

GENE FLOW AMONG POPULATIONS OF THE MAYFLY *Epeorus pleuralis* (BANKS
1910) (EPHEMEROPTERA:HEPTAGENIIDAE) IN THREE ADJACENT
APPALACHIAN HEADWATER STREAMS

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Thesis Prepared for the Degree of
MASTER OF SCIENCE

UNIVERSITY OF NORTH TEXAS

May 2006

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Dunlap, Rebecca, Gene Flow among Populations of the Mayfly *Epeorus pleuralis* (Banks 1910) (Ephemeroptera: Heptageniidae) in Three Adjacent Appalachian Headwater Streams. Master of Science (Biology), May 2005, 105 pp., 8 tables, 2 illustrations, references, 42 titles.

Dispersal of aquatic insects is difficult to measure with traditional direct trapping methodologies. However, genetic markers are an ideal surrogate to indirectly infer dispersal and gene flow. For this research, a portion of the cytochrome oxidase I gene was used to evaluate gene flow and dispersal of *Epeorus pleuralis* located in the northern Appalachian headwater streams of the Allegheny, Genesee, and Susquehanna watersheds. A total of 536 basepairs from 16 individual insects were used for analysis. Thirteen haplotypes were discovered, two of which were shared between the Allegheny and Genesee streams. Although no shared haplotypes were found in the Susquehanna, analysis of molecular variance results suggest that there is not a significant genetic difference between the three populations and attributes the majority of variation to within population differences.

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INTRODUCTION

Aquatic Insect Dispersal

Aquatic insect dispersal has been largely unstudied, despite its importance in the colonization of new habitats, entry of aquatic species into the terrestrial food web, and association with species-specific developmental or reproductive behavior (Kovats et al. 1996). According to Muller (1982), aquatic insect flight movements can be interpreted to be a consequence of food search, copulation rituals, or propagation. Furthermore, propagation flight and subsequent colonization cycles are thought to be either overwintering strategies, compensations for downstream drift, or simply exploitations of different biotypes.

Aquatic insect flight dispersal can be measured directly with attracting traps (i.e. light traps), non-attracting traps (i.e. Malaise traps), or capture-mark-recapture methods (Schultheis 2000). Kovats et al. (1996) utilized light traps to examine the inland dispersal of hydroptychid caddisflies and species of the *Hexagenia* mayfly. The authors determined that inland dispersal of these adult aquatic insects was limited, because most specimens were collected within 100 meters of the water. Similar results were found by Peterson et al. (1999) who measured movement of stoneflies and caddisflies with bilateral Malaise traps. The majority of captured adult stoneflies traveled less than 16 and 51 meters, respectively, from the stream. Caddisfly adults showed a similar pattern, and the authors concluded that this may be a reason why recolonization of disturbed streams is sometimes prolonged (Peterson et al. 1999). A problem associated with measuring dispersal with attracting and non-attracting traps is

that the proportion of individuals caught may not represent the entire population since small trap size limits the number captured (Schultheis 2000).

Another direct method to measure dispersal as well as levels of gene flow between populations is with capture-mark-recapture techniques. This involves the capture of insects, giving each a characteristic mark or number, and subsequently releasing and recapturing them at varying time intervals. This technique was used to estimate gene flow of eight Belgium populations of the adult damselfly *Lestes viridis* (Linden, 1825) (Geenen et al. 2000). *L. viridis* were released and recaptured daily or at 2-day intervals during a 1-month peak flying period. Forty-seven percent of the marked individuals were recaptured, but none were found to move between water bodies (0.2 to 28.4 km apart). These results indicate that *L. viridis* adults do not disperse but demonstrate a high fidelity to breeding ponds.

Potential problems of the capture-mark-recapture method are the possibility of behavior change of the marked individuals (Bilton et al. 2001) and the inability to mark fragile, teneral adults. Indirect measures of dispersal (allozyme electrophoresis and isoelectric focusing) of *L. viridis* revealed high gene flow estimates in three of the aforementioned study sites. This illustrates that dispersal of *L. viridis* is primarily by teneral adults, since they could not be included in the capture-mark-recapture study (Geenen et al. 2000).

Indirect measures of dispersal infer patterns of movement between populations by using the relationship between dispersal and population genetic structure. This technique is valuable, because it can be used to determine both the extent and likely mechanism (i.e. adult or larval) for dispersal (Schultheis 2000). If movement is primarily

by immature insects, most genetic differentiation will occur between populations in different drainages but if primary dispersal is by adults, populations in nearby but independent drainages will show either little or no genetic differentiation (Gibbs et al. 1998). Furthermore, population genetic theory states that high levels of dispersal among species will result in the mixing of alleles and produce homogeneity of gene frequencies (Hughes et al. 1999). Conversely, lack of a dispersal mechanism or failure to distribute between adjacent areas will result in the absence of appreciable levels of gene flow and perhaps reproductive isolation followed by speciation (Bilton et al. 2001).

When indirectly measuring dispersal, it is often assumed that the population in question has reached equilibrium between gene flow and genetic drift (Hughes et al. 2003). This requires that the rate at which gene frequencies become homogenized is equal to the rate at which they become differentiated. It has been estimated that the time it takes a population to reach the halfway point to equilibrium is $t_{1/2} = (\ln 2) / (2m + 1/N_e)$ where $t_{1/2}$ is time in generations, m is the probability of migration per generation, and N_e is the effective population size (Hughes et al. 2003). Therefore, small populations will meet the equilibrium between gene flow and genetic drift at a faster rate than will large populations.

A synthesis of observed relationships between genetic population structure and dispersal modes was proposed by Monaghan et al. (2002). The authors explain that species experiencing equilibrium between genetic drift and gene flow will show an increase in genetic differentiation with spatial distance and that two spatial dependent types of equilibrium are possible. The first, drainage equilibrium, is reached when there is sufficient gene flow within and among streams but not between drainages. This

equilibrium would be expected for populations of strong fliers or those who can readily disperse. Drainage equilibrium has been observed in the mayfly *Rithrogenia loyolaea* (Navas) (Monaghan et al. 2002) and caddisfly *Helicopsyche borealis* (Hagen) (Jackson and Resh, 1992). The second, stream equilibrium, is reached when there is sufficient gene flow within streams but not at larger scales. Taxa with limited dispersal capabilities are the most likely to be at this equilibrium (Monaghan et al. 2002). Stream equilibrium has been observed in populations of the water strider *Aquaris remigis* (Gerris) (Preziosi and Fairbairn, 1992) and the atyid shrimp *Paratya australiensis* (Kemp) (Hughes et al. 1995).

According to Monaghan et al. (2002), species that have not reached gene flow and genetic drift equilibrium will not demonstrate an increase in genetic differentiation as spatial distance increases. One type of non-equilibrium is at the reach level where reduced genetic differentiation is seen at a large scale (i.e. drainages and streams) but markedly higher differentiation is found at small scales (i.e. within streams). Small-scale differences are thought to be the product of recent evolutionary events such as repeated bottlenecks or oviposition by a limited number of females. Reach non-equilibrium has been observed in the mayfly *Baetis alpinus* (Pictet) (Monaghan et al. 2002) and the caddisfly *Tasiagma ciliata* (Neboiss) (Hughes et al. 1998).

When high genetic differentiation is found only among streams, the species is considered to be at stream non-equilibrium (Monaghan et al. 2002). These species are believed to be biogeographically widespread, have recently colonized areas from large populations, and maintain low gene flow among streams. Stream non-equilibrium is a possible explanation of the genetic structure of *Yoraperla brevis* (Banks) (Hughes et al.

1999) populations. Dispersal and population genetic structure have been indirectly quantified using molecular markers such as allozymes (Hughes et al. 1999, Jackson and Resh 1991), and mitochondrial DNA (Ravel et al. 2001, Gibbs et al. 1998, Schultheis 2000, Zhang et al. 1995, Simon et al. 1994).

Allozyme Electrophoresis

Allozymes generate variants of an isozyme enzyme system that are encoded by different alleles of the same gene locus (Karp *et al.*, 1998). Isozymes mark allelic variation at a single locus causing different alleles to express different electrophoretic mobility (Karp *et al.*, 1998). Allozyme electrophoresis is considered a relatively cheap and effective way to measure genetic variation because results can often be achieved from 0.5-2.0 μ l of extract or from specimens whose body length does not exceed 0.5 mm (Herbert and Beaton 1993).

Allozyme electrophoresis has been used to successfully quantify dispersal and genetic structure of several aquatic insects (Hughes et al. 2003, Monaghan et al. 2002, Wilcock et al. 2001, Monaghan et al. 2001, Hughes et al. 1999, Jackson and Resh 1992, 1991, and Zurwerra et al. 1986). For example, in 1991, Jackson and Resh analyzed populations of *H. borealis* in three northern California streams. The authors concluded that *H. borealis* from geographically isolated sites may be genetically distinct and speculated that genetic structure may contribute to the differences in life history traits noted in the widely distributed species (Jackson and Resh 1991). A subsequent study compared the genetically differentiated *H. borealis* species of California to specimens collected from sites in Indiana, Oklahoma, and Pennsylvania (Jackson and

Resh, 1992). As expected, small genetic differences were found in populations located in the same drainage basins and large differences were found between populations from different basins. Allozyme electrophoresis revealed four different species of *H. borealis* that were validated by differences in the male genitalia and in 14 other morphological characteristics on the 9th and 10th abdominal segments (Jackson and Resh, 1992).

Similar results were found by Hughes et al. (1999), who measured genetic variation of the stonefly *Y. brevis* inhabiting seven streams in Montana. Due to the synchronous emergence of *Y. brevis* and the low regional temperatures restricting flight periods, the authors predicted little genetic differentiation between sites in the same stream as well as between the streams themselves. Allozyme electrophoresis results demonstrated small genetic variation within streams as predicted, but large variation between streams. The authors determined that the dispersal of *Y. brevis* is limited to within streams despite the insect's ability to move between catchments (Hughes et al. 1999).

Populations of the mayfly *Baetis alpinus* (Pictet) were also determined to be genetically differentiated among habitat fragments in alpine streams (Monaghan et al. 2001). The authors determined that *B. alpinus* dispersal over lakes was limited but that genetic differentiation was not limited to lake size. Instead, genetic differentiation occurred only if the lakes were located in valleys that were ice-free throughout the Holocene (Monaghan et al. 2001).

Monaghan et al. (2002) investigated how habitat fragmentation affected population genetics at multiple spatial scales using populations of the mayfly *B. alpinus*,

and *R. loyolae*, and the caddisfly *Allogamus auricollis* (Pictet). The authors measured genetic diversity within streams, among streams, and within major drainages of the Swiss Alps. Interestingly, results showed that *R. loyolae* was genetically distinct between drainages but not within or among streams, while *B. alpinus* showed significant genetic distance within and among streams but not drainages, and *A. auricollis* showed little or no difference among drainages or within streams (Monaghan et al. 2002).

The role of local and long distance dispersal by the adult caddisfly *Plectoncnemia conspersa* (Curtis) was examined in order to infer a pattern of recolonization of Britain after glacial refugium (Wilcock et al. 2001). The authors assumed that if *P. conspersa* exhibited limited dispersal, genetic structure would follow local drainage networks and that genetic structure at the large scale would reflect climate change. It was predicted that the populations did not have sufficient time to reach equilibrium between genetic drift and gene flow since the last glacial expansion. Results showed that the highest genetic variation was found between Britain and Mainland Europe, more so than within either of the areas themselves. Furthermore, Britain had considerable lower levels of heterozygosity, percent polymorphic loci, and allele numbers. In addition, observed and expected heterozygosity showed a significant negative relationship with northern latitudes. Surprisingly, genetic differentiation did not relate to the pattern of the drainage network, and the authors concluded that *P. conspersa* has the ability to colonize sites up to 10 km away (Wilcock et al. 2001).

Although allozymes have been successfully used to determine genetic distances in many populations, they are biparentally inherited and subject to selection, indicating that the genetic variability levels required for fine-scale dispersal studies may not be

achieved with their use (Schultheis, 2000). Therefore, other molecular markers (i.e. mitochondrial DNA) may be more suited for population genetic studies.

Microsatellite and Mitochondrial DNA

Equilibrium between gene flow and genetic drift is reached faster in microsatellite and mitochondrial DNA because it requires a smaller effective population size (N_e) than does nuclear DNA (Hughes et al. 2003). To date, genetic differentiation has been determined using microsatellites and several portions of the mitochondrial genome (Gibbs et al. 1998) including the control region (Simon et al. 1994, Zhang et al. 1995, Schultheis 2000) and the cytochrome oxidase I gene (Hughes et al. 2003).

Microsatellite DNA is a tandem repeating sequence unit generally less than 5 base pairs (Bruford and Wayne, 1993). Microsatellites are found in a wide range of eukaryotic organisms and are often highly polymorphic due to their variation in number of repetitive units. Because this DNA is non-coding, it is ideal for sequence variation without interference from selection pressure (Haymer, 1994). Microsatellite DNA has been successfully used in a range of applications including genetic disease identification as well as population genetic studies (Jarne and Lagoda, 1996).

Specifically, this type of assay has been used to genetically differentiate populations of the mayfly *Siphonisca aerodromia* (Needham) (Gibbs et al. 1998).

According to Gibbs et al. (1998), *S. aerodromia* is a threatened species in Maine and has been listed as a candidate for the US Federal Endangered Species List (category 2), because only 13 populations have been located despite extensive search efforts. Populations of *S. aerodromia* were analyzed for genetic differentiation by

comparing microsatellite DNA sequences, and it was determined that all populations were genetically distinct from each other, even those located in the same river drainage. In fact, populations only 4 km apart in the same stream showed significant differences in allele frequencies (Gibbs et al. 1998).

Despite the relative success of microsatellites in population genetic studies, only a few publications have characterized this region in aquatic invertebrates (Williams et al., 2002). A potential problem associated with sequencing microsatellite DNA is that the presence of large and complicated GC-rich satellite sequences leave little or no single copy sequence in the insert in which to locate primers (Gibbs et al. 1998).

The mitochondrial control region (A + T rich region) is the only major non-coding portion of the insect genome. It is high in adenine and thymine nucleotides and is thought to evolve under a strong directional mutation pressure (Zhang et al., 1997). The mitochondrial control region has been successfully amplified and sequenced in several insects, including the fruit fly *Drosophila yakuba* (Simon et al., 1994), the locust *Schistocerca gregaria gregaria* (Zhang et al., 1995), and the stonefly *Peltoperla tarteri* (Schultheis, 2000).

Schultheis (2000) used the control region of mtDNA to study gene flow and dispersal among populations of *Peltoperla tarteri* (Needham & Kondratieff). *P. tarteri* demonstrates a semivoltine lifecycle denoting that the cohorts should reproduce every other year. Samples were collected from two consecutive summers and were assumed to be from different cohorts. Surprisingly, comparison of control regions showed that the cohorts were leaky or that there was enough gene flow between them to counteract

the effects of genetic drift and selection, making the two populations homogeneous (Schultheis 2000).

Recently, the cytochrome oxidase I gene has also proved useful in population genetic studies of aquatic insects. Hughes et al. (2003) used this region of the mitochondrial genome to determine if it shows the same pattern as allozymes in populations of the mayfly *Bungona narilla* (Harker). In a previous study, allozyme data showed the most genetic variation among pools within a stream, and, effectively, no variation among subcatchments and catchments (Hughes et al. 2003). The authors predicted that adult dispersal was actually limited and that this had not been detected with allozymes, because there was insufficient time to reach gene flow and genetic drift equilibrium. Surprisingly, the authors concluded that the mitochondrial DNA results did not differ from the allozymes and that genetic differentiation was non-significant at the largest spatial scale (Hughes et al. 2003).

Hughes et al. (2004) again used allozymes and a portion of the cytochrome oxidase I gene, this time to evaluate the dispersal of the montane mayfly *Baetis bicaudatus* (Dodds). Results from allozyme electrophoresis indicated that there was no evidence of isolation by distance. Conversely, results from a portion of the cytochrome oxidase I gene produced genetic differentiation an order of magnitude higher between different streams than among streams. The authors suggest that the difference in results may reflect different evolutionary rates of the markers or the difference in male and female dispersal, since allozymes are bi-parentally inherited and mtDNA is maternally inherited (Hughes et al. 2004).

For this research, the 1) the best molecular techniques were identified for DNA extraction and PCR clean-up of a portion of the cytochrome oxidase I gene of *Epeorus pleuralis* and 2) sequences from the cytochrome oxidase I gene were used to infer patterns of gene flow and dispersal of *E. pleuralis* populations in the northern Appalachian headwater streams of the Allegheny, Genesee, and Susquehanna watersheds.

E. pleuralis populations in these waters are hypothesized to be genetically similar, since the insect possesses a flight mechanism and the headwaters are closely located.

Ho: *E. pleuralis* populations in the Allegheny, Genesee, and Susquehanna headwaters are not genetically similar.

Study Organism

A larval description of *Epeorus pleuralis* has not been published. However, the genus *Epeorus* is known to be a Heptageniidae mayfly found in lotic erosional streams across the United States. They are classified trophically as collector gathers, specifically, scrapers (Merritt and Cummins, 1996) indicating that they feed on periphyton and algae that encrust stream debris. *Epeorus* sp. possess a distinctly depressed body and a prognathous head capsule that forms the entire dorsal surface. The frontal and anterolateral margins of the head are expanded with dense marginal setae. The labrum is 0.25 to 0.2 as wide as the head and the maxillary and labial palpi are two-segmented. Eyes and antennae are dorsally located, and two caudal filaments are present. Abdominal gills, composed of plate-like lamellae and a reduced fibrilliform

portion, are present on segments 1-7. Nymphs are found in shallow, cool rapidly flowing water in the southeast and northeast United States where they are attached to rocks, sticks, or other firmly anchored material (Edmunds, et al., 1976).

Nymphal development of *Epeorus* sp. has been observed to take 7 to 8 months. Upon completion of development, nymphs rise to the surface, and the subimagos immediately escape from the water. They have been observed crawling within a few centimeters of the water surface, emerging from their skin, and breaking the surface with wings fully exposed. Adults emerge in the early morning and late afternoon with wings measuring 7-19mm. Males possess large eyes that are usually dorsally contiguous with forelegs as long or slightly longer than the body (Edmunds, et al. 1976).

Epeorus pleuralis is reported to be univoltine in Erie Co., Pennsylvania with emergence occurring from late April to late May with peak emergence in mid-May (Grant et al. 1997).

Site Locations

Headwaters of the Allegheny, Genesee, and Susquehanna watersheds (Figure 1) are located in the Appalachian Plateau physiographic province, which extends from the western border of Pennsylvania to the Allegheny Front. The waters are located at the southern boundary of the Glaciated High Plateau Section of the province. This section consists of elongate uplands separated in most places from the adjacent Glaciated Low Plateau Section by a steep-sloped, well-defined escarpment. The southern boundary of the Glaciated High Plateau Section is by definition, the limit of the late Wisconsinan glaciation extent (Fenneman, 1938). Radiocarbon dating has

established that the late Wisconsinan glaciation occurred between 25,000 and 12,000 ybp, and that the ice reached its maximum extent in the Glaciated High Plateau approximately 20,000 ybp (Sevon and Fleeger, 1999). The Allegheny, Genesee, and Susquehanna rivers began flowing in their present day drainages with the retreat of the glacier (< 20,000 ybp).

Samples sites are located within a short distance from each other (Tables 1 and 2). Interestingly, despite their close location, each stream empties into a unique major water body. Specifically, the waters of the Allegheny flow into the Mississippi River, the Genesee into the St. Lawrence River, and the Susquehanna into the Chesapeake Bay.

Table 1. Spatial Location of the Allegheny, Genesee, and Susquehanna Watershed Headwaters

Headwater	N	W
Allegheny	41° 52.288	77° 50.236
Genesee	41° 51.711	77° 52.561
Susquehanna	41° 56.777	77° 41.124

Table 2. Approximate Distances Between the Allegheny, Genesee, and Susquehanna Headwaters (Kilometers)

	Allegheny	Genesee	Susquehanna
Allegheny	X	3.4	9.1
Genesee	3.4	x	7.7
Susquehanna	9.1	7.7	x



Figure 1. Headwaters of the Allegheny, Genesee, and Susquehanna Watersheds

METHODS

Larvae Collection

A qualitative collection method was implemented in which *E. pleuralis* larvae were hand picked off rocks and boulders and preserved in ethanol. Specimens were shipped to the University of North Texas on ice and stored at either -6°C or -80°C. Insects were collected from the headwaters of the Susquehanna in December of 2003 and insects from the Allegheny and Genesee waters were collected in April of 2004. The insects were identified as *E. pleuralis* based on previous species level descriptions made for the area and similarities in nymph morphology.

DNA Extraction

Several protocols for DNA extraction, PCR amplification, and PCR cleanup were evaluated using a variety of mayfly species (*Stenacron interpunctatum*, *Stenonema femoratum*, *Stenonema* sp., and *Stenonema viacarium*) (n=50), because a limited number of suitable *E. pleuralis* (n=37) were available.

A version of the guanidium thiocyanate (GTC) DNA extraction method (Hammond et al. 1996) was used to attempt DNA extraction from 32 individual insects. This process involves homogenization of single insects in a microcentrifuge tube with liquid nitrogen and a disposable polypropylene pestle. 0.5 ml of GITC/EDTA (0.5 M guanidium thiocyanate and 0.1 M EDTA) and 250 µl of ice cold ammonium acetate were added and the tubes mixed. Tubes were left on ice for 10 minutes and then mixed with 500 µl of a 24:1 chloroform:pentanol mix. Samples were centrifuged at 13,000 rpm for 10 minutes in a microcentrifuge after which the upper aqueous phase was recovered

and placed into a new tube. DNA was precipitated with the addition of 500 μ l of cold isopropanol; the tubes were mixed and stored at -80° C for 20 minutes. Tubes were centrifuged at 13,000 rpm at 4° C for 20 minutes. The supernatant was removed, and the DNA pellet was washed with 1 ml of 70% ethanol and centrifuged at 13,000 rpm at 4° C for 2 minutes. The supernatant was removed, and the DNA pellet was dried in a speed vacuum system. The pellet was resuspended in 100 μ l TE buffer and heated in a 65° C water bath for 5 minutes. The sample was then placed in a -80° C freezer for 2 minutes and mixed. 20 μ l of sample and 5 μ l of loading dye were run on a 0.8% agarose gel to ensure that the DNA had been properly isolated. The remainder of the sample was kept frozen until PCR protocols could be performed.

QIAGEN® DNeasy® tissue kit (QIAGEN, Inc.) methodology was used to isolate DNA from 54 individual specimens. Individual insects were homogenized in a microcentrifuge tube with liquid nitrogen. Buffer ATL (180 μ l) and 20 μ l of proteinase K was added to each tube. Samples were vortexed and incubated at 55° C until the tissue was completely lysed (1-3 hours). 200 μ l of Buffer AL was added to each tube, and samples were vortexed and incubated at 70° C for 10 minutes. 200 μ l of ethanol (96-100%) was added and samples were again vortexed. Tube contents were then removed with a pipette and placed into a DNeasy® spin column and a 2ml collection tube. The spin column was centrifuged at 8,000 rpm for 1 minute, and flow-through and collection tubes were discarded. The DNeasy® spin column was placed in a new 2ml collection tube and 500 μ l of Buffer AW1 was added. Samples were again centrifuged at 8,000 rpm for 1 minute and flow-through and collection tubes were discarded. The DNeasy® spin column was again placed in a new 2ml collection tube and 500 μ l of

Buffer AW2 was added. Samples were centrifuged for 3 minutes at full speed to dry the membrane. The DNeasy® spin column was placed in a clean 1.5 ml microcentrifuge tube, and 200µl of Buffer AE was added directly to the membrane. Samples were incubated at room temperature for 1 minute and centrifuged at 8,000 rpm for an additional minute. 20 µl of sample and 5 µl of loading dye were run on a 0.8% agarose gel to ensure that the DNA had been properly isolated.

Polymerase Chain Reaction (PCR) and Amplification

During polymerase chain reaction (PCR) two synthetic oligonucleotides (replication primers) are designed to be complementary to the sequences on the opposite strands of target DNA. The 3' end of the probes are oriented toward each other and the portion of DNA to be amplified is denatured by heating. A brief cooling period allows the target DNA and the oligonucleotide primers to bind. Four deoxynucleoside triphosphates (dNTP) are added and the primed DNA is selectively replicated. The cycling of this process 25 or 30 times allows minute amounts of DNA to be replicated to measurable size (Nelson and Cox, 2000).

The primers used by Hughes et al. (2003) (Table 3) to amplify 720 base pairs of the cytochrome oxidase I gene of *B. narilla* were used to amplify a similar size and region of this gene in *E. pleuralis*.

Table 3. Primers for Amplification of Mitochondrial Cytochrome C Oxidase Subunit I (Folmer et al. 1994)

<u>Name</u>	<u>Sequence</u>
LCO1490	5'-ggtaacaaatcataaagatattgg-3'
HCO2198	5'-taaacttcagggtgaccaaataatca-3'

Prior to PCR, a concentrated oligonucleotide stock (10x or 100x) was made from lyophilized primers. 1µl of each primer (10µM) was used in each 25µl PCR reaction that contained 22µl ddH₂O and 1µl undiluted template. The PCR program (Table 4) was cycled 30 times. 3 µl of PCR product was run on a 0.8% agarose gel and stained with ethidium bromide to ensure that the DNA was properly amplified.

Table 4. PCR Program (30 cycles)

Temperature	Time	Cycles
94°C	3 minutes	
94°C	30 seconds	5
44°C	30 seconds	
	1:15	
72°C	minutes	
94°C	30 seconds	25
46°C	30 seconds	
	1:15	
72°C	minutes	
72°C	4 minutes	

PCR Clean-Up

PCR reaction buffers, enzymes, primers, and dNTPs were removed from PCR products via 1) microCLEAN methodologies and 2) QIAquick Gel Extraction methodologies. 80µl of each 100µl PCR reaction and 80µl of microCLEAN was required to complete MicroCLEAN procedures. MicroCLEAN and PCR reactions were placed into a clean microcentrifuge tube, and contents were briefly vortexed. The solution was incubated at room temperature for 5 minutes and subsequently centrifuged on high speed for 5 minutes. The supernatant was removed and the solution was vortexed for an additional five minutes at high speed. Any remaining supernatant was removed and the tube contents were resuspended in 20-25µl of ddH₂O. 4µl of microcleaned product was added to a tube containing 2µl loading dye and 4µl of ddH₂O.

This mixture was run on a 0.8% agarose gel stained with ethidium bromide to ensure that the PCR reactants were properly removed.

The QIAquick Gel Extraction Protocol using a microcentrifuge was designed to extract and purify DNA of 70bp to 10kb from standard low-melt agarose gels in TAE or TBE buffer. Target DNA bands were excised from agarose gel with a clean razor blade and a black light. DNA fragments were placed into a pre-weighed colorless microcentrifuge tube. The mass of the agarose slice containing the target DNA was determined by weighing the tube and its contents and subsequently subtracting the empty weight of the tube from this result. Three volumes of Buffer QG was added for each one volume of gel (100 mg ~ 100 μ l). Microcentrifuge tubes were incubated at 50°C for 10 minutes or until the agarose gel was completely melted. Samples were vortexed every 2-3 minutes during incubation to help dissolve the agarose. Additionally, each sample was checked for a yellow color. The adsorption of DNA to the QIAquick membrane is efficient only at pH equal to or greater than 7.5. Buffer QG contains a pH indicator which is yellow at pH greater than 7.5 and orange or violet at a high pH (QIAGEN, 2002).

A QIAquick spin column was placed in a 2ml collection tube and 800 μ l of sample was applied. The spin column and collection tube were centrifuged at high speed for one minute to bind the DNA to the column. The flow-through was discarded, and the procedure was repeated for any remaining sample. Trace amounts of agarose were removed from the spin column with the addition of 0.5ml of Buffer QG and an additional spin at high speed for 1 minute. Samples were washed with the application of 0.75ml of Buffer PE to the spin column and centrifuged on high for one minute. The flow through

was discarded and the QIAquick column was centrifuged for an additional one minute at 13,000 rpm. The QIAquick column was placed into a clean microcentrifuge tube and DNA was eluted in 50µl of Buffer EB.

Sequencing and Alignment

An aliquot of cleaned DNA and stocks of 5µmol primers were forwarded to a University of North Texas technician for sequencing with an ABI 373 Automated Sequencer. Sequencing results (APPENDIX I) were viewed with Chromas Version 1.5 (Chromas, 1996) to verify a suitable sequencing product. Forward and reverse sequences were visually aligned with BioEdit Version 5.0.9 (Hall, T.A. 1999) and a 536 bp contiguous sequence, a consensus sequence from the region of overlap between the 5' and 3' sequences, was created for each insect.

Related sequences were obtained with a BLAST search (Altschul et al 1990), and a bit-score, E-value, and related species were recorded. A bit score is calculated by assigning a one to each match site and a zero to each mismatch site and subtracting penalties for gaps. Thus, the higher the bit score the more closely related the two individuals. An E-value represents the number of matches to the current nonredundant sequence that are expected by chance alone. The lower the E-value the higher the likelihood that the similarity is not a result of chance (Hall, 2001).

Genetic Variation

Arelquin population genetic analysis software version 2000 (Schneider et al. 2000) was used to determine haplotypes, calculate transition transversion ratios and

pairwise difference statistics, and create a minimum spanning tree. The tree was printed with TreeView Version 1.6.6 (Roderic D. M. et al. 2001) and rooted with the cytochrome oxidase I sequence from *Epeorus fragilis* obtained from the BLAST search (Altschul et al. 1990).

RESULTS

DNA Extraction

Of the 32 isolations completed with GTC techniques, approximately 35% did not produce visible bands of DNA. It is unclear whether the lack of DNA bands was a result of technician error or faulty reagents. QIAGEN DNeasy® methodology consistently produced visible bands of DNA in all 54 individual samples.

PCR Clean-Up

Procedures to remove PCR reactants with microCLEAN techniques were used successfully for eight individual insects and proved to be quick and efficient. QIAquick Gel Extraction procedures were used to purify PCR product from 46 insects. Although this procedure was more time consuming, it also consistently produced a clean, usable elution for sequencing.

Sequencing and Alignment

Related sequences for 21 insects were obtained with a BLAST search on the NCBI website. All sequences with a high Bit-score (>690) and low E-value (=0) relationship with known *Epeorus fragilis* sequences were kept for further analysis. Four sequences were considered contaminated and were subsequently eliminated because of their close relationship to known coleopteran or algal sequences. An additional sample was eliminated from further analysis because of the degraded quality of its sequence (Table 5).

Table 5. NCBI Blast Results from the 21 Insects Analyzed

Sample I.D.	Related Sequence	Bit-Score	E-Value
A1	Ephemeroptera: <i>Epeorus fragilis</i>	835	0
A2	Ephemeroptera: <i>Epeorus fragilis</i>	868	0
A3	Ephemeroptera: <i>Epeorus fragilis</i>	1233	0
A4	Ephemeroptera: <i>Epeorus fragilis</i>	781	0
A5	Ephemeroptera: <i>Epeorus fragilis</i>	860	0
A6	Coleoptera: <i>Cychnus brezinai</i>	48	5.00E-04
A7	Ephemeroptera: <i>Epeorus fragilis</i>	985	0
A8	Ephemeroptera: <i>Epeorus fragilis</i>	868	0
S1	Ephemeroptera: <i>Epeorus fragilis</i>	860	0
S2	Ephemeroptera: <i>Epeorus fragilis</i>	694	0
S3	Ephemeroptera: <i>Epeorus fragilis</i>	868	0
S4	Ephemeroptera: <i>Epeorus fragilis</i>	839	0
S5	Ephemeroptera: <i>Epeorus fragilis</i>	860	
G1	<i>P.littoralis</i> (algae)	90	7.00E-15
G2	Ephemeroptera: <i>Epeorus fragilis</i>	868	0
G3	Ephemeroptera: <i>Epeorus fragilis</i>	860	0
G4	Ephemeroptera: <i>Epeorus fragilis</i>	868	0
G5	Ephemeroptera: <i>Epeorus fragilis</i>	878	0
G6	Ephemeroptera: <i>Heptagenia adaequata</i>	343	3.00E-91
G7	Ephemeroptera: <i>Epeorus fragilis</i>	876	0
G8	<i>P.littoralis</i> (algae)	90	7.00E-15

Genetic Variation

Of the 536 base pairs that were analyzed, 67 sites were variable (Table 6) and 13 haplotypes were identified. Haplotype A3, from the Allegheny headwaters, had considerably more variable sites (n=43) than did the other 12 haplotypes (n= 1 to 16) (Table 6). Two haplotypes were shared between the Allegheny and Genesee Headwaters and eleven were unique (found only in a single individual). Shared haplotypes between the Allegheny and Genesee headwaters indicate that either 1) *E. pleuralis* are likely to disburse between the two streams or 2) there has not been sufficient time for the isolated populations to reach the equilibrium between gene flow and genetic drift.

Table 6. Variable Sites for 13 Haplotypes of the Cytochrome Oxidase I Gene from 16 *E.pleuralis* Individuals. Dashes Indicate Similarity to Halpotype B2. N Indicates an Undecipherable Site.

Haplotype	4	6	9	12	15	33	36	65	66	87	93	95	96	111	114	118	119	122	125	131
B2	T	A	C	G	T	T	A	C	A	T	C	G	T	-	G	T	T	A	G	G
B4			T														-			
B1					G												-			
B5																	-			
B3																	-			
A3		G	T	A			G				A		G	T	-		-		T	A
A2																	-			
A5							G										-			
A8	-																-			
A1									G				C			C	-			
A4						C		A		C		A					-			
G3																	-	G		
G5							G										-			

	140	148	149	155	167	174	191	197	202	206	215	218	221	231	236	265	274	278	281	284
B2	T	C	G	A	T	C	A	C	T	A	T	T	T	A	A	A	C	T	C	G
B4																				
B1		G						T												A
B5																				
B3						T	G													
A3	C			G	C	T			A	G	G	C	C	G	T	G				T
A2																				
A5																				
A8																				
A1									A		N					G				N
A4			A			T										G	A			
G3																				
G5																				

Table 6 (continued).

Haplotype	Position																			
	287	294	299	305	308	311	317	325	341	362	374	376	389	392	398	401	410	445	452	457
B2	G	T	T	A	A	A	G	C	A	A	C	A	T	T	G	T	T	T	C	A
B4													C	C						
B1													C				C			
B5													C	C						
B3													C							
A3	A		A	G	C	T	A	T	G	G	T		C		A			C	T	G
A2													C							
A5													C							
A8													C							
A1													C						C	
A4		C			T							G	C			C	C			
G3													C							
G5													C							

Haplotype	Position						
	461	473	488	505	515	521	532
B2	A	C	C	C	T	A	A
B4							
B1			T				
B5							
B3							
A3	G	T	T	T	A	G	
A2							
A5							
A8							
A1		T					T
A4		T			G		
G3							
G5			N				

The transition/transversion ratio for the Allegheny, Genesee, and Susquehanna samples was 42:13, 2:0, and 9:2, respectively. The ratio of the Allegheny was 2:1 upon removal of haplotype A3 from the analysis. Hughes et al. (2003) reports a transition/transversion ratio of 8:1 in the same region of the cytochrome oxidase I gene in samples of *B. bicaudatus*. Transitions, or the substitution of purine (A or G) for another purine or a pyrimidine (T or C) for another pyrimidine, are commonly thought to outnumber transversions, or the substitution of a purine for a pyrimidine or a pyrimidine for a purine, by at least 2:1 (Freeman et al. 2004).

Of the 67 variable sites, 29% (n=20) were determined to have occurred at 3rd position. This percentage increased to 67% (n=45) with removal of haplotype A3 from the analysis. Hughes et al. (2003) reports 85% (n=16) 3rd position changes in 19 haplotypes in the same region of the cytochrome oxidase I gene in samples of *B. bicaudatus*.

No significant pairwise difference was found ($F_{ST} = 0.02088$) among the three populations studied. Furthermore, 97.91% of the variation in the samples analyzed was attributed to within population variation (Table 7). However, removal of Haplotype A3 from the analysis resulted in a F_{ST} of 0.05087 and 94.9% of within population variation (Table 8). Although there was a F_{ST} of 0.05087 in the comparison among and within populations, no significant F_{ST} values were found between the populations.

Table 7. Pairwise Difference (F_{ST}) for all Haplotypes

Source of Variation	d.f.	SS	Variance Components	% of Variation	F_{ST}
Among Populations	2	11.104	0.10633	2.09	0.02088
Within Populations	13	64.833	4.91878	97.91	
Total	15	75.938	5.09351		

Table 8. Pairwise Difference (F_{ST}) for all Haplotypes Minus A3

Source of Variation	d.f.	SS	Variance Components	% of Variation	F_{ST}
Among Populations	2	6.467	0.13667	5.09	0.05087
Within Populations	12	30.6	2.55	94.9	
Total	14	37.067	2.8667		

The minimum spanning tree, (Figure 2) does not group haplotypes from the three different headwaters into distinct clades further supporting the results that there is no difference between the populations studied.

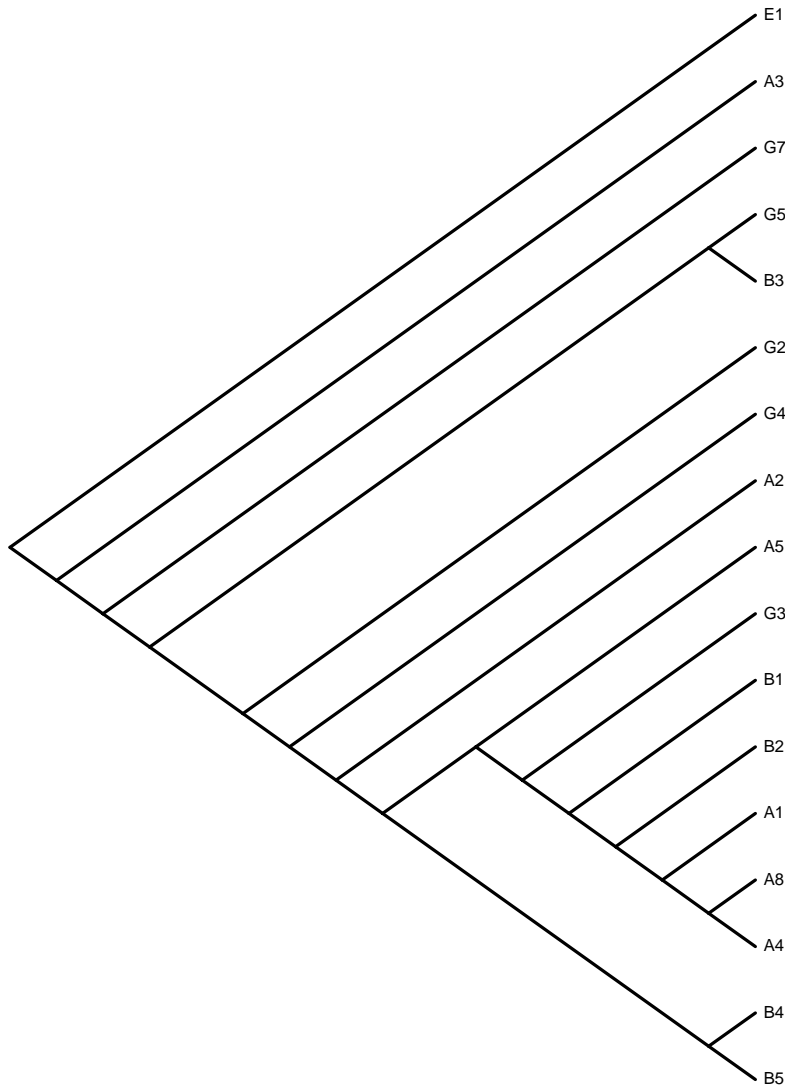


Figure 2. Minimum Spanning Tree for 13 Haplotypes from the 3 Headwaters (*E. fragilis* (E 1) = Outgroup).

DISCUSSION

Results of this study failed to reject the hypothesis that populations of *E. pleuralis* located in the headwaters of the Allegheny, Genesee, and Susquehanna are genetically similar. 536 basepair sequences of the cytochrome oxidase I gene from 16 *E. pleuralis* were used to infer patterns of gene flow and dispersal in the northern Appalachian headwater streams of the Allegheny, Genesee, and Susquehanna watersheds. Of the 13 haplotypes discovered, two were shared between Allegheny and Genesee. Although no shared haplotypes were found in the Susquehanna, analysis of molecular variance results suggest that there is not a significant genetic difference between the three populations and attributes most variation to within population differences.

A considerable amount of haplotype variability was attributed to Haplotype A3 and removal of this specimen from the pairwise statistical analysis increased the resulting F_{ST} . These results suggest that this specimen may possibly be a sibling species.

The Allegheny, Genesee, and Susquehanna headwaters began flowing in their present day drainages with the retreat of the ice from the late Wisconsinan glaciation period approximately 20,000 ybp. Historical populations of *E. pleuralis* are predicted to be homogenous in the three headwaters before the glacial retreat occurred. Results from this study indicate that the present day populations of *E. pleuralis* are also genetically similar. This implies that either 1) the populations are isolated and have not yet reached equilibrium between gene flow and genetic drift 2) the isolation was undetected due to the evolutionary rate of the cytochrome oxidase I gene and/or the restricted power of the analysis of molecular variance resulting from small sample size

or 3) *E. pleuralis* populations have been homogenous since the late Wisconsinan glaciation.

Gibbs et al. (1998) detected differences in populations of the mayfly *S. aerdormia* located only 4 km apart using microsatellite DNA. Since microsatellites are non-coding and do not experience selection pressure, they have a higher ability to detect population differences than does the maternally inherited cytochrome oxidase I gene. Therefore, it is possible that the populations of *E. pleuralis* became isolated as the headwaters began flowing into their respective drainages after the Wisconsinan glaciation and the isolation was undetected in this study, because of small sample size or the slower evolutionary rate of the cytochrome oxidase I gene.

It is also possible that the populations have experienced historical isolation and that there has not been sufficient time for the equilibrium between gene flow and genetic drift to be met. One explanation for this scenario is that *E. pleuralis* could not efficiently disperse between the headwaters until the area was heavily logged in the late 19th and early 20th centuries. During the period of heavy logging, *E. pleuralis* could have easily exchanged genes between the headwaters, and the populations could have experienced partial or complete homogenization. The ability of the insects to disperse may have decreased with increased forest regeneration, and there may not have been sufficient time for the cytochrome oxidase I gene to detect the recent re-isolation. This type of stream non-equilibrium has been cited as a possible explanation for the genetic structure of the stonefly *Yoraperla brevis* (Hughes et al. 1999).

It is likely that the populations of *E. pleuralis* in the three headwaters are in fact genetically similar given the close spatial relationship of the streams and the flight

mechanism of the insect. Similar results were found using the same region of the cytochrome oxidase I gene by Hughes et al. (2003) in populations of the mayfly *Bungona narilla*.

This likely scenario is of particular importance to the region. Peterson et al. (1999) concluded that recolonization of disturbed streams is sometimes prolonged due to the short distances traveled by some adult aquatic insects. In 2001, construction on the Wending Creek Golf Club, adjacent to the headwaters of the Allegheny, was halted leaving the stream vulnerable to high inputs of sediment and loss of aquatic life. It is unknown if populations of *E. pleuralis* were affected by the construction. However since the results of this study indicate that the populations of *E. pleuralis* in the three headwaters are genetically similar, it is likely that the Allegheny headwaters would be recolonized by populations of *E. pleuralis* from the Genesee and Susquehanna streams if its population were jeopardized.

APPENDIX

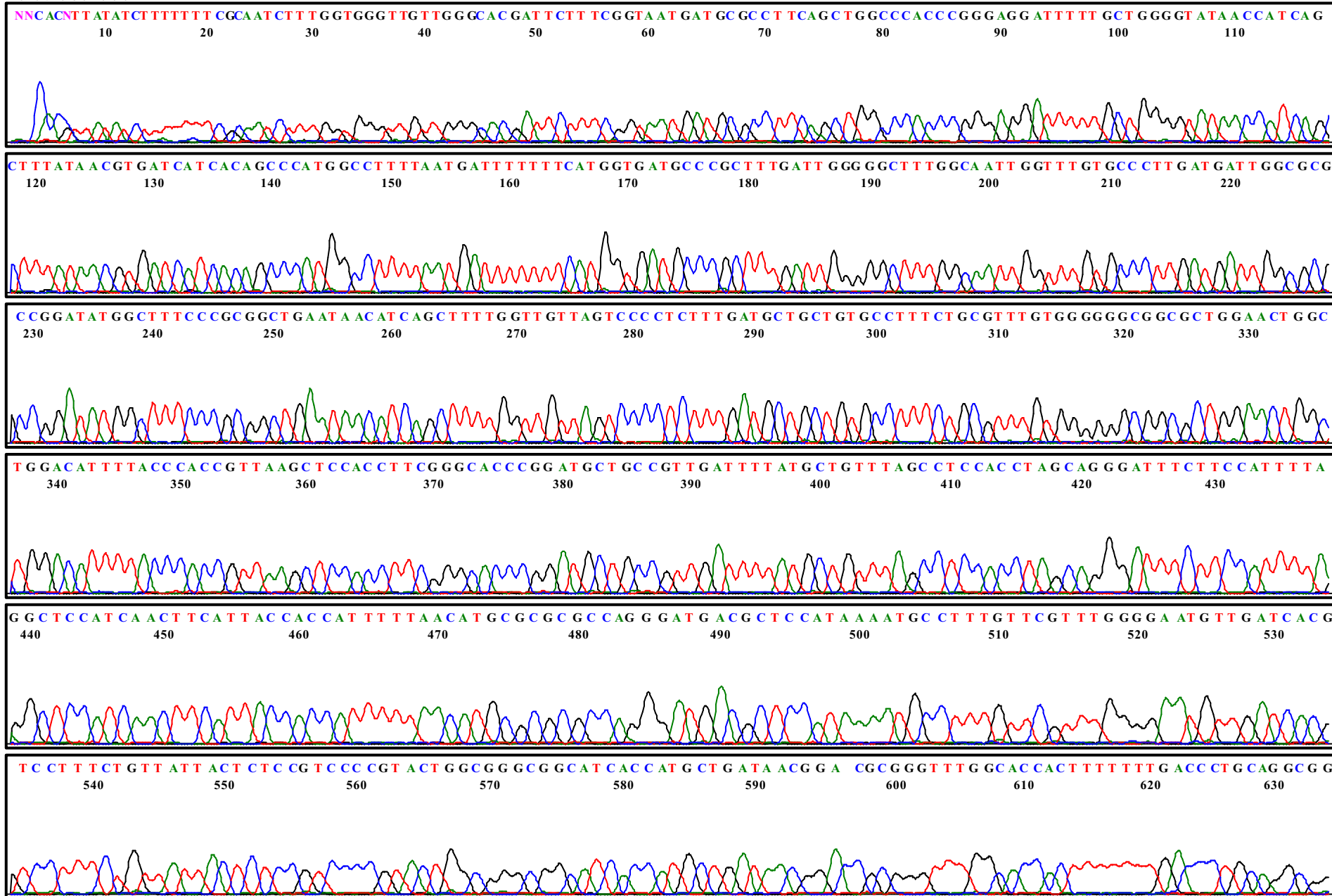


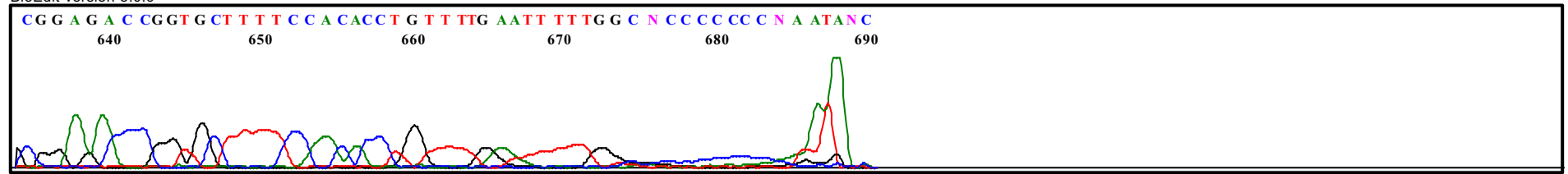
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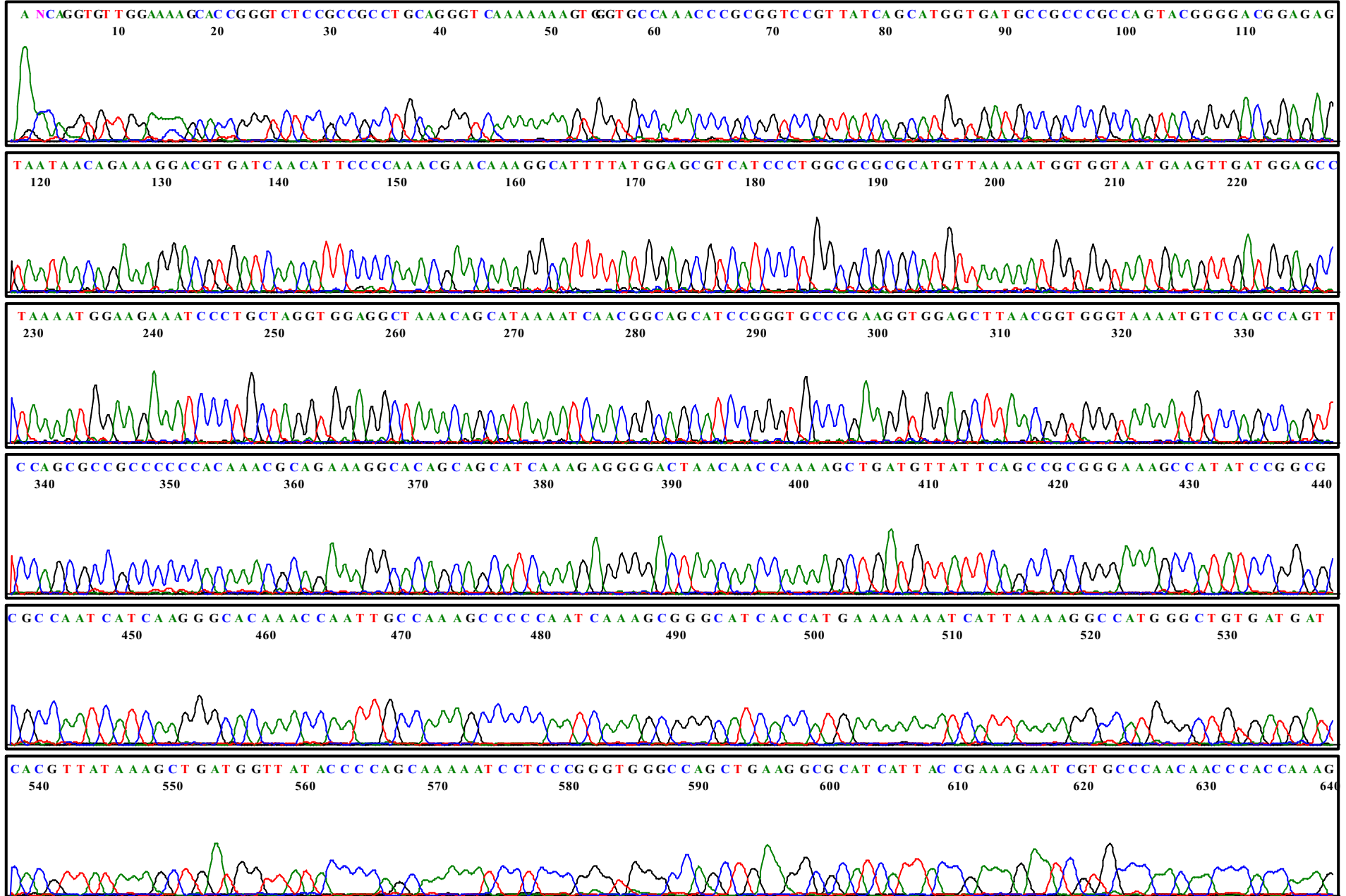
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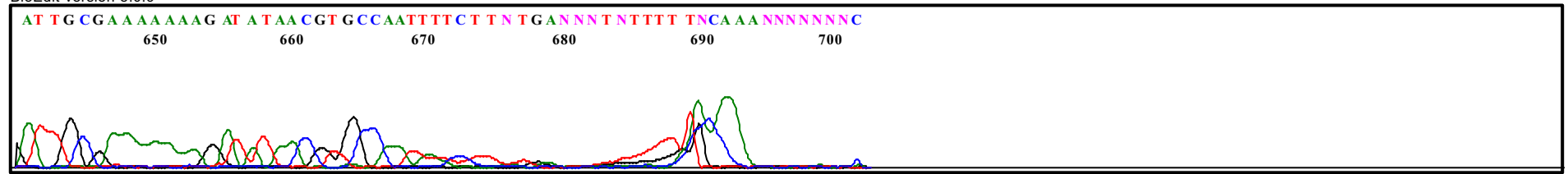
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Points 1087 to 9796

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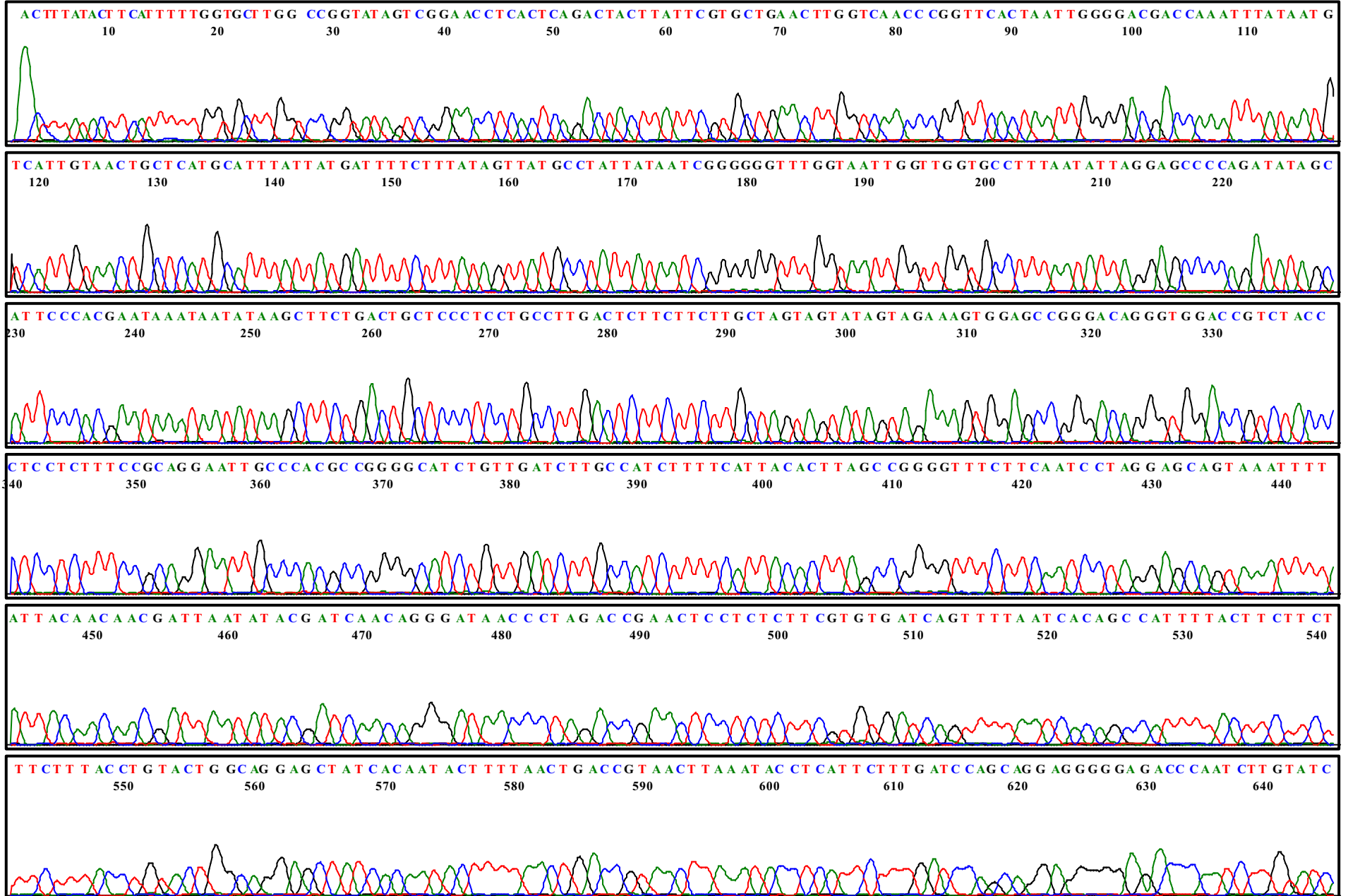
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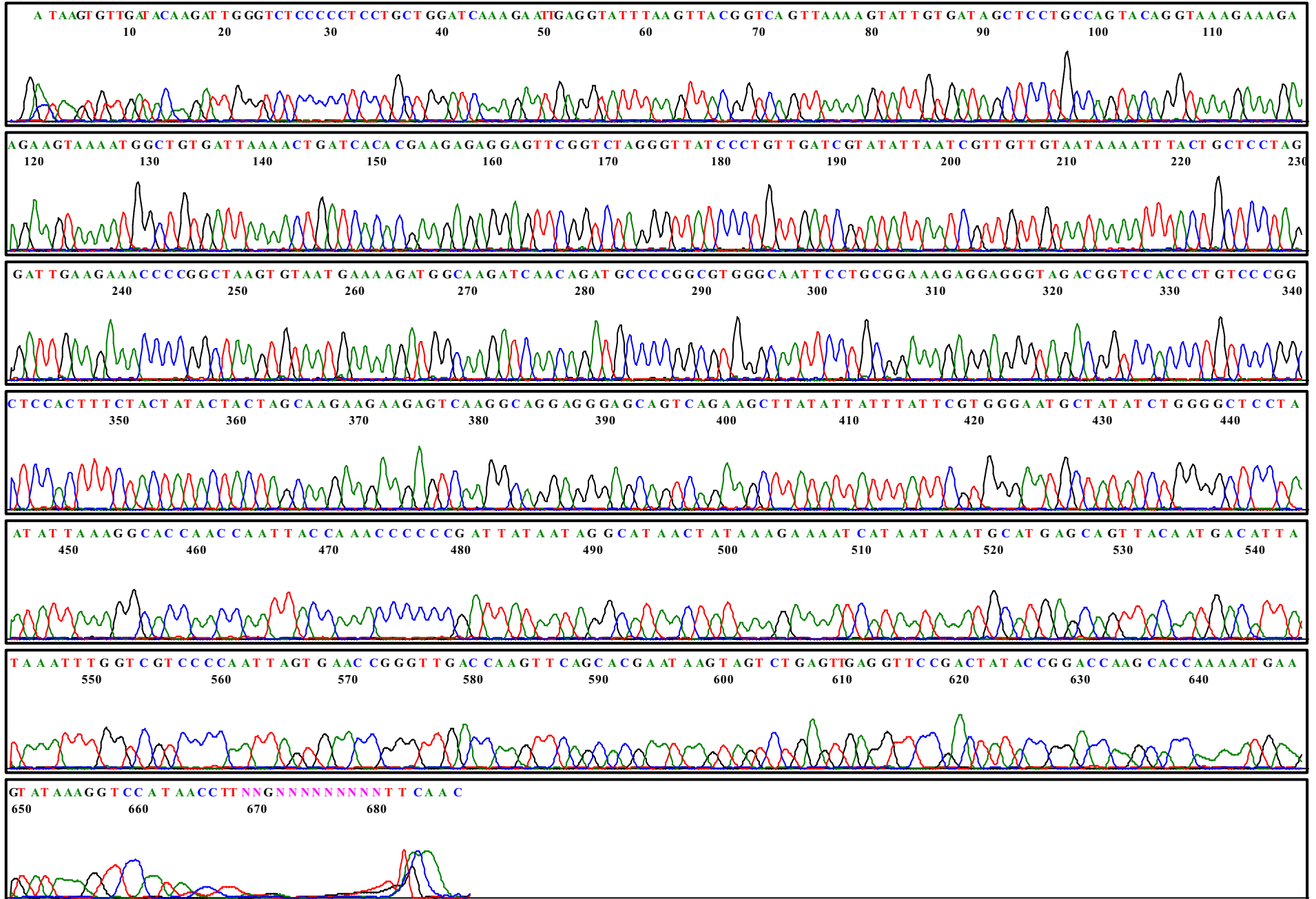
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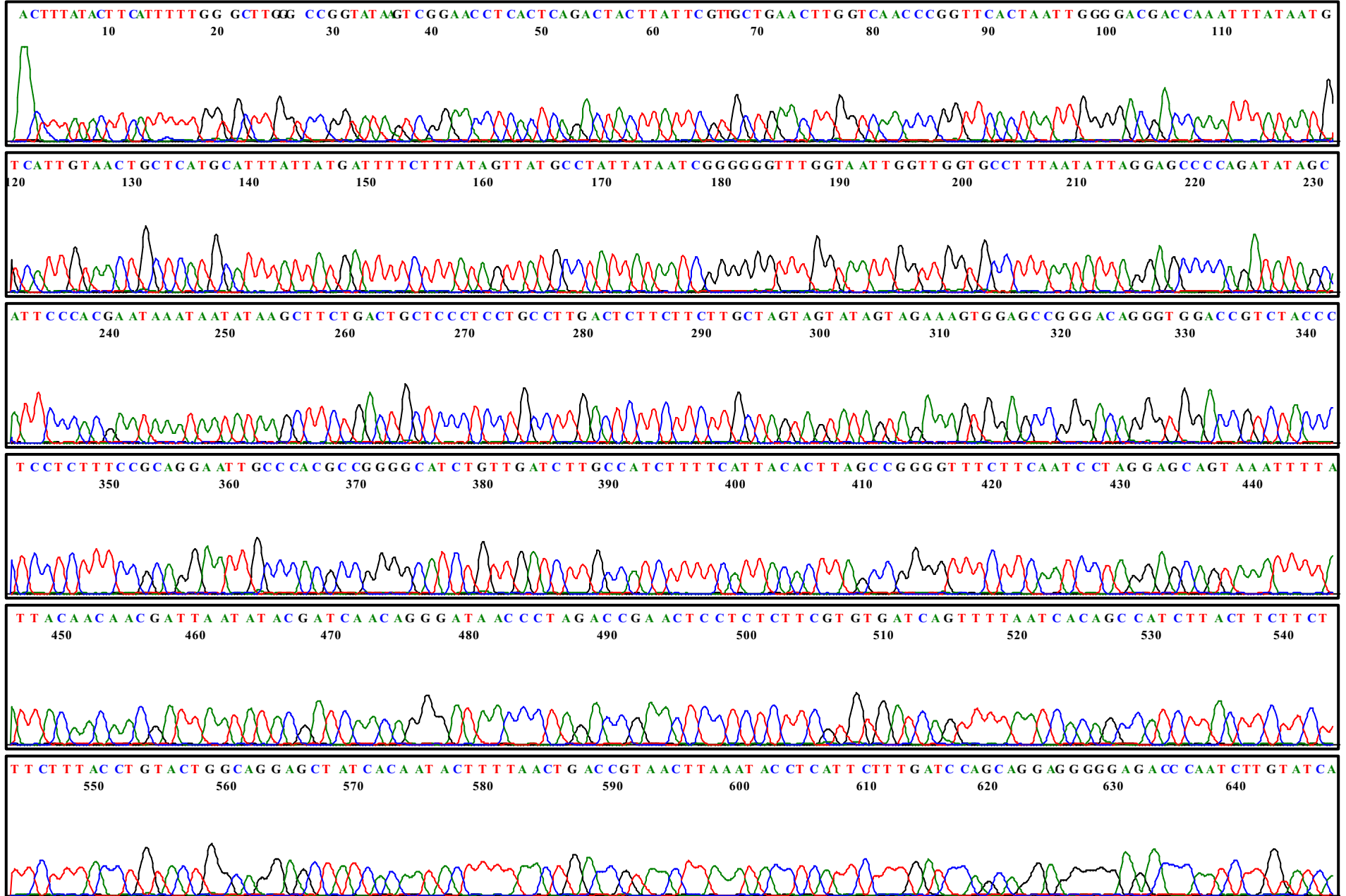
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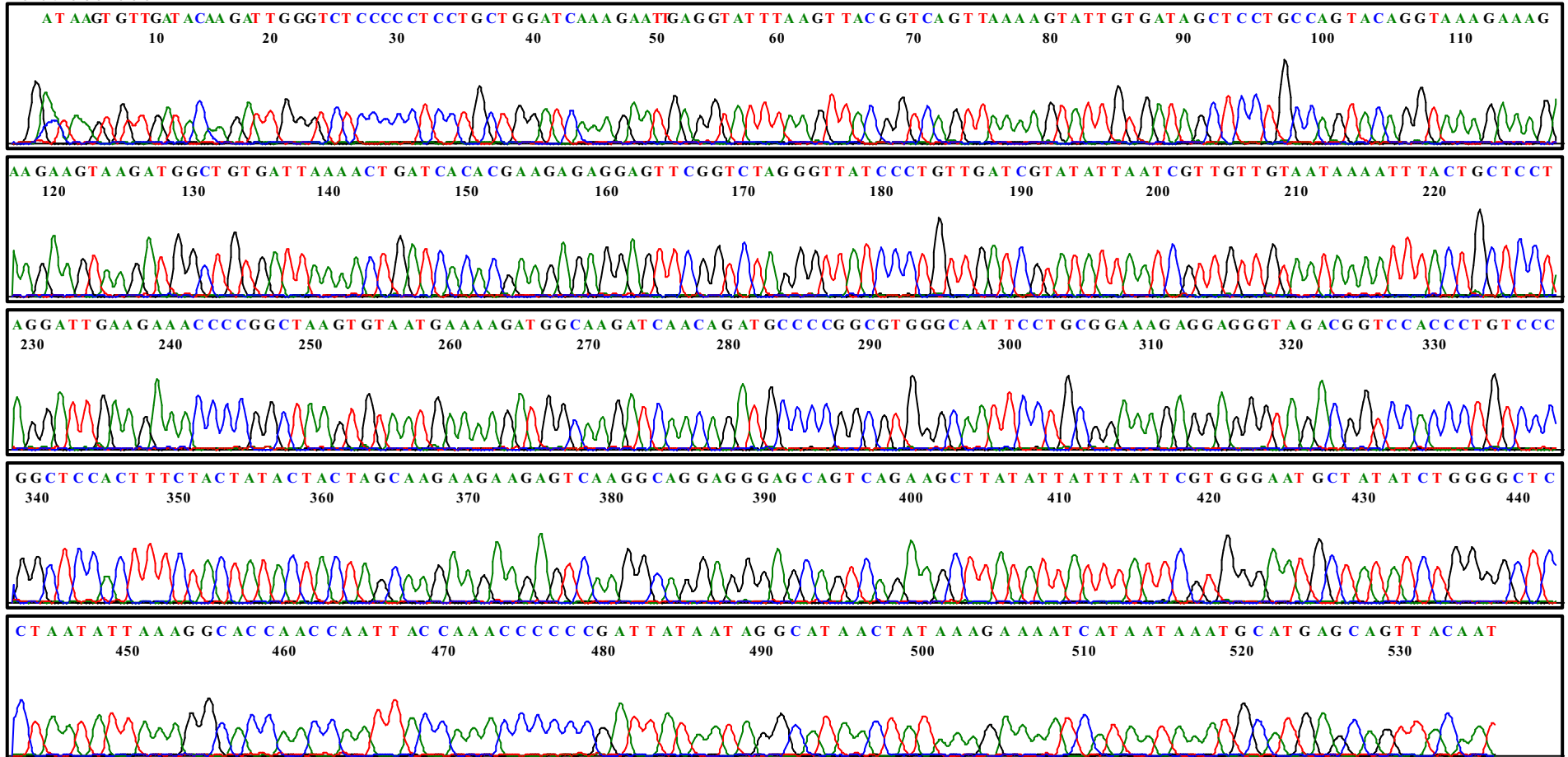














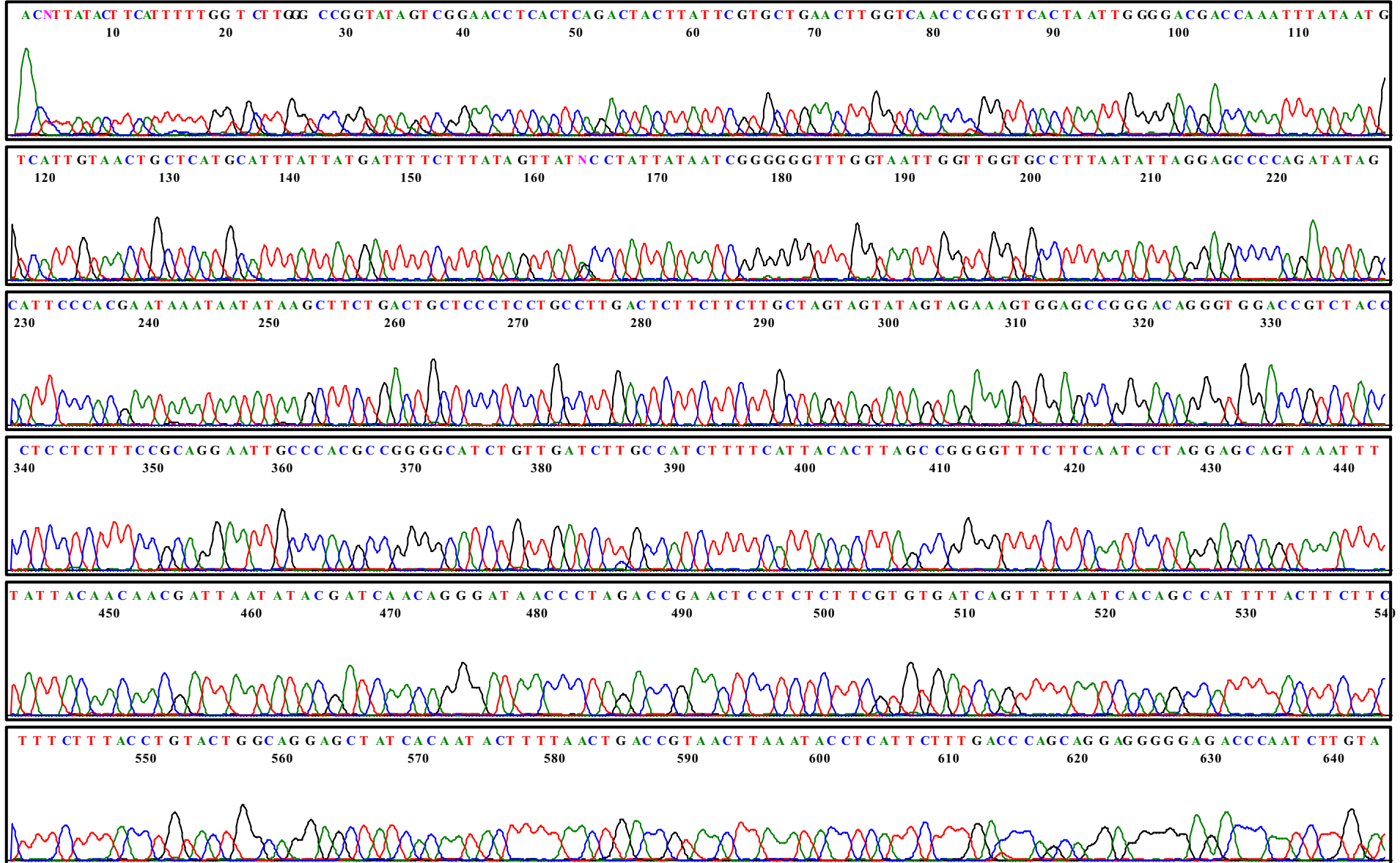
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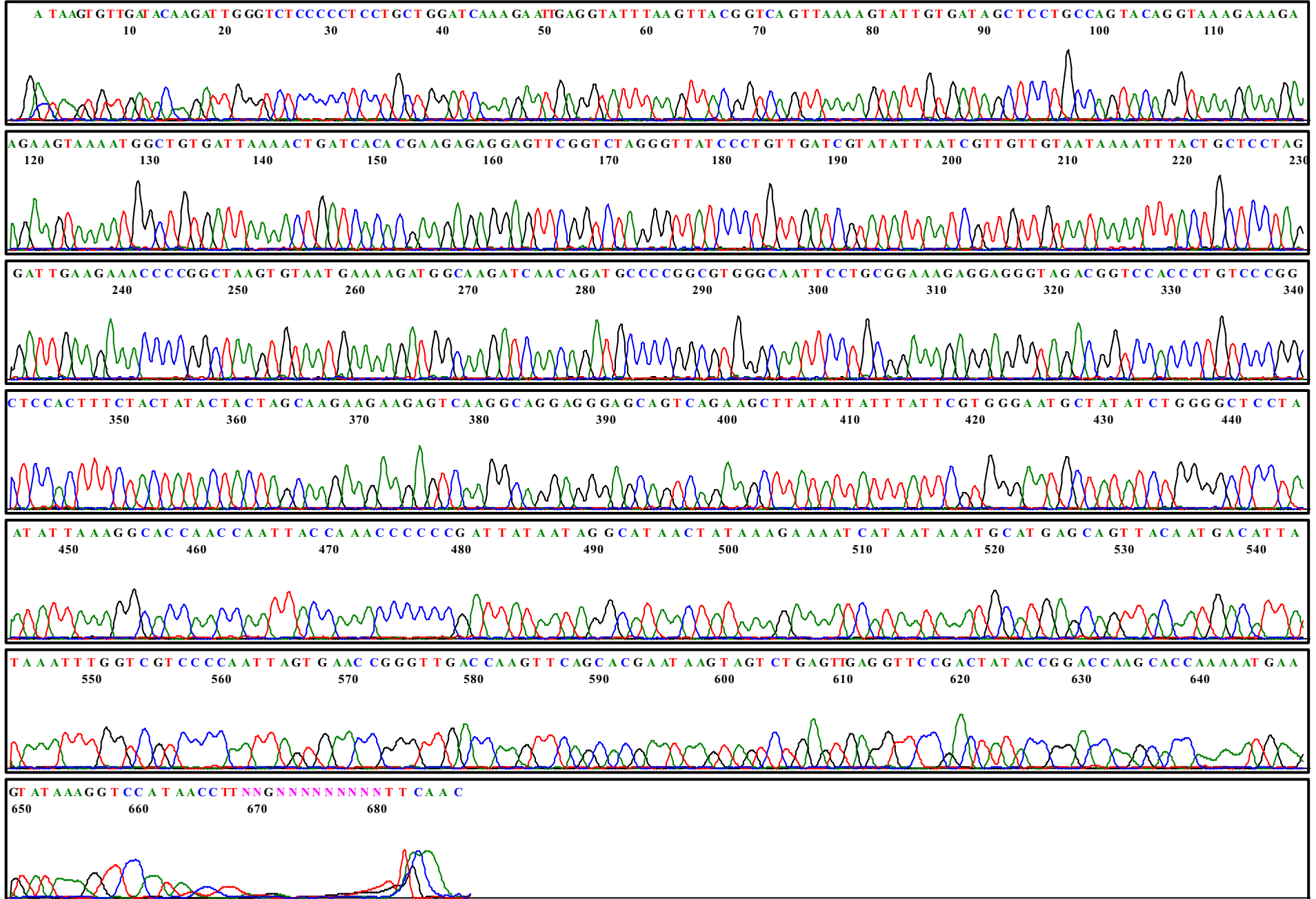
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dRhod2
Points 1099 to 9796

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Spacing: 9.64000034332275









BioEdit version 5.0.9

Model 373

ABI50

Version 3.0

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BIF

G6 LCO1490

Lane 23

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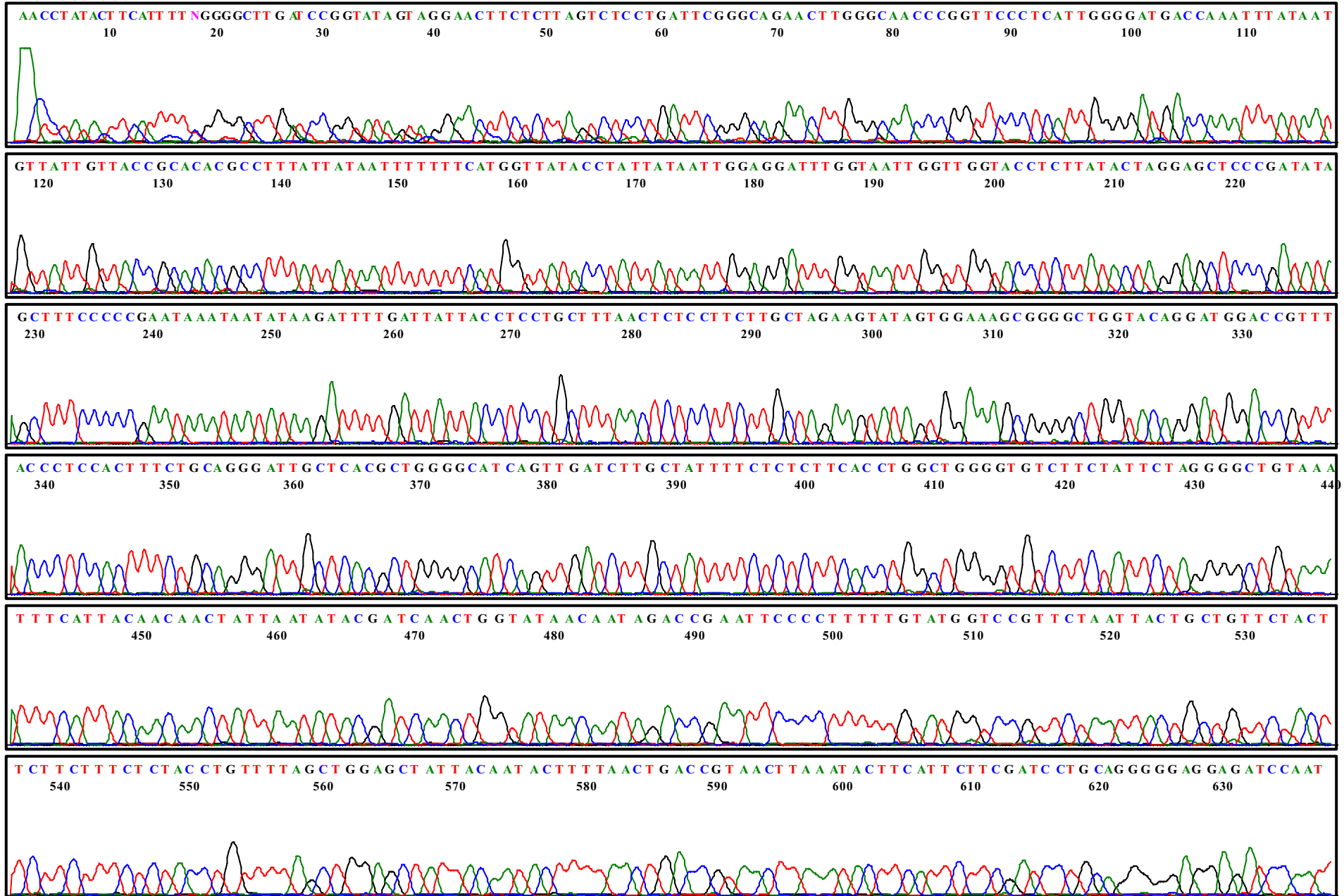
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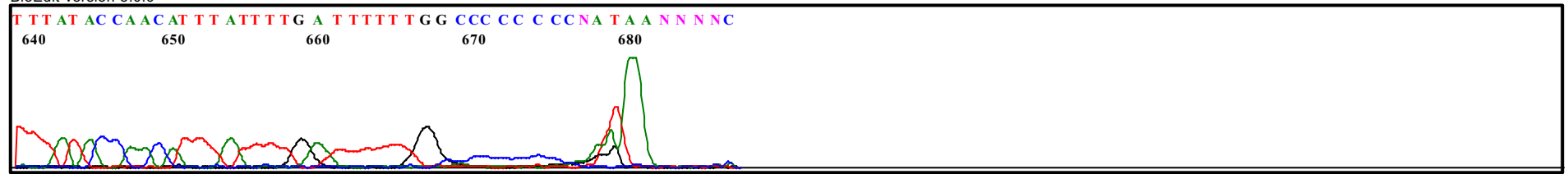
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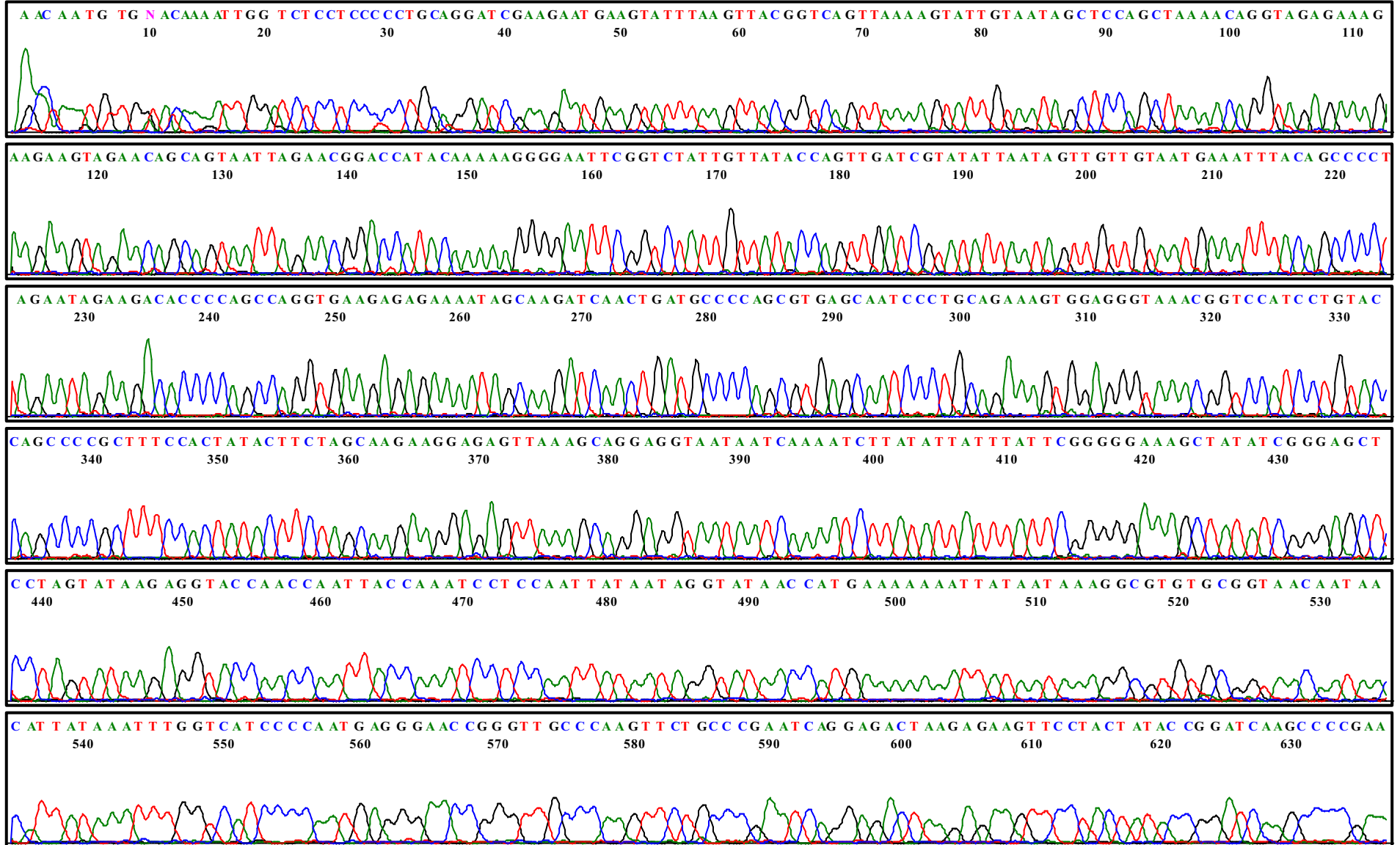
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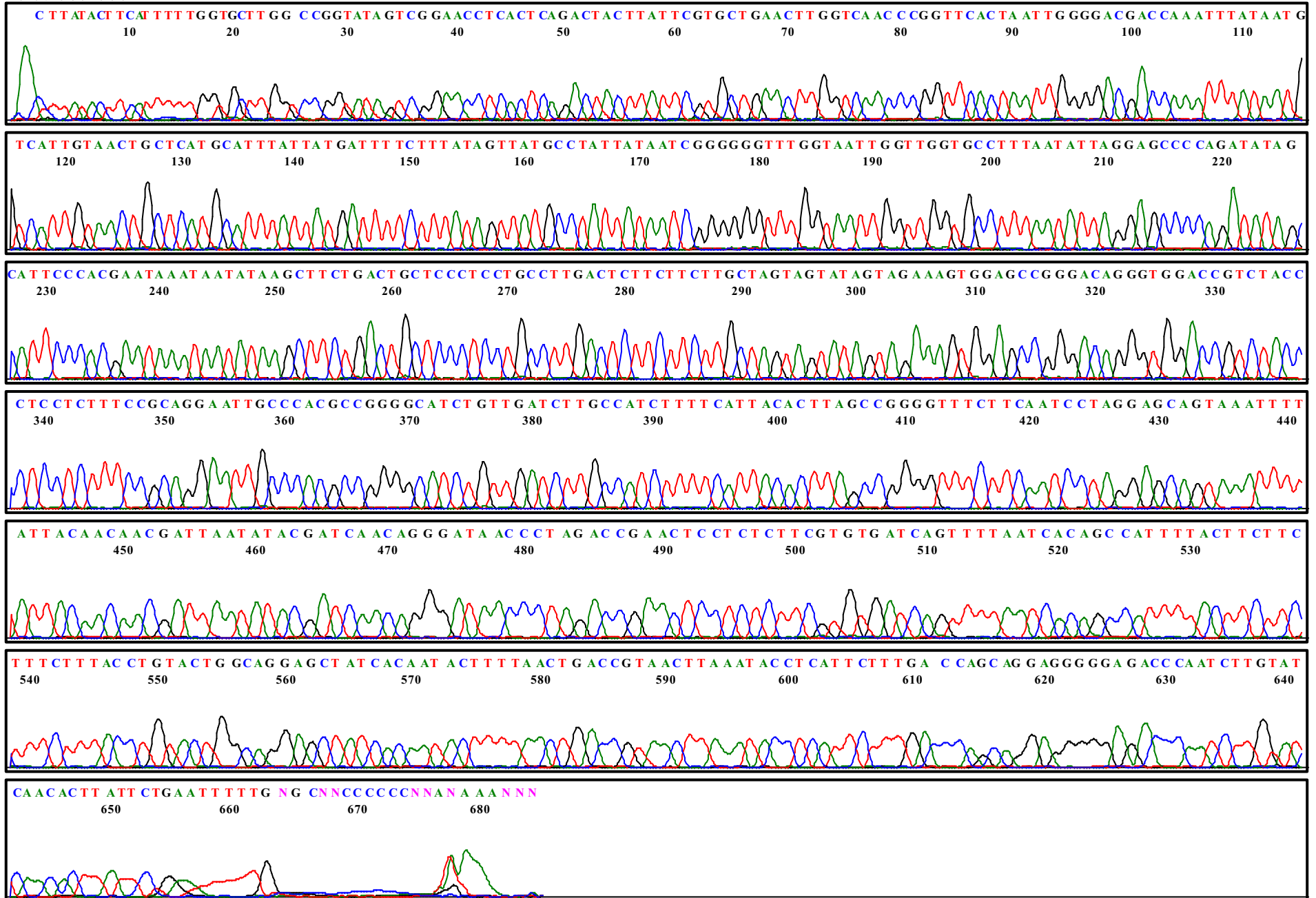
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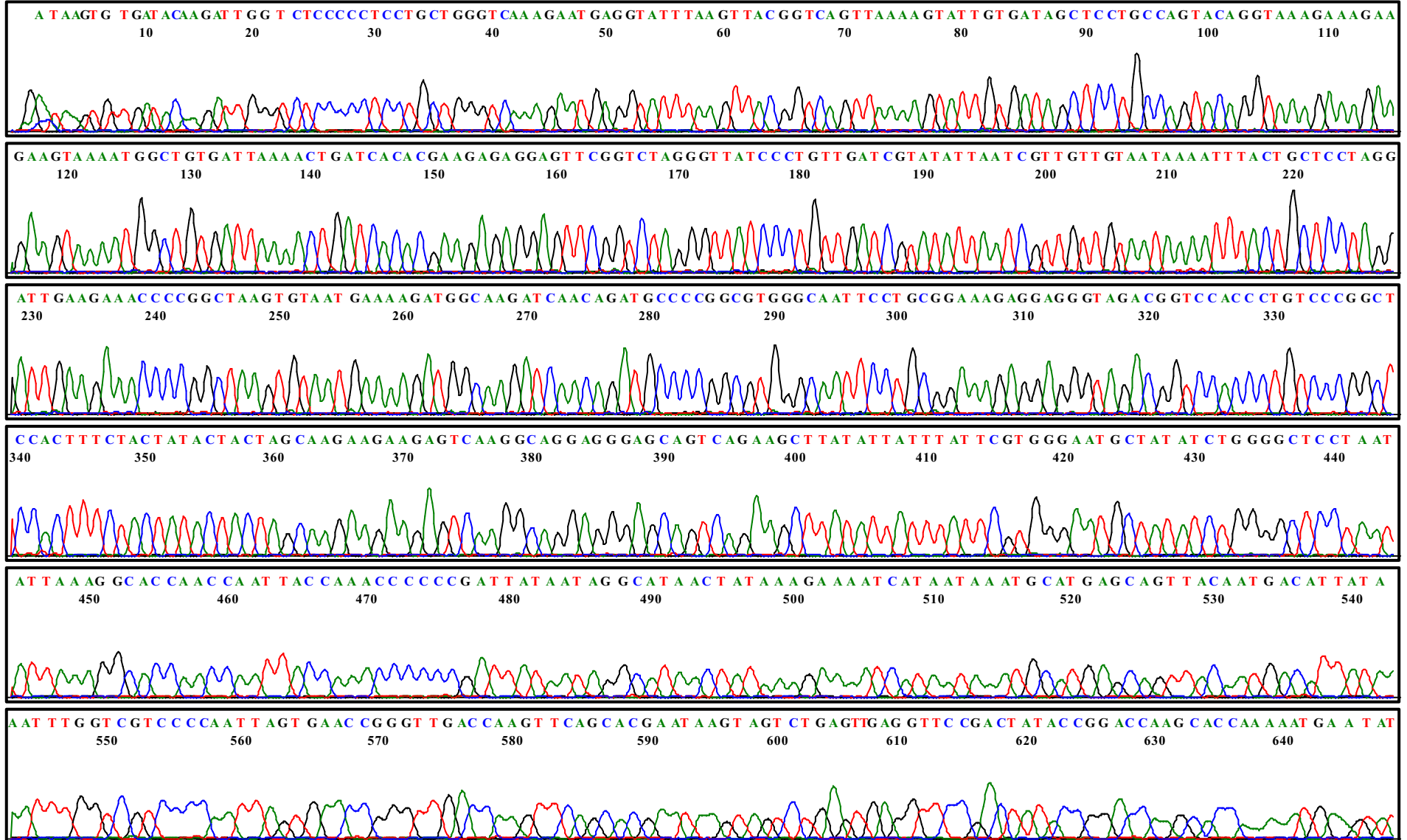
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Points 1098 to 9796

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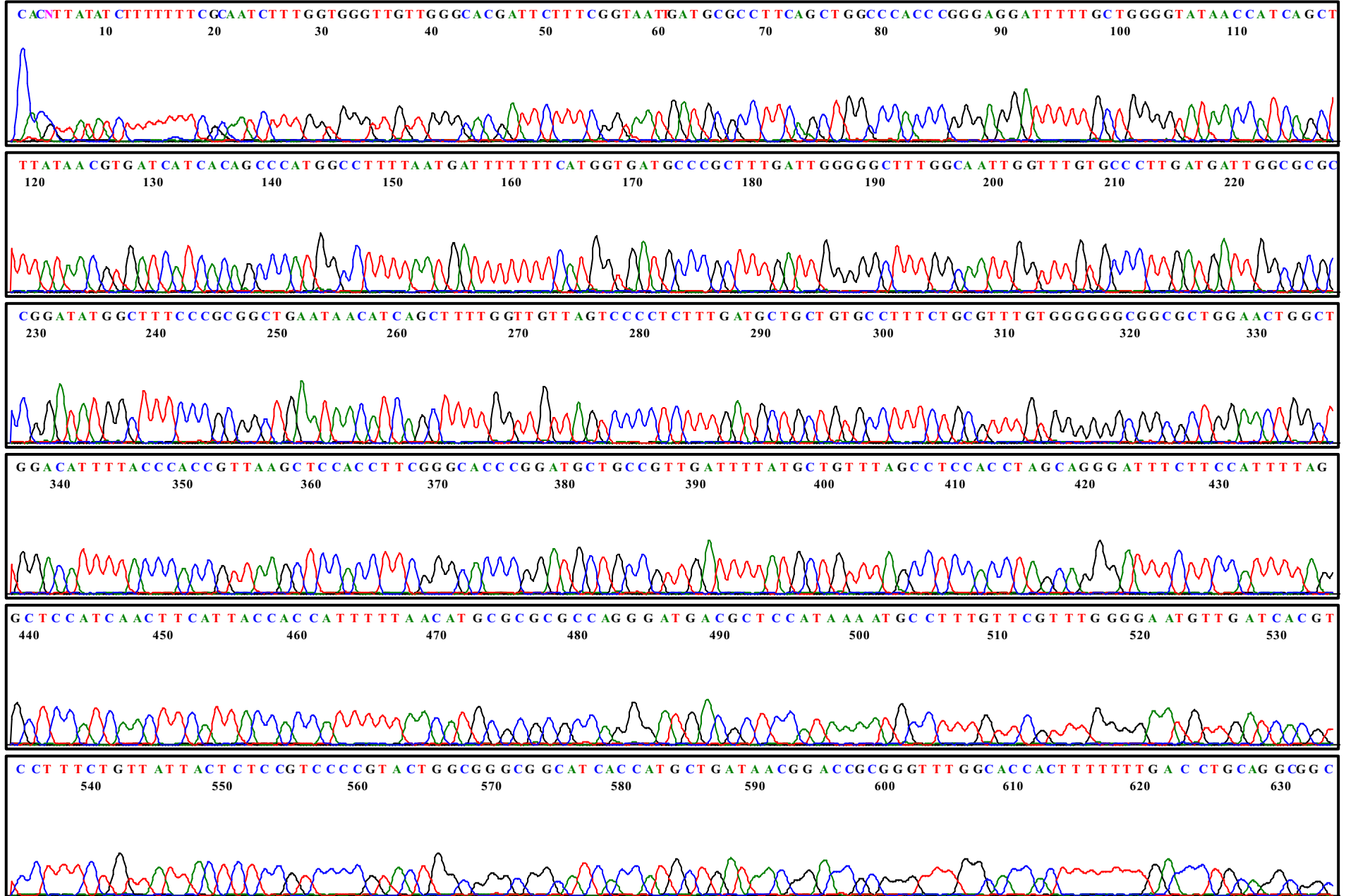
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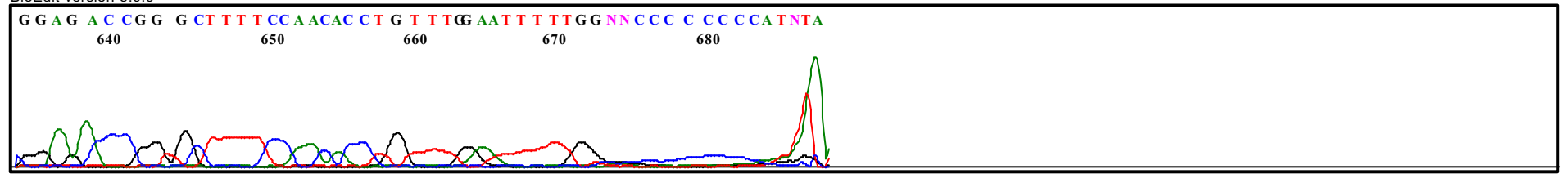
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BIF
G8 LCO1490
Lane 25

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dRhod2
Points 1096 to 9796

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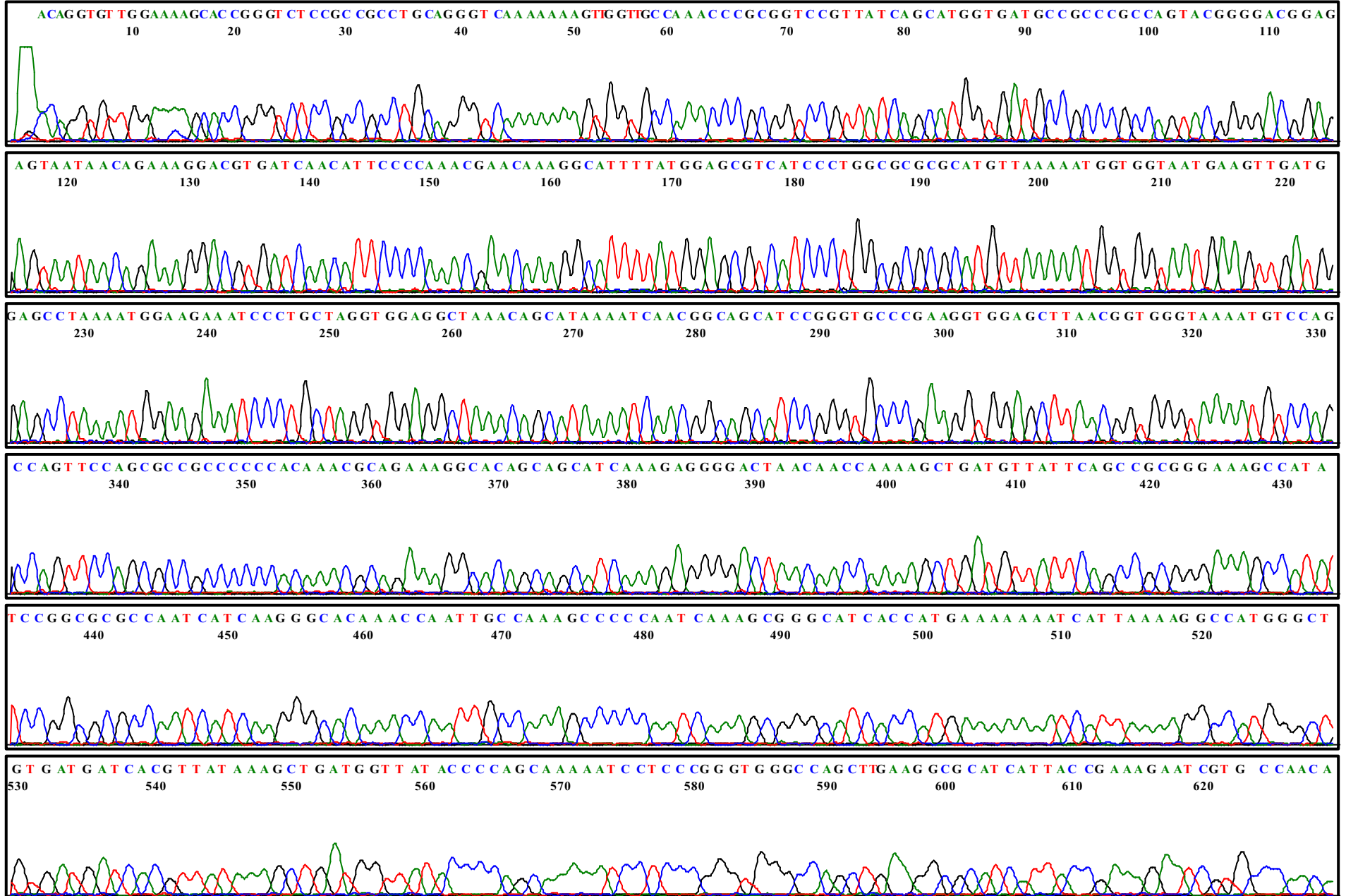
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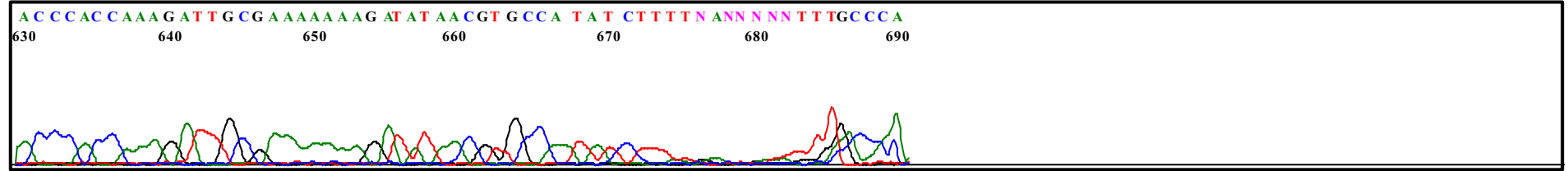
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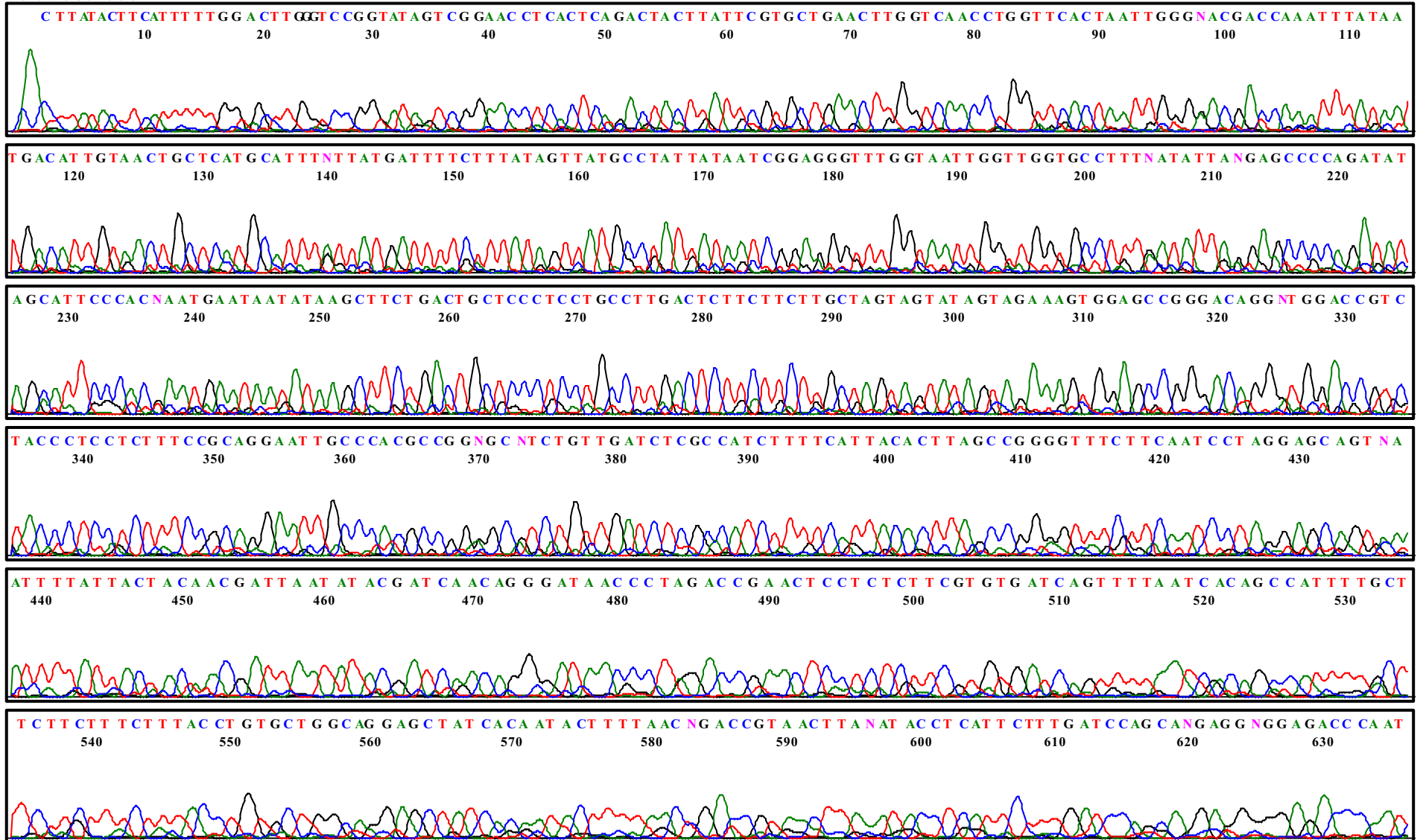
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Points 1098 to 9796

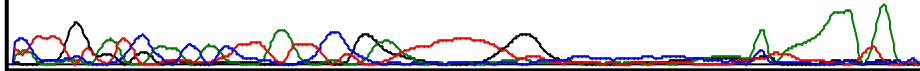
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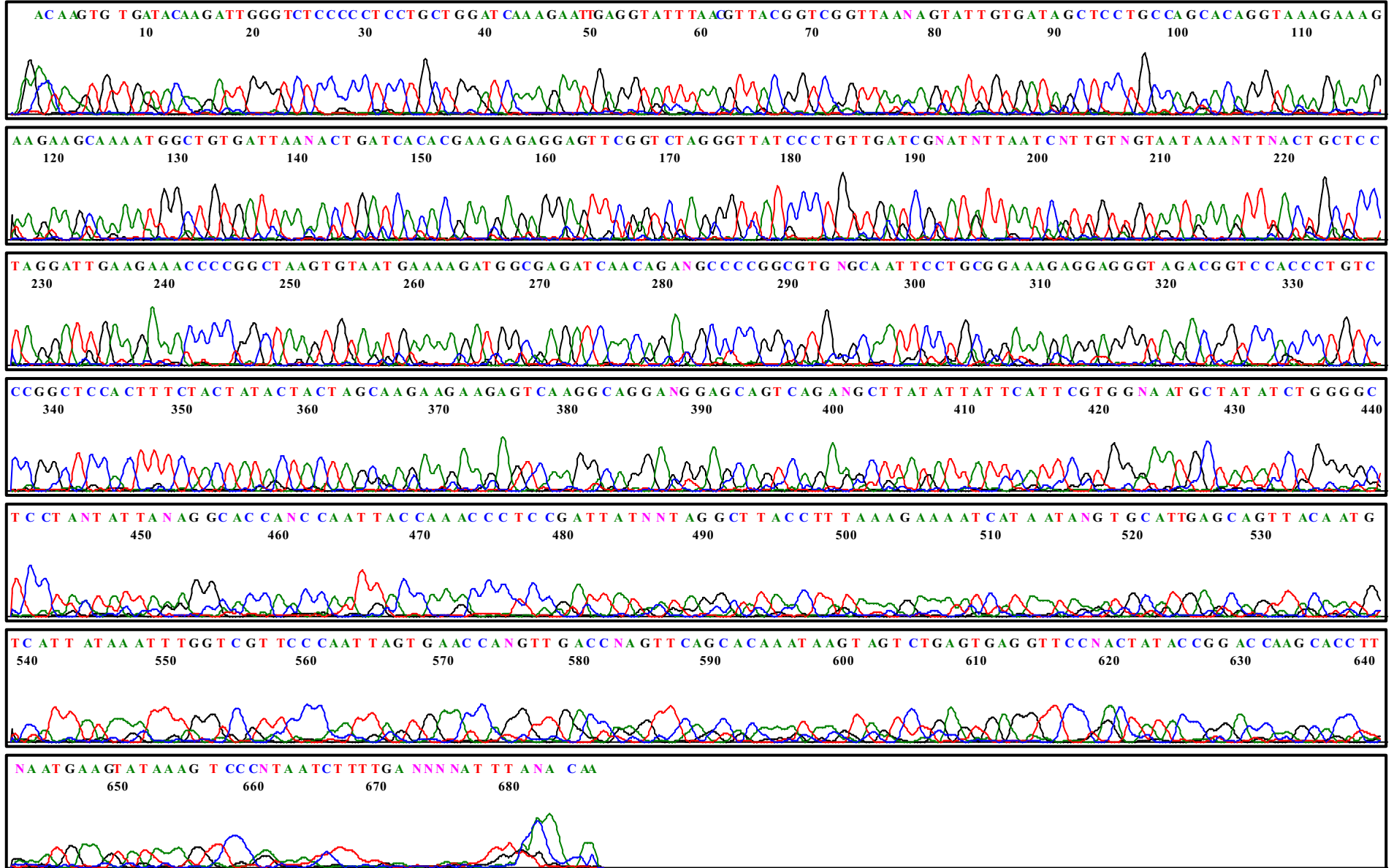
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BIF
A1 HCO2198
Lane 42

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DT373{BDv3}v1.mob
dRhod2
Points 1098 to 9796

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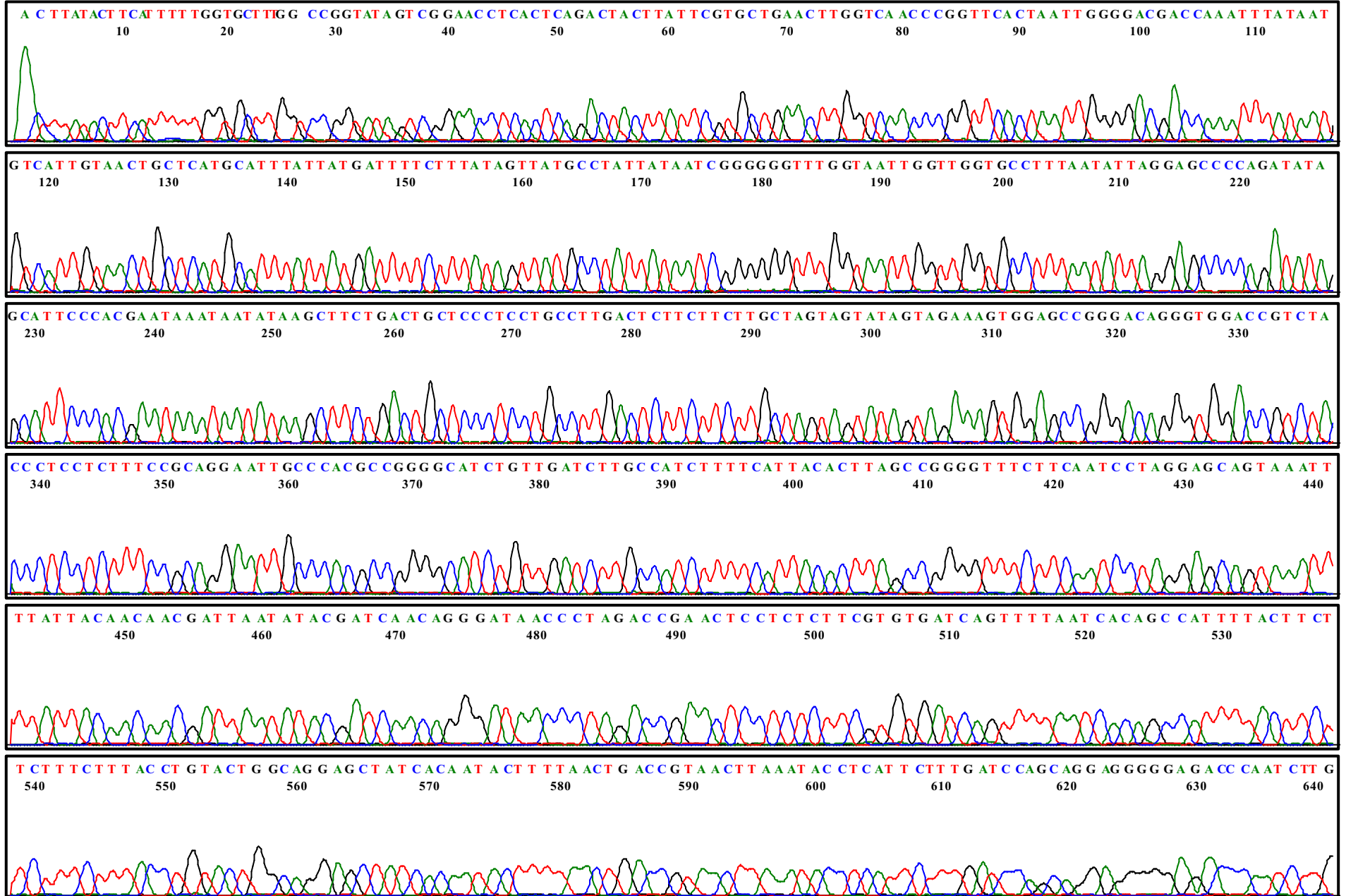
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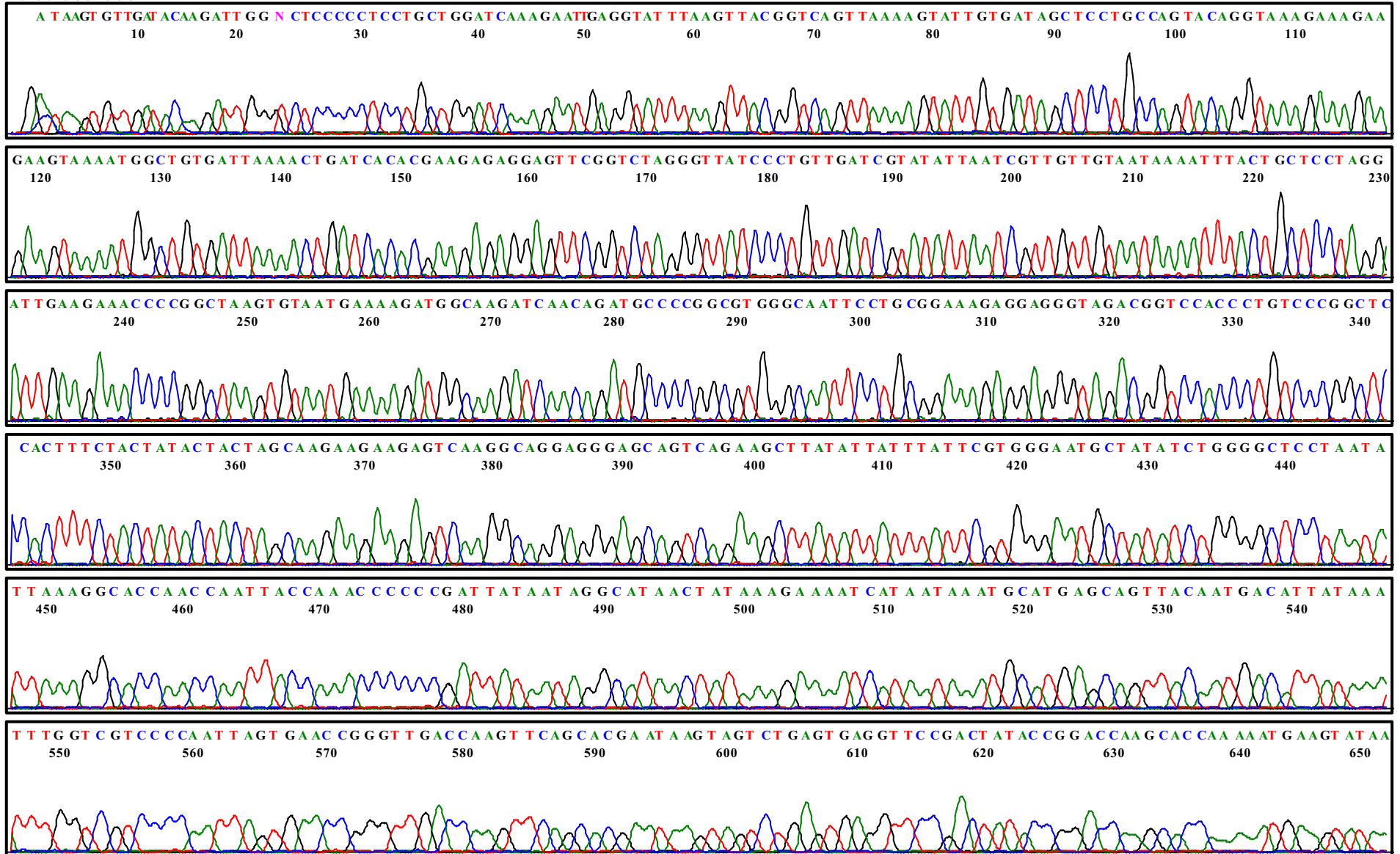
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BIF
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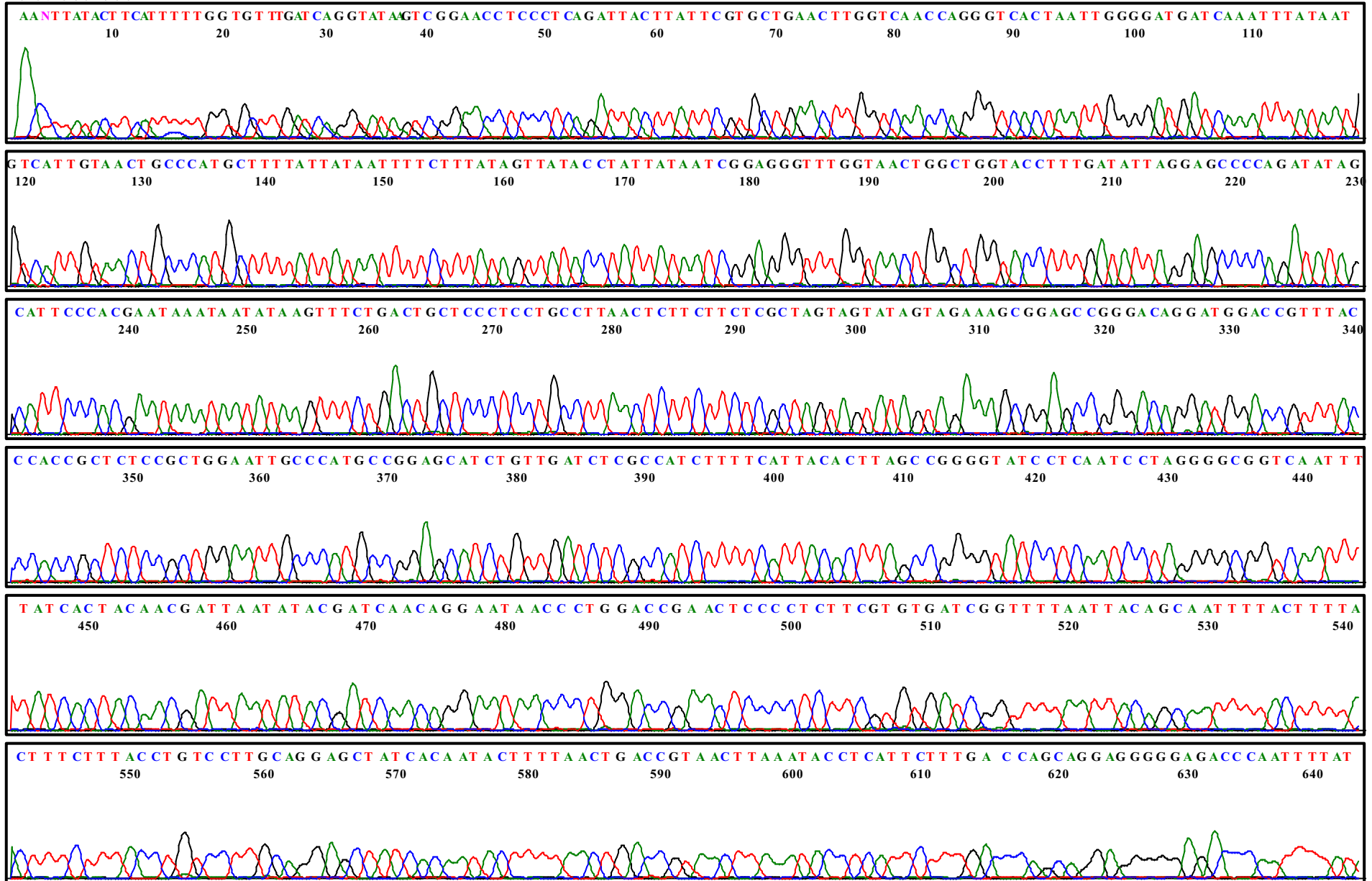


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dRhod2
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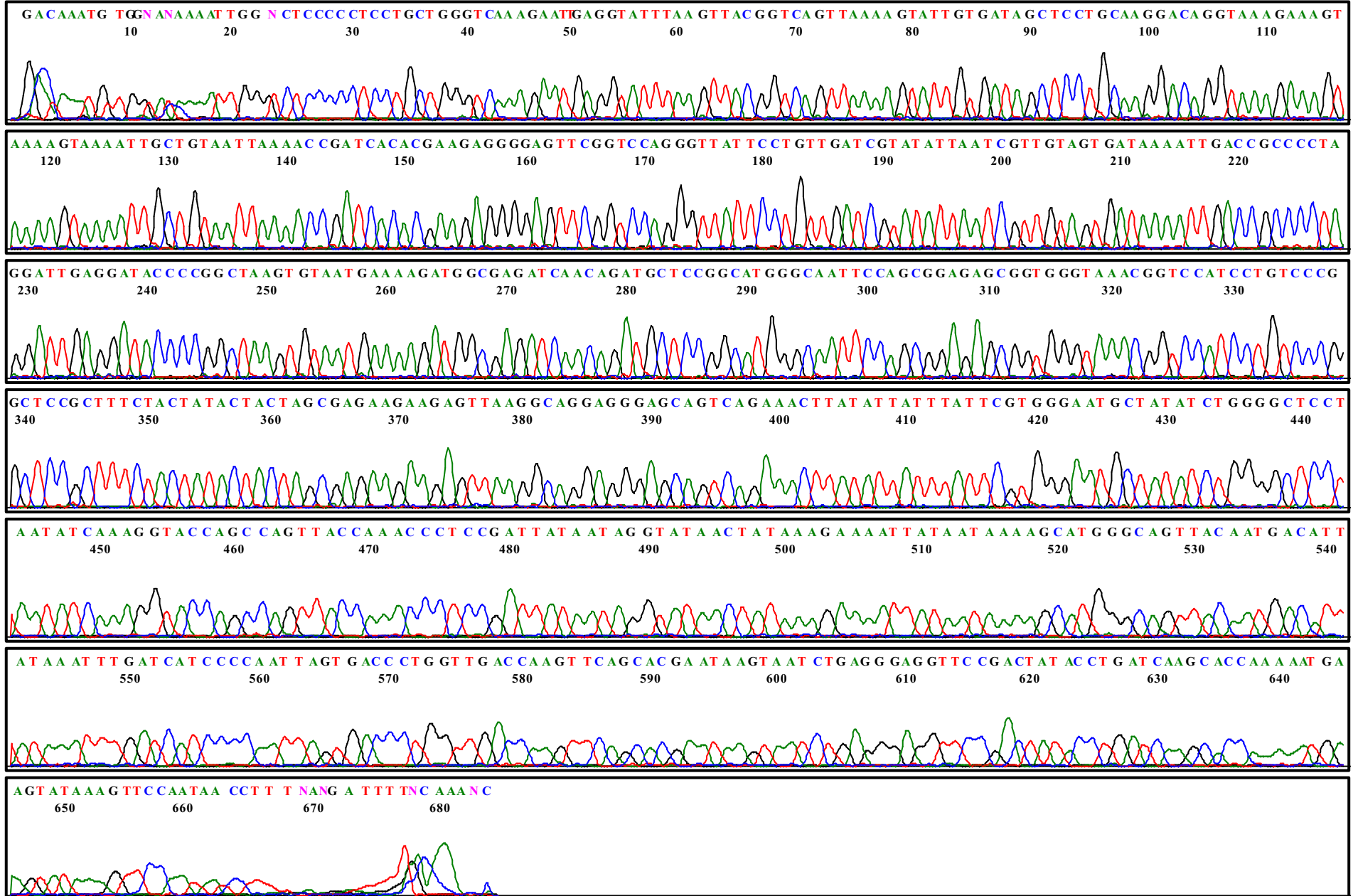
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DT373{BDv3}v1.mob
dRhod2
Points 1098 to 9796

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BioEdit version 5.0.9

Model 373

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Version 3.0

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BIF

A5 LCO1490

Lane 30

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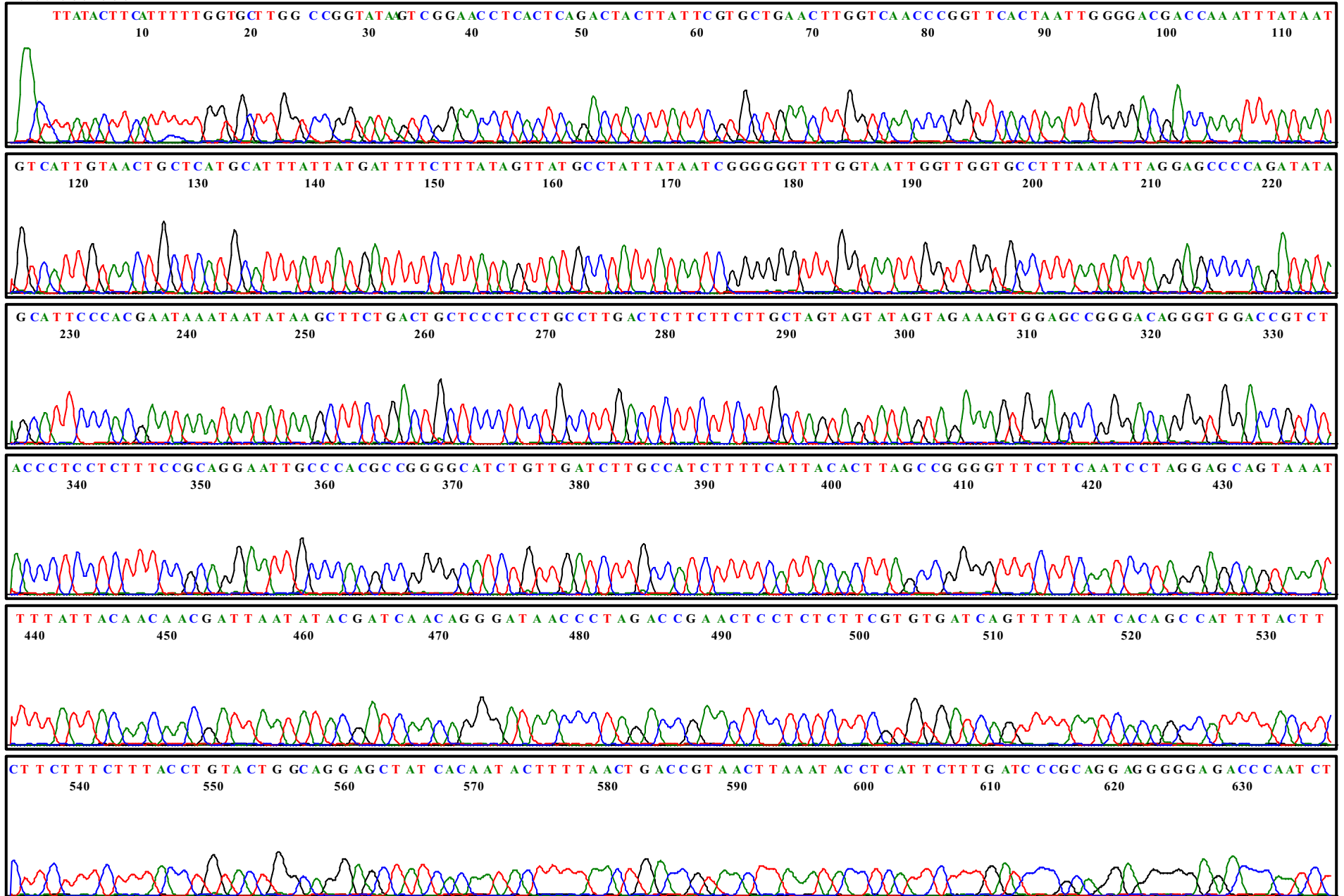
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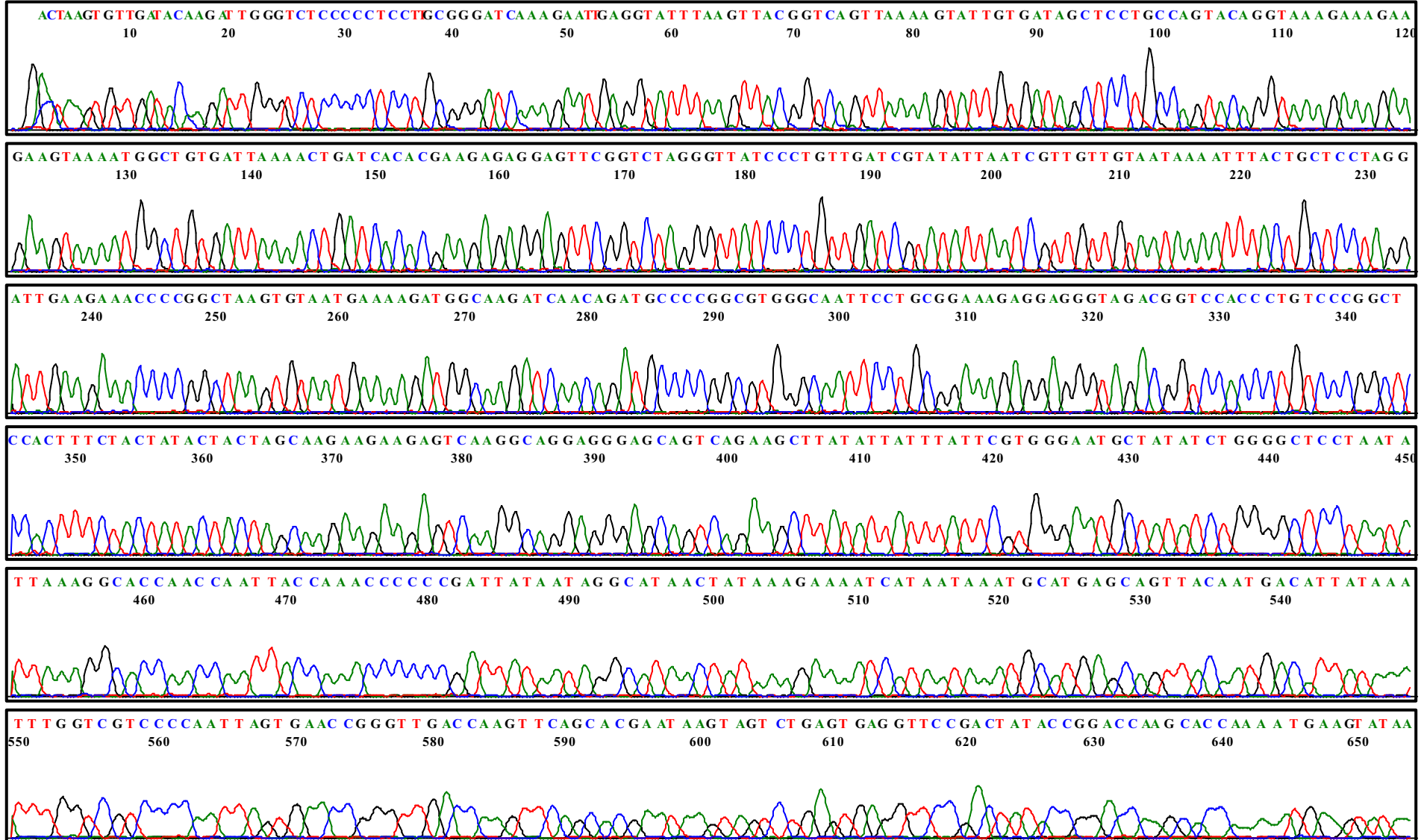
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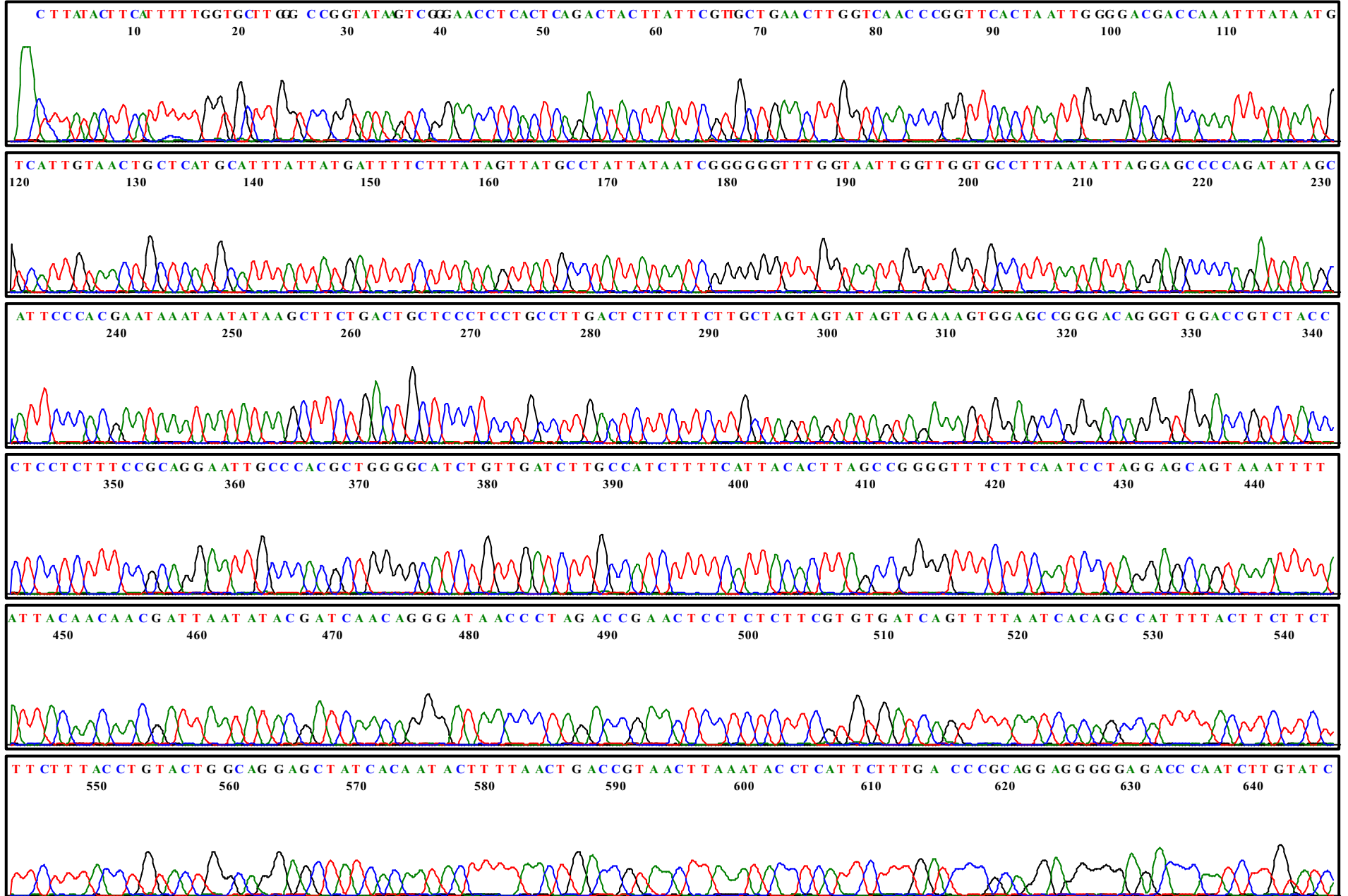
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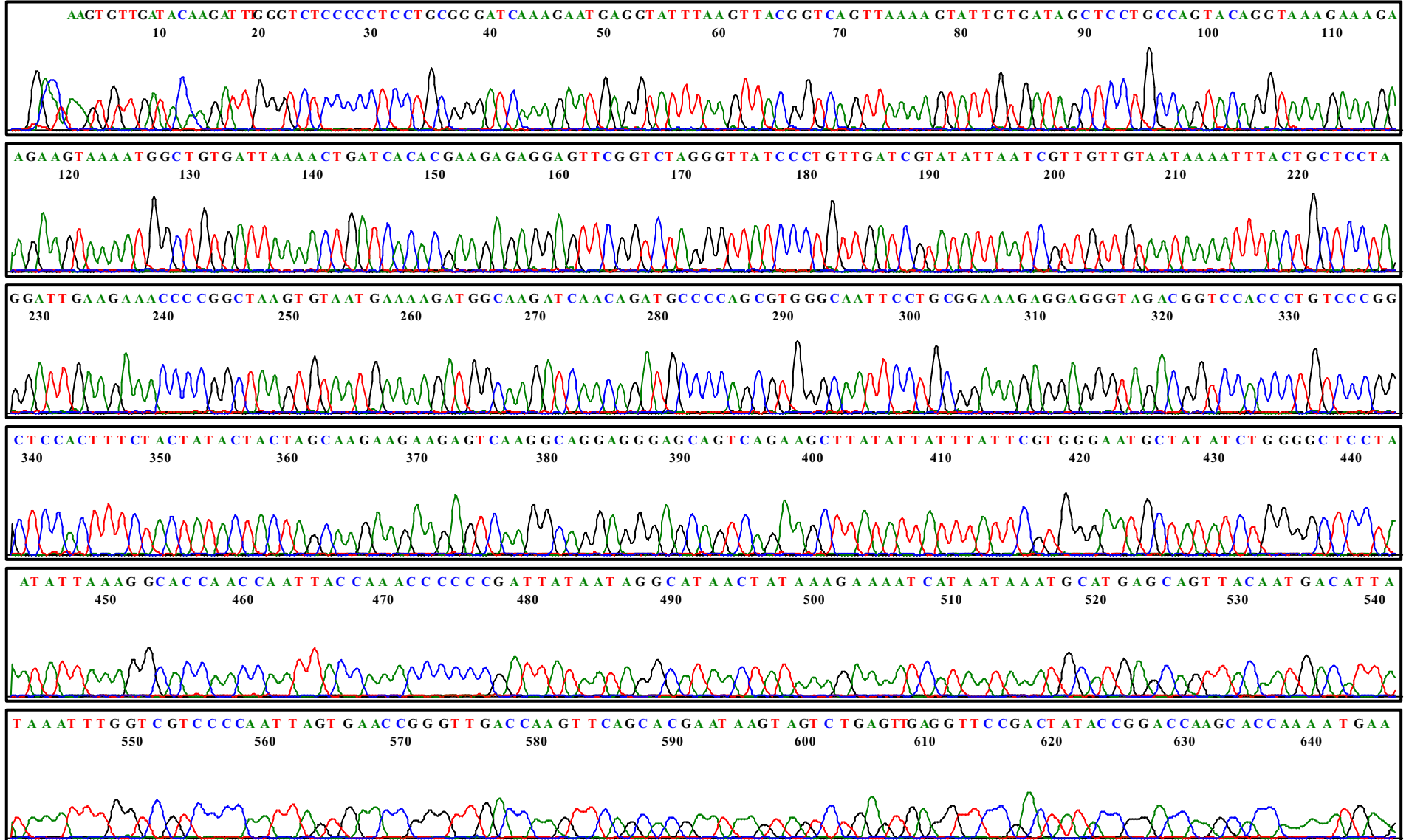














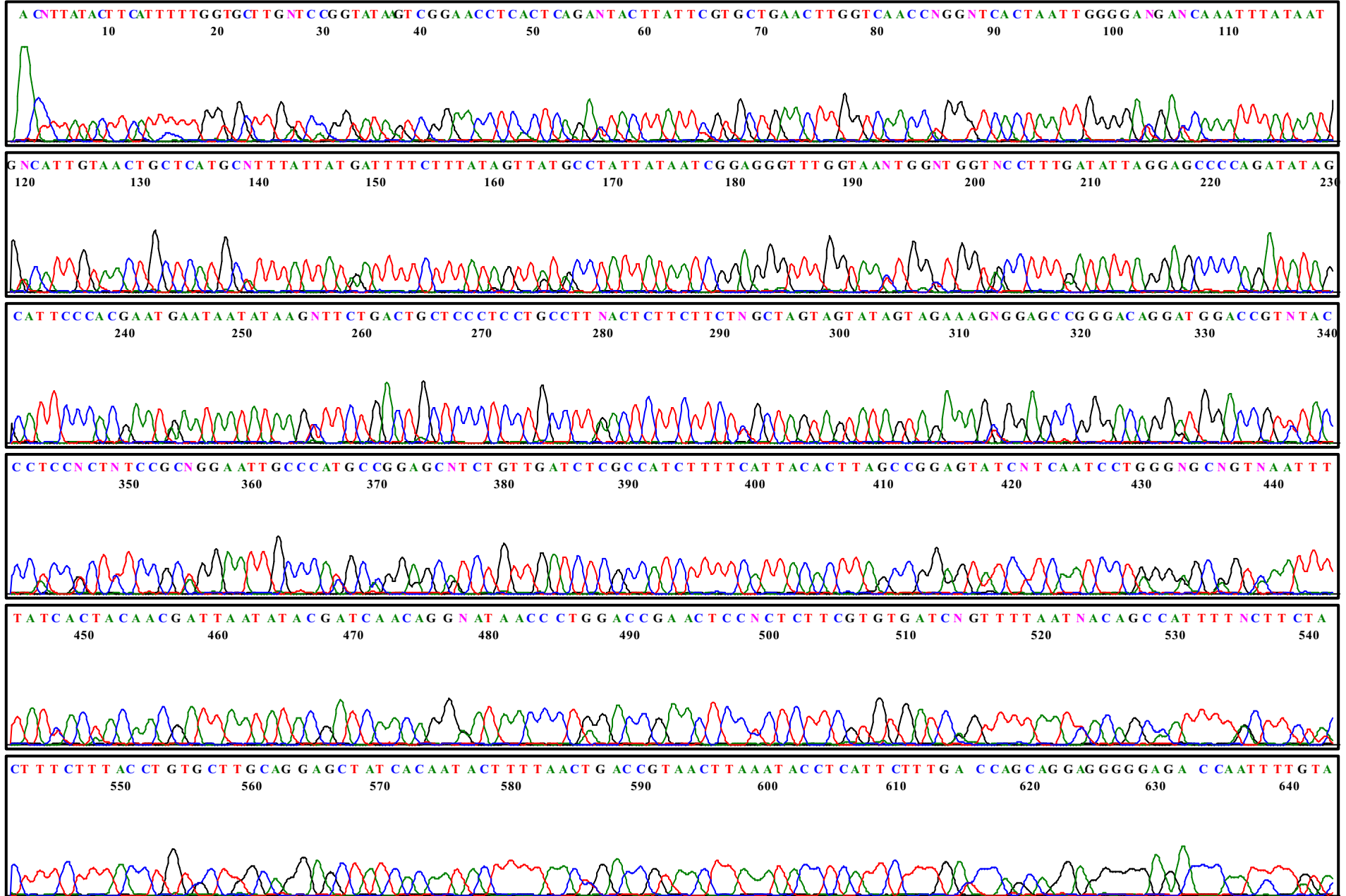


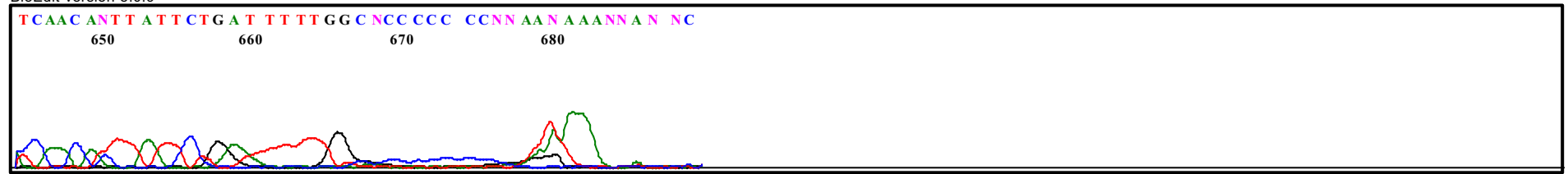
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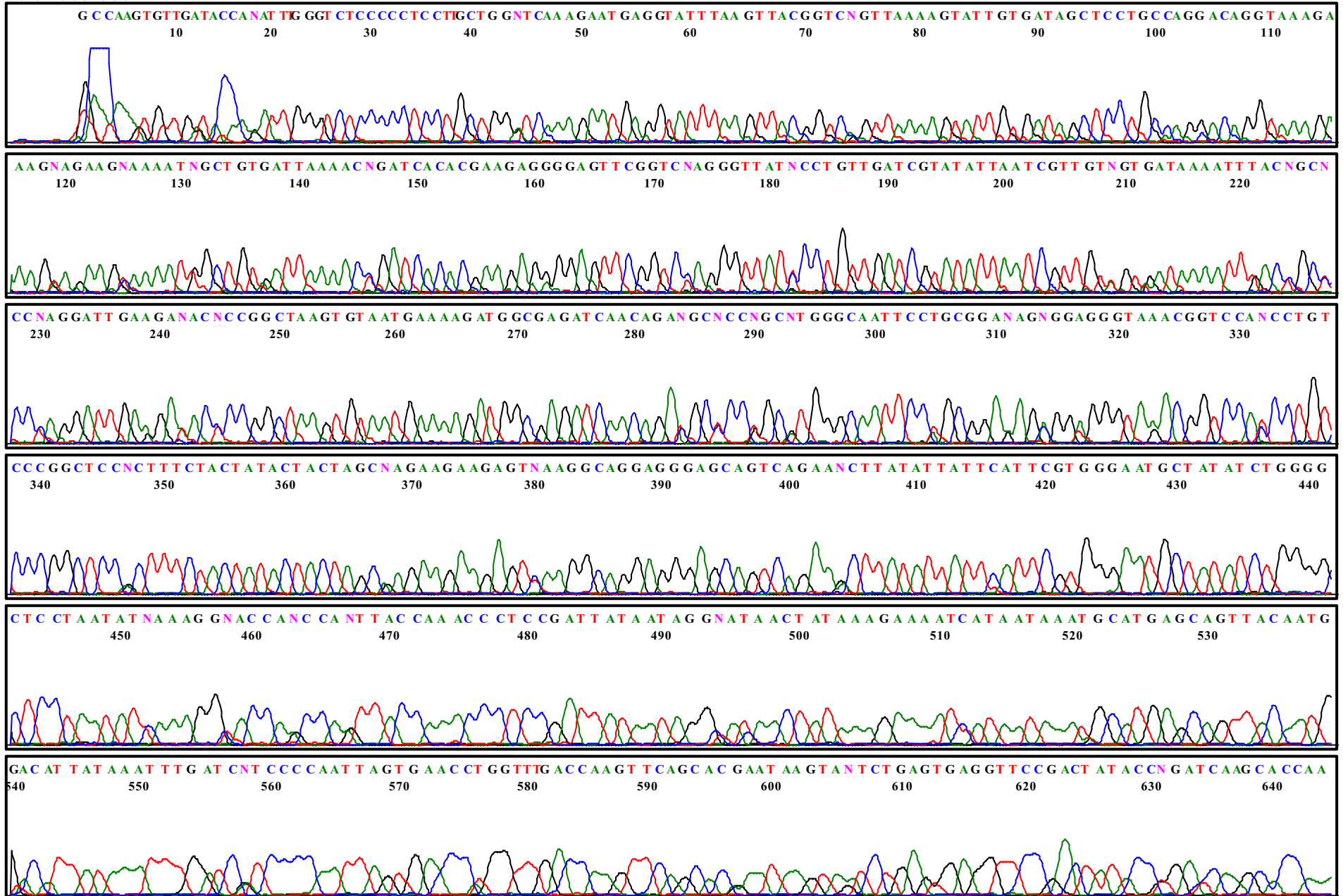
Model 373
ABI50
Version 3.0
File: 32•A7 LCO1490
BIF
A7 LCO1490
Lane 32

Signal G:644 A:427 T:590 C:474
DT373{BDv3}v1.mob
dRhod2
Points 1098 to 9796

Page 1 of 2
5/10/2004
Spacing: 9.59000015258789









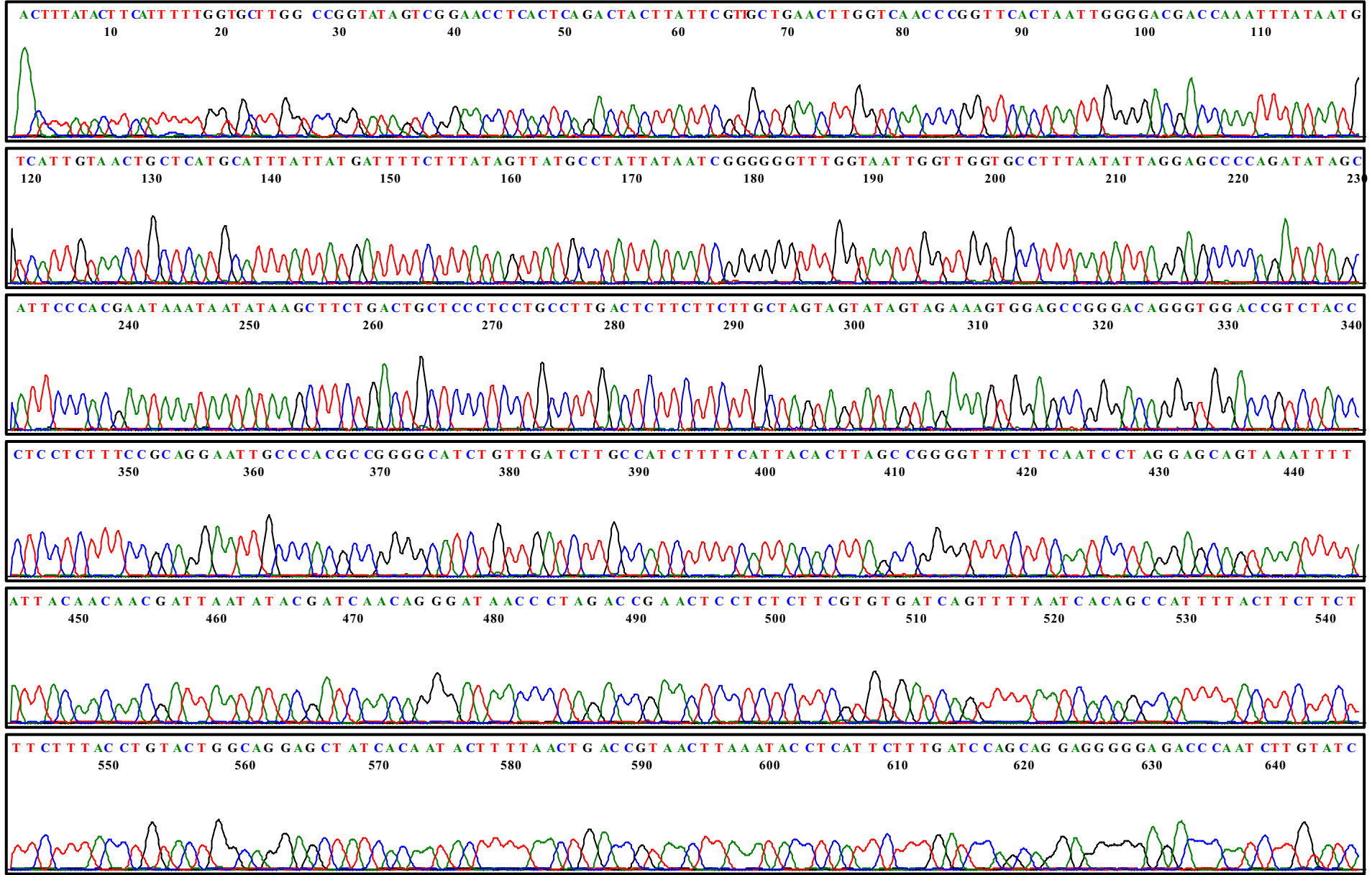


BioEdit version 5.0.9

Model 373
ABI50
Version 3.0
File: 33•A8 LCO1490
BIF
A8 LCO1490
Lane 33

Signal G:727 A:502 T:744 C:576
DT373{BDv3}v1.mob
dRhod2
Points 1098 to 9796

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5/10/2004
Spacing: 9.61999988555908







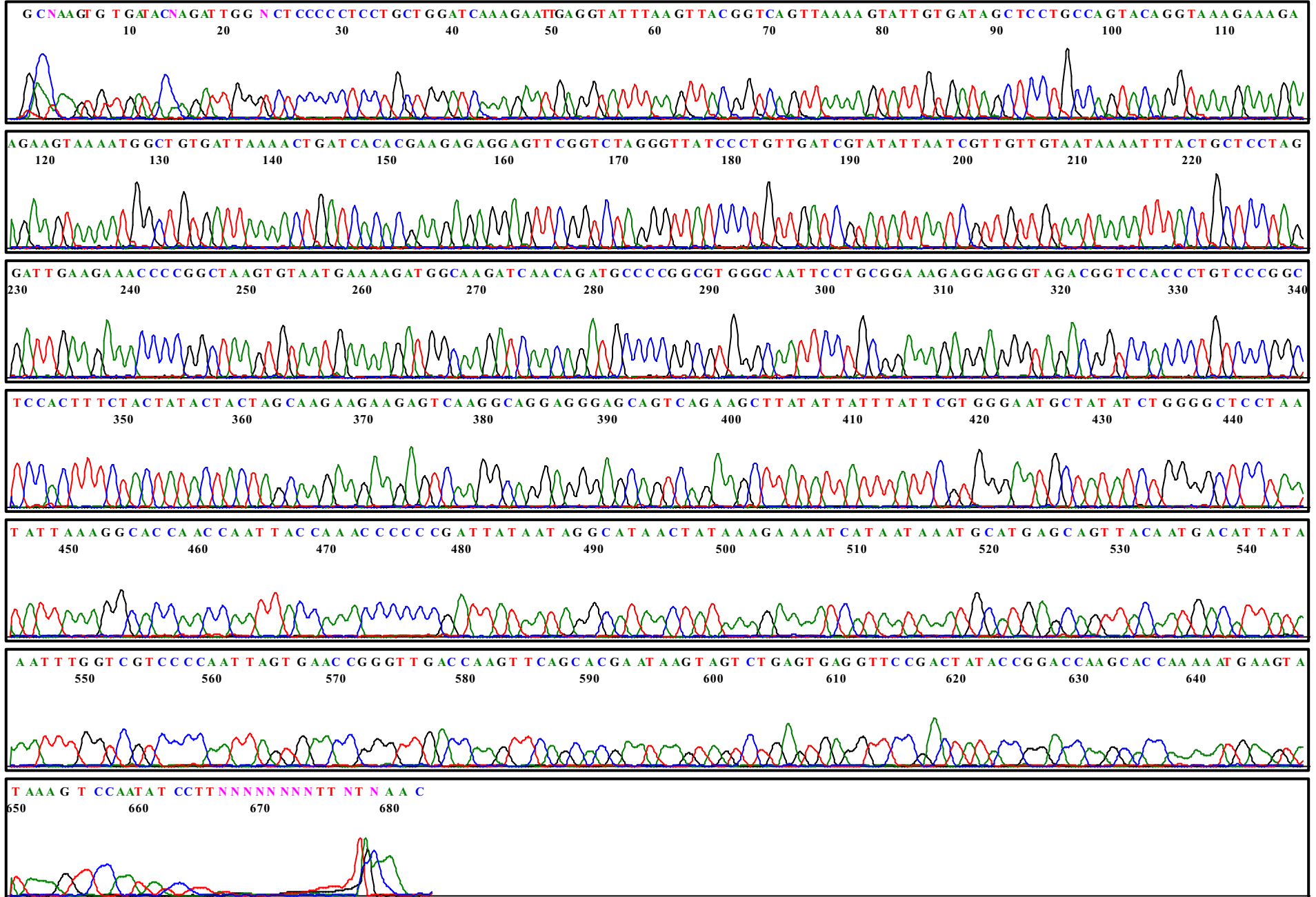
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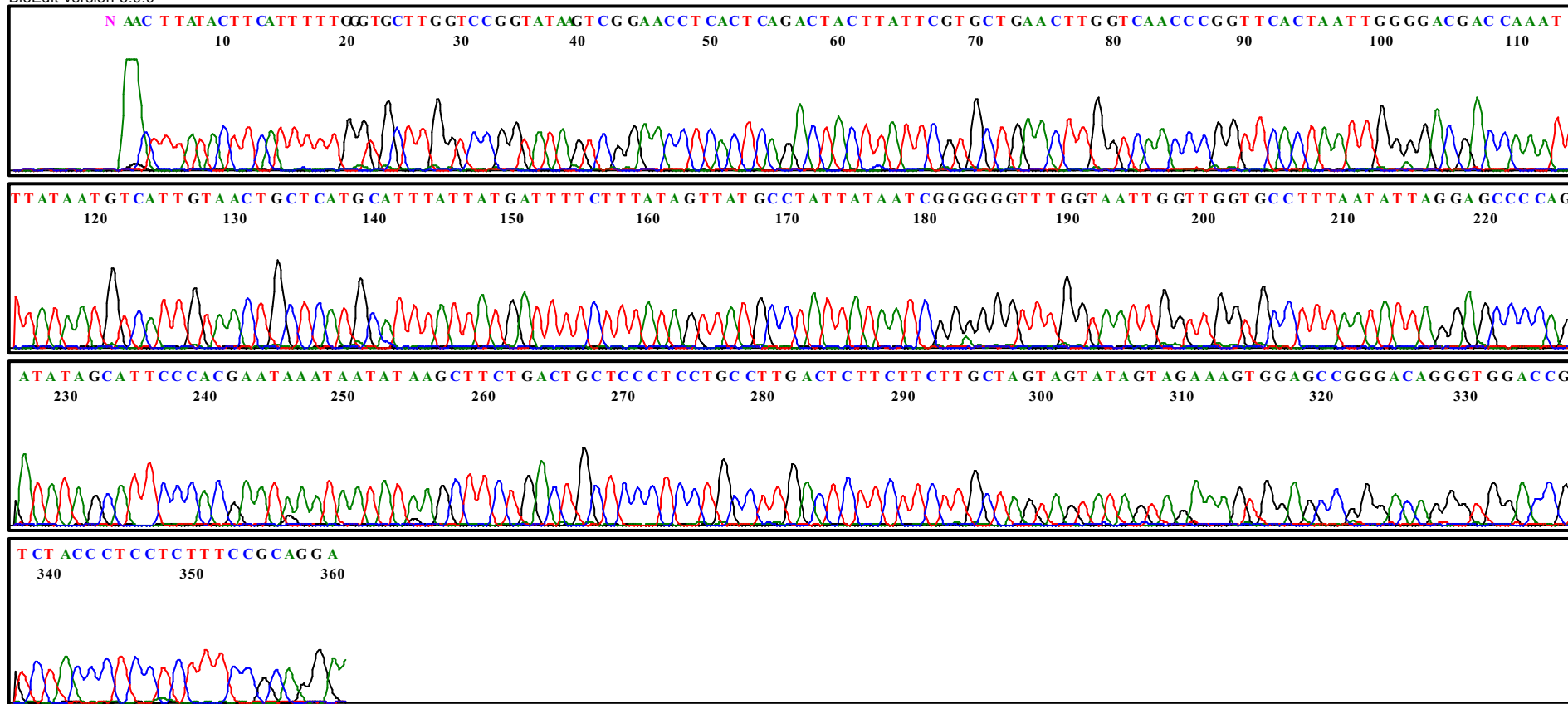
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Version 3.0

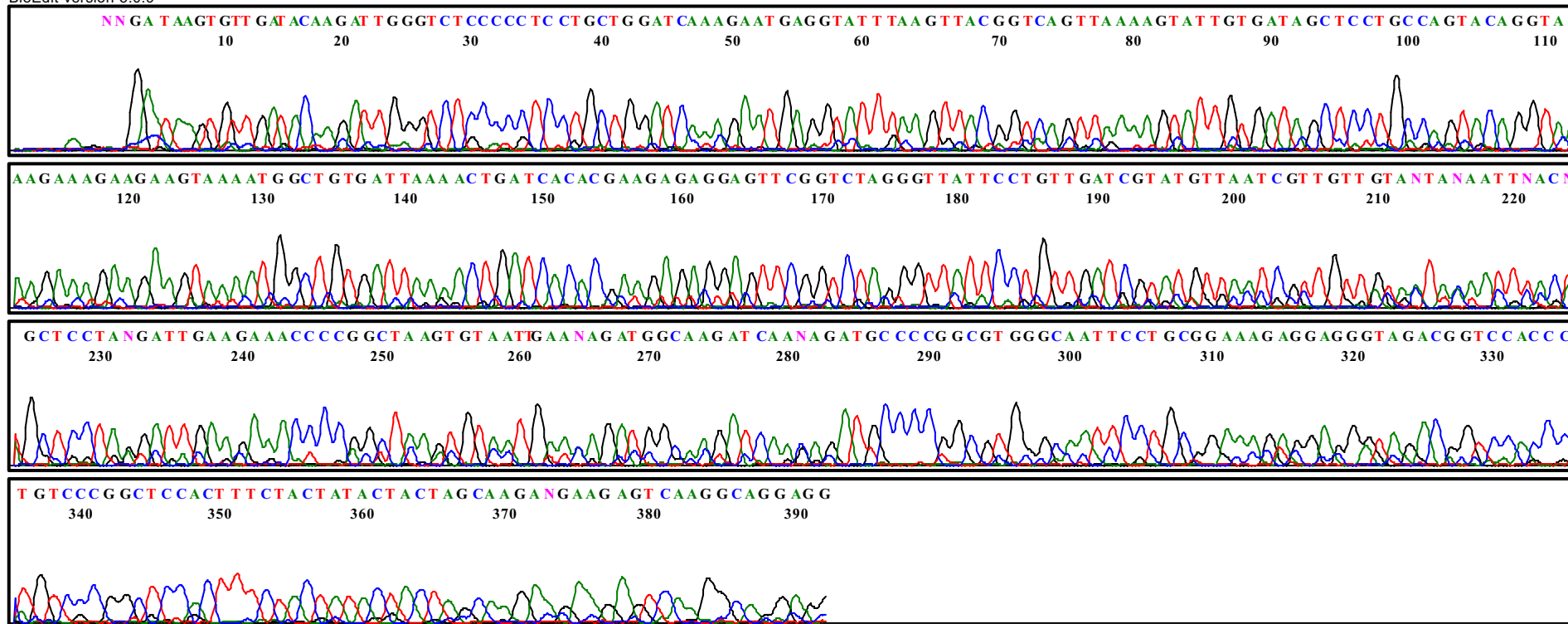
File: 49•A8 HCO2198
BIF
A8 HCO2198
Lane 49

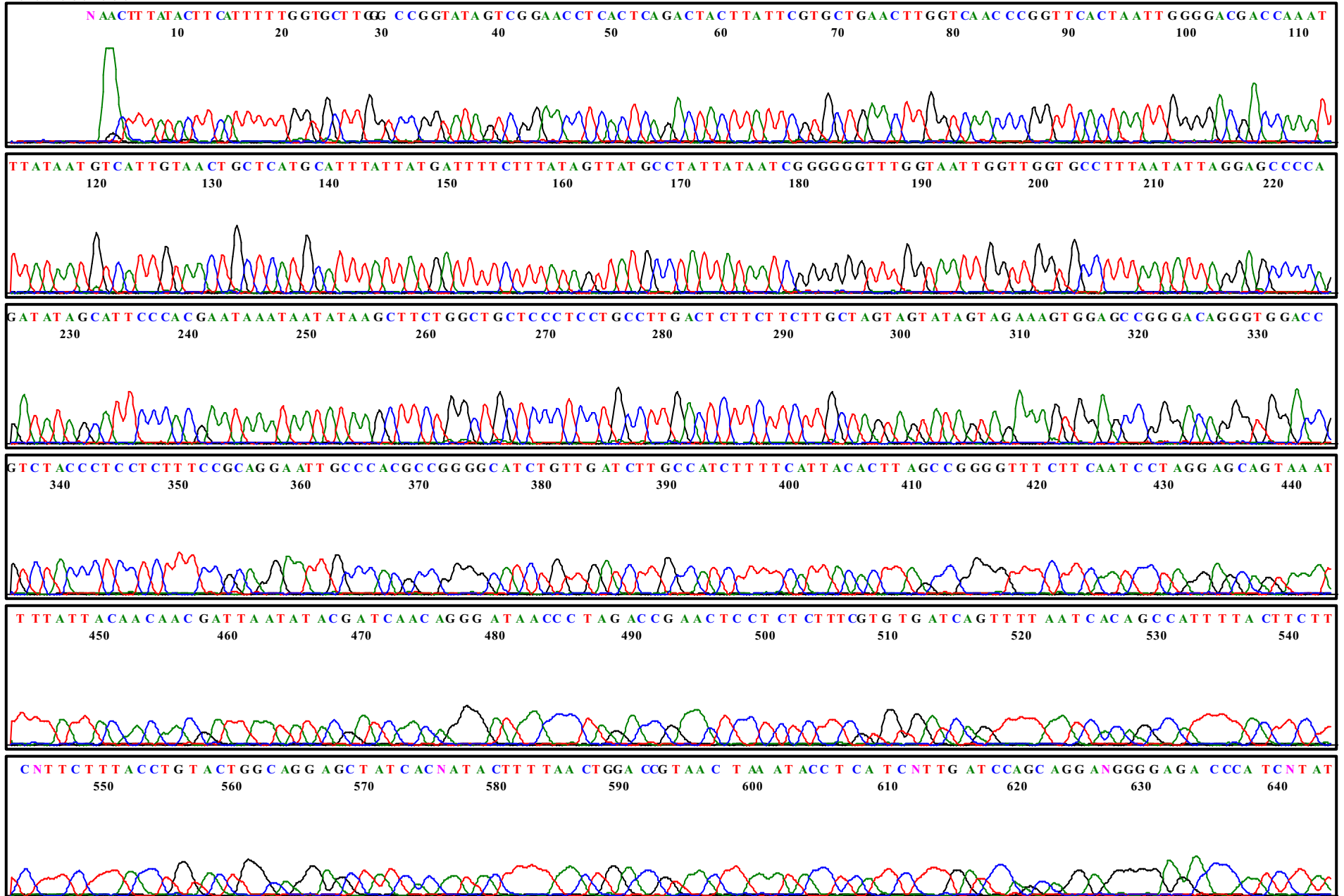
Signal G:790 A:576 T:557 C:497
DT373{BDv3}v1.mob
dRhod2
Points 1099 to 9796

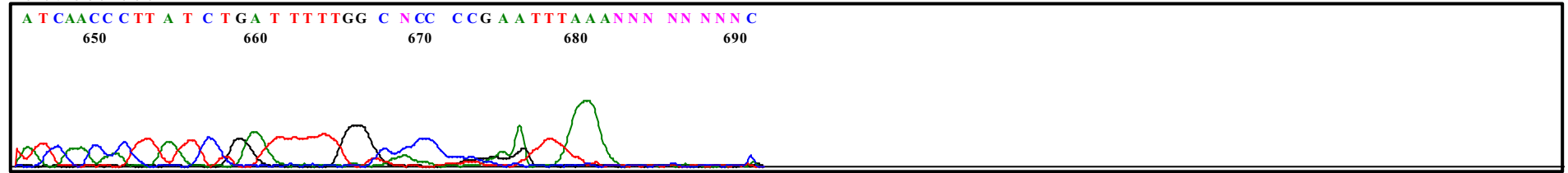
Page 1 of 1
5/10/2004
Spacing: 9.63000011444092

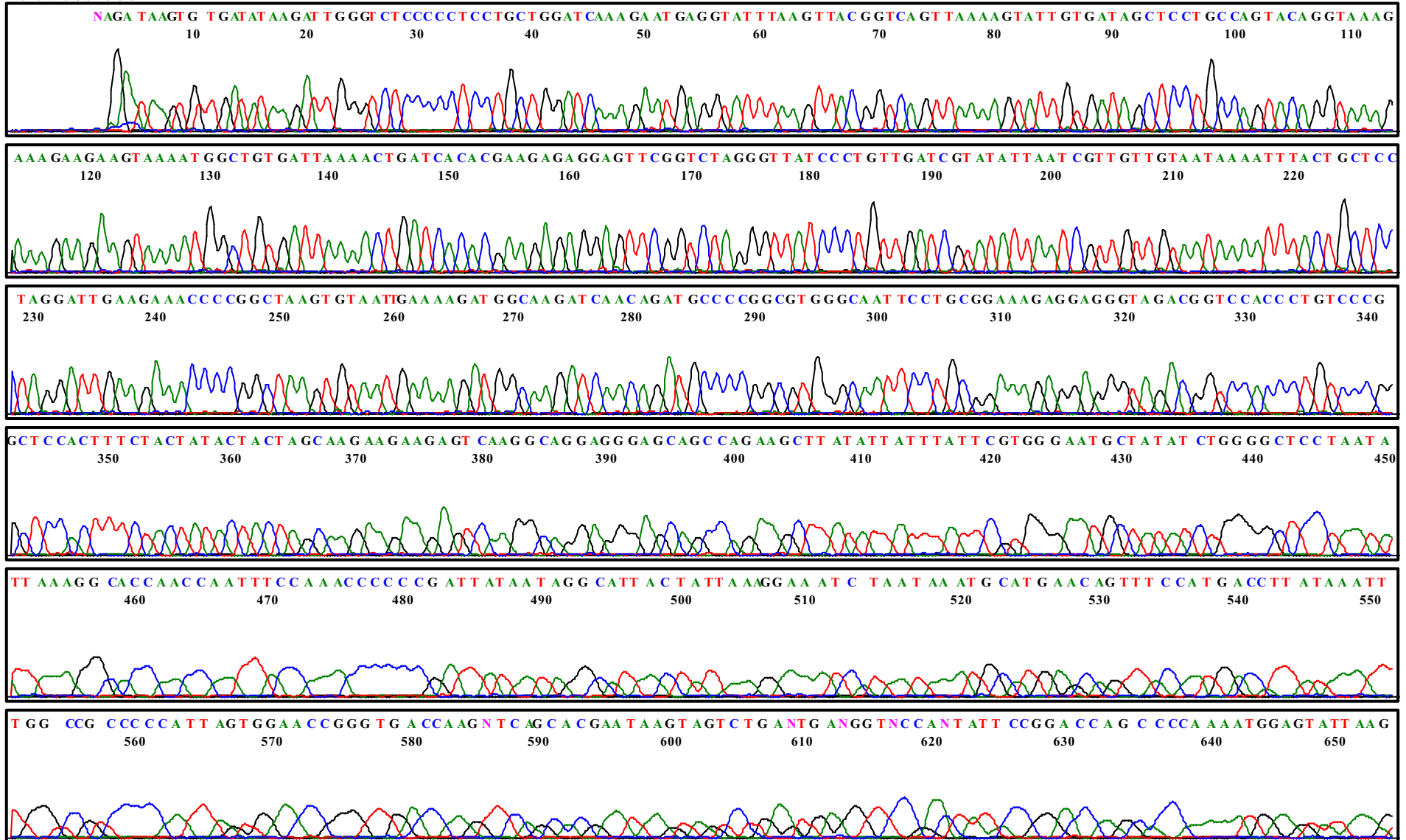




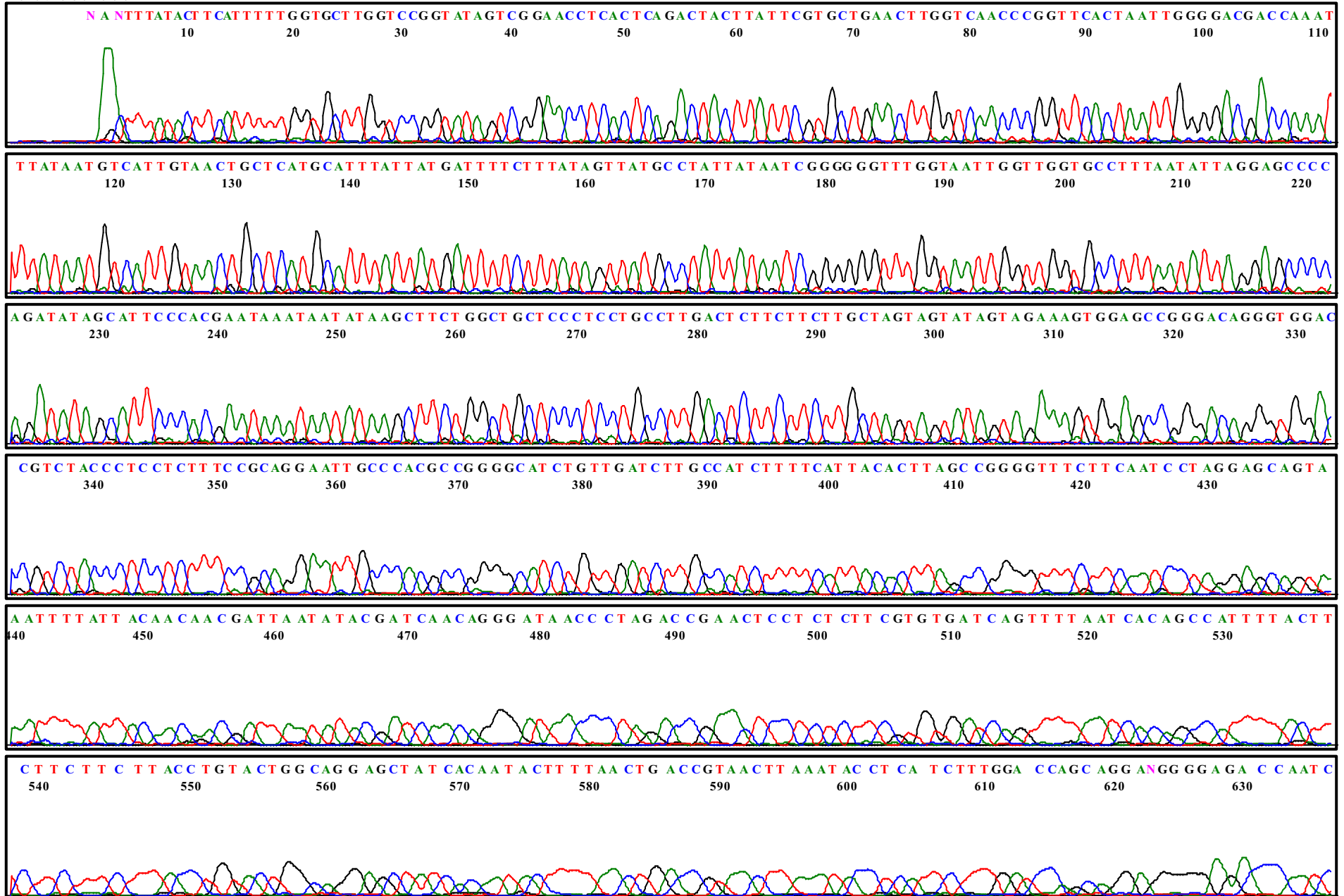


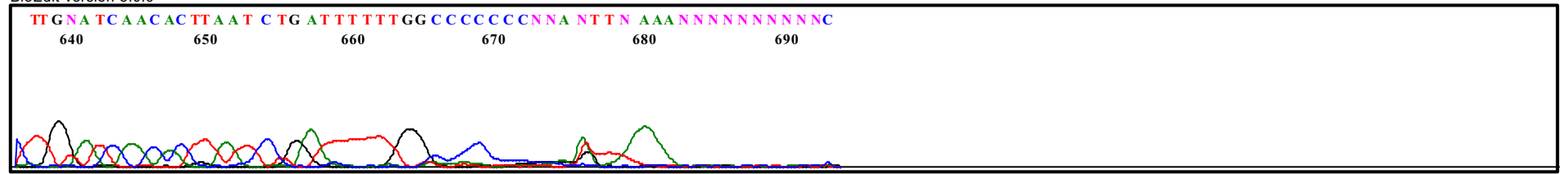


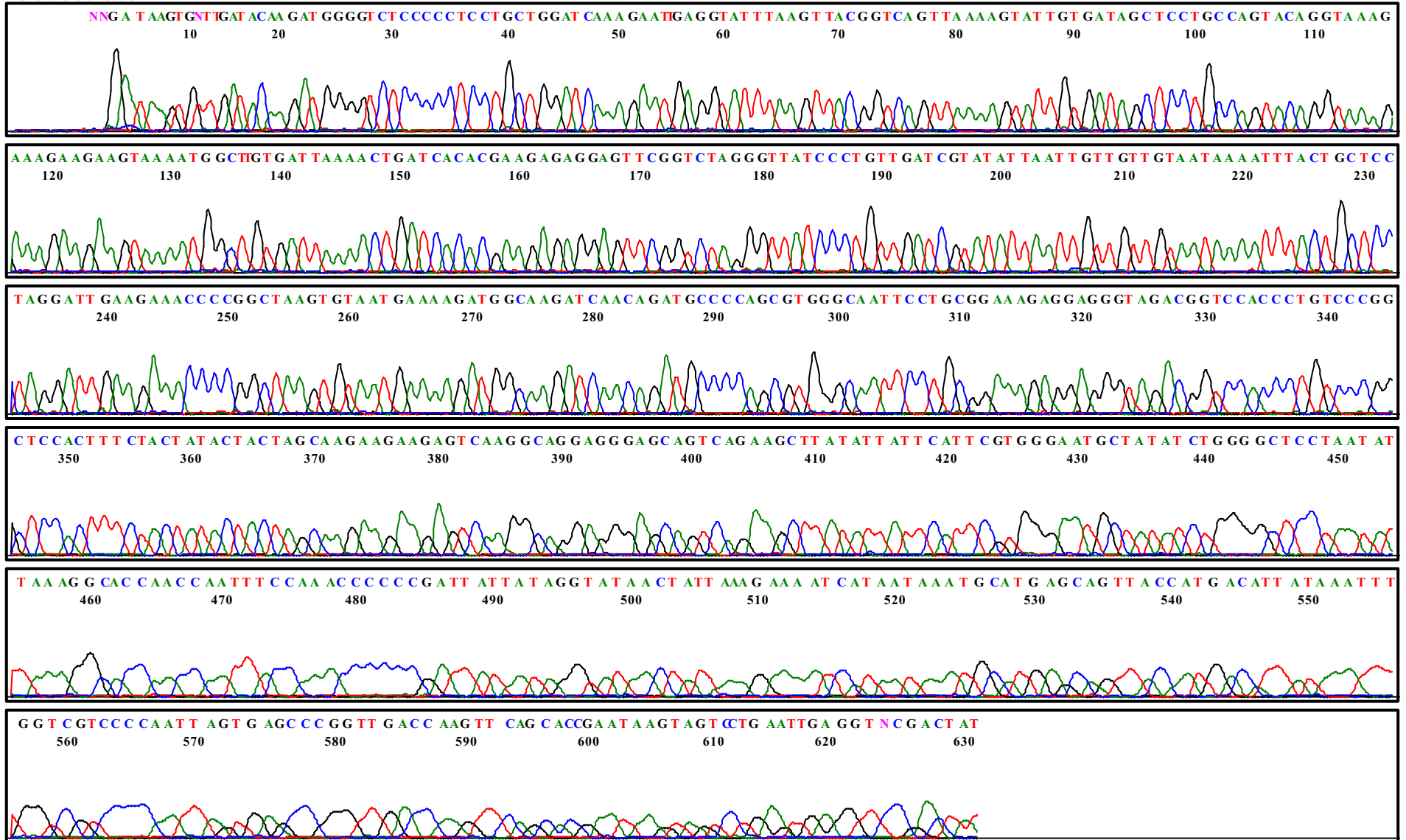


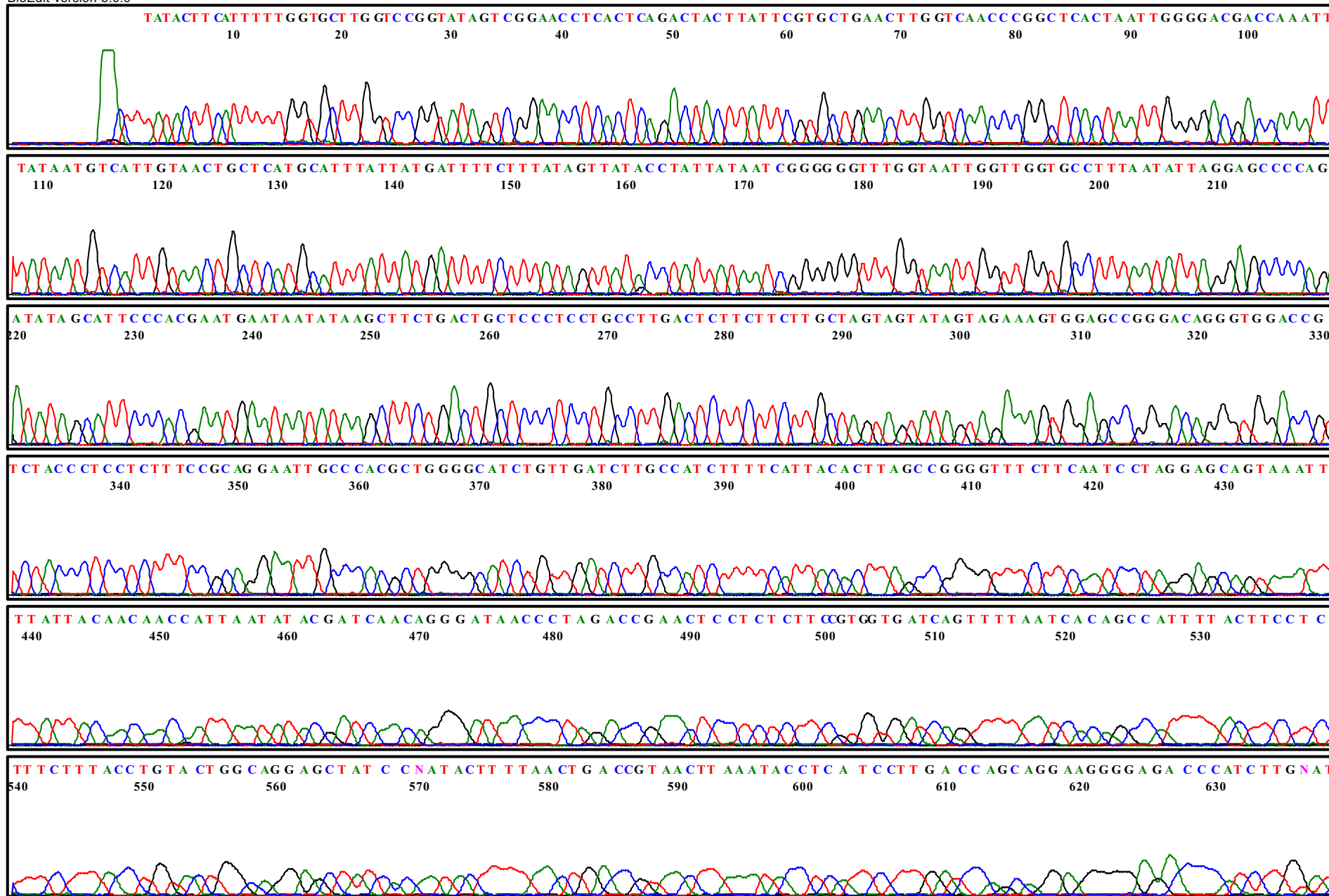


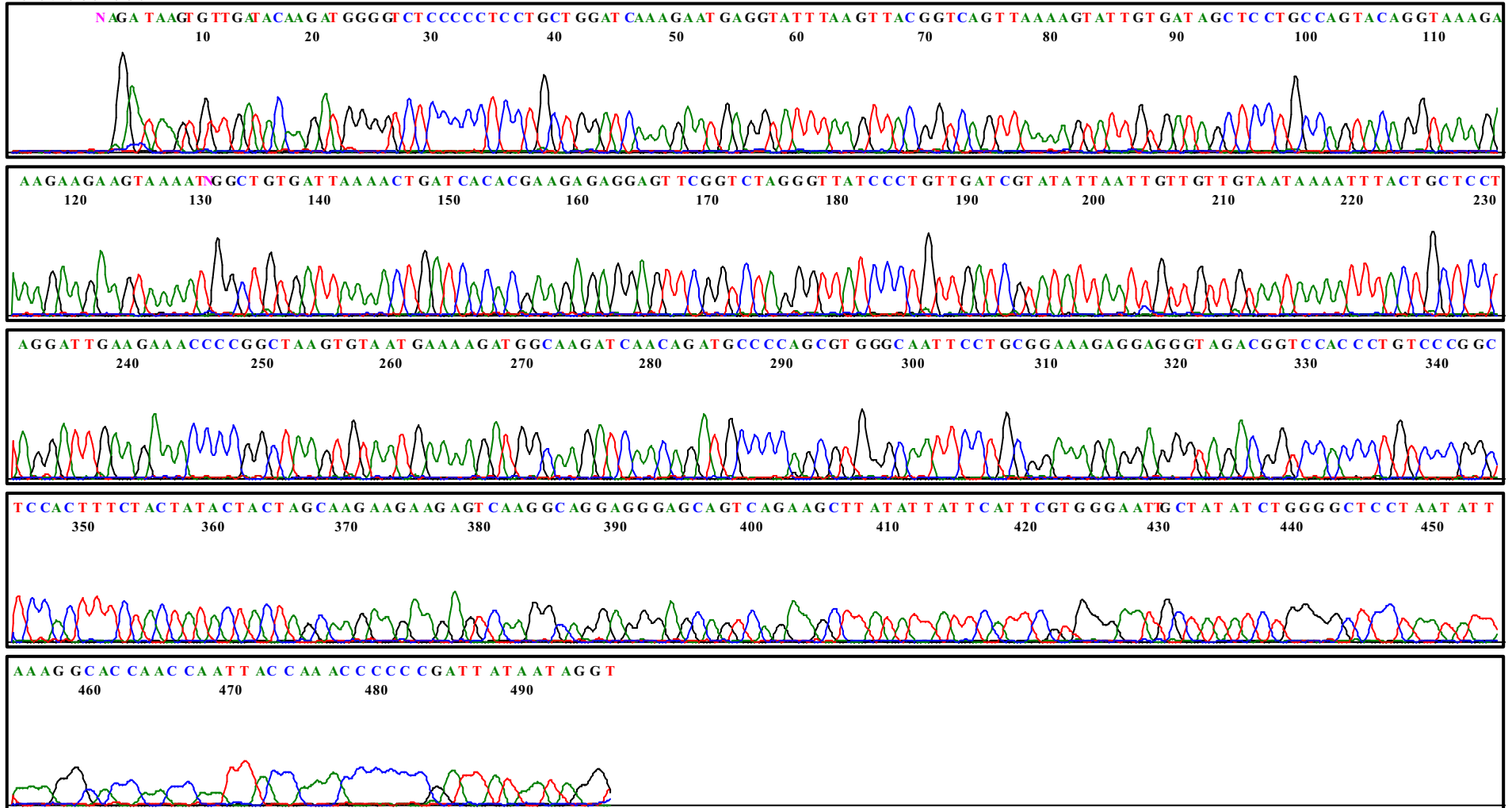


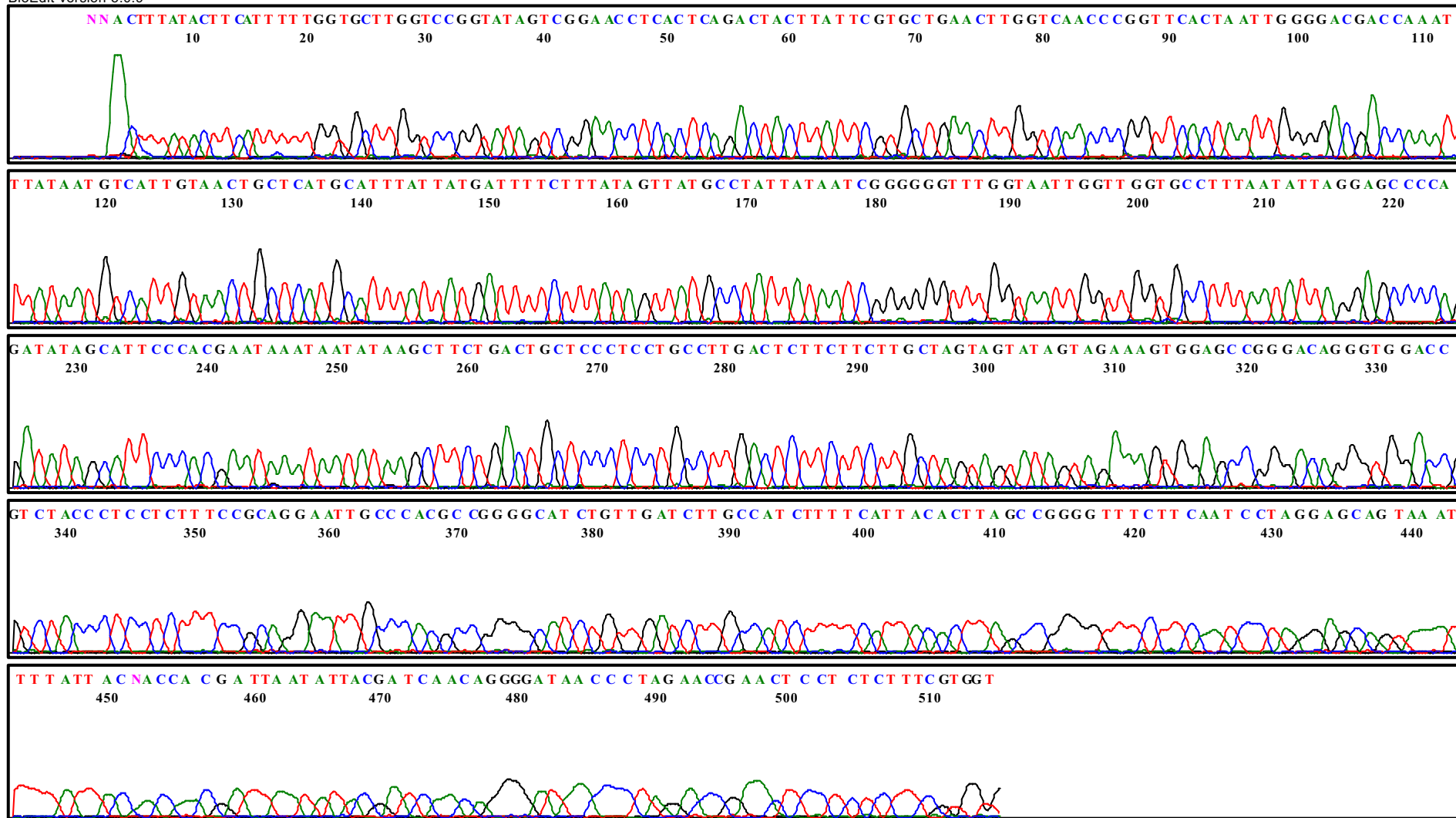


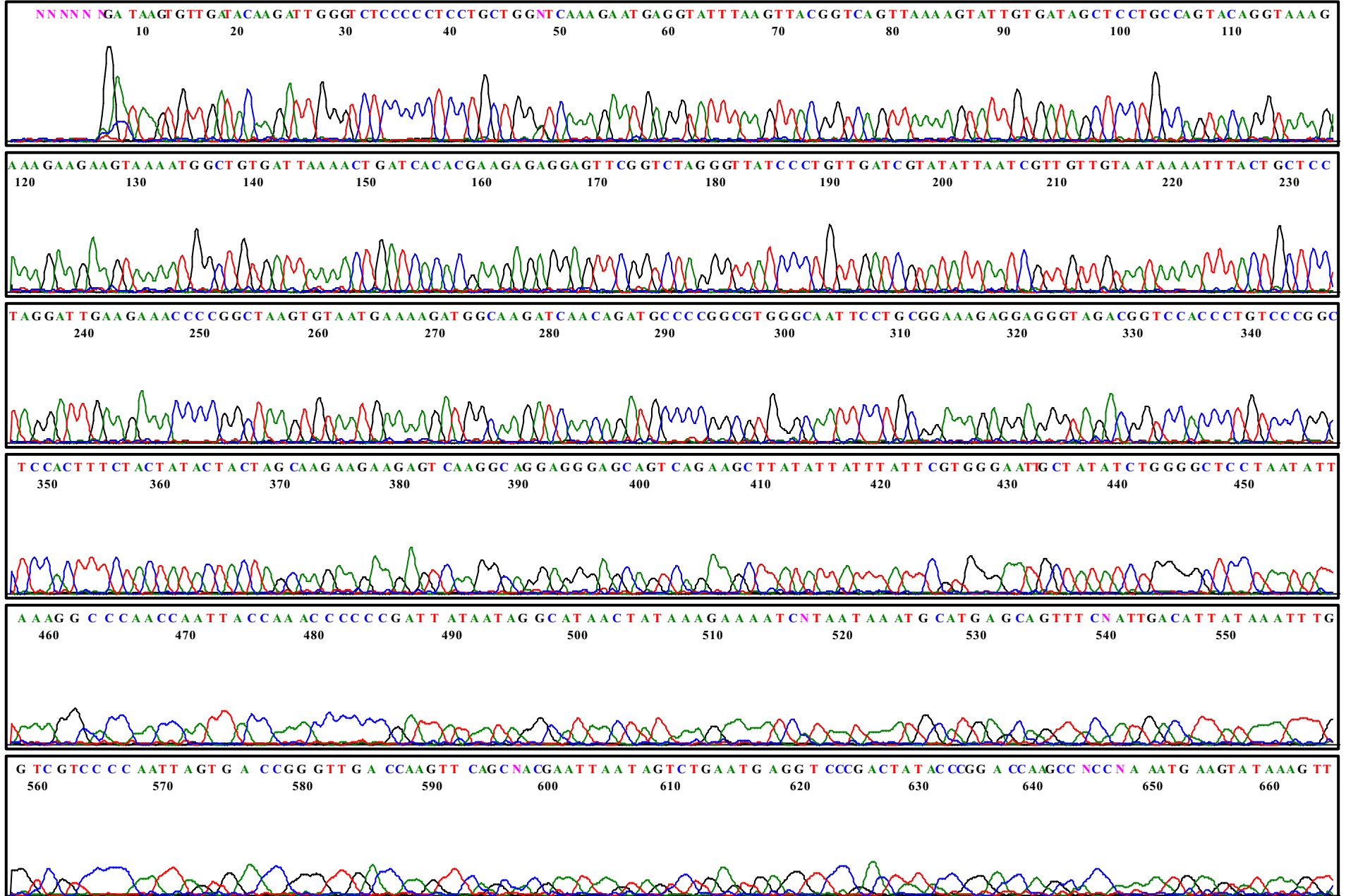




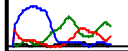


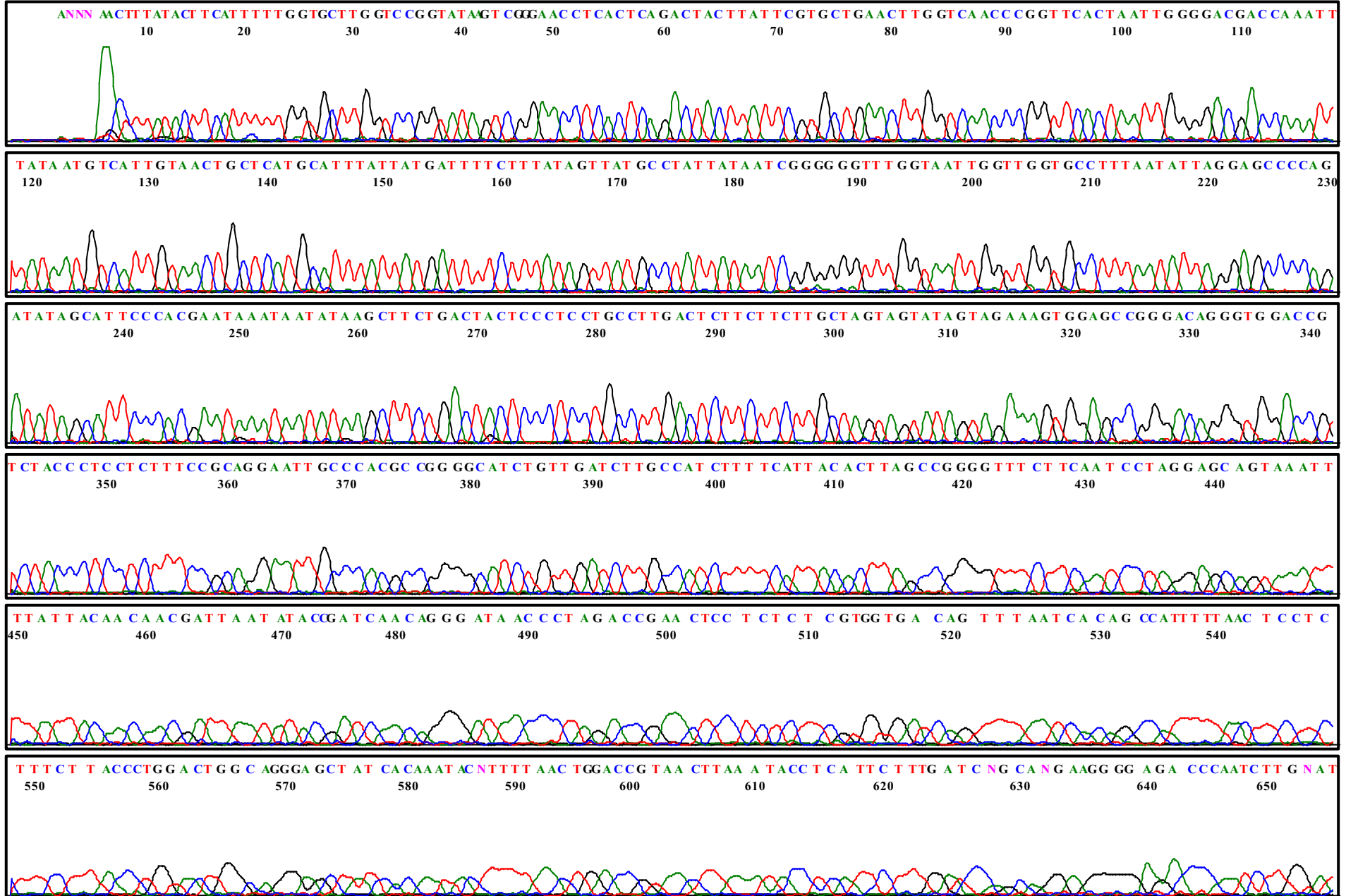


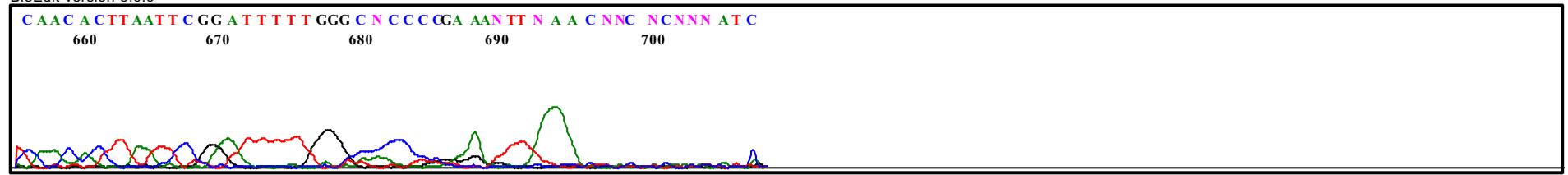


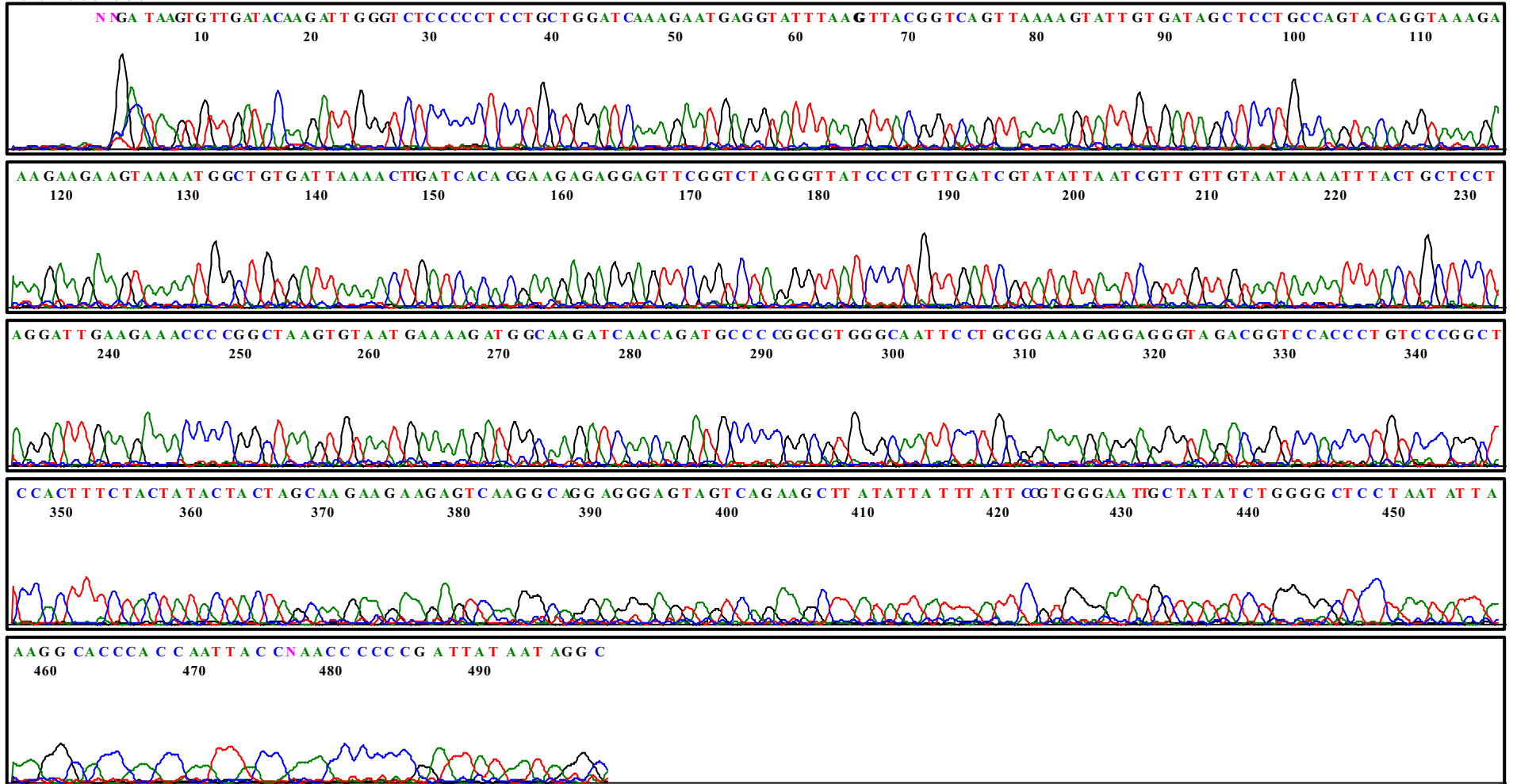


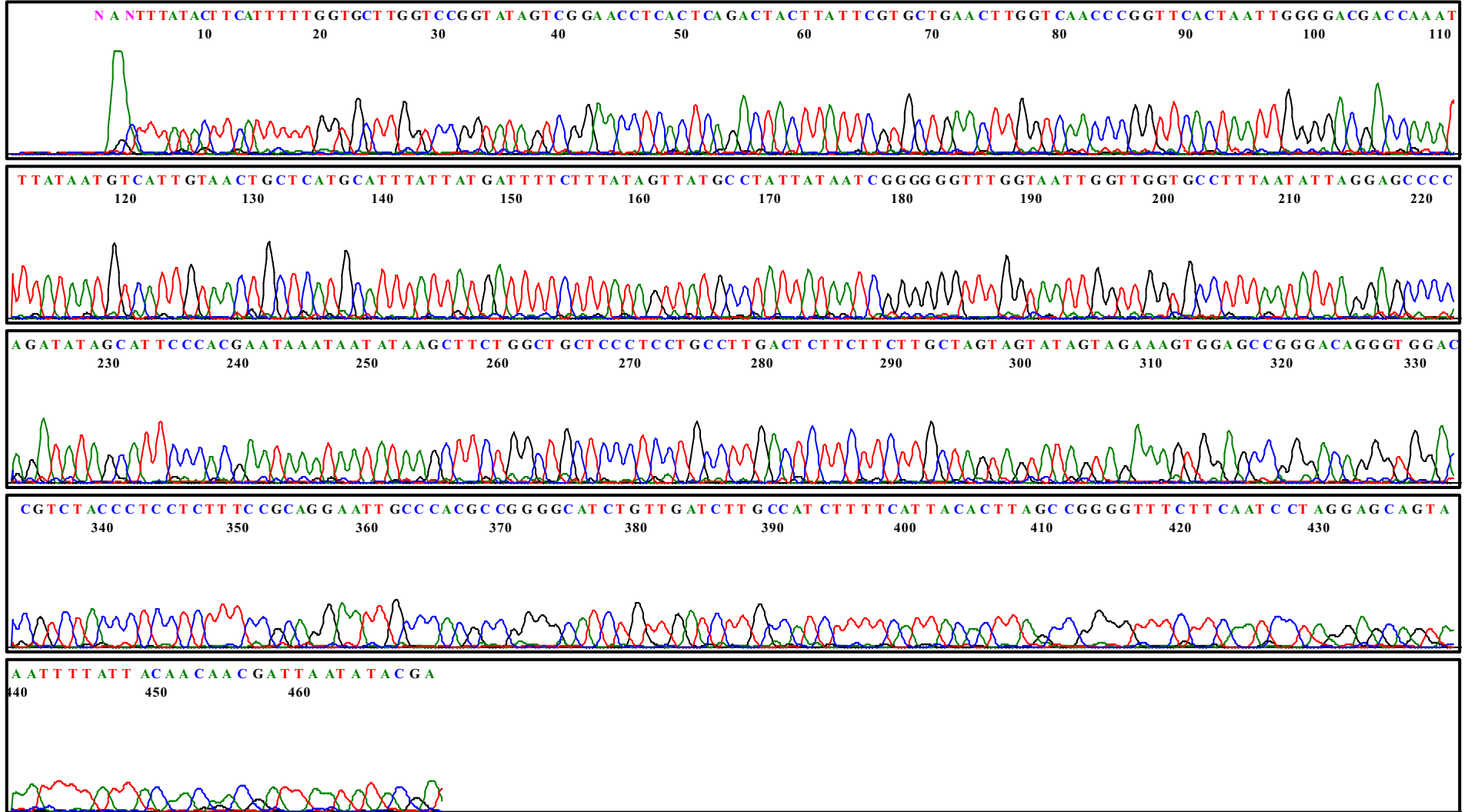
C C A T A
670

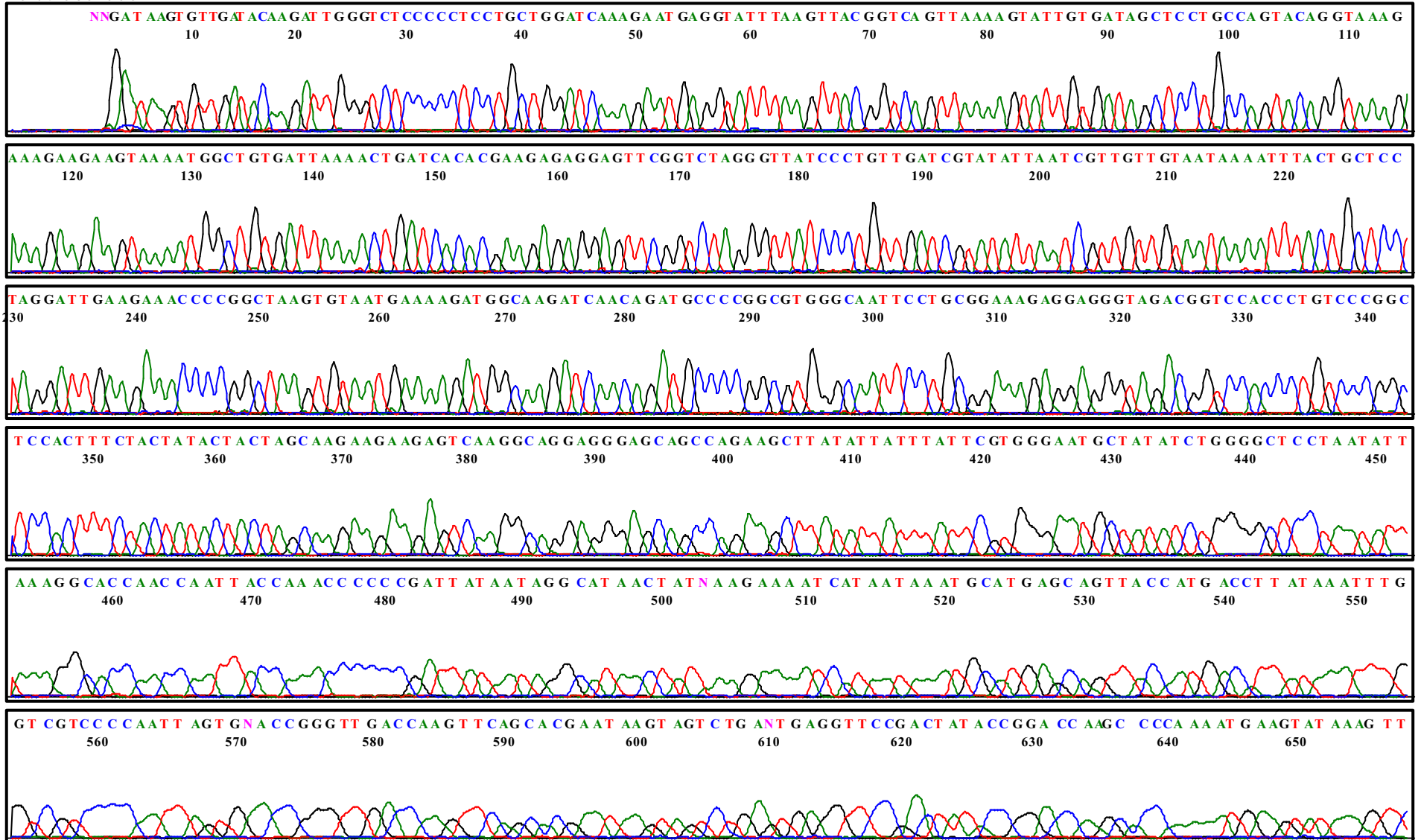














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