptation to the individual treatments in earlier passages, with adaptation in the later generations relying on beneficial mutations which were fewer in number, or smaller in effect, or both (de Visser and Lenski, 2002).

When comparing between coefficient of variations of passages 71-128 to that of 128-153, a slight decrease could be observed, indicating the possibility of the deceleration of the rate of adaptation as the cells reach optimized, and stabilised growth. This further supports the deceleration of the rate of adaptation of cells at later generations.

### ***5.3 Ecological Specialisation in Adaptation of Cells to Their Individual Treatments***

Comparing of generation time trends of High to Low treatment swaps of earlier passages, to that of later passages, it could be observed that generation time trends of the earlier passages showed a decreasing trend, while later passages showed an increasing trend for all 4 individual treatments. This could suggest that the cells, which have already adapted to their high individual treatments, have lost their ability to adapt to low treatment media. It was interpreted that such ecological specialization was caused by mutation accumulation. This was due to the fact that high treatment cells of earlier passages did not show a rapid rate of functional decay, which refers to the rapid loss of ability of the cells to adapt in media other than their individual treatments through the mutation of unused functions. Functional decay, which may be signified by drastic increases in generation time through the swaps therefore, rules out the possibility of antagonistic pleiotropy. Generation time trends comparison of High treatment to High combination treatment between earlier and later passages also showed similar results, where drastically decreasing generation time in earlier passages differ to a large extent to increasing generation time trends in later passages. In low to high treatment

swaps, it was observed that only L MSG and L COMB cells had an increasing generation time trend, which supports the possibility of mutation accumulation.

When organisms adapt genetically to one environment, they may lose fitness in other environments through ecological specialization processes such as mutation accumulation and antagonistic pleiotropy (Cooper et al., 2000, Cooper et al., 2001). Mutation accumulation refers to the process whereby mutations become fixed by genetic drift in genes that are not maintained by selection; adaptation to one environment and loss of adaptation to another are caused by different mutations. In contrast, antagonistic pleiotropy is a process by which adaptation to one environment and loss of adaptation to another arise from genetic trade-offs, such that the same mutations that are beneficial in one environment are detrimental in another. Therefore, in the case of antagonistic pleiotropy, it should be expected that if adaptation to a selective environment is initially fast but decelerates over time (as in earlier passages), the functional decay should initially be more rapid. Functional decay caused by mutation accumulation should be at a constant rate, since it only depends on the mutation rates of neutral alleles through genetic drift (Cooper et al., 2000).

However, in low to high treatment swaps of L BA and L SALT cells, decreasing generation time trends were observed, in contrast to L MSG, and L COMB cells. This could be a result of mutation accumulation which does not occur at genes that causes decay in the corresponding high treatments and thus, not affecting the adaptation of cells in the high treatment media.

Ecological specialization was not observed in Low single treatment to low combination swaps, whereby there was a decline in the rate at which the generation time was increasing. A declining rate shows that Low single treatment cells in later passages could grow comparatively better in L COMB treatment than in cells of the earlier passages, indicating that adaptation of low treatment cells to their individual treatments could assist them to grow better in L COMB media.

#### **5.4 Different Concentrations of Food Additives Render Different Types, Instead of Levels of Chemical Stress**

In observation of generation time trends of Low/High Salt cells swapped into Higher (2.7%) Salt treatment, it could be seen that the rate of decline in generation time was faster in Low salt cells than in High salt cells. This was in contrast to our hypothesis, whereby it was expected that High Salt cells were able to adapt to Higher salt treatment at a rate faster than low salt cells, due to the assumed, lesser difference in chemical stress.

This was similar to High/Low treatment swaps of the earlier passages (Lee et al., 2010), whereby Low treatment cells swapped into high treatment media had a faster rate of decrease in generation time as compared to high treatment cells swapped into low treatment media. This could further prove that different concentrations of food additives may render different types of chemical stresses to the cells, instead of just different levels of chemical stress. In the case of different salt concentrations, all 3 salt concentrations render different types of chemical stress, rather than just different levels. This could suggest that adaptive mechanisms for each of the 3 salt concentrations are different.

Such a possibility exists in microbial populations. An example would be the adaptation of *Pseudomonas aeruginosa* cells to dinitrophenol (DNP) and other chemical stressors (Ray and Peters, 2010), whereby different concentrations of DNP caused different types of chemical stress, and different adaptations.

#### **5.5 Similar Cell Densities Regardless of Aerobic or Anaerobic Conditions**

*Escherichia coli* were cultured in 4 different media with 2 varying levels of stress (H, L). After growing for 70 passages in an aerobic condition (Lee et al., 2010) and subsequently, 83 passages in anaerobic condition, the total generation time for each interval of 10 passages were calculated. Our results demonstrated that both aerobic and anaerobic cultures result in 6.6 to 6.8 generations per passage of 2 to 3 days, suggesting that anaerobic conditions did not impact on the number of logarithmic generations even though the generation time is extended in anaerobic conditions. However, culturing the cells in aerobic conditions resulted in a slightly larger fluctuation of generation time (Lee et al., 2010) while a more stable generation time occurred in anaerobic culture as demonstrated in this study. The fluctuation might be due to the cells being grown in strict aerobic conditions, while a micro-aerobic culture might

be more suitable for growth and colonisation; therefore, producing a more stable generation time (Jones et al., 2007).

Similar trends were shown in the Day 7 / Day 5 cell ratio density readings. The cells were observed to grow slower but obtained similar cell densities. This suggests that the cells were not limited by the lack of oxygen in the media which is expected as *E. coli* is well-established to be a facultative anaerobe (Finegold et al., 1983).

### **5.6 Increased Growth Ability in Specialised Media**

At the early phase of the experiment (Passage 71 to 83), all treatments showed a stabilised coefficient of variation. This stability was not present when the cells were first introduced at the very beginning (before passage 25) of the experiment (Lee et al., 2010). This stability suggests that cells had prior adaptation to their specialised growth media from the initial 70 passages.

Throughout the later 83 passages (Passage 70 to 153), the coefficient of variation showed an increase in minimal percentage at around 6% to 8% (Figure 4.2 and Table 4.1) as compared to the 3% to 5% for the first 70 passages (Lee et al., 2010). The overall increase may suggest that the stationary phase (Day 5) was longer than the death phase (Day 7). This could be due to the cells being able to maintain a longer growth time as compared to the first 70 passages. The ability of the cells to maintain a longer growth time may imply that their adaptation to its own specialised media have improved further over time so as to sustain longer survivability.

Nearing the end of the experiment (after passage 128), the coefficient of variation for all treatments started to decrease to around 1% to 4%. The decrease may be linked to the cells reaching optimised growth for their media as seen from the generation time graph (Figure 4.7). The graphs shows a flattening of the linear curve which means that the generation time is getting more stable which could imply that growth was optimised.

### **5.7 Occurrence of Stepwise Adaptation**

The coefficient of variation from passage 84 to passage 113 shows a huge jump in percentage, from around 6% to around 25% (Figure 4.2). This huge jump could be due to a sudden surge in adaptation rates. Adaptation does not occur linearly, but could instead occur

in a step-wise manner (Clune et al., 2008). The step-wise adaptation is due to the ruggedness of the environment, a more rugged landscape will result in a steeper adaptation occurrence (Clune et al., 2008). A more rugged landscape will mean a harsher environment or an increase in amount of variation of stress in the environment. As compared to the values across other passage intervals, the percentage of the coefficient of variation is almost tripled. At the same time, the number of generations for this period fell significantly (Figure 4.1). The sudden decrease in generation number may be caused by the action of the adaptation mechanism. With the sudden change in cell adaptation, unfit cells are unable to survive; thus, eliminated from the ecosystem. The large number of cell deaths result in a drop in generation number; thus, the multiple ‘0’ in the tabulation of the number of generations (Appendix A). A ‘0’ would mean that there was no net increase in population from the previous passage, indicating that the numbers of cell deaths were higher than cell division with the number of new cells unable to replace the population that were lost.

If adaptation occurs linearly, one would expect a gradual increase in coefficient of variation and not a sudden increase in the coefficient of variation. However, from the results of the generation time and swap experiment, a linear adaptation is seen. This may suggest that there are both gradual adaptation (Wu et al., 2006) and stepwise adaptation jumps, whereby the linear adaptation may serve as a bridge between each adaptation jump to allow the gradual adaptation of the organism before each jump. This can be a mechanism to prevent the loss of a huge population of cells, so much that the remaining population is unable to proliferate further (Jiang et al., 2007).

### **5.8 Cells of different treatment shows genetic divergence**

The results from the PCR/RFLP show a slight divergence in DI (Figure 4.15) indicating that the *E. coli* of different treatments are getting genetically different. The experimental results contradict with our hypothesis, which suggested that the rate of adaptation will slow down after the 150<sup>th</sup> passage and the genetic variation will decrease. The decrease would have resulted in a convergence in the DI graph but experimental results show a divergence. This suggests that they may have evolved specialised adaptation mechanism to their own type of stress environment (Miller et al., 2009), also known as niche adaptation, which had been reported in a number of microorganisms such as *Pseudomonas putida* (Wu et al., 2010), *Lactobacillus plantarum* (Siezen et al., 2010) and *Streptococcus uberis* (Ward et al., 2009).

*E. coli* exposed to stress will respond to counter the effects. A study had shown that *E. coli* will utilise its ArcAB global regulatory system in aerobic conditions to counter the effects caused by reactive oxygen stress (ROS) (Loui et al., 2009). In another study (Wang et al., 2009), *E. coli* are shown to be able to utilise the Poly- $\beta$ -hydroxybutyrate (PHB) mobilisation mechanism to increase their survivability under stress conditions

The slight divergence of the genetic similarity of *E. coli* is contrasting to the convergence of genetic similarity as stated in the first 70 passages (Lee et al., 2010). The first 70 passages showed a converging trend in the DI calculations with the DI values ending off at around 0.05 to 0.2 at the end of 70 passages (Lee et al., 2010).

From the statistical test (Table 4.15), the p-value obtained from the tabulation of the correlation coefficient of the DI of the 6 selected treatment pairs shows that all pairs are statistically significant. Each pair of treatments has a common resulting effect; such as L MSG/L SALT, L BA/L COMB; having MSG as the common resulting effect. The statistical significance shows that although both adaptations are towards MSG, due to the different media the cells were originally grown in, they have chosen different types of adaptation pathways to adapt to the same source of stress. The action of the cells choosing different pathways for adaptation results in the divergence of their genetic material across all 8 different treatments which explains the slight divergence of DI values from passage 82 onwards (Figure 4.15) due to ecological specialisation. A study conducted on *Listeria monocytogenes* shows that it can change its membrane fluidity based on the temperature of the environment it is residing in (Badaoui Najjar et al., 2007). In another study, *Lactobacillus sakei* 21k, *Lactobacillus. sakei* LTH5590 and *Lactobacillus. sakei* FLEC01 can survive the transition from conventional mice gastrointestinal tracts to the GI tract of axenic mice by inducing morphological changes and modified protein expressions levels causing the plasma membrane to appear rough for better colonisation of the GI of axenic mice (Chiaramonte et al., 2010).

However, the adaptation responses and divergence of DI cannot be applied for the whole *E. coli* genome. This is due to the use of only 3 primers (Primer 5, 6, 7) during the study only amplify 0.37% of the total *E. coli* genome. Hence, only the area of the genomes that are being amplified by the 3 primers can account for the divergence. The global stress response genes that are activated may or may not lie within the amplified region. From the DI values of

passage 1 to passage (Lee et al., 2010) and the DI values of passage 83 to passage 153 (Figure 4.15), the DI value may slowly approach 0 through a series of convergence and divergence but it will not reach a DI value of 0. A DI value of 0 will mean that there is no difference in the amplified region of the genome which is not possible due to each cells having unique mutations.

## **6 Recommendations**

There are some areas for improvements in this study. Firstly, a comparison of the same treatment between different passages can be established to analyze the effects of the treatment as the cells are being subcultured into more passages. Such mutation may not be detected by the PCR/RFLP profile comparisons between treatment samples of the same passage. We hypothesize that the profiles of the different passages will differ from each other due to mutations occurring to adapt to the selective environment.

Secondly, we can do swap experiment where both concentrations of the remaining treatments (H/L MSG, H/L BA, H/L COMB) are swapped into a higher concentration. This could be done to determine if other treatments will exhibit similar trends as H/L SALT being swapped to Higher Salt.

Thirdly, more enzymes and primers can be included in the study to allow more coverage of the *E. coli* genome to allow for a more detailed analysis to be done on a global genomic scale.

Lastly, we can include more combinations of treatments to use, such as growing the *E. coli* in MSG and BA in the absence of nutrient broth. Other types of combination may include combination treatments of both H and L treatments, such as using H MSG and L BA together. These can show the different effect of the different chemicals at different concentration in combination.

## **7 Conclusion**

Our results demonstrated that the cells of the later passages were better adapted to their individual treatment as compared to cells of earlier passages, indicated by generally lower generation times and the ability of cells to maintain longer growth. The rate of adaptation was decreasing, as observed from the declined rate of decrease in generation time. Apart from cells adapting to their individual treatments, ecological specialization was observed, whereby there was a loss of ability of cells to adapt to other environments other than their selected individual treatments. This was indicated in the results of swap experiments where cells were introduced into an environment other than their own. Ecological specialization was also observed because there was significant difference in the dissimilarity index values when comparing 2 different comparisons with the identical resulting effect, indicating that there is a slight diverging linear regression.

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## Appendix A – Number of Generations

This appendix is the tabulation of the number of log phase generation of the main culture experiment for the 8 different treatments. OD600 readings were taken on every passage at Day 2 to estimate the number of log phase generations within the passage. In total, the number of log phase generation across 83 passages is estimated.

Passage Number	High Monosodium Glutamate (MSG)		Low Monosodium Glutamate (MSG)	
	OD 600 at Start of next Passage	Logarithmic Phase Generations	OD 600 at Start of next Passage	Logarithmic Phase Generations
71	0.345		0.644	
72	0.426	6.88	0.886	6.87
73	0.402	6.58	0.511	6.22
74	0.525	6.90	0.487	6.60
75	0.424	6.44	0.430	6.52
76	0.522	6.84	0.517	6.82
77	0.520	6.64	0.516	6.64
78	0.619	6.79	0.547	6.70
79	0.890	6.91	0.703	6.85
80	0.601	6.35	0.451	6.27
81	0.552	6.57	0.457	6.66
82	0.399	6.33	0.554	6.82
83	0.431	6.72	0.469	6.49
84	0.675	7.04	0.524	6.75
85	0.470	6.34	0.538	6.67
86	0.505	6.71	0.202	0.00
87	0.425	6.48	0.442	7.82
88	0.448	6.70	0.444	6.65
89	0.213	0.00	0.215	0.00
90	0.206	0.00	0.202	0.00
91	0.214	0.00	0.228	0.00
92	0.261	0.00	0.259	0.00
93	0.281	0.00	0.270	0.00
94	0.368	7.15	0.356	7.17
95	0.378	6.67	0.364	6.67
96	0.517	6.95	0.379	6.69
97	0.498	6.61	0.452	6.83
98	0.391	6.40	0.388	6.49
99	0.416	6.71	0.429	6.75

100	0.433	6.68	0.435	6.66
101	0.440	6.65	0.507	6.79
102	0.461	6.69	0.448	6.53
103	0.470	6.66	0.459	6.67
104	0.436	6.57	0.408	6.53
105	0.471	6.72	0.461	6.77
106	0.593	6.85	0.564	6.83
107	0.464	6.43	0.438	6.41
108	0.498	6.71	0.445	6.66
109	0.492	6.63	0.463	6.68
110	0.349	6.29	0.370	6.41
111	0.456	6.93	0.473	6.90
112	0.508	6.74	0.489	6.67
113	0.486	6.60	0.499	6.66
114	0.477	6.63	0.486	6.62
115	0.492	6.67	0.492	6.66
116	0.524	6.70	0.573	6.78
117	0.550	6.69	0.558	6.62
118	0.489	6.54	0.501	6.55
119	0.505	6.67	0.507	6.65
120	0.533	6.69	0.542	6.70
121	0.487	6.56	0.505	6.58
122	0.553	6.76	0.523	6.68
123	1.137	7.16	0.488	6.58
124	0.495	6.03	0.434	6.53
125	0.497	6.65	0.537	6.84
126	0.501	6.65	0.516	6.61
127	0.450	6.54	0.456	6.53
128	0.507	6.76	0.468	6.67
129	0.509	6.65	0.535	6.77
130	0.564	6.73	0.479	6.54
131	0.523	6.58	0.508	6.70
132	0.630	6.80	0.551	6.72
133	0.483	6.42	0.517	6.59
134	0.508	6.69	0.541	6.68
135	0.526	6.67	0.545	6.65
136	0.519	6.63	0.539	6.63
137	0.488	6.59	0.466	6.51
138	0.462	6.59	0.476	6.66
139	0.458	6.64	0.521	6.73
140	0.497	6.72	0.534	6.67
141	0.538	6.71	0.581	6.72

142	0.453	6.49	0.479	6.47
143	0.479	6.70	0.500	6.68
144	0.471	6.63	0.499	6.64
145	0.486	6.67	0.531	6.69
146	0.465	6.60	0.496	6.58
147	0.630	6.91	0.550	6.74
148	0.503	6.45	0.511	6.58
149	0.507	6.65	0.541	6.69
150	0.453	6.54	0.496	6.57
151	0.509	6.75	0.525	6.69
152	0.555	6.72	0.575	6.72
153	0.609	6.72	0.620	6.71
Cumulative Generations		512.80		506.98

Passage Number	High Benzoic Acid (BA)		Low Benzoic Acid (BA)	
	OD 600 at Start of next Passage	Logarithmic Phase Generations	OD 600 at Start of next Passage	Logarithmic Phase Generations
71	0.467		0.343	
72	0.817	7.10	0.839	7.44
73	0.503	6.26	0.395	6.00
74	0.443	6.52	0.427	6.72
75	0.324	6.30	0.428	6.65
76	0.474	7.06	0.515	6.82
77	0.393	6.46	0.487	6.59
78	0.448	6.78	0.478	6.63
79	0.582	6.88	0.665	6.92
80	0.350	6.14	0.680	6.66
81	0.386	6.75	0.471	6.33
82	0.468	6.84	0.545	6.78
83	0.432	6.57	0.477	6.52
84	0.440	6.66	0.505	6.70
85	0.468	6.70	0.515	6.66
86	0.168	0.00	0.314	6.12
87	0.368	7.89	0.468	7.08
88	0.389	6.71	0.463	6.63
89	0.211	0.00	0.217	0.00
90	0.221	0.00	0.193	0.00
91	0.215	0.00	0.176	0.00
92	0.205	0.00	0.253	0.00
93	0.265	0.00	0.288	0.00
94	0.385	7.29	0.374	7.13
95	0.423	6.74	0.606	7.10

96	0.387	6.55	0.394	6.24
97	0.456	6.81	0.391	6.64
98	0.420	6.56	0.394	6.65
99	0.405	6.61	0.465	6.81
100	0.405	6.64	0.457	6.63
101	0.404	6.64	0.470	6.67
102	0.415	6.67	0.496	6.69
103	0.792	7.19	0.518	6.68
104	0.371	5.97	0.462	6.54
105	0.432	6.81	0.509	6.73
106	0.578	6.91	0.561	6.73
107	0.569	6.63	0.493	6.53
108	0.388	6.28	0.506	6.67
109	0.468	6.83	0.506	6.64
110	0.330	6.27	0.379	6.36
111	0.396	6.85	0.453	6.83
112	0.425	6.72	0.539	6.80
113	0.437	6.67	0.596	6.73
114	0.423	6.61	0.506	6.50
115	0.453	6.71	0.490	6.61
116	0.550	6.82	0.550	6.75
117	0.545	6.64	0.555	6.65
118	0.509	6.58	0.507	6.56
119	0.505	6.64	0.506	6.64
120	0.549	6.72	0.549	6.72
121	0.504	6.57	0.496	6.55
122	0.515	6.66	0.511	6.67
123	0.442	6.50	0.482	6.59
124	0.379	6.48	0.473	6.63
125	0.494	6.91	0.526	6.74
126	0.466	6.59	0.519	6.63
127	0.421	6.54	0.457	6.53
128	0.501	6.81	0.526	6.77
129	0.478	6.60	0.517	6.63
130	0.466	6.62	0.474	6.56
131	0.496	6.70	0.523	6.73
132	0.500	6.65	0.530	6.66
133	0.461	6.57	0.512	6.61
134	0.495	6.71	0.540	6.69
135	0.466	6.59	0.487	6.55
136	0.525	6.75	0.531	6.72
137	0.429	6.45	0.499	6.59

138	0.441	6.67	0.472	6.59
139	0.491	6.75	0.545	6.77
140	0.492	6.65	0.552	6.65
141	0.550	6.74	0.600	6.71
142	0.468	6.50	0.504	6.49
143	0.498	6.70	0.533	6.69
144	0.482	6.61	0.511	6.61
145	0.440	6.56	0.491	6.61
146	0.441	6.65	0.481	6.62
147	0.490	6.74	0.521	6.72
148	0.472	6.61	0.495	6.60
149	0.499	6.70	0.537	6.72
150	0.438	6.52	0.581	6.71
151	0.481	6.73	0.687	6.78
152	0.511	6.70	0.562	6.48
153	0.543	6.70	0.585	6.68
Cumulative Generations		507.18		512.77

Passage Number	High Salt		Low Salt	
	OD 600 at Start of next Passage	Logarithmic Phase Generations	OD 600 at Start of next Passage	Logarithmic Phase Generations
71	0.557		0.342	
72	0.974	7.06	0.896	7.49
73	0.438	6.01	0.511	6.21
74	0.680	7.03	0.372	6.33
75	0.489	6.37	0.417	6.77
76	0.571	6.78	0.510	6.84
77	0.549	6.61	0.529	6.68
78	0.515	6.59	0.311	6.08
79	0.641	6.83	0.385	6.90
80	0.474	6.38	0.610	7.07
81	0.923	7.17	0.418	6.30
82	0.745	6.50	0.558	6.91
83	0.549	6.40	0.491	6.53
84	0.595	6.71	0.485	6.63
85	0.525	6.54	0.521	6.71
86	0.335	6.18	0.261	0.00
87	0.447	6.96	0.412	7.38
88	0.492	6.73	0.451	6.73
89	0.154	0.00	0.213	0.00
90	0.175	0.00	0.201	0.00
91	0.146	0.00	0.219	0.00

92	0.307	7.88	0.263	0.00
93	0.349	6.80	0.313	7.05
94	0.397	6.79	0.410	6.96
95	0.446	6.76	0.431	6.69
96	0.343	6.36	0.436	6.66
97	0.492	7.02	0.538	6.84
98	0.437	6.53	0.449	6.48
99	0.485	6.74	0.487	6.72
100	0.505	6.68	0.511	6.69
101	0.460	6.56	0.469	6.56
102	0.528	6.77	0.510	6.72
103	0.526	6.64	0.518	6.66
104	0.484	6.57	0.475	6.56
105	0.553	6.76	0.699	6.97
106	0.699	6.83	0.587	6.51
107	0.561	6.47	0.700	6.78
108	0.509	6.56	0.502	6.37
109	0.561	6.73	0.593	6.79
110	0.453	6.45	0.393	6.26
111	0.518	6.77	0.479	6.84
112	0.538	6.68	0.523	6.72
113	0.559	6.68	0.645	6.82
114	0.526	6.59	0.515	6.46
115	0.495	6.59	0.486	6.59
116	0.513	6.68	0.524	6.71
117	0.584	6.76	0.577	6.73
118	0.661	6.74	0.509	6.54
119	0.481	6.37	0.517	6.66
120	0.470	6.62	0.559	6.71
121	0.524	6.74	0.493	6.53
122	0.554	6.69	0.533	6.71
123	0.468	6.49	0.474	6.54
124	0.538	6.77	0.467	6.63
125	0.606	6.74	0.542	6.78
126	0.498	6.47	0.533	6.63
127	0.504	6.65	0.461	6.51
128	0.550	6.72	0.522	6.76
129	0.538	6.62	0.569	6.72
130	0.521	6.62	0.543	6.60
131	0.565	6.71	0.664	6.81
132	0.542	6.61	0.541	6.48
133	0.553	6.66	0.495	6.56

134	0.585	6.69	0.540	6.72
135	0.550	6.59	0.545	6.65
136	0.569	6.67	0.514	6.59
137	0.560	6.63	0.508	6.63
138	0.507	6.56	0.498	6.63
139	0.549	6.71	0.542	6.72
140	0.555	6.65	0.516	6.60
141	0.597	6.70	1.082	7.19
142	0.518	6.52	0.498	6.07
143	0.558	6.71	0.509	6.66
144	0.521	6.58	0.514	6.65
145	0.526	6.65	0.493	6.61
146	0.532	6.65	0.479	6.62
147	0.565	6.70	0.544	6.76
148	0.543	6.61	0.519	6.60
149	0.591	6.72	0.542	6.68
150	0.501	6.50	0.479	6.53
151	0.556	6.74	0.532	6.74
152	0.604	6.71	0.584	6.72
153	0.620	6.66	0.598	6.66
Cumulative Generations		526.66		513.87

Passage Number	High Combination		Low Combination	
	OD 600 at Start of next Passage	Logarithmic Phase Generations	OD 600 at Start of next Passage	Logarithmic Phase Generations
71	0.503		0.456	
72	0.448	6.53	0.470	6.67
73	0.520	6.78	0.502	6.70
74	0.504	6.62	0.600	6.80
75	0.358	6.29	0.430	6.34
76	0.960	7.48	0.573	6.91
77	0.779	6.50	0.834	6.93
78	0.474	6.24	0.540	6.31
79	0.575	6.82	1.297	7.26
80	0.414	6.34	0.473	5.91
81	0.539	6.89	0.446	6.59
82	0.467	6.51	0.618	6.93
83	0.453	6.61	0.489	6.44
84	0.609	6.91	0.637	6.87
85	0.477	6.43	0.487	6.41
86	0.289	0.00	0.305	6.13
87	0.373	7.13	0.423	7.02

88	0.423	6.78	0.452	6.71
89	0.240	0.00	0.220	0.00
90	0.227	0.00	0.211	0.00
91	0.241	0.00	0.185	0.00
92	0.324	7.22	0.299	0.00
93	0.354	6.75	0.304	6.83
94	0.358	6.66	0.369	6.88
95	0.435	6.85	0.278	0.00
96	0.419	6.61	0.440	7.36
97	0.487	6.79	0.519	6.80
98	0.437	6.54	0.392	6.37
99	0.468	6.71	0.809	7.27
100	0.475	6.66	0.483	6.23
101	0.460	6.61	0.458	6.59
102	0.641	6.93	0.469	6.67
103	0.482	6.40	0.469	6.64
104	0.433	6.54	0.419	6.53
105	0.494	6.77	0.488	6.79
106	0.506	6.67	0.551	6.75
107	0.439	6.51	0.459	6.48
108	0.450	6.67	0.495	6.71
109	0.464	6.67	0.482	6.62
110	0.477	6.67	0.370	6.37
111	0.438	6.56	0.515	6.97
112	0.631	6.97	0.525	6.66
113	0.453	6.35	0.518	6.63
114	0.438	6.61	0.505	6.62
115	0.465	6.70	0.487	6.61
116	0.556	6.81	0.515	6.69
117	0.522	6.59	0.555	6.71
118	0.473	6.55	0.496	6.54
119	0.688	6.96	0.493	6.64
120	0.661	6.61	0.562	6.76
121	0.487	6.39	0.499	6.54
122	0.500	6.67	0.511	6.67
123	0.421	6.48	0.485	6.60
124	0.423	6.65	0.486	6.65
125	0.484	6.77	0.516	6.70
126	0.476	6.63	0.527	6.66
127	0.429	6.54	0.452	6.50
128	0.491	6.77	0.518	6.77
129	0.489	6.64	0.542	6.68

130	0.466	6.60	0.487	6.55
131	0.494	6.70	0.521	6.71
132	0.508	6.67	0.561	6.71
133	0.486	6.60	0.486	6.52
134	0.519	6.70	0.545	6.75
135	0.478	6.57	1.159	7.18
136	0.408	6.49	0.708	6.32
137	0.429	6.70	0.490	6.34
138	0.700	7.07	0.470	6.60
139	0.618	6.55	0.500	6.70
140	0.416	6.28	0.519	6.68
141	0.455	6.73	0.557	6.71
142	0.469	6.67	0.460	6.47
143	0.620	6.89	0.481	6.69
144	0.462	6.39	0.480	6.64
145	0.475	6.67	0.490	6.66
146	0.444	6.58	0.446	6.55
147	0.512	6.78	0.543	6.83
148	0.576	6.75	0.492	6.56
149	0.497	6.51	0.530	6.71
150	0.421	6.48	0.469	6.53
151	0.514	6.83	0.516	6.73
152	0.551	6.70	0.546	6.69
153	0.558	6.65	0.599	6.72
Cumulative Generations		519.89		512.94

## Appendix B – Generation time Estimation

This is the tabulation of the estimated average log phase generation time of the 8 different treatment culture in the main experiment. Every 3 passages, OD readings will be taken for the first 5 hours after subculture. The log phase generation time is tabulated and the result can be used to monitor the adaptability of the cells. The blank cells in the table below (High BA of Passage 76 and 79, High Comb of Passage 76) indicate that the generation time could not be calculated due to abnormality in OD readings.

Passage	Generation Time in Minutes (Geometric Mean) of each Treatment							
	High MSG	Low MSG	High BA	Low BA	High Salt	Low Salt	High Comb	Low Comb
<b>73</b>	77.316	90.487	74.846	98.475	98.852	97.048	82.559	97.764
<b>76</b>	78.513	76.899		91.464	71.389	74.631		77.266
<b>79</b>	82.674	98.949		67.741	55.288	54.171	188.112	68.185
<b>85</b>	79.432	74.289	78.292	75.094	77.831	90.653	83.402	73.006
<b>88</b>	73.914	88.525	49.614	74.911	185.753	82.867	52.742	82.092
<b>94</b>	35.143	34.047	69.085	38.934	38.138	46.575	98.258	45.009
<b>97</b>	87.97	69.074	62.941	73.09	55.442	86.628	122.759	83.972
<b>100</b>	72.225	66.614	65.812	83.568	75.758	80.973	81.082	93.107
<b>103</b>	73.134	65.546	78.259	74.544	75.311	76.105	78.129	73.765
<b>106</b>	101.624	81.278	71.716	102.055	74.49	91.121	80.835	70.697
<b>109</b>	69.585	60.177	47.131	71.336	57.846	75.617	41.254	66.365
<b>111</b>	83.395	82.783	46.092	75.763	80.444	77.434	90.895	84.446
<b>114</b>	93.034	74.532	79.355	79.702	66.147	80.19	125.355	72.735
<b>117</b>	62.665	79.68	59.64	63.822	51.903	107.808	50.507	61.422
<b>120</b>	98.639	82.968	92.490	112.460	86.612	116.816	113.109	123.437
<b>123</b>	81.873	71.514	73.223	81.127	72.438	86.679	68.325	88.642
<b>126</b>	61.102	60.746	57.184	67.898	61.725	77.988	55.348	78.480
<b>129</b>	72.205	63.976	70.742	71.162	102.927	106.660	66.295	79.341
<b>132</b>	89.039	69.224	76.337	83.596	69.010	87.713	71.259	85.195
<b>135</b>	61.999	71.964	66.685	68.784	58.635	56.992	57.170	88.859
<b>141</b>	87.367	83.918	84.834	108.509	68.454	92.140	70.670	102.350
<b>144</b>	70.808	64.936	41.096	71.609	54.553	69.166	59.566	53.082
<b>147</b>	66.541	66.811	70.024	96.109	77.067	74.868	78.172	78.154
<b>150</b>	86.954	94.156	72.072	84.918	97.091	127.137	71.710	127.483
<b>153</b>	82.612	76.178	77.595	85.686	76.845	78.760	72.863	93.345

## Appendix C – Cell Density at Stationary Phase

This is the appendix for cell density of every 5 and 7 days of every passage. The cell density is estimated from the OD600 reading taken on 5 and 7 days of the passage. This could estimate the growth rate which is used to estimate the adaptability of the E. coli during stationary phase.

### Ratio of Day 7/ Day 5 Cell Density of High MSG Treatment

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.644	0.733	9.58E+07	1.03E+08	107.05%
72	0.601	0.626	9.22E+07	9.43E+07	102.31%
74	0.693	0.698	9.96E+07	1.00E+08	100.38%
75	0.561	0.463	8.86E+07	7.86E+07	88.70%
77	0.636	0.802	9.51E+07	1.07E+08	112.71%
78	0.753	0.740	1.04E+08	1.03E+08	99.13%
80	0.659	0.749	9.70E+07	1.04E+08	106.88%
81	0.638	0.729	9.53E+07	1.02E+08	107.30%
83	0.588	0.632	9.10E+07	9.48E+07	104.13%
84	0.753	0.448	1.04E+08	7.69E+07	73.95%
86	0.660	1.809	9.70E+07	1.50E+08	154.21%
87	0.513	0.574	8.39E+07	8.98E+07	106.98%
89	0.299	0.368	5.58E+07	6.66E+07	119.41%
90	0.271	0.406	5.06E+07	7.17E+07	141.61%
92	0.316	0.430	5.87E+07	7.47E+07	127.38%
93	0.412	0.495	7.25E+07	8.21E+07	113.20%
95	0.508	0.595	8.34E+07	9.16E+07	109.88%
96	0.590	0.761	9.12E+07	1.04E+08	114.55%
98	0.537	0.612	8.63E+07	9.31E+07	107.90%
99	0.491	0.638	8.16E+07	9.53E+07	116.73%
101	0.541	0.583	8.67E+07	9.06E+07	104.50%
102	0.524	0.602	8.50E+07	9.23E+07	108.51%
104	0.583	0.656	9.06E+07	9.67E+07	106.79%
105	0.609	0.707	9.29E+07	1.01E+08	108.38%
107	0.643	1.965	9.57E+07	1.54E+08	160.86%
108	0.612	0.519	9.31E+07	8.45E+07	90.80%
110	0.515	0.605	8.41E+07	9.25E+07	109.98%

111	0.661	0.730	9.71E+07	1.02E+08	105.33%
113	0.501	0.672	8.27E+07	9.80E+07	118.52%
114	0.632	0.762	9.48E+07	1.05E+08	110.29%
116	0.598	0.749	9.19E+07	1.04E+08	112.77%
117	0.736	0.843	1.03E+08	1.10E+08	106.89%
119	0.601	0.706	9.22E+07	1.01E+08	109.11%
120	0.735	0.786	1.03E+08	1.06E+08	103.41%
122	0.647	0.791	9.60E+07	1.06E+08	110.91%
123	1.083	1.110	1.23E+08	1.24E+08	101.04%
125	0.691	0.790	9.94E+07	1.06E+08	107.02%
126	0.650	0.831	9.63E+07	1.09E+08	113.31%
128	0.710	0.846	1.01E+08	1.10E+08	109.06%
129	0.680	0.831	9.86E+07	1.09E+08	110.60%
131	0.722	0.828	1.02E+08	1.09E+08	107.02%
132	0.765	0.837	1.05E+08	1.09E+08	104.48%
134	0.668	0.795	9.77E+07	1.07E+08	109.29%
135	0.692	0.837	9.95E+07	1.09E+08	109.97%
137	0.657	0.728	9.68E+07	1.02E+08	105.53%
138	0.628	0.747	9.45E+07	1.04E+08	109.58%
140	0.705	0.790	1.00E+08	1.06E+08	105.91%
141	0.729	0.903	1.02E+08	1.13E+08	110.92%
143	0.633	0.734	9.49E+07	1.03E+08	108.14%
144	0.693	0.745	9.96E+07	1.03E+08	103.79%
146	0.636	0.727	9.51E+07	1.02E+08	107.33%
147	0.826	0.986	1.09E+08	1.18E+08	108.49%
149	0.646	0.793	9.59E+07	1.07E+08	111.14%
150	0.639	0.763	9.54E+07	1.05E+08	109.70%
152	0.714	0.819	1.01E+08	1.08E+08	107.07%
153	0.793	0.916	1.07E+08	1.14E+08	107.05%

#### Ratio of Day 7/ Day 5 Cell Density of Low MSG Treatment

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.686	0.805	9.91E+07	1.07E+08	108.42%
72	0.919	0.906	1.14E+08	1.14E+08	99.35%
74	0.674	0.710	9.81E+07	1.01E+08	102.76%
75	0.613	0.701	9.32E+07	1.00E+08	107.50%
77	0.674	0.742	9.81E+07	1.03E+08	105.11%
78	0.700	0.692	1.00E+08	9.95E+07	99.40%
80	0.664	0.711	9.74E+07	1.01E+08	103.66%
81	0.562	0.781	8.87E+07	1.06E+08	119.35%

83	0.640	0.497	9.55E+07	8.23E+07	86.19%
84	0.602	0.463	9.23E+07	7.86E+07	85.16%
86	0.436	0.784	7.54E+07	1.06E+08	140.55%
87	0.537	0.610	8.63E+07	9.29E+07	107.70%
89	0.227	0.306	4.14E+07	5.70E+07	137.60%
90	0.248	0.356	4.60E+07	6.49E+07	140.95%
92	0.420	0.431	7.35E+07	7.48E+07	101.83%
93	0.381	0.449	6.84E+07	7.70E+07	112.52%
95	0.484	0.572	8.09E+07	8.96E+07	110.77%
96	0.506	0.663	8.32E+07	9.73E+07	116.93%
98	0.548	0.644	8.74E+07	9.58E+07	109.63%
99	0.521	0.674	8.47E+07	9.81E+07	115.84%
101	0.661	0.715	9.71E+07	1.01E+08	104.22%
102	0.626	0.773	9.43E+07	1.05E+08	111.66%
104	0.609	0.662	9.29E+07	9.72E+07	104.69%
105	0.612	0.704	9.31E+07	1.00E+08	107.84%
107	0.600	2.139	9.21E+07	1.58E+08	171.97%
108	0.607	0.518	9.27E+07	8.44E+07	91.08%
110	0.515	0.625	8.41E+07	9.42E+07	112.00%
111	0.584	0.685	9.07E+07	9.90E+07	109.17%
113	0.508	0.648	8.34E+07	9.61E+07	115.22%
114	0.655	0.752	9.67E+07	1.04E+08	107.45%
116	0.622	0.723	9.40E+07	1.02E+08	108.35%
117	0.721	0.840	1.02E+08	1.10E+08	107.83%
119	0.616	0.716	9.35E+07	1.01E+08	108.39%
120	0.590	0.796	9.12E+07	1.07E+08	117.12%
122	0.624	0.798	9.41E+07	1.07E+08	113.62%
123	0.615	0.736	9.34E+07	1.03E+08	110.03%
125	0.729	0.838	1.02E+08	1.10E+08	107.11%
126	0.667	0.870	9.76E+07	1.11E+08	114.19%
128	0.740	0.851	1.03E+08	1.10E+08	107.07%
129	0.648	0.857	9.61E+07	1.11E+08	115.17%
131	0.725	0.848	1.02E+08	1.10E+08	108.01%
132	0.732	0.510	1.02E+08	8.36E+07	81.61%
134	0.712	0.848	1.01E+08	1.10E+08	109.02%
135	0.743	0.896	1.03E+08	1.13E+08	109.46%
137	0.644	0.720	9.58E+07	1.02E+08	106.07%
138	0.667	0.784	9.76E+07	1.06E+08	108.63%
140	0.734	0.863	1.03E+08	1.11E+08	108.23%
141	0.849	1.000	1.10E+08	1.19E+08	107.75%
143	0.684	0.826	9.89E+07	1.09E+08	109.94%
144	0.743	0.838	1.03E+08	1.10E+08	106.08%

146	0.707	0.823	1.01E+08	1.09E+08	107.87%
147	0.735	0.872	1.03E+08	1.12E+08	108.68%
149	0.694	0.847	9.97E+07	1.10E+08	110.42%
150	0.707	0.849	1.01E+08	1.10E+08	109.48%
152	0.796	0.901	1.07E+08	1.13E+08	106.05%
153	0.783	0.947	1.06E+08	1.16E+08	109.36%

### Ratio of Day 7/ Day 5 Cell Density of High BA Treatment

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.668	0.755	9.77E+07	1.04E+08	106.53%
72	0.889	0.910	1.13E+08	1.14E+08	101.08%
74	0.630	0.657	9.46E+07	9.68E+07	102.31%
75	0.507	0.668	8.33E+07	9.77E+07	117.26%
77	0.609	0.745	9.29E+07	1.03E+08	111.32%
78	0.674	0.670	9.81E+07	9.78E+07	99.68%
80	0.571	0.710	8.95E+07	1.01E+08	112.69%
81	0.565	0.696	8.90E+07	9.98E+07	112.22%
83	0.590	0.635	9.12E+07	9.50E+07	104.20%
84	0.563	0.418	8.88E+07	7.32E+07	82.51%
86	0.476	0.620	8.00E+07	9.38E+07	117.22%
87	0.525	0.615	8.51E+07	9.34E+07	109.69%
89	0.219	0.293	3.95E+07	5.47E+07	138.39%
90	0.253	0.371	4.71E+07	6.70E+07	142.41%
92	0.360	0.397	6.55E+07	7.06E+07	107.79%
93	0.411	0.540	7.24E+07	8.66E+07	119.67%
95	0.543	0.583	8.69E+07	9.06E+07	104.27%
96	0.502	0.668	8.28E+07	9.77E+07	117.99%
98	0.601	0.681	9.22E+07	9.87E+07	107.07%
99	0.551	0.705	8.76E+07	1.00E+08	114.66%
101	0.609	0.652	9.29E+07	9.64E+07	103.83%
102	0.546	0.687	8.72E+07	9.91E+07	113.74%
104	0.582	0.637	9.05E+07	9.52E+07	105.20%
105	0.577	0.685	9.00E+07	9.90E+07	109.93%
107	0.878	2.009	1.12E+08	1.55E+08	138.55%
108	0.679	0.495	9.85E+07	8.21E+07	83.28%
110	0.525	0.636	8.51E+07	9.51E+07	111.75%
111	0.530	0.639	8.56E+07	9.54E+07	111.39%
113	0.555	0.661	8.80E+07	9.71E+07	110.35%
114	0.641	0.733	9.55E+07	1.03E+08	107.32%
116	0.584	0.708	9.07E+07	1.01E+08	111.07%

117	0.742	0.781	1.03E+08	1.06E+08	102.59%
119	0.589	0.674	9.11E+07	9.81E+07	107.71%
120	0.692	0.785	9.95E+07	1.06E+08	106.61%
122	0.562	0.728	8.87E+07	1.02E+08	115.22%
123	0.599	0.721	9.20E+07	1.02E+08	110.51%
125	0.692	0.790	9.95E+07	1.06E+08	106.94%
126	0.655	0.866	9.67E+07	1.11E+08	115.06%
128	0.715	0.858	1.01E+08	1.11E+08	109.39%
129	0.689	0.865	9.93E+07	1.11E+08	111.94%
131	0.694	0.856	9.97E+07	1.11E+08	110.97%
132	0.709	0.822	1.01E+08	1.08E+08	107.65%
134	0.694	0.846	9.97E+07	1.10E+08	110.36%
135	0.694	0.837	9.97E+07	1.09E+08	109.80%
137	0.665	0.786	9.74E+07	1.06E+08	108.94%
138	0.669	0.807	9.78E+07	1.08E+08	110.00%
140	0.722	0.888	1.02E+08	1.13E+08	110.61%
141	0.845	1.030	1.10E+08	1.20E+08	109.39%
143	0.677	0.828	9.84E+07	1.09E+08	110.67%
144	0.770	0.880	1.05E+08	1.12E+08	106.62%
146	0.656	0.792	9.67E+07	1.07E+08	110.15%
147	0.740	0.845	1.03E+08	1.10E+08	106.72%
149	0.618	0.840	9.36E+07	1.10E+08	117.09%
150	0.670	0.837	9.78E+07	1.09E+08	111.86%
152	0.726	0.823	1.02E+08	1.09E+08	106.41%
153	0.713	0.851	1.01E+08	1.10E+08	109.13%

#### Ratio of Day 7/ Day 5 Cell Density of Low BA Treatment

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.698	0.656	1.00E+08	9.67E+07	96.76%
72	0.832	0.791	1.09E+08	1.06E+08	97.59%
74	0.628	0.613	9.45E+07	9.32E+07	98.67%
75	0.530	0.675	8.56E+07	9.82E+07	114.73%
77	0.633	0.766	9.49E+07	1.05E+08	110.48%
78	0.650	0.660	9.63E+07	9.71E+07	100.83%
80	0.739	0.765	1.03E+08	1.05E+08	101.75%
81	0.562	0.682	8.87E+07	9.88E+07	111.38%
83	0.648	0.689	9.61E+07	9.93E+07	103.33%
84	0.611	0.419	9.30E+07	7.34E+07	78.86%
86	0.516	0.703	8.42E+07	1.00E+08	119.14%
87	0.685	0.722	9.90E+07	1.02E+08	102.77%

89	0.240	0.282	4.43E+07	5.27E+07	118.97%
90	0.240	0.339	4.43E+07	6.23E+07	140.63%
92	0.297	0.365	5.54E+07	6.62E+07	119.39%
93	0.419	0.431	7.34E+07	7.48E+07	102.01%
95	0.772	0.882	1.05E+08	1.12E+08	106.60%
96	0.551	0.684	8.76E+07	9.89E+07	112.86%
98	0.605	0.679	9.25E+07	9.85E+07	106.50%
99	0.575	0.747	8.99E+07	1.04E+08	115.18%
101	0.748	0.730	1.04E+08	1.02E+08	98.77%
102	0.603	0.705	9.23E+07	1.00E+08	108.82%
104	0.646	0.665	9.59E+07	9.74E+07	101.58%
105	0.654	0.740	9.66E+07	1.03E+08	106.67%
107	0.635	2.110	9.50E+07	1.58E+08	165.87%
108	0.645	0.574	9.59E+07	8.98E+07	93.66%
110	0.559	0.659	8.84E+07	9.70E+07	109.71%
111	0.618	0.718	9.36E+07	1.01E+08	108.35%
113	0.617	0.707	9.35E+07	1.01E+08	107.59%
114	0.682	0.797	9.88E+07	1.07E+08	108.23%
116	0.613	0.706	9.32E+07	1.01E+08	107.90%
117	0.746	0.805	1.03E+08	1.07E+08	103.84%
119	0.614	0.693	9.33E+07	9.96E+07	106.76%
120	0.599	0.750	9.20E+07	1.04E+08	112.74%
122	0.580	0.817	9.03E+07	1.08E+08	119.78%
123	0.626	0.705	9.43E+07	1.00E+08	106.57%
125	0.694	0.773	9.97E+07	1.05E+08	105.64%
126	0.680	0.843	9.86E+07	1.10E+08	111.36%
128	0.751	0.848	1.04E+08	1.10E+08	106.10%
129	0.690	0.816	9.94E+07	1.08E+08	108.80%
131	0.703	0.875	1.00E+08	1.12E+08	111.37%
132	0.705	0.796	1.00E+08	1.07E+08	106.30%
134	0.718	0.816	1.01E+08	1.08E+08	106.58%
135	0.705	0.836	1.00E+08	1.09E+08	108.84%
137	0.680	0.782	9.86E+07	1.06E+08	107.39%
138	0.679	0.826	9.85E+07	1.09E+08	110.37%
140	0.724	0.867	1.02E+08	1.11E+08	109.22%
141	0.800	0.989	1.07E+08	1.18E+08	110.33%
143	0.668	0.813	9.77E+07	1.08E+08	110.48%
144	0.781	0.883	1.06E+08	1.12E+08	106.05%
146	0.642	0.740	9.56E+07	1.03E+08	107.75%
147	0.708	0.822	1.01E+08	1.08E+08	107.73%
149	0.653	0.793	9.65E+07	1.07E+08	110.49%
150	0.749	0.849	1.04E+08	1.10E+08	106.30%

152	0.707	0.838	1.01E+08	1.10E+08	108.81%
153	0.779	0.911	1.06E+08	1.14E+08	107.72%

### Ratio of Day 7/ Day 5 Cell Density of High Salt Treatment

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.797	0.823	1.07E+08	1.09E+08	101.57%
72	0.880	0.949	1.12E+08	1.16E+08	103.51%
74	0.777	0.716	1.06E+08	1.01E+08	95.96%
75	0.595	0.754	9.16E+07	1.04E+08	113.47%
77	0.706	0.782	1.01E+08	1.06E+08	105.30%
78	0.683	0.781	9.88E+07	1.06E+08	107.07%
80	0.707	0.745	1.01E+08	1.03E+08	102.71%
81	0.761	0.913	1.04E+08	1.14E+08	109.09%
83	0.650	0.652	9.63E+07	9.64E+07	100.17%
84	0.619	0.728	9.37E+07	1.02E+08	109.02%
86	0.597	0.605	9.18E+07	9.25E+07	100.76%
87	0.543	0.626	8.69E+07	9.43E+07	108.54%
89	0.154	0.259	2.12E+07	4.83E+07	227.98%
90	0.186	0.350	3.10E+07	6.40E+07	206.25%
92	0.399	0.655	7.08E+07	9.67E+07	136.49%
93	0.400	0.509	7.09E+07	8.35E+07	117.71%
95	0.506	0.563	8.32E+07	8.88E+07	106.69%
96	0.431	0.568	7.48E+07	8.92E+07	119.23%
98	0.635	0.728	9.50E+07	1.02E+08	107.50%
99	0.535	0.734	8.61E+07	1.03E+08	119.15%
101	0.586	0.630	9.09E+07	9.46E+07	104.15%
102	0.546	0.664	8.72E+07	9.74E+07	111.70%
104	0.599	0.606	9.20E+07	9.26E+07	100.66%
105	0.595	0.697	9.16E+07	9.99E+07	109.00%
107	0.646	1.967	9.59E+07	1.54E+08	160.51%
108	0.613	0.493	9.32E+07	8.18E+07	87.79%
110	0.550	0.614	8.75E+07	9.33E+07	106.56%
111	0.590	0.664	9.12E+07	9.74E+07	106.75%
113	0.537	0.625	8.63E+07	9.42E+07	109.17%
114	0.643	0.774	9.57E+07	1.05E+08	110.10%
116	0.567	0.663	8.91E+07	9.73E+07	109.15%
117	0.706	0.797	1.01E+08	1.07E+08	106.29%
119	0.500	0.607	8.26E+07	9.27E+07	112.24%
120	0.686	0.752	9.91E+07	1.04E+08	104.83%
122	0.554	0.711	8.79E+07	1.01E+08	114.79%

123	0.610	0.604	9.29E+07	9.24E+07	99.45%
125	0.696	0.740	9.98E+07	1.03E+08	103.20%
126	0.620	0.797	9.38E+07	1.07E+08	113.96%
128	0.689	0.782	9.93E+07	1.06E+08	106.65%
129	0.675	0.800	9.82E+07	1.07E+08	109.02%
131	0.655	0.778	9.67E+07	1.06E+08	109.28%
132	0.682	0.755	9.88E+07	1.04E+08	105.37%
134	0.668	0.771	9.77E+07	1.05E+08	107.65%
135	0.696	0.833	9.98E+07	1.09E+08	109.38%
137	0.695	0.825	9.97E+07	1.09E+08	108.96%
138	0.734	0.839	1.03E+08	1.10E+08	106.79%
140	0.671	0.783	9.79E+07	1.06E+08	108.22%
141	0.771	0.915	1.05E+08	1.14E+08	108.49%
143	0.638	0.772	9.53E+07	1.05E+08	110.43%
144	0.741	0.802	1.03E+08	1.07E+08	104.00%
146	0.659	0.757	9.70E+07	1.04E+08	107.45%
147	0.749	0.849	1.04E+08	1.10E+08	106.30%
149	0.704	0.853	1.00E+08	1.10E+08	109.97%
150	0.754	0.879	1.04E+08	1.12E+08	107.69%
152	0.715	0.766	1.01E+08	1.05E+08	103.55%
153	0.708	0.808	1.01E+08	1.08E+08	106.84%

#### Ratio of Day 7/ Day 5 Cell Density of Low Salt Treatment

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.576	0.716	9.00E+07	1.01E+08	112.61%
72	0.896	0.898	1.13E+08	1.13E+08	100.10%
74	0.586	0.628	9.09E+07	9.45E+07	103.97%
75	0.501	0.654	8.27E+07	9.66E+07	116.80%
77	0.736	0.850	1.03E+08	1.10E+08	107.31%
78	0.494	0.658	8.20E+07	9.69E+07	118.24%
80	0.770	0.823	1.05E+08	1.09E+08	103.30%
81	0.557	0.774	8.82E+07	1.05E+08	119.45%
83	0.623	0.676	9.40E+07	9.83E+07	104.53%
84	0.596	0.560	9.17E+07	8.85E+07	96.46%
86	0.532	0.594	8.58E+07	9.16E+07	106.70%
87	0.541	0.639	8.67E+07	9.54E+07	110.01%
89	0.203	0.221	3.56E+07	4.00E+07	112.45%
90	0.231	0.323	4.23E+07	5.98E+07	141.30%
92	0.331	0.383	6.11E+07	6.87E+07	112.46%
93	0.452	0.515	7.73E+07	8.41E+07	108.80%

95	0.570	0.677	8.94E+07	9.84E+07	110.03%
96	0.594	0.769	9.16E+07	1.05E+08	114.70%
98	0.625	0.666	9.42E+07	9.75E+07	103.52%
99	0.595	0.760	9.16E+07	1.04E+08	113.92%
101	0.678	0.740	9.85E+07	1.03E+08	104.63%
102	0.691	0.773	9.94E+07	1.05E+08	105.88%
104	0.659	0.718	9.70E+07	1.01E+08	104.61%
105	0.754	0.822	1.04E+08	1.08E+08	104.33%
107	0.631	2.313	9.47E+07	1.62E+08	171.51%
108	0.681	0.537	9.87E+07	8.63E+07	87.43%
110	0.590	0.664	9.12E+07	9.74E+07	106.75%
111	0.623	0.713	9.40E+07	1.01E+08	107.48%
113	0.635	0.673	9.50E+07	9.81E+07	103.19%
114	0.705	0.782	1.00E+08	1.06E+08	105.38%
116	0.648	0.707	9.61E+07	1.01E+08	104.73%
117	0.748	0.807	1.04E+08	1.08E+08	103.82%
119	0.623	0.725	9.40E+07	1.02E+08	108.41%
120	0.685	0.791	9.90E+07	1.06E+08	107.58%
122	0.592	0.714	9.14E+07	1.01E+08	110.69%
123	0.635	0.725	9.50E+07	1.02E+08	107.27%
125	0.741	0.833	1.03E+08	1.09E+08	105.92%
126	0.696	0.878	9.98E+07	1.12E+08	112.13%
128	0.758	0.859	1.04E+08	1.11E+08	106.25%
129	0.743	0.920	1.03E+08	1.14E+08	110.79%
131	0.774	0.848	1.05E+08	1.10E+08	104.52%
132	0.712	0.842	1.01E+08	1.10E+08	108.66%
134	0.702	0.858	1.00E+08	1.11E+08	110.43%
135	0.721	0.880	1.02E+08	1.12E+08	110.22%
137	0.690	0.860	9.94E+07	1.11E+08	111.56%
138	0.704	0.811	1.00E+08	1.08E+08	107.35%
140	0.738	0.893	1.03E+08	1.13E+08	109.66%
141	1.103	1.184	1.24E+08	1.28E+08	102.98%
143	0.634	0.809	9.50E+07	1.08E+08	113.38%
144	0.739	0.824	1.03E+08	1.09E+08	105.51%
146	0.686	0.869	9.91E+07	1.11E+08	112.44%
147	0.741	0.883	1.03E+08	1.12E+08	108.87%
149	0.643	0.815	9.57E+07	1.08E+08	112.91%
150	0.697	0.854	9.99E+07	1.10E+08	110.60%
152	0.730	0.893	1.02E+08	1.13E+08	110.27%
153	0.777	0.931	1.06E+08	1.15E+08	108.93%

**Ratio of Day 7/ Day 5 Cell Density of High Combination Treatment**

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.538	0.633	8.64E+07	9.48E+07	109.72%
72	0.596	0.654	9.17E+07	9.65E+07	105.19%
74	0.696	0.705	9.98E+07	1.00E+08	100.61%
75	0.522	0.667	8.48E+07	9.75E+07	114.98%
77	0.776	0.813	1.05E+08	1.08E+08	102.27%
78	0.600	0.678	9.21E+07	9.84E+07	106.84%
80	0.595	0.657	9.16E+07	9.67E+07	105.56%
81	0.573	0.677	8.97E+07	9.83E+07	109.62%
83	0.571	0.633	8.95E+07	9.48E+07	105.91%
84	0.632	0.414	9.48E+07	7.26E+07	76.57%
86	0.516	0.669	8.42E+07	9.77E+07	115.99%
87	0.522	0.645	8.48E+07	9.58E+07	112.91%
89	0.235	0.336	4.32E+07	6.17E+07	142.68%
90	0.264	0.444	4.93E+07	7.62E+07	154.70%
92	0.474	0.481	7.98E+07	8.04E+07	100.79%
93	0.475	0.603	7.99E+07	9.23E+07	115.45%
95	0.713	0.965	1.01E+08	1.17E+08	115.60%
96	0.517	0.656	8.43E+07	9.67E+07	114.63%
98	0.579	0.634	9.02E+07	9.49E+07	105.15%
99	0.537	0.693	8.63E+07	9.95E+07	115.33%
101	0.645	0.689	9.59E+07	9.92E+07	103.52%
102	0.632	0.717	9.48E+07	1.01E+08	106.88%
104	0.627	0.660	9.44E+07	9.70E+07	102.75%
105	0.580	0.672	9.03E+07	9.79E+07	108.42%
107	0.591	2.049	9.13E+07	1.56E+08	171.14%
108	0.599	0.538	9.20E+07	8.63E+07	93.76%
110	0.621	0.886	9.39E+07	1.12E+08	119.71%
111	0.560	0.664	8.85E+07	9.73E+07	109.95%
113	0.516	0.609	8.42E+07	9.28E+07	110.15%
114	0.630	0.714	9.46E+07	1.01E+08	106.83%
116	0.551	0.653	8.76E+07	9.64E+07	110.02%
117	0.622	0.707	9.40E+07	1.01E+08	107.04%
119	0.586	0.691	9.09E+07	9.94E+07	109.39%
120	0.705	0.687	1.00E+08	9.91E+07	98.59%
122	0.502	0.653	8.28E+07	9.64E+07	116.47%
123	0.582	0.638	9.05E+07	9.52E+07	105.20%
125	0.623	0.693	9.40E+07	9.95E+07	105.83%
126	0.606	0.769	9.26E+07	1.05E+08	113.36%
128	0.622	0.716	9.40E+07	1.01E+08	107.75%

129	0.410	0.772	7.22E+07	1.05E+08	145.61%
131	0.621	0.724	9.39E+07	1.02E+08	108.46%
132	0.663	0.730	9.73E+07	1.02E+08	105.10%
134	0.616	0.714	9.35E+07	1.01E+08	108.17%
135	0.645	0.803	9.59E+07	1.07E+08	111.88%
137	0.532	0.639	8.58E+07	9.53E+07	111.04%
138	0.744	0.705	1.03E+08	1.00E+08	97.22%
140	0.507	0.577	8.33E+07	8.99E+07	107.98%
141	0.576	0.633	9.00E+07	9.48E+07	105.38%
143	0.849	0.859	1.10E+08	1.11E+08	100.53%
144	0.685	0.774	9.90E+07	1.05E+08	106.39%
146	0.594	0.767	9.16E+07	1.05E+08	114.50%
147	0.748	0.837	1.04E+08	1.09E+08	105.63%
149	0.657	0.817	9.68E+07	1.08E+08	111.70%
150	0.672	0.829	9.80E+07	1.09E+08	111.14%
152	0.661	0.715	9.71E+07	1.01E+08	104.15%
153	0.678	0.761	9.85E+07	1.04E+08	106.07%

#### Ratio of Day 7/ Day 5 Cell Density of Low Combination Treatment

Passage	OD at Day 5	OD at Day 7	Cell Density at Day 5 (Cells/mL)	Cell Density at Day 7 (Cells/mL)	Ratio of Day 7, Day 5 Cell Density
71	0.646	0.673	9.59E+07	9.81E+07	102.23%
72	0.623	0.364	9.40E+07	6.60E+07	70.21%
74	0.765	0.764	1.05E+08	1.05E+08	99.93%
75	0.572	0.705	8.96E+07	1.00E+08	112.17%
77	0.869	0.970	1.11E+08	1.17E+08	105.15%
78	0.740	0.823	1.03E+08	1.09E+08	105.38%
80	0.682	0.796	9.88E+07	1.07E+08	108.16%
81	0.581	0.775	9.04E+07	1.05E+08	116.62%
83	0.640	0.701	9.55E+07	1.00E+08	104.97%
84	0.695	0.478	9.97E+07	8.02E+07	80.44%
86	0.551	0.628	8.76E+07	9.45E+07	107.78%
87	0.556	0.663	8.81E+07	9.73E+07	110.41%
89	0.263	0.316	4.91E+07	5.87E+07	119.50%
90	0.247	0.359	4.58E+07	6.53E+07	142.56%
92	0.370	0.427	6.69E+07	7.44E+07	111.17%
93	0.427	0.499	7.44E+07	8.25E+07	110.93%
95	0.534	0.603	8.60E+07	9.23E+07	107.37%
96	1.036	0.979	1.21E+08	1.18E+08	97.55%
98	0.617	2.081	9.35E+07	1.57E+08	167.76%
99	0.841	0.928	1.10E+08	1.15E+08	104.68%

101	0.633	0.699	9.49E+07	1.00E+08	105.45%
102	0.595	0.787	9.16E+07	1.06E+08	115.91%
104	0.644	0.711	9.58E+07	1.01E+08	105.39%
105	0.645	0.736	9.59E+07	1.03E+08	107.18%
107	0.585	2.234	9.08E+07	1.61E+08	176.97%
108	0.625	0.554	9.42E+07	8.79E+07	93.35%
110	0.551	0.650	8.76E+07	9.63E+07	109.83%
111	0.657	0.710	9.68E+07	1.01E+08	104.18%
113	0.554	0.646	8.79E+07	9.59E+07	109.11%
114	0.674	0.769	9.81E+07	1.05E+08	107.00%
116	0.630	0.682	9.46E+07	9.88E+07	104.37%
117	0.729	0.823	1.02E+08	1.09E+08	106.18%
119	0.621	0.724	9.39E+07	1.02E+08	108.52%
120	0.675	0.829	9.82E+07	1.09E+08	110.91%
122	0.588	0.722	9.10E+07	1.02E+08	111.76%
123	0.651	0.756	9.63E+07	1.04E+08	108.09%
125	0.728	0.795	1.02E+08	1.07E+08	104.49%
126	0.676	0.886	9.83E+07	1.12E+08	114.35%
128	0.733	0.655	1.03E+08	9.67E+07	94.28%
129	0.674	1.004	9.81E+07	1.19E+08	121.17%
131	0.708	0.848	1.01E+08	1.10E+08	109.34%
132	0.719	0.837	1.02E+08	1.09E+08	107.80%
134	0.705	0.833	1.00E+08	1.09E+08	108.66%
135	1.245	1.215	1.30E+08	1.29E+08	99.02%
137	0.659	0.825	9.70E+07	1.09E+08	112.08%
138	0.688	0.789	9.92E+07	1.06E+08	107.20%
140	0.714	0.838	1.01E+08	1.10E+08	108.25%
141	0.756	0.940	1.04E+08	1.15E+08	110.91%
143	0.623	0.790	9.40E+07	1.06E+08	113.17%
144	0.673	0.752	9.81E+07	1.04E+08	105.90%
146	0.640	0.801	9.55E+07	1.07E+08	112.26%
147	0.743	0.880	1.03E+08	1.12E+08	108.55%
149	0.630	0.785	9.46E+07	1.06E+08	112.12%
150	0.689	0.806	9.93E+07	1.07E+08	108.24%
152	0.690	0.824	9.94E+07	1.09E+08	109.31%
153	0.756	0.869	1.04E+08	1.11E+08	106.97%

## Appendix D – Swap Treatments

This is the tabulation of the generation time of swap experiment. On every 5<sup>th</sup> to 7<sup>th</sup> passage, OD600 readings were taken regularly after subculture to monitor the log phase. This measures the time taken for 1 generation to take place which is used to estimate the adaptability of the cells in the media. Below are the geometric means of the generation time for every swap experiment.

### Experiment 1: Low Salt to Different treatments

Swap Count	Generation Time in Minutes (Geometric Mean) of each Treatment					
	H MSG	L MSG	H BA	L BA	H COMB	L COMB
1 (15/4)	68.34	71.43	91.15	64.09	77.50	86.80
2 (30/4)	1704.76	762.39	123.58	640.03	70.76	287.54
3 (14/5)	68.90	70.54	68.42	68.13	58.46	66.10
4 (28/5)	42.63	27.83	29.83	38.04	33.33	26.22
5 (11/6)	80.71	76.42	69.03	68.49	322.97	89.75
6 (25/6)	64.82	75.08	63.62	78.08	58.40	120.22
7 (9/7)	81.95	85.47	30.99	71.79	44.74	86.61
8 (23/7)	75.91	81.88	73.71	70.77	104.30	69.27
9 (6/8)	103.95	116.22	112.09	120.14	115.91	113.22
10 (20/8)	82.23	80.74	82.14	85.52	75.88	74.15
11(3/9)	47.83	45.28	50.84	42.28	22.45	49.70
12(17/9)	71.40	66.29	68.28	67.91	64.07	79.01
13(1/10)	145.15	125.02	153.60	88.02	144.94	133.32
14(15/10)	78.67	67.72	68.77	65.29	63.69	68.06

### Experiment 2: High to Low, Low to High

Swap Count	Generation Time in Minutes (Geometric Mean) of each Treatment							
	H MSG Cells to L MSG	L MSG Cells to H MSG	H BA Cells to L BA	L BA Cells to H BA	H Salt Cells to L Salt	L Salt Cells to H Salt	H Comb Cells to L Comb	L Comb Cells to H Comb
1 (15/4)	73.12	81.17	64.61	170.33	71.51	86.74	64.09	73.27
2 (30/4)	756.94	1224.14	556.85	37.42	239.59	4809.81	22.22	234.91
3 (14/5)	58.95	66.89	44.89	67.58	54.46	129.02	43.65	60.76
4 (28/5)	52.39	30.59	30.42	67.25	38.18	44.19	36.86	51.68
5 (11/6)	69.77	72.10	68.25	83.99	73.87	64.71	64.23	56.67
6 (25/6)	83.09	82.04	83.82	64.80	85.79	59.59	75.32	100.20
7 (9/7)	69.55	81.98	68.67	103.90	72.97	67.57	87.82	103.88
8 (23/7)	77.57	77.27	103.28	80.65	56.80	60.13	68.41	54.44
9 (6/8)	118.08	117.26	137.02	122.15	105.94	88.60	123.61	113.29
10 (20/8)	78.52	98.01	83.65	87.55	91.15	66.09	76.56	78.79

### Experiment 3: High Single Treatments to High Combination Treatment

Swap Count	Generation Time in Minutes (Geometric Mean) of each Treatment		
	H MSG Cells to High Comb Media	H BA Cells to High Comb Media	High Salt Cells to High Comb Media
1 (15/4)	97.79	-	57.88
2 (30/4)	60.00	34.54	53.40
3 (14/5)	63.46	74.49	67.18
4 (28/5)	29.21	43.68	51.13
5 (11/6)	90.51	93.47	76.72
6 (25/6)	76.40	83.78	94.06
7 (9/7)	56.67	79.26	88.38
8 (23/7)	61.78	51.91	53.20
9 (6/8)	188.01	178.67	91.13
10 (20/8)	77.20	75.00	62.78
11(3/9)	51.81	51.10	58.02
12(17/9)	75.81	70.15	61.66
13(1/10)	375.05	159.76	192.21
14(15/10)	88.02	61.85	69.98

### Experiment 4: Low Single Treatments to Low Combination Treatment

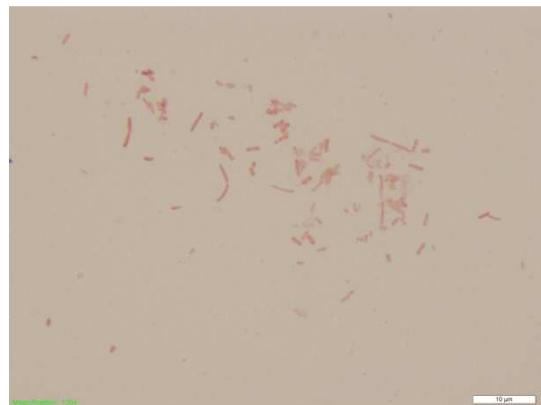
Swap Count	Generation Time in Minutes (Geometric Mean) of each Treatment		
	L MSG Cells to Low Comb Media	L BA Cells to Low Comb Media	L Salt Cells to Low Comb Media
1 (15/4)	376.00	63.02	47.71
2 (30/4)	60.38	397.31	380.68
3 (14/5)	18.20	67.62	62.41
4 (28/5)	87.72	50.84	42.78
5 (11/6)	77.60	94.69	86.30
6 (25/6)	94.94	72.49	89.94
7 (9/7)	65.36	77.91	72.79
8 (23/7)	124.37	75.96	59.17
9 (6/8)	89.11	118.83	111.39
10 (20/8)	56.61	156.19	83.57
11(3/9)	90.07	47.68	57.31
12(17/9)	156.12	97.48	93.04
13(1/10)	70.90	130.02	140.04
14(15/10)	376.00	72.15	67.30

## Appendix E – Gram Staining Pictures

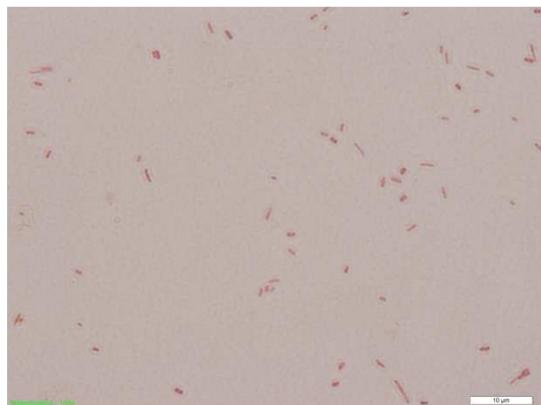
Contamination check is conducted by Gram staining to ensure that only Gram negative bacteria are grown in the media. Below are the Gram staining pictures on passage 145.



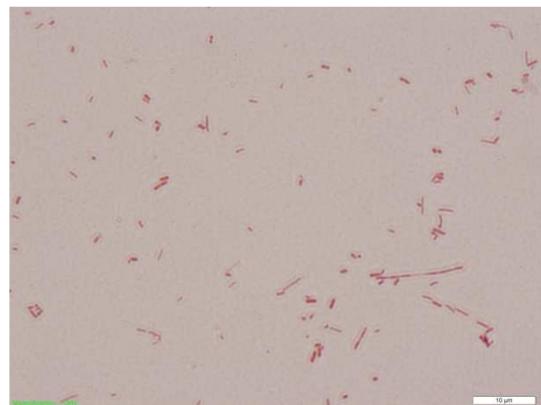
H MSG



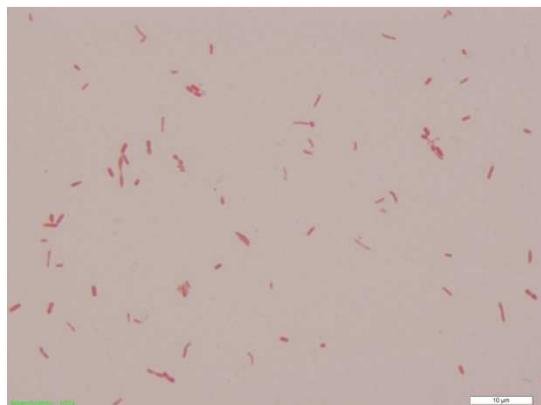
L MSG



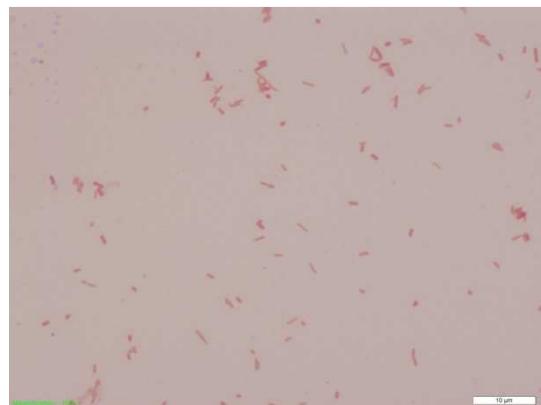
H BA



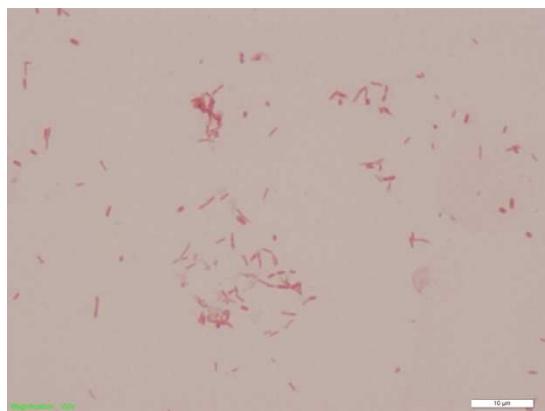
L BA



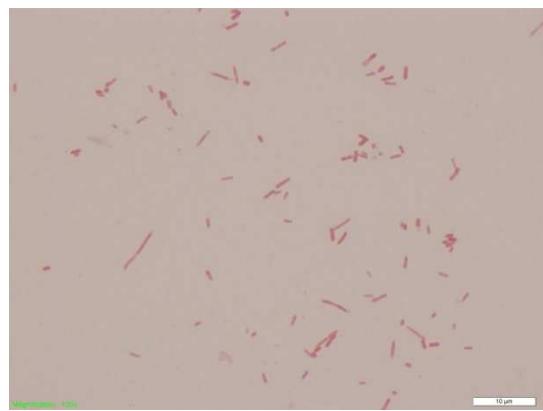
H SALT



L SALT



H COMB



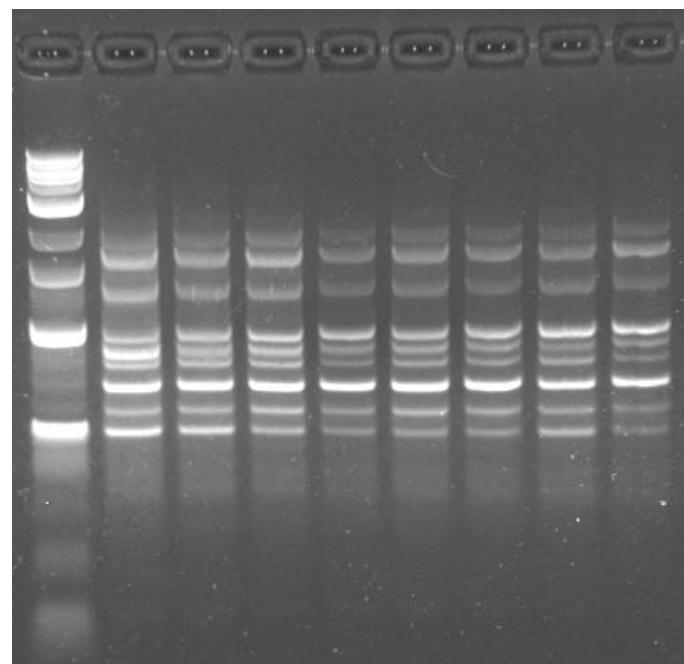
L COMB

## Appendix F – Agarose Gel Electrophoresis of PCR-RFLP

For every 12<sup>th</sup> passage, genetic works were conducted on the eight treatment cells. Below shows the gel pictures of *E. coli* on every 12<sup>th</sup> passage.

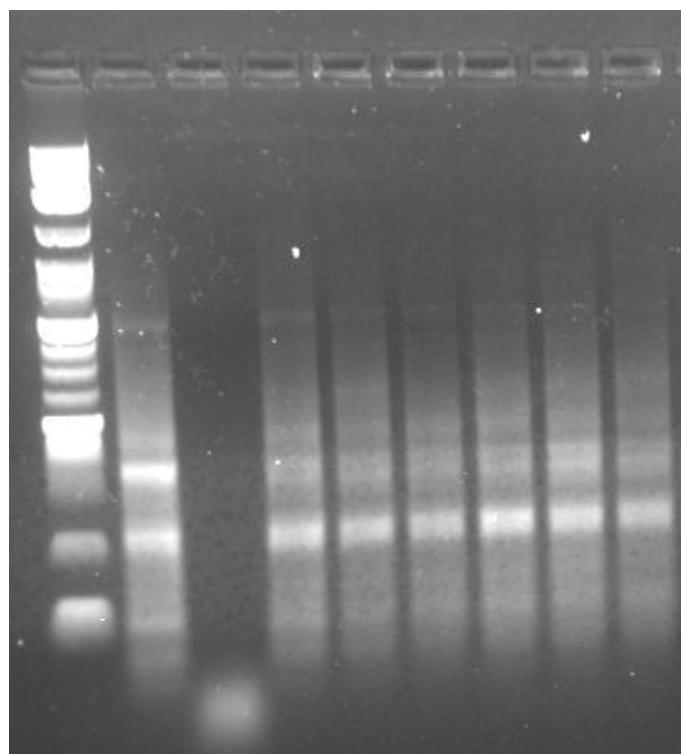
### Passage 82

Agarose Gel 1: Passage 82 PCR, Primer 5



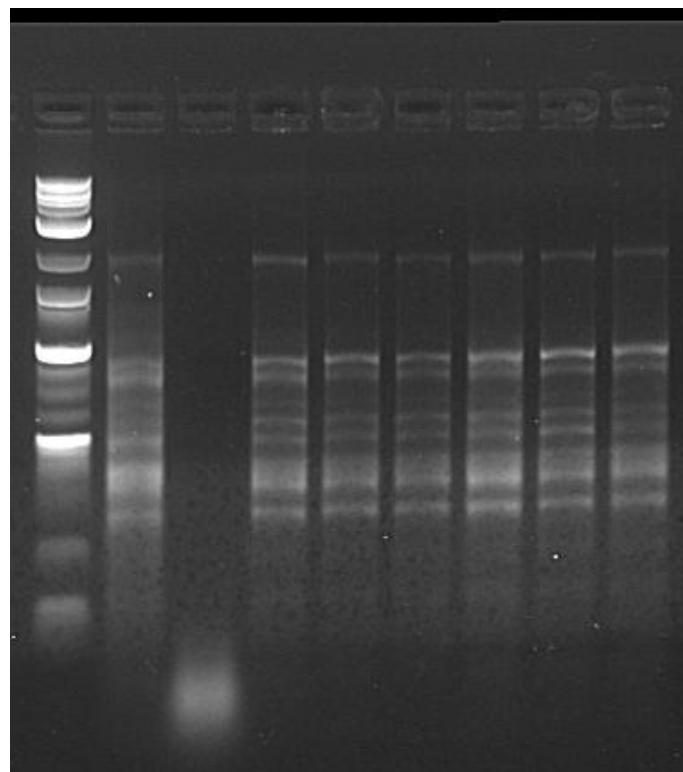
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	19	23	23	23	23	23	23	23	23
1000	24	26	26	26	26	26	26	26	26
900	31	30	30	30	30	30	30	30	30
600	45	35	35	35	35	35	35	35	35
500	48	37	37	37	37	37	37	37	37
		39	39	39	39	39	39	39	39
		42	42	42	42	42	42	42	42
		45	45	45	45	45	45	45	45
		48	48	48	48	48	48	48	48

Agarose Gel 2: Passage 82 PCR product, Primer 5, digested by MspI



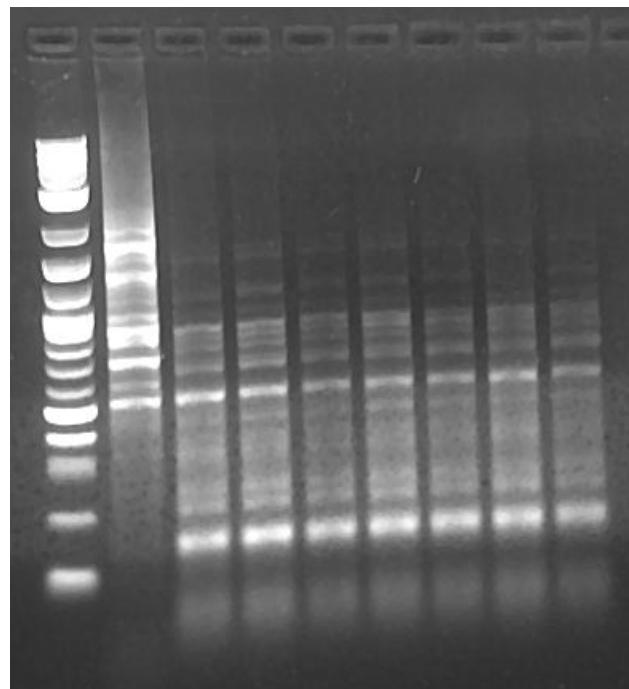
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	17	32		32	32	32	32	32	32
700	39			40	40		40	40	40
600	42	46		46	46	46	46	46	46
500	45	52		52	52	52	52	52	52
200	63	60		60	60	60	60	60	60
		70		70	70	70	70	70	70

Agarose Gel 3: Passage 82 PCR product, Primer 5, digested by *HinfI*



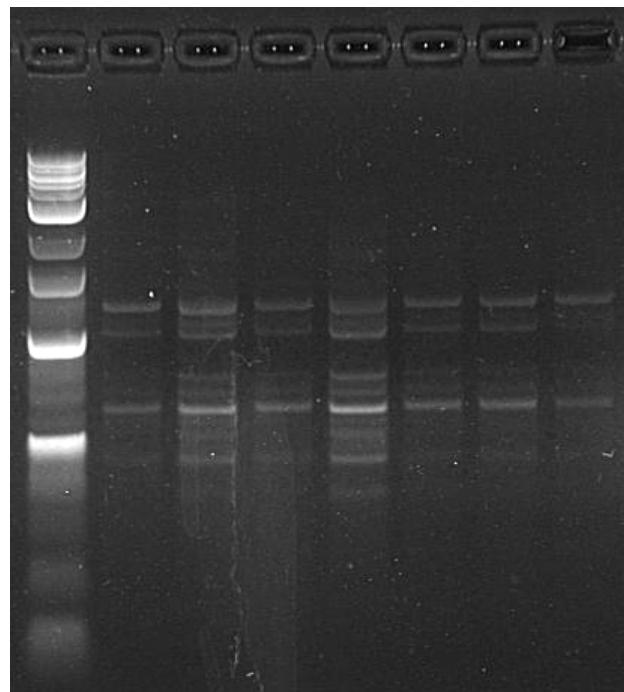
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	14	18	30	18	18	18	18	18	18
1000	30	31		31	31	31	31	31	31
900	32	33		33	33	33	33	33	33
600	34	36		36	36	36	36	36	36
500	42	39		39	39	39	39	39	39
		48		48	48	48	48	48	48
		52		52	52	52	52	52	52

Agarose Gel 4: Passage 82 PCR product, Primer 5, digested by TaqI



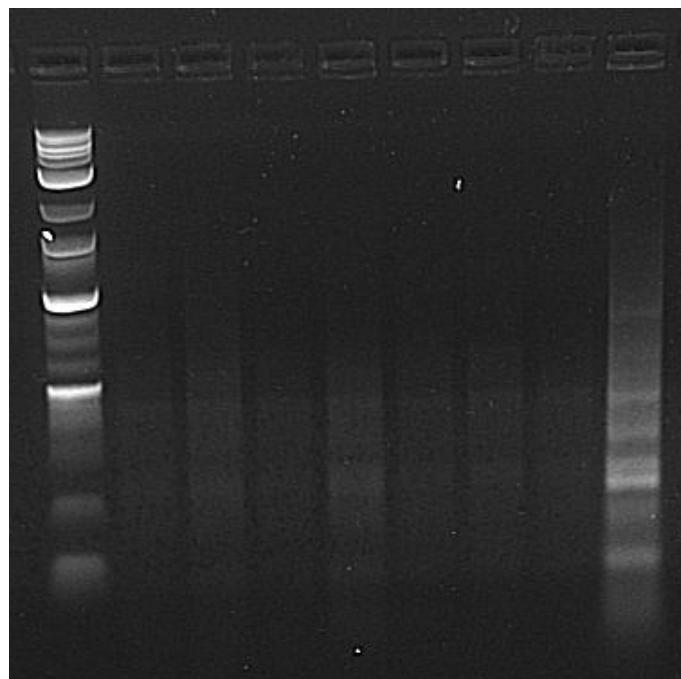
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	21	23							
1500	30	26							
1000	36		28	28	28	28	28	28	28
900	38	30							
500	49	32.5	32.5	32.5	32.5	32.5	32.5	32.5	32.5
		35	35	35	35	35	35	35	35
		37	37	37	37	37	37	37	37
		38	38	38	38	38	38	38	38
			39.5	39.5	39.5	39.5	39.5	39.5	39.5
		44							
			42	42	42	42	42	42	42
			47	47	47	47	47	47	47
			49	49	49	49	49	49	49
			53	53	53	53		53	
				58	58	58	58	58	58
				61	61	61	61	61	61
				65	65	65	65	65	65

Agarose Gel 5: Passage 82 PCR, Primer 6



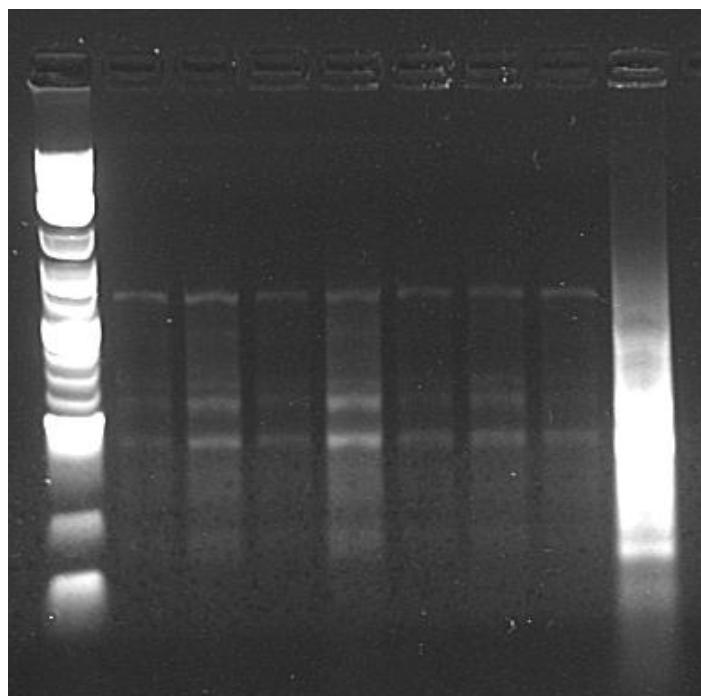
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	20	33	33	33	33	33	33	33	33
1000	25		34	34	34	34	34		
900	37	36	36	36	36				
600	46		41	41	41				
500	50	46	46	46	46	46	46	46	46
			52		52				52
			53	53	53				53
			58		58				58

Agarose Gel 6: Passage 82 PCR product, Primer 6, digested by MspI



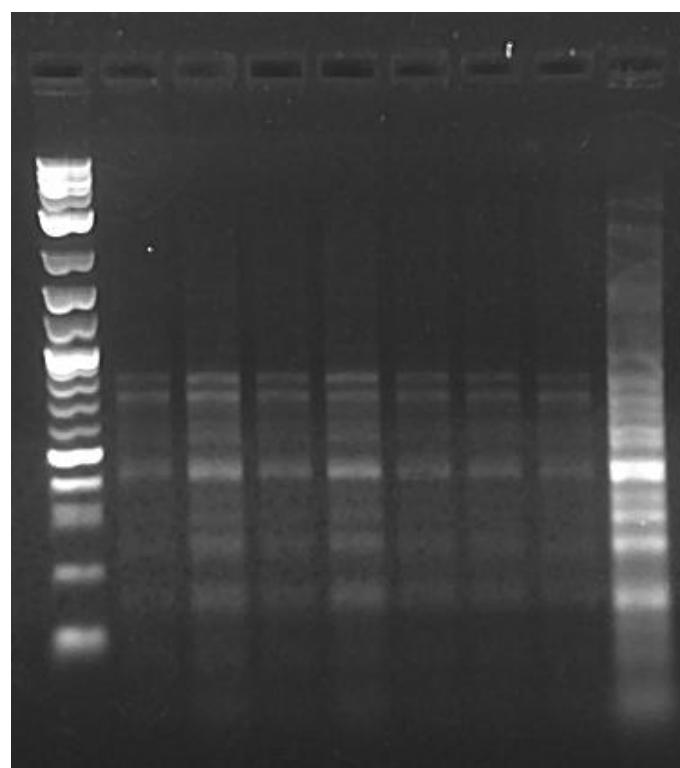
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	14		38		38				
1000	30	43	43	43	43	43	43	43	43
700	35	55	55	55	55	55	55	55	55
500	41	67	67	67	67	67	67	67	67
200	57								

Agarose Gel 7: Passage 82 PCR product, Primer 6, digested by HinfI



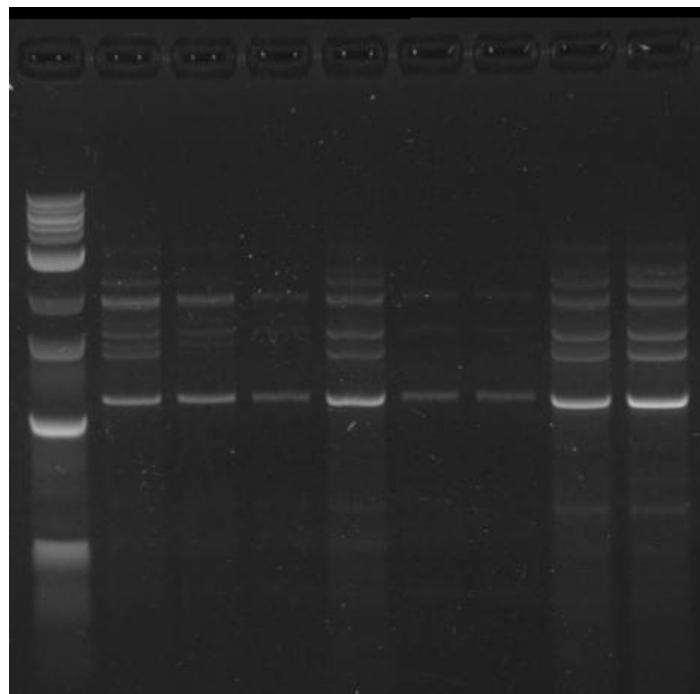
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	16	28	28	28	28	28	28	28	28
2000	21				32				
1000	32		38		38		38	38	
600	41	42	42	42	42	42	42	42	
500	43	44	44	44	44	44	44	44	

Agarose Gel 8: Passage 82 PCR product, Primer 6, digested by TaqI



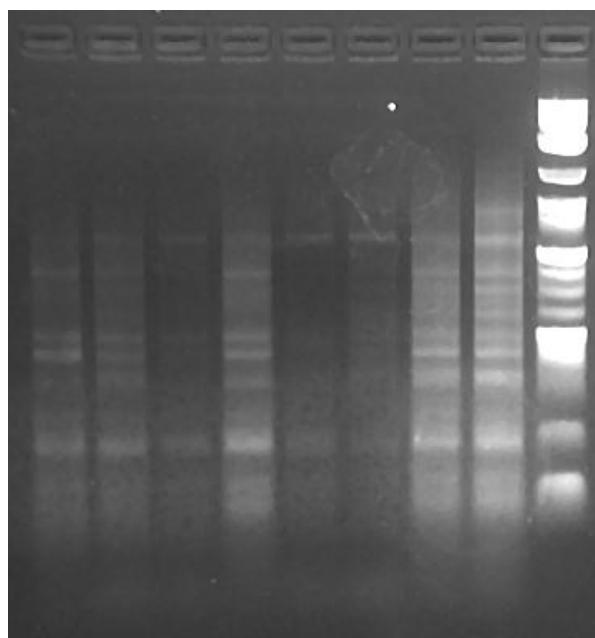
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	20	39	39	39	39	39	39	39	39
2000	25	41.5	41.5	41.5	41.5	41.5	41.5	41.5	
1500	30								37
1200	34								43
500	50								45
		47	47	47	47	47			47
									48.5
		51.5	51.5	51.5	51.5	51.5	51.5	51.5	51.5
			55.5		55.5				55.5
			58	58	58				58
		62	62	62	62	62	62	62	62
		69	69	69	69	69	69	69	69
									73.5
									77

Agarose Gel 9: Passage 82 PCR, Primer 7



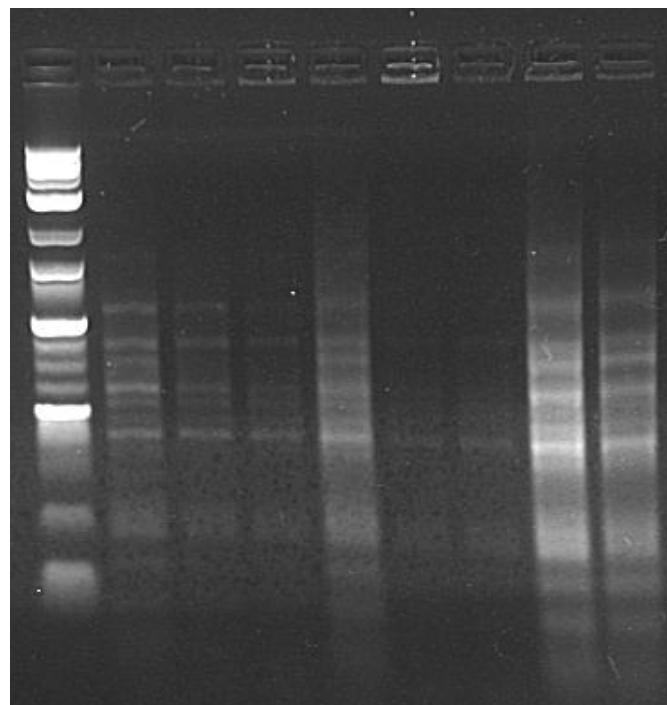
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	19	23			23			23	23	
1000	20	30	30	30	30	30	30	30	30	
900	25	35	35	35	35	35	35	35	35	
600	37	37			37			37	37	
500	47	49	49	49	49	49	49	49	49	

Agarose Gel 10: Passage 82 PCR product, Primer 7, digested by MspI



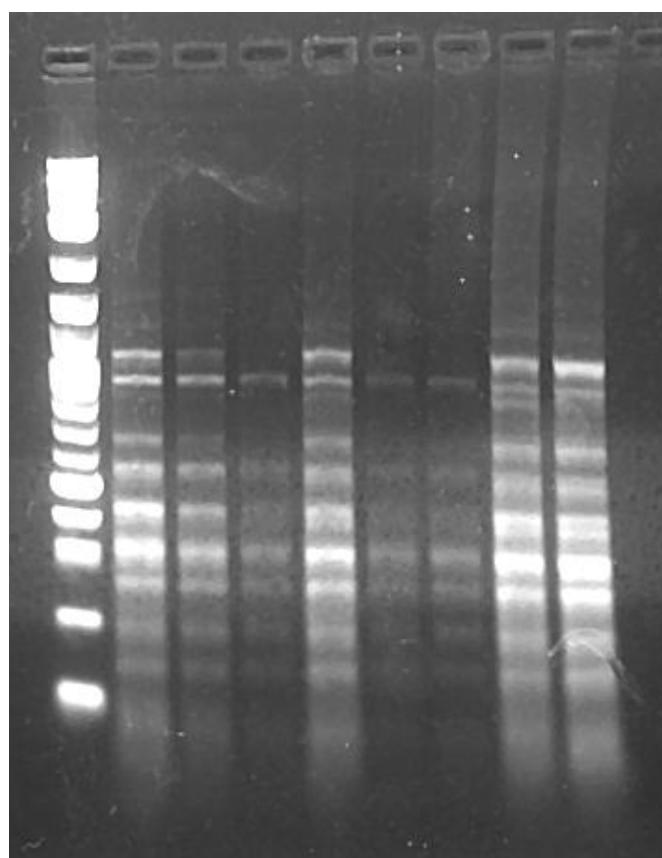
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
1000	27								20
600	34	24	24	24	24	24	24	24	24
500	37	28	28	28		28	28	28	28
200	51		30				30	30	30
100	59		33					33	33
		36	36	36	36			36	36
		39	39		39			39	39
		42	42		42			42	42
		51	51	51	51	51	51	51	51
		56	56	56	56	56	56	56	56
		59	59	59	59			59	59

Agarose Gel 11: Passage 82 PCR product, Primer 7, digested by *HinfI*



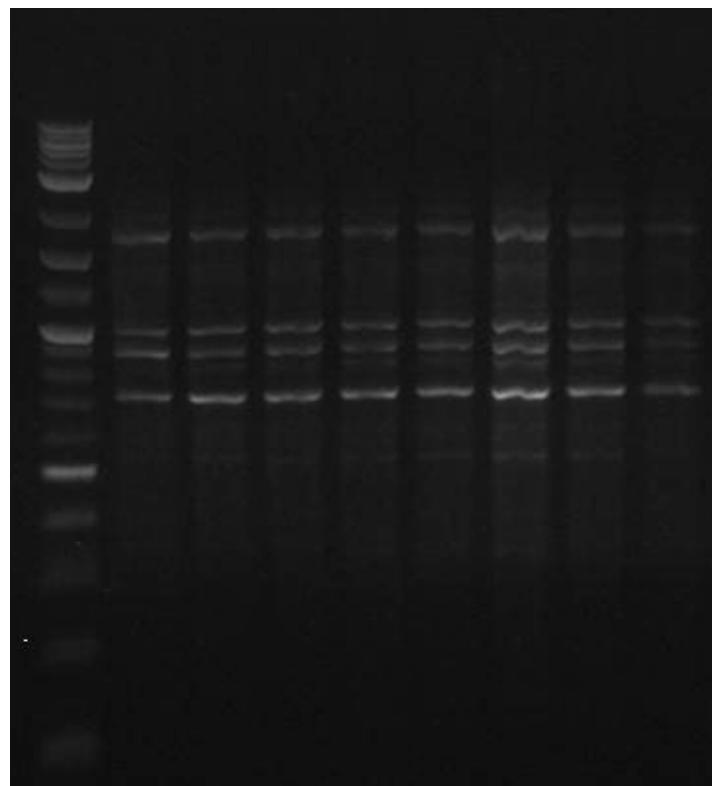
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	17				23			23	23
2000	22	29	29					29	29
1000	34	34	34	34	34			34	34
600	42	36			36			36	36
500	45	41	41	41	41			41	41
		46	46	46	46			46	46
		48	48	48	48	48	48	48	48
		65	65	65	65	65	65	65	65
		68			68			68	68

Agarose Gel 12: Passage 82 PCR product, Primer 7, digested by TaqI



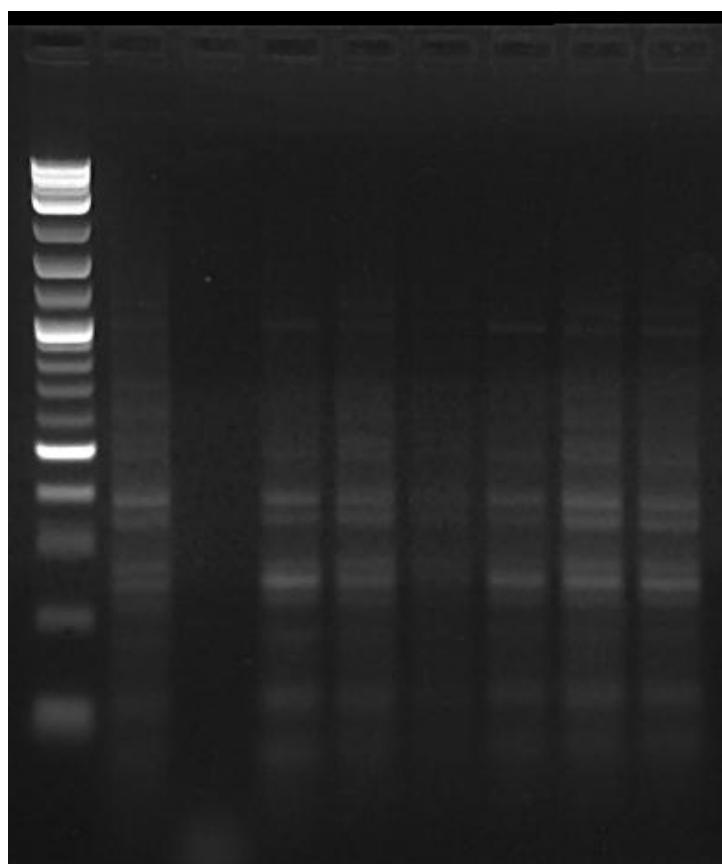
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	27	30	30					30	30
1500	32.5	34	34		34			34	34
900	46	37	37		37			37	37
700	52	41	41	41	41	41	41	41	41
500	60	42.5	42.5		42.5			42.5	42.5
		49	49		49			49	49
		53	53	53	53	53	53	53	53
		59	59	59	59	59	59	59	59
				61					
		65	65	65	65	65	65	65	65
		68.5	68.5	68.5	68.5	68.5	68.5	68.5	68.5
		75	75	75	75	75	75	75	75
		80	80	80	80	80	80	80	80

Agarose Gel 13: Passage 94 PCR, Primer 5



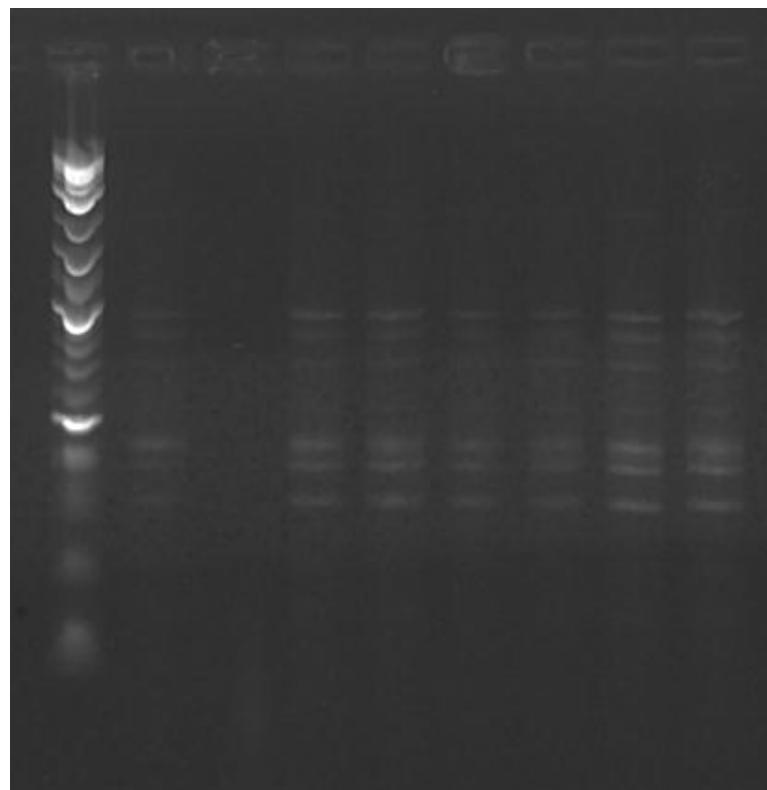
		Migration Distance (mm)								
Log 2 Marker		Samples								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	20						23			
1000	25	28	28	28	28	28	28	28	28	
900	40	39	39	39	39	39	39	39	39	
600	55	41	41	41	41	41	41	41	41	
500	58						43	43		
		47	47	47	47	47	47	47	47	
						55	55	55		

Agarose Gel 14: Passage 94 PCR product, Primer 5, digested by MspI



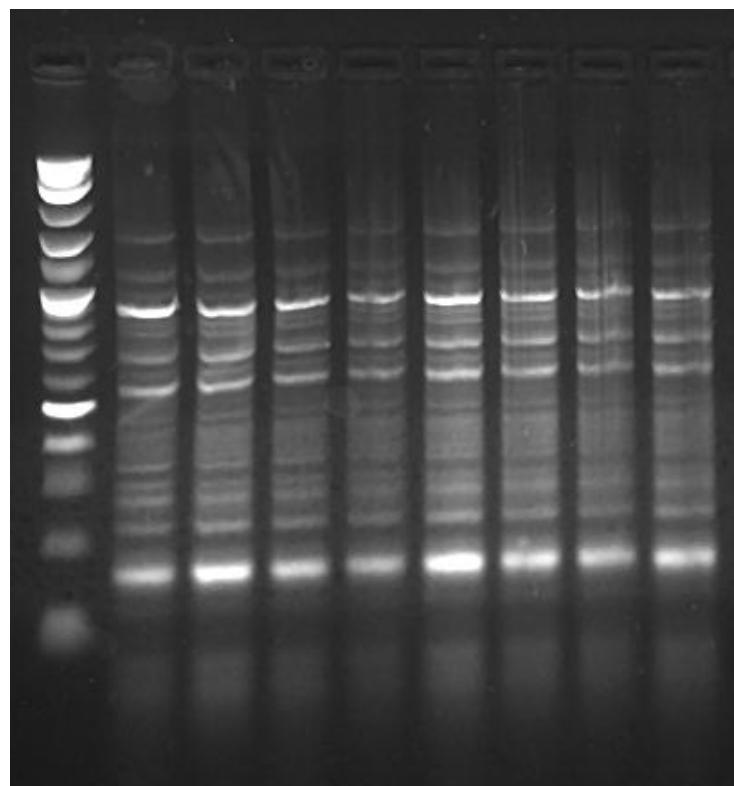
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	20	33			33	33		33	33
1500	29	36		36	36	36	36	36	36
1200	33	45						45	
800	41	49						49	
500	53	53			53	53	53	53	53
		59			59	59	59	59	59
		62			62	62	62	62	62
		68			68	68	68	68	68
		70			70	70	70	70	70
		73			73	73		73	73
		78			78	78		78	78
		85			85	85	85	85	85
		93			93	93	93	93	93

Agarose Gel 15: Passage 94 PCR product, Primer 5, digested by *HinfI*



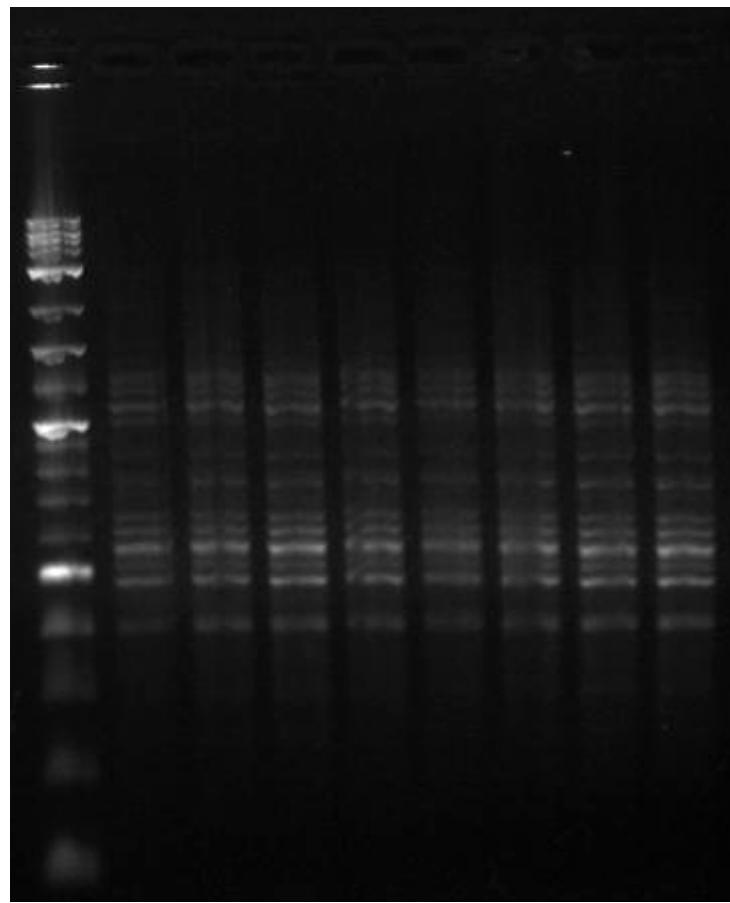
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	19	33		33	33	33	33	33	33
2000	23	36		36	36	36	36	36	36
1000	35	40		40	40	40	40	40	40
600	44	41		41	41	41	41	41	41
500	48							45	45
		50		50	50	50	50	50	50
		53		53	53	53	53	53	53
		58		58	58	58	58	58	58

Agarose Gel 16: Passage 94 PCR product, Primer 5, digested by TaqI



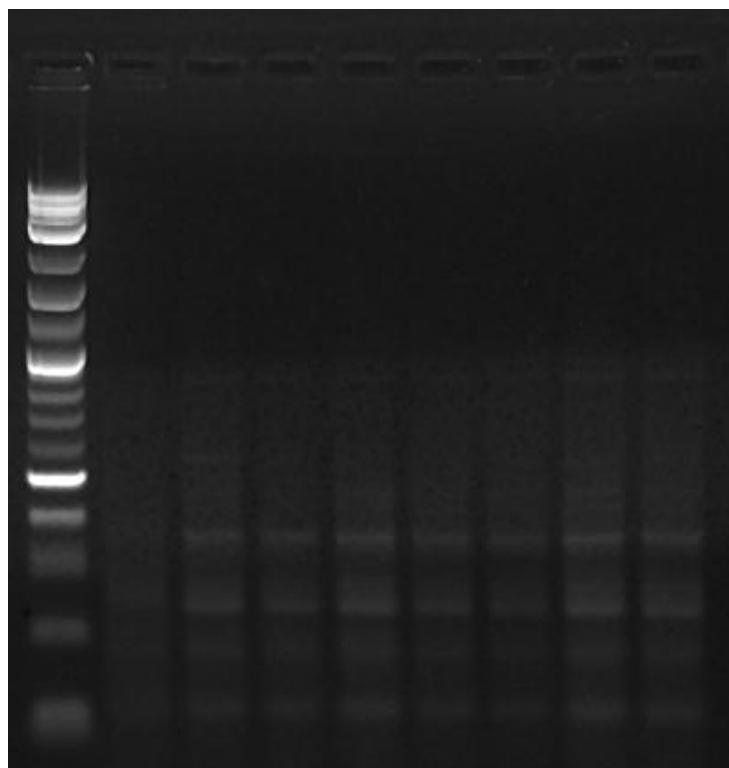
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	20	22	22	22	22	22	22	22	22
1500	24	27	27	27	27	27	27	27	27
800	33.5	29	29	29	29	29	29	29	29
500	45	31.5	31.5	31.5	31.5	31.5	31.5	31.5	31.5
400	49.5	33	33	33	33	33	33	33	33
		34	34	34	34	34	34	34	34
		38	38	38	38	38	38	38	38
		40	40	40	40	40	40	40	40
		42	42	42	42	42	42	42	42
		46	46	46	46	46	46	46	46
		47.5	47.5	47.5	47.5	47.5	47.5	47.5	47.5
		48	48	48	48	48	48	48	48
		50	50	50	50	50	50	50	50
		51.5	51.5	51.5	51.5	51.5	51.5	51.5	51.5
		54.5	54.5	54.5	54.5	54.5	54.5	54.5	54.5
		56	56	56	56	56	56	56	56
		66.5	66.5	66.5	66.5	66.5	66.5	66.5	66.5

Agarose Gel 17: Passage 94 PCR, Primer 6



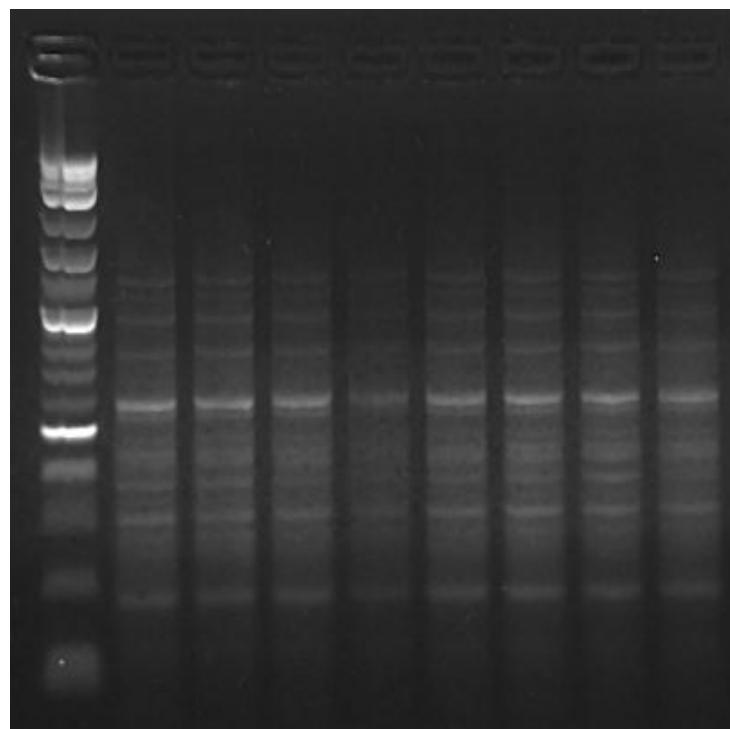
		Migration Distance (mm)								
Log 2 Marker		Samples								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	27	40	40	40	40	40	40	40	40	
1000	32	42	42	42	42	42	42	42	42	
900	46	44	44	44	44	44	44	44	44	
600	61	46	46	46	46	46	46	46	46	
500	66	53	53	53	53	53	53	53	53	
		58	58	58	58	58	58	58	58	
		60	60	60	60	60	60	60	60	
		62	62	62	62	62	62	62	62	
		64	64	64	64	64	64	64	64	
		66	66	66	66	66	66	66	66	
		72	72	72	72	72	72	72	72	

Agarose Gel 18: Passage 94 PCR product, Primer 6, digested by MspI



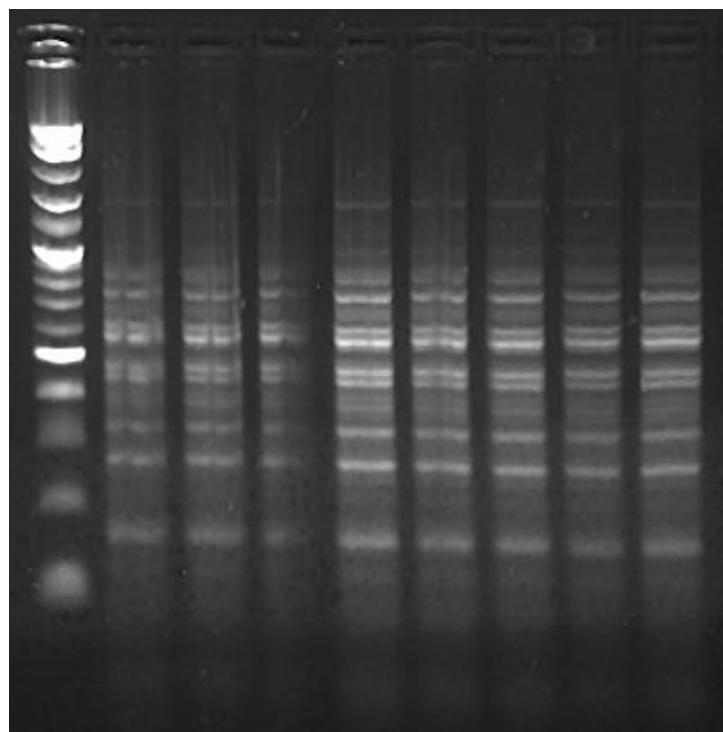
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	21	40	40	40	40	40	40	40	40
2000	25	50		50	50	50	50	50	50
1200	33	55		55	55		55	55	55
800	42	57		57	57		57	57	57
500	53	62	62	62	62	62	62	62	62
400	58	71	71	71	71	71	71	71	71
		77	77	77	77	77	77	77	77
		84	84	84	84	84	84	84	84

Agarose Gel 19: Passage 94 PCR product, Primer 6, digested by HinfI



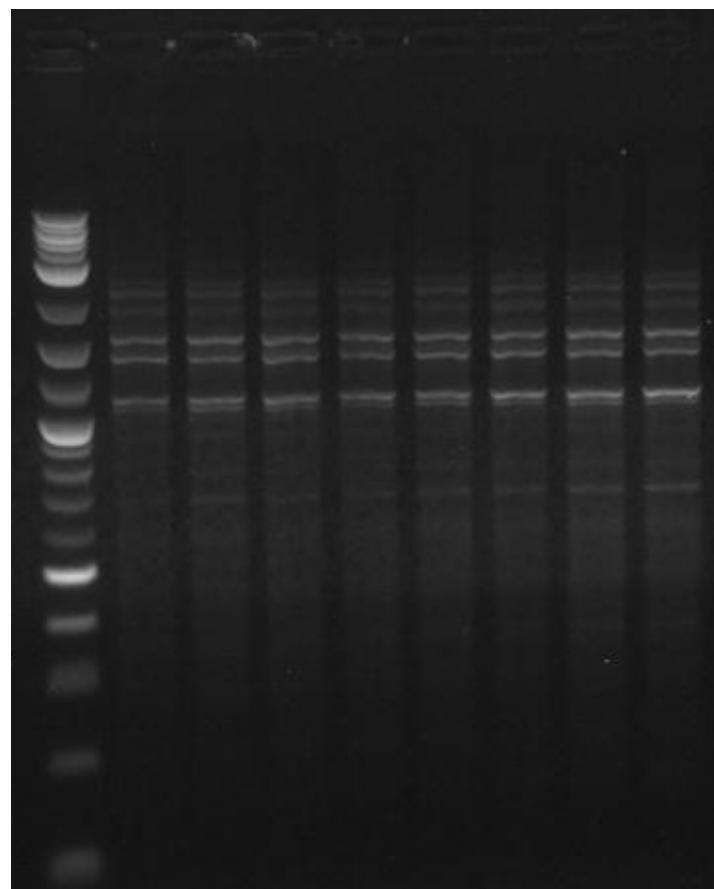
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	17					25			
2000	21	27	27	27	27	27	27	27	27
1000	34	29	29	29		29	29	29	29
600	44	31	31	31	31	31	31	31	31
500	48	36	36	36	36	36	36	36	36
		40	40	40		40	40	40	40
		43	43	43	43	43	43	43	43
		44	44	44		44	44	44	44
		47	47	47		47	47	47	47
		49	49	49		49	49	49	49
		51	51	51		51	51	51	51
		53	53			53	53	53	53
		57	57	57	57	57	57	57	57
		69	69	69	69	69	69	69	69

Agarose Gel 20: Passage 94 PCR product, Primer 6, digested by TaqI



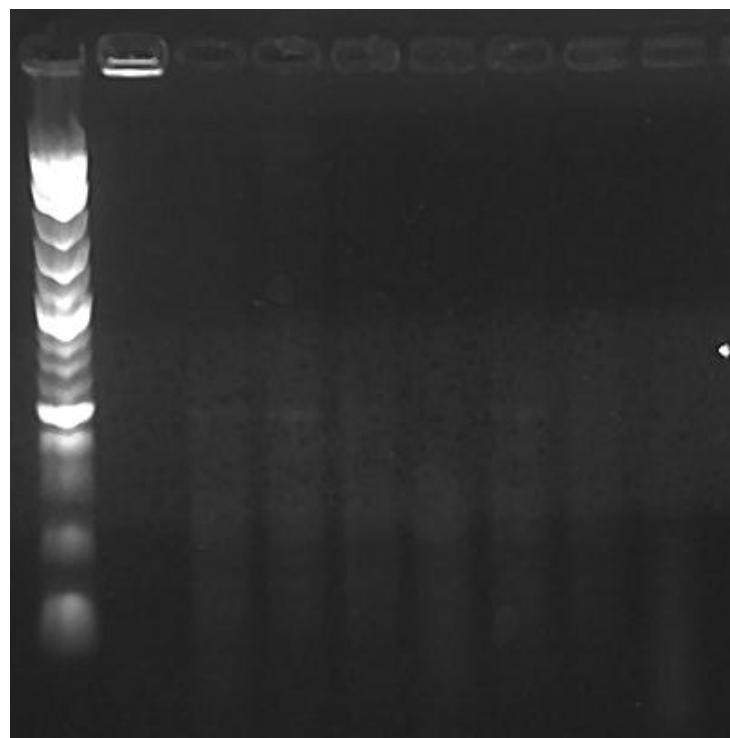
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	17	19.5	19.5	19.5	19.5	19.5	19.5	19.5	19.5
1500	21	26	26	26	26	26	26	26	26
800	31	29	29	29	29	29	29	29	29
500	41	30	30	30	30	30	30	30	30
400	45	33	33	33	33	33	33	33	33
		35	35	35	35	35	35	35	35
		36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5
		38	38	38	38	38	38	38	38
		41.5	41.5	41.5	41.5	41.5	41.5	41.5	41.5
		43	43	43	43	43	43	43	43
		45	45	45	45	45	45	45	45
		46	46	46	46	46	46	46	46
		47							
		50	50	50	50	50	50	50	50
		54	54	54	54	54	54	54	54
					56.5	56.5	56.5	56.5	56.5
					61	61	61	61	61
		65	65	65	65	65	65	65	65
		70	70	70	70	70	70	70	70

Agarose Gel 21: Passage 94 PCR, Primer 7



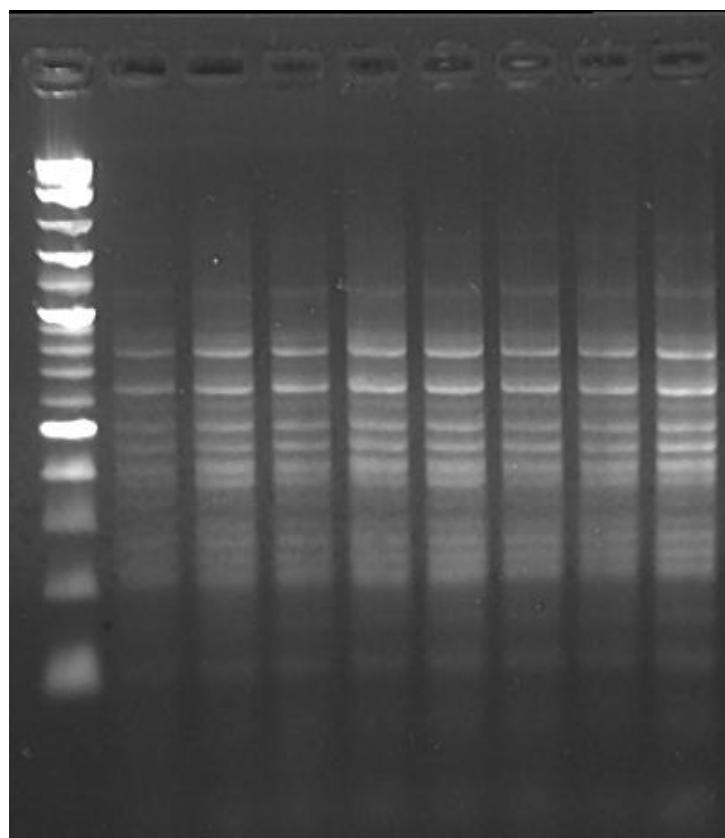
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	30	24	24	24	24	24	24	24	24
1000	35	30	30	30	30	30	30	30	30
900	51	32	32	32	32	32	32	32	32
600	65	35	35	35	35	35	35	35	35
500	70	38	38	38	38	38	38	38	38
		41	41	41	41	41	41	41	41
		46	46	46	46	46	46	46	46
		55		55		55	55	55	55
		59	59	59	59	59	59	59	59

Agarose Gel 22: Passage 94 PCR product, Primer 7, digested by MspI



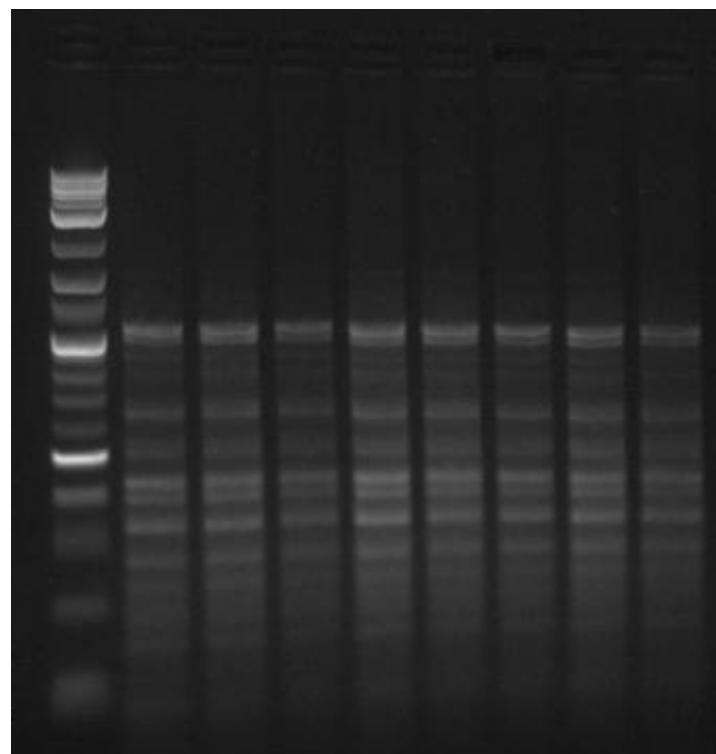
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	24		46	46			46	46	
1000	36								
800	38								
500	47								
400	50								

Agarose Gel 23: Passage 94 PCR product, Primer 7, digested by *HinfI*



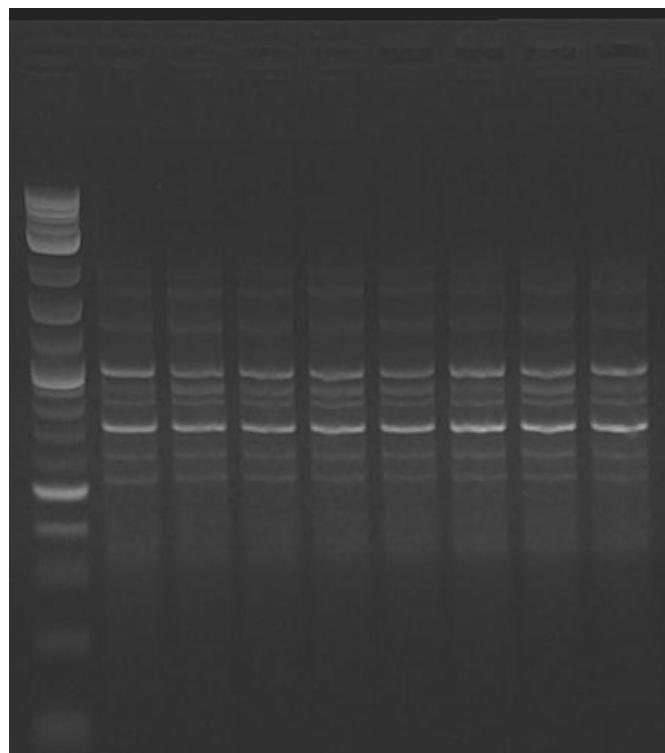
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	24				21	21	21	21	21
2000	27								24
1000	31	28	28	28	28	28	28	28	28
600	42	32	32	32	32	32	32	32	32
500	47	41	41	41	41	41	41	41	41
		43	43	43	43	43	43	43	43
		46	46	46	46	46	46	46	46
		49	49	49	49	49	49	49	49
		51	51	51	51	51	51	51	51
		53	53	53	53	53	53	53	53
		56	56	56	56	56	56	56	56
		57	57	57	57	57	57	57	57
		60	60	60	60	60	60	60	60
		63	63	63	63	63	63	63	63
		65	65	65	65	65	65	65	65

Agarose Gel 24: Passage 94 PCR product, Primer 7, digested by TaqI



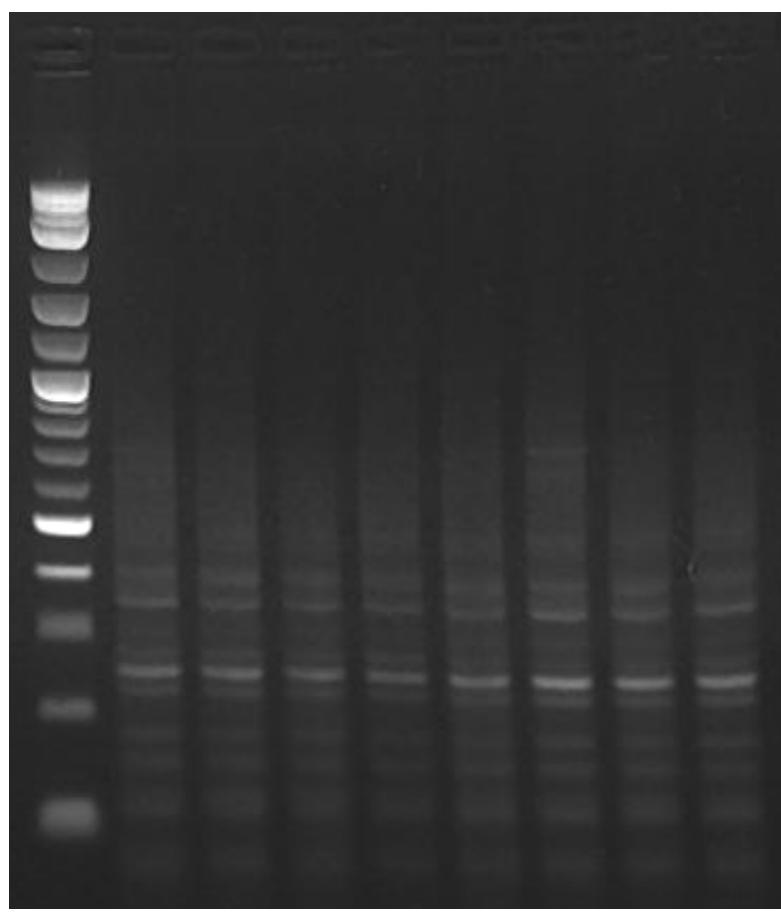
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	21	27	27	27	27	27	27	27	27
1500	29.5	35	35	35	35	35	35	35	35
700	44	36	36	36	36	36	36	36	36
500	52	38	38	38	38	38	38	38	38
400	57	40	40	40	40	40	40	40	40
		45	45	45	45	45	45	45	45
		49	49	49	49	49	49	49	49
		50	50	50	50	50	50	50	50
		53.5	53.5	53.5	53.5	53.5	53.5	53.5	53.5
		56	56	56	56	56	56	56	56
		60	60	60	60	60	60	60	60
		64	64	64	64	64	64	64	64
		67	67	67	67	67	67	67	67
		70	70	70	70	70	70	70	70
		72	72	72	72	72	72	72	72
		75	75	75	75	75	75	75	75

Agarose Gel 25: Passage 106 PCR, Primer 5



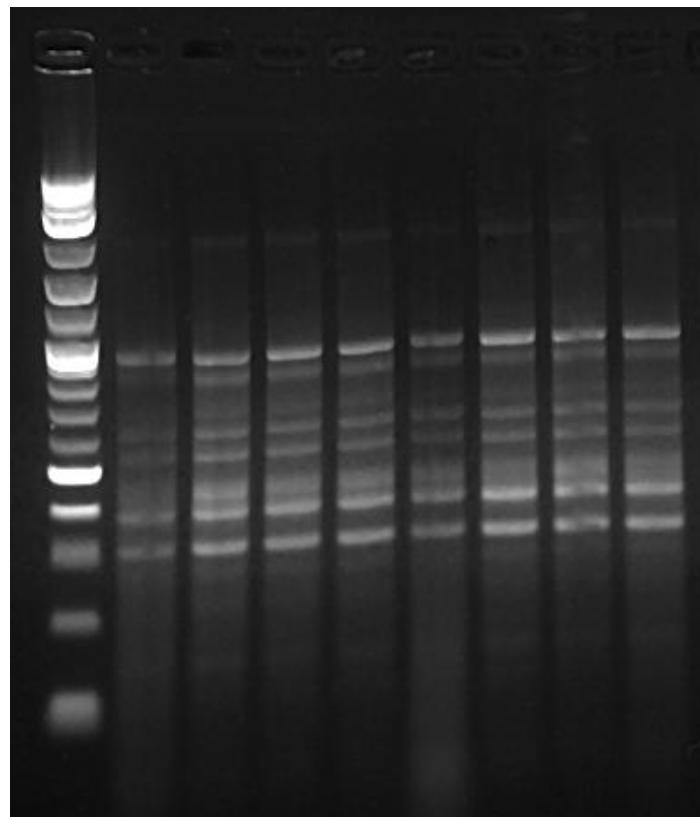
Log 2 Marker	Distance	Migration Distance (mm)							
		Samples							
Molecular Weight (bp)		H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	26	31	31	31	31	31	31	31	31
1000	30	36	36	36	36	36	36	36	36
900	44	42	42	42	42	42	42	42	42
600	54	44	44	44	44	44	44	44	44
500	58	46	46	46	46	46	46	46	46
		49	49	49	49	49	49	49	49
		53	53	53	53	53	53	53	53
		56	56	56	56	56	56	56	56

Agarose Gel 26: Passage 106 PCR product, Primer 5, digested by MspI



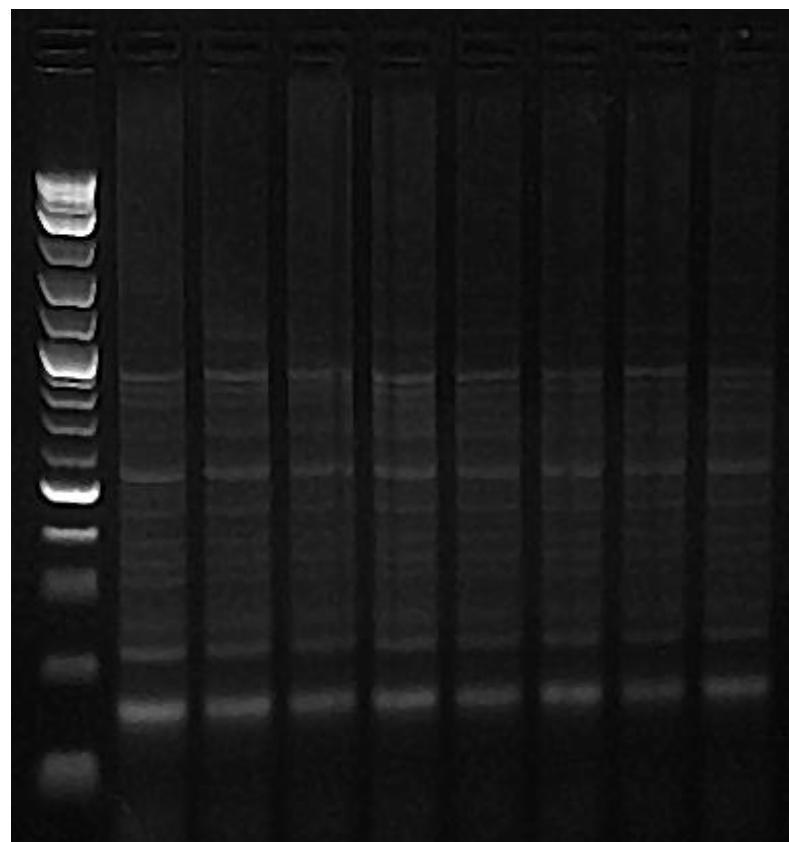
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	25		43		43	43	43	43	43
2000	29	54					54		
1500	35	56					56		
1200	39	67	67	67	67	67	67	67	67
1000	45	71	71	71	71	71	71	71	71
500	63	75	75	75	75	75	75	75	75
		78	78	78	78	78	78	78	78
		81	81	81	81	81	81	81	81
		84	84	84	84	84	84	84	84
		86	86	86	86	86	86	86	86
		92	92	92	92	92	92	92	92
		96	96	96	96	96	96	96	96
		102	102	102	102	102	102	102	102
		109	109	109	109	109	109	109	109

Agarose Gel 27: Passage 106 PCR product, Primer 5, digested by Hinfl



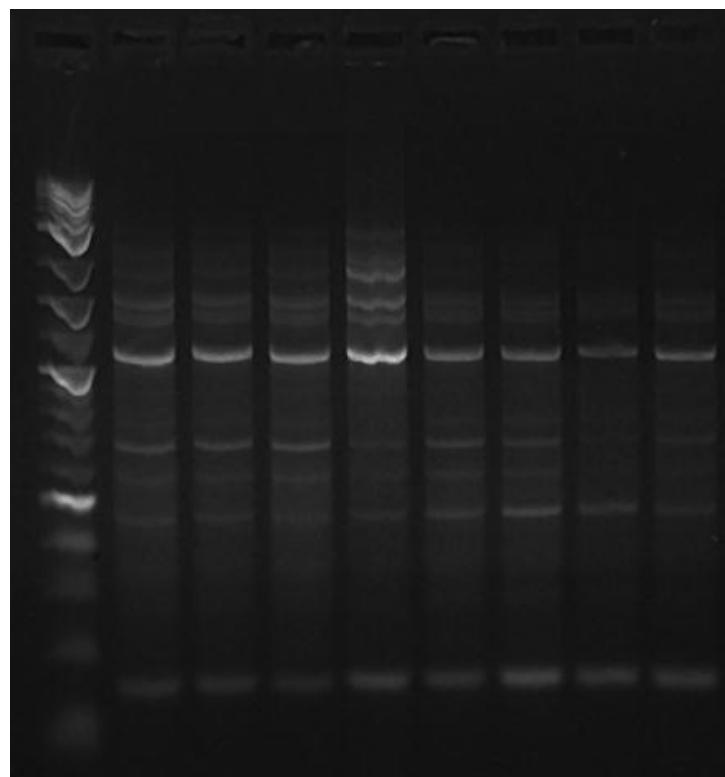
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	22	21	21	21	21	21	21	21	21
2000	26	36	36	36	36	36	36	36	36
1000	39	39	39	39	39	39	39	39	39
600	51	46	46	46	46	46	46	46	46
500	55	49	49	49	49	49	49	49	49
		57	57	57	57	57	57	57	57
		62	62	62	62	62	62	62	62

Agarose Gel 28: Passage 106 PCR product, Primer 5, digested by TaqI



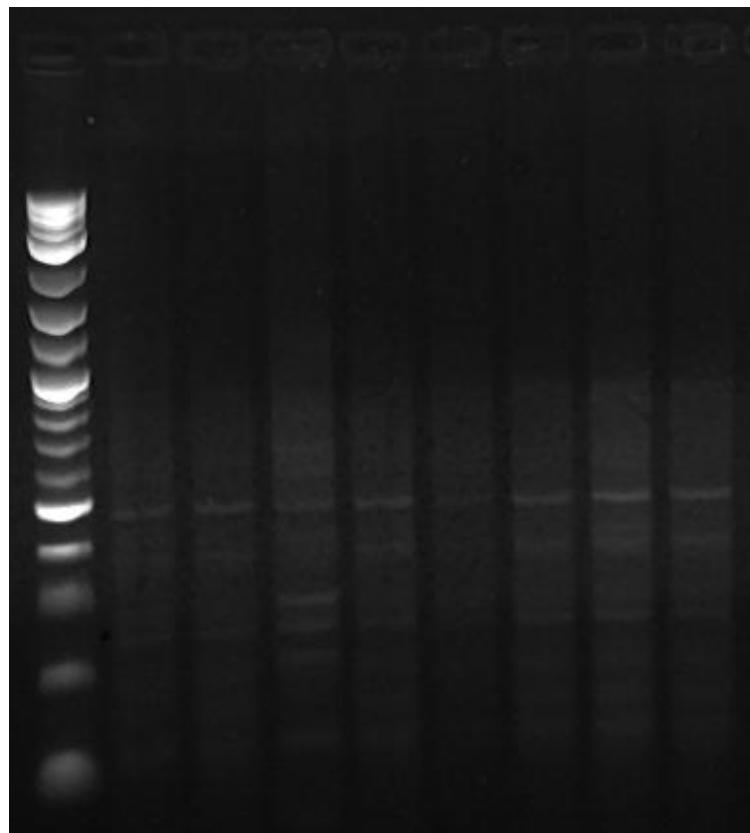
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	27	29	29	29	29	29	29	29	29
1500	32.5		36	36	36	36	36	36	36
1000	42	42	42	42	42	42	42	42	42
500	58.5	44	44	44	44	44	44	44	44
400	64	45	45	45	45	45	45	45	45
					46.5	46.5	46.5	46.5	46.5
		48.5	48.5	48.5	48.5	48.5	48.5	48.5	48.5
		55	55	55	55	55	55	55	55
		60	60	60	60	60	60	60	60
		64	64	64	64	64	64	64	64
		67	67	67	67	67	67	67	67
		69.5	69.5	69.5	69.5	69.5	69.5	69.5	69.5
		76	76	76	76	76	76	76	76
		79	79	79	79	79	79	79	79
		86.5	86.5	86.5	86.5	86.5	86.5	86.5	86.5

Agarose Gel 29: Passage 106 PCR, Primer 6



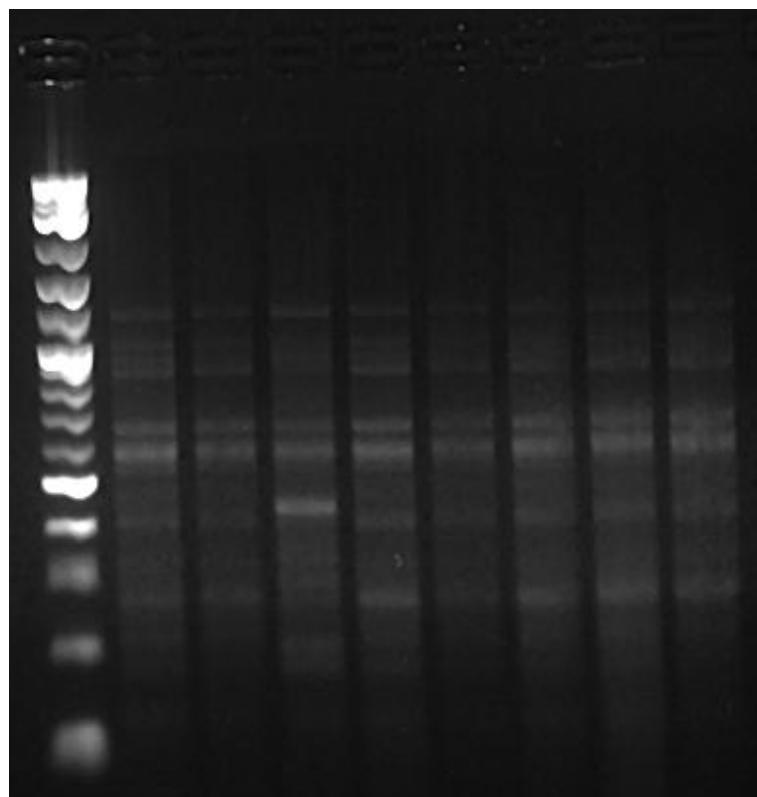
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	27				26				
1000	33				31				
900	46	35	35	35	35				
600	58	37	37	37	37	37	37	37	37
500	61	42	42	42	42	42	42	42	42
									51
		54	54	54		54	54	54	54
		58	58	58		58	58		58
		63	63	63	63	63	63	63	63
		85	85	85	85	85	85	85	85

Agarose Gel 30: Passage 106 PCR product, Primer 6, digested by MspI



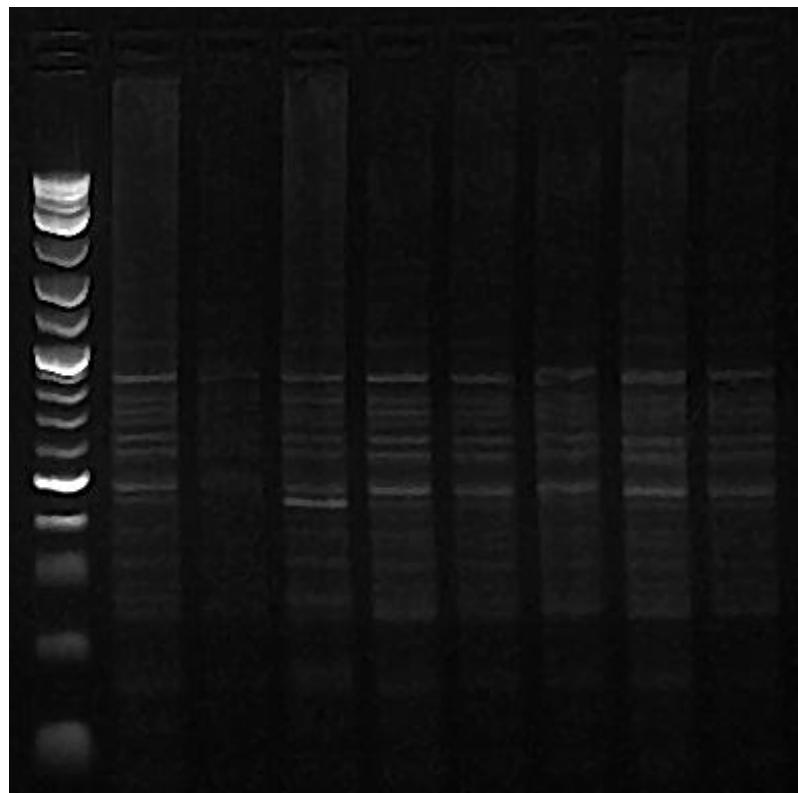
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	26	43	43	43	43	43	43	43	43
2000	30			51					
1500	35			54					
1200	40	59	59	59	59	59	59	59	59
800	48	65	65		65		65	65	65
500	60			72					
400	65	75	75	75	75	75	75	75	75
		79	79	79	79	79	79	79	79
			85		85	85	85	85	85
		91	91	91	91	91	91	91	91

Agarose Gel 31: Passage 106 PCR product, Primer 6, digested by Hinfl



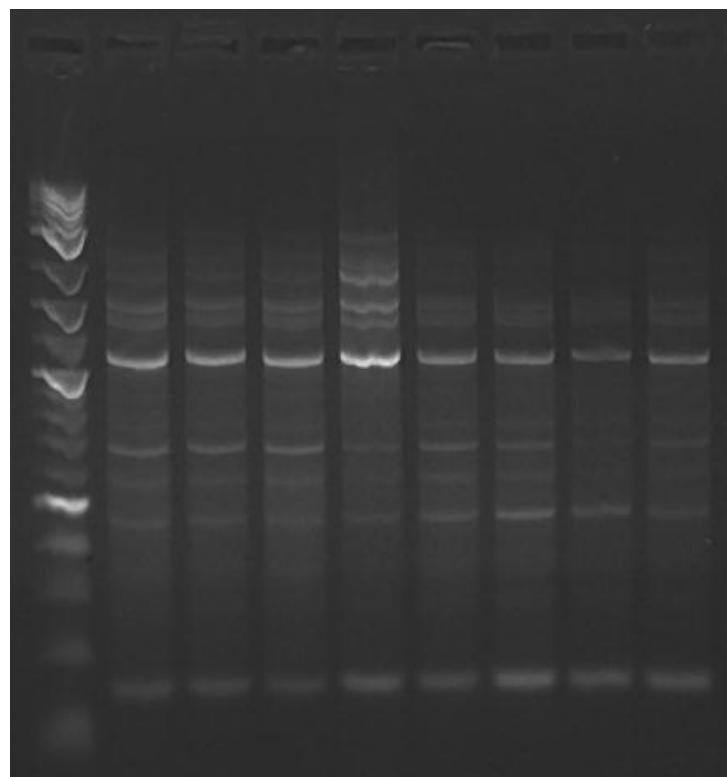
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	20	22	22	22	22	22	22	22	22
2000	24	36	36	36	36	36	36	36	36
1000	39	38	38	38	38	38	38	38	38
600	50	40	40	40	40	40	40	40	40
500	54	47	47	47	47	47	47	47	47
		50	50	50	50	50	50	50	50
		57	57	57	57	57	57	57	57
		60	60		60	60	60	60	60
		70	70	70	70	70	70	70	70
		75	75	75	75		75	75	75

Agarose Gel 32: Passage 106 PCR product, Primer 6, digested by TaqI



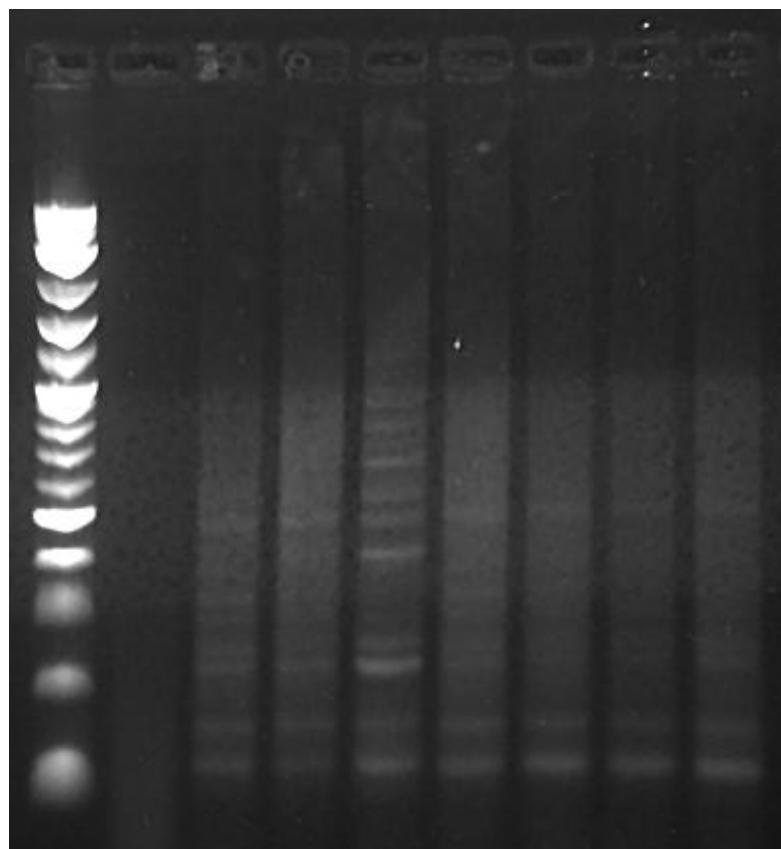
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	23	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
1200	36	42	42	42	42	42	42	42	42
1000	41	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5
500	56	46	46	46	46	46	46	46	46
400	61	47.5	47.5	47.5	47.5	47.5	47.5	47.5	47.5
		49.5	49.5	49.5	49.5	49.5	49.5	49.5	49.5
		51	51	51	51	51	51	51	51
		56	56	56	56	56	56	56	56
		57	57	57	57	57	57	57	57
								62.5	62.5
		63	63	63	63	63	63	63	63
								66	66
		68.5	68.5	68.5	68.5	68.5	68.5	68.5	68.5
		71	71	71	71	71	71	71	71
		72.5	72.5	72.5	72.5	72.5	72.5	72.5	72.5
		83	83	83	83	83	83	83	83

Agarose Gel 33: Passage 106 PCR, Primer 7



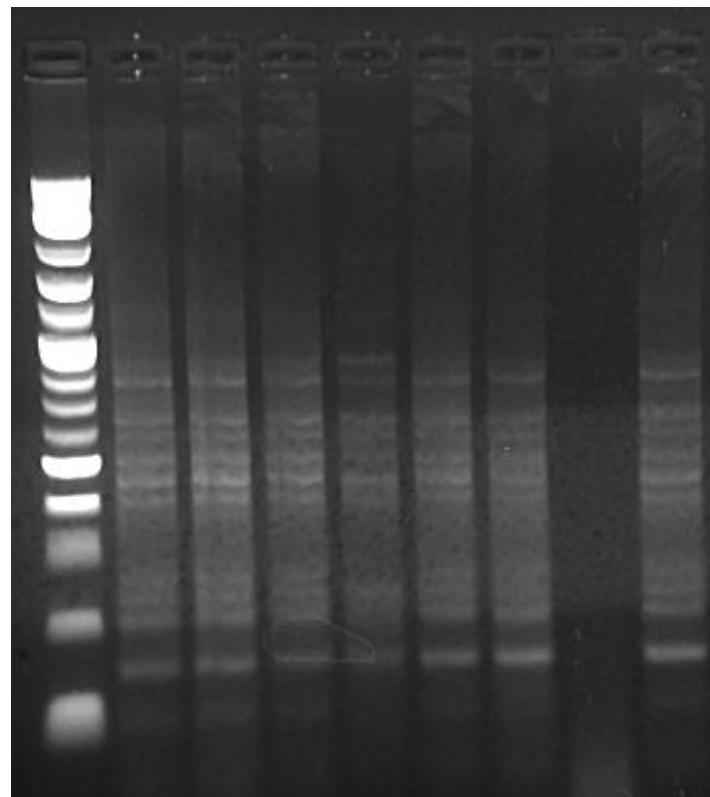
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	26	25	25	25	25				
1000	31	29			29				
900	45	30			30				
600	56	34	34	34	34	34	34	34	34
500	59	36	36	36	36	36	36	36	36
		41	41	41	41	41	41	41	41
					49	49			
		52	52	52	52	52	52	52	52
		57	57	57		57	57		57
		62	62	62	62	62	62	62	62
		84	84	84	84	84	84	84	84

Agarose Gel 34: Passage 106 PCR product, Primer 7, digested by MspI



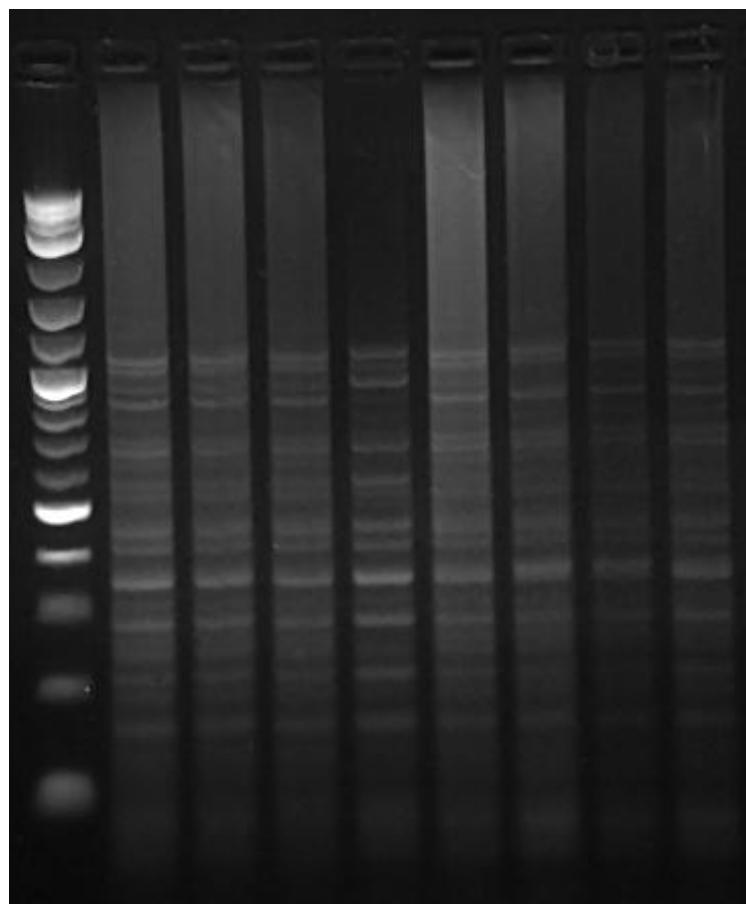
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	26				43				
2000	30				47				
1500	35				51				
800	48				56				
700	51	60	60	60	60	60	60	60	60
500	60				64				
400	65	69			69	69			
		72	72	72	72	72	72	72	72
		75	75	75	75	75	75	75	75
		79	79	79	79	79	79	79	79
		86	86	86	86	86	86	86	86
		92	92	92	92	92	92	92	92

Agarose Gel 35: Passage 106 PCR product, Primer 7, digested by HinfI



Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	20	38	38	38	38	38	38		38
2000	24	40	40	40	40	40	40		40
1000	37	45	45	45	45	45	45		45
600	48	47	47	47	47	47	47		47
500	52	50	50	50	50	50	50		50
		53	53	53	53	53	53		53
		67	67	67		67	67		67
		69	69	69	69	69	69		69
		72	72	72		72	72		72
		78	78	78	78	78	78		78

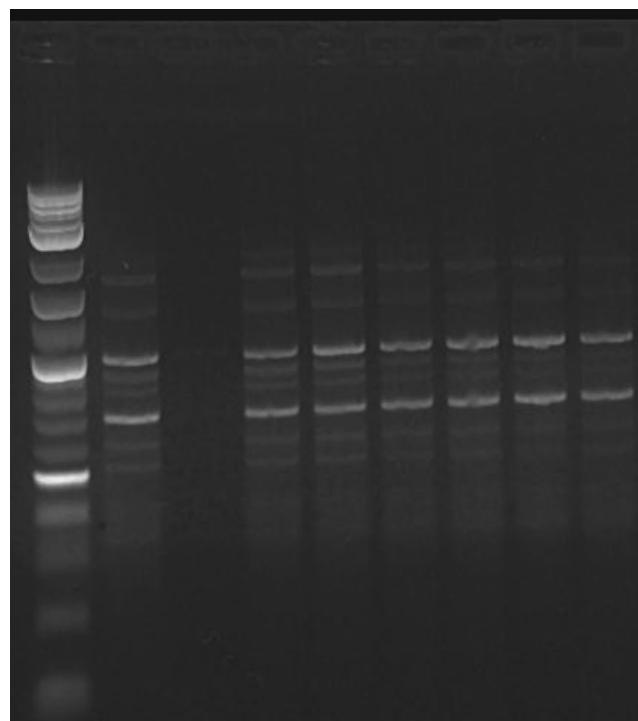
Agarose Gel 36: Passage 106 PCR product, Primer 7, digested by TaqI



Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	24	37	37	37	37	37	37	37	37
1500	34	39	39	39	39	39	39	39	39
1000	43	41	41	41	41	41		41	41
500	59	43	43	43	43	43	43	43	43
300	72	46	46	46	46	46	46	46	46
		48	48	48	48	48	48	48	48
		50	50	50	50	50	50	50	50
		51.5	51.5	51.5		51.5	51.5		
		54	54	54	54	54	54		54
		56	56	56	56	56	56	56	56
					58				
		60	60	60	60	60	60	60	60
		62	62	62	62	62	62	62	62
		67	67	67	67	67	67	67	67
		70	70	70	70	70	70		70
		72.5	72.5	72.5	72.5	72.5	72.5	72.5	72.5

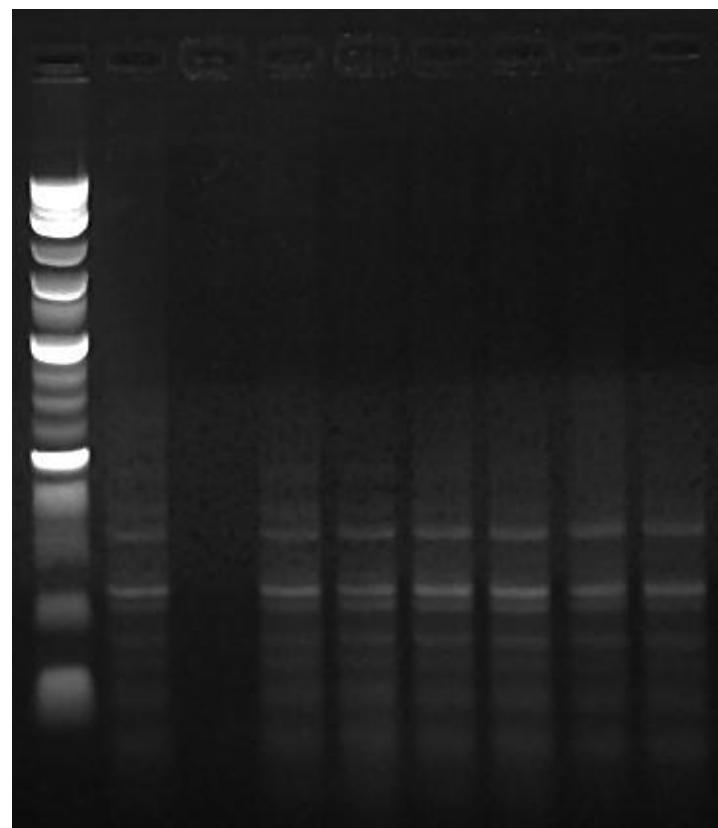
		74	74	74		74	74	74	74
		76	76	76	76	76	76	76	76
		79.5	79.5	79.5	79.5	79.5	79.5	79.5	79.5
		82	82	82	82	82	82	82	82
		87	87	87	87	87	87	87	87

Agarose Gel 37: Passage 117 PCR, Primer 5



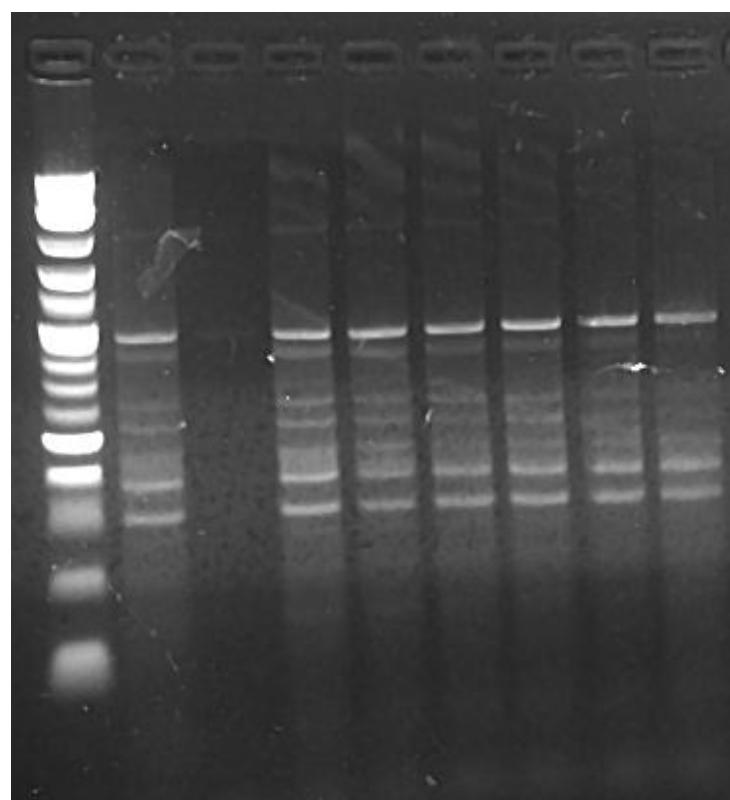
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
25	3000			27	27				
30	2000	29		29	29	29	29	29	29
34	1500	34		34	34	34	34	34	34
38	1200	40		40	40	40	40	40	40
43	1000	42		42	42	42	42	42	42
		44		44	44	44	44	44	44
		48		48	48	48	48	48	48
		51		51	51	51	51	51	51
		54		54	54	54	54	54	54

Agarose Gel 38: Passage 117 PCR product, Primer 5, digested by MspI



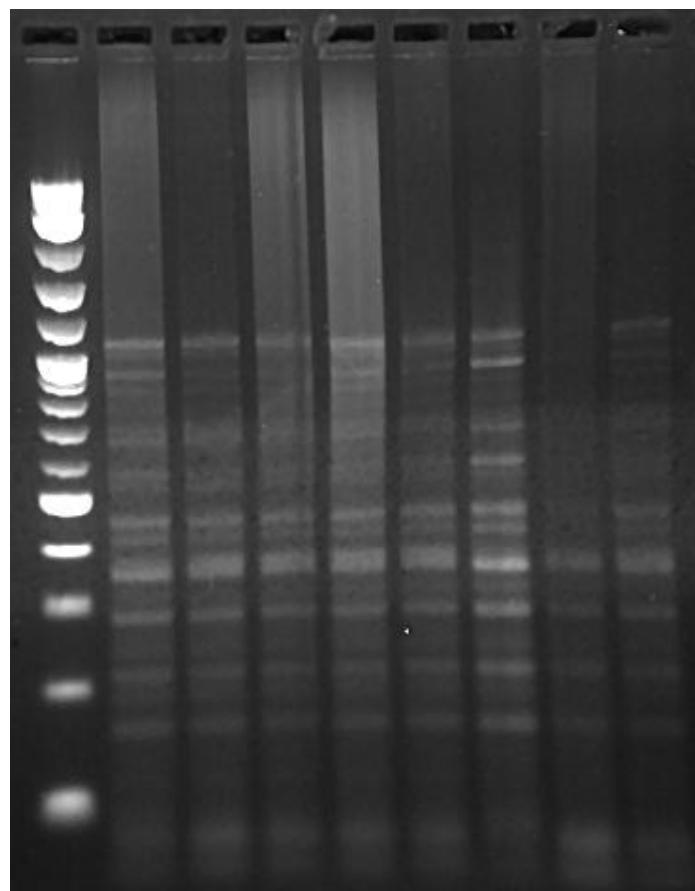
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	26	55		55	55	55	55		55
1500	30	60		60	60	60	60	60	60
1000	39	62		62	62	62	62	62	62
700	45	70		70	70	70	70	70	70
600	49	72		72	72	72	72	72	72
500	53	77		77	77	77	77	77	77
		80		80	80	80	80	80	80
		85		85	85	85	85	85	85
		91		91	91	91	91	91	91

Agarose Gel 39: Passage 117 PCR product, Primer 5, digested by HinfI



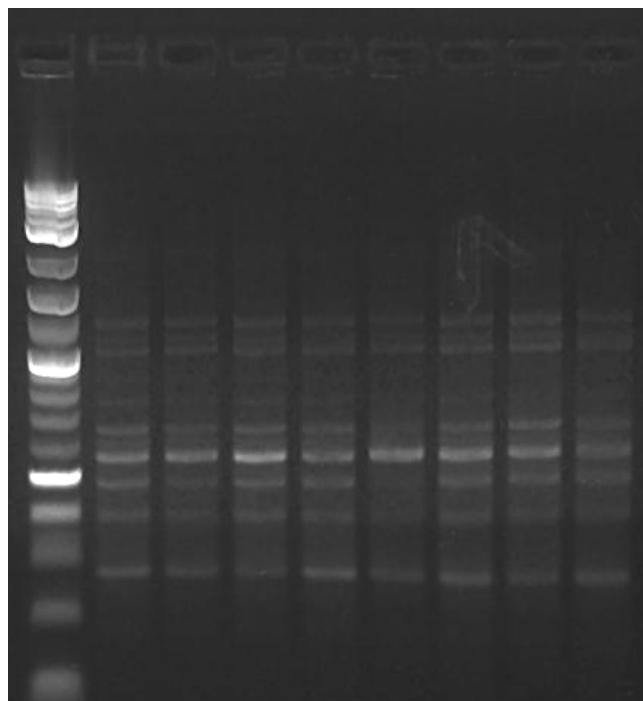
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	20	34		34	34	34	34	34	34
2000	23	37		37	37	37	37	37	37
1000	31	43		43	43	43	43	43	43
600	46	46		46	46	46	46	46	46
500	49	50		50	50	50	50	50	50
		53		53	53	53	53	53	53
		58		58	58	58	58	58	58

Agarose Gel 40: Passage 117 PCR product, Primer 5, digested by TaqI



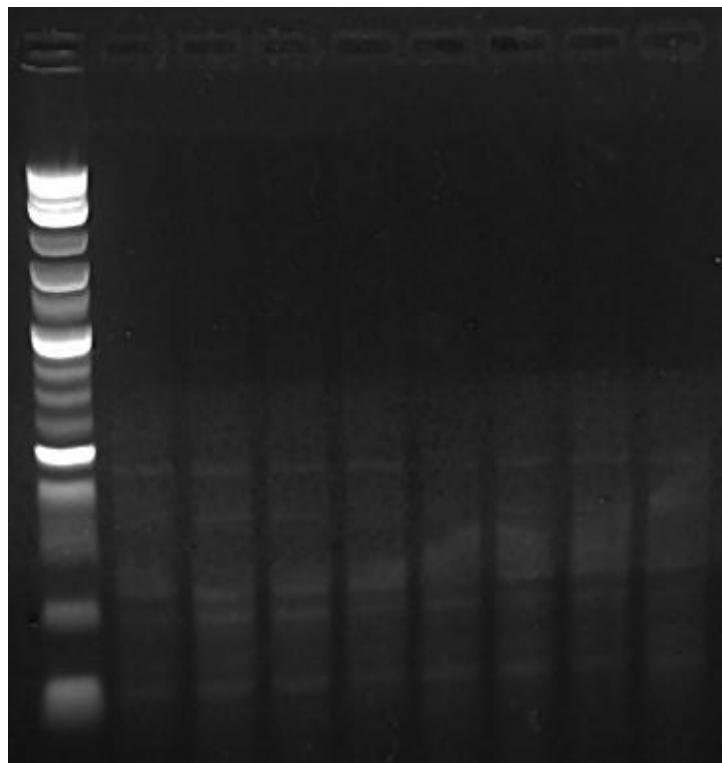
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	30.5	46		46	46	46	46		
1000	46			49	49				
700	54	55.5		55.5	55.5	55.5	55.5		
500	64.5	60		60	60	60	60	60	60
400	70	66		66	66	66	66	66	66
		80		80	80	80	80	80	80
		83		83	83	83	83	83	83
		88		88	88	88	88	88	88

Agarose Gel 41: Passage 117 PCR, Primer 6



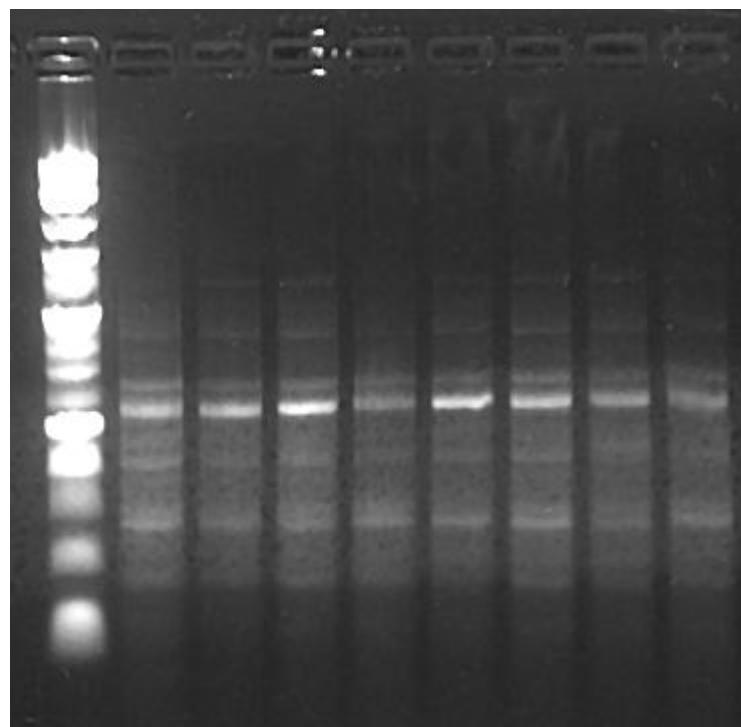
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
23	3000	33	33	33	33	33	33	33	33
27	2000	37	37	37	37	37	37	37	37
32	1500	44	44	44	44	44	44	44	44
25	1200	48	48	48	48	48	48	48	48
40	1000	51	51	51	51	51	51	51	51
44	800	55	55	55	55	55	55	55	55
		59	59	59	59	59	59	59	59
		66	66	66	66	66	66	66	66

Agarose Gel 42: Passage 117 PCR product, Primer 6, digested by MspI



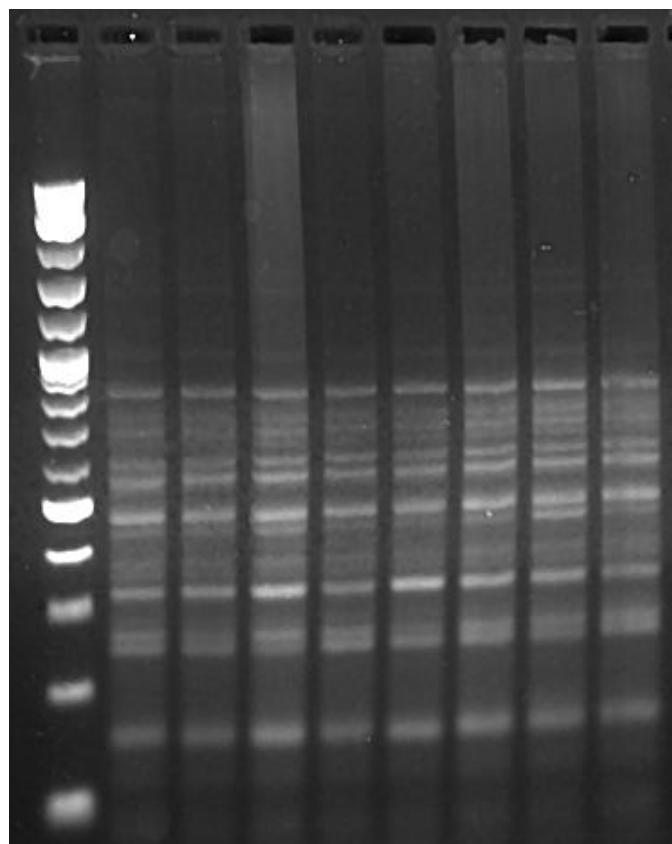
		Migration Distance (mm)								
Log 2 Marker		Samples								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	22	55	55	55	55		55	55	55	
2000	26		61	61			61			
1500	30	71	71	71	71	71	71	71	71	
1000	39	74	74	74	74	74	74	74	74	
700	46	80	80	80	80	80	80	80	80	
500	54	82	82	82	82	82	82	82	82	

Agarose Gel 43: Passage 117 PCR product, Primer 6, digested by HinfI



Log 2 Marker		Migration Distance (mm)							
		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	18	28	28	28		28	28	28	28
2000	22	35	35	35	35	35	35	35	35
1000	33	42	42	42	42	42	42	42	42
600	44	45	45	45	45	45	45	45	45
500	48	51	51	51	51	51	51	51	51
		60	60	60	60	60	60	60	60
		68	68	68	68	68	68	68	68

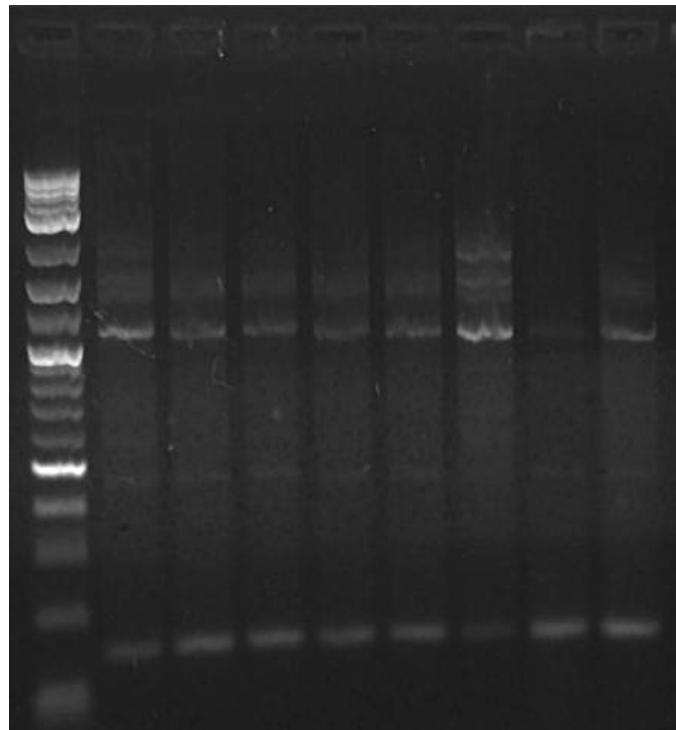
Agarose Gel 44: Passage 117 PCR product, Primer 6, digested by TaqI



		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
800	48							28.5	28.5
500	62	31	31	31	31	31	31	31	31
400	67	41	41	41	41	41	41	41	41
300	75	45	45	45	45	45	45	45	45
200	86	49	49	49	49	49	49	49	49
		51	51	51	51	51	51	51	51
				52	52	52	52	52	52
		55.5	55.5	55.5	55.5	55.5	55.5	55.5	55.5
		58	58	58	58	58	58	58	58
		62	62	62	62	62	62	62	62
		64	64	64	64	64	64	64	64
		67.5	67.5	67.5	67.5	67.5	67.5	67.5	67.5
		69	69	69	69	69	69	69	69
		72	72	72	72	72	72	72	72
		75	75	75	75				
		77	77	77	77	77	77	77	77
		79	79	79	79	79	79	79	79

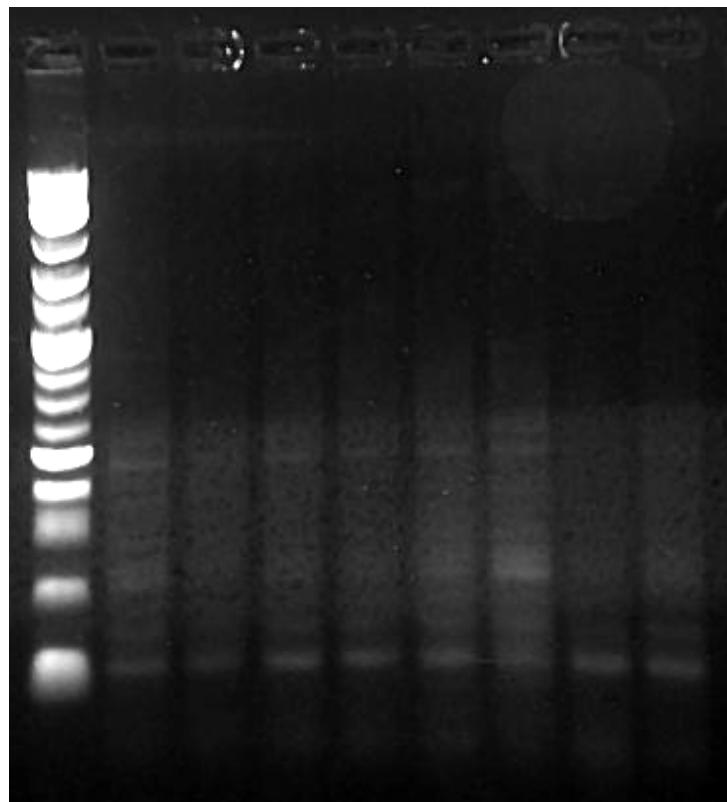
		91	91	91	91	91	91	91	91
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Agarose Gel 45: Passage 117 PCR, Primer 7



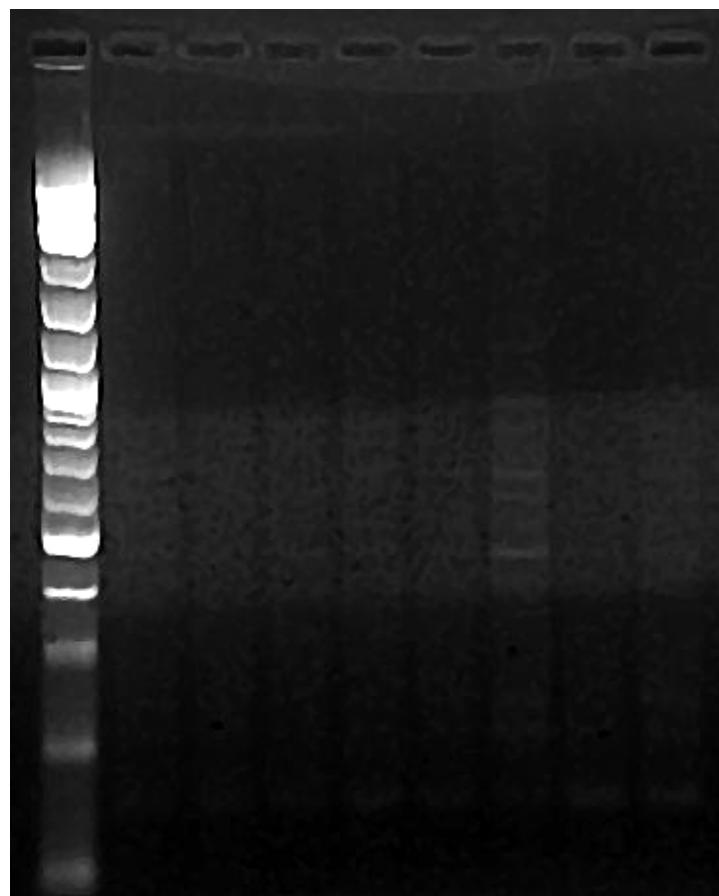
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
25	3000	28					28		
29	2000	34	34	34	34	34	34		34
34	1500	39	39	39	39	39	39	39	39
38	1200	58	58	58	58	58	58	58	58
42	1000	79	79	79	79	79	79	79	79

Agarose Gel 46: Passage 117 PCR product, Primer 7, digested by MspI



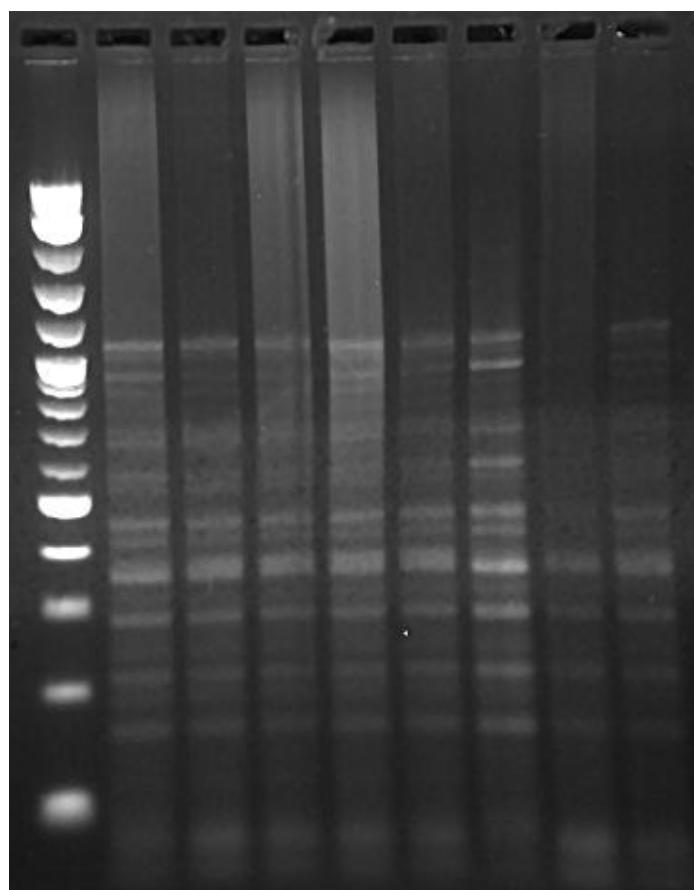
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
2000	25	38		38			38			
1500	30	49					49			
1200	33	52		52	52	52	52		52	
600	49	68		68	68	68	68	68	68	
500	52	75		75	75	75	75		75	
400	56	80	80	80	80	80	80	80	80	

Agarose Gel 47: Passage 117 PCR product, Primer 7, digested by HinfI



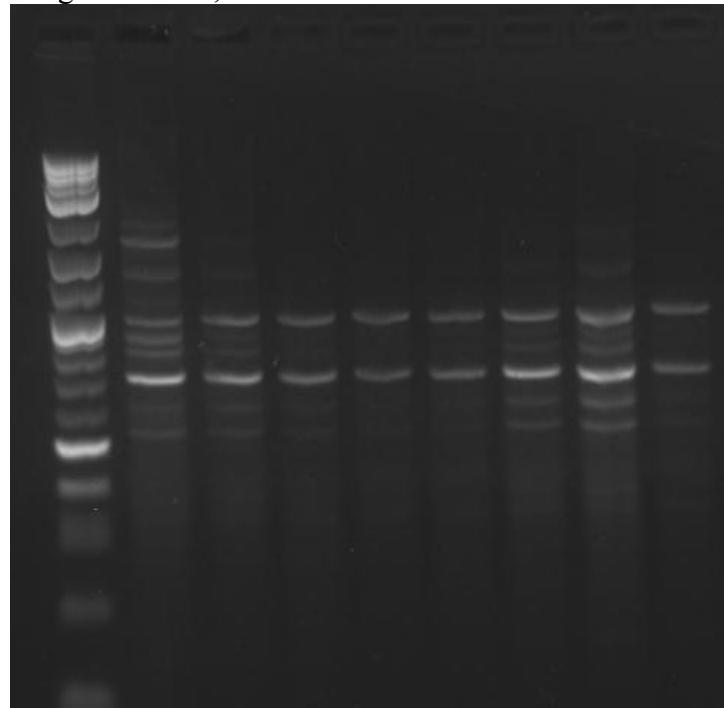
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	24						39		
2000	28						53		
1000	44						56		
600	58						64		
500	64								

Agarose Gel 48: Passage 117 PCR product, Primer 7, digested by TaqI



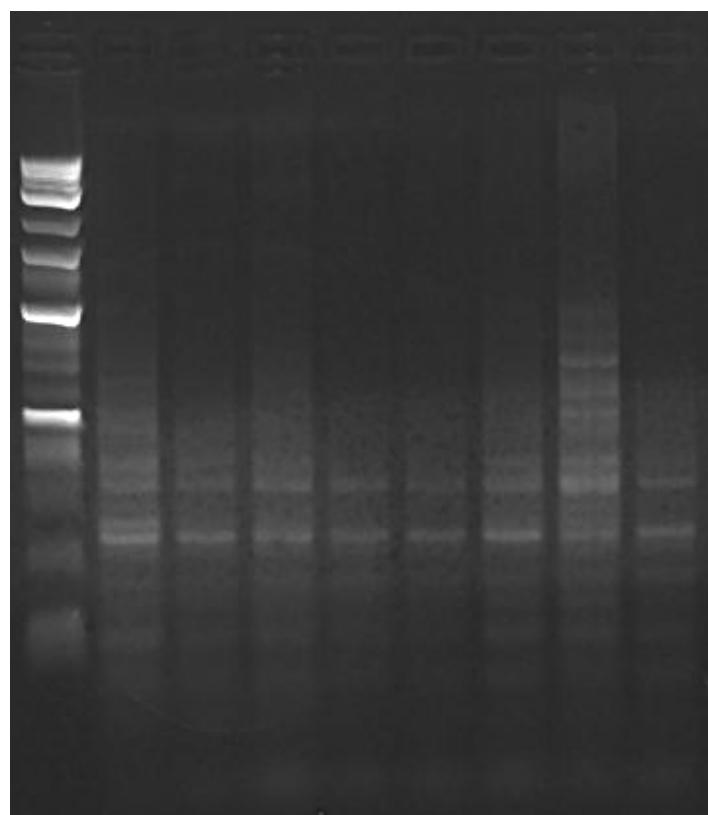
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
2000	28.5	38	38	38	38	38	38		38	
1000	43.5	39	39	39	39	39	39		39	
700	51	42	42	42	42	42	42	42	42	
500	60.5	45	45	45	45	45	45		45	
400	66	47	47	47	47	47	47		47	
	50	50								
	51	51	51	51		51			51	
	56	56	56	56	56	56			56	
	57.5	57.5	57.5	57.5	57.5	57.5	57.5		57.5	
	61	61	61	61	61	61	61	61	61	
	64	64	64	64	64	64			64	
	66.5	66.5	66.5	66.5	66.5	66.5			66.5	
	68.5	68.5	68.5	68.5	68.5	68.5	68.5	68.5	68.5	
	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.5	71.5	
	74	74	74	74	74	74	74	74	74	
	82	82	82	82	82	82	82	82	82	
	89	89	89	89	89	89	89	89	89	

Agarose Gel 49: Passage 129 PCR, Primer 5



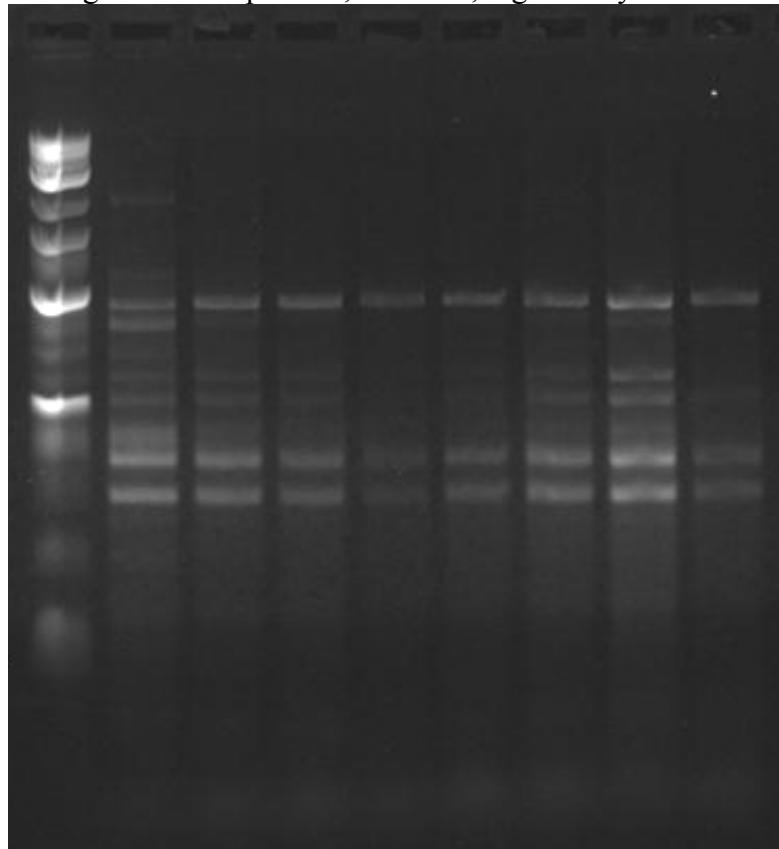
		Migration Distance (mm)								
Log 2 Marker		Samples								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
22	3000	23								
30	1500	26	26				26	26		
29	1000	30	30				30	30		
46	700	34	34							
54	500	36	36	36	36	36	36	36	36	
59	400	39	39	39		39	39	39		
		41	41	41		41	41	41		
		44	44	44	44	44	44	44	44	
		48	48	48	48	48	48	48	48	
		51	51	51	51	51	51	51	51	

Agarose Gel 50: Passage 129 PCR product, Primer 5, digested by MspI



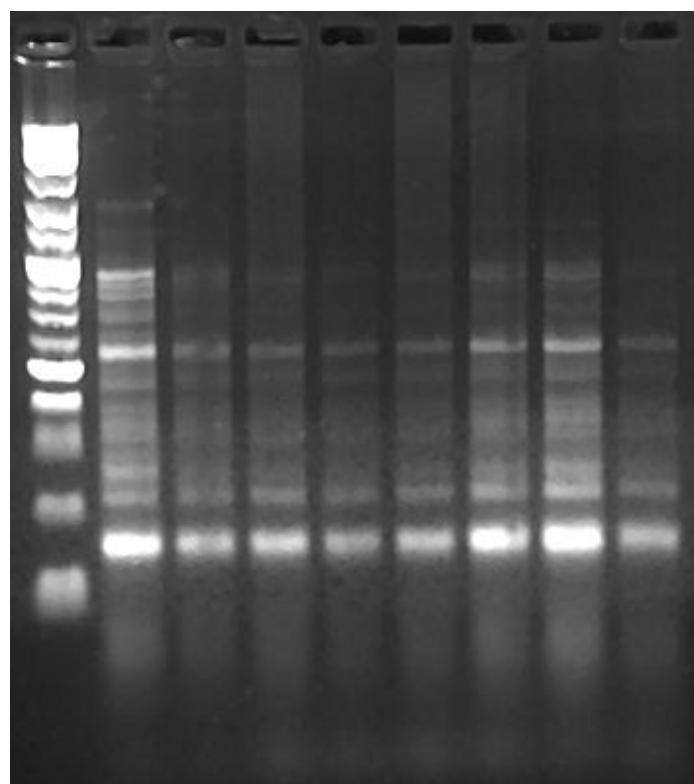
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	19	34				34	34		
2000	22						37		
1500	27	40					40		
1000	34	44					44		
500	48	47					47		
		54	54			54	54	54	
		57	57	57	57	57	57	57	57
		62					62		
		63	63	63	63	63	63	63	63
		69	69	69	69	69	69	69	69
		72				72	72	72	
		77	77	77	77	77	77	77	77
		82	82	82	82	82	82	82	82

Agarose Gel 51: Passage 129 PCR product, Primer 5, digested by HinfI



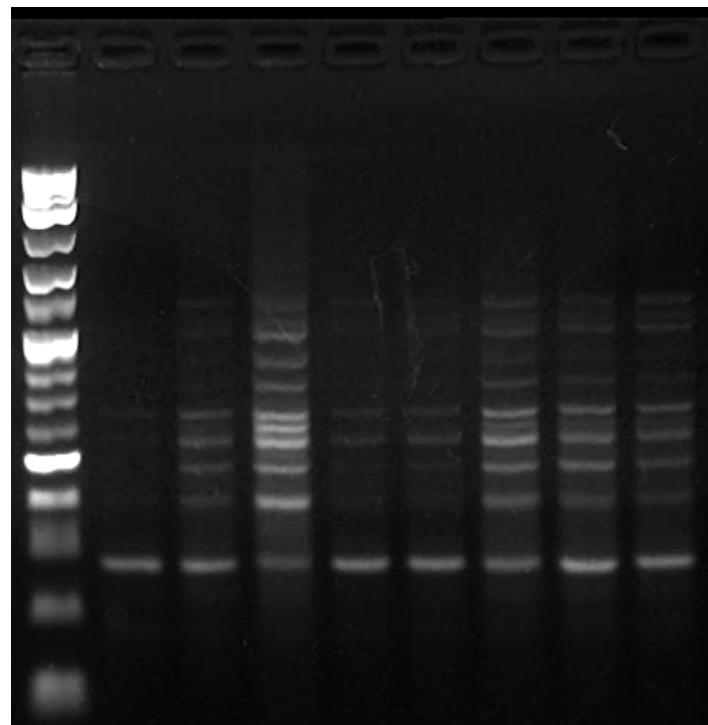
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	19	21								
2000	23	31								
1000	36	35	35	35	35	35	35	35	35	
600	45	38								
500	48	45								
		48	48	48	48		48	48		
		53	53	53	53		53	53		
		56	56	56	56	56	56	56	56	
		61	61	61	61	61	61	61	61	

Agarose Gel 52: Passage 129 PCR product, Primer 5, digested by TaqI



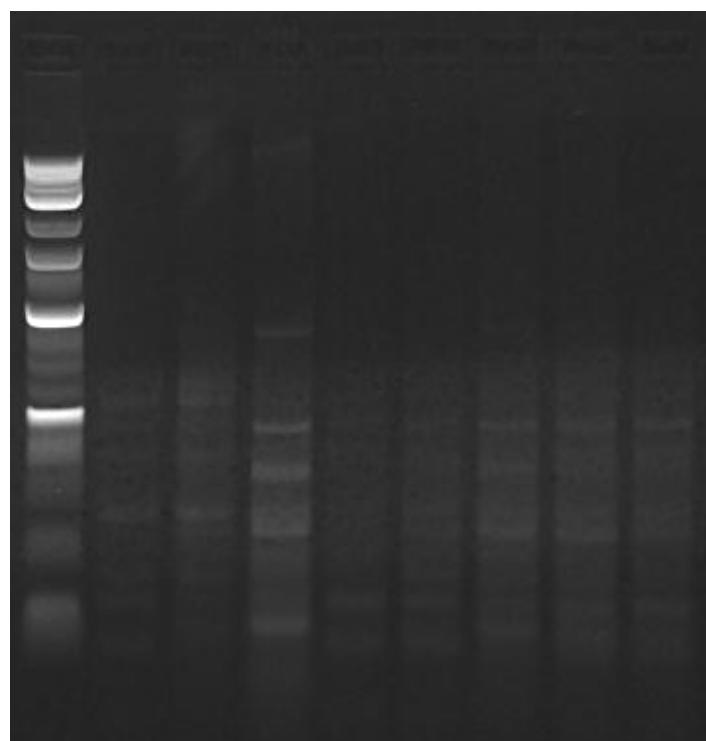
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
800	33	20							
500	43	23		23	23	23	23		
400	47	29	29	29	29	29	29	29	29
300	53	30	30	30	30	30	30	30	30
100	74	32				32	32		
		35	35	35	35	35	35	35	
		40	40	40	40	40	40	40	40
		44	44	44	44	44	44	44	44
		47.5	47.5			47.5	47.5		
		49	49			49	49		
		51	51	51	51	51	51	51	51
		55.5	55.5	55.5	55.5	55.5	55.5	55.5	55.5
		59	59	59	59	59	59	59	59
		66	66	66	66	66	66	66	66

Agarose Gel 53: Passage 129 PCR, Primer 6



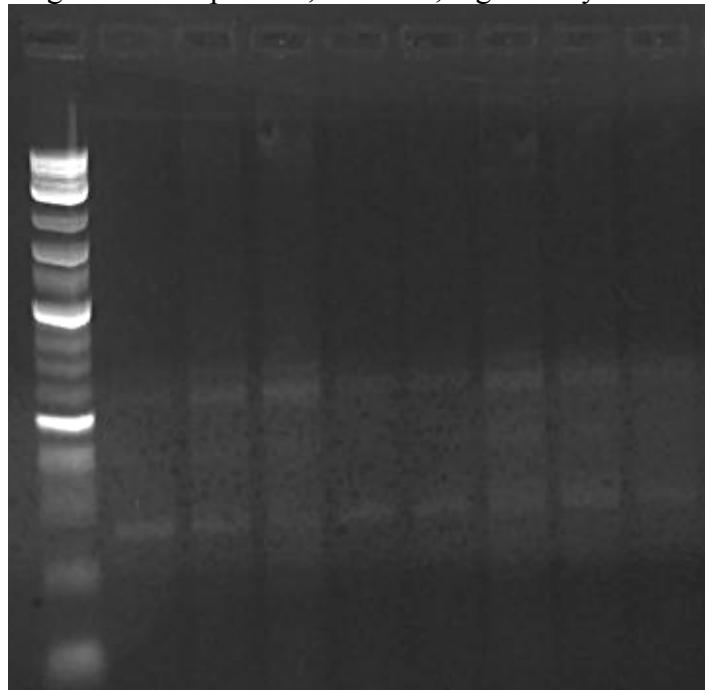
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
25	2000			23					
30	1500			27					
33	1200		32	32	32	32	32	32	32
42	800		34	34			34	34	34
45	700		36	36	36	36	36	36	36
54	500		40	40			40	40	40
			43	43			43	43	43
		47	47	47	47	47	47	47	47
			49	49			49	49	49
		51	51	51	51	51	51	51	51
			54	54	54	54	54	54	54
			59	59	59	59	59	59	59
		67	67	67	67	67	67	67	67
		70	70	70	70	70	70	70	70
		75	75	75	75	75	75	75	75

Agarose Gel 54: Passage 129 PCR product, Primer 6, digested by MspI



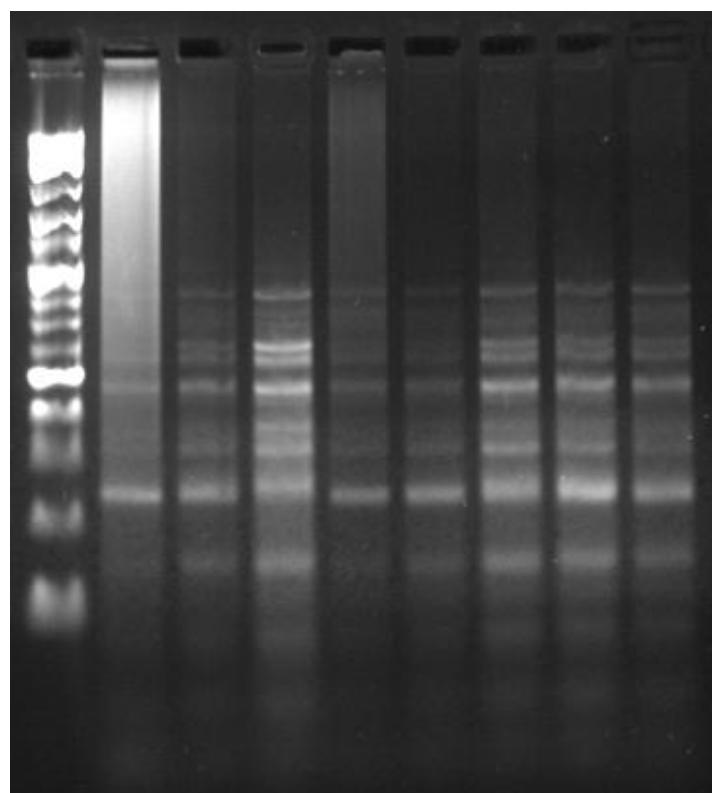
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	19			36					
2000	23	45	45						
1500	27			49		49	49	49	49
1000	35	52	52						
500	48			55			55	55	55
		60	60			60	60	60	60
				63		63	63	63	63
		68	68			68	68	68	68
		72	72	72	72	72	72	72	72
			76	76			76	76	
		78			78	78			

Agarose Gel 55: Passage 129 PCR product, Primer 6, digested by HinfI



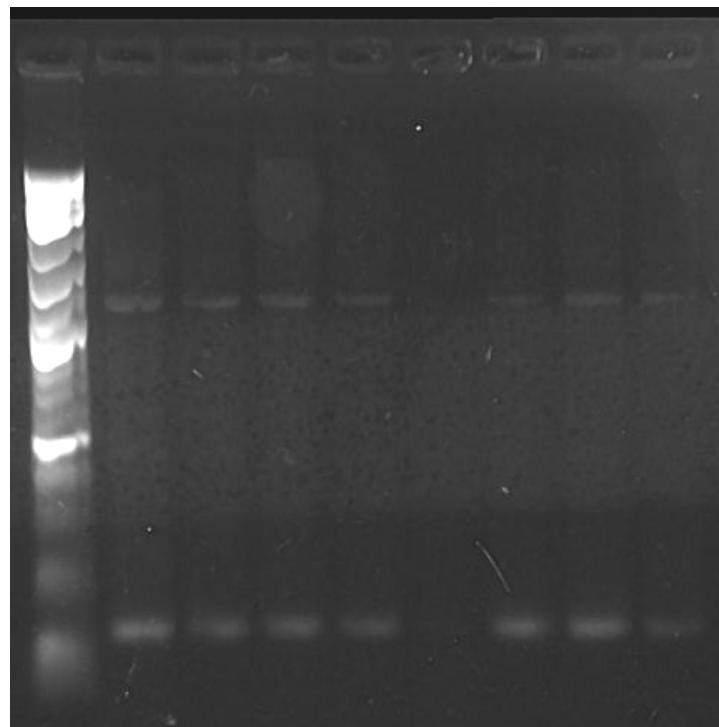
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	20	45	45	45	45	45	45	45	45
2000	23	62	62	62	62	62	62	62	62
1000	31								
600	46								
500	50								

Agarose Gel 56: Passage 129 PCR product, Primer 6, digested by TaqI



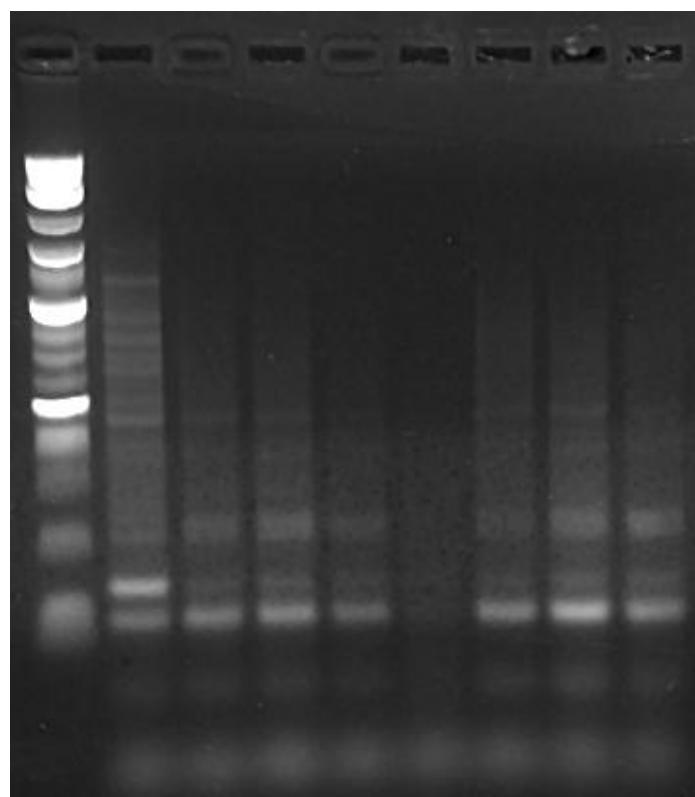
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
1500	22	30.5	30.5	30.5	30.5	30.5	30.5	30.5	30.5
800	32	37.5	37.5	37.5	37.5	37.5	37.5	37.5	37.5
500	42	39	39	39	39	39	39	39	39
400	46	42.5	42.5	42.5	42.5	42.5	42.5	42.5	42.5
200	61	48	48	48	48	48	48	48	48
		51	51	51	51	51	51	51	51
		57	57	57	57	57	57	57	57
		67	67	67	67	67	67	67	67

Agarose Gel 57: Passage 129 PCR, Primer 7



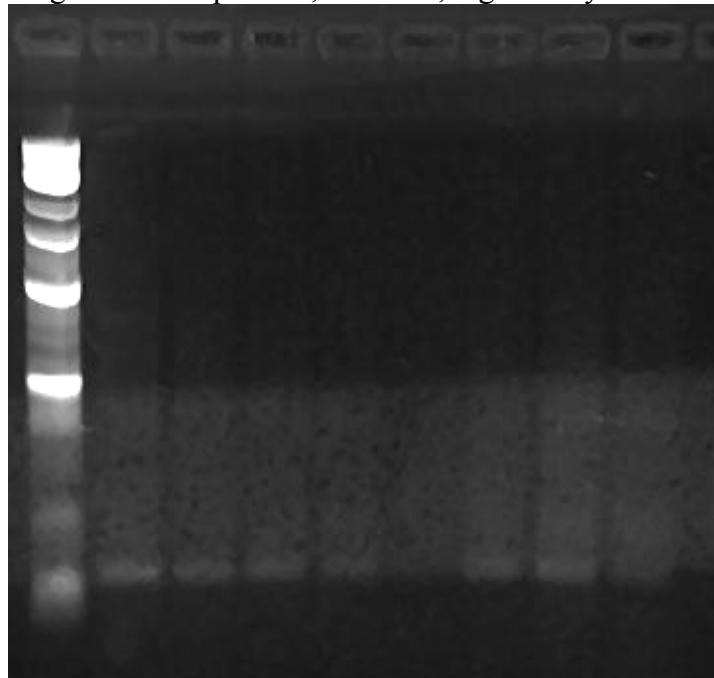
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
22	3000	31	31	31	31		31	31	31
39	1000	75	75	75	75		75	75	75
51	500								
55	400								
60	0.3								

Agarose Gel 58: Passage 129 PCR product, Primer 7, digested by MspI



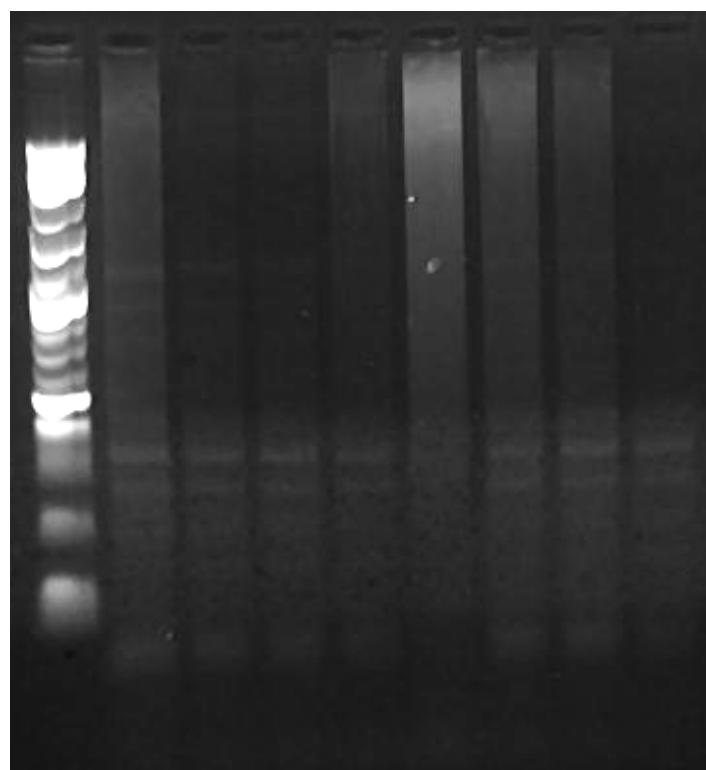
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	18	29								
2000	22	33								
1500	26	36								
1000	34	38								
800	36	40								
500	46	45								
		47	47	47	47			47	47	47
		62	62	62	62			62	62	62
		69	69	69	69			69	69	69
		73	73	73	73			73	73	73
		82	82	82	82			82	82	82

Agarose Gel 59: Passage 129 PCR product, Primer 7, digested by HinfI



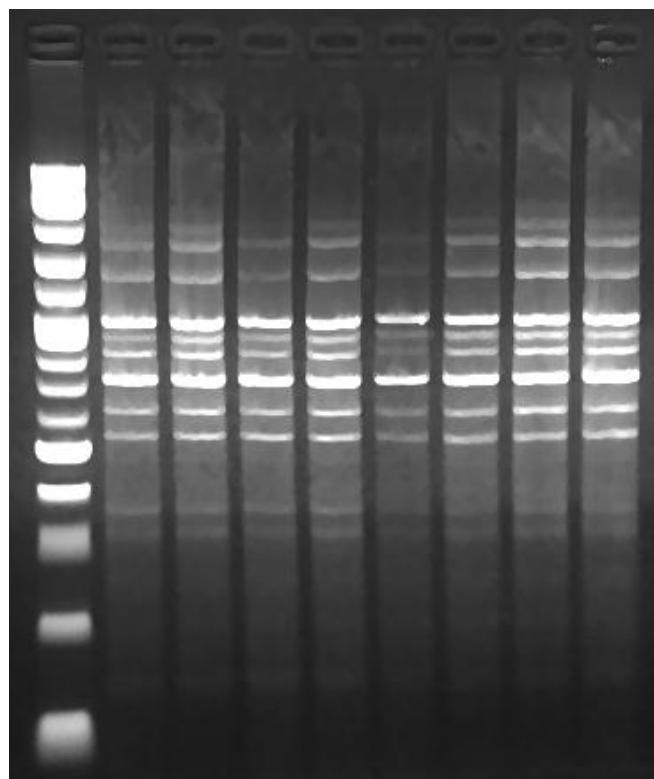
		Migration Distance (mm)								
Log 2 Marker		Samples								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	19	70	70	70	70	70	70	70	70	
2000	22									
1000	33									
600	44									
500	46									

Agarose Gel 60: Passage 129 PCR product, Primer 7, digested by TaqI



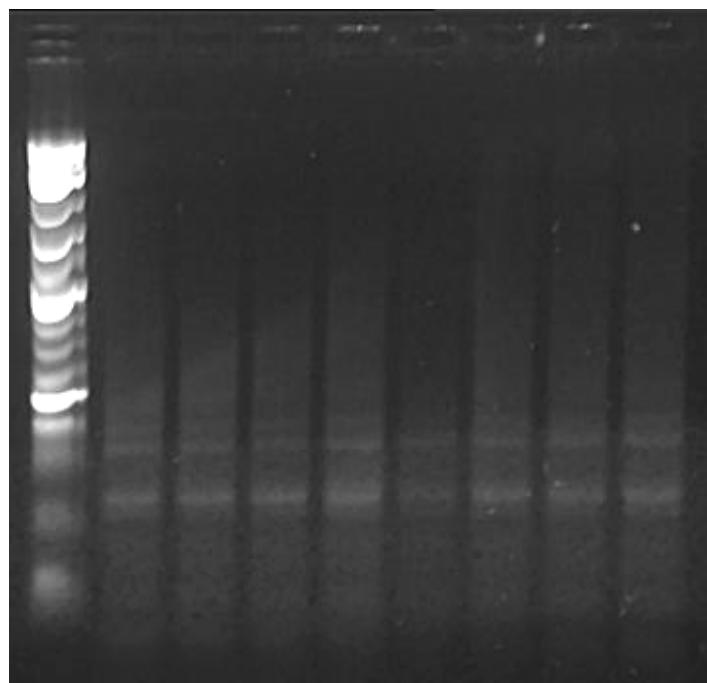
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
700	41.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5	29.5
600	44	34.5	34.5	34.5	34.5	34.5	34.5	34.5	34.5
500	48.5	54	54	54	54	54	54	54	54
300	57	57.5	57.5	57.5	57.5	57.5	57.5	57.5	57.5
200	64								

Agarose Gel 61: Passage 141 PCR, Primer 5



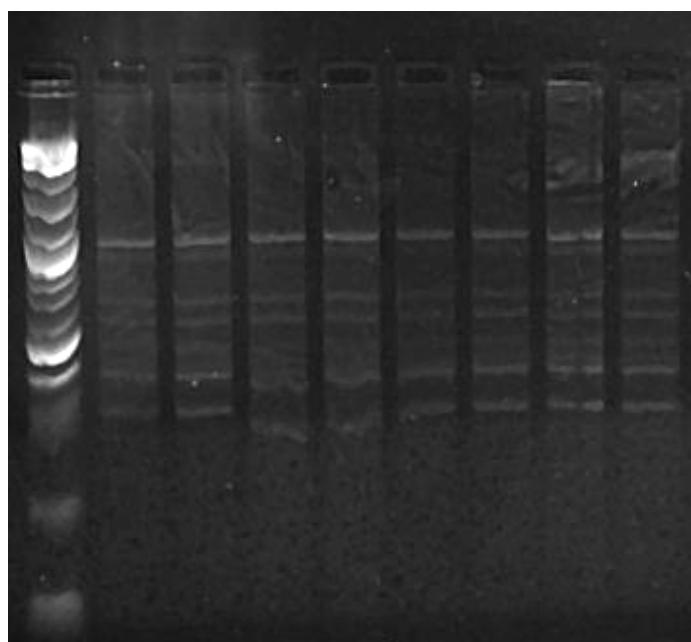
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
28	1500	21	21					21	21
32	1200	23	23	23	23		23	23	23
40	800	26	26	26	26	26	26	26	26
44	700	30	30	30	30	30	30	30	30
53	500	36	36	36	36	36	36	36	36
58	400	38	38	38	38	38	38	38	38
76	200	40	40	40	40	40	40	40	40
		44	44	44	44	44	44	44	44
		48	48	48	48	48	48	48	48
		51	51	51	51	51	51	51	51
		61	61	61	61	61	61	61	61
		64	64	64	64	64	64	64	64
		70	70	70	70	70	70	70	70
		76	76	76	76	76	76	76	76
		84	84	84	84	84	84	84	84

Agarose Gel 62: Passage 141 PCR product, Primer 5, digested by MspI



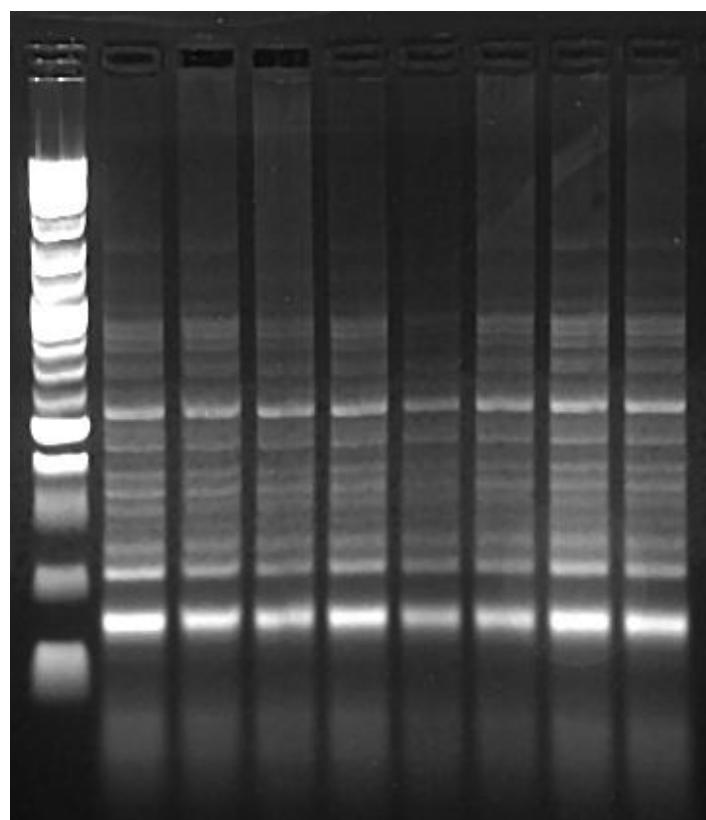
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	24	51	51	51	51	51	51	51	51
1500	28	54	54	54	54	54	54	54	54
1000	36	60	60	60	60	60	60	60	60
800	39								
700	42								
500	48								

Agarose Gel 63: Passage 141 PCR product, Primer 5, digested by HinfI



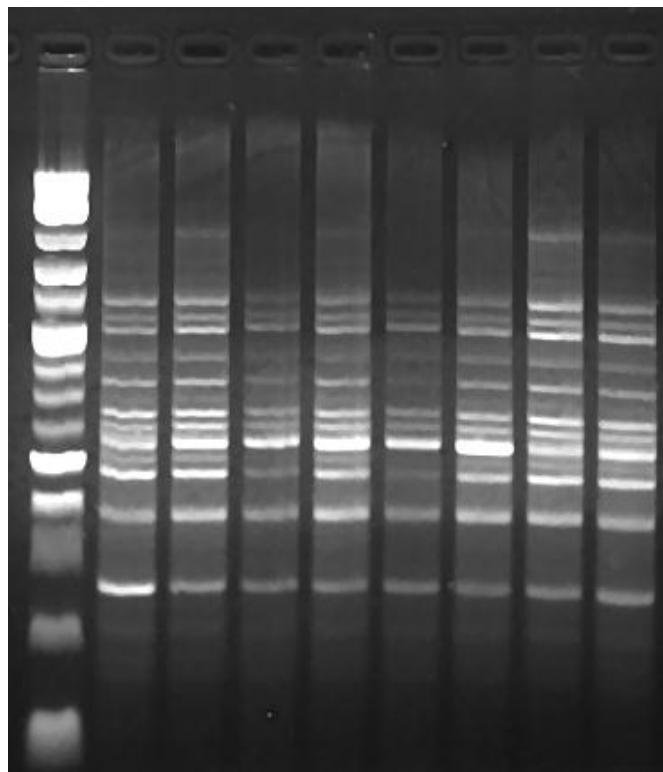
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	12	21	21					21	21
2000	14	23	23	23	23	23	23	23	23
1000	25	28	28	28	28	28	28	28	28
600	33	31	31	31	31	31	31	31	31
500	42	38	38	38	38	38	38	38	38
		43	43	43	43	43	43	43	43

Agarose Gel 64: Passage 141 PCR product, Primer 5, digested by TaqI



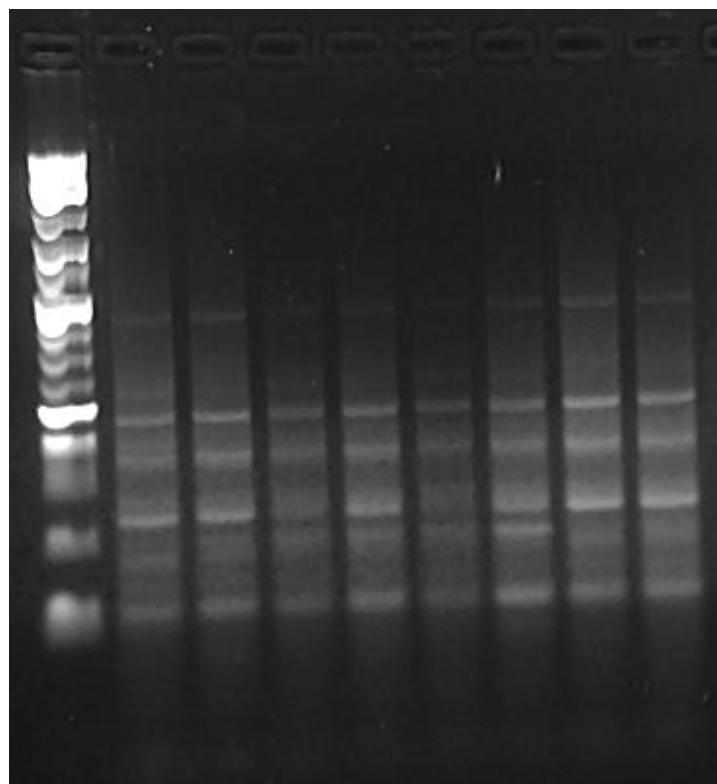
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
800	32.5	24	24	24	24	24	24	24	24	
700	41.5	28.5	28.5	28.5	28.5	28.5	28.5	28.5	28.5	
600	45.5	32	32	32	32	32	32	32	32	
400	53	34	34	34	34	34	34	34	34	
200	70	35	35	35	35	35	35	35	35	
		37	37	37	37	37	37	37	37	
		41	41	41	41	41	41	41	41	
		45.5	45.5	45.5	45.5	45.5	45.5	45.5	45.5	
		50	50	50	50	50	50	50	50	
		54	54	54	54	54	54	54	54	
		56	56	56	56	56	56	56	56	
		59	59	59	59	61			59	
									61	
		63	63	63	63	63	63	63	63	
		67	67	67	67	67	67	67	67	
		75	75	75	75	75	75	75	75	

Agarose Gel 65: Passage 141 PCR, Primer 6



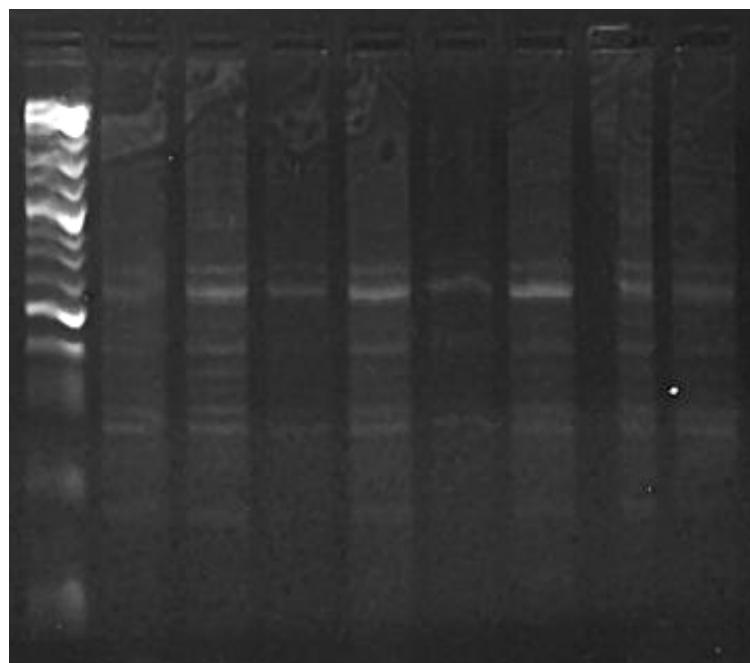
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
24	2000	23	23		23		23	23	23	
29	1500	32	32	32	32	32	32	32	32	
33	1200	34	34	34	34	34	34	34	34	
42	800	36	36	36	36	36	36	36	36	
45	700	39	39	39	39	39	39	39	39	
54	500	43	43	43	43	43	43	43	43	
		47	47	47	47	47	47	47	47	
		49	49	49	49	49	49	49	49	
		51	51	51	51	51	51	51	51	
		53	53		53		53	53	53	
		55	55	55	55	55	55	55	55	
		60	60	60	60	60	60	60	60	
		70	70	70	70	70	70	70	70	
		75	75	75	75	75	75	75	75	

Agarose Gel 66: Passage 141 PCR product, Primer 6, digested by MspI



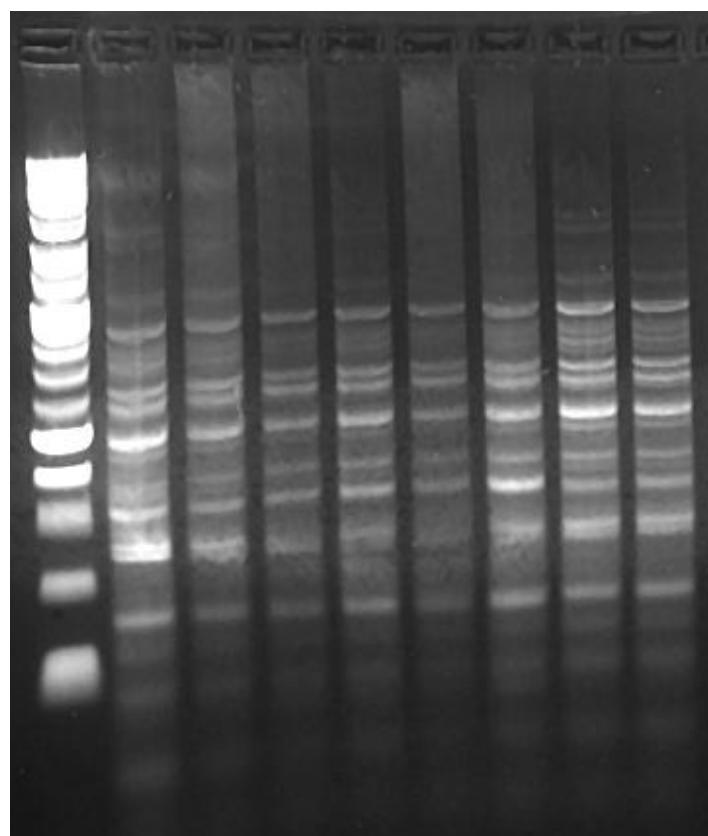
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	23	33	33	33	33	33	33	33	33
1500	28	44	44	44		44	44		
800	38	47	47	47	47	47	47	47	47
700	41	52	52	52	52	52	52	52	52
600	44	60	60	60	60	60	60	60	60
500	47	63	63	63	63	63	63	63	63
		72	72	72	72	72	72	72	72

Agarose Gel 67: Passage 141 PCR product, Primer 6, digested by HinfI



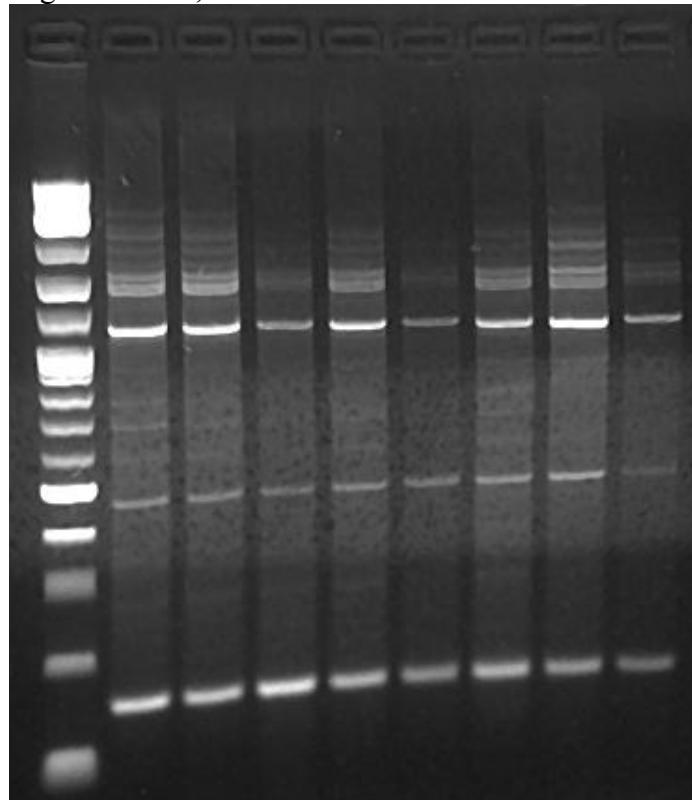
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	10	28	28	28	28	28	28	28	28
2000	14	32	32	32	32	32	32	32	32
1000	22	39	39		39		39	39	39
600	30		41		41		41	41	41
500	34		47		47		47	47	47
		50	50	50	50	50	50	50	50
		62	62		62		62	62	62

Agarose Gel 68: Passage 141 PCR product, Primer 6, digested by TaqI



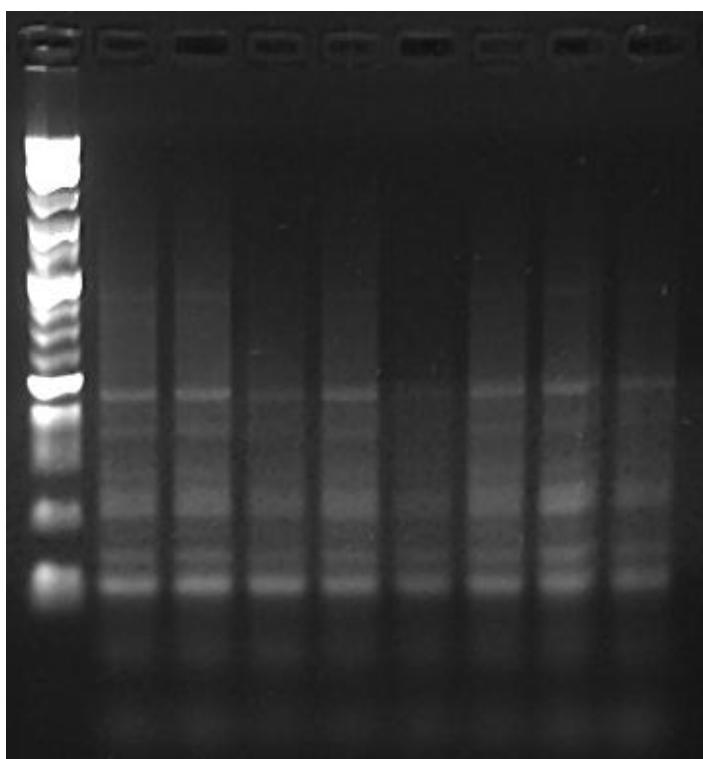
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
700	44	23	23		23		23	23	23
500	51	25	25		25		25	25	25
400	57	32	32	32	32		32	32	32
300	63	37	37	37	37	37	37	37	37
200	71.5	40	40	40	40		40	40	40
		42	42	42	42		42	42	42
		44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5
		47	47	47	47	47	47	47	47
		51	51	51	51	51	51	51	51
		53	53	53	53	53	53	53	53
		56	56	56	56	56	56	56	56
		57.5	57.5					57.5	57.5
		60	60	60	60	60	60	60	60
		65	65		65		65	65	65
		66	66	66	66			66	66
		75	75	75	75	75	75	75	75
		80	80	80	80	80	80	80	80

Agarose Gel 69: Passage 141 PCR, Primer 7



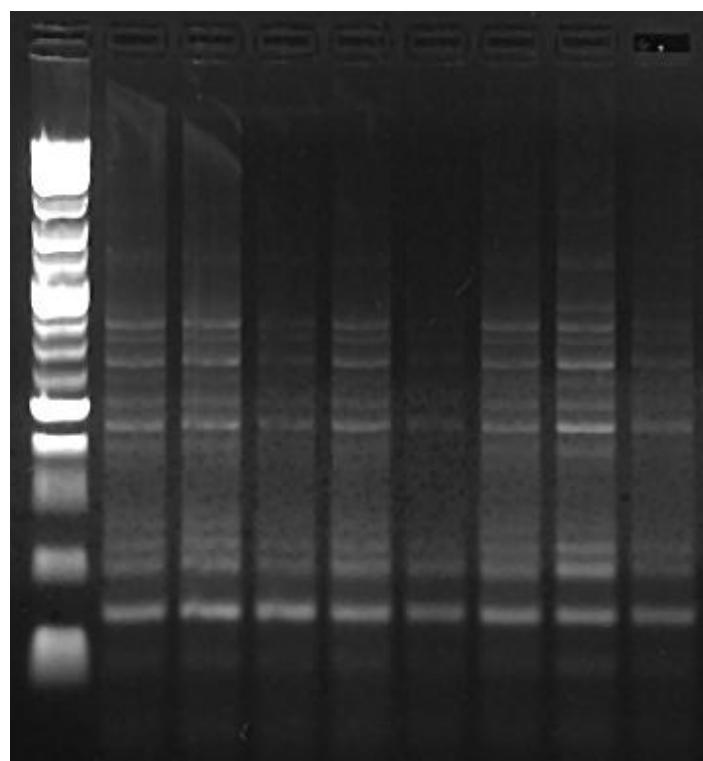
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
27	2000	22	22		22		22	22	22
32	1500	25	25	25	25		25	25	25
36	1200	27	27	27	27	27	27	27	27
46	800	29	29	29	29	29	29	29	29
49	700	30	30	30	30	30	30	30	30
53	600	32	32	32	32	32	32	32	32
58	500	38	38	38	38	38	38	38	38
63	400	42	42		42		42	42	
		47	47		47		47		
		50	50		50		50		
		54	54		54		54		
		60	60	60	60	60	60	60	60
		72	72	72	72		72		
		84	84	84	84	84	84	84	84

Agarose Gel 70: Passage 141 PCR product, Primer 7, digested by MspI



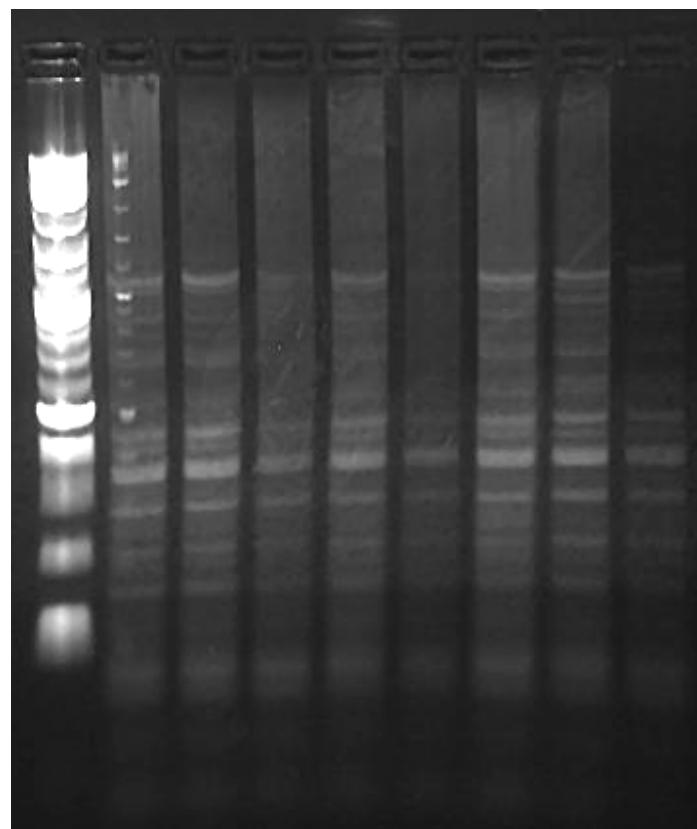
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	21	31	31		31		31	31	
1500	25	45	45	45	45	45	45	45	45
800	35	50	50		50		50	50	
700	38	55	55				55	55	55
600	41	60	60	60	60	60	60	60	60
500	45	66	66	66	66	66	66	66	66
		70	70	70	70	70	70	70	70
		80	80	80	80	80	80	80	80

Agarose Gel 71: Passage 141 PCR product, Primer 7, digested by HinfI



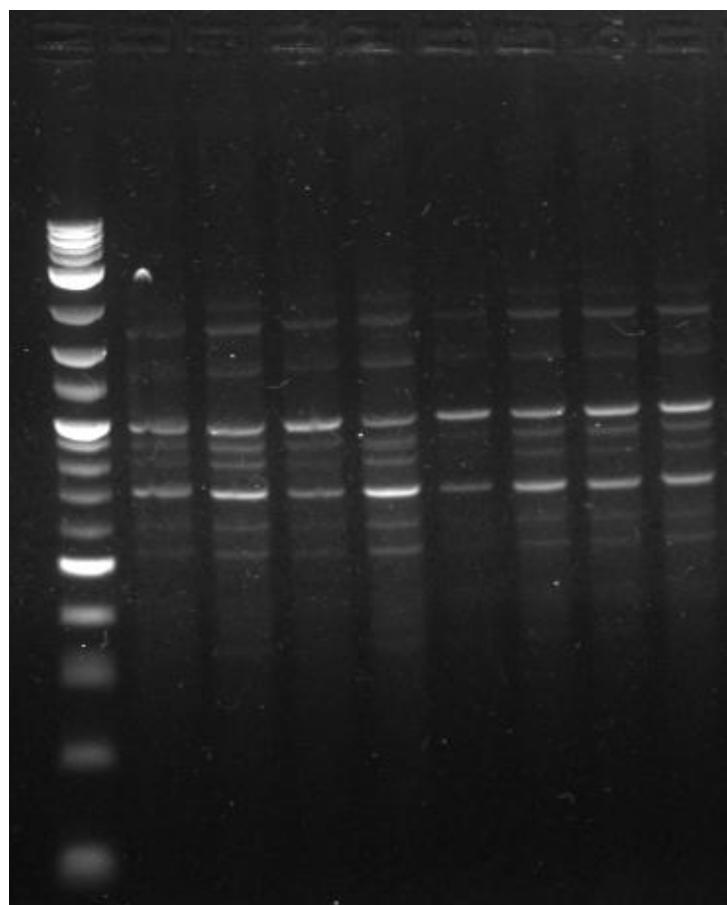
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	17	27	27	27	27		27	27	27
2000	20							33	
1000	33	35	35	35	35	35		35	
600	43	37	37	37	37	37	37	37	37
500	47	40	40	40	40	40	40	40	40
		42	42	42	42	42		42	42
		45	45	45	45	45	45	45	45
		51	51	51		51	51	51	
		61	61	61	61		61	61	
		65	65	65	65	65	65	65	65
		67	67	67	67	67	67	67	67
		74	74	74	74	74	74	74	74

Agarose Gel 72: Passage 141 PCR product, Primer 7, digested by TaqI



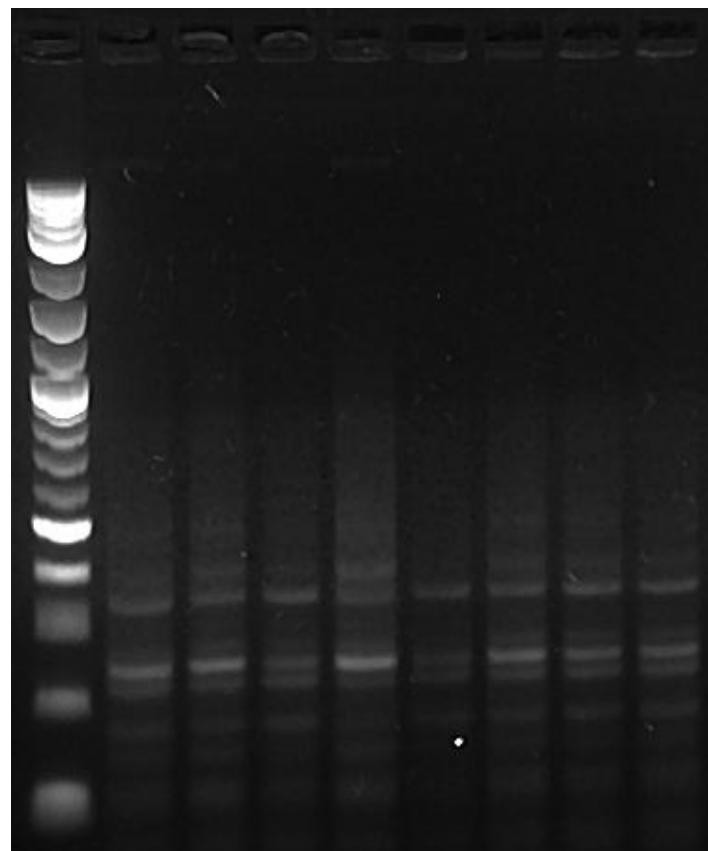
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
700	40	28	28	28	28	28	28	28	28
600	43	29	29	29	29		29	29	29
500	47							31.5	31.5
400	51	32.5	32.5		32.5		32.5	32.5	32.5
300	58	39	39		39		39	39	39
200	66						41.5	41.5	41.5
		44	44		44		44	44	44
		48.5	48.5	48.5	48.5	48.5	48.5	48.5	48.5
		51	51		51	51	51	51	51
		54	54	54	54	54	54	54	54
		58	58	58	58	58	58	58	58
		63	63	63	63	63	63	63	63
		69	69	69	69	69	69	69	69

Agarose Gel 73: Passage 153 PCR, Primer 5



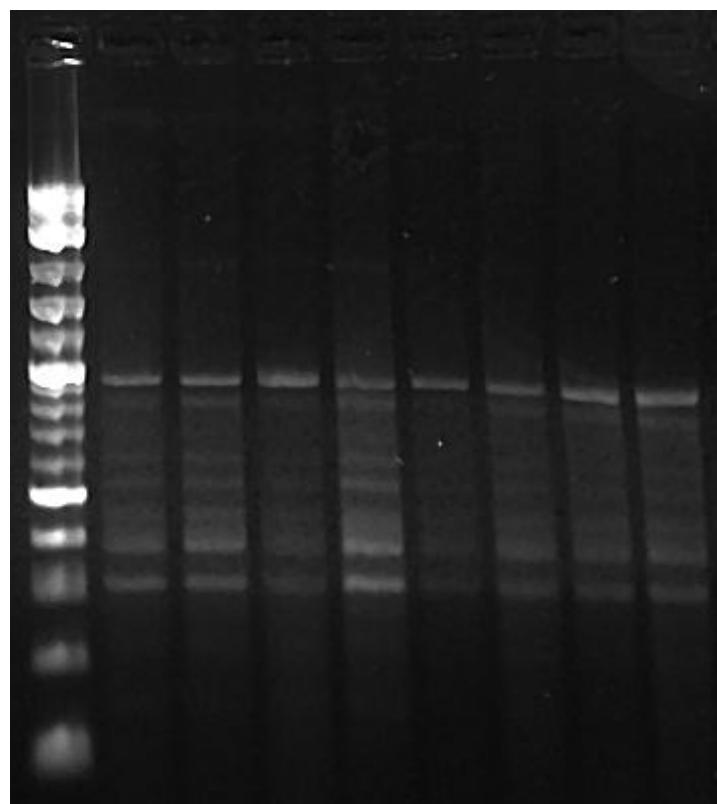
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	31	34	34	34	34				
2000	36	37	37	37	37	37	37	37	37
1000	50	42	42	42	42	42	42	42	42
600	53	49	49	49	49	49	49	49	49
500	73	51	51	51	51		51	51	51
		54	54	54	54		54	54	54
		58	58	58	58	58	58	58	58
		62	62	62	62		62	62	62
		66	66	66	66		66	66	66

Agarose Gel 74: Passage 141 PCR product, Primer 5, digested by MspI



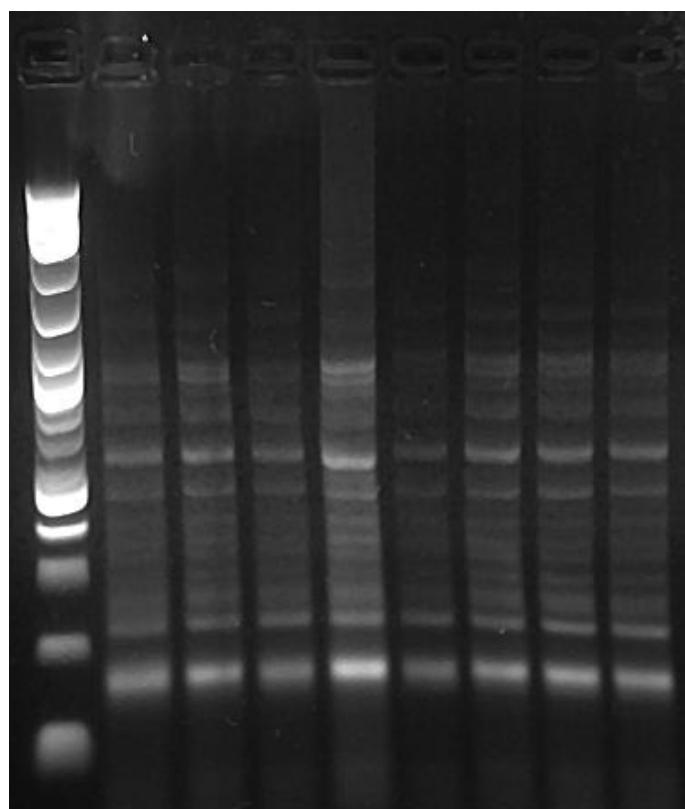
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	26		60		60		60	60	
2000	30		66	66	66		66	66	66
1500	36	70	70	70	70	70	70	70	70
1000	46	76	76	76	76		76	76	76
800	50	78	78	78	78	78	78	78	78
		81	81	81	81	81	81	81	81
		86	86	86	86	86	86	86	86
		90	90		90		90	90	90
		96	96	96	96		96	96	96

Agarose Gel 75: Passage 141 PCR product, Primer 5, digested by Hinfl



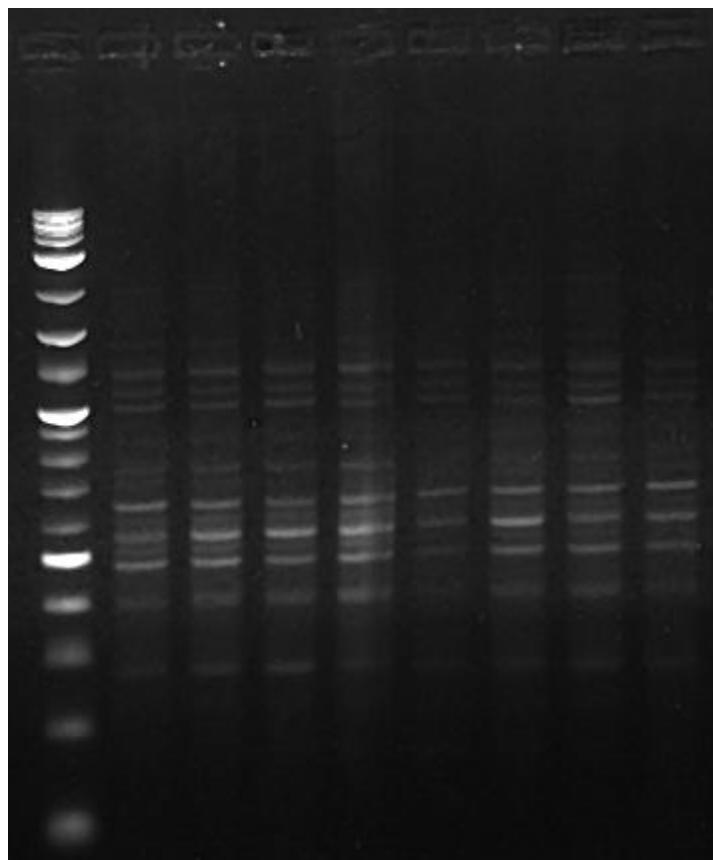
		Migration Distance (mm)								
Log 2 Marker		Samples								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	24	42	42	42	42	42	42	42	42	
2000	28	44	44		44		44	44	44	
1000	41	52	52		52		52	52	52	
600	52	54	54		54		54	54	54	
500	57	62	62	62	62	62	62	62	62	
		67	67	67	67	67	67	67	67	

Agarose Gel 76: Passage 141 PCR product, Primer 5, digested by TaqI



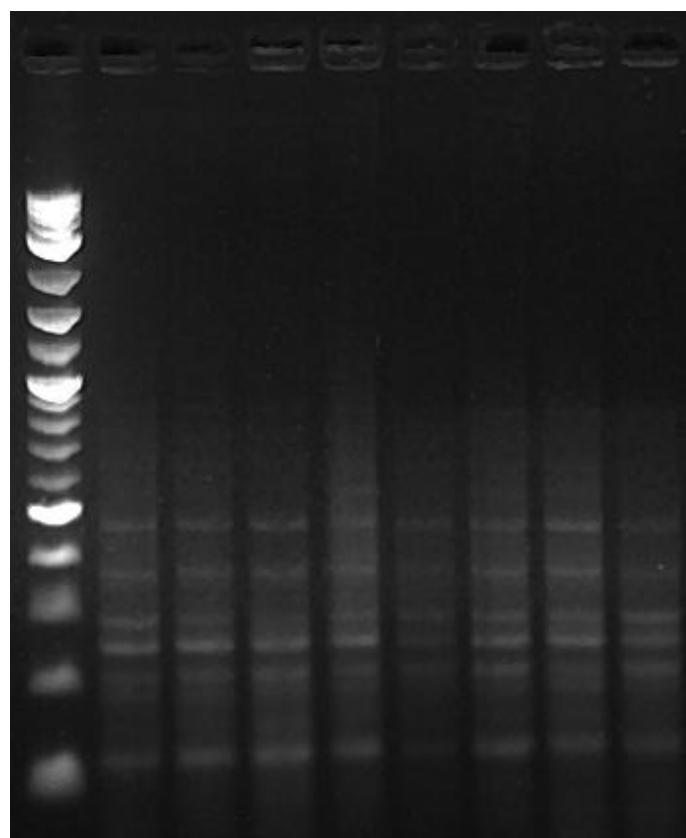
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
2000	30	27	27	27	27	27	27	27	27
1200	40	32	32	32	32	32	32	32	32
800	48	38	38	38	38	38	38	38	38
500	58	39	39	39	39	39	39	39	39
200	77	41	41	41	41	41	41	41	41
		43	43		43		43	43	43
		47	47	47	47	47	47	47	47
		52	52	52	52	52	52	52	52
		56	56	56	56	56	56	56	56
		59	59	59	59	59	59	59	59
		62	62		62	62	62	62	62
		64	64	64	64		64	64	64
			66	66	66	66	66	66	66
		70	70	70	70	70	70	70	70
		72	72	72	72	72	72	72	72
		80	80	80	80	80	80	80	80

Agarose Gel 77: Passage 153 PCR, Primer 6



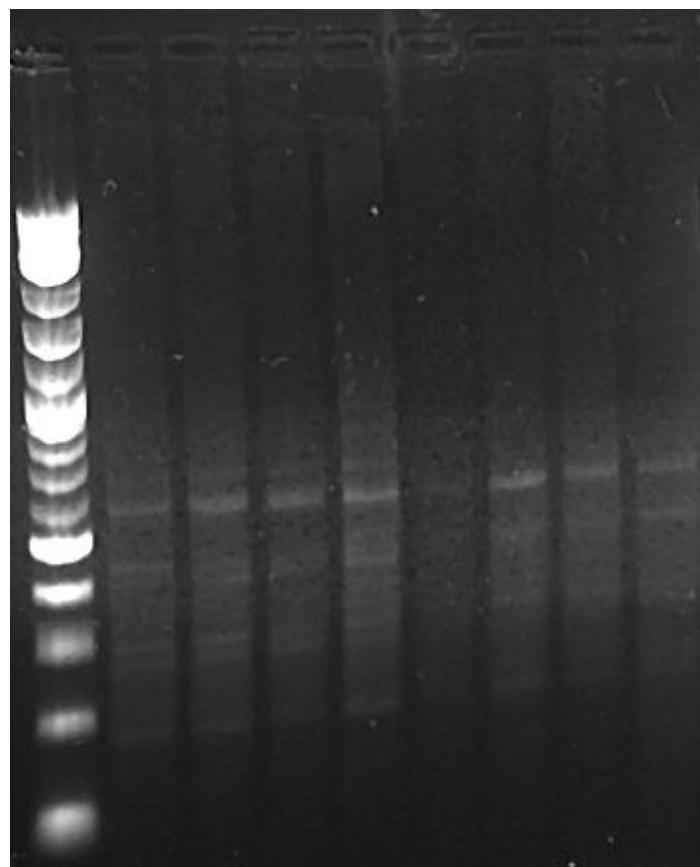
Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	27	40	40	40	40	40	40	40	40
2000	32	43	43	43	43	43	43	43	43
1000	42	45	45	45	45	45	45	45	45
600	61	53	53	53	53				
500	65	59	59	59	59	59	59	59	59
		61	61	61	61				
		63	63	63	63	63	63	63	63
		65	65	65	65	65	65	65	65
		70	70	70	70			70	70
		79	79	79	79			79	79

Agarose Gel 78: Passage 153 PCR product, Primer 6, digested by MspI



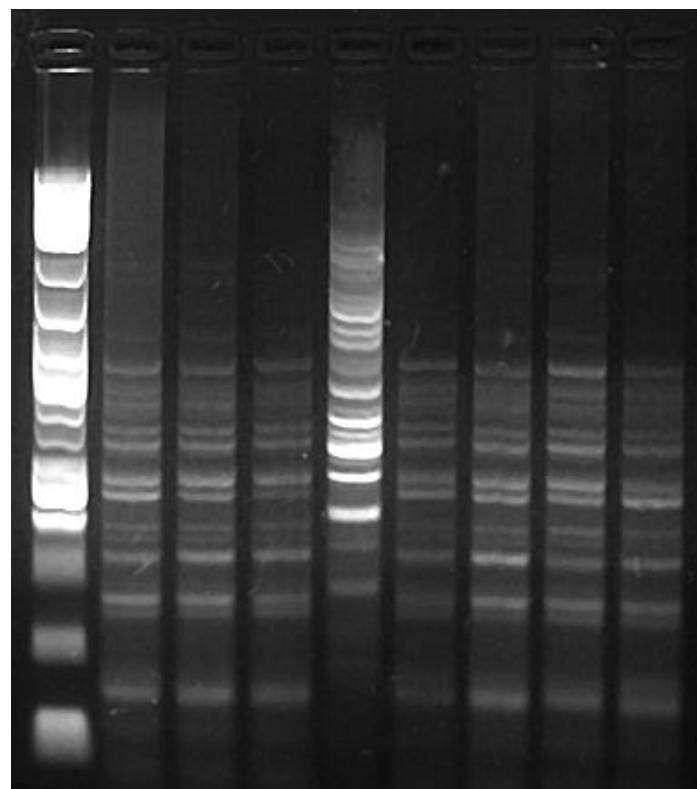
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	24				54		54	54		
2000	28	59	59	59	59	59	59	59	59	
1500	33	65	65	65	65	65	65	65	65	
1200	38	70	70	70	70	70	70	70	70	
1000	42	74	74	74	74	74	74	74	74	
		78	78	78	78	78	78	78	78	
		80	80	80	80		80	80		
				85	85		85	85		
		88	88	88	88	88	88	88	88	

Agarose Gel 79: Passage 153 PCR product, Primer 6, digested by HinfI



Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
3000	27	58	58	58	58		58	58	58	
2000	32	65	65	65	65		65	65	65	
1000	48	75	75	75	75		75			
600	59	78	78	78	78		78			
500	63									

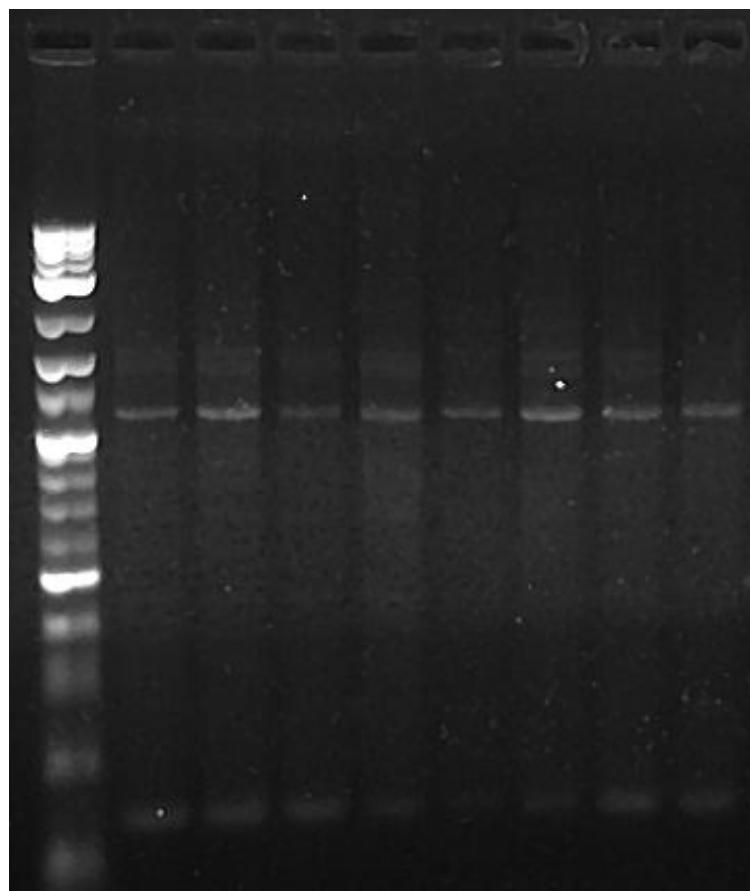
Agarose Gel 80: Passage 153 PCR product, Primer 6, digested by TaqI



Log 2 Marker		Migration Distance (mm)							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
1500	36				21				
1200	42				25				
700	52				27				
400	62	28	28	28	28		28	28	
300	69	30	30	30	30		30	30	30
					32				
					33.5				
					35				
		36.5	36.5	36.5	36.5	36.5	36.5	36.5	36.5
					38				
		42			42	42		42	42
		44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5
					48				
		49	49	49	49	49	49	49	49
		51	51	51	51	51	51	51	51
					53				
		56	56	56	56	56	56	56	56
		58	58	58		58	58	58	58
					61				

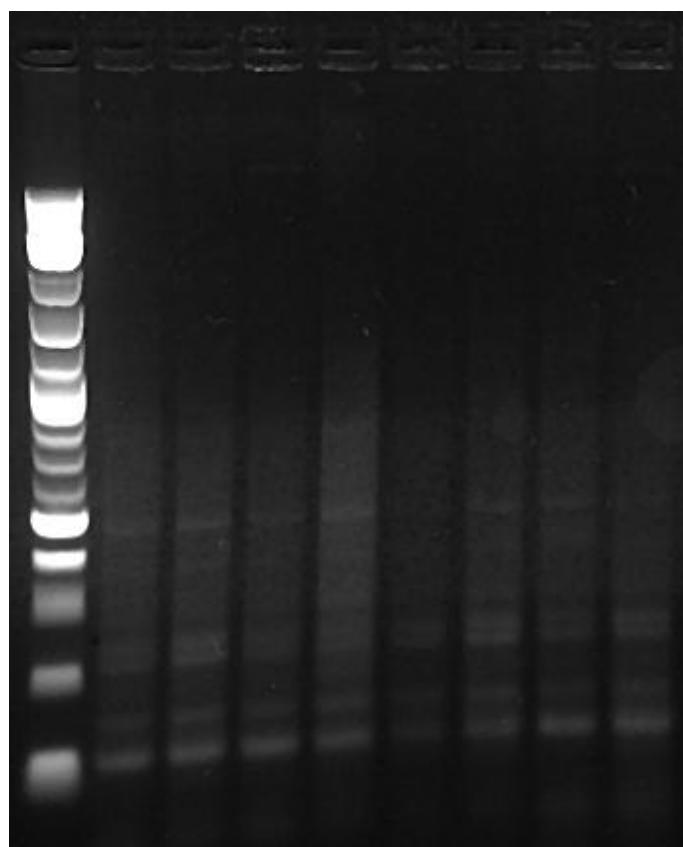
		62	62	62		62	62	62	62
		63	63	63		63	63	63	63
					64				
		65	65	65		65	65	65	65
		69				69	69		
					70				
		71	71	71		71	71	71	71
		73	73	73		73	73	73	73
		83.5	83.5	83.5		83.5	83.5	83.5	83.5
		89	89	89		89	89	89	89

Agarose Gel 81: Passage 153 PCR, Primer 7



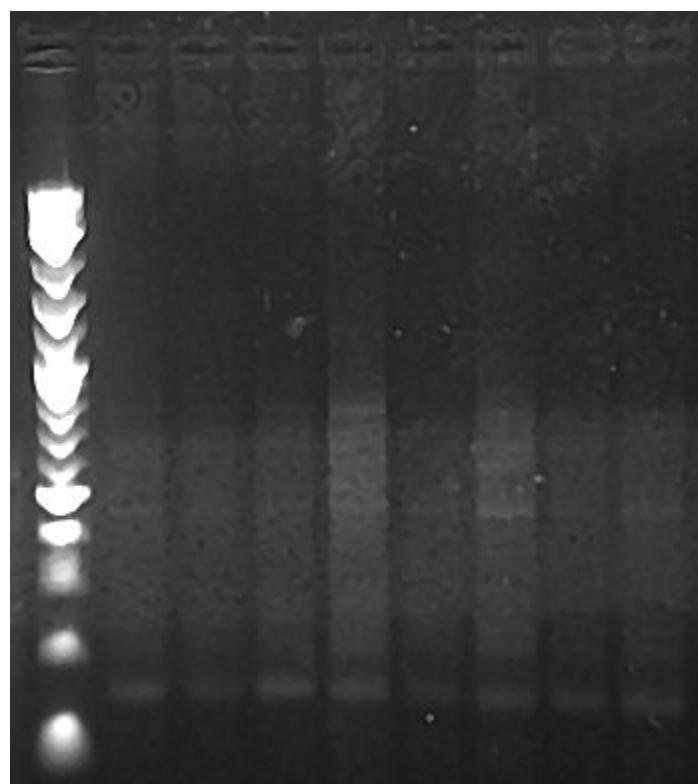
		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
3000	32	46	46	46	46	46	46	46	46
2000	36								
1000	53								
600	64								
500	69								

Agarose Gel 82: Passage 153 PCR product, Primer 7, digested by MspI



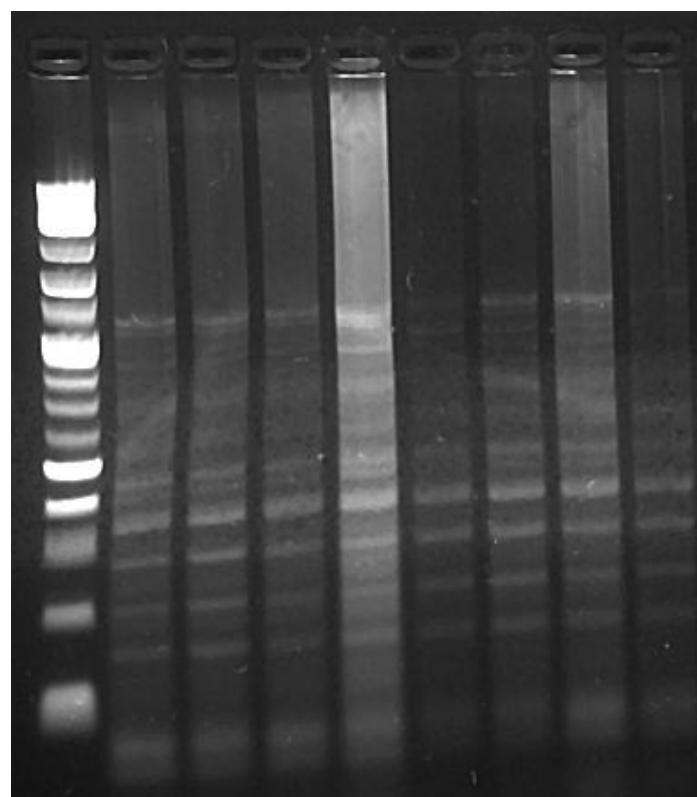
Log 2 Marker		Migration Distance (mm)								
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb	
2000	30	58	58	58	58		58	58	58	
1500	36		63		63		63	63	63	
1200	40	72	72	72	72	72	72	72	72	
800	49	74	74	74	74	74	74	74	74	
700	52	82	82	82	82	82	82	82	82	
		87	87	87	87	87	87	87	87	

Agarose Gel 83: Passage 153 PCR product, Primer 7, digested by HinfI



Log 2 Marker		Migration Distance (mm)									
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb		
3000	25	43	43	43	43	43	43				
2000	34	47	47	47	47	47	47	47	47		
1000	43	54	54	54	54		54				
600	52										
500	57										

Agarose Gel 84: Passage 153 PCR product, Primer 7, digested by TaqI



		Migration Distance (mm)							
Log 2 Marker		Samples							
Molecular Weight (bp)	Distance	H MSG	L MSG	H BA	L BA	H Salt	L Salt	H Comb	L Comb
1500	31	34	34	34	34	34	34	34	34
700	46	35	35	35	35		35	35	
400	59	38	38	38	38	38	38	38	38
300	65	40	40	40	40	40	40	40	
		45	45	45	45	45	45	45	45
		51	51	51	51	51	51	51	51
		55	55	55	55	55	55	55	55
		57	57	57	57		57	57	
		61	61	61	61	61	61	61	61
		65	65	65	65	65	65	65	65
		71	71	71	71	71	71	71	71
		77	77	77	77	77	77	77	77

## Appendix G – Dissimilarity Index

The Dissimilarity Index (DI) obtained from the Nei-Li Dissimilarity Index calculation is tabulated into the following matrixes according to PCR/RFLP count.

### PCR/RFLP #1 (Passage 82)

	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
H MSG	0							
L MSG	0.180	0						
H BA	0.150	0.110	0					
L BA	0.148	0.091	0.131	0				
H SALT	0.183	0.207	0.094	0.221	0			
L SALT	0.220	0.196	0.115	0.216	0.040	0		
H COMB	0.093	0.142	0.155	0.117	0.175	0.144	0	
L COMB	0.160	0.138	0.181	0.101	0.247	0.235	0.097	0

### PCR/RFLP #2 (Passage 94)

	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
H MSG	0							
L MSG	0.151	0						
H BA	0.118	0.033	0					
L BA	0.094	0.155	0.145	0				
H SALT	0.085	0.124	0.144	0.063	0			
L SALT	0.158	0.051	0.047	0.160	0.125	0		
H COMB	0.156	0.030	0.069	0.150	0.115	0.035	0	
L COMB	0.072	0.014	0.052	0.050	0.032	0.051	0.029	0

### PCR/RFLP #3 (Passage 106)

	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
H MSG	0							
L MSG	0.038	0						
H BA	0.059	0.042	0					
L BA	0.079	0.085	0.117	0				
H SALT	0.072	0.030	0.063	0.111	0			
L SALT	0.053	0.028	0.070	0.109	0.019	0		
H COMB	0.091	0.052	0.084	0.091	0.053	0.051	0	
L COMB	0.069	0.034	0.064	0.100	0.037	0.035	0.027	0

**PCR/RFLP #4 (Passage 117)**

	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
H MSG	0							
L MSG	0.131	0						
H BA	0.044	0.104	0					
L BA	0.052	0.118	0.025	0				
H SALT	0.063	0.153	0.058	0.047	0			
L SALT	0.019	0.135	0.035	0.060	0.065	0		
H COMB	0.135	0.154	0.133	0.123	0.123	0.133	0	
L COMB	0.061	0.126	0.056	0.045	0.038	0.058	0.090	0

**PCR/RFLP #5 (Passage 129)**

129	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
H MSG	0							
L MSG	0.148	0						
H BA	0.239	0.094	0					
L BA	0.204	0.129	0.104	0				
H SALT	0.216	0.152	0.117	0.106	0			
L SALT	0.166	0.056	0.071	0.138	0.130	0		
H COMB	0.153	0.075	0.089	0.156	0.153	0.020	0	
L COMB	0.244	0.126	0.102	0.134	0.092	0.092	0.112	0

**PCR/RFLP #6 (Passage 141)**

141	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
H MSG	0							
L MSG	0.014	0						
H BA	0.105	0.117	0					
L BA	0.043	0.029	0.109	0				
H SALT	0.146	0.159	0.055	0.152	0			
L SALT	0.044	0.030	0.122	0.034	0.154	0		
H COMB	0.047	0.034	0.131	0.050	0.165	0.057	0	
L COMB	0.073	0.059	0.125	0.069	0.152	0.076	0.033	0

**PCR/RFLP #7 (Passage 153)**

153	H MSG	L MSG	H BA	L BA	H SALT	L SALT	H COMB	L COMB
H MSG	0							
L MSG	0.022	0						
H BA	0.058	0.053	0					
L BA	0.074	0.060	0.111	0				
H SALT	0.188	0.206	0.152	0.259	0			
L SALT	0.041	0.020	0.074	0.071	0.192	0		
H COMB	0.183	0.107	0.153	0.143	0.202	0.079	0	
L COMB	0.127	0.127	0.161	0.164	0.187	0.103	0.024	0