Analysis of the Kekkon Family in Neuronal development

by

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I dedicate this work to the designer and creator of life.

May the knowledge and understanding never diminish the awe.

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ABSTRACT

Adhesion Molecules have been associated with a number of neurological and psychological disorders (humans), and implicated in various developmental processes (animals). Better understanding the development of the nervous system and the roles of adhesion molecules in it may be crucial to better understanding these disorders. LIGs, Leucine Rich Repeat and ImmunoGlobulin containing transmembrane proteins, represent a novel class of such adhesion molecules and have been implicated in various neuronal processes, including neurite outgrowth, axonal pathfinding, neuronal regeneration and survival. Two such LIGs are Kek1 and Kek2, members of a Drosophila LIG family, which have been reported to function in axonal pathfinding and synaptic plasticity, respectively. It is unclear what their roles in these processes are, as well as if other members of the Drosophila LIG family have similar roles. Current studies aim to survey the Kekkon family function in the nervous system, looking to identify new phenotypes and/or to elucidate the mechanisms underlying previously identified phenotypes.

To achieve this goal, tissue specific inducible RNAi technique was employed. Validating of a number of transgenic RNAi stocks obtained was necessary and showed that all stocks obtained promoted specific and efficient knock down of target gene. Next an assessment of RNAi knockdown efficacy in developing nervous system was carried out and knockdown was

shown to be weak if not in the presence of Dicer-2 co-misexpression. A number of screens for general behavioral phenotypes were performed including ubiquitous, neural, and imaginal discs knockdown. These uncovered possible effects of *kek1* neural knockdown, as well as possible interaction of Kek1 with neurotactin, neuroglian and kek2. NMJ analysis of Kek5 and Kek6 was also carried out and preliminary results indicate possible interaction of kek5 in NMJ, although no statistical significance was detected.

INTRODUCTION

The nervous system is arguably the most complex biological system. Proper function relies greatly on a pattern of highly stereotyped neural projections and very precise connections formed during the development, as well as the proper transmission and receipt of signals that direct neuronal activity. As a result, enormous emphasis has been placed in understanding the molecular mechanisms that control, regulate and modulate these processes. Consequently, significant progress in elucidating these processes has been made in recent decades. Despite this progress, however, our overall understanding of neural development and brain function is still in its infancy.

NEURAL CELL ADHESION MOLECULES

At the molecular level, our understanding of the processes involved in neural development has been enhanced by the identification of neural adhesion molecules (Van Vactor, 1998). Such molecules are transmembrane proteins, often with defined extracellular motifs, that have been demonstrated to be essential for various aspects of neural development and linked to a variety of neural diseases (Katidou et al., 2008). For example, one such molecule, Neural Cell Adhesion Molecule (NCAM), is a key adhesion molecule in the vertebrate nervous system and contains 5 Immunoglobulin-like (IG) domains and 2 fibronectin (FN) type III domains within its extracellular domain (Cunningham et al., 1987). NCAM mutant mice have

diminished overall brain and olfactory bulb size, but otherwise normal nervous system structure (Campos-Ortega, 1997; Van Vactor, 1998). NCAM has been associated with various neurological disorders, such as schizophrenia, bipolar disorder, depression and anxiety disorders (Katidou et al., 2008). While no direct link has been found between genetic mutations in NCAM and the aforementioned diseases, differential regulation of NCAM isoforms has been clearly associated with these disorders. It is possible, therefore, that differences in NCAM regulation reflects a feature of these disorders, rather than represents an underlying cause. Additional links come from the use of a NCAM derived peptide for therapeutic treatment of Alzheimer's Disease (AD) due to its neuro-protective role in the pathology of AD (Klementiev et al., 2007).

The L1 family, with six IG domains, followed by three to five FN II domains, represent another important class of neural adhesion molecules (Maness and Schachner, 2007). Mutations in L1, which is known to interact with NCAM in the nervous system, has been clearly linked to an X-linked neurological syndrome of broad spectrum called CRASH syndrome (acronym for corpus collosum hypoplasia, retardation, adducted thumbs, spastic paraplegia and hydrocephalus)(Fransen et al., 1996). L1 activity may be associated with other neurological disorders as well. Fetal alcohol disorder, for instance, seems to be in part due to alcohol inhibition of some L1 functions during fetal development(Bearer, 2001). Furthermore, modification

of L1 activity has shown promising results in treatment of spinal cord injury in mouse models. Additional members of the L1 subfamily have also been associated with neural disorders, including schizophrenia, mental retardation, autism, multiple sclerosis and vulnerability to drug addiction.

LIGS REPRESENT A NEW CLASS OF NEURAL MOLECULES

In addition to the aforementioned IG and FN motifs, adhesion molecules may contain additional sequence elements governing their function. These include Leucine rich repeats (LRRs), which represent a protein-protein interaction motif consisting of a β-strand and a α-helix connected by loops, that together often form a curved, horseshoe-shaped structure. As with other NCAMs, LRR containing molecules have also been implicated in neurological disorders. For instance, SLITRK1 seems to be associated with Tourette's syndrome (Abelson et al., 2005), LGI1 is connected to temporal lobe epilepsy (Kalachikov et al., 2002) and NYCTALOPIN with congenital stationary night blindness (Bech-Hansen et al., 2000; Pusch et al., 2000).

A more recently identified class of adhesion molecules contains both **L**RRs and **IG** domains and is referred to as the **LIG** superfamily (MacLaren et al., 2004). Although individually, both the IG domain and the LRR motifs are very common, relatively few molecules contain both together and these molecules are often associated with enriched or exclusive expression in the

nervous system. Since LIGs have only recently been a focus of research, information on the functional significance of these molecules is limited (Fig. 1).

One of the most characterized sub-families of LIGs is LINGO, a family of 4 molecules with 12 LRRs and 1 IG domain (Chen et al., 2006). LINGO-1 interacts with the Nogo receptor and $p75^{NRT}$ and acts through RhoA to inhibit neurite outgrowth, and therefore has become an important target for axonal regeneration research. AMIGO/ALIVIN, another subfamily of LIGs, contains 6 LRRs and 1 IG domain and likewise has been linked to neurite outgrowth. However, AMIGO-1 (ALIVIN-2) acts to promote neurite outgrowth in vitro, in contrast to the inhibitory activity associated with LINGO-1. Also linked to neural development, AMIGO-2 (ALIVIN-1) was shown to mediate synaptic activity dependent-cell survival (Ono et al., 2003), potentially playing a role in apoptotic cell selection during neural development. AMIGO-2 also has been mapped to the

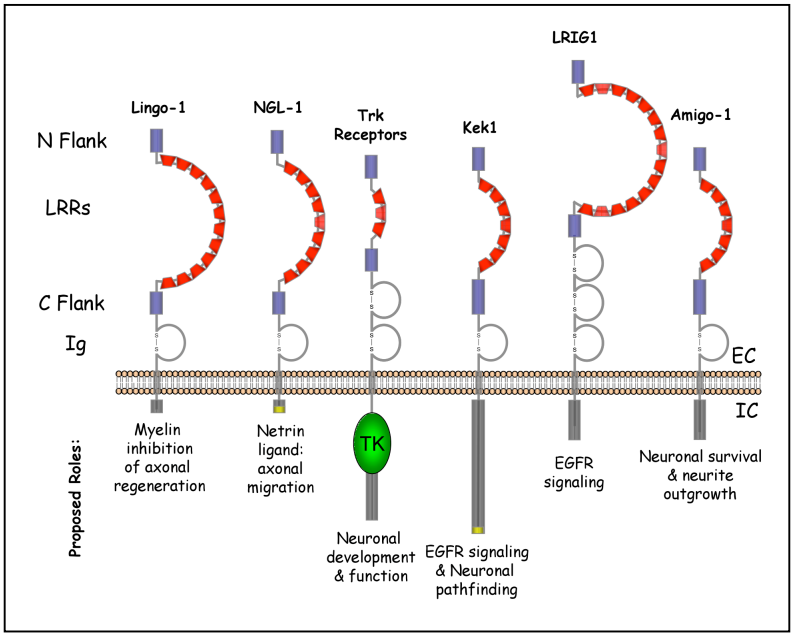


Figure 1: Members of the LIG superfamily. LIGs have variable numbers of LRRs (illustrated in red) and Ig domains (gray horseshoe), a transmembrane and an intracellular region. Only the Trks, which are receptor tyrosine kinases, have an identified enzymatic domain (green).

same region as loci associated with Alzheimer's disease type 5 and Parkinson's disease type 8 were mapped, however the molecular nature of these disease variants has not yet been determined.

Other subfamilies of LIGs include Netrin G1 ligand (NGL-1), NLRR and FLRT, all of which again appear to have associations with neural development. NGL-1 promotes growth of neurons in the thalamus during embryogenesis (Lin et al., 2003). For the NLRR family, NLRR-3 was shown to be upregulated when damage is afflicted to the brain cortex of mouse and NLRR-4 seems to have a role in hippocampal-dependent memory retention(Bando et al., 2005; Ishii et al., 1996). Finally, FLRT3 has been shown to promote neurite outgrowth and is upregulated during peripheral nerve injury(Tsuji et al., 2004).

While various links have been established between adhesion molecules and diseases, the exact mechanisms by which these molecules contribute to nervous system development and to the pathology of neural disorders is largely unknown. An improved understanding of how adhesion molecules function in the formation of the nervous system and the mechanisms through which they accomplish their various roles is an important step in unraveling the basis of neurological disorders and developing effective therapeutic strategies.

Ethical and moral complications make animal model organisms vital to the study of neuronal developmental process. The inherent tractability and

the genetic tools available in *Drosophila melanogaster*, in addition to a simplified, but largely homologous developmental mechanism, makes *Drosophila* instrumental in understanding the basic interaction of adhesion mechanisms in neural processes.

EMBRYOGENESIS

Early embryonic development of the fly is marked by the formation of a syncytium, a single multinucleated cell. Subsequently, as the embryo develops, membranes form creating a cellular blastoderm stage, and gastrulation starts along with formation of the germ band. Soon after the start of gastrulation, the germ band elongates, folding internally and extending anteriorally, thereby causing the posterior extremity of the embryo to approach the anterior extremity. Later in embryogenesis the germ band retracts until the posterior extremity of embryo reaches the posterior end

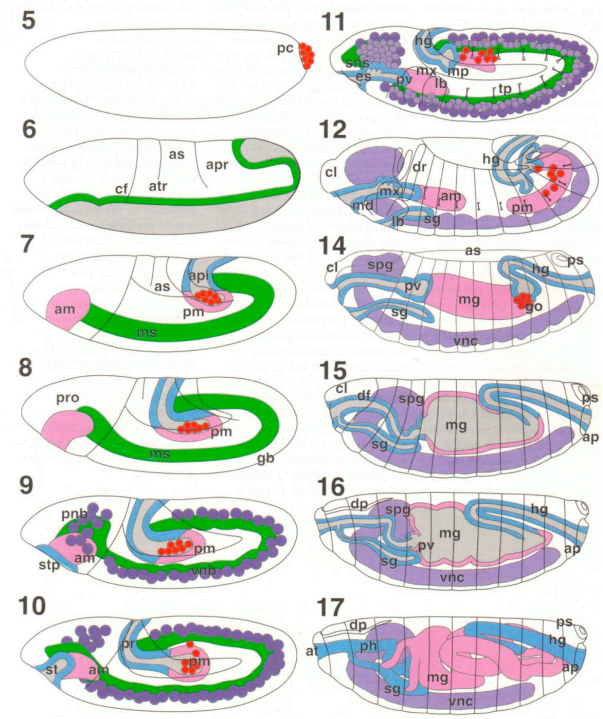


Fig 2: schematic illustration of embryonic staging and main events of embryonic development. Number at top left indicates staging. Stage 6 starts gastrulation. Stage 9 indicates neural progenitor segregation (purple) (vnb-ventral neuroblasts, pnb – procephalic neuroblasts). Stage 10 shows further segregation of neuroblasts and first neuroblast division and appearance of ganglion mother cells. Germ band elongation stops. Stage 11 epidermal segmentation becomes evident and neuroblasts division continues. Stage 12 shows ventral nerve cord entirely separated from epidermis and appearance of first neural processes and fibers. Stage 13 signifies well-differentiated ventral nerve cord and supraoesophageal ganglion and head involution begins. Fiber connectives and commissures linking the different neuromeres and muscle cells are visible at this stage. In stage 16 synapse formation starts and lasts well into time of hatching. During stage 17 ventral cord further retracts. (Campos-Ortega, 1997)

proper.

To better understand embryonic development, a classification system involving stages based on prominent features of the embryo has been developed. Figure 2 illustrates the main stages of embryonic development and its staging classification (Campos-Ortega, 1997).

NEUROGENESIS

There are three main processes in *Drosophila* neurogenesis:

1. Acquisition of neural identity,
2. Axonal guidance/fasciculation, and
3. Synapse formation.

The first process is the specification of neuroblasts followed by the formation and differentiation of neurons. Once cells have adopted a neuronal identity neurite outgrowth is initiated, thus starting the process of axonal guidance and pathfinding. The first differentiated neurons are called pioneer neurons and their axons lay out a scaffold for further development of the nervous system. Subsequently, differentiated neurons then extend their axons which fasciculate to pioneer neurons' axons. The axons of these neurons reach their appropriate target by constantly selecting and fasciculating with correct axons among many choices, a process often referred to as selective fasciculation (Goodman and Doe, 1993). The final process is the formation of synapse, which consists of three distinct steps.

The first step is appropriate synaptic target recognition, which is then followed by structural, molecular and physiological changes that characterize synapse formation. Synapses reach functional maturity by the time embryos hatch, however synapse maintenance, growth and plasticity, the third step of synapse formation, continues.

1. Acquisition of neural identity

The *Drosophila* nervous system develops in a bilateral symmetrical segmented pattern forming a sequence of repeated units called neuromeres (Campos-Ortega, 1993; Campos-Ortega, 1997). At each neuromere, clusters of neuroectodermal cells are formed and generate only one neural progenitor cell per cluster – the neuroblast. Neural fate determination is conferred by expression of proneural genes, such as members of the *achete-scute* complex, and is controlled by Delta/Notch signaling (Campos-Ortega, 1995; Duffy and Gergen, 1994). Proneural genes encode transcription factors and promote accumulation of Delta. Delta interacts with the receptor Notch on neighboring cells and induces down-regulation of the proneural genes. As a result the cell with highest levels of proneural genes expression becomes a neuroblast and delaminates from the cluster. Segmentation genes, such as the pair-rule genes *fushi tarazu* and *even skipped* and the segment polarity genes *wingless*, *hedgehog*, *patched*, *gooseberry* and *engrailed*, along with apicobasal polarity genes are also involved with specifying neuroblast

identity(Siller and Doe, 2009). Specification of a neuroblast's particular identity by the patterning genes determines its lineage, ultimately determining the identify of daughter neurons.

After a neuroblast delaminates from the ectodermal layer, it undergo several rounds of asymmetric divisions, generating a ganglion mother cell (GMC) at each division while still preserving its stem cell properties. GMCs then undergo one division to generate two distinct neurons and/or glial cells. Within *Drosophila* the number and identity of neurons generated by each neuroblast is invariant and highly reproducible.

2. Axonal guidance and fasciculation

During the second phase of neural development, the first set of differentiated neurons elongate axonal projections that are directed by guidance cues throughout the nervous system (Goodman and Doe, 1993). These cues may be released (by glial cells) and diffusible as is the case of Slits and Netrins, or they may be membrane bound molecules such as Ephrins. Semaphorins can be membrane bound or released. Guidance molecules can act as attractants or repellents depending on cell type, context and timing. Slits and Semaphorins act principally as repellent signals, and Netrin acts primarily as an attractive cue, while Ephrin can act either way.

A paradigm for axonal guidance is midline axonal crossing in *Drosophila* (Chilton, 2006; Sanchez-Soriano et al., 2007). In *Drosophila*, Slit and Netrin are both expressed and released by the midline glial cells. Expression of Slit in the midline repels growth cones thereby preventing ipsilateral projecting neurons from crossing the midline. This repulsion is mediated by Slit's receptor Roundabout (Robo), by interaction of Slit's LRR with Robo's Ig domain. In neurons that project contralaterally across the midline, however, the repulsive effect of Slit is negated by Commissureless (Comm) expression, which prevents Robo transport to the growth cone. Then the growth cone is able to cross the midline in response to Netrin attraction though the Frazzled receptor. Once growth cones cross the midline, Comm is down-regulated and Slit/Robo mediated repulsion takes place, allowing axons to proceed past the midline (Fig. 3). Independently Netrin also mediates repulsion from midline though receptor UNC-5, which is upregulated after midline crossing.

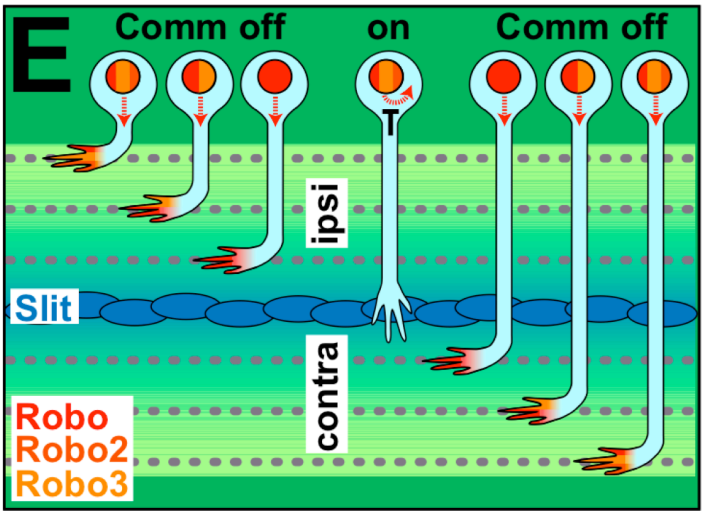


Figure 3: Illustration of Robo and Slit signaling in midline crossing. Robo expression profile determines the axonal track that axons select as they join the ventral nerve chord. Comm expression blocks Slit mediated repulsion and enable axons to cross midline. Once across midline, Comm down-regulation reinstates Slit-mediated repulsion and axon proceed to the axonal track of choice via Robo signaling. (Sanchez-Soriano et al., 2007)

Once pioneer neurons have established their axonal tracks, remaining neurons extend their axons through selective fasciculation (Goodman and Doe, 1993). Although some molecules involved in this process have been identified, the signaling mechanisms involved are not well understood. For instance FasciclinII (FasII), the *Drosophila* homolog of Neural Cell Adhesion Molecule (NCAM), has been shown to mediate axonal fasciculation. In animals overexpressing Fas II, over-fasciculation is observed to the point where axons do not defasciculate and diverge from pathways when they normally would. Furthermore, in *fasII* mutants axonal fascicles are not bundled together as tightly as wild type, indicating lack of proper fasciculation. However, no defective projections are observed in these mutants (Lin et al., 1994; Lin and Goodman, 1994). These results indicate that the mechanisms that govern selective fasciculation may be distinct from guidance cues mechanisms (Goodman and Doe, 1993; Van Vactor, 1998).

Neuroglian (NRG), a homolog of the vertebrate L1 adhesion molecule, (Bieber et al., 1989; Hall and Bieber, 1997), N-type Cadherin (DN-cadherin) (Iwai et al., 1997), matrix metalloproteinase (Miller et al., 2008) and Neurotactin (NRT) (Speicher et al., 1998) have also been implicated in axonal fasciculation. In addition, substrate adhesion molecules (SAMs), such as integrins, were shown to be involved in neuronal migration and axonal fasciculation in *C. elegans* (Baum and Garriga, 1997). Considering the more limited family of integrins in *C. elegans*, this result is believed to be

indicative of a novel role for integrins in other species as well (Baun & Garriga, 1997).

Two other major families of molecules also implicated in axonal fasciculation are receptor tyrosine kinases (RTKs), such as Derailed (Drl), and receptor protein tyrosine phosphatases (RPTPs), such as DPTP69D and DPTP99A (Van Vactor, 1998). Both kinases and phosphatases seem to modulate both axonal fasciculation as well as axonal guidance, indicating that although these processes may have separate mechanisms, they appear to share a common pathway. Figure 4 depicts several molecules involved in axonal fasciculation.

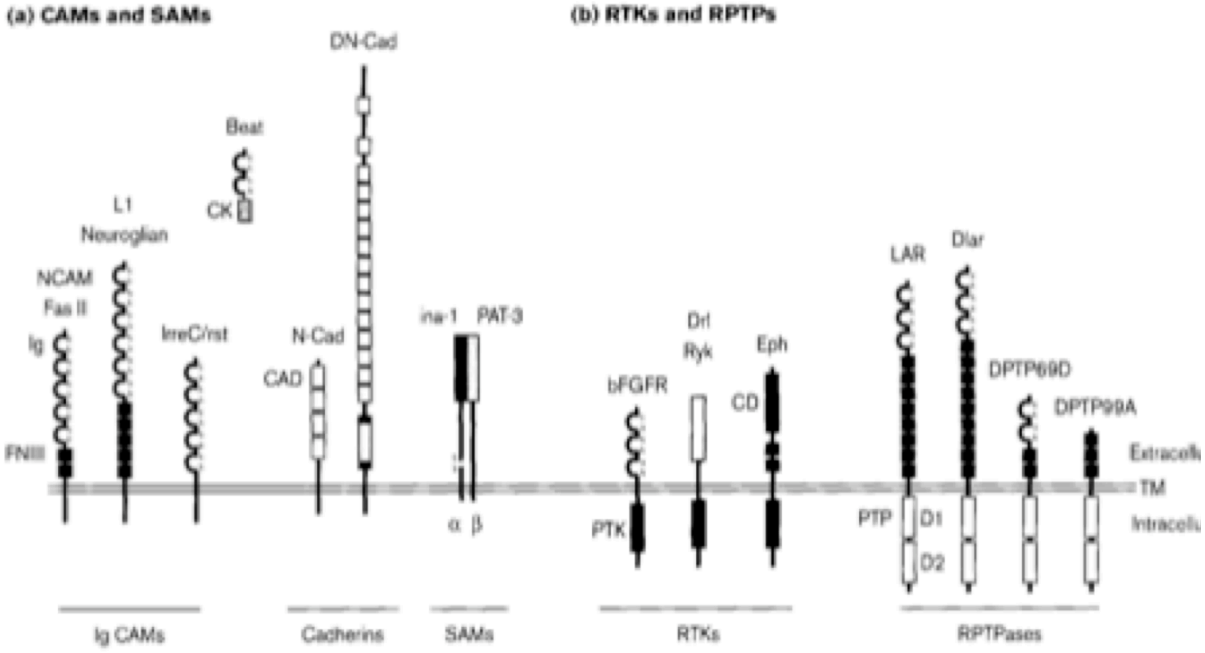


Figure 4: Molecules involved in selective fasciculation. Cell adhesion molecules featuring IG and fibronectin domains represent the most characterized molecules involved in axonal fasciculation. Substrate adhesion molecules represent a more recent class of molecules that also seems to be involved in this process. RTKs and RPTPs are seems to modulate both fasciculation and guidance. (Van Vactor, 1998)

Semaphorin Ia and Beaten Path Ia (Beat) are also believed to act as repellents in axonal fasciculation (Van Vactor, 1998). For instance, Beat Ia accumulates in high concentration on specific choice points where fascicles divide. It has been proposed that Beat Ia decreases adhesion of FasII and other CAMs to allow branching of nerves.

As a result of the mechanisms in place for axonal guidance and selective fasciculation, very clear axonal patterns are formed. Different aspects of this pattern can be observed with different markers. For instance upon staining with the antibody BP102, which targets an epitope on CNS axons, a ladder like structure can be observed in the ventral nerve cord, where the two commissures per segment can be distinguished (Fig.5A). Alternatively detecting expression of FasII in embryos with the 1D4 antibody yields a view of the three axonal tracks on each side of the midline, as well as some of the peripheral nervous system (Fig. 5B).

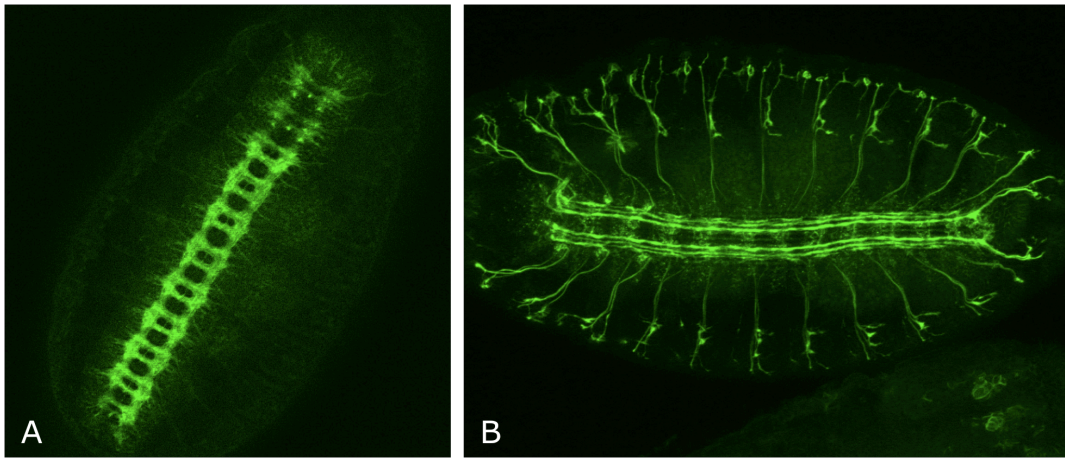


Figure 5: Axonal patterns of *Dosophila* embryo. Panel A shows CNS upon staining with BP102 antibody, which marks all CNS axons. Panel B shows staining with ID04 antibody against FASII, which marks the three major axonal tracks on each side of the midline as well as some of the peripheral nervous system. Both images were taken at stage 15/16 at 100x magnification, using Zeiss apotome processing. Right panel is a z-stack maximum image projection.

3. Synapse formation – NMJ as a model

Structure of the NMJ

The last phase of neural development involves synapse formation, the study of which is limited to a few *in vivo* models, due to the scale and complexity of synapses. Of these, the Neuromuscular Junction (NMJ) in *Drosophila* has long been used as a model for synapse formation, due to the relative ease of its manipulation and observation, as compared to synapses in the CNS. Furthermore, general aspects of NMJ formation are stereotypical and reproducible. In each hemisegment (the lateral half of each segment),

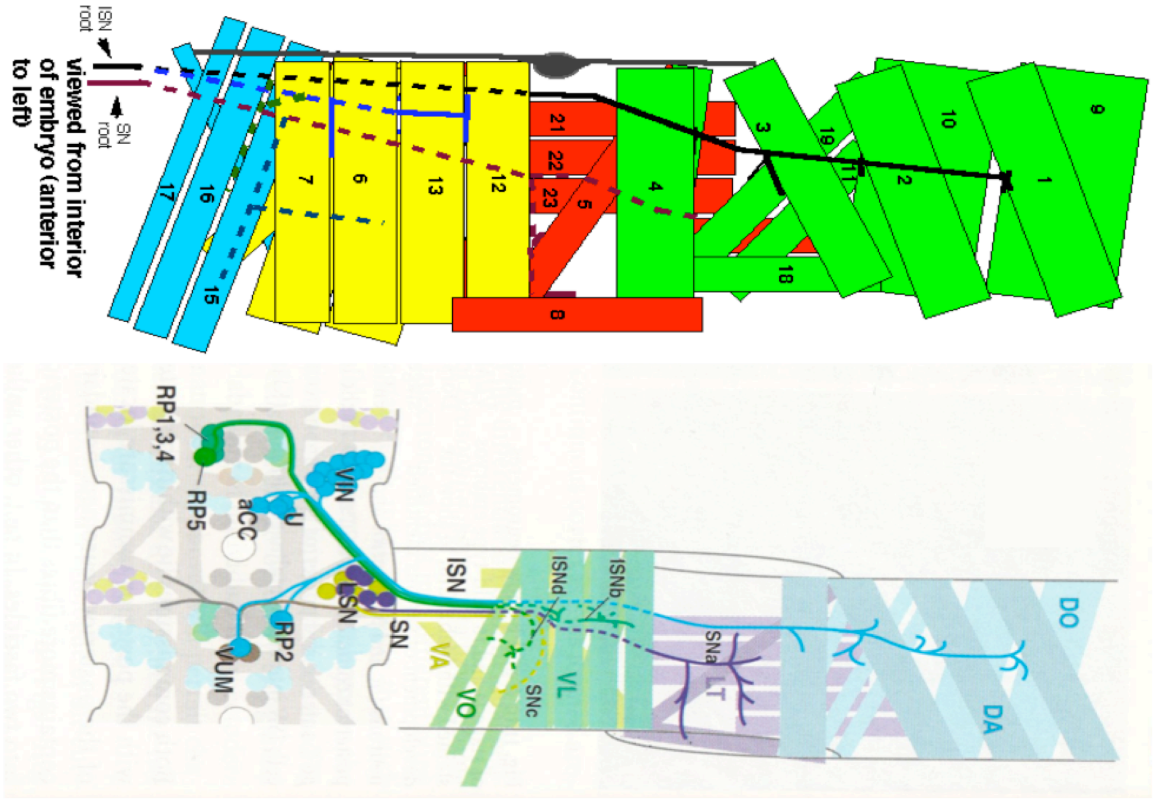


Figure 6: Muscle and motor neuron patterns. Schematic illustrations of a hemisegment muscle system. Muscle groups and nerve branches are color coded. Top panel indicates muscle pattern and muscle numbering nomenclature. Bottom panel shows section of vnc and MN cell bodies as well as labelling of nerve branches. (Campos-Ortega, 1997)

30 muscles are innervated by 33-40 motor neurons. Figure 6 shows a schematic of the observed muscle pattern; the top panel indicates the numerical nomenclature used, while the bottom panel shows the innervating neurons and their trajectories through the various nerve branches.

Generally speaking, each motor neuron (MN) innervates one or more muscle fiber and forms a specific type of synapse, Ib, Is, II, or III. Since only a few neurons have been identified to innervate specific muscle fibers, synapses are identified by bouton size. Each fiber is innervated by only one neuron of each type (Hoang and Chiba, 2001). Structurally, type Ib (for big) boutons are the largest, measuring 3-6μm; they are glutamatergic, are present in all muscles and tend to be in short and minimally branched terminals. The majority of MNs form type Ib boutons and innervate only one or immediately adjacent muscle fibers (as is the case of muscle 6/7, 21-24 and 15-17). Type Is (small) boutons are slightly smaller than Ib, about 2-4μm, also glutamatergic and exist in longer and more elaborate terminals. They are believed to be present in all muscles, though in some cases the exact bouton type present is not clear. Type II boutons are small (1 to 2μm), they use glutamate and octopamine as neurotransmitters, and are present in most muscle in very long and elaborate terminals. The innervation pattern of Is and II are similar, with one MN innervating multiple muscle fibers in a given muscle group. Finally, type III boutons are of a medium size, about 2-

3μm, are believed to be present only on muscle 12, and contain both glutamate and insulin.

TARGET RECOGNITION AND SYNAPTOGENESIS

A key aspect of synapse formation involves target recognition. Axonal guidance mechanisms collaborate in achieving synaptic specificity as they lead the growth cone toward the appropriate synaptic target. The final step before synapse formation is the selection of the target cell from among its many neighbors. Filopodial processes in presynaptic growth cones probe the environment to identify the appropriate target, following the same strategy as used during axonal pathfinding. Likewise, filopodia-like processes are also present in postsynaptic cells, both in dendrites and muscles (Ritzenthaler and Chiba, 2001). These processes in muscles are called myopodia and they enable direct and dynamic interaction between the growth cone and possible muscle targets at relatively long distances. The interaction between filopodia and myopodia is sufficient for target recognition and formation of a stable connection between growth cone and muscle.

Target identification is believed to occur through molecules with very specific expression patterns. Arguably, one of the most well-characterized examples is FasIII (Rose and Chiba, 2000). During synapse formation within the PNS, FasIII is expressed in the RP3 motor neurons and its synaptic targets - muscles 6 and 7. In *fasIII* null mutants, incorrect innervation is observed, albeit at low frequency – 9% of RP3 neurons form connections

with other muscles. In Fas3 overexpression in muscles, however, incorrect innervation is observed in 72% of the cases, indicating that gain-of-function experiments can have stronger phenotypes than loss-of-function experiments, most likely because of redundant mechanisms that exist to ensure precise connectivity. When FasIII is overexpressed in muscles in a null background (no FasIII in RP3 neurons), the frequency of incorrect innervation decreases to 14%, demonstrating that phenotype is potentially conferred primarily through a homophilic interaction.

Another important molecule linked to synapse formation is the LRR containing immune receptor Toll, which is also a synaptic repellent molecule expressed in muscles 15, 16, 17 and 29, a muscle group that the RP3 growth cone crosses on its way to M6/7 (Rose and Chiba, 1999; Rose et al., 1997). In a *toll* knockout, only 10% of RP3 growth cones reach the appropriate target. Upon misexpression of Toll in muscle, only 14% of RP3 growth cones form appropriate connections, while the other 86% form incorrect innervations or no innervations at all. Interestingly, misexpression of both Toll and Fas3, repellent and attractant respectively, in muscle results in few defects, indicating that growth cones receive and integrate multiple inputs to generate a decision regarding synaptic target recognition.

Likewise, temporal regulation is also key, as expression of Toll in proximal muscles ensures that inappropriate synapses are not formed by MNs that will innervate more distal muscles. Subsequently, Toll is then

down-regulated by the time the appropriate MNs should synapse in these proximal muscles.

Not surprisingly, ligands (Wnt4, Netrin), receptors (Frizzled and Derailed), LRR containing adhesion molecules (Capricious) and transcription factors have all been implicated in specific aspects of synaptogenesis. From work on these and other molecules, it has become clear that synaptic specificity is not only conferred by attractant signals, but also by repellent signals from surrounding inappropriate targets. In support of this latter notion, several synaptogenic inhibitory molecules have been identified to date, such as Dishevelled, Beaten path, and D-semaphorin. Moreover, it appears that some molecules may have dual roles. For instance, Netrin and Wnt-4 have been shown to have synaptogenic and anti-synaptogenic properties in a context dependent manner. Such effects are likely mediated by distinct receptors as observed in axonal guidance.

One model proposes that synaptogenesis occurs through two different classes of molecules (Hoang and Chiba, 1999). The first class includes molecules with very narrow expression patterns and promotes specific target recognition, such as FasIII. Manipulation of these molecules would generate very specific single cell level abnormalities. The second class includes molecules with broader expression profiles that promote synaptogenesis after initial target recognition steps. Manipulation of these molecules would cause more generalized defects, although these could be subtle due to

possible functional redundancy of molecules of this class. Consistent with this model, there are several molecules identified as general adhesion molecules that promote synaptic formation in a general manner, including Neuroglian, Integrin and Dn-Cadherin.

Upon target recognition, the relatively flat growth cone swells and forms large prevaricosities, which then constrict to form smaller varicosities or boutons, typical of a mature synaptic connection. At this point in time, clusters of presynaptic and postsynaptic apparatus is formed and co-localized across the synaptic cleft in distinct active zones. It is largely unknown how adhesion molecules promote the molecular changes associated with synaptogenesis, and what are the components/pathways involved in this process. It is known however that pre- and postsynaptic growth is tightly regulated and coordinated, particularly during larval development, when muscles grow to become about 150X their original volume.

A predictable series of events has been observed during the development of the synapse between muscle 6 and the RP3 motor neuron. Prior to synaptogenesis, neither neurotransmitter nor a functional receptor is present on MN or muscle. As the motor neuron growth cone contacts the muscle, transmitter expression starts, and myotubes uncouple soon after. Immediately after uncoupling of myotubes, a small number of functional glutamate receptors are evenly distributed on the muscle surface. Shortly

after motor neuron filopodia localize at the developing synaptic zone, functional receptor localization occurs. At this point a functional synapse forms, endogenous muscle activity begins and nerve stimulation leads to muscle contraction. Then presynaptic specialization develops, giving rise to the mature morphology. After that a second motor neuron contacts muscle 6 at the pre-established synaptic zone, a second stage of functional receptor expression emerges and vigorous neuromuscular activity characteristic of larval locomotory movement initiates.

Presynaptic activity, although not required for initial synapse formation is required for post synaptic clustering of Glutamate Receptors (GlutR) and regulates structure and strength during NMJ growth (Budnik, 1996; Nakayama et al., 2006; Prokop and Meinertzhagen, 2006). Pre and postsynaptic FasII levels are also tightly coregulated (Ashley et al., 2005). In fact, upregulating FasII activity only on one side of the synapse decreases synaptic size and bouton number and generates abnormal NMJ morphology. However, equal upregulation of FasII on both pre and post-synaptic cells increases NMJ size significantly, stimulating new bouton formation.

An additional mechanism to coordinate pre and postsynaptic growth is retrograde Bone Morphogenic Protein (BMP) signaling (Keshishian and Kim, 2004; Nakayama et al., 2006; Prokop and Meinertzhagen, 2006). BMP signaling is a conserved signaling cascade that controls many developmental processes. In *Drosophila,* members of the BMP pathway are required for

proper NMJ development and manipulations that decrease BMP signaling negatively affects synapse size, stability and homeostasis. It is believed that type II receptor Wishful thinking (Wit) as well as both type I receptors, Saxphone (Sax) and Thickvein (Tkv) are involved in retrograde signaling at the NMJ (Keshishian and Kim, 2004). Specifically, the ligand Glass bottom boat (Gbb) is released by the postsynaptic muscle terminal onto the synaptic cleft, which promotes phosphorylaton of the downstream transcription factor Mad in the motor presynaptic terminal (Goold and Davis, 2007). BMP component mutants, such as BMP receptor *wishful thinking* (*wit*), show decreased NMJ size and bouton number to approximately 40% of wild type (Aberle et al., 2002). On the other hand, overexpression of BMP components or mutants of negative regulators of BMP components exhibit larger NMJ size. For instance *highwire* (*hiw*) mutant, a putative E3 ligase that negatively regulates levels of co-Smad Medea shows synaptic bouton number increase of up to 200% over wild type (Aberle et al., 2002; McCabe et al., 2004).

THE KEKKON FAMILY

Our lab has been studying a set of proteins called the Kekkon family. This class of LIG family members are transmembrane proteins identified in invertebrates with seven leucine-rich repeats and an Immunoglobulin domain (Fig.7) (MacLaren et al., 2004). Embryonic expression profiles for family members indicates they are expressed in the nervous system,

however, to date little is known about the functional role of these molecules in neural development.

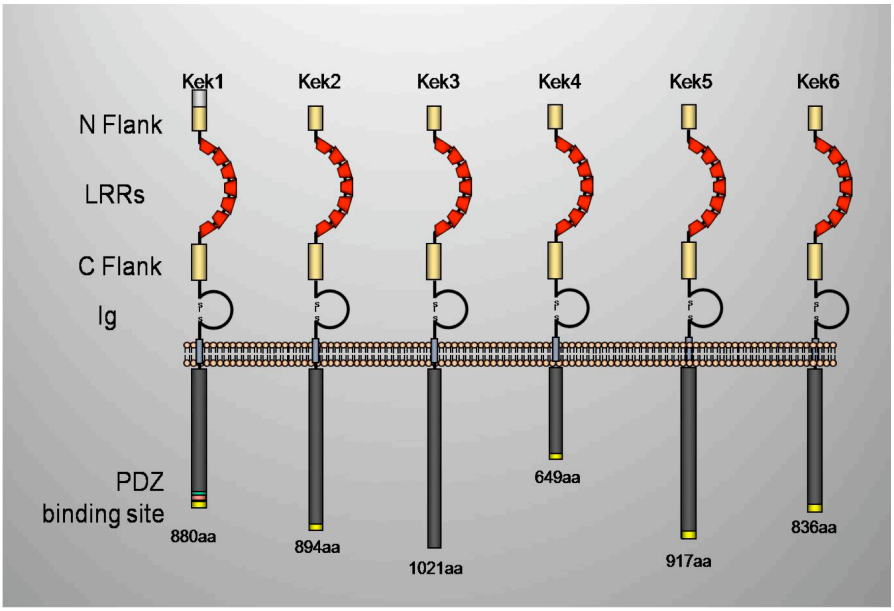


Figure 7: The Kekkon Family. Constituted of 6 transmembrane molecules containing 7 LRR and one Ig domain. Their intracellular region does not contain a catalytic activity domain but most display a PDZ binding site.

Kekkon1 (Kek1) was initially identified on the basis of its expression in the nervous system (Musacchio and Perrimon, 1996) and has subsequently been shown to interact with Epidermal Growth Factor receptor (EGFR) as a negative regulator (Ghiglione et al., 1999). In addition, in a study of *neurotactin (nrt)* role in axonal fasciculation, the *nrt* and *kek1* double mutant shows ventral nerve cord fasciculation defects not seen in the single mutants, indicating a possible role of *kek1* in axonal fasciculation (Speicher et al., 1998). Synergistic genetic interaction were also observed between *nrt* and *neuroglian* and *derailed,* but not with *DPTP69D* and *DPTP99A*. However, no mechanisms for these interactions have been proposed, leaving open the significance of these findings.

Kekkon5, another member of the Kek family, has recently been demonstrated to modulate Bone Morphogenesis protein (BMP) signaling in

the crossvein development in the *Drosophila* wing (Evans et al., 2009). Misexpression and loss-of-function of *kek5* was shown to affect the profile of phosphorylated Mad and dSRF in presumptive crossvein cells. Furthermore, Kek5 phenotypes are similar to those obtained by manipulation of Short gastrulation (Sog), a secreted modulator of BMP signaling, but unlike phenotypes of dominant negative receptors, indicating Kek5 may be acting upstream of BMP receptors. Additionally Kek5 was shown to antagonize Glass bottom boat (gbb), a BMP ligand, supporting this claim.

Recently, Kekkon2 was identified in a microarray screen for genes involved in synaptic plasticity (Guan et al., 2005). Kek2 was upregulated and downregulated in mutants in which synaptic activity levels were increased and decreased long-term, respectively. It was then shown that the absolute level of *kek2* expression modulates the extent of innervation in the NMJ, where both increase and decrease in Kek2 levels caused a decrease of bouton number in the NMJ (30-50% decrease), thereby supporting a role for *kek2* in synaptic plasticity.

The goal of my thesis research project was to perform a broad survey of the role of the Kek family in neural development. For family members this included expression profiling, functional tests using RNAi-mediated knockdown, and attempts to reproduce the reported phenotypes of axonal fasciculation and synaptic plasticity to further characterize these interactions and possibly determine specificity of the involved Kek family member.

RESULTS

KNOCKDOWN STRATEGY

To enable a survey of the function of Kek family members in *Drosophila*, RNA interference (RNAi) was used. In *Drosophila*, transgenic hairpin constructs capable of producing gene specific RNAi triggers can be coupled to the existing GAL4/UAS system to promote inducible knockdown of genes in specific tissues (Duffy, 2002). Manny lines have already been established which induce expression of the GAL4 transcription factor in a variety of tissues and patterns. When expressed, GAL4 binds its recognition site – upstream activating sequences (UAS), promoting expression of the desired target sequence. This system is often used to promote misexpression of genes in a tissue of interest, by inserting the coding region of the genes of interest downstream of the UAS recognition site. In this bipartite system, UAS responder lines are created, and then flies are mated with GAL4 drivers of interest to promote expression of the gene of interest.

Recently, the Vienna Drosophila RNAi Center (VDRC) has created a library of transgenic stocks with inducible RNAi constructs targeting 88.2% of the *Drosophila* genome (Fig.8) (Dietzl et al., 2007). In this library, the UAS promoter is attached to an inverted repeat sequence of approximately 300-400bp that matches the target gene. Upon transcription, the inverted repeat folds over creating a hairpin RNA (hpRNA). The hpRNA is recognized by the RNA interference machinery of the cell and the Dicer enzyme (Dcr-2 in *Drosophila*) cuts the hpRNA into short stretches of RNA of about 19-23bps each, thereby forming a set of silencing RNAs (siRNA) all matching the target mRNA (Ghildiyal and Zamore, 2009). With the help of Dcr-2 and the double stranded RNA binding protein R2D2 the siRNAs are subsequently loaded onto the RNA-induced silencing complex (RISC), which with the help of Argonaute2 (Ago2) selects the guide strand from the siRNA.

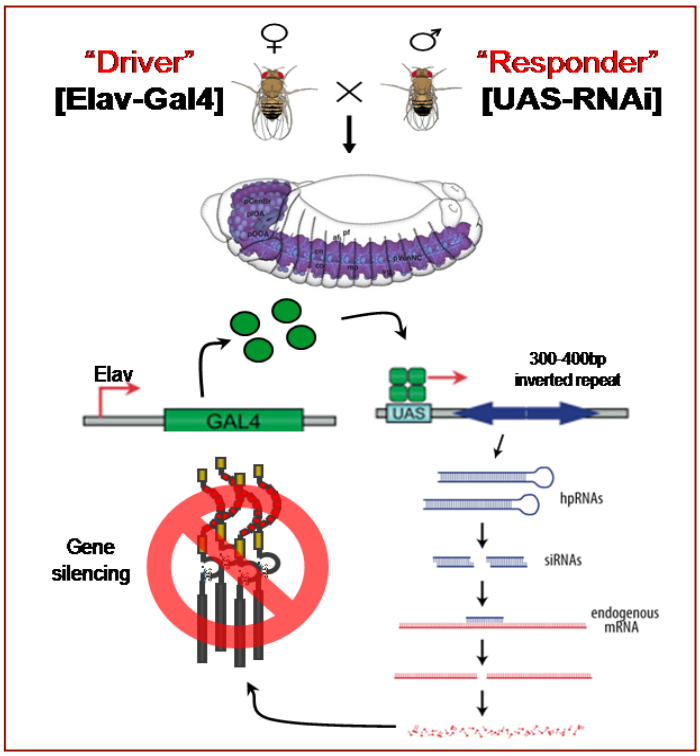


Figure 8: The GAL4-UAS.RNAi system. Parental flies have driver constructs directing GAL4 expression in tissue specific manner (e.g. nervous system), or a responder construct that directs RNAi trigger transcription under control of UAS sites. Progeny express GAL4 in the nervous system, which then directs transcription of the RNAi trigger in that tissue. RNAi trigger forms hpRNAi, which is processed by Dicer-2, generating siRNAs. The gene specific siRNAs then couple with the RISC complex to promote degradation of the target gene mRNA, thereby silencing target gene expression.

At this stage, Ago2 cleaves the passenger strand of the siRNA, forming mature RISC loaded with a single stranded RNA (guide strand). This guide strand then directs identification of mRNAs that have complementary sequence, promoting their degradation, and effectively preventing translation of target mRNA.

In theory, then, with the VDRC strains and existing GAL4 lines, the function of ~88% of the genes in the *Drosophila* genome can quickly be assessed in the tissue of interest (Fig. 8). Transgenic RNAi responder lines targeting each of the *keks* were available and obtained from VDRC. The sequences used to generate the hairpin for each *kek* family member are shown in Appendix 2. To examine further the published genetic interactions between Kek1 and Neurotactin (Nrt) and to study the possibility of an interaction between Neuroglian (Nrg) and Kek1, VDRC RNAi lines targeting *nrt* and *nrg* were also obtained.

A significant concern when using RNAi mediated knockdown is possible OFF target effects; that is adverse effects caused by knockdown of non-target genes whose sequence matches possible siRNA sequences derived from the hairpin sequence, but which are not the intended target. To address this concern, the VDRC has created a scoring system to evaluate their constructs for possible off-target effects (Dietzl et al., 2007). For each line created, the number of ON targets is published, as well as the number of OFF targets, and a relative specificity ranking called the S19 score.

Definition of an ON target is any gene that contains a perfect match to at least 50% of the 19-mer siRNAs generated by a hairpin construct. In contrast, an OFF target is defined as any gene that contains sequence similarity with at least one 19-mer, but less than 50% of all 19-mers possibly generated by the construct. To calculate a the S19 relative specificity score, the following formula is used:

S19 = Σ ON target matches /(Σ ON target matches + Σ OFF target matches).

Hence, in a line that has only one ON target and no OFF target, S19 equals 1. This is the most desirable scenario to insure that only the desired target gene is knocked down, and it is the case with most of the VDRC lines obtained for this work. One exception is *kek5* RNAi strain 47770, which has one ON target and 5 OFF target genes. However, the calculated S19 for this line is 0.98, which means that although there are 5 OFF targets, 98% of the possible 19-mer siRNAs produced are complementary to the *kek5* mRNA, while only 2% of the 19-mer siRNAs have complementarity with OFF targets. Moreover, for the five OFF target genes, four genes are targeted by only a single 19-mer siRNA and the fifth gene is only targeted by three possible 19-mer siRNAs. This is in contrast to *kek5*, which is targeted by all of the possible 290 19-mer siRNAs produced by the hairpin. This indicates that even though OFF target effects are possible for this line, such effects are unlikely to reduce expression of the OFF target genes to a level that would

be physiologically significant. Table 1 lists all lines obtained from VDRC, including number of OFF targets and S19 score for each line.

Table 1: RNAi lines obtained from VDRC and validation data

Target Gene	Line #	Inserted chromo.	OFF target	S19	PCR	GFP	Phen.	Tested against
Kek 1	36252	3	0	1	✓	✓	✓	-
Kek 1	43521	2	0	1	✓	✓	✓	Kek 6
Kek 1	4761	2	0	1	-	✓	✓	-
Kek 2	42449	2	1	1	-	✓	-	Kek 1, 6
Kek 3	6354	3	0	1	✓	-	-	-
Kek 3	6356	2	0	1	✓	-	-	-
Kek 4	915	2	0	1	-	✓	-	-
Kek 5	27249	1	5	0.98	✓	✓	✓	-
Kek 5	47770	2	5	0.98	✓	✓	✓	Kek 1, 2, 4
Kek 6	27164	0	0	1	✓	✓	-	Kek 1
Kek 6	27165	0	0	1	✓	✓	-	Kek 1, 2, 5
NRT	8495	2	0	1	✓	-	-	-
NRG	27201	3	0	1	✓	-	-	-

OFF target - indicates number of OFF target genes for each line
S19 - score calculated as Σ ON matches / (Σ ON matches + Σ OFF matches)
PCR - indicates result of construct validation by PCR
GFP - result of functional validation by knockdown of GFP-tagged target gene
Phen. - functional validation by suppression of misexpression phenotype of target gene
✓ - indicates positive validation was obtained
"**-**" - indicates no validation data was obtained
Tested against - indicates target genes against which indicated RNAi line was tested as a negative controls in functional validation assays. No cross activity was observed with any controls tested

RNAi Line Validation

Since the project was largely reliant on the RNAi lines obtained, it was crucial to confirm their identity and efficacy. Initially, to verify the presence of the indicated construct, PCR was performed using a primer designed to

match a short sequence in the pUAST transgene vector within the UAS binding region and a gene specific primer to amplify a section of the construct excluding the inverted repeat. Lines that were validated by use of PCR are also indicated in Table 1. To verify that the lines were capable of effecting silencing of the desired target gene, functional validation was also carried out. This was assayed in two ways. First, the RNAi lines were crossed to strains in which a GFP tagged version of the desired target gene is expressed in the eye using the GMRGAL4 driver. The presence of the RNAi trigger should then lead to degradation of the mRNA for the GFP tagged target gene resulting in a loss of GFP fluorescence in the adult eye. For *keks 1,2,4,5,* and *6*, gene specific loss of GFP fluorescence was observed, thereby demonstrating that each respective RNAi line is capable of effectively reducing expression of the desired target gene (Fig. 9 and 10 and Table 1). In addition, RNAi effects are limited to single family members with no cross family effects observed (Fig. 9, Table 1). Because of the lack of a GFP-tagged version this assay was not carried out for *kek3*.

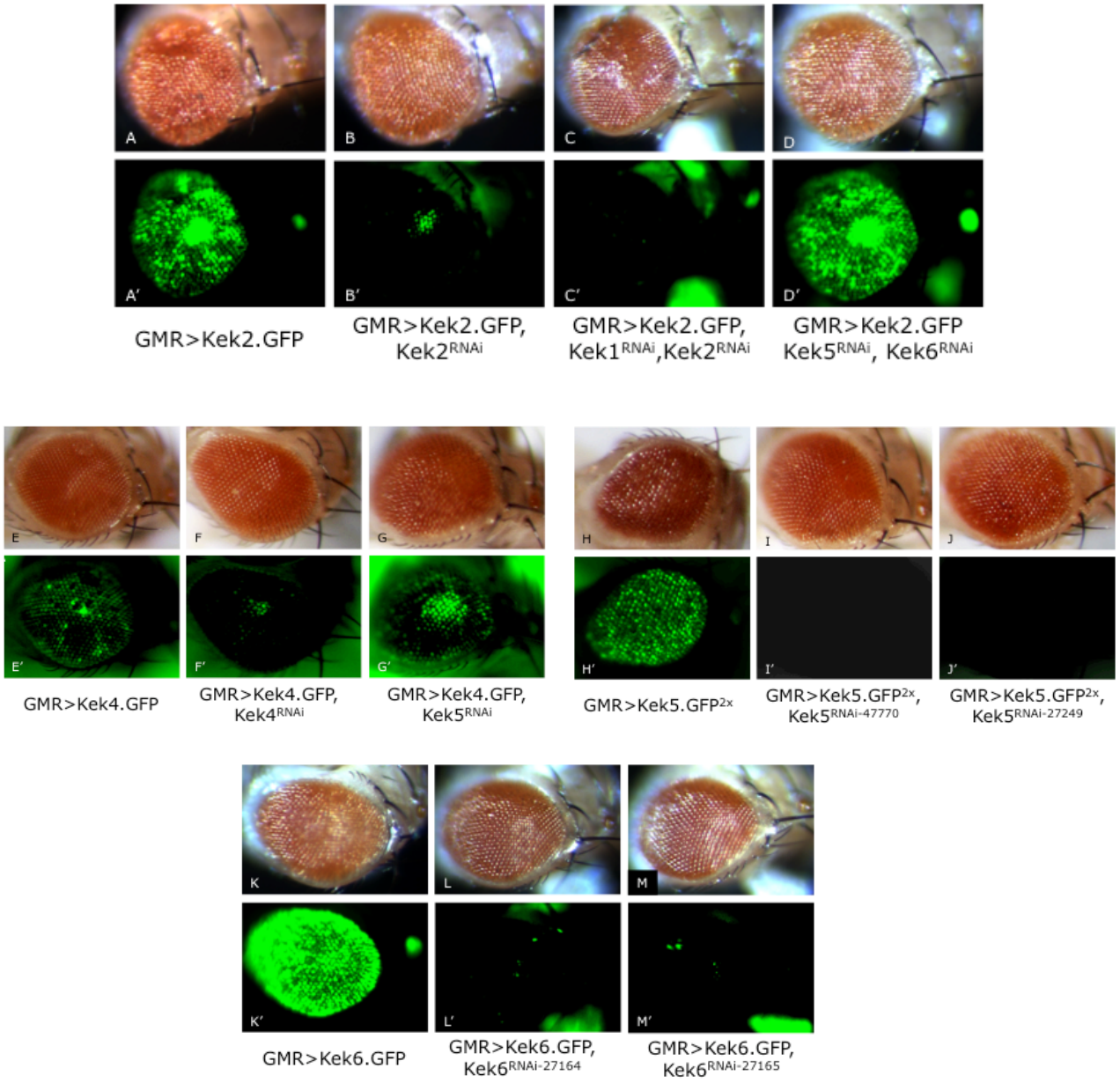


Figure 9: Functional validation of *kek* family RNAi lines through Kek-GFP knockdown. Brightfield (A-M) and epifluorescent (A'-M') micrographs of adult compound eyes. Upon GMRGAL4 mediated misexpression of Kek-GFPs, significant GFP expression is observed in the adult eye (panels A', E', H' and K'. Introducing the presence of gene specific RNAi results in efficient knockdown of appropriate target gene as observed by the loss of Kek-GFP expression in the adult eye (B', C', F', I',J', K' and L').

Another method to functionally validate the lines obtained was through suppression of misexpression phenotypes. In the case of Kek1 and Kek5, misexpression has known phenotypes. Therefore, RNAi lines could be validated by their ability to suppress the misexpression phenotypes when co-expressed with the target gene (Table 1). Misexpression of Kek1 in the eye with the GMRGAL4 driver causes a rough eye phenotype and *kek1* RNAi lines were able to fully suppress this phenotype (Fig. 10). Likewise, *kek5* RNAi lines suppress the effects of misexpression of Kek5 in the wing (severe wing blisters) by the apterousGAL4 (apGAL4) driver (Fig. 10).

In the case of Kek1, less GFP is detected in the eye, relative to the other Keks, even in the absence of the RNAi trigger. This is due to inhibition of the EGFR by misexpression of GFP-tagged Kek1, which leads to a loss of the Kek1-GFP expressing photoreceptor cells and thus the rough eye phenotype (Alvarado et al., 2004).

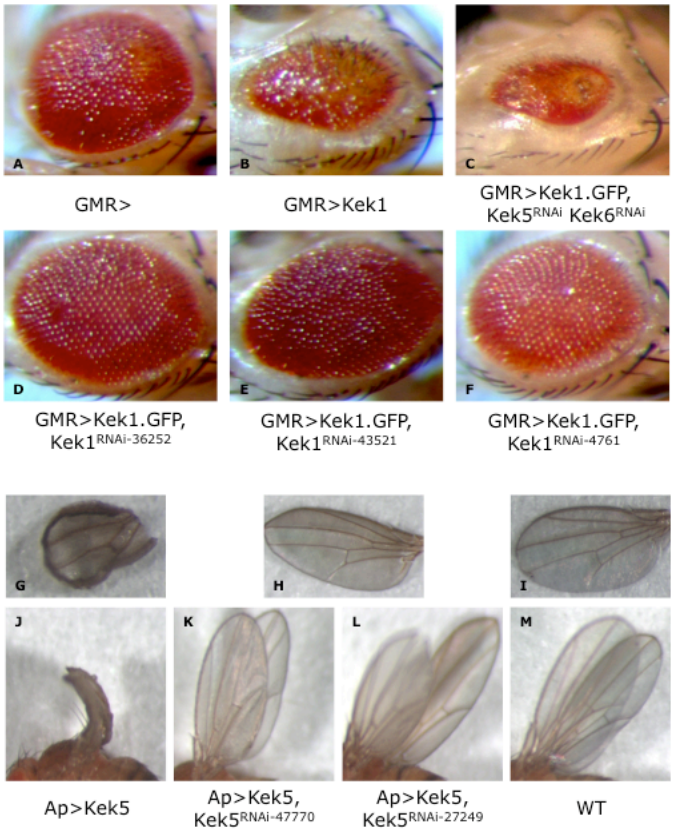


Figure 10: Functional validation of RNAi lines by suppression of misexpression phenotypes. Brightfield micrographs of adult compound eyes (A-F) and wings (G-M). GAL4 mediated misexpression of Kek1 and Kek5 leads to rough eye and abnormal wing phenotypes, respectively (B, G, J). Introducing the presence of gene specific RNAi results in suppression of these phenotypes (D-F, H, K, and L).

However, coexpression of the *kek1* RNAi hairpin triggers knockdown of Kek1-GFP expression, thereby restoring EGFR activity and the presence of photoreceptor cells as observed by suppression of rough eye phenotype. However, although photoreceptor cells are restored, GFP expression is not present also confirming knockdown of the *kek1-GFP* mRNA (data not shown). Thus, all of the assayed *kek* family RNAi lines appear to promote efficient, gene specific knockdown in the tissues tested. It should be noted however, some non-specific effects are observed, as expression of RNAi lines using apGAL4 driver appears to promote a held out wing phenotype.

DEVELOPMENTAL ASSESSMENT OF RNAI KNOCKDOWN EFFICACY

The validation tests above confirmed effectiveness and specificity of the RNAi lines in the developing eye and wing. However, the effectiveness of transgenic RNAi in the *Drosophila* nervous system has not been addressed to date. Moreover, communication with others in the field indicated that the efficacy of transgenic RNAi in the embryonic nervous system was questionable.

Hence, I aimed to validate the use of the transgenic RNAi technique in this context. To that end we expressed GFP tagged Kek5 in the nervous system using the pan neural C155GAL4 driver, with and without co-expression of the *kek5* RNAi trigger. Levels of GFP in the nervous system in embryo, 1st instar and 3rd instar larva were then compared among the

genotypes. Some knockdown was observed in the presence of RNAi; knockdown was more robust in 3rd instar larva than in embryos or 1st instar, but not as significant as might be necessary in order to carry out functional studies (Fig. 11). Therefore, I attempted to improve efficiency of knockdown either by expressing 2 copies of *kek5* RNAi trigger and/or by co-expressing Dcr2. Expressing 2 copies of Kek5 RNAi gave a minimal increase in knockdown efficiency. One possibility is that Dcr2, which is required to generate siRNA triggers from the hairpin RNA, is limiting in the nervous system. If so, then increasing Dcr2 levels should lead to increased knockdown effects in the nervous system. Consistent with this, significant knockdown of Kek5-GFP was observed in the presence of Dcr2 co-expression (Fig. 11). It was observed that the co-expression of Dcr2 increases the variability of Kek5-GFP expression levels in the nervous system. Furthermore, even in the presence of Dcr2 and RNAi, GFP expression varied and very often some GFP can still be observed in the embryo and 1st instar larva, indicating that knockdown of Kek5-GFP is not complete in early stages of development. In contrast, GFP levels in dissected 3rd instar larval brains were not variable and seemed to indicate complete GFP knockdown.

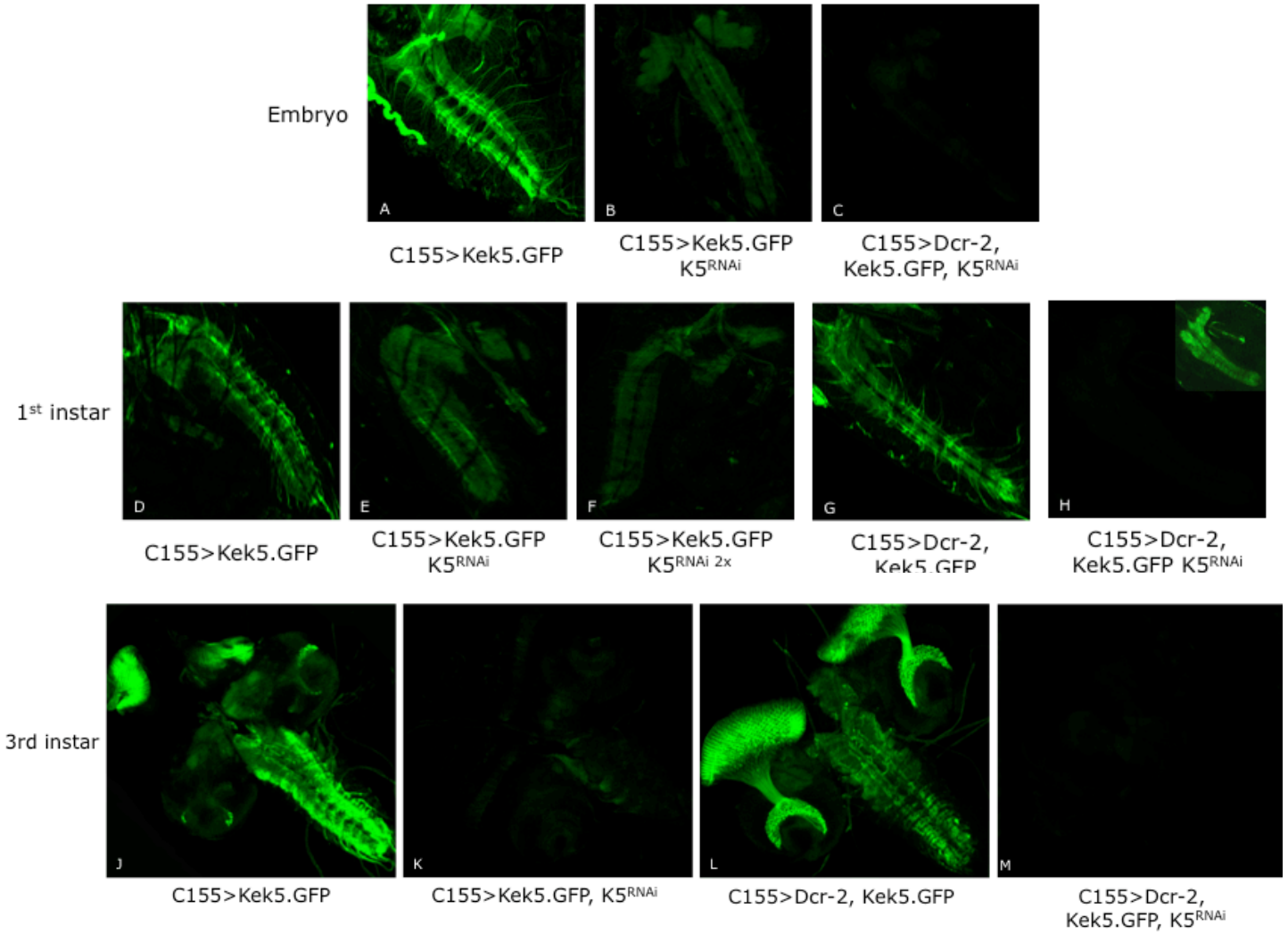


Figure 11: Assessment of RNAi knockdown in developing nervous system. Epifluorescent micrographs of the nervous system of embryos (A-C), early 1st instar larva (D-I) and 3rd instar larva (J-M). Bright GFP can be observed in the ventral nerve cord when Kek5-GFP is misexpressed by C155GAL4 (A, D, J). Some, albeit not robust, knockdown is observed when Kek5 RNAi triggers are also expressed (B, E, K). Having two RNAi trigger constructs is not sufficient to increase knockdown significantly (F). Adding Dicer does slightly decrease GFP intensity in the absence of RNAi (G, L) but in the presence of RNAi, it enhances knockdown appreciably (C, H, M). However some GFP is still visible in early development even in the presence of Dcr and RNAi. All images are taken with similar exposure and adjusted to similar brightness, contrast and color levels, except inset in H, which has been adjusted in brightness and contrast to become visible.

CREATING COMBINATORIAL KNOCKDOWN STRAINS

Overt neuronal phenotypes have not yet been reported for single mutants of Kek1 and Kek5. In addition, Kek2 phenotypes reported in NMJ are quite subtle. It is believed that functional redundancy in the nervous system is prevalent which may be particularly relevant in the study of the Kek family function since the Keks share strong structural homology. Hence, combinatorial knockdown may be an important component of the Kek functional investigation. Prior phylogenetic analyses in the lab revealed that the Kek family is divided into 2 clades where Keks1, 2 and 3 form a clade separate from Keks4, 5 and 6 (MacLaren et al., 2004) (Fig. 12), suggesting an initial combination for multiple knockdowns. Furthermore, this phylogenetic analysis shows that although the Kek family has been evolutionarily conserved for approximately 500 million years, Kek4 arose

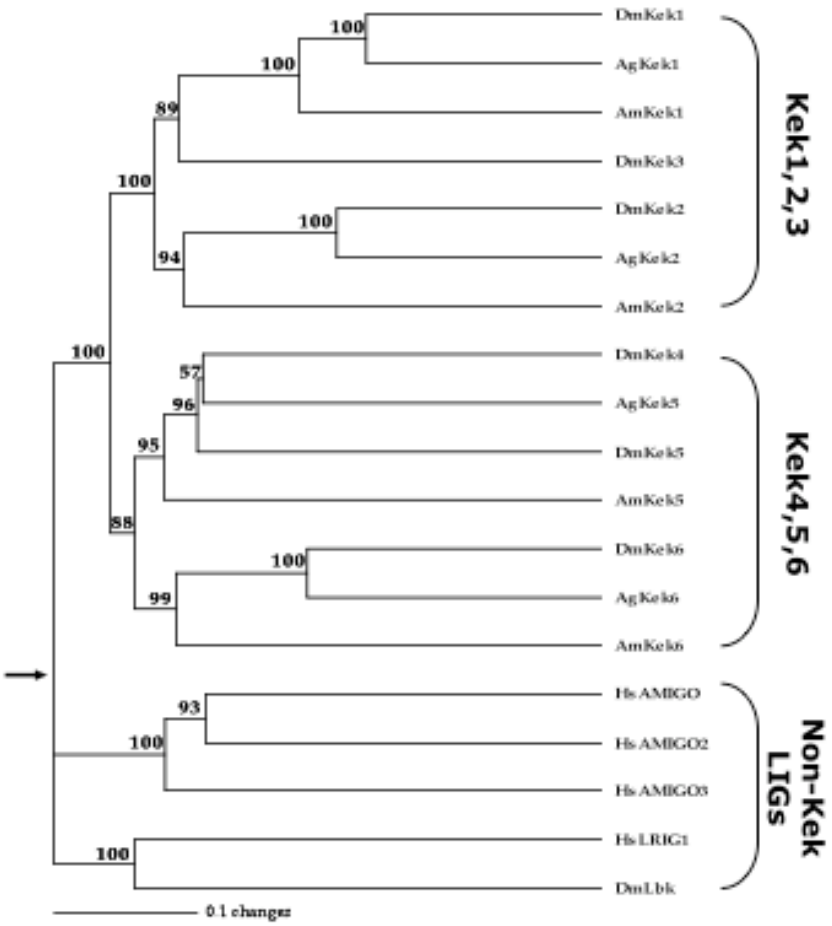


Figure 12. Phylogenetic analysis of Kek family (from T. Evans)

more recently than that, and Kek3 has been lost in some species after its emergence (*A. mellifera* and *A. gambiae*; Fig. 13), indicating they may be more dispensable for the organism.

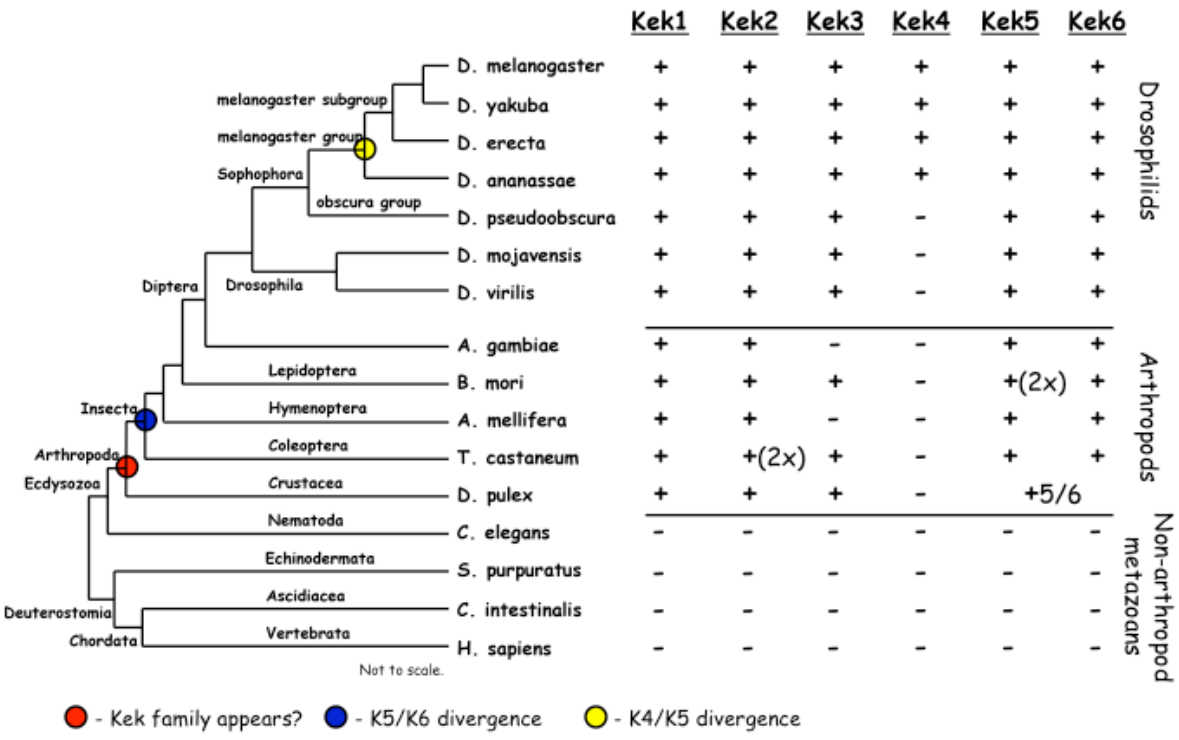


Figure 13. Analysis of Kek family evolution (from T. Evans).

Therefore, as an initial strategy, lines were created to generate pairwise knockdowns for the most well conserved loci in the two clades, namely a *kek1* and *kek2* recombinant and a *kek5 and kek6* recombinant. These lines were validated using similar strategies as the original RNAi lines as is summarized in Table 2. Recombinant lines were also created with *kek1, nrt, nrg* and *kek2* to further investigate the reported interaction between *kek1* and *nrt* and to study the specificity of this interaction.

Table 2: Recombinant RNAi lines created and validation data

Target Genes	Line #	1st Line	2nd Line	Chrom.	PCR		GFP		Phen.	
					1st	2nd	1st	2nd	1st	2nd
Kek1 Kek2	43	43521	42449	2	✓	-	-	✓	✓	-
Kek1 Kek2	47	4761	42449	2	-	-	-	✓	✓	-
Kek5 Kek6	64	47770	27164	2	✓	✓	✓	✓	✓	-
Kek5 Kek6	65	47770	27165	2	✓	✓	✓	✓	✓	-
Kek1 NRT	43	43521	8495	2	✓	✓	✓	-	✓	-
Kek1 NRT	47	4761	8495	2	-	✓	✓	-	✓	-
Kek1 NRG		36252	27201	3	✓	✓	✓	-	✓	-
Kek2 NRT		42449	8495	2	-	✓	✓	-	-	-

PCR - indicates result of construct validation by PCR
GFP - result of functional validation by knockdown of GFP-tagged target gene
Phen. - functional validation by suppression of misexpression phenotype of target gene
✓ - indicates positive validation was obtained
"**-**" - indicates no validation data was obtained

PRIMARY SCREEN FOR KEK FAMILY FUNCTION

Ubiquitous knockdown

Expression of the *kek* family has been demonstrated to be principally in neural tissue, but expression in additional tissues has also been observed. For example, *kek1* expression has been reported in the wing and ovary, in addition to the eye, where it is a downstream target of the EGFR, while *kek5* expression appears to be more ubiquitious with some enrichment in the embryonic nervous system. Therefore, to assess the effect of global knockdown, the role of each *kek* in the organism was assessed using two ubiquitous drivers, tubulinGAL4 (tubGAL4) and actinGAL4 (actGAL4). No

overt phenotype was observed other than reduced viability, which was observed for Kek1, Kek3 and Kek5 (Tables 3 and 4). Viability for each cross was calculated based on the following formula:

(UAS-*kek*^{RNAi}/GAL4 driver) / #(UAS-RNAi/Balancer), n equals the total number of flies scored. Although percent viability for each *kek* family member varied between both drivers, effects with tubGAL4 appear more severe with lower viability numbers.

Table 3: Screen of Tubulin driven RNAi knockdown

Target Gene	Line #	Viability	n	Observation
Kek1	43521	0%	55	
Kek1	4761	0%	52	
Kek2	42449	215%	41	
Kek3	6354	0%	45	pupal lethality
Kek3	6356	0%	58	pupal lethality
Kek4	915	159%	75	
Kek5	27249	5%	22	
Kek5	47770	4%	29	
Kek6	27164	120%	8	
Kek6	27165	104%	47	
Kek5 Kek6	64V	50%	57	
Kek5 Kek6	65V	53%	26	
Kek5 Kek6	64IV	29%	79	

Table 4: Screen of Actin driven RNAi knockdown

Target Gene	Line #	Viability	n	Observation
Kek1	43521	2%	59	Walk up deficiency
Kek1	4761	2%	86	
Kek2	42449	109%	90	
Kek4	915	118%	61	
Kek5	27249	29%	40	Walk up deficiency
Kek5	47770	15%	38	
Kek6	27164	106%	55	Jumpy
Kek6	27165	80%	88	

Based on the RNAi results, *kek1, kek3,* and *kek5* appear to be vital loci, essential for viability, while no major effects are seen for *keks 2,4* and *6*, with the latter results arguing against lethality as a non-specific result of RNAi induction. For *kek5*, the reduced viability with RNAi is consistent with reported data for a *kek5* null mutant, albeit more severe in the case of the tubGAL driver. In contrast, the lethality observed with the *kek1* RNAi was somewhat unexpected as deletion of *kek1* is viable (Musacchio and Perrimon, 1996). Although this raises suspicion over the 0% viability observed with the tubGAL4 driver, the relative viability of the *kek1* null mutant has not been reported and thus could indeed be low. Similarly, although circumstantial evidence suggests that loss of *kek3* is not lethal, *kek3* null mutants have not yet been isolated, so it remains possible that knockdown of *kek3* could lead to effects on viability. Consistent with this, lethality observed with the *kek3* RNAi occurred during the pupal stage, which was not observed in *kek1* RNAi, supporting the possibility of a distinct functions for these genes.

It is important to note that for both *kek1* and *kek3* there appear to be no OFF target genes based on the VDRC scoring system. However, if lethality associated with GAL4 mediated knockdowns is non-specific - caused by soaking up essential cellular components of the RNAi processing machinery, increasing levels of this component would restore levels, thereby yielding more accurate results. Since Dcr2 seems to be a limiting factor for

RNAi processing in the nervous system, it is possible that it may be a limiting factor in other contexts as well. Therefore, knockdowns of *kek1* and *kek3* were tested again using actGAL4 in the presence of additional Dcr2 (genotypes - *UASkek1*^{RNAi}, *actGAL4 UASdcr2* and *UASkek3*^{RNAi}, *actGAL4 UASdcr2*). In addition, an RNAi trigger for GFP, which is not present in the *Drosophila* genome and for which knockdown should have no effect, was used as a control. Surprisingly, in the presence of additional Dcr2, no RNAi expressing flies were recovered, including the GFP RNAi control (although it's not clear if OFF targets for the GFP RNAi hairpin exist). The number of control (balancer) flies recovered was 15, 21, 31 and 34 for *UASkek1*$^{RNAi-4761}$, *UASkek3*$^{RNAi-6354}$, *UASkek3*$^{RNAi-6356}$ and *UASGFP*$^{RNAi-9331}$ respectively. Although only a small number of progeny were scored, the data obtained with GFP$^{RNAi-9331}$ indicates that simply activating the RNAi mechanism in the presence of Dcr2 misexpression by actGAL4 greatly reduces viability.

Thus, although RNAi can trigger gene specific knockdown with no obvious non-specific or detrimental effects in the eye, interpretation of results with ubiquitious drivers appears much more complex.

Neuronal knockdown I

Given that the results with a tissue specific driver (GMRGal4) provided clear-cut results and expression of *kek* family members is principally in the nervous system, I limited the remainder of my RNAi studies to tissue-specific

drivers. For this I utilized pan-neural drivers C155Gal4 and ElavGAL4, which express Gal4 under the control of a gene, *elav*, expressed predominantly in all neurons. Initially, knockdown of *keks* in the nervous system was performed without the presence of additional Dcr2 as an enhancer. Flies were then assayed at the cellular and organismal levels. First, the overall pattern of the embryonic nervous system was assessed with antibodies that identify the longitudinals and commissures (anti-FasII and BP104). Using these antibodies, no obvious defects were uncovered for any of the *keks* tested, as well as for the *kek5/kek6* and *kek1/nrt* recombinants (Fig. 5 and data not shown).

At the organismal level, three assays (tap, vortex, and flight) were used to evaluate the behavior of adult flies. In the tap assay flies are tapped down in the vial and their behavior observed. Wild type flies climb up the vial immediately after being tapped down. The vortex assay was performed to evaluate flies response to physical stress. After wild type flies in a vial are vortexed for 5 seconds they quickly recover – regain balance, move up, and groom. Finally, a flight assay was done to evaluate flies' flight response. Upon being dropped into a graduated cylinder, wild type flies fly and alight onto the walls, without falling to the bottom.

After running all the adult assays, no significant behavioral anomaly was observed. It was noted that RNAi expressing flies groomed less, particularly after vortex assay, and that they jumped around more.

However, this phenotype was observed in all genotypes and does not seem to reflect a specific gene function. Nonetheless, in view of my results indicating the limited RNAi knockdown efficiency in developing nervous system in the absence of additional Dcr2, I can only conclude that the *keks* are not essential for basic adult nervous system function. This conclusion is supported in part by another study in the lab that indicates that without additional dicer, RNAi mediated knockdown may be effective in the nervous system as early as late pupae stage. In this case it can also be concluded that the *Keks* function is also not vital in late pupal development.

Viability data was also obtained using ElavGAL4 driver in a similar method as viability data was obtained for ubiquitous drivers. This line has the same expression profile as C155, but it was observed to induce lower levels of responder expression. Slightly decreased viability was observed for a few lines, particularly Kek4, with no effects observed for GFP RNAi trigger in contrast to the viability results obtained with the ubiquitious drivers (Table 5, data not shown).

Table 5: Viability data for Elav driven RNAi knockdown

Target gene	Line #	Viability	n
Kek1	36252	148%	82
Kek1	43521	79%	93
Kek1	4761	89%	70
Kek2	42449	102%	107
Kek3	6354	142%	29
Kek3	6356	111%	154
Kek4	915	66%	128
Kek5	47770	111%	74
Kek5	27249	103%	201
Kek6	27164	235%	124
Kek6	27165	81%	107

Neuronal knockdown II: addition of Dicer2

To better address the role of *keks* during neural development, RNAi lines were also crossed to a strain containing both the C155GAL4 driver and UASDcr2. Since the driver and most RNAi lines were homozygous, the lack of control classes in the progeny precluded the ability to obtain accurate viability data for most lines (Table 6). However, all crosses (including *wild type* and *UASGFP*^{RNAi} controls) were set up with the same number of parental males and females of approximately the same age. Thus, between cross comparisons provide an approximate estimate of the number of progeny that should be recovered. Although not standard, in the absence of sib control classes this comparison provides one simple measure for determining if large effects on viability occur. For lines without control sibs, estimated viability was calculated as: # gene specific RNAi progeny/116 (the average for the two control crosses). Where Balancer (control) sibs were present and standard percent viability could be calculated, the % of control recovered should be approximately half of the viability for that line.

Although the results of the behavioral assays were not quantified, behavioral observations were noted. In particular it was observed that in a few lines most larva pupated near the food or in the food itself. Upon increases in levels of the molting hormone ecdysone, wild type larva crawl out of the food and pupate up on the sides of the vial. Inability to do this may denote a larval behavioral deficit, either in locomotor activity, the

ecdysone pathway, or in environment sensing. This was most severe in the *nrg*, *kek1* and *kek1/nrg* recombinant lines, and also occurred in the *kek5/kek6* recombinant line, albeit less severely. Other observations worth noting were that in the *nrg/kek1* recombinant knockdown, flies did not walk up the vial normally and were visibly uncoordinated, which likely resulted in the large numbers of adults stuck in the food. Large numbers of adults stuck in the food, consistent with a locomotion deficit, was also observed in other lines, including the *nrg* and *kek1* individual knockdowns. Additionally, in the *kek1/kek2* combinatorial knockdown, flies did not walk up the vial normally and appeared visibly uncoordinated. Although no clear phenotype was observed for any of the single *kek* knockdowns, *kek1* knockdown shows evidence of a locomotor deficit and decreased viability. Furthermore, 2 of the *kek1* recombinant knockdowns (*kek1/nrg* and *kek1/kek2*) demonstrated increased locomotor deficit. It is still unclear if the increased deficit is due to a synergistic interaction or an additive effect. Either way, this is the first time that any interaction has been observed between *kek1* and *kek2* or *kek1* and *nrg.* Likewise, this is also the first time that any indications of a neuronal phenotype has been reported for single *kek1* knockdown. In comparison no phenotype was observed for the other *keks*. Nonetheless, this could be a result of inefficient knockdown early in development or due to a lack of specific and sensitive assays to detect behavioral anomalies.

Table 6: Screen of neuronal RNAi knockdown with Dicer missexpression

Target Gene	Line #	RNAi	Balancer	Viability	% control	% males	% Stuck	Observations
K1	35252	70			60%	39%	6%	-
K1	43521	23			20%	0%	100%	PCF
K1	4761	34			29%	26%	62%	PCF
K2	42449	89			76%	56%	2%	-
K3	6354	83			71%	40%	5%	-
K3	6356	47			40%	19%	4%	1 missing 1/2 thorax
K4	915	72			62%	52%	1%	-
K5	27249	80			69%	50%	3%	-
K5	47770	111			95%	42%	0%	-
K6 (cyo)	27164	41	53	77%	35%	44%	0%	-
K6	27165	56			48%	57%	0%	-
Lambik	42570	88			76%	50%	2%	-
LIG	7993	113			97%	44%	0%	-
NRG	27201	37			32%	46%	32%	MPCF, 2 CW, 40% SU, uncoordinated
NRT	8495	52	44	118%	45%	35%	21%	-
GFP	9331	111			95%	36%	10%	-
K1K2	47I	61			52%	6%	20%	PCF, 1 curly wing
K1K2	47II	24			21%	13%	58%	PCF
K1K2	43II	10			9%	0%	60%	MPCF, very uncoordinated
K1K2	43III	5			4%	0%	80%	MPCF, very uncoordinated
K1 NRT	47I	47			40%	15%	57%	MPCF
K1 NRT	47II	41			35%	7%	10%	PCF, 1 SU
K1 NRT (cyo)	43I	2	82	2%	2%	N/A	100%	PCF
K1NRG (TM3)	I	0	37	0%	0%	N/A	N/A	PCF, uncoordicated
K1NRG (TM3)	II	15	N/A		13%	0%	67%	MPCF, uncoordicated
K1NRG (TM3)	III	8	40	20%	7%	0%	0%	MPCF, uncoordicated
K5K6 (cyo)	64IV	32	27	119%	27%	23%	19%	PCF
K5K6	64V	63			54%	38%	16%	PCF
K5K6	65II	53			45%	25%	0%	PCF
K5K6	65IV	49			42%	27%	2%	PCF
WT	W1118	122			105%	48%	2%	-

Average control progeny # 116.5 Control groups are W1118 and GFP^{RNAi}

Legend: PCF = some pupae cases close to or in food SU = stuck unfurled

MPCF = most pupae cases in food itself CW = curly wing

Imaginal discs knockdown

One of the best-characterized members of the Kek family is Kek5, which has been shown to interact with BMP signaling in wing crossvein development. Mutants in *kek5* exhibit crossvein defects, both in anterior (ACV) and posterior crossvein (PCV), in approximately 30% of adults. Defects include ectopic crossvein around ACV, truncated, missing or meandering PCV and ectopic PCV material. Crossvein signaling is also disrupted when *kek5* is misexpressed using engrailedGAL4 (enGAL4), a segment polarity gene that drives expression on the posterior compartment of each segment, including the region of presumptive ACV and PCV in the developing wing. This allowed me to explore the efficacy of RNAi in a different tissue for which a *kek* null phenotype has been well characterized. RNAi lines against *kek5*, *kek6* and their recombinant were crossed to enGAL4. Wings were then scored for crossvein defects.

Some defects were observed with enGAL4 and the *kek5* RNAi line, including ectopic ACV material and truncated PCV (Table 7). Knockdown with one *kek6* RNAi line gave a high penetrance of crossvein defects, but not in the other. In this combinatorial knockdown, defects were mostly missing or truncated ACV. When both *kek5* and *kek6* were simultaneously knocked down, defects in ACV increased to 100%. Moreover, the percent of missing ACV increased as compared to *kek6* alone and defects in PCV also increased

when compared to *kek5* alone, supporting a possible interaction between *kek5* and *kek6*.

Table 7: Crossvein Screen using En driven RNAi knockdown

Target Gene	Line #	n	% defects	
			ACV	PCV
Kek5	47770	98	1	5
Kek6	65	112	69	0
Kek6	64	88	6	1
Kek5 Kek6	65 II	61	100	44
Kek5 Kek6	64V	148	1	3

However, additional results cause concern regarding the validity of this phenotype. To start with, the frequency of defects observed with the *kek5* line is significantly lower than that observed with the null allele (20-30% missing or truncated PCV). Second, only one of the *kek6* lines presented any phenotype or interaction with *kek5*, while no significant phenotype or interaction was observed in the other *kek6* RNAi line. This could be explained by a difference in expression levels of the corresponding *UASkek6*^{RNAi} lines due to position effects on the transgenes, however both lines were equally effective in knocking down Kek6-GFP expression in the eye with GMRGal4. *kek5*^{RNAi}, *kek6*^{RNAi} and the *kek5/kek6* RNAi recombinants were also tested with the neural driver ScabrousGAL4 and no overt phenotypes were observed.

Neuronal Misexpression

Although the true benchmark for characterizing gene function *in vivo* is the analysis of loss-of-function effects, important information can often be derived from gain-of-function studies as well. Thus, as a complement to the RNAi approach to assessing Kek function in the nervous system, I also performed a screen in which members of the *kek* family were individually misexpressed in neural tissues, with the exception of *kek3* for which no transgenic misexpression lines exist. Specifically, *kek1, 2, 4, 5* and *6* were misexpressed with the pan-neural driver C155GAL4. No overt abnormalities in adult behavior were observed upon misexpression of the single responders. However, misexpression of *kek5* with a strain containing two responders led to an extremely high percentage of flies stuck in the food (82%) and flies that were not stuck on the food showed clear behavioral defects: they did not climb up the vial, walk around, groom, attempt flight or display any movement typical of wild type flies and typically were dead in a few days. In addition, many flies had wings that remained uninflated, consistent with a lack of motor coordination since wings require stroking for inflation. Thus, Kek5 expression must be appropriately regulated for wild type neural development to occur.

To gain better insight into the mechanism underlying the misexpression effect of Kek5, an intracellular variant - $Kek5^{ICΔ123}$, which lacks three conserved motifs within the intracellular domain, was also tested.

Misexpression of this Kek5 variant appears to have increased activity in other misexpression assays, producing phenotypes often more severe than 2X misexpression of wild type Kek5. Similar effects to 2X wild type Kek5 misexpression were observed with respect to viability and flies stuck in the food, although all stuck flies had uninflated wings. In contrast, however, the $Kek5^{IC\Delta 123}$ flies that did not get stuck exhibited behavior much more typical of wild type flies than those with 2X Kek5 misexpression. Hence, it seems that at least in the context of neural misexpression this variant does not produce increased activity relative to wild type Kek5.

NMJ ANALYSIS

No clear and overt phenotype was observed in the general screen, but it is possible that *kek* neural phenotypes are highly specific and would only be detected at the tissue or cellular level. For instance a *kek2* phenotype has only been observed in the NMJ (Guan et al., 2005). *kek5* was also reported to be downregulated by chronically increased synaptic activity by the same study that reported a *kek2* function in synaptic structure. Furthermore, Kek5's interaction with BMP signaling, a known modulator of synaptic structure, makes it a likely effector in elaborating NMJ structure and would be a plausible explanation for the overt behavioral phenotypes observed with high levels of Kek5 misexpression. Thus, given the technical demands of NMJ analysis and the putative likelihood of uncovering a role for Kek5, I focused

primarily on Kek5 in the interest of uncovering a novel phenotype. Analysis was carried out in *kek5* null mutant, *kek5* RNAi-mediated neural knockdown (with Dcr2 coexpression), and misexpression of both $Kek5^{WT}$ and $Kek5^{IC\Delta123}$. To test the hypothesis of functional complementation, Kek5/Kek6 combinatorial knockdown was also tested, as well as Kek6 RNAi neural knockdown (with Dicer) and neural misexpression.

Initial analysis of the *kek5* null mutant, $kek5^{fe148}$, suggested muscle pattern and innervation defects. Defects in the uncoupling of muscles 6 and 7 were observed in 2 out of 5 larva, and complete lack of innervation of muscle 6 and 7 was also observed in the same larvae, in a total of 3 hemi-segments. However, similar defects were not observed in larva from a *kek5* null mutation with a different genetic background ($Df(1)JA27/kek5^{fe148}$) and were occasionally observed in wild type larva, indicating that this is unlikely to represent a *kek5* phenotype.

In the *Drosophila* neurobiology field, standard analysis of NMJ structure relies on quantification of boutons and is often normalized over muscle surface area. Thus, quantification of boutons in the NMJ of muscles 6 and 7 in segment A2 was carried out at 10x magnification (representative images in Fig. 14). Value from both sides were averaged and analysis of variance (ANOVA) was carried out among genotypes comparing raw values of bouton number, as well as bouton number normalized over muscle surface area (Table 8 and 9 respectively). Because of developmental

correlations between physiological activity and overall muscle size, I also tested bouton number normalized by average muscle length.

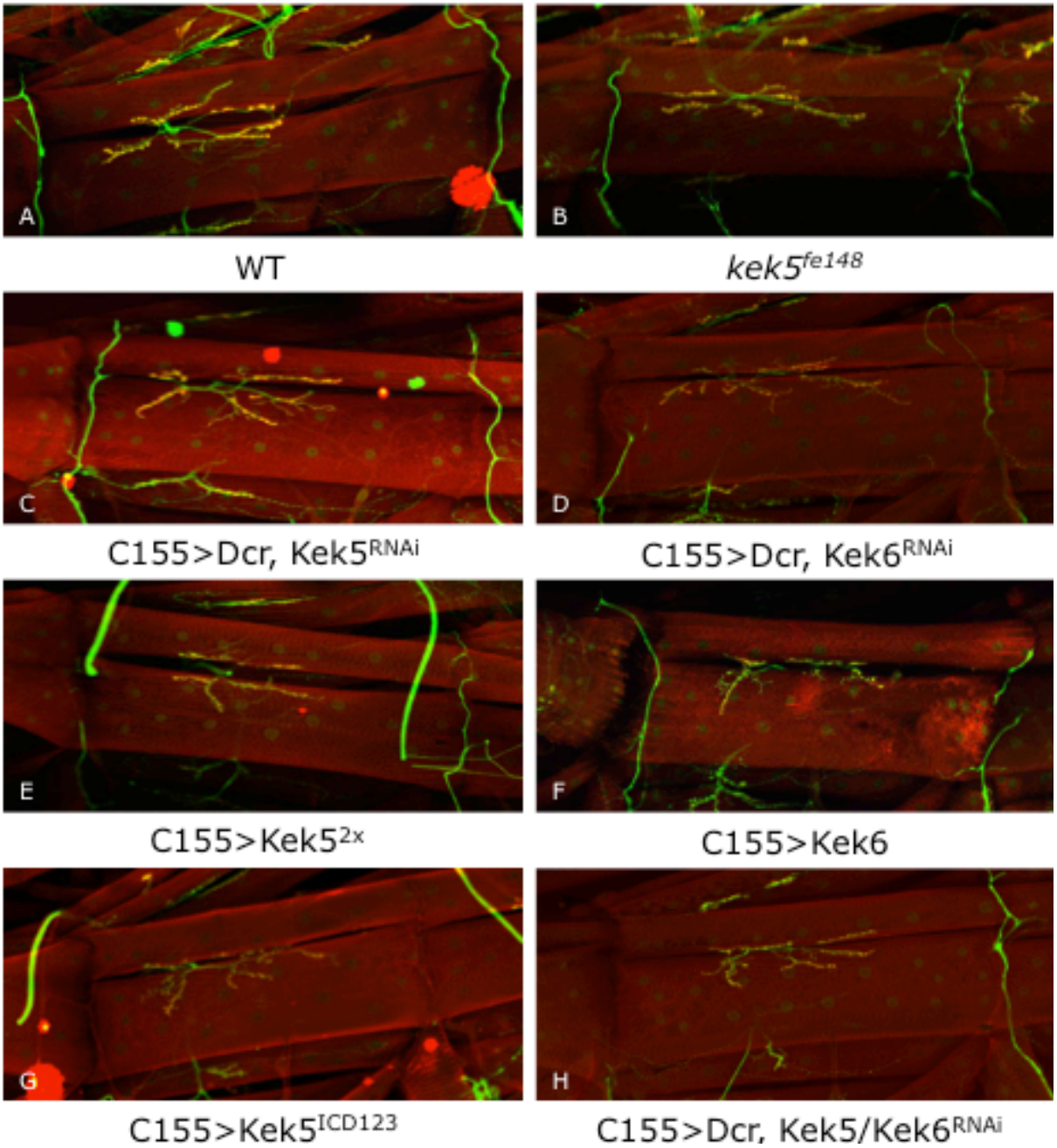


Figure 14: Representative images of NMJ. Epifluorescent micrographs of the NMJ of muscle 6/7 of 3rd instar larva for indicated genotypes. Anti-HRP (nervous system) stained in green and post-synaptic marker, Discs large, in red. Images captured at 10x with apotome processing.

Table 8: Analysis of Variance for bouton number

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Mean relative to wild type*	*Variance*
W1118	17	2264.5	133	100.0%	496
K5Fe148	10	1151.5	115	86.4%	1236
C155>Dcr,K5RNAi	10	1520.5	152	114.1%	423
C155>Dcr,K5K6RNAi	8	1160.5	145	97.9%	542
C155>K52x	12	1667.495	139	102.3%	690
C155>K5IC?123	5	681.5	136	114.0%	1111
C155>Dcr,K6RNAi	6	911.5	152	106.7%	1335
C155>K6	4	568.5	142	108.9%	770

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	9239.1	7	1319.882	1.772	10.84%	2.1564
Within Groups	47671.7	64	744.8709			
Total	56910.9	71				

Table 9: Analysis of Variance for bouton per muscle surface area

Summary

Groups	*Sample size*	*Sum*	*Mean*	*Mean relative to wild type*	*Variance*
W1118	17	0.0279	0.0016	100.0%	0.
K5Fe148	10	0.0168	0.0017	102.6%	0.
C155>Dcr,K5RNAi	10	0.0178	0.0018	108.7%	0.
C155>Dcr,K5K6RNAi	8	0.015	0.0019	97.2%	0.
C155>K52x	12	0.0206	0.0017	114.0%	0.
C155>K5IC?123	5	0.0093	0.0019	109.8%	0.
C155>Dcr,K6RNAi	6	0.0108	0.0018	92.5%	0.
C155>K6	4	0.0061	0.0015	114.7%	0.

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.	7	0.	0.5106	82.32%	2.1564
Within Groups	0.	64	0.			
Total	0.	71				

Table 10: Analysis of Variance for bouton per muscle length

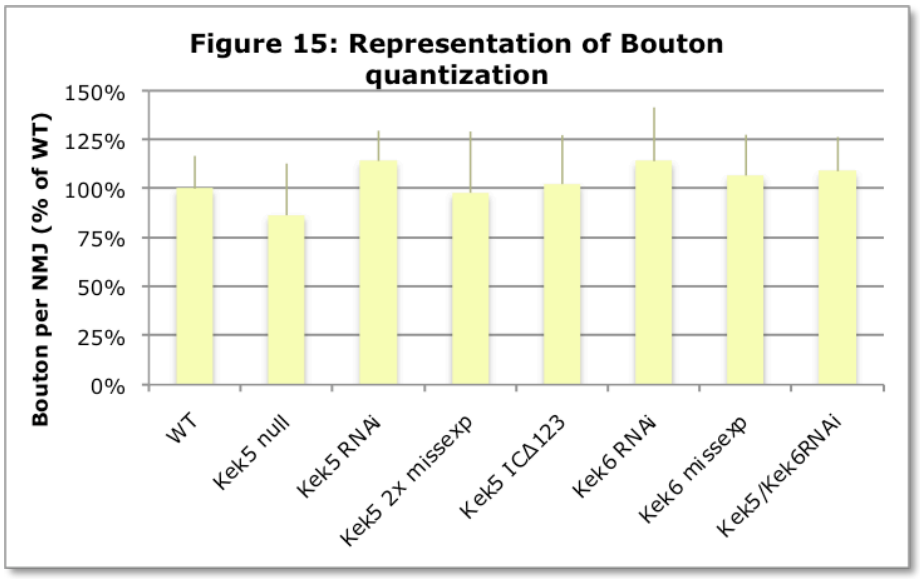
Summary

Groups	*Sample size*	*Sum*	*Mean*	*Mean relative to wild type*	*Variance*
W1118	17	4.8277	0.284	99.9%	0.0026
K5Fe148	10	2.6092	0.2609	91.9%	0.0082
C155>Dcr,K5RNAi	10	3.1399	0.314	110.6%	0.0017
C155>Dcr,K5K6RNAi	8	2.534	0.3167	97.0%	0.0065
C155>K52x	12	3.6693	0.3058	107.4%	0.0028
C155>K5IC?123	5	1.5245	0.3049	110.6%	0.0054
C155>Dcr,K6RNAi	6	1.8846	0.3141	104.0%	0.0044
C155>K6	4	1.1817	0.2954	111.5%	0.0034

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*p-level*	*F crit*
Between Groups	0.0249	7	0.0036	0.8697	53.53%	2.1564

No statistically significant difference was detected in NMJ size, irrespective of the normalization performed. Trends were observed, particularly in the number of boutons per NMJ (Fig. 15). However their relevance is unclear, for example, while bouton number in the *kek5* null is approximately 14% lower than wild type, RNAi knockdown of *kek5* resulted in an increase in the number of boutons (approximately 14%).



Although we might expect similar trends in both genotypes, it is important to note that there is a tissue-specific difference in the two loss-of-function approaches. In the null allele, *kek5* activity is removed both pre and post synaptically, while in the RNAi knockdown it was only removed pre-synaptically. Thus, it is possible that relative levels of Kek5 in neural tissue and muscle tissue are important for proper NMJ size and structure. In fact, this is exactly the case for FasII and maybe consistent with the role of BMP signaling in pre and post-synaptic co-regulation. Although no effect was observed in response to Kek5 misexpression pre-synaptically, there was a general impression that NMJ structure seemed less developed with smaller boutons. The quantification of bouton size was not carried out, but indicates that further analysis is warranted to establish Kek5 function in NMJ development. Furthermore, higher resolution analysis (such as 40x magnification) may be required to obtain more accurate and possibly less variable results (Fig. 16).

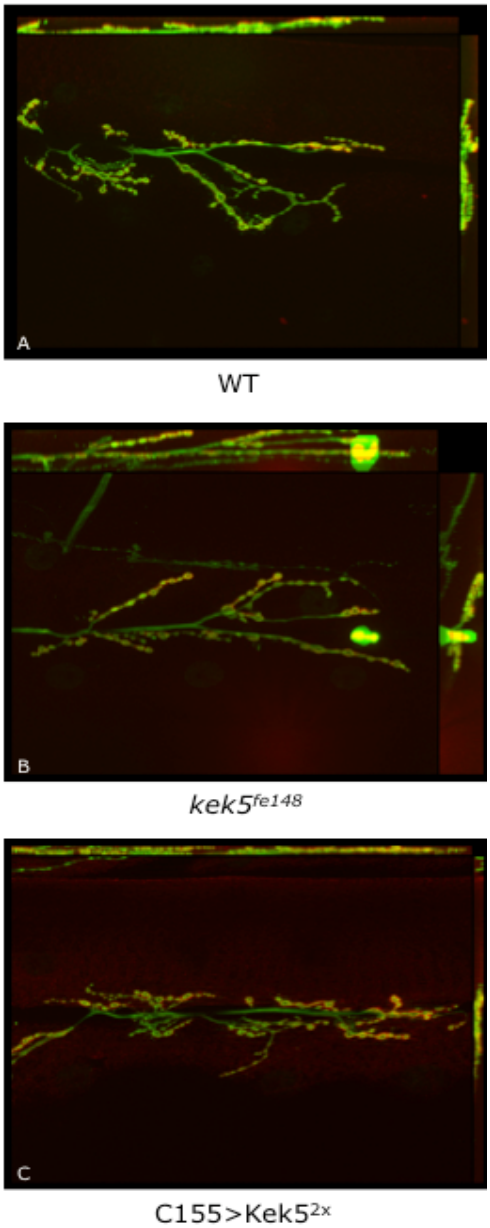


Figure 16: High resolution (40X) images of NMJ. Epifluorescent micrographs of the NMJ of muscle 6/7 of 3rd instar larva as in Fig. 14, z-stack MIP projection, apotome. processing.

DISCUSSION

It is imperative to take advantage of the emerging tools and techniques to keep research at the forefront of science. Small silencing RNA was first discovered 15 years ago, and our knowledge of RNA has boomed in the last decade. RNAi has since been the focus of much interest and press and promises to be a powerful and convenient technique to promote gene silencing in both research and therapeutic fronts.

With genome-wide transgenic RNAi libraries made available from the VDRC and similar projects (i.e. TRIP at Harvard), the *Drosophila* community has a new set of tools to advance genetic inquiries (Ni et al., 2009). Here I have validated the identity and knockdown effectiveness of several RNAi lines targeted against the members of the Kek family for current and future investigations of their function. Furthermore, effectiveness of RNAi mediated knockdown in the developing nervous system was assessed, making this information available for the first time. Although some knockdown is observed in the developing nervous system, this effect is unlikely to be significant enough to result in detectable phenotypes, particularly considering that neuronal phenotypes are often subtle.

In addition our data indicates that coexpression of *dcr2* greatly increases the effectiveness of RNAi mediated knockdown, promoting significant knockdown in the embryo and early larva, and virtually complete knockdown in late larval stages. This data suggests that Dcr2 processing is

the limiting step in the processing of RNAi in the nervous system; hence future neuronal studies using transgenic RNAi must incorporate UASDcr2 for effective results.

It should be noted that effectiveness of knockdown only of Kek5 RNAi was tested in the developing nervous system. Although all lines presented similar knockdown effectiveness in adult eye, it is possible that different lines may have different effectiveness temporally in the nervous system.

SCREEN FOR KEK FAMILY FUNCTION

Neural knockdown of the *keks* with *dcr* coexpression shows no effect on adult behavior with the exception of *kek1* knockdown, which based on preliminary analyses shows aspects of locomotor deficits. Observations from this screen also indicate possible interactions between *kek1* and *kek2*, *nrt* and *nrg.* Since no interaction between *kek1* and *nrg* has been published to date, this is a novel result worth further investigating. *kek1* has been previously shown to interact with *nrt* in axonal guidance and fasciculation as detected in the axonal pattern of the CNS. It would be of interest to investigate if *kek1*/*nrg* double knockdown would also generate a phenotype in the same tissue.

Since *kek2* was previously shown to interact in the NMJ structure (Guan et al., 2005), it would also be worthwhile investigating if *kek1*/*kek2* double knockdown shows a synergistic effect in the NMJ. Furthermore, since

various molecules such as FasII have been shown to interact both in axonal guidance and fasciculation as well as NMJ structure, the potential for an interaction of *kek1* with *nrg* and *nrt* should also be investigated in the context of NMJ structure.

KEK5 ROLE IN THE NMJ

BMP signaling regulates NMJ size and structure via retrograde signaling from the muscle to the pre-synaptic MN (Keshishian and Kim, 2004). An increase in BMP signaling promotes growth of the NMJ as can be observed by increased bouton numbers. Given the *kek5* inhibitory interaction with BMP signaling in wing pattern formation (Evans et al., 2009), it was proposed that *kek5* null mutant would increase NMJ size due to increased BMP signaling. Likewise, *kek5* misexpression would inhibit BMP signaling, thereby inhibiting NMJ growth.

Preliminary analysis of NMJ indicates that this is not the case; albeit no statistically significant effect was observed, decreased bouton numbers was seen in the loss of function, while no trend was detected in the *kek5* gain of function. Furthermore pre-synaptic knockdown of *kek5* shows increased bouton number, indicating that if these trends are real, *kek5* is involved in a different mechanism of NMJ regulation. NMJ analysis at a higher resolution should be carried out to support and extend preliminary results. In addition, manipulating levels of *kek5* post-synaptically may provide further insight

into any possible involvement of *kek5* in NMJ regulation. Similarly, although a possible synergistic interaction was observed between *kek5* and *kek6* in the wing cross vein formation, no interaction was detected in the NMJ structure, nor was any effect of *kek6* itself observed on the NMJ.

RNAi AS A SCREENING TOOL

The screening of the Kekkon family function produced some results that need to be reconciled with previous data, such as the effects on viability of *kek1* and *kek3* ubiquitous knockdown. A meticulous evaluation of single *kek1* and *kek3* null mutant viability may confirm this novel phenotype, and would also validate transgenic RNAi as a legitimate tool for phenotypic screens.

Another unexpected result was the cross vein defect observed with *kek6* knockdown in imaginal disc. A *kek6* mutant has been previously characterized in the lab and no wing phenotype was observed. However, it is not clear that the characterized mutant was indeed a null mutation. Another observation of concern is the variation of penetrance between different RNAi lines. Although position effects due to chromosomal location of the transgenes can be expected among lines, such drastic variation is unusual.

Much is still unknown about the pathways controlling RNAi and it is uncertain what other possible artifacts may arise from using this technique. New classes of small RNAs are constantly being discovered and many still

have unclear biosynthesis, functions and pathway components. Moreover, cross talk among the different small RNA pathways have recently been detected. This is particularly concerning given that many classes of small RNA regulate chromatin states and general transcription. Furthermore, various components interact in the processing of different types of small RNA, and expressing high levels of exogenous RNAi triggers may titrate these components from other endogenous functions.

Another serious concern regards the use of Dcr2 to promote knockdown in the developing nervous system. Although efficiency of knockdown is significantly increased, raising the levels of Dcr2 may have detrimental effects to the organism and to the nervous system itself. For instance, in addition to exogenous dsRNA, Dicer is also involved in endogenous small silencing RNA (Chung et al., 2008; Okamura et al., 2008) and small nucleolar RNAs (snoRNAs) (Taft et al., 2009), some of which are actively regulated. Altering Dcr2 levels may affect the regulation of other genes, with unpredictable outcomes. Hence RNAi can be used as a powerful tool to screen and identify relevant phenotypes, but careful confirmation and validation of phenotypes with conventional and cleaner genetic techniques must follow initial identification.

MATERIALS AND METHODS

GENETICS

Fly stocks were kept at room temperature (24°C) and raised on standard media. Experimental crosses involving the Gal4/UAS system were raised on 28°C on standard media. Flies used for NMJ analysis were raised at 28°C in brown media. w^{1118} was used as the wild type strain, $Kek5^{2x}$ is [UASKek5GFP][16, 52] and the $Kek5^{IC\Delta123}$ line used was CE1-12F-1M.

RNAi transgenic lines were obtained from Vienna *Drosophila* Research Center (VDRC) (table 1) and presence of each construct was verified by PCR using standard procedures and gene specific primers as indicated in appendix 2. *Drosophila* genomic DNA templates were prepared by squishing a single fly in 50μl a solution of 10mM Tris buffer pH8, 1mM EDTA, 2.5mM NaCl and 0.2mg/ml proteinase K. This was then incubated at room temperature for 20min., heated to 95°C in a hot block for 2min. and centrifuged at maximum speed for 5 minutes in a microfuge. 4-8μl of this genomic prep was then used for PCR reactions.

Recombinant lines were created according scheme in Fig17 and validated according to table2. Table 2 also includes line number used to identify different lines. Roman numerals in these recombinants lines are used to identify various copies (different recombination events) of the same genotype.

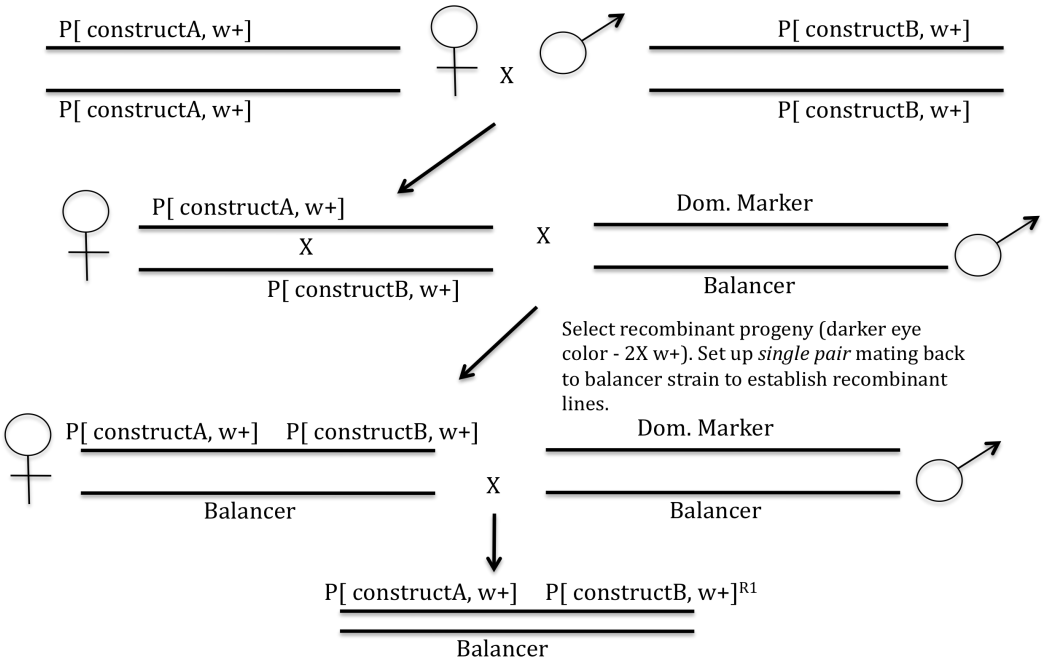


Figure 17: Schematic for creation of recombinants.

Recombinants lines used for validation of lines are listed below. GMR recombinants with Kek1 and Kek5 had been previously created Remaining recombinant lines were created by scheme in figure below, and selected by expression of GFP or PCR.

- GMRGAL4, [UAS.Kek1.GFP]^{59II}
- GMRGAL4, [UAS.Kek2.GFP]$^{R6\text{-}1a}$
- GMRGAL4, [UAS.Kek4.GFP]$^{7\text{-}4,\ 38\text{-}5}$
- GMRGAL4, [UAS.Kek5.GFP]$^{16,\ 52}$
- GMRGAL4, [UAS.Kek6.GFP]^{12}
- C155GAL4,UASkek5$^{RNAi\text{-}47770}$
- [UAS.kek5.GFP]^{11} ,UASkek5$^{RNAi\text{-}27249}$

Driver lines used are listed below with a Stock number. Full genotype and further information can be found at Flybase or Bloomington Drosophila Stock Center:

- GMRGAL4 - 1104
- apGAL4 - 3041
- tubGAL4 - 5138
- actGAL4 - 4414
- actGAL4; UASdcr-2 - 25708
- ElavGAL4 - 8765
- C155GAL4 - 458
- C155GAL4; UASdcr-2 -25750
- enGAL4 - 6356
- ScabrousGAL4 -6479

ASSESSMENT OF KNOCKDOWN IN DEVELOPING NERVOUS SYSTEM

The lines used for RNAi knockdown assessment in the developing nervous system were *C155GAL4;UASDcr2*^{25750}, [*C155GAL4*,*UASkek5*^{RNAi}]^{III}, [*UASkek5-GFP*,*UASkek5*^{RNAi}]^{V} and *UASkek5-GFP*^{16}.

Embryos – Live embryos were washed into collection nets, placed into petri dishes, dechorionated using 50% bleach. The bleach was removed and vitelline membranes were then removed by popping embryos with a sharp tungstein needle in water after adhering embryos to the petri dish plate by allowing them to dry briefly. Embryos were then mounted in water and imaged immediately.

1st instar larva – live larva 24-30hrs after egg lay were incubated in PBT with 4% formaldehyde for 15-20 min, then mounted in water and imaged immediately.

3rd instar larva – wandering larval brains were dissected and fixed in PBT with 4% formaldehyde for 15 min, then mounted in mounting media (50% glycerol in PBS).

IMMUNOHISTOCHEMISTRY - EMBRYOS

Embryos were collected overnight, dechorionated with 50% bleach and fixed for 20 minutes in a mixture of 6mls heptane and 1ml fixative (10% formaldehyde and 50mM EGTA in PBS). Fixative was then removed (bottom layer), 8mls of methanol added and solution was shaken vigorously for 1 min. Embryos were then extensively rinsed with 100% methanol and stored in methanol at -20°C. Embryos were rehydrated in PBS with 0.1% Tween20 and blocked for 30minutes in PBT with 5% NGS at room temperature. Primary antibody was incubated overnight at 4°C in PBT with 5% normal goat serum (NGS). Primary antibodies and dilutions were as follows: anti-GFP (BD Bioscience) 1:500, BP102 (DSHB) 1:500, and 1D4 (DSHB) 1:100. Secondary antibody was incubated for two hours in room temperature in PBT with 5% normal goat serum. The secondary antibody was anti-mouse or rabbit Alexa 488 used at 1:500. Samples were mounted in 70% Glycerol in PBS.

IMMUNOHISTOCHEMISTRY - LARVA

Dissecting magnetic chambers were constructed similar to published protocol (Bellen & Budnik, 2000), with the necessary adaptations as indicated in Appendix 1. Third instar wandering larva were selected and dissected in Ca^{+} free saline (128mM NaCl, 2mMKCl, 4mM $MgCl_2$, 35.5mM Sucrose, 5mM Hepes, 1mM EGTA). Larvae were pinned down by posterior and anterior extremities and opened dorsally along midline using dissecting scissors (Roboz RS5618). Interior organs and gut were removed with fine forceps and larval cuticle (with attached muscles) were opened and pinned at the 4 extremities so that cuticle of larva is flat against chamber surface. While pinned down, larvae were fixed with 4% formaldehyde (alcohol free) in 0.1M phosphate buffer pH7.2. Samples were then transferred to round bottom wells (maximum 4/well), washed and stained according to standard immunostaining procedures using 0.1M phosphate buffer pH7.2 with 0.2% TritonX-100. Samples were arranged in desired orientation and mounted cuticle downward in glycerol/PBS. Primary antibodies used were rabbit anti-HRP (Jackson ImmunoResearch) – 1:1000, mouse anti-Discs large (DSHB) – 1:2000 and rabbit Anti-GFP – 1:1000. Secondary antibodies used were goat anti mouse and rabbit Alexa 488 or 568 at 1:500.

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APPENDIX

APPENDIX 1

MAGNETIC CHAMBERS AND PINS - ASSEMBLY

Materials

- Glass slides – Glass cut into 2''x3'', approx 1/16-1/8'' thickness obtained from a window store. Edges were polished for safety.
- Adhesive magnet business card size obtained from office supply store.
- Silicone sealant
- Beaded stainless steel insect pins size 00 obtained from Carolina Biological Supply Co. item#65-4331
- Prong fasteners from office supply store
- Epoxy glue (5 min.)
- Fine thin-nose pliers
- Sharp cutting pliers
- Snap blade knife
- Sand paper grit 200 and 400 (silicone carbide recommended).

Chambers Assembly

1) Using the adhesive in the magnetic sheet, glue 2 magnets sheets together.

2) Cut a hole around a nickel coin as a template with a snap blade knife.

4) Remove the adhesive protector and place a bead of silicone sealant around the perimeter of the strip and then again right at the rim of the cut hole (see diagram). Make sure there are no breaks around the inner rim.

5) Place the 2" x 3” glass slide over the back side of the magnetic strip and press into the adhesive. Some sealant should leak into the hole. Use the excess sealant to seal the inner edge of the magnetic assembly, to make sure no liquid will penetrate in between the magnetic sheets. Carefully place a heavy, flat object over the chambers for several hours (12-24hrs). A heavy book lined with aluminum foil or wax paper works well. Some sealants release acetic acid, so fill chambers with water and let sit 12-24hrs.

Pins assembly

1) Form beaded end insect pins as in Fig. 1 by using a thin-nosed pair of pliers. Rinse the formed end with 95% alcohol.

2) Cut prong fastener approx. 3-5mm before fold (the thinner side) and about 15mm after fold (bottom part), as indicated in the diagram above figures. Using pliers fold handle end in the middle, then fold again the edges 90° to have it stand up on its own. For safety and comfort, also fold raised edge from the tab base. Final assembly should look like figure 2.

3) Sand down the surfaces to be glued. This makes epoxy adhere better. Clean and rinse with alcohol to remove any oils from fingers, etc.

4) Using quick setting epoxy, glue pins to tabs as in Fig. 2. Apply one drop of glue from a toothpick or wooden applicator stick. Middle section left from prong fasteners works well. The pin tip should be raised about 2-3mm (set tip on a strip of cardboard, etc. before gluing, diagram on bottom of figures). This raised angle will prevent the solution from

spreading all over the top of the chamber via capillary action. Let glue sit overnight to harden completely.

5) After glue has hardened, cut the two center pins to ~16 mm and the 4 corner pins to ~18-20 mm (for 2^{nd}-3^{rd} instars) , measured from the end of the tab (the end with the glue). Pin tips should be sanded flat and thin with silicon carbide paper - grit #220. Finish with grit # 400-600. See diagram. Tip can be sharp but not too narrow. It's convenient for your index fingernail to have 2 mm of white showing.

5) Bend the pins as in Fig. 3. Dimensions are in millimeters. The last bend (0.5-1mm) is best done with old and somewhat dull forceps under a low power dissecting scope.

6) Corner pins should also be bent from the side as in Fig. 4. Pin tips should have moderate to light tension when bent to position on surface of glass. Too much tension rips cuticle, especially when larvae are very young. Not enough tension will not keep larva in place. All dimensions are approximate and may need to be adjusted for chamber.

Tab assembly

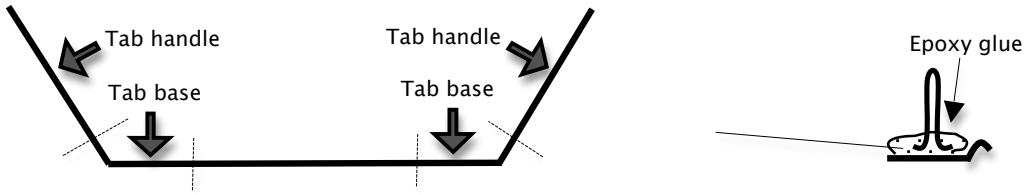


Fig. 1

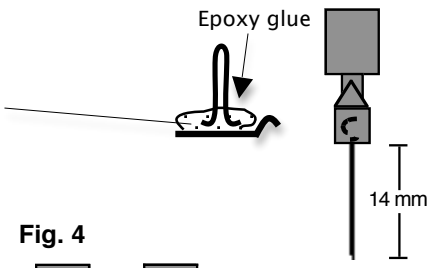
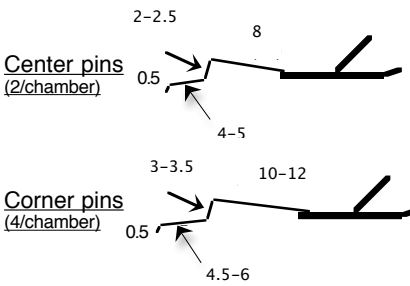


Fig. 2

Fig. 3

Length is measured from end of tab. See Fig. 2, bottom right.



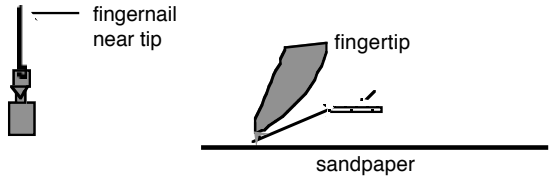
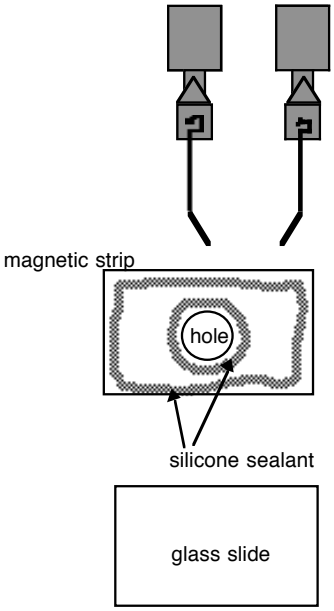


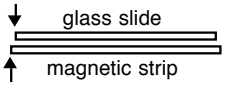
Fig. 4



8" x 1" strip of 220 silicone carbide paper; 2-3 passes, top and bottom of pin; get tip quite thin

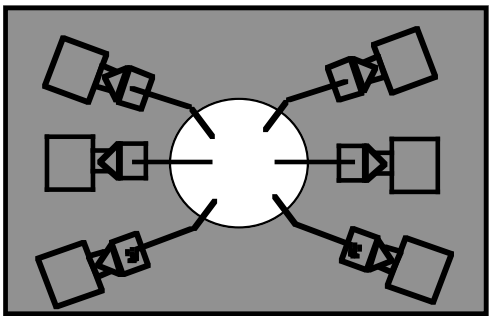
Then do likewise with 400-600 grit paper

Place glass slide over strip and position carefully to allow proper 1mm overlap of strip.

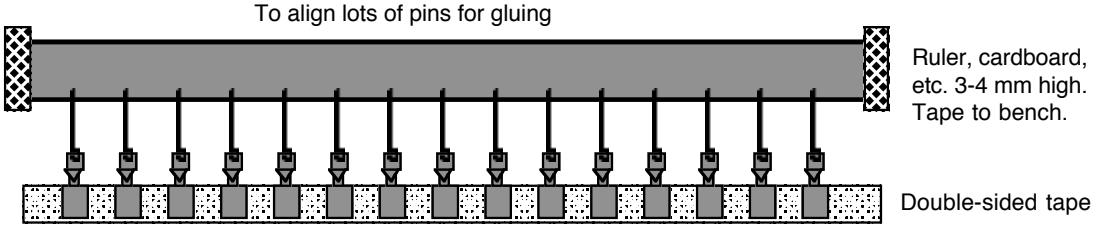


Invert above assembly and bend pins to provide the correct amount of pressure on slide.

Finished Chamber



To align lots of pins for gluing



APPENDIX 2

GENETIC SEQUENCES

The sequences of the vectors used to clone individual keks are indicated here. Within each sequence is the open reading frame (ORF) of each kek, and within it, some structures are marked (such as LRRs and transmembrane region). Also marked (in dark yellow) are the trigger sequence of the RNAi lines, labeled as RNAi-#### (line number), and the oligo used in PCR to identify presence of construct, marked in black, labeled with oligo number starting with W (e.g. W65). RNai trigger and oligos are also marked on ORF of *nrt* and *nrg*.

UAS Kek1 GFP

```
   1 GGCCAGACCCACGTAGTCCAGCGGCAGATCGGCGGCGGAGAAGTTAAGCGTCTCCAGGATGACCTTGCCCGAACT
  76 GGGGCACGTGGTGTTCGACGATGTGCAGCTAATTTCGCCCGGCTCCACGTCCGCCCATTGGTTAATCAGCAGACC
 151 CTCGTTGGCGTAACGGAACCATGAGAGGTACGACAACCATTTGAGGTATACTGGCACCGAGCCCGAGTTCAAGAA
 226 GAAGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAA
 301 CCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCC
 376 GCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCT
 451 CAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTT
 526 ATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAG
 601 GATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAG
 676 GACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAA
 751 ACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGA
 826 AGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAG
 901 ATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGA
 976 GTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATC
1051 CATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAAT
1126 GATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAG
1201 AAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCC
1276 AGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTC
1351 ATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTT
1426 CGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTC
1501 TCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTG
1576 TATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGT
1651 GCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTA
1726 ACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAG
1801 GCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTA
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1951 GGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAA
2026 AAATAGGCGTATCACGAGGCCCTTTCGTCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCT
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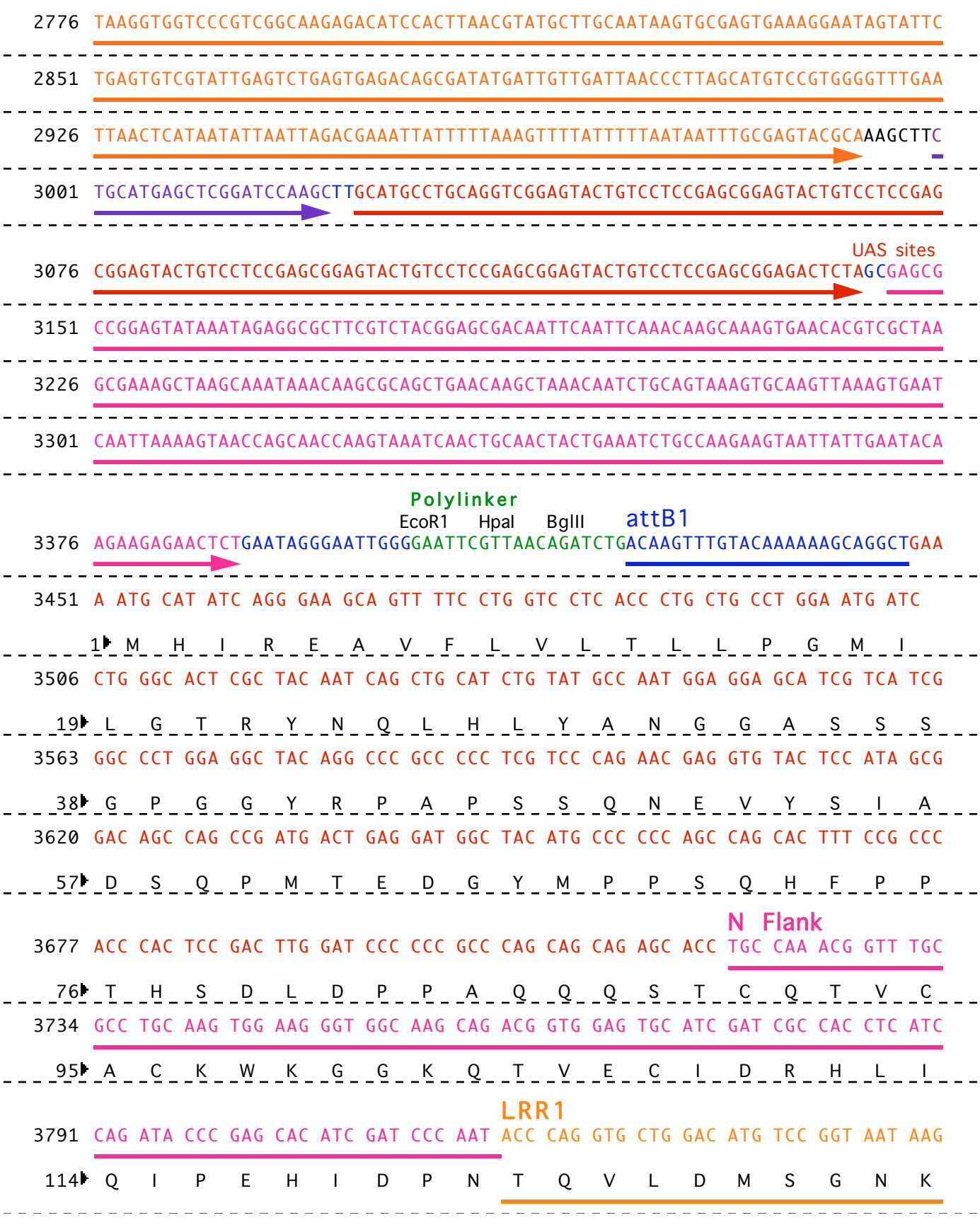
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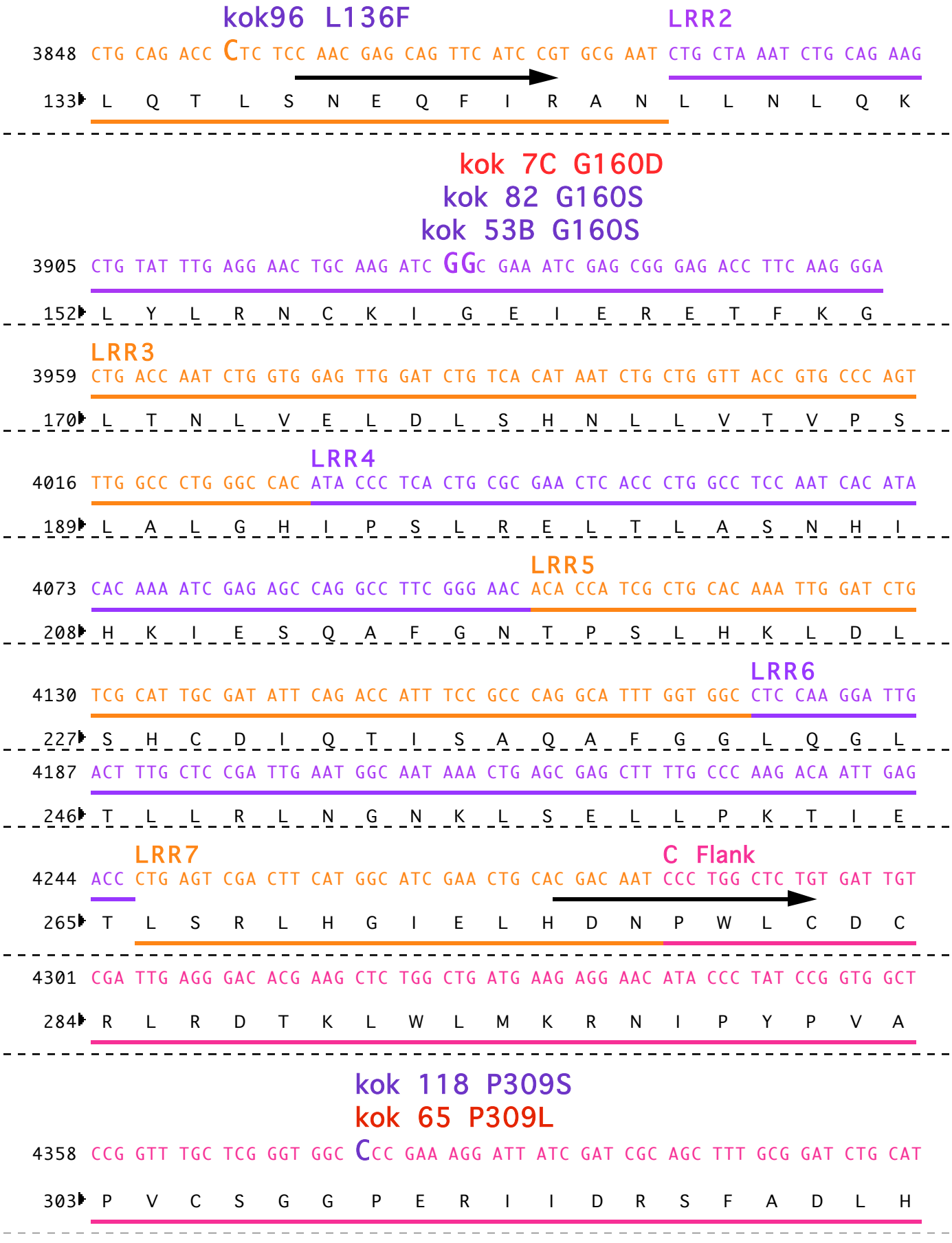
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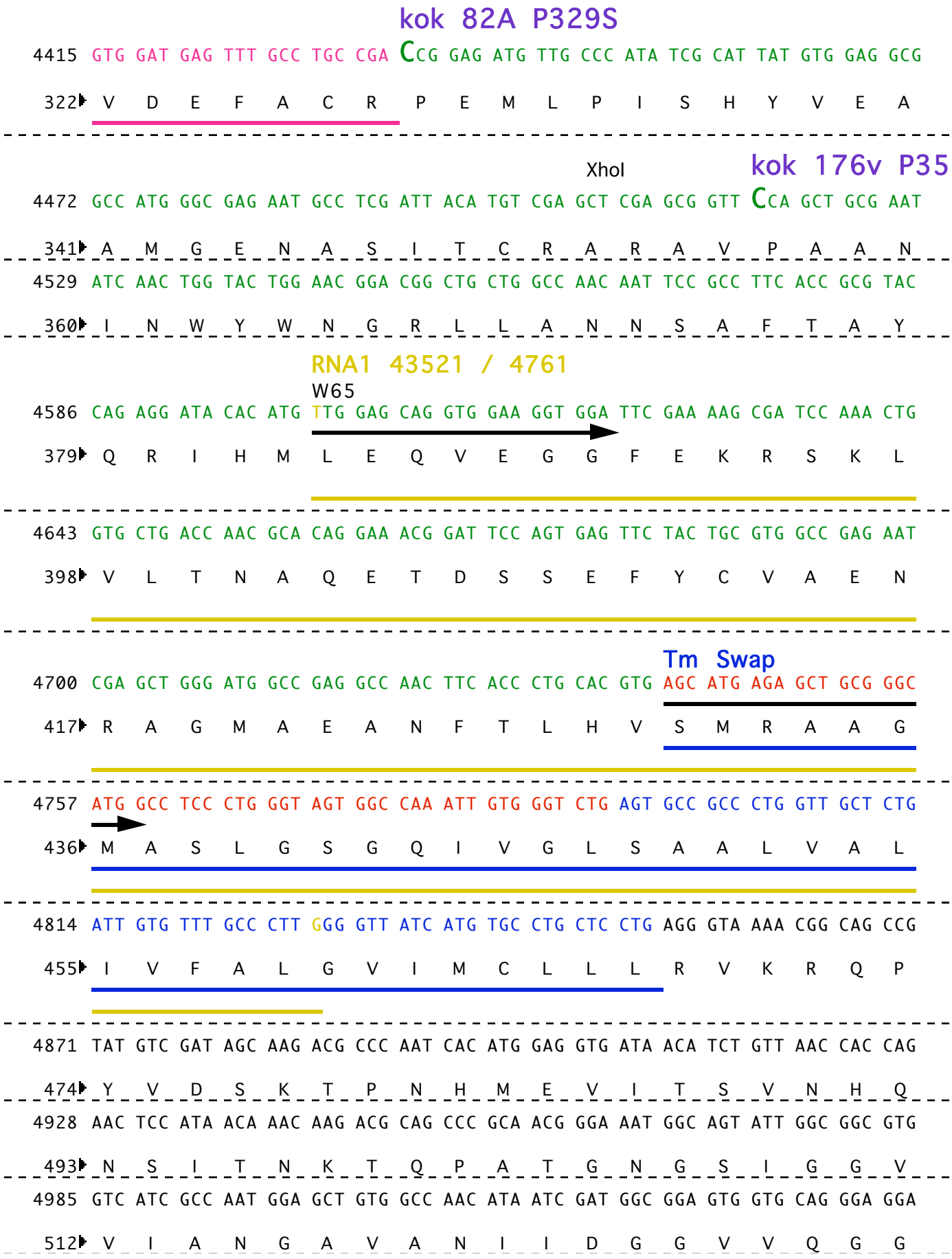
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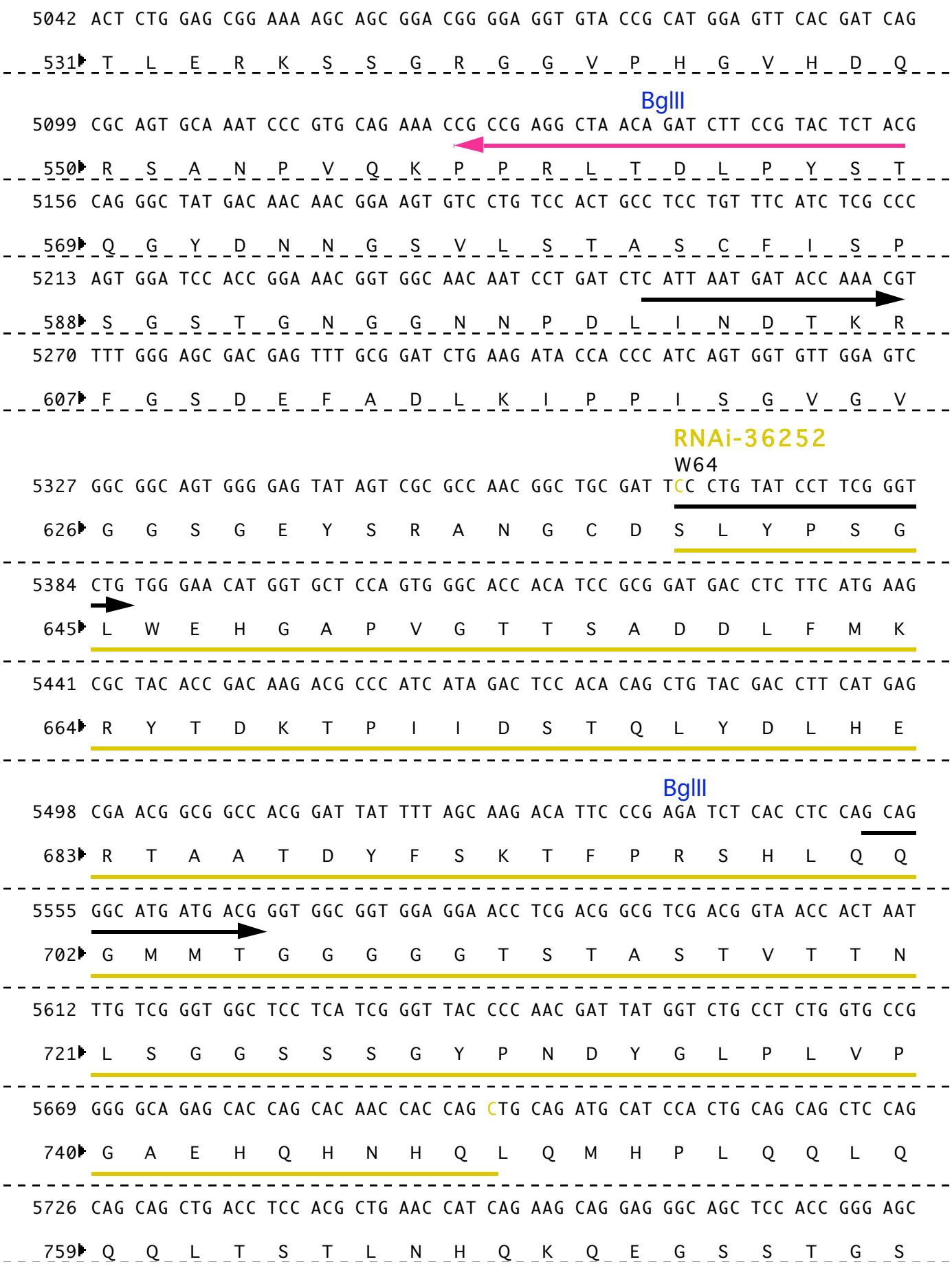
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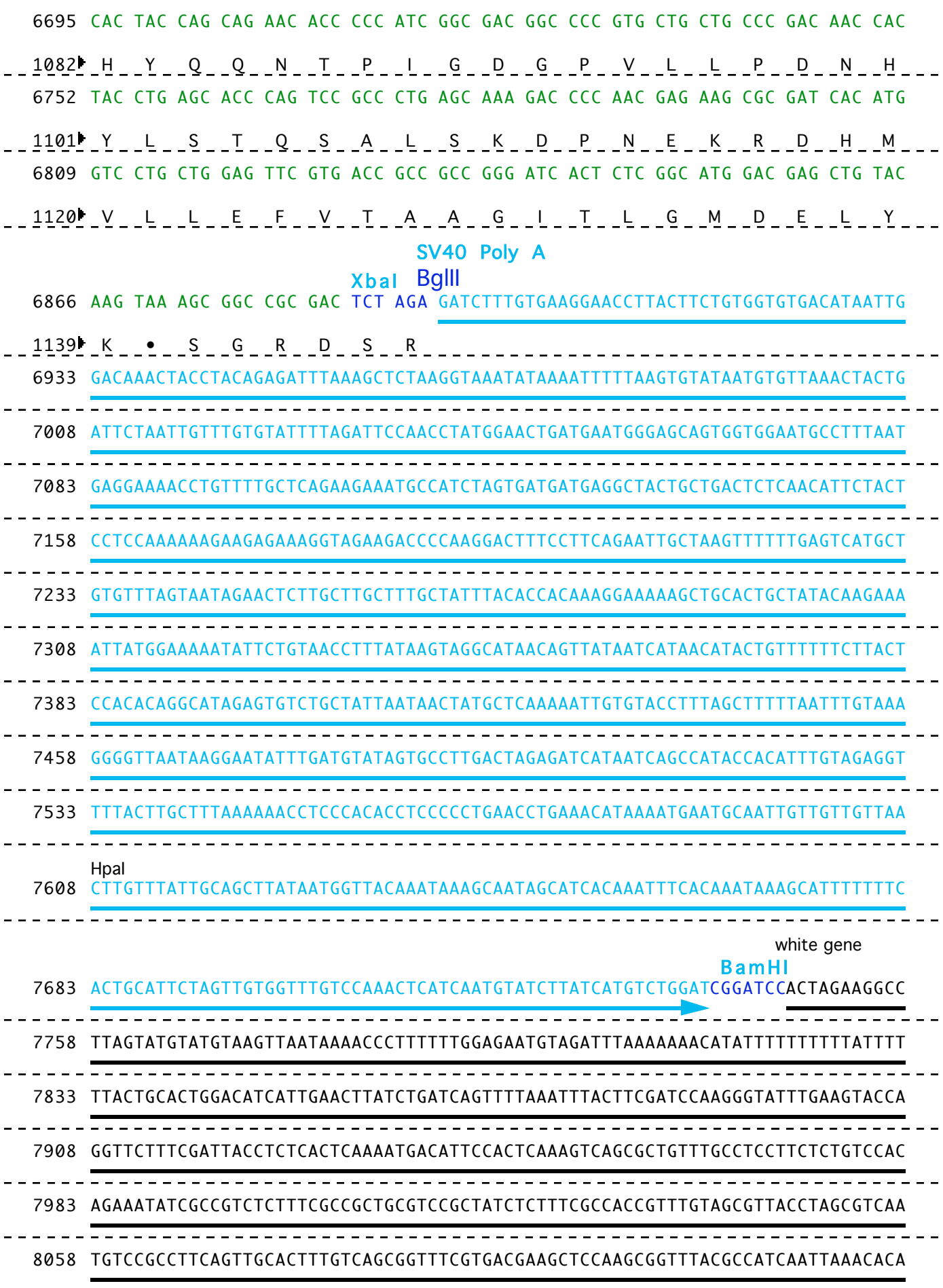
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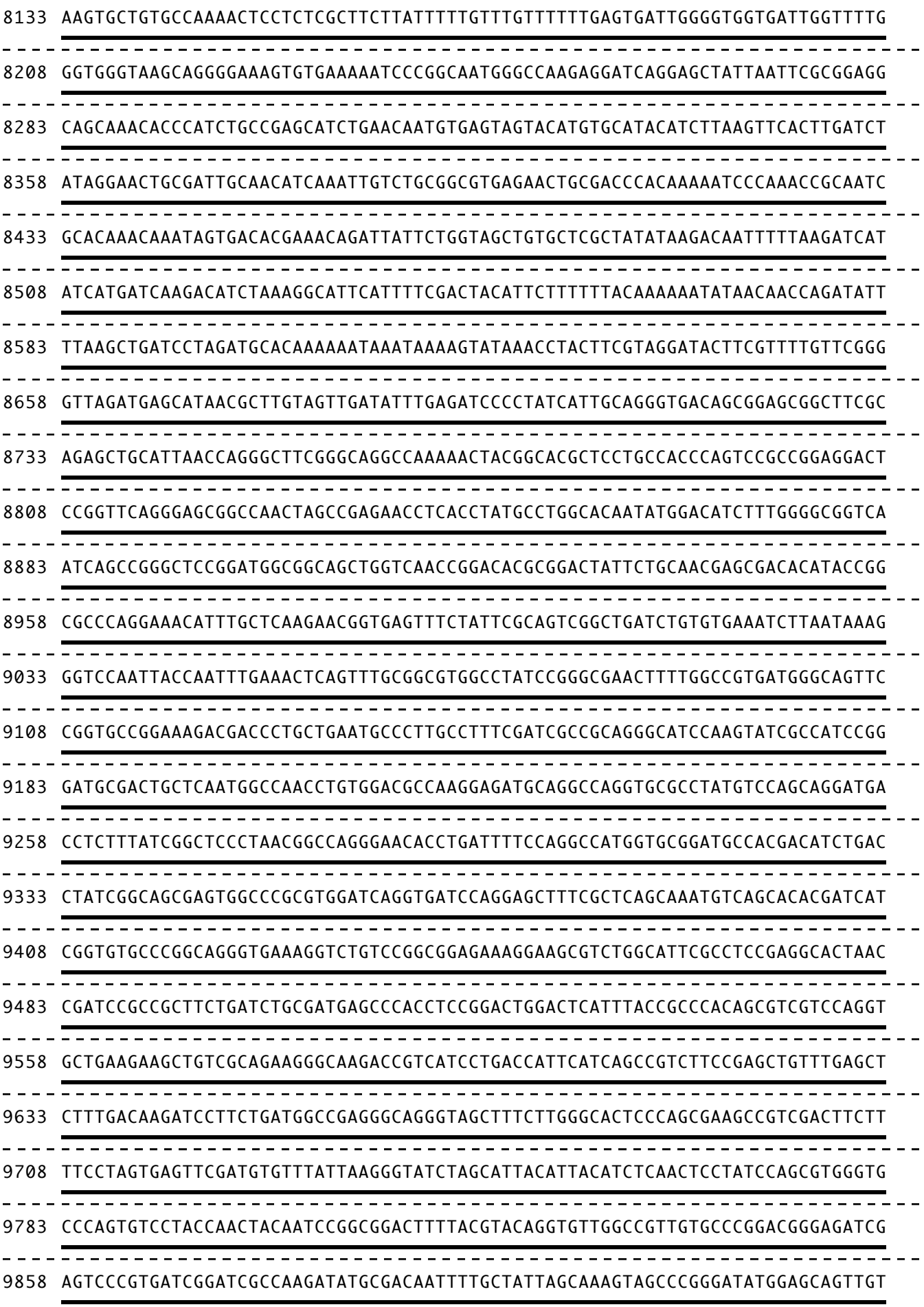
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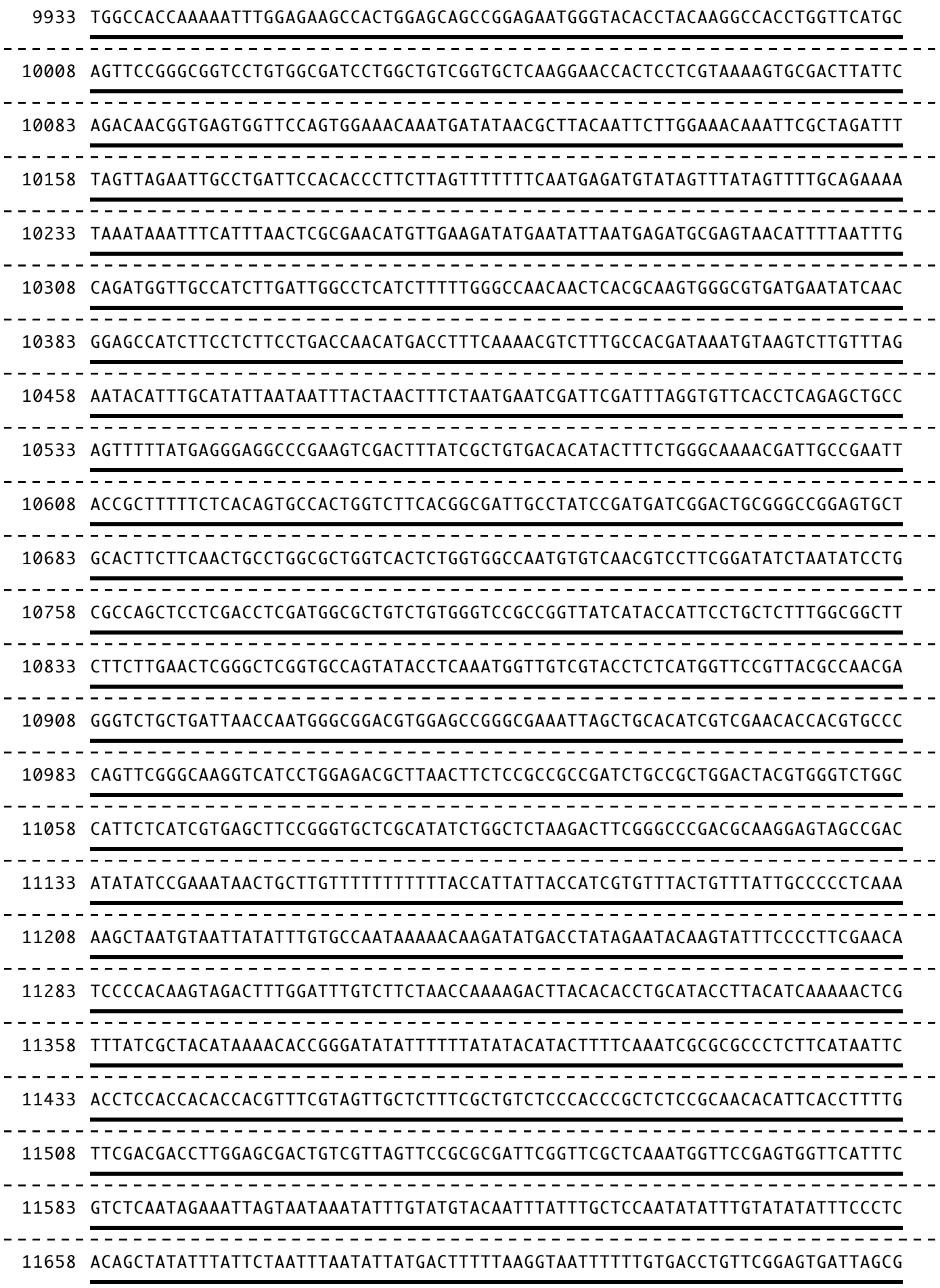
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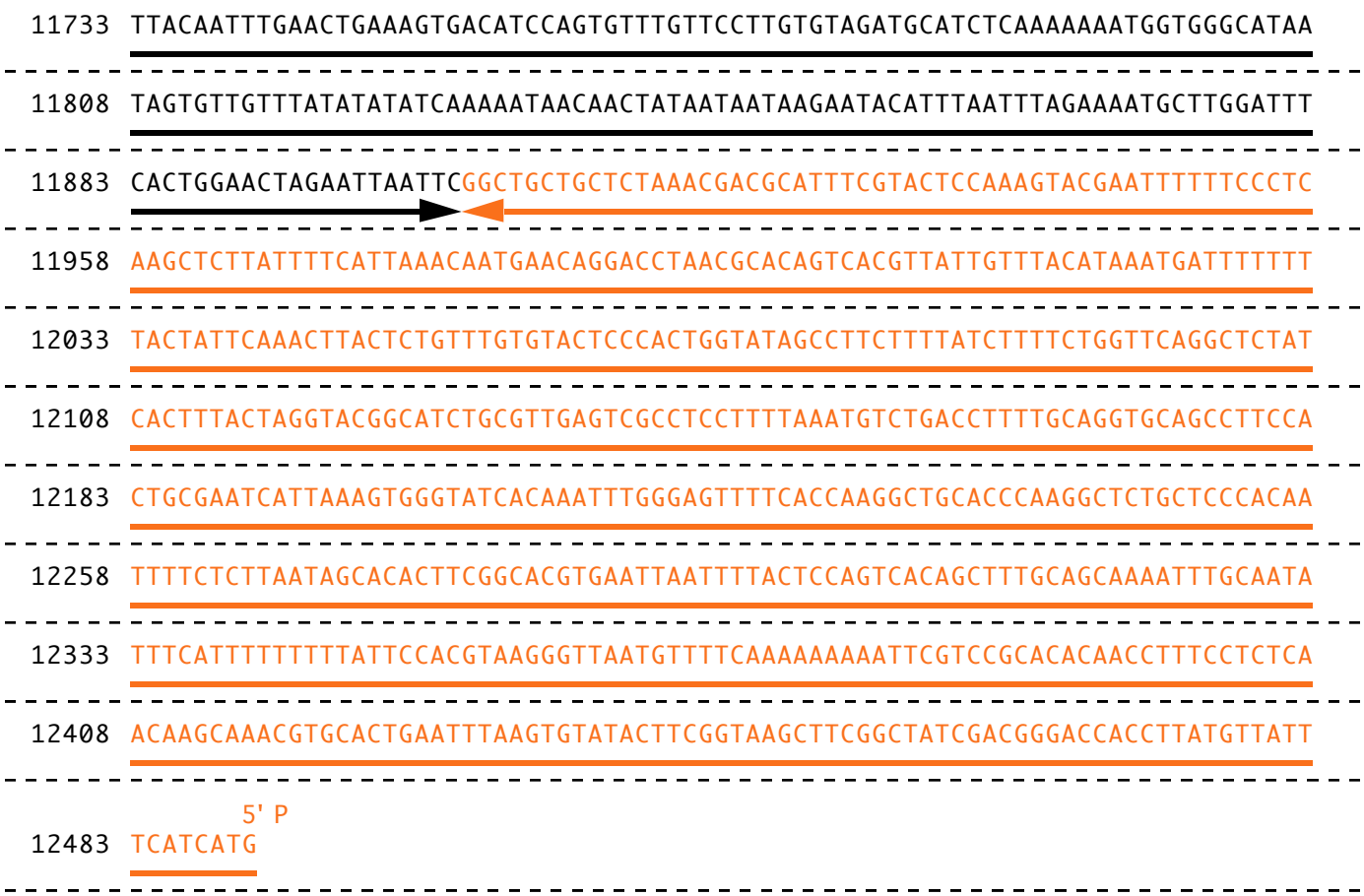
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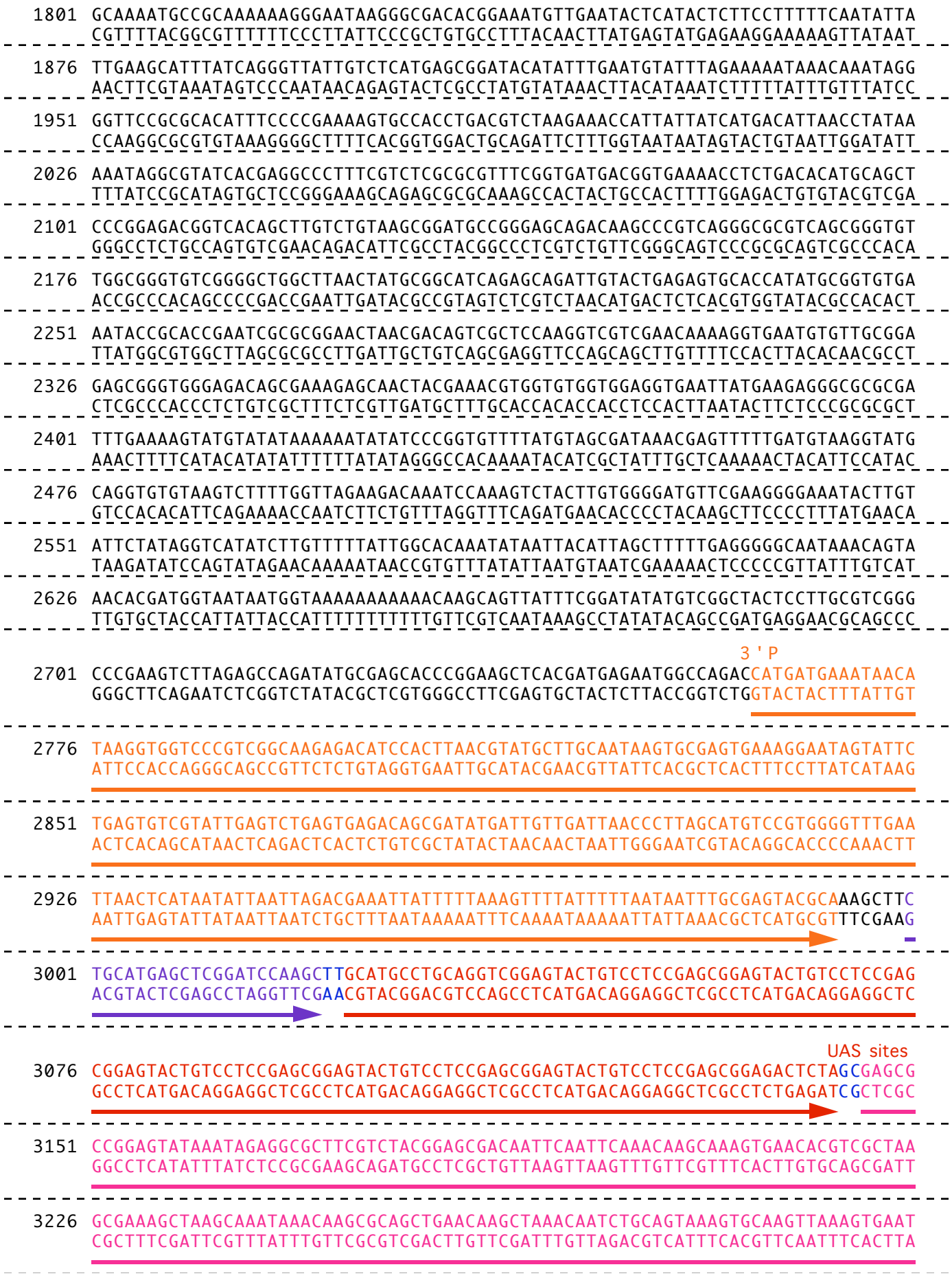
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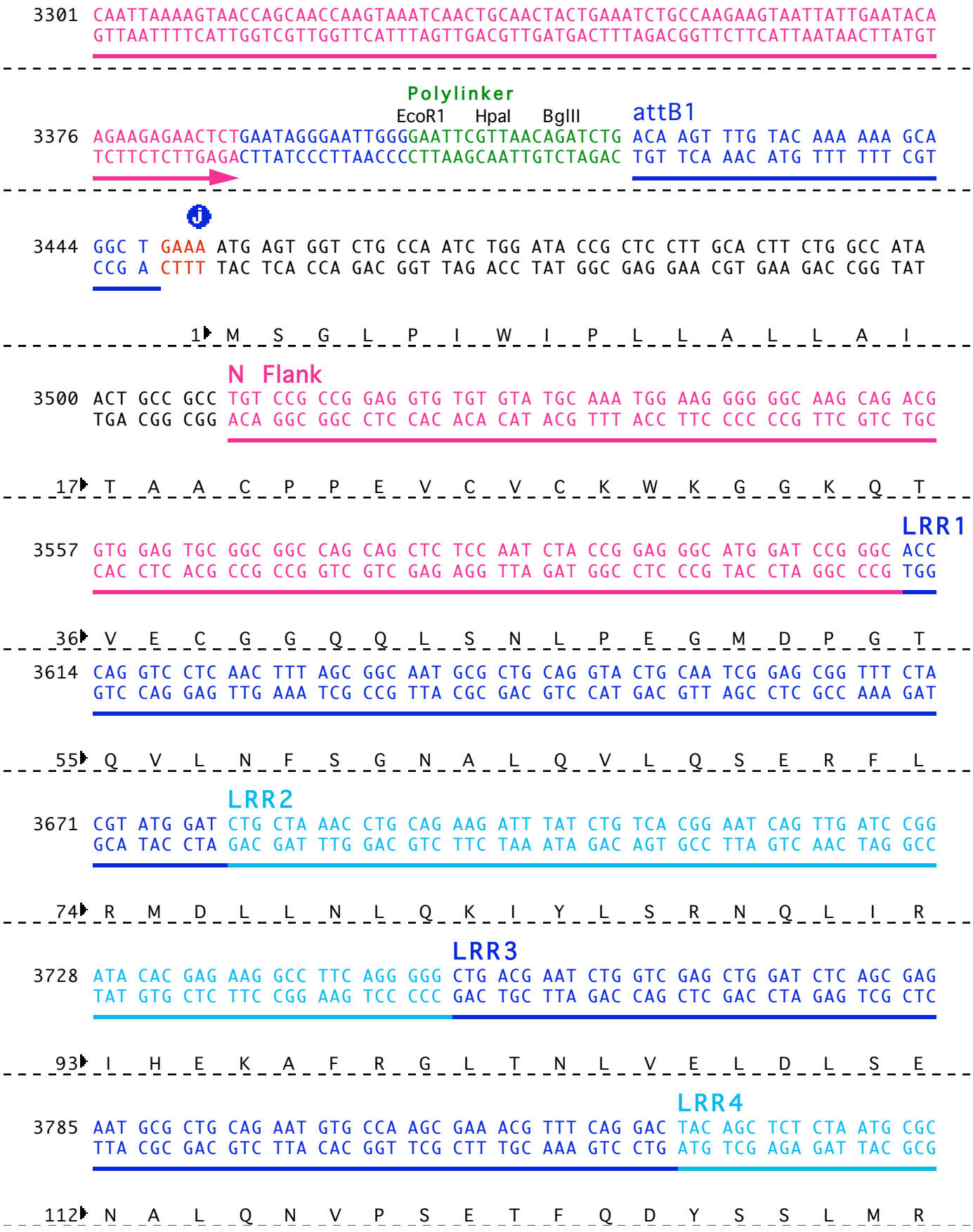
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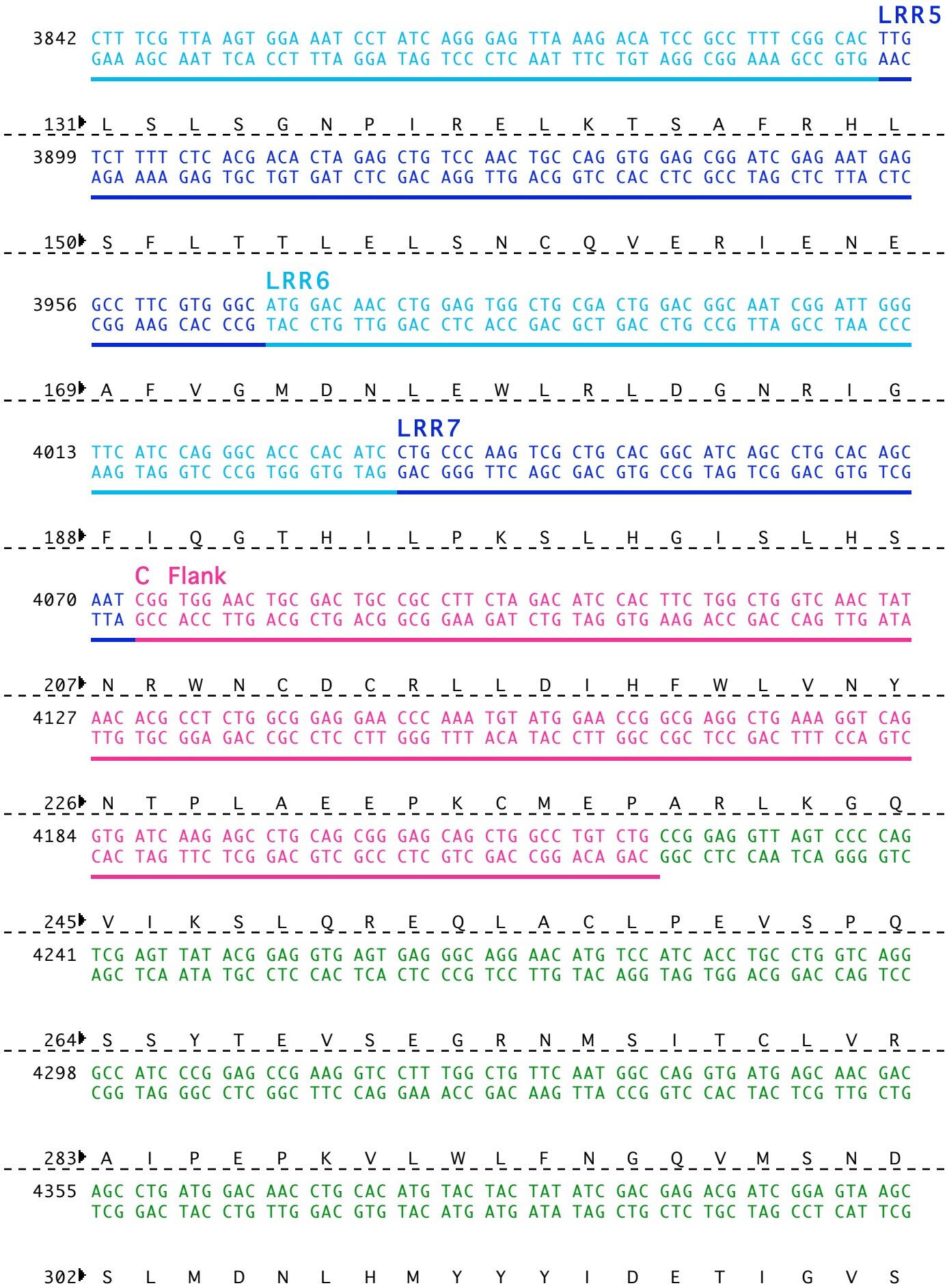
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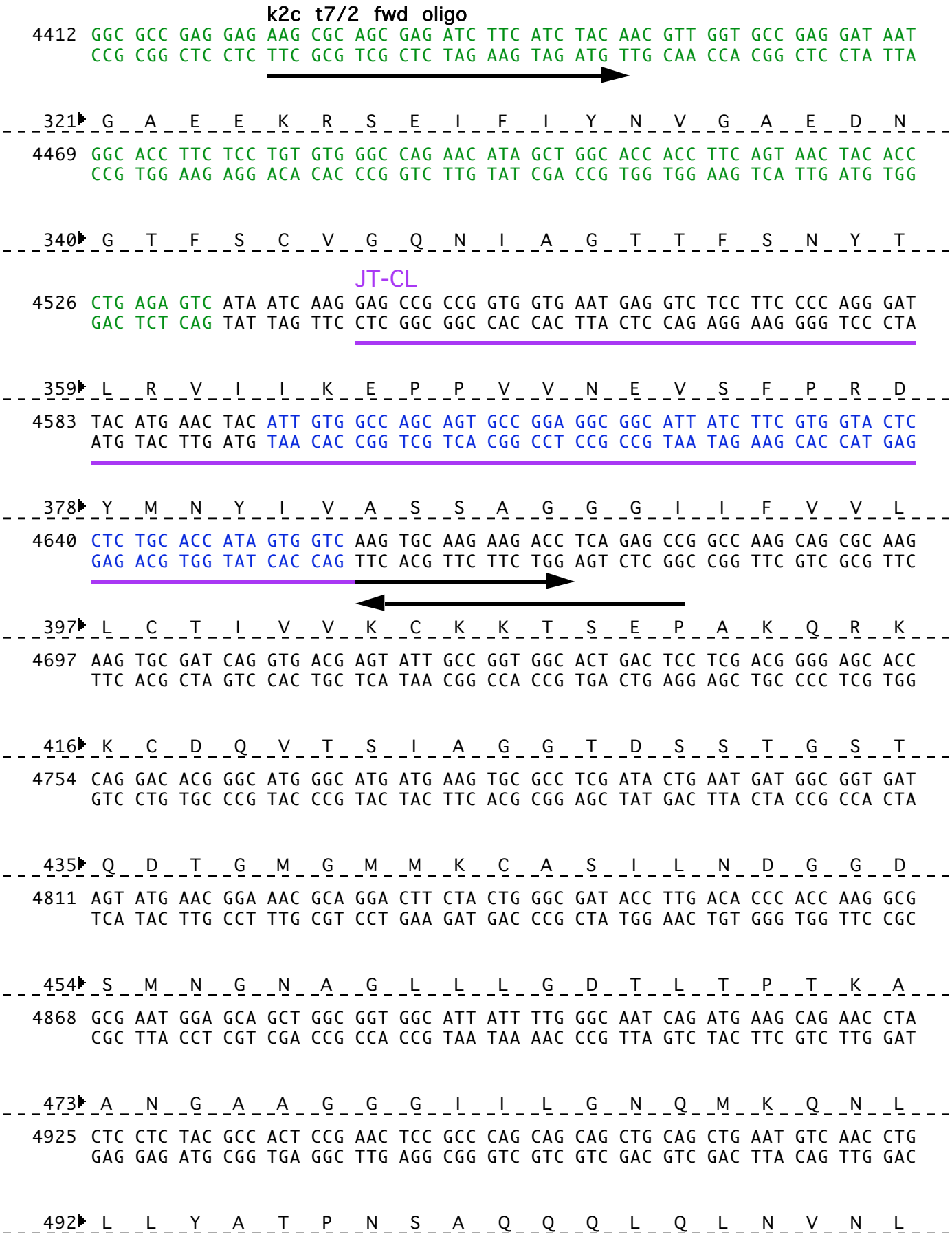
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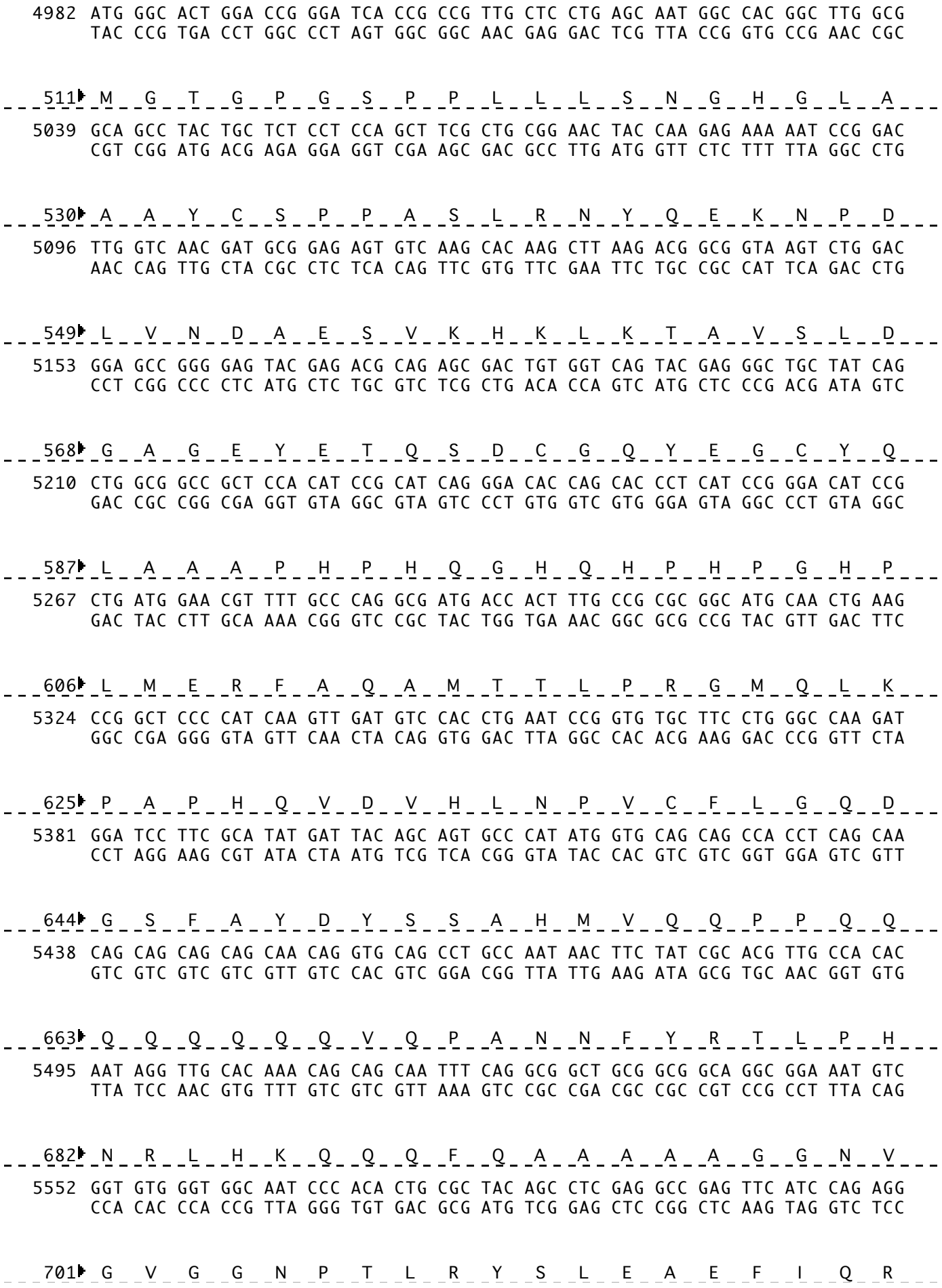
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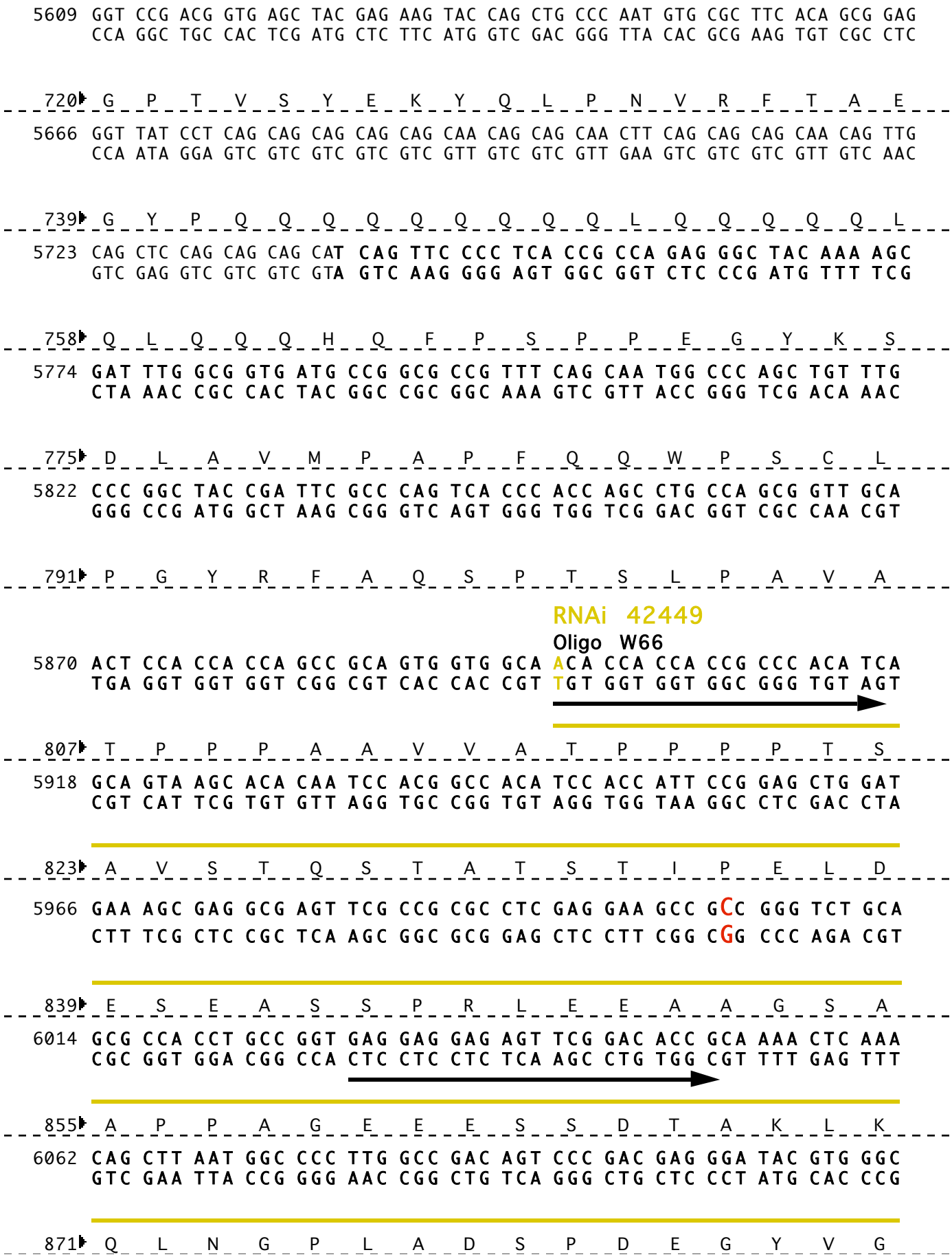
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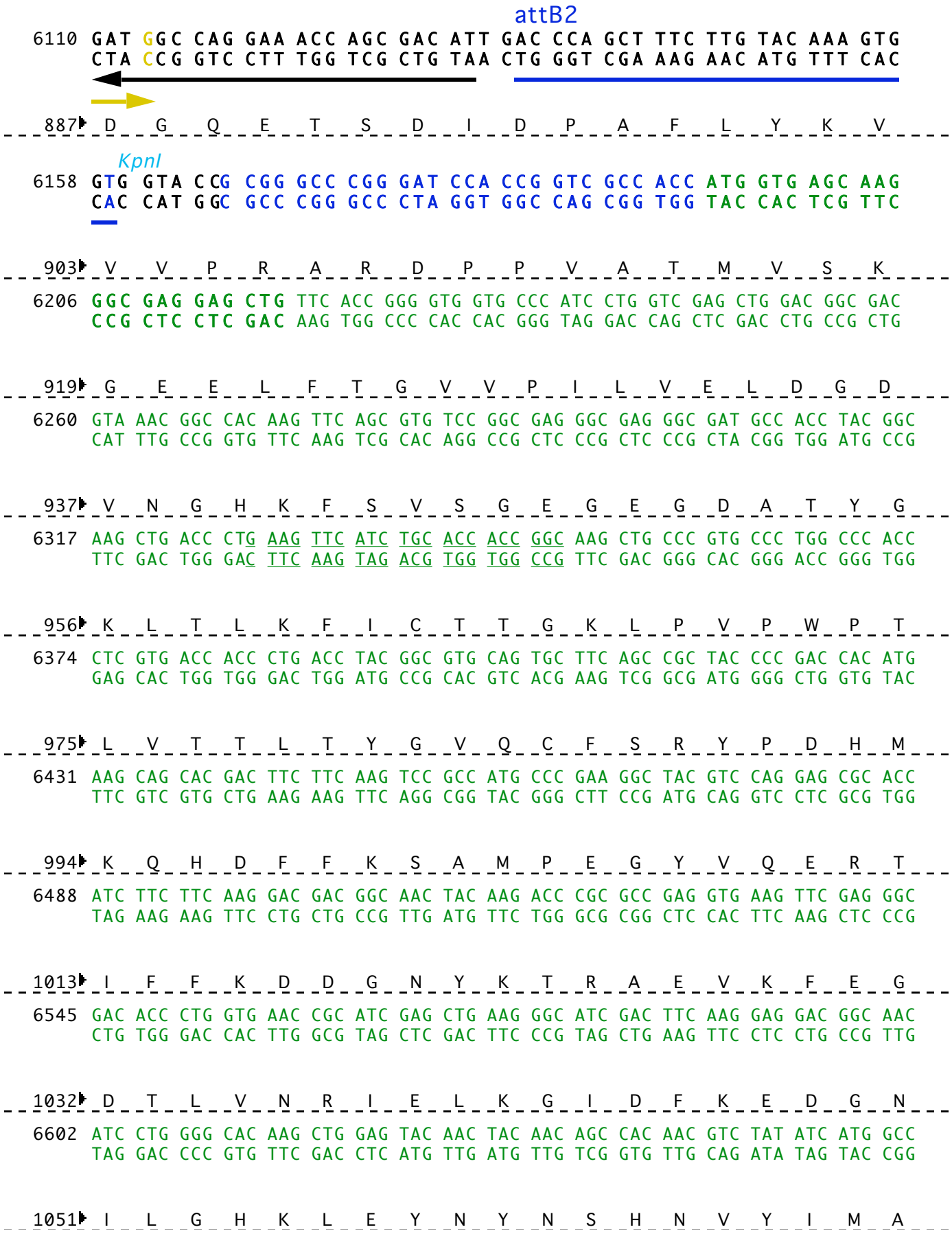
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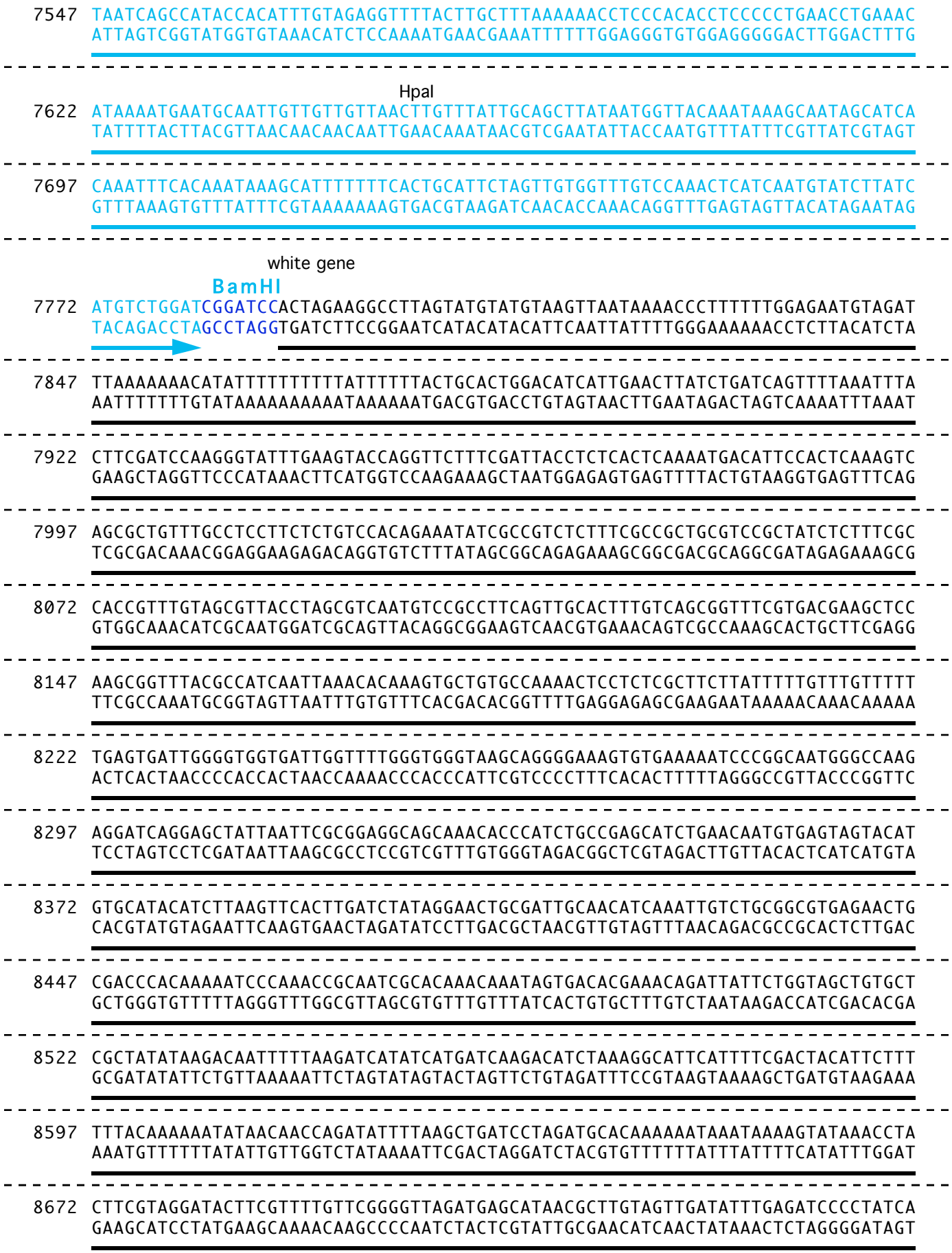
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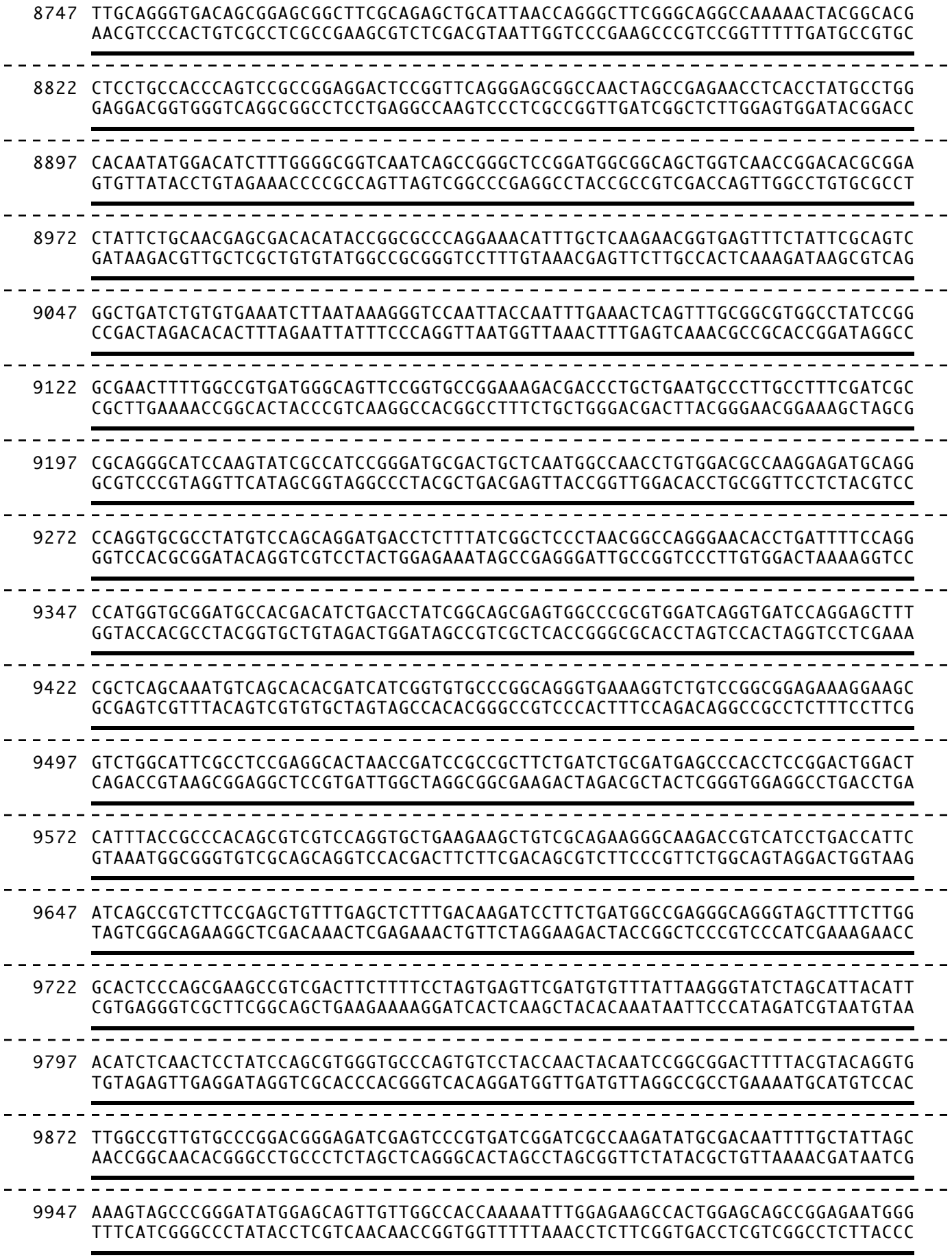
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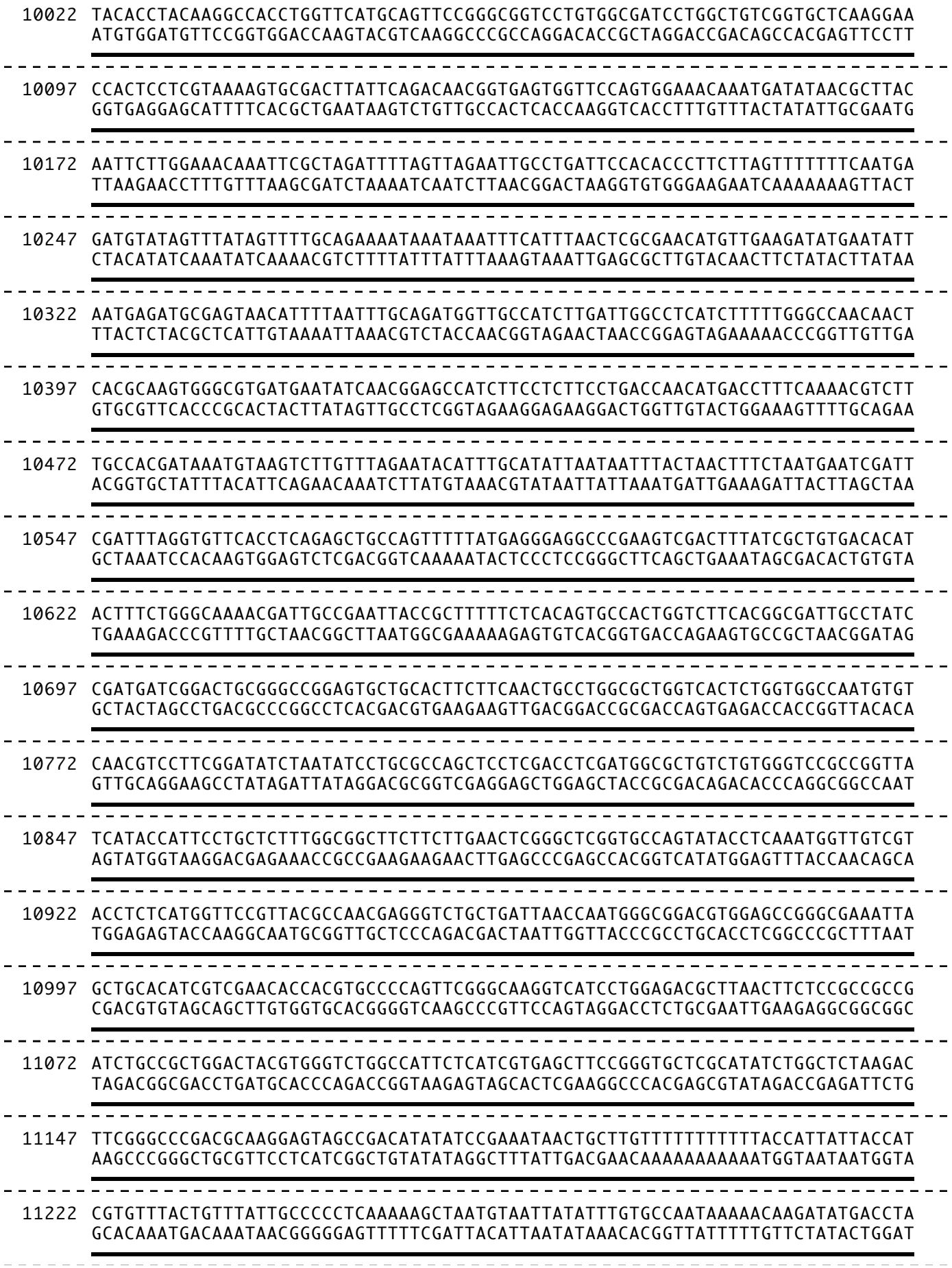
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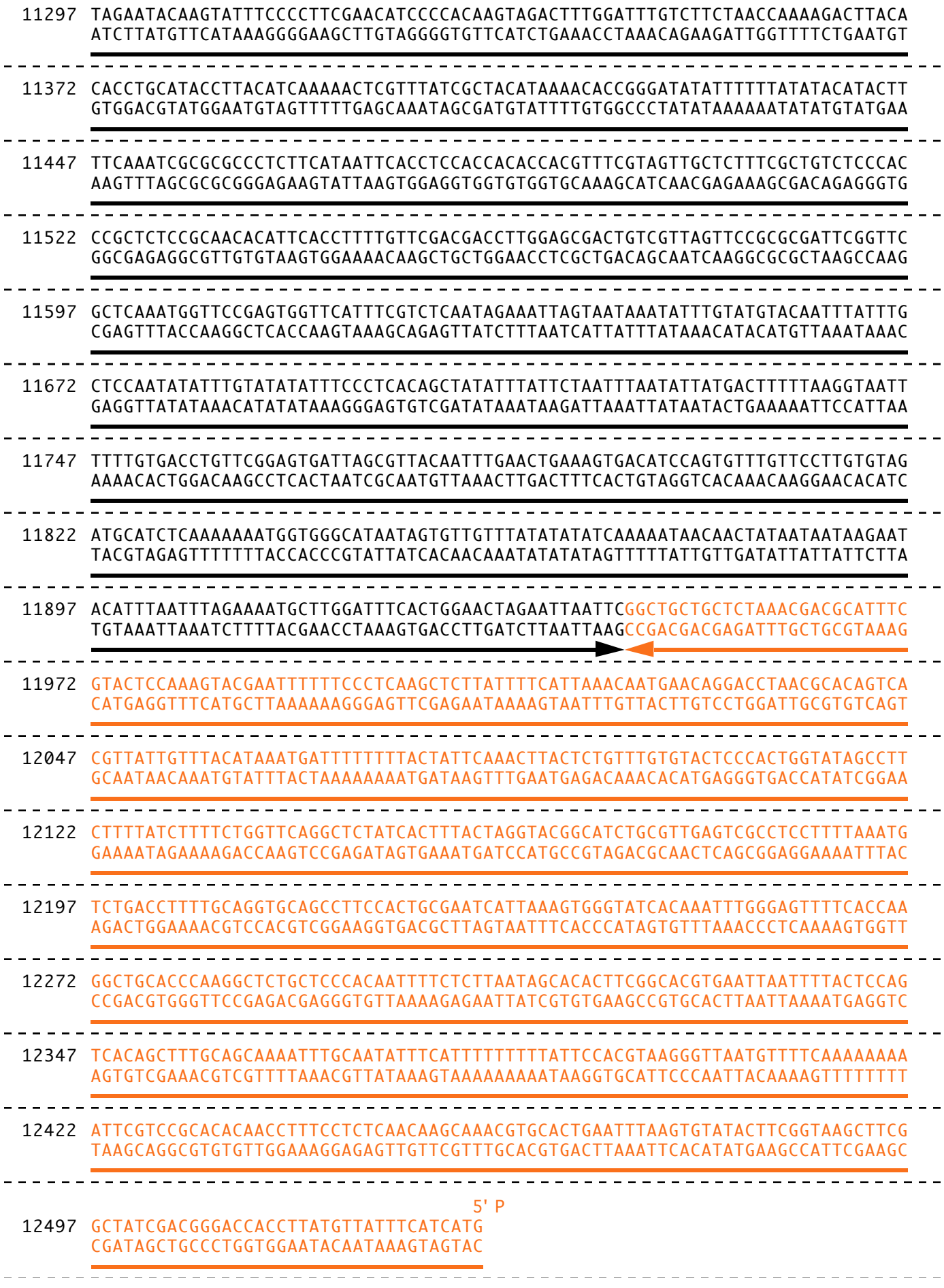
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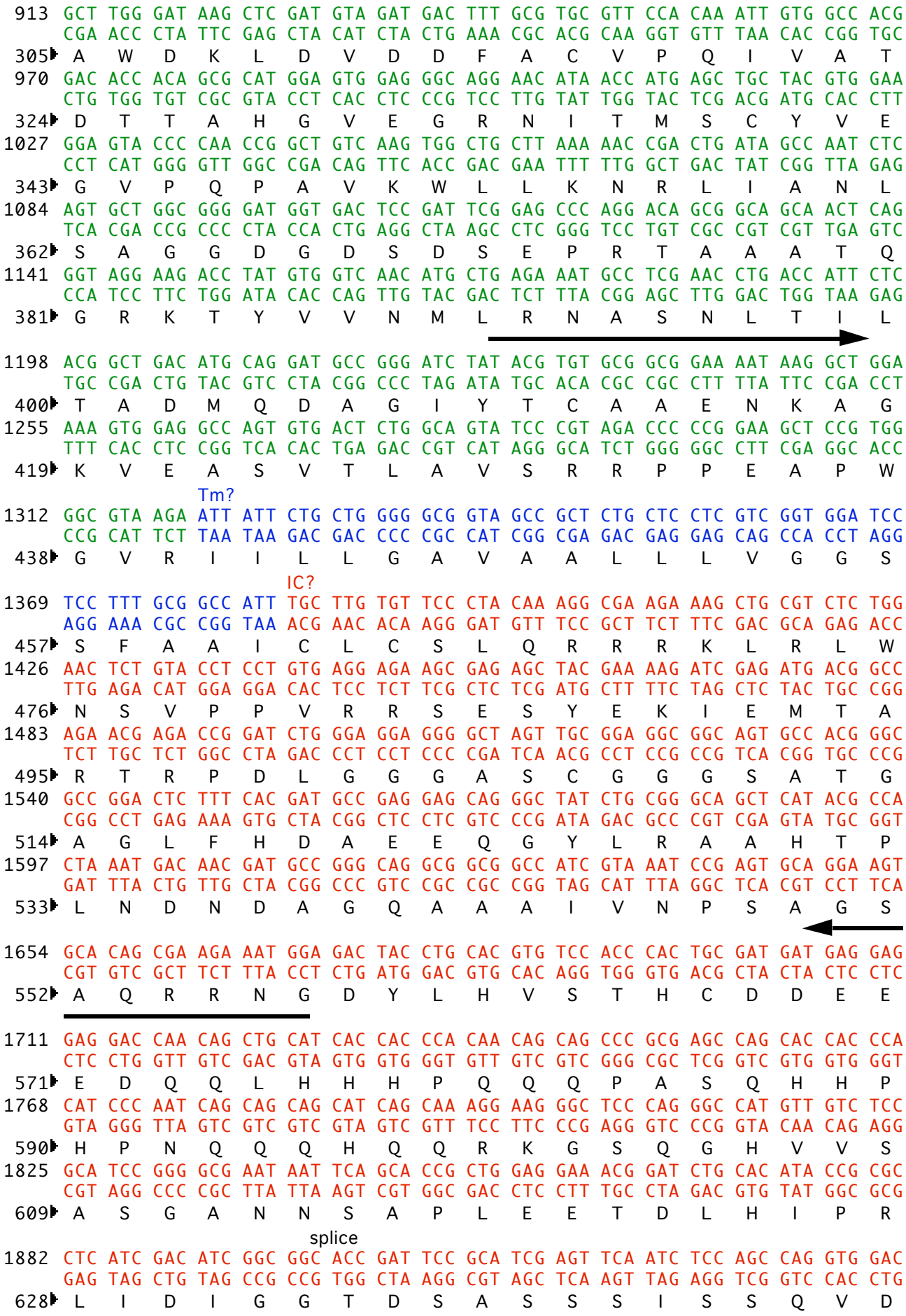
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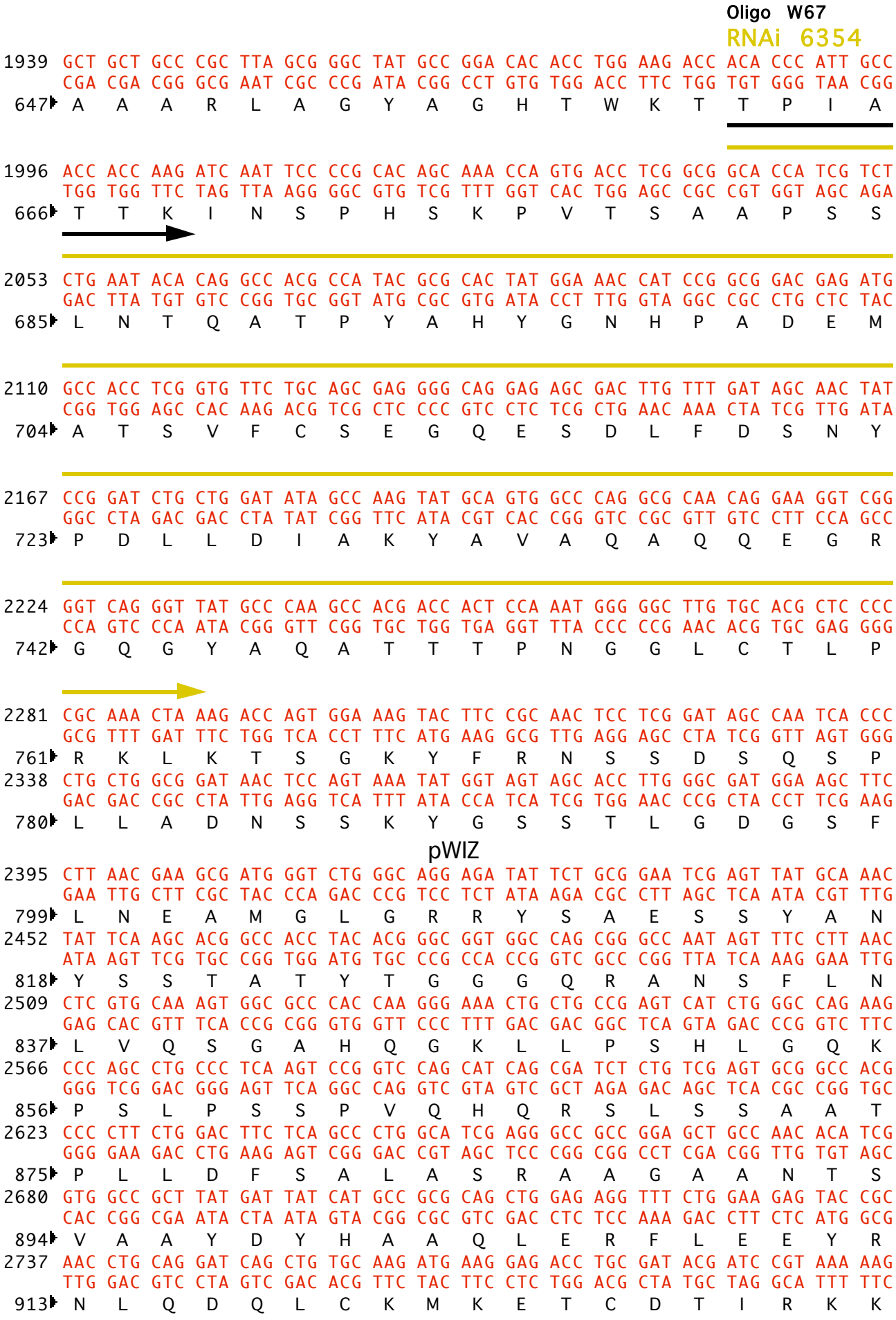
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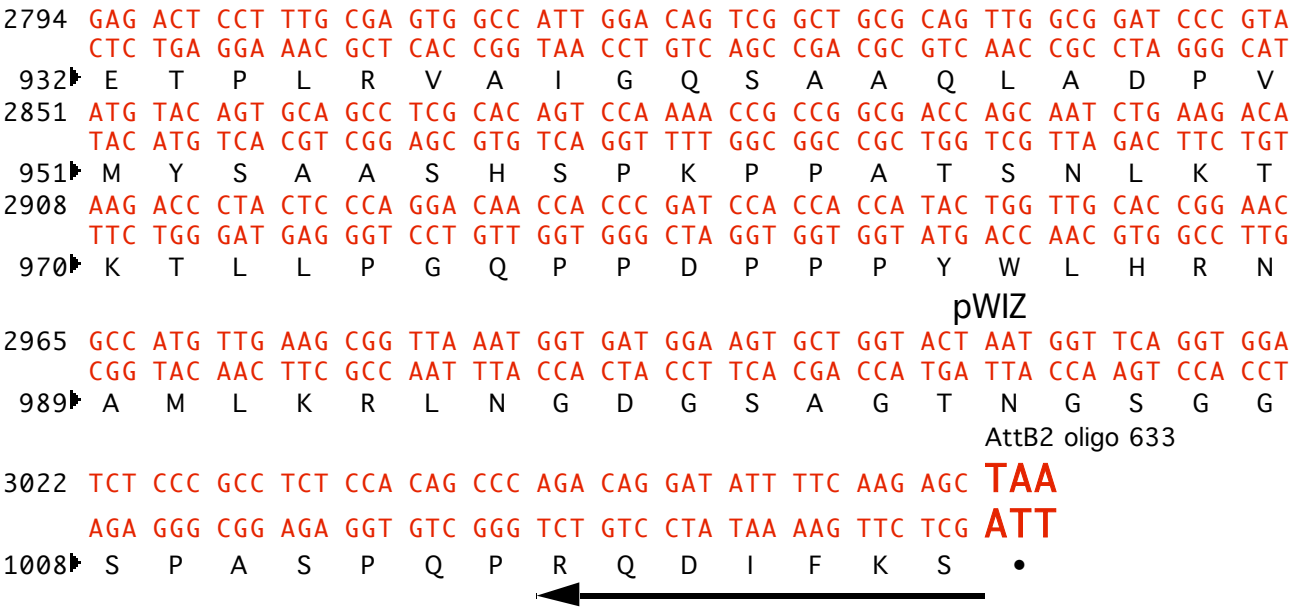
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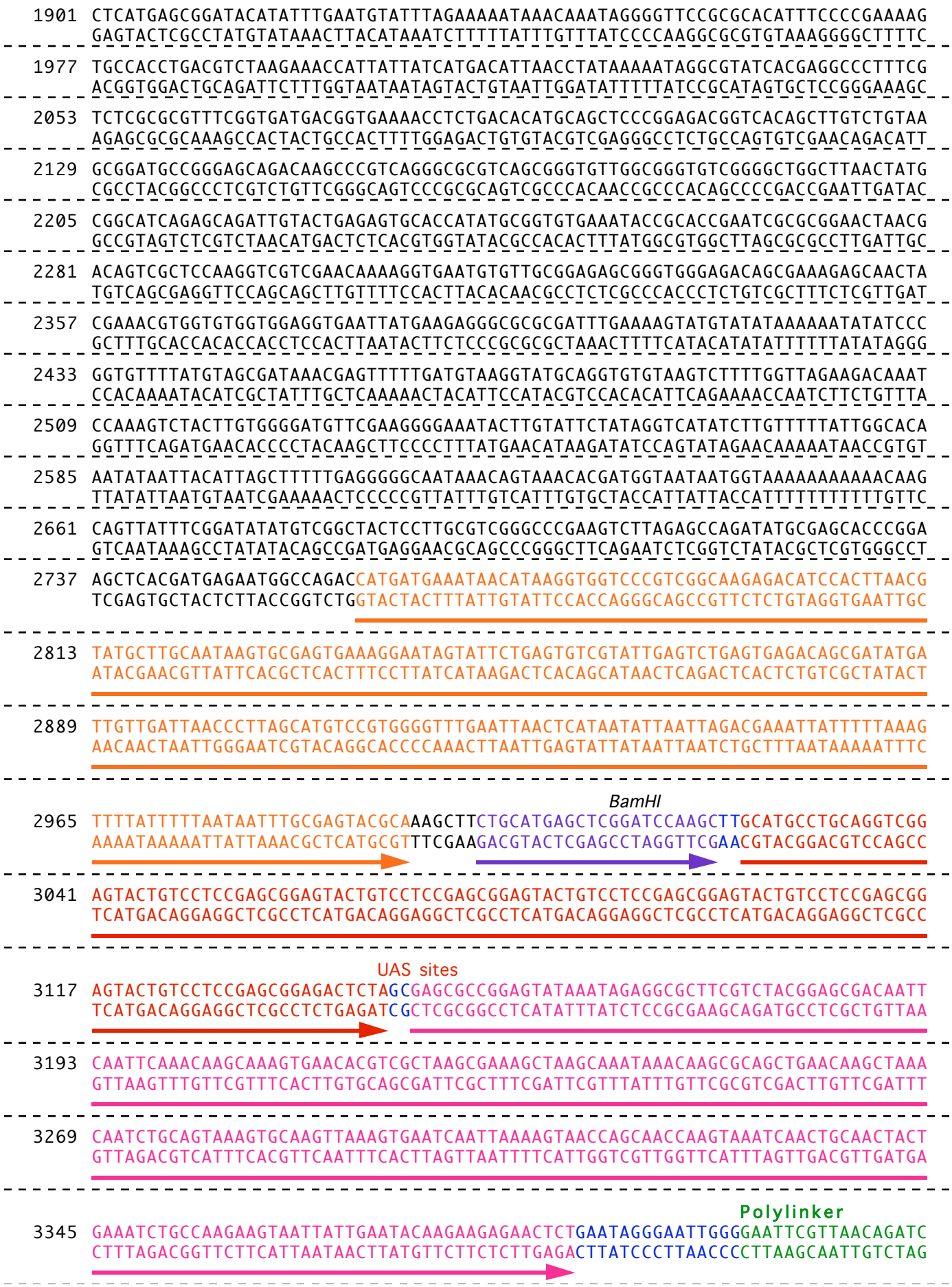
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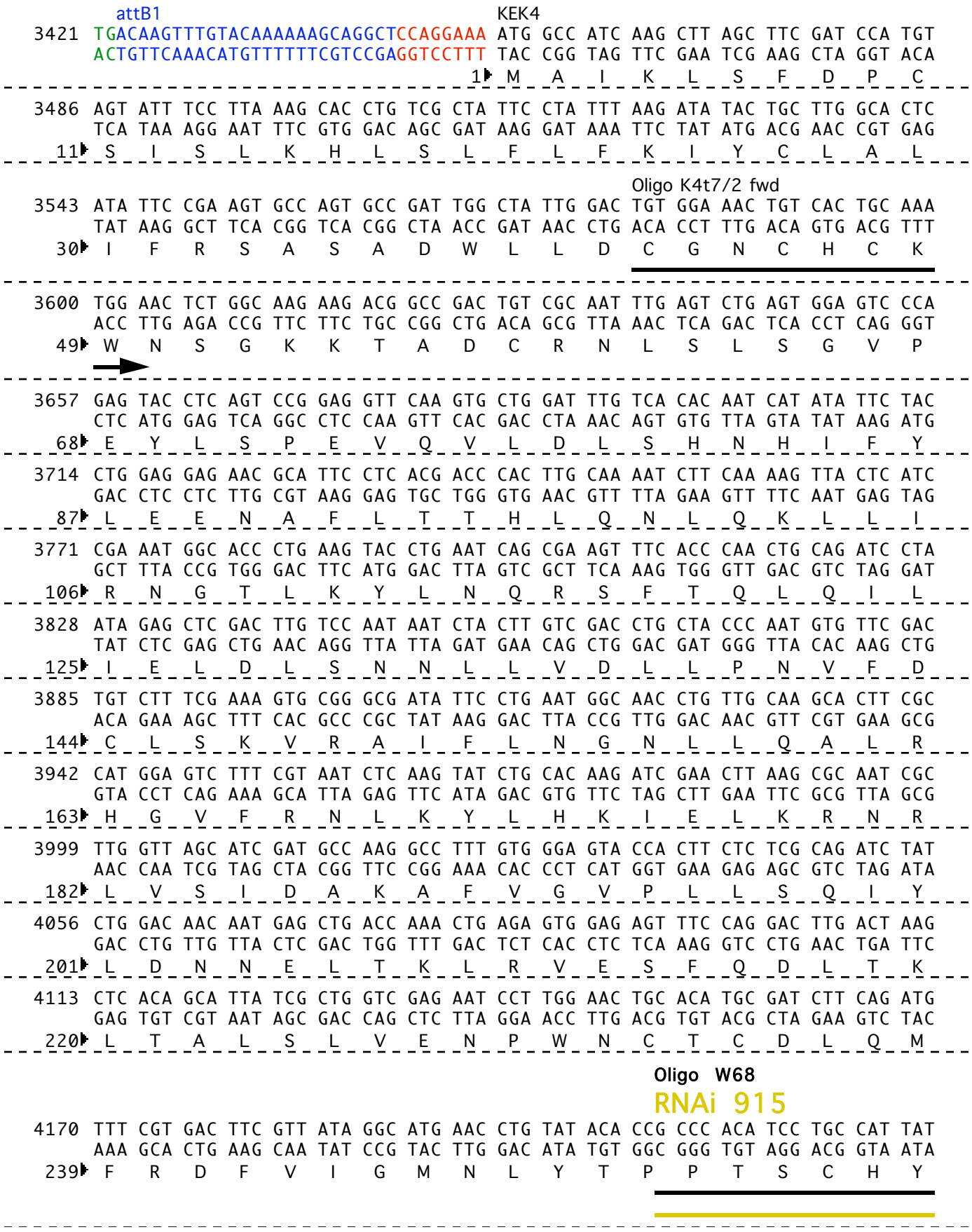
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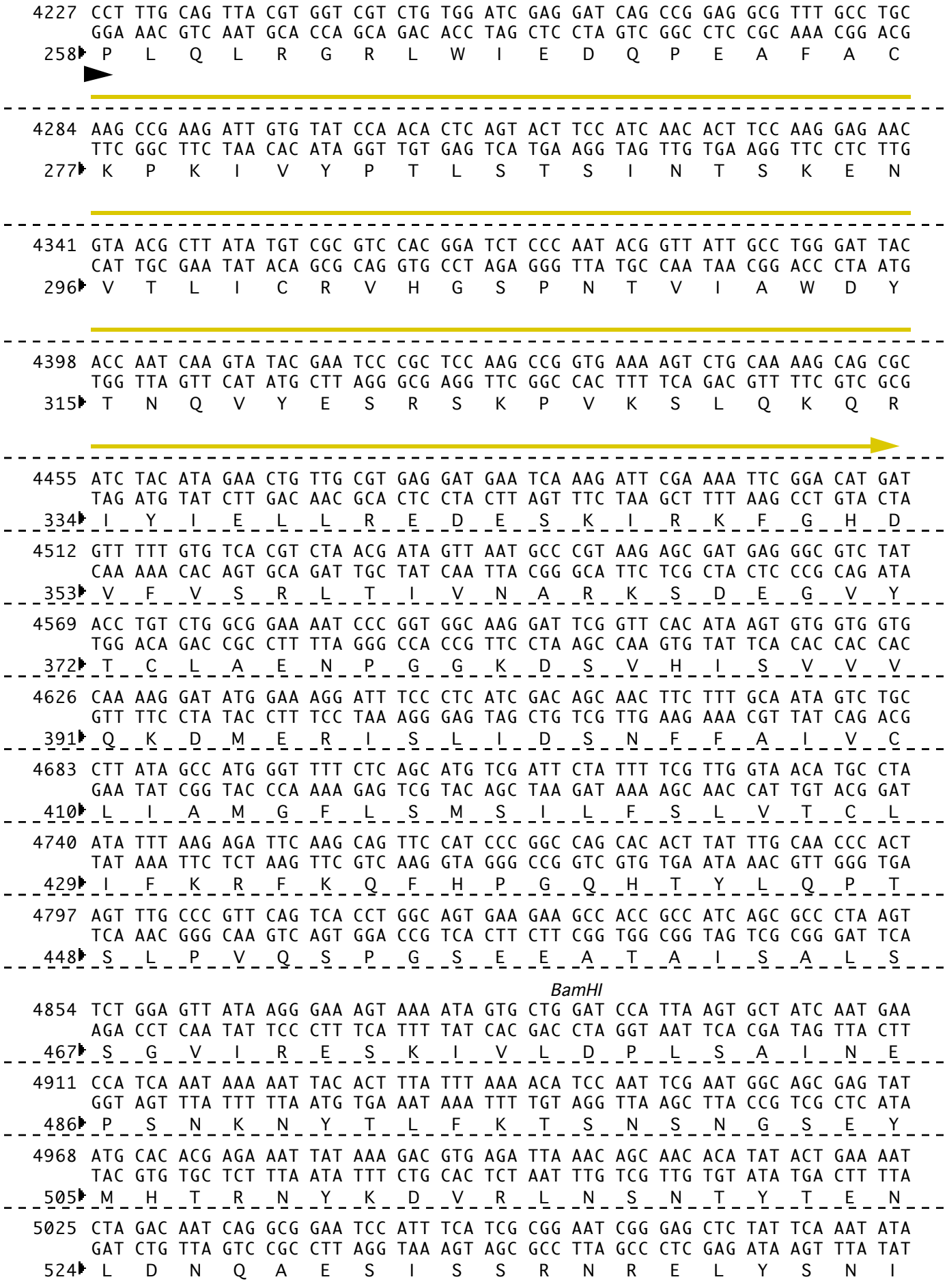
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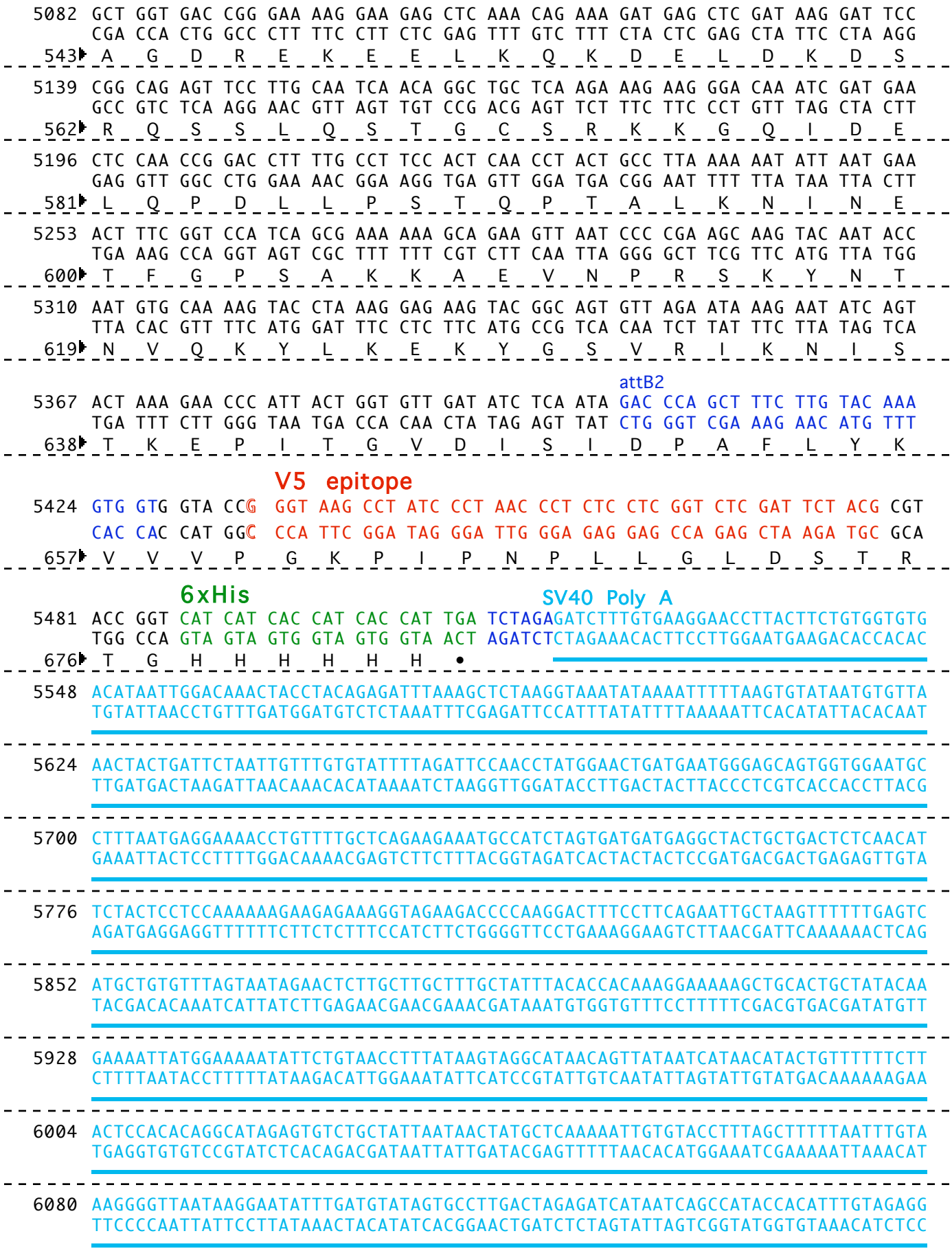
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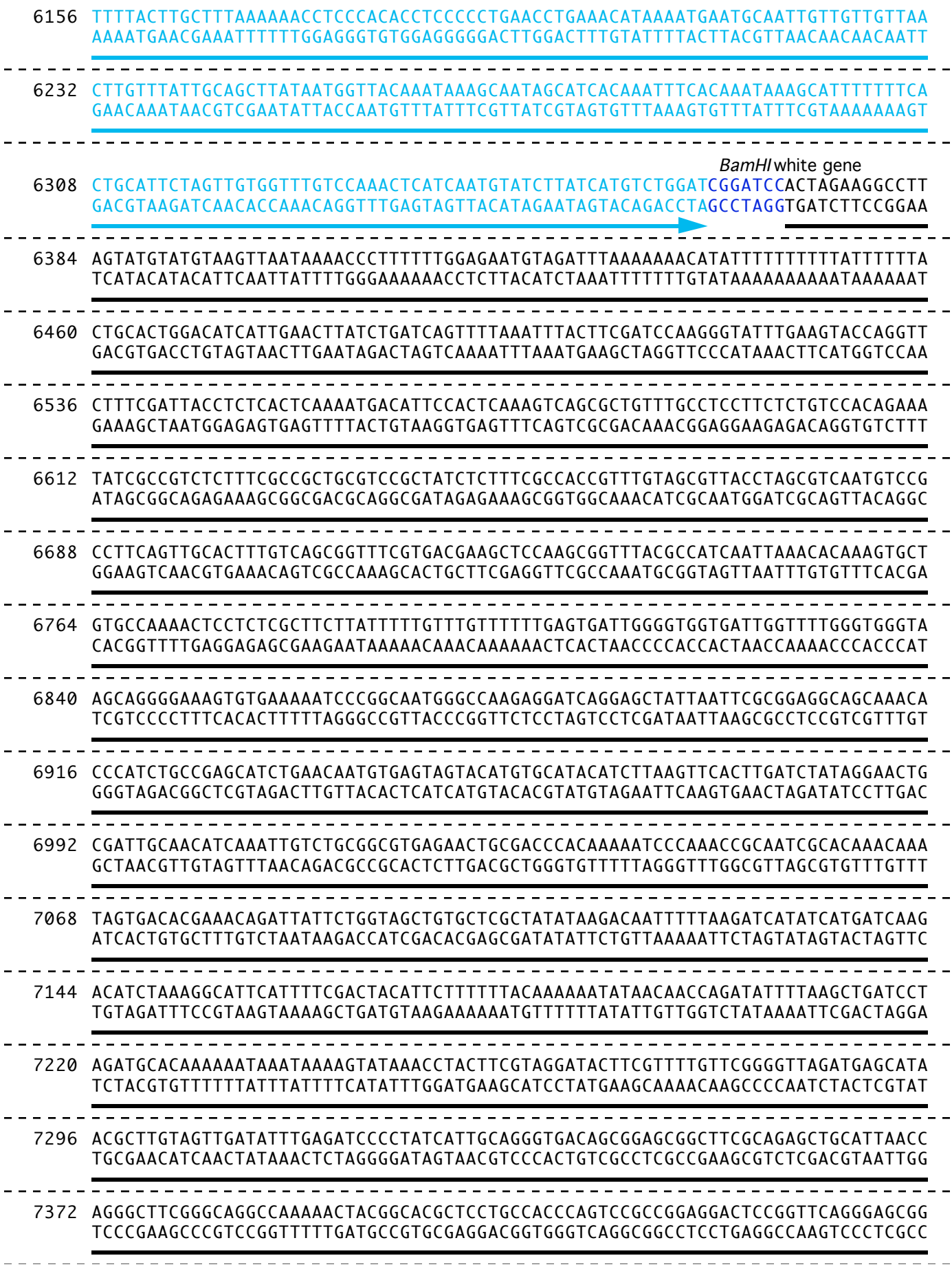
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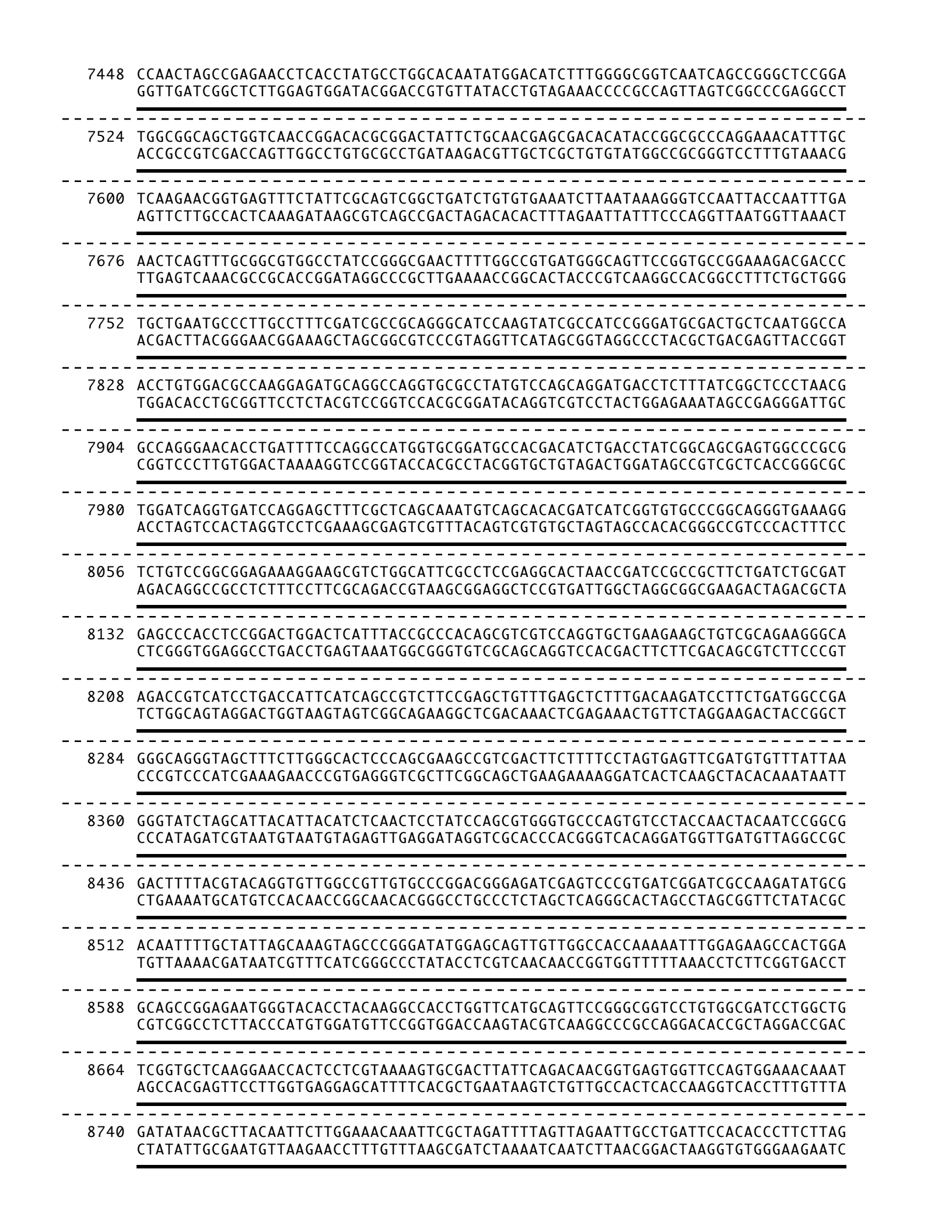
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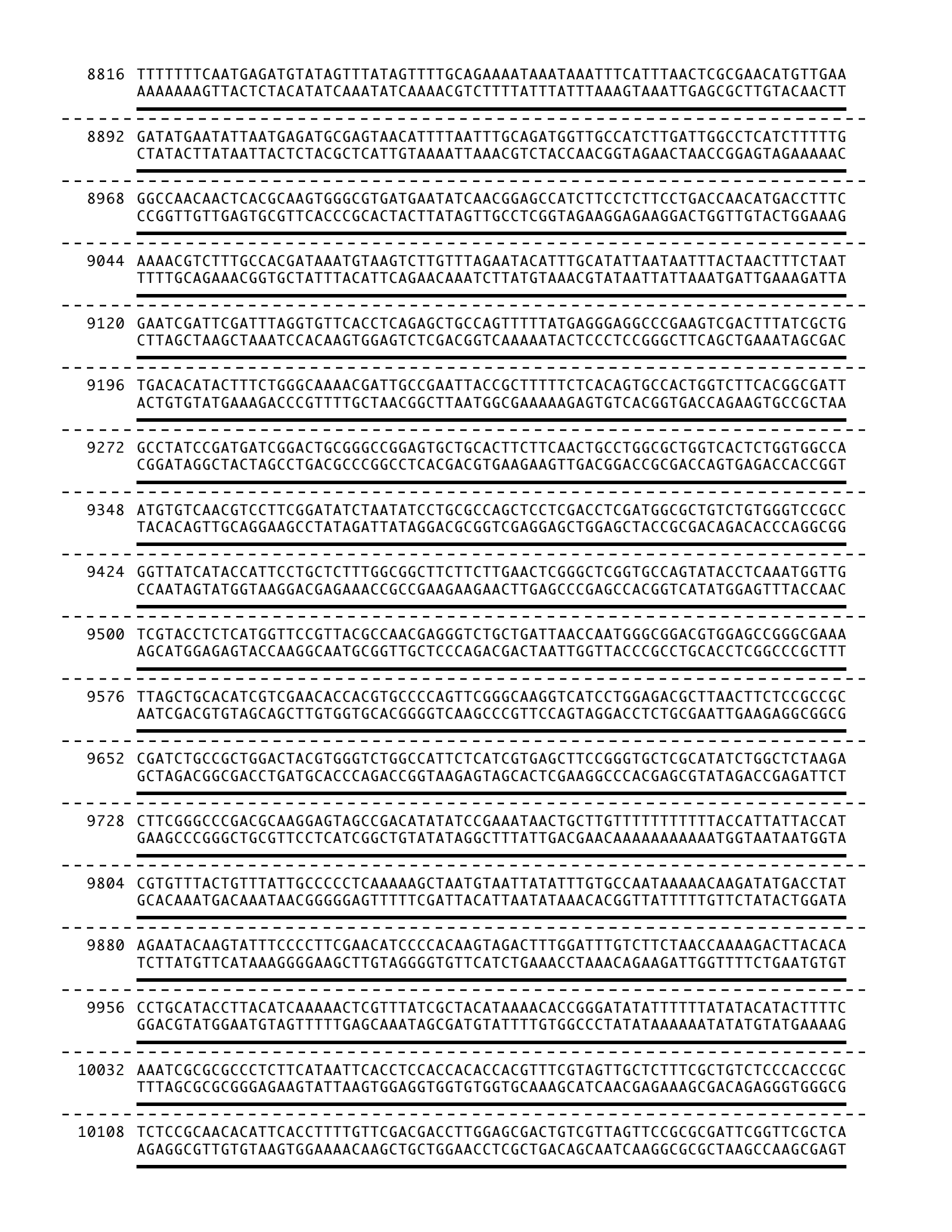
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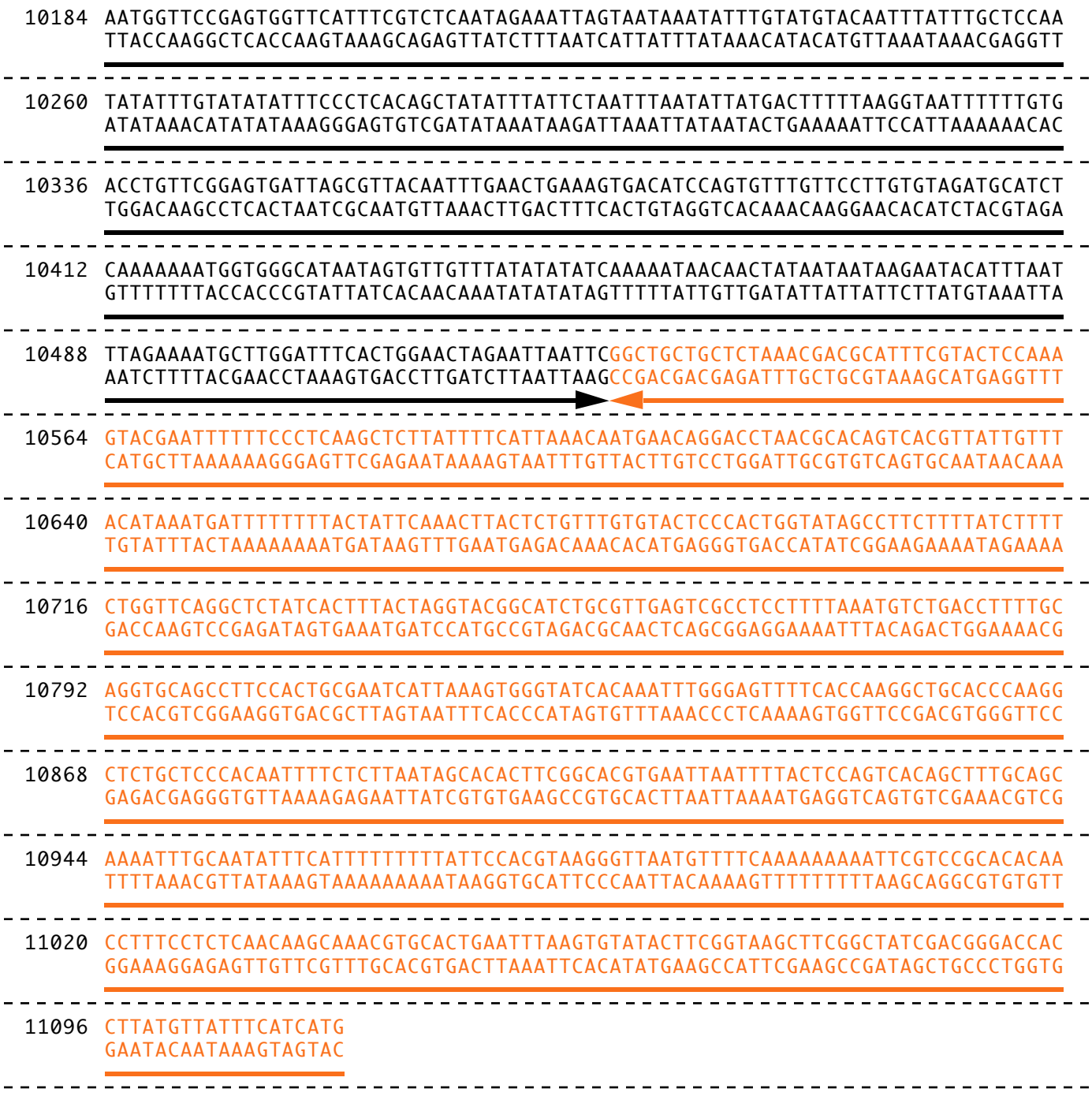
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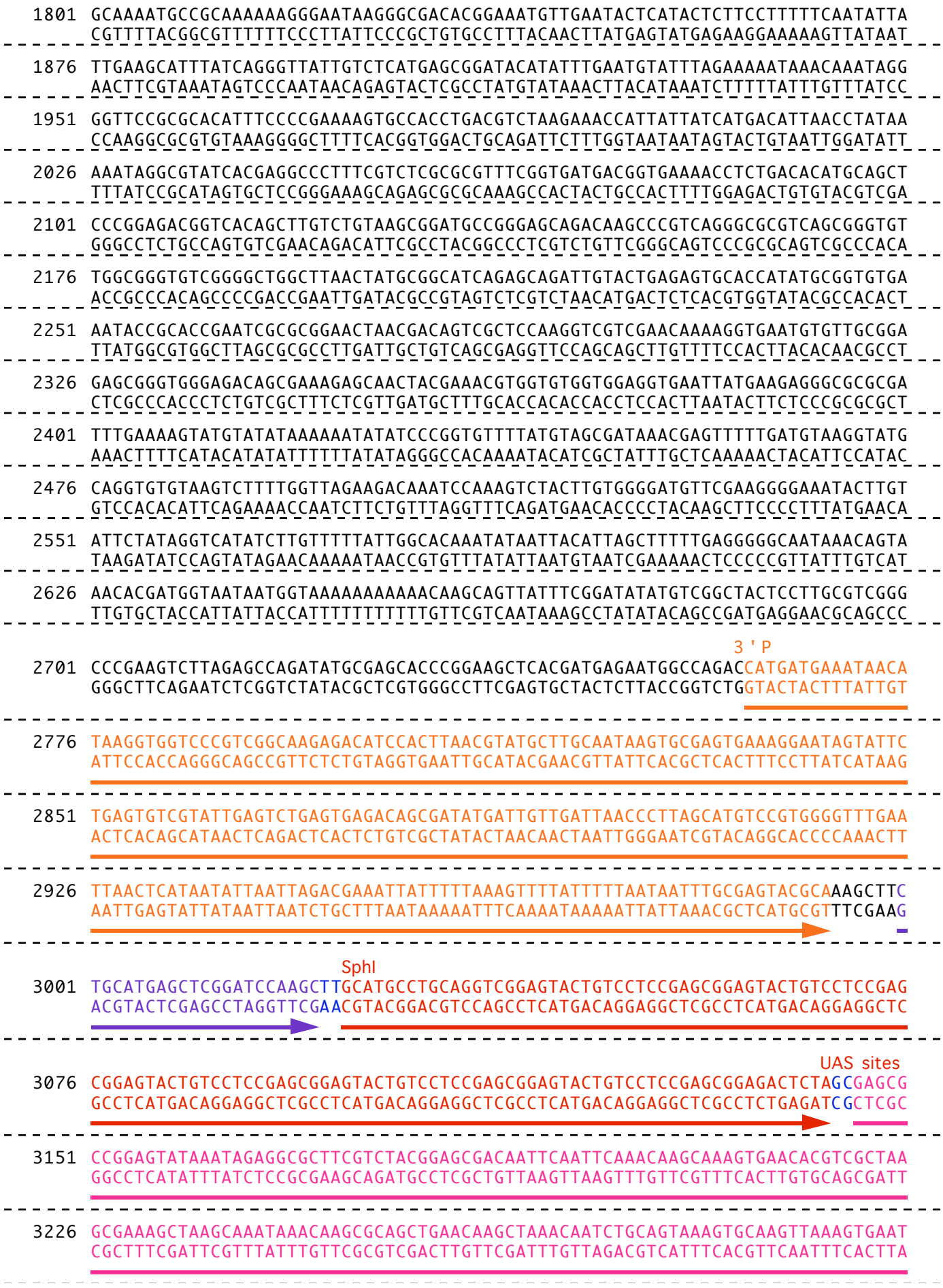
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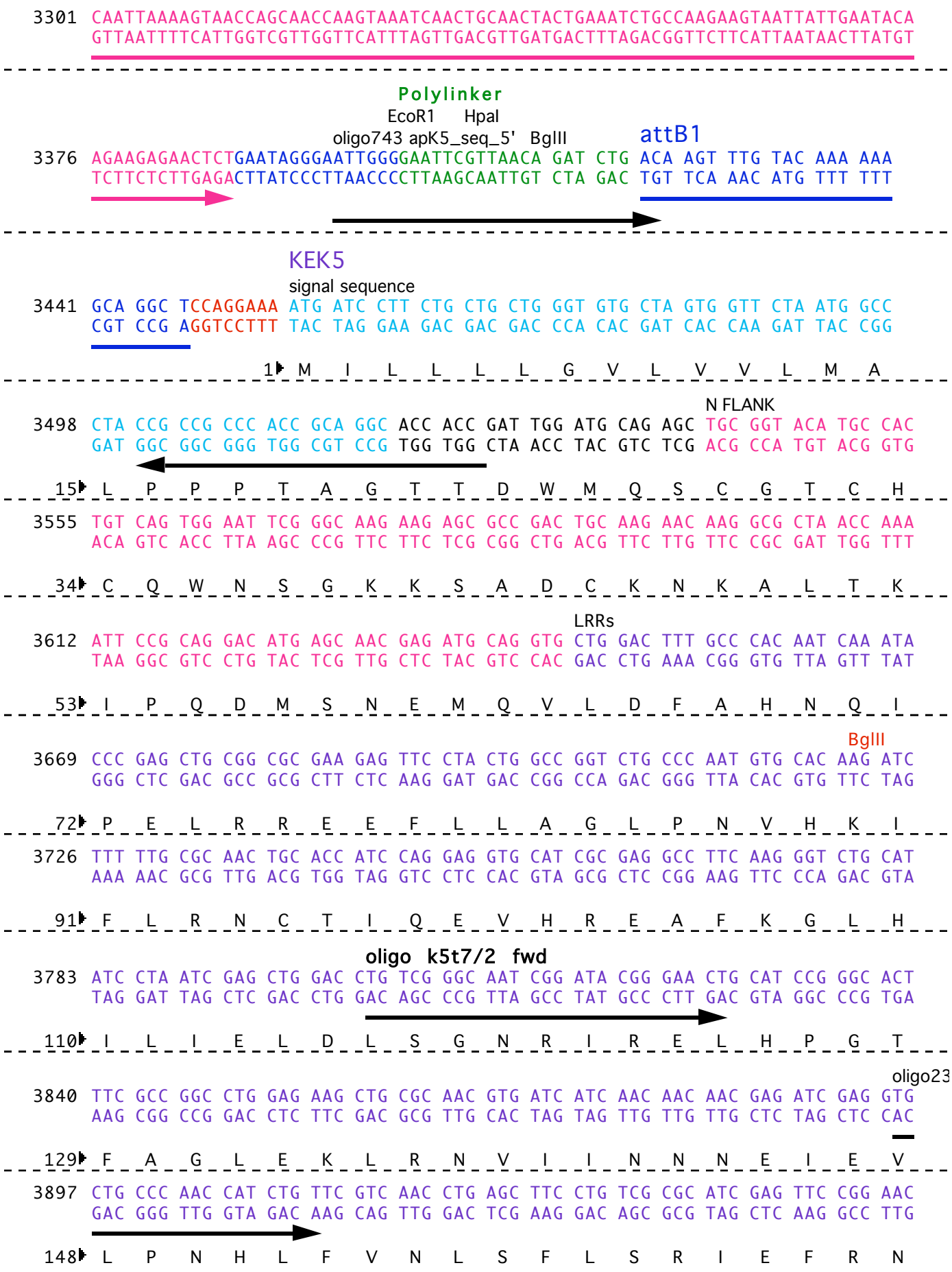
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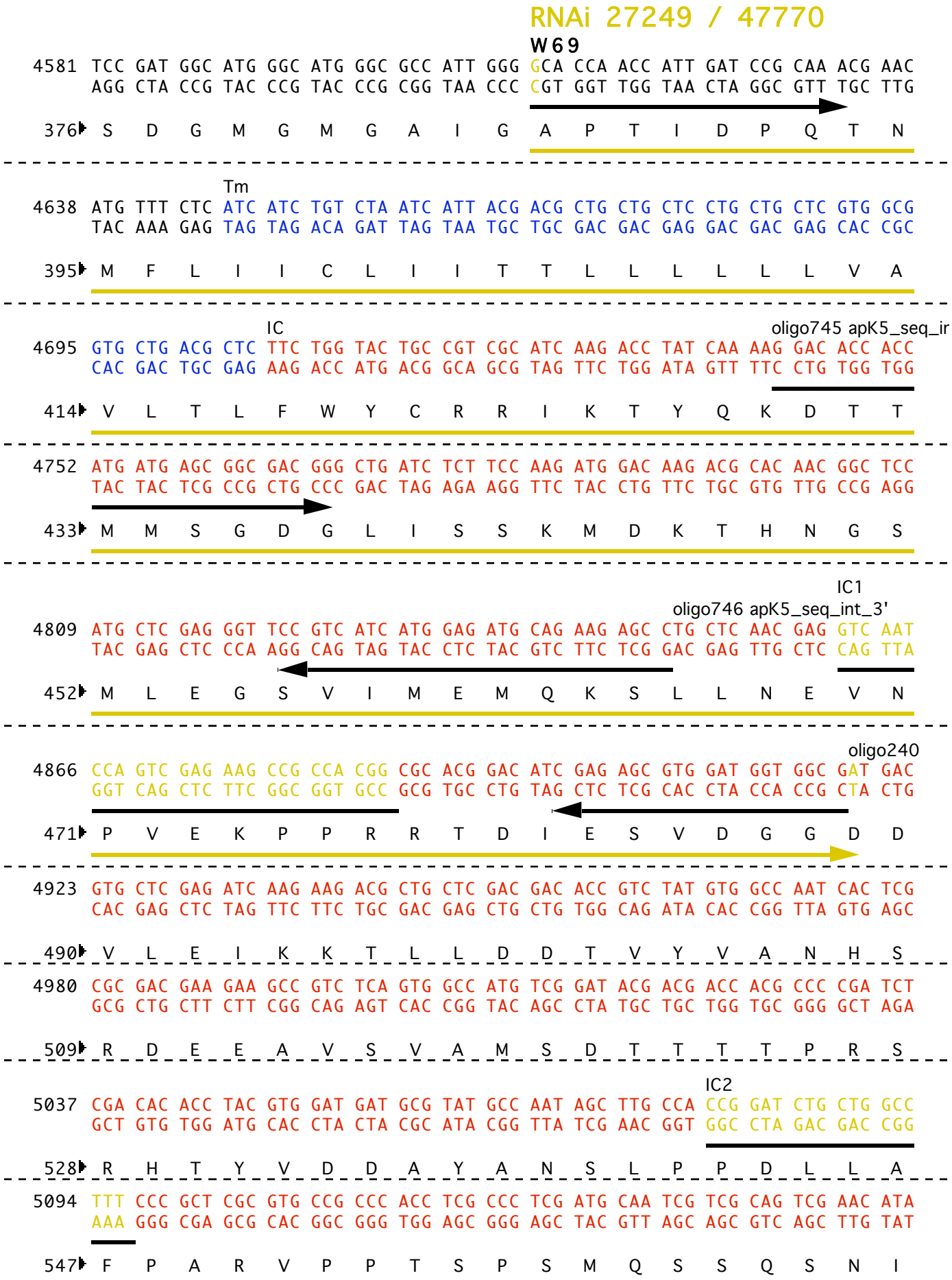
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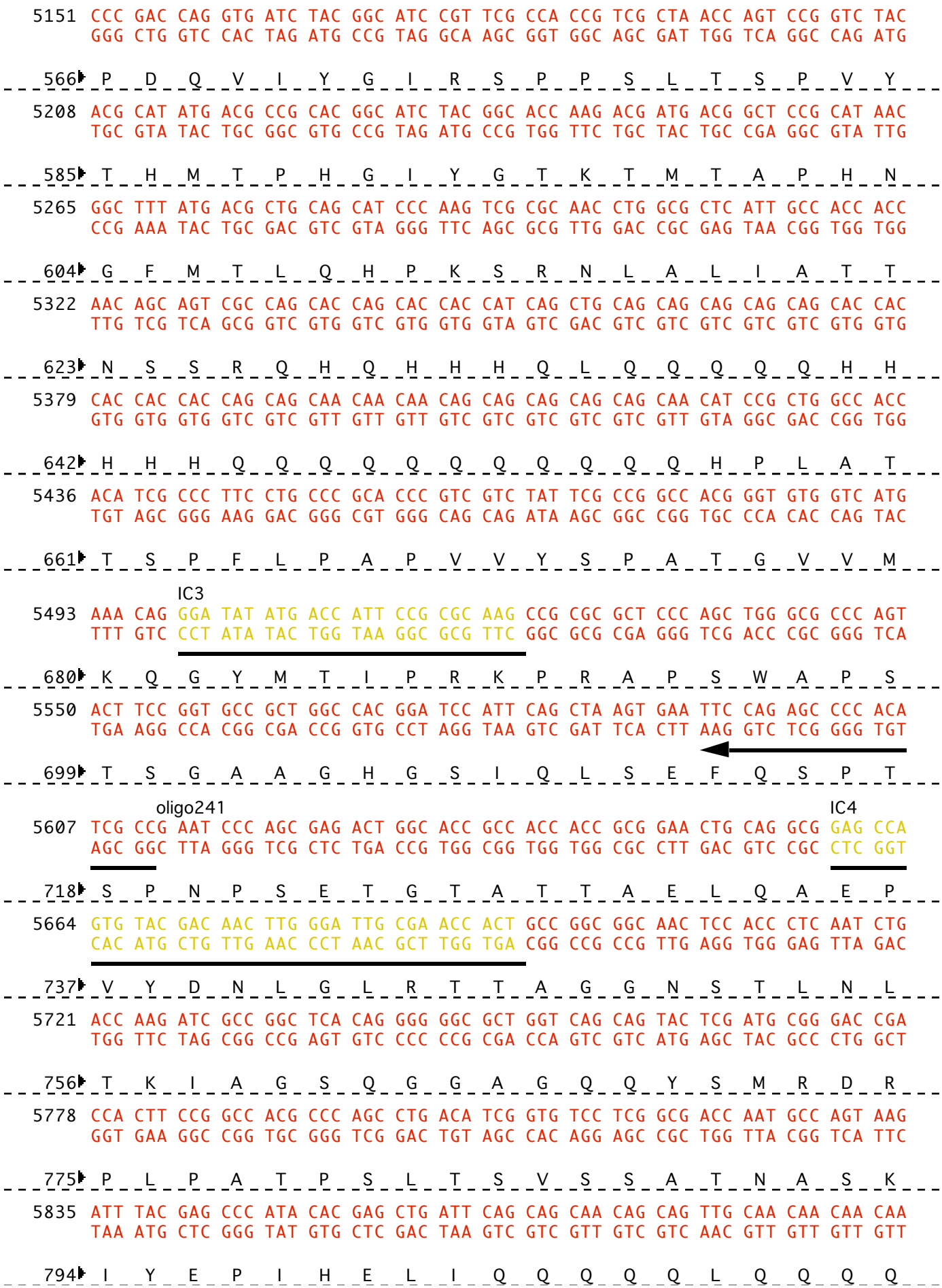
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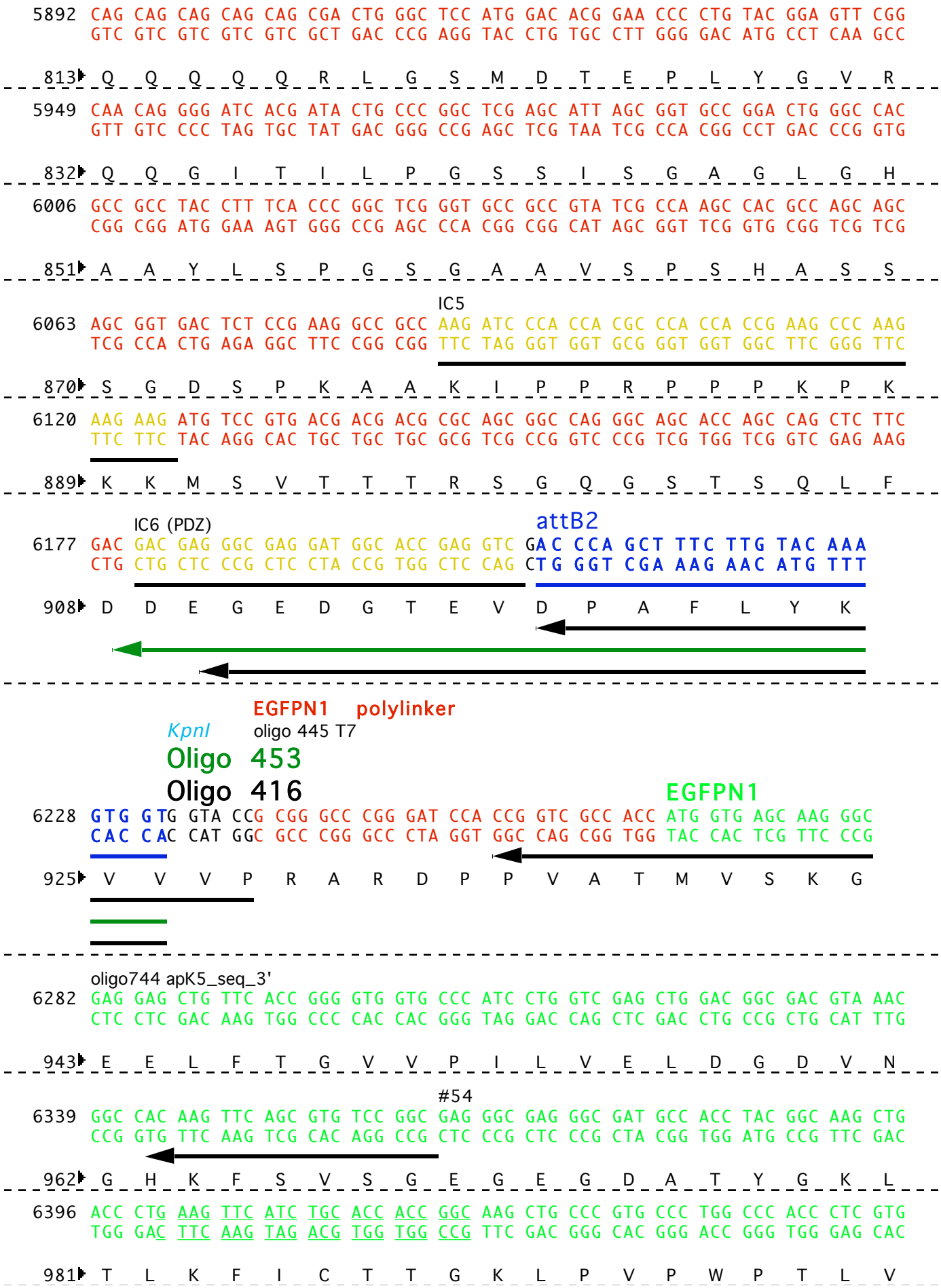
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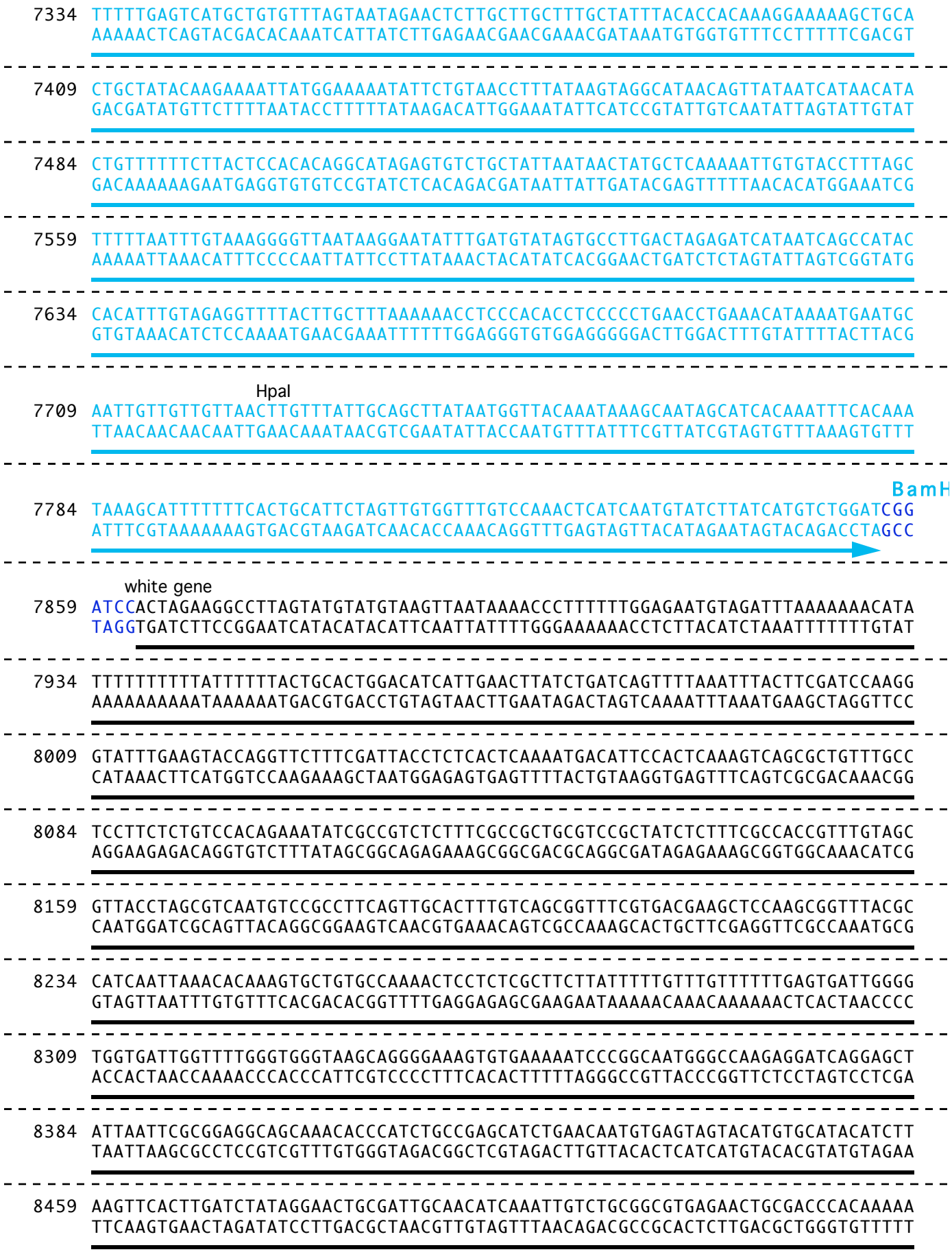
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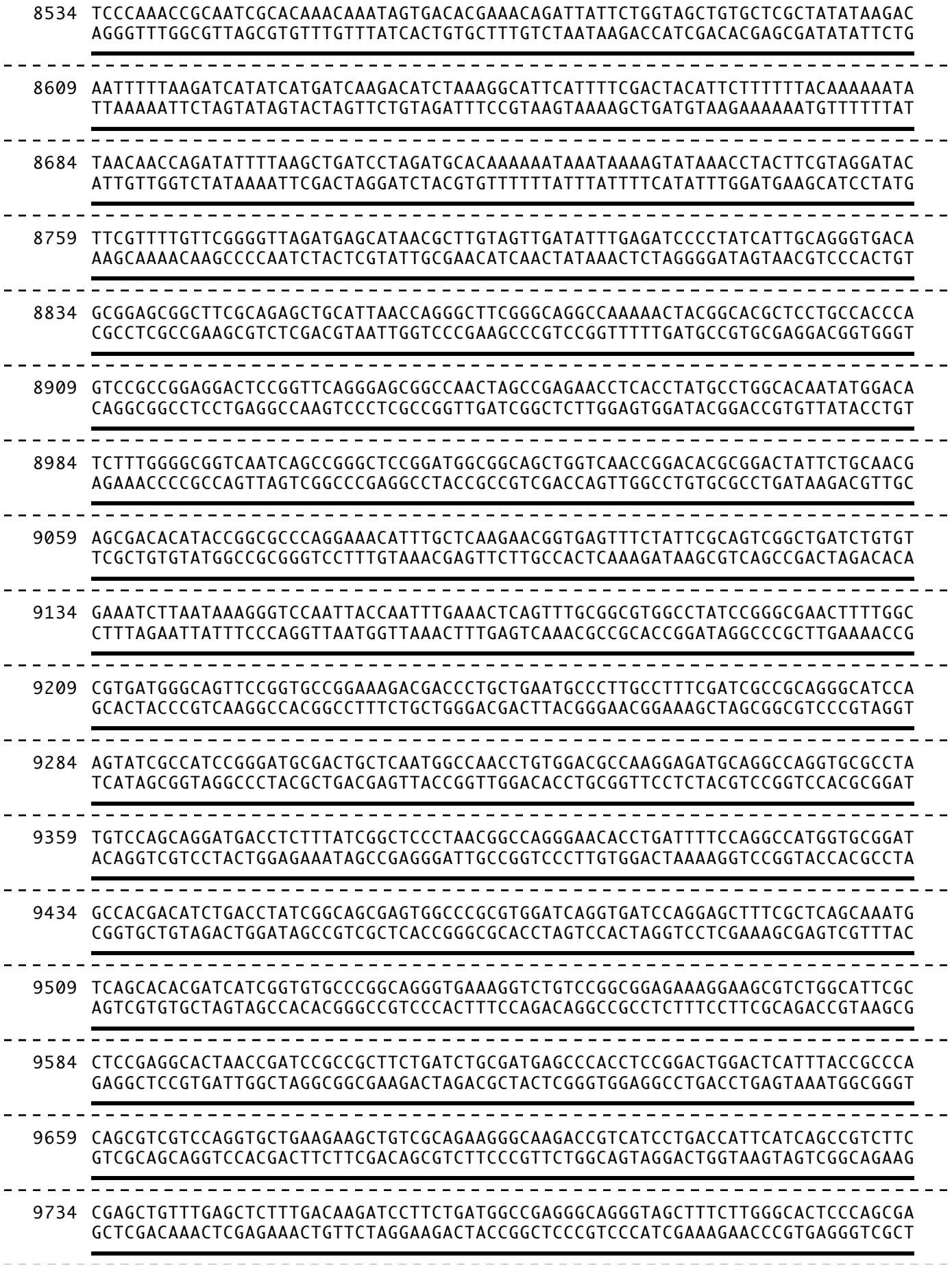
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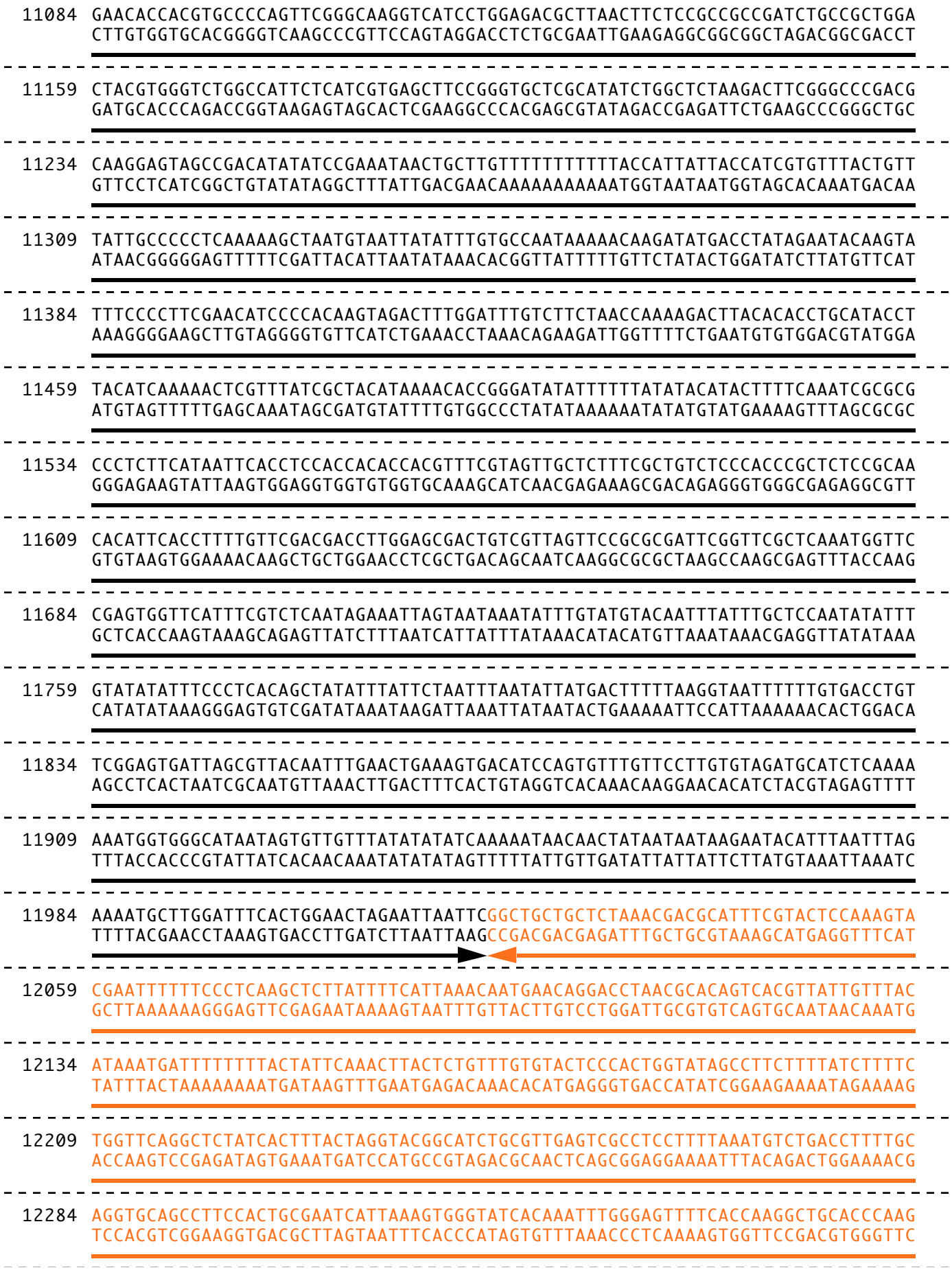
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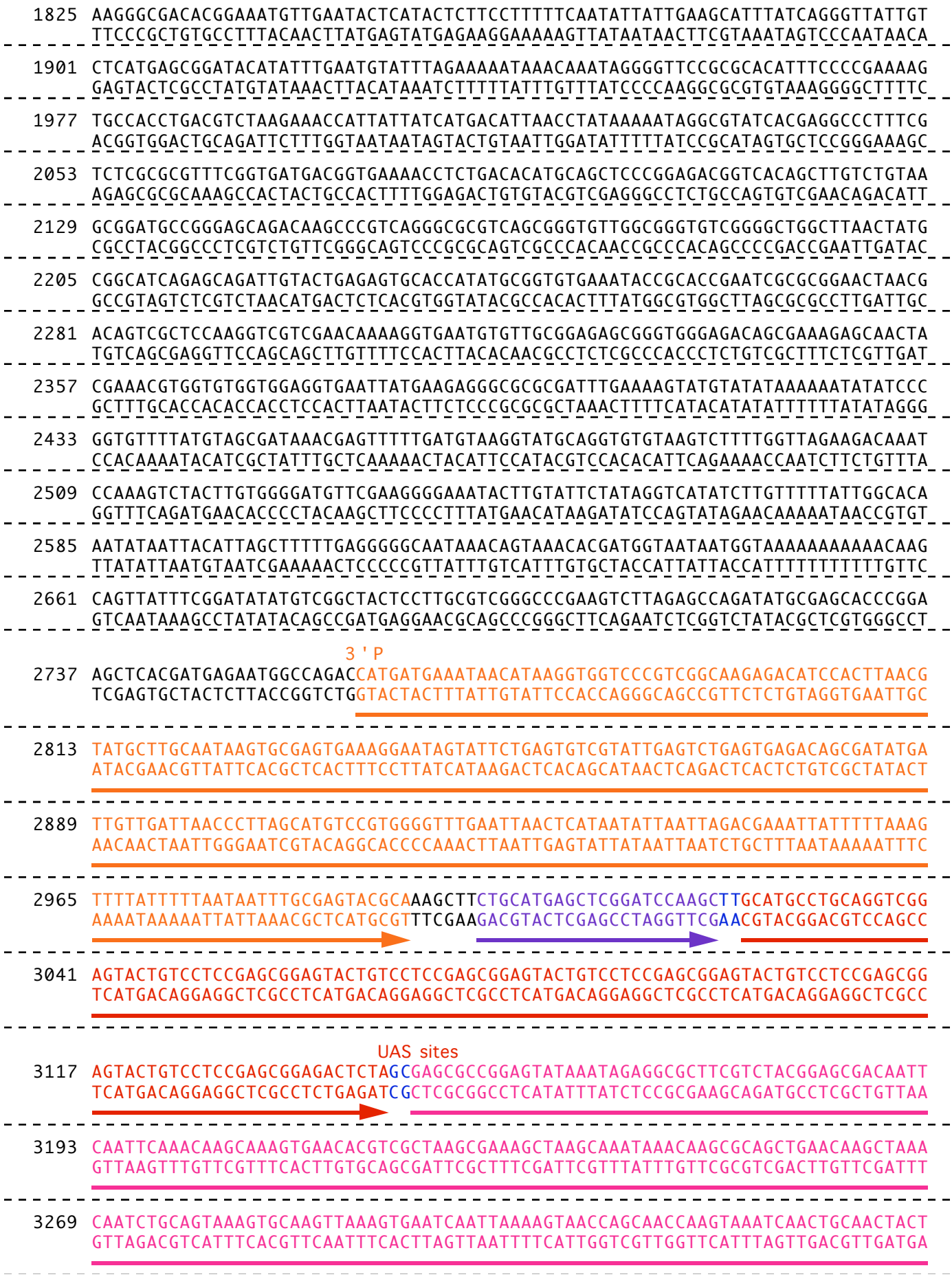
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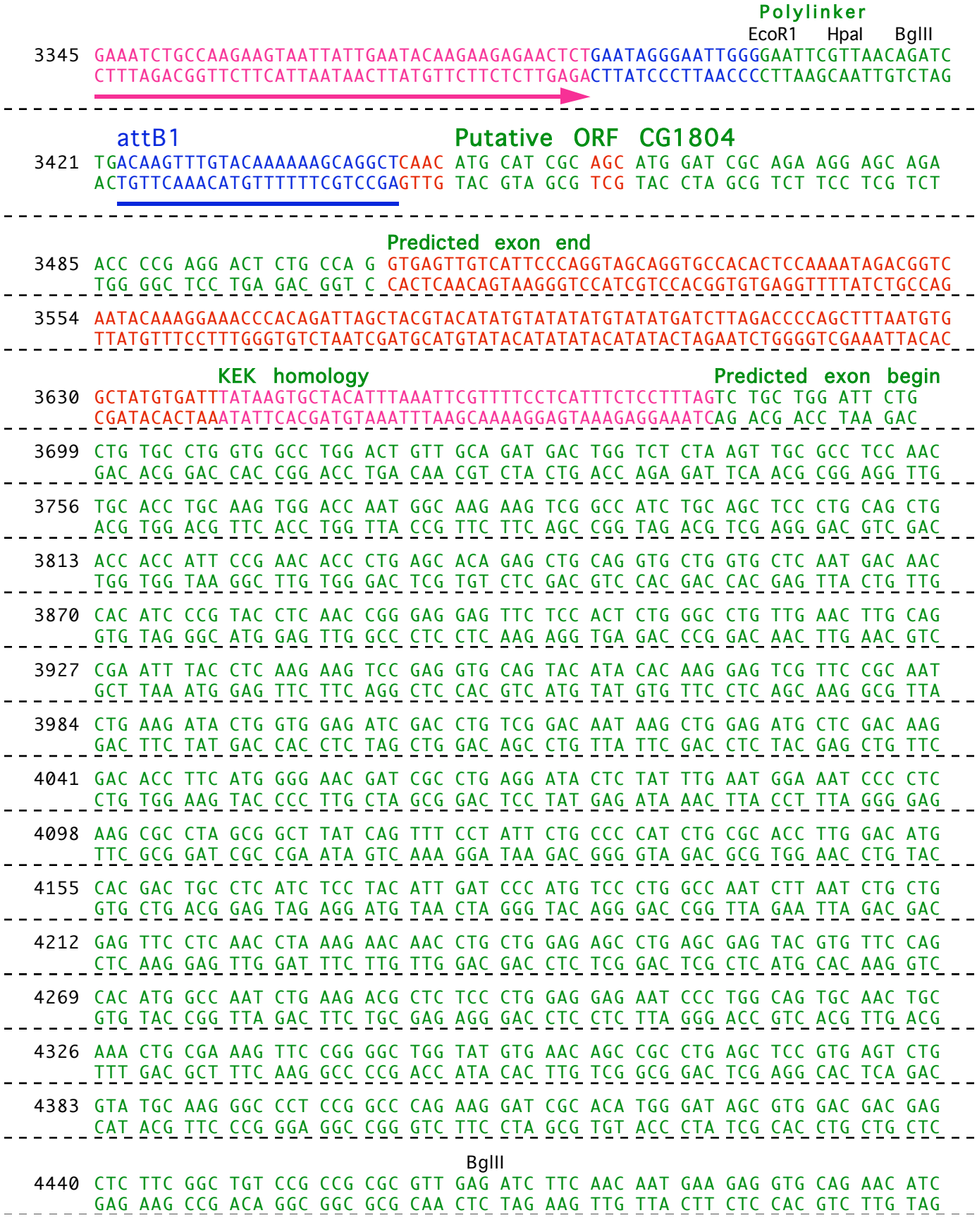
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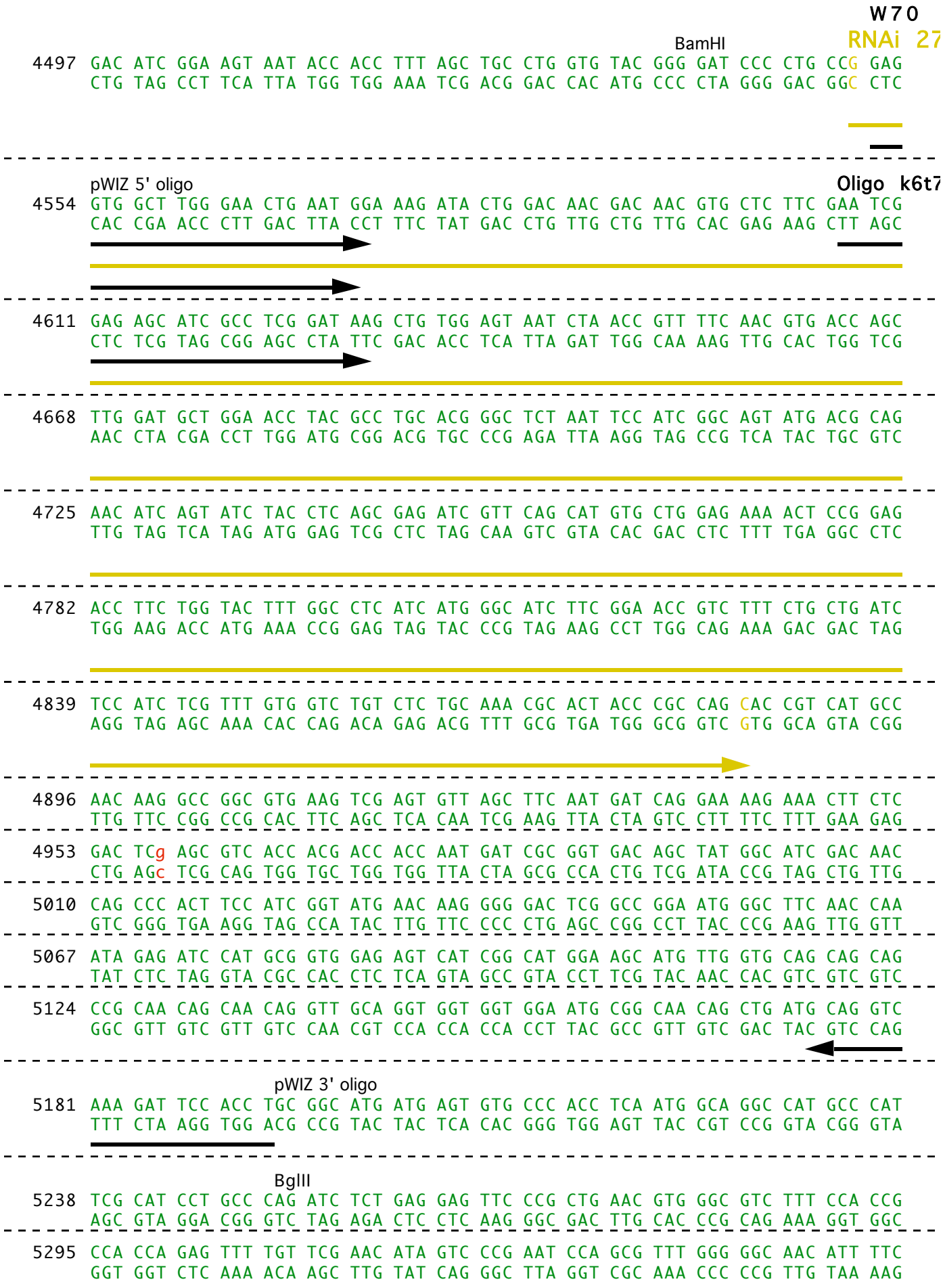
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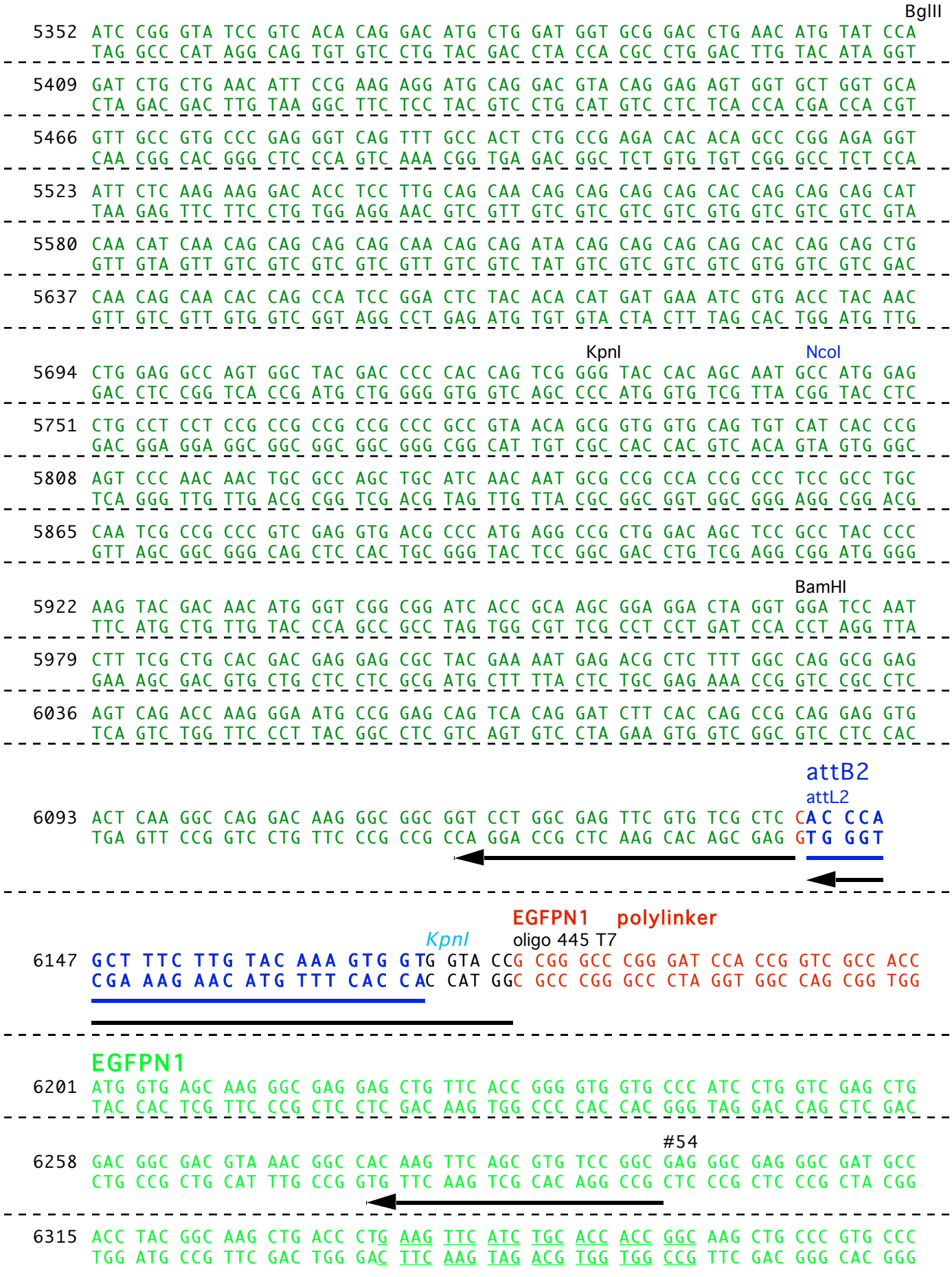
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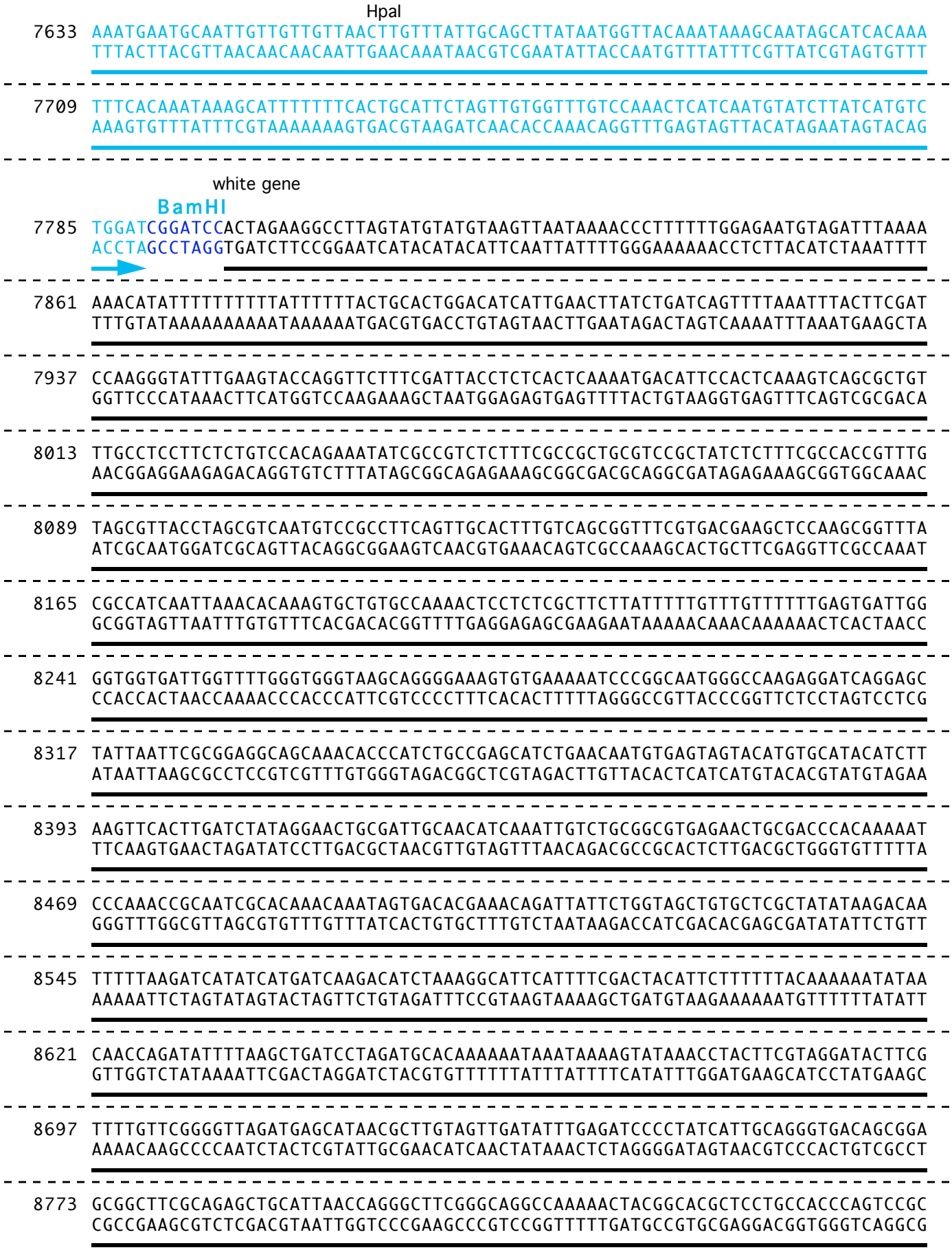
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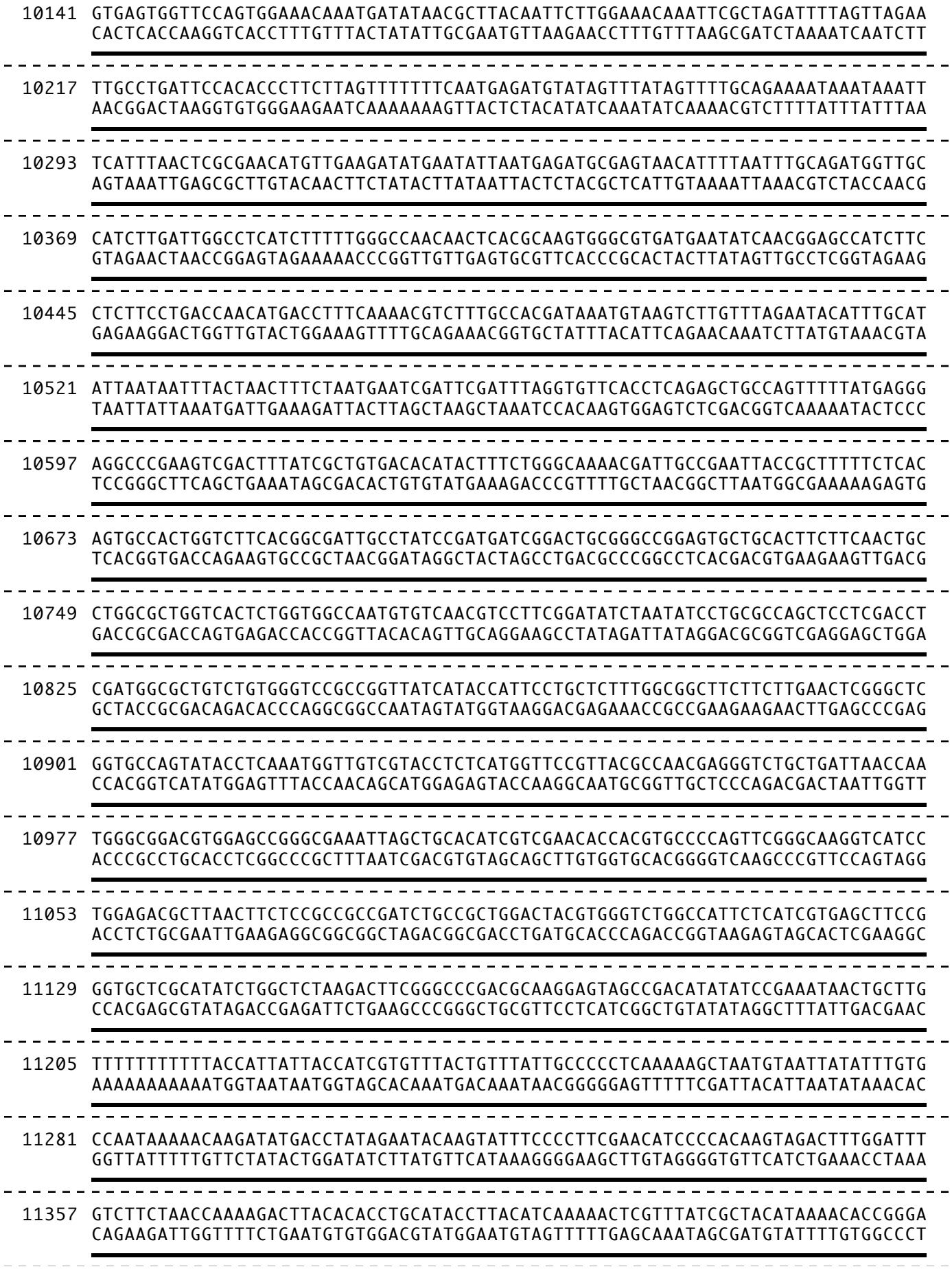
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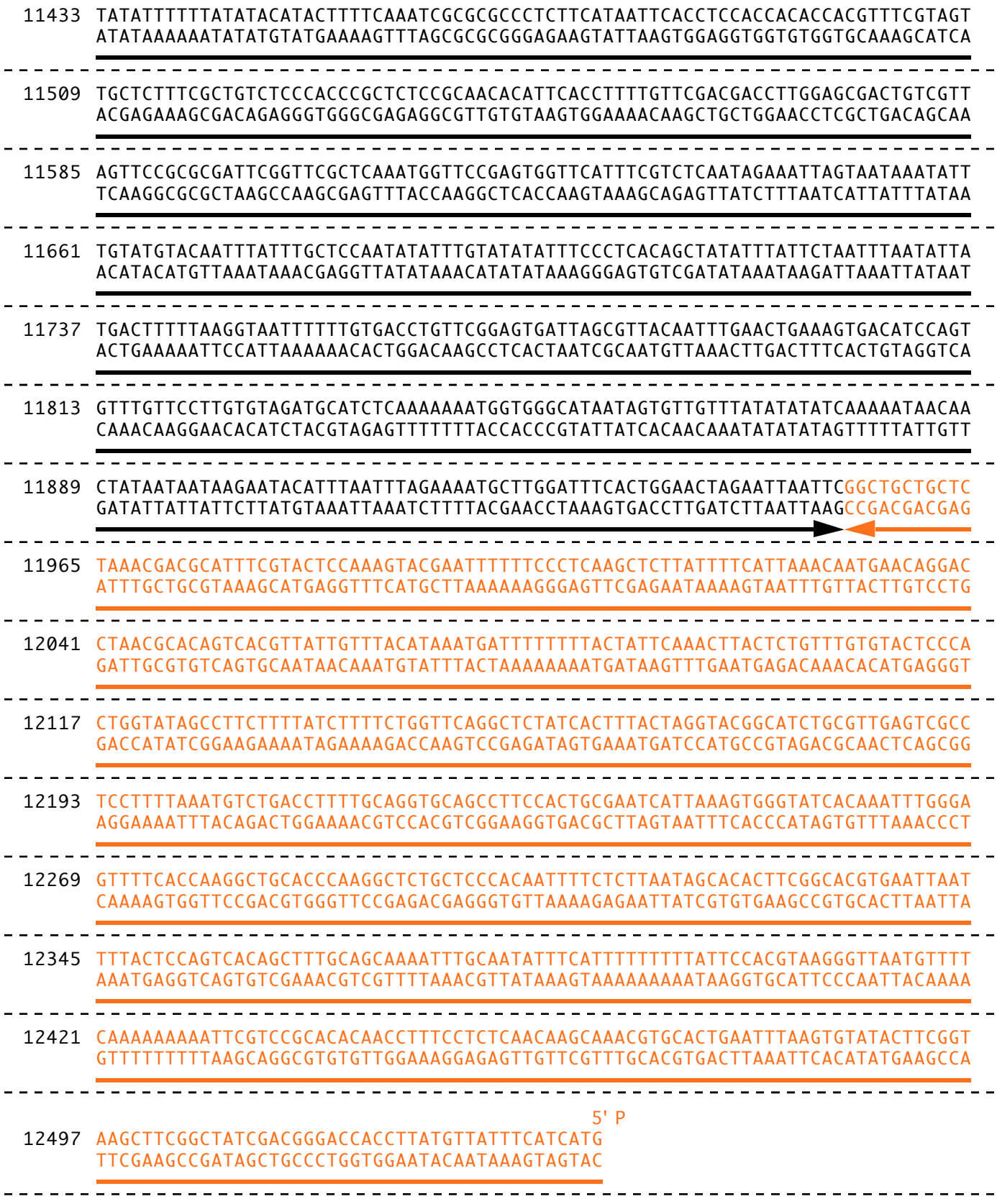
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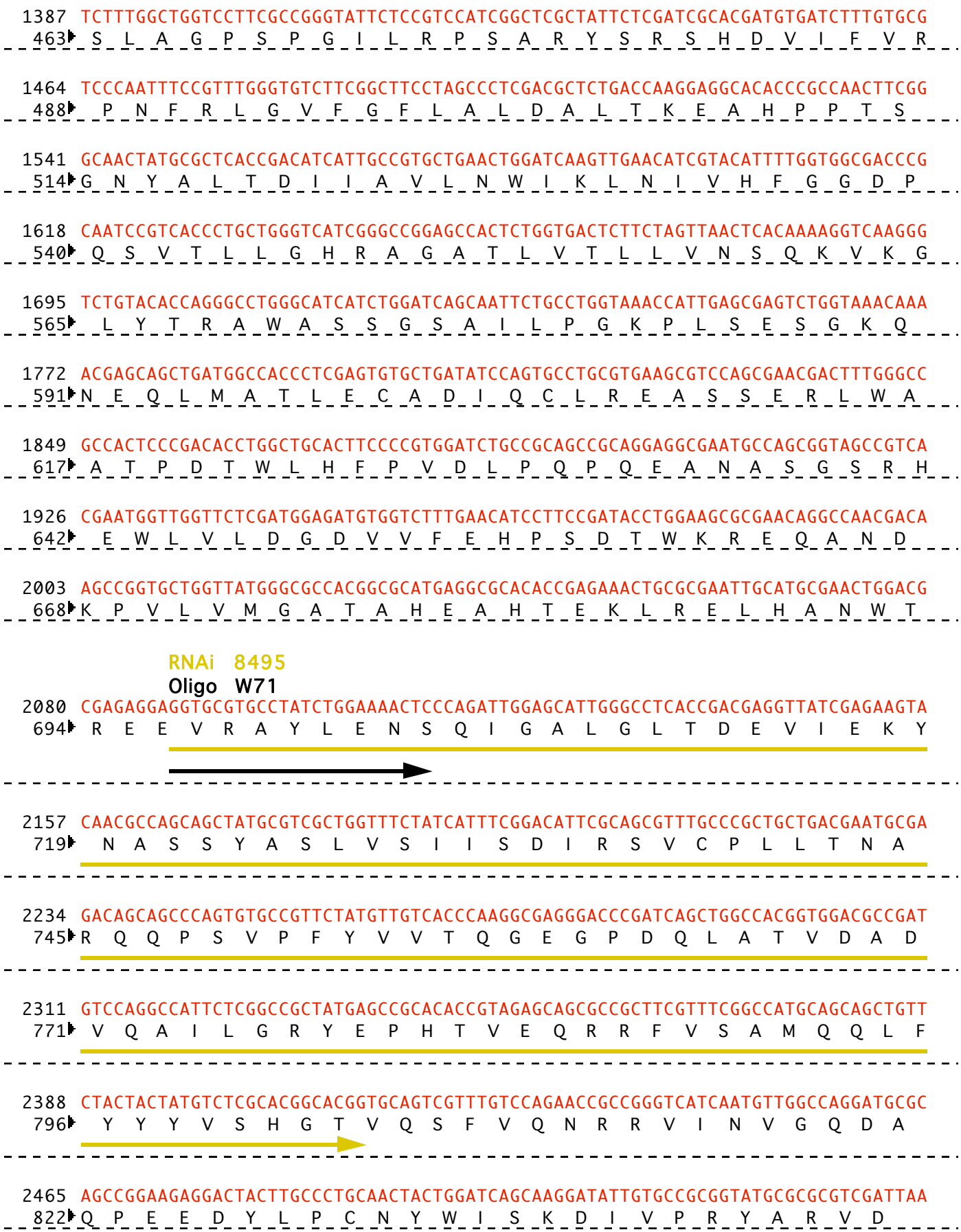
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2157 CAACGCCAGCAGCTATGCGTCGCTGGTTTCTATCATTTCGGACATTCGCAGCGTTTGCCCGCTGCTGACGAATGCGA
719 N A S S Y A S L V S I I S D I R S V C P L L T N A
2234 GACAGCAGCCCAGTGTGCCGTTCTATGTTGTCACCCAAGGCGAGGGACCCGATCAGCTGGCCACGGTGGACGCCGAT
745 R Q Q P S V P F Y V V T Q G E G P D Q L A T V D A D
2311 GTCCAGGCCATTCTCGGCCGCTATGAGCCGCACACCGTAGAGCAGCGCCGCTTCGTTTCGGCCATGCAGCAGCTGTT
771 V Q A I L G R Y E P H T V E Q R R F V S A M Q Q L F
2388 CTACTACTATGTCTCGCACGGCACGGTGCAGTCGTTTGTCCAGAACCGCCGGGTCATCAATGTTGGCCAGGATGCGC
796 Y Y Y V S H G T V Q S F V Q N R R V I N V G Q D A
2465 AGCCGGAAGAGGACTACTTGCCCTGCAACTACTGGATCAGCAAGGATATTGTGCCGCGGTATGCGCGCGTCGATTAA
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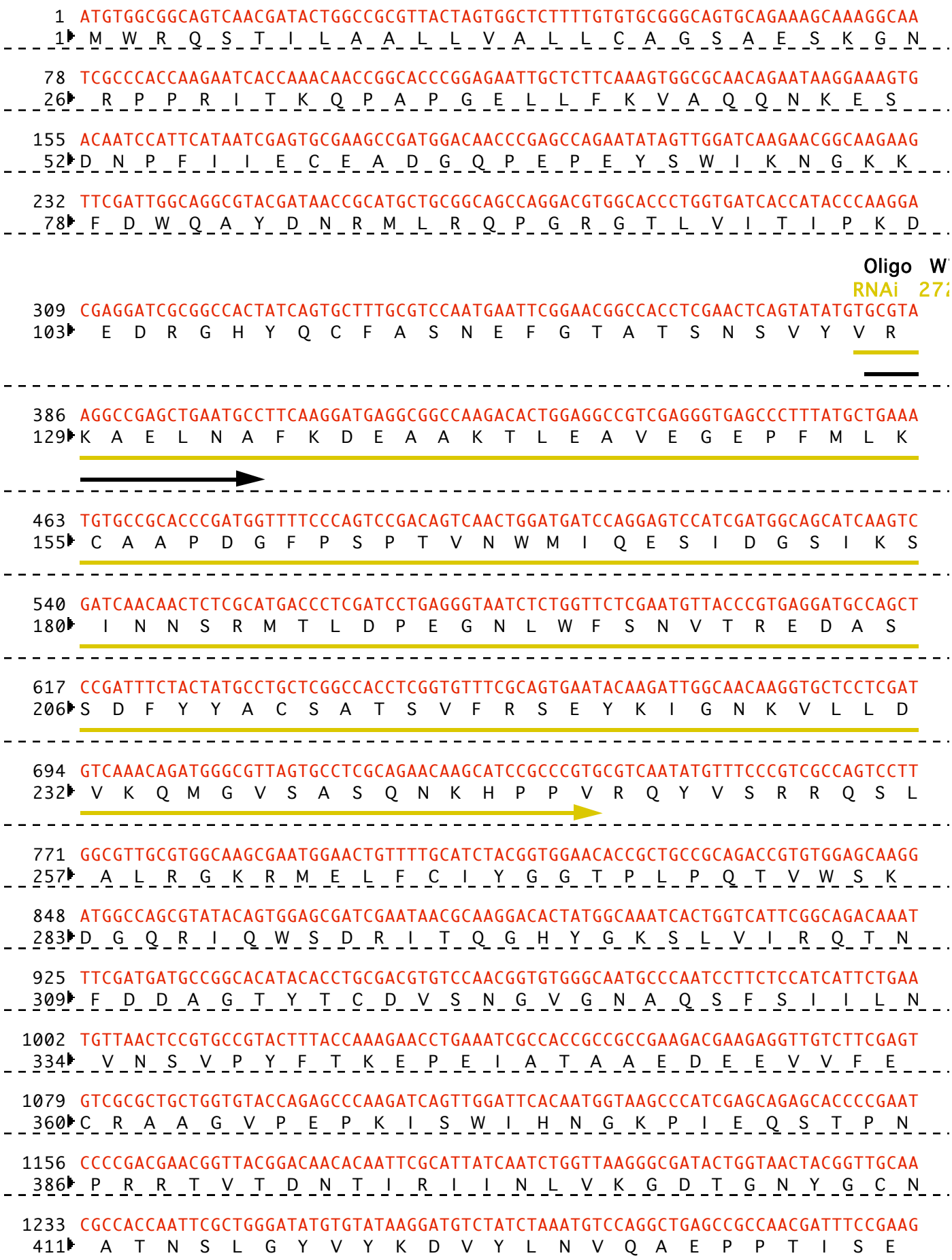
NRG ORF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1 M W R Q S T I L A A L L V A L L C A G S A E S K G N
78 TCGCCCACCAAGAATCACCAAACAACCGGCACCCGGAGAATTGCTCTTCAAAGTGGCGCAACAGAATAAGGAAAGTG
26 R P P R I T K Q P A P G E L L F K V A Q Q N K E S
155 ACAATCCATTCATAATCGAGTGCGAAGCCGATGGACAACCCGAGCCAGAATATAGTTGGATCAAGAACGGCAAGAAG
52 D N P F I I E C E A D G Q P E P E Y S W I K N G K K
232 TTCGATTGGCAGGCGTACGATAACCGCATGCTGCGGCAGCCAGGACGTGGCACCCTGGTGATCACCATACCCAAGGA
78 F D W Q A Y D N R M L R Q P G R G T L V I T I P K D
Oligo W
RNAi 27
309 CGAGGATCGCGGCCACTATCAGTGCTTTGCGTCCAATGAATTCGGAACGGCCACCTCGAACTCAGTATATGTGCGTA
103 E D R G H Y Q C F A S N E F G T A T S N S V Y V R
386 AGGCCGAGCTGAATGCCTTCAAGGATGAGGCGGCCAAGACACTGGAGGCCGTCGAGGGTGAGCCCTTTATGCTGAAA
129 K A E L N A F K D E A A K T L E A V E G E P F M L K
463 TGTGCCGCACCCGATGGTTTTCCCAGTCCGACAGTCAACTGGATGATCCAGGAGTCCATCGATGGCAGCATCAAGTC
155 C A A P D G F P S P T V N W M I Q E S I D G S I K S
540 GATCAACAACTCTCGCATGACCCTCGATCCTGAGGGTAATCTCTGGTTCTCGAATGTTACCCGTGAGGATGCCAGCT
180 I N N S R M T L D P E G N L W F S N V T R E D A S
617 CCGATTTCTACTATGCCTGCTCGGCCACCTCGGTGTTTCGCAGTGAATACAAGATTGGCAACAAGGTGCTCCTCGAT
206 S D F Y Y A C S A T S V F R S E Y K I G N K V L L D
694 GTCAAACAGATGGGCGTTAGTGCCTCGCAGAACAAGCATCCGCCCGTGCGTCAATATGTTTCCCGTCGCCAGTCCTT
232 V K Q M G V S A S Q N K H P P V R Q Y V S R R Q S L
771 GGCGTTGCGTGGCAAGCGAATGGAACTGTTTTGCATCTACGGTGGAACACCGCTGCCGCAGACCGTGTGGAGCAAGG
257 A L R G K R M E L F C I Y G G T P L P Q T V W S K
848 ATGGCCAGCGTATACAGTGGAGCGATCGAATAACGCAAGGACACTATGGCAAATCACTGGTCATTCGGCAGACAAAT
283 D G Q R I Q W S D R I T Q G H Y G K S L V I R Q T N
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309 F D D A G T Y T C D V S N G V G N A Q S F S I I L N
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334 V N S V P Y F T K E P E I A T A A E D E E V V F E
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360 C R A A G V P E P K I S W I H N G K P I E Q S T P N
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386 P R R T V T D N T I R I I N L V K G D T G N Y G C N
1233 CGCCACCAATTCGCTGGGATATGTGTATAAGGATGTCTATCTAAATGTCCAGGCTGAGCCGCCAACGATTTCCGAAG
411 A T N S L G Y V Y K D V Y L N V Q A E P P T I S E

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1387 GTTAAATGGCTAAGGGCCAGCAACTGGCTGACCGGAGGTCGTTACAATGTCCAAGCTAACGGTGACCTGGAGATCCA
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1464 AGATGTGACATTCTCGGATGCCGGCAAATACACATGCTATGCGCAGAACAAGTTTGGTGAAATTCAAGCCGATGGTT
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1541 CGCTGGTGGTCAAGGAGCATACGAGAATTACCCAAGAGCCGCAAAACTACGAGGTGGCCGCCGGACAATCGGCCACG
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1618 TTCCGCTGTAACGAGGCCCACGACGATACGCTGGAGATTGAGATCGATTGGTGGAAGGATGGCCAGTCCATTGACTT
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1695 TGAGGCCCAGCCGCGATTCGTGAAGACCAATGATAATTCCCTGACGATTGCCAAGACAATGGAGTTGGATTCTGGCG
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1772 AATATACGTGCGTGGCCCGGACGCGTTTGGATGAGGCAACGGCCAGGGCGAATTTGATTGTCCAGGATGTGCCGAAT
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1849 GCACCAAAACTGACCGGCATCACCTGCCAGGCCGACAAGGCCGAGATCCACTGGGAACAGCAGGGTGACAATCGTTC
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1926 GCCCATTCTGCACTACACCATTCAGTTCAATACATCGTTCACGCCCGCCTCCTGGGATGCCGCCTACGAGAAGGTGC
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2003 CCAACACGGACTCCTCGTTCGTCGTCCAGATGTCACCGTGGGCCAACTATACGTTCCGTGTGATTGCCTTCAACAAG
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2157 TGTCGTTGGCCAGGGCACTGAGCCCAACAATCTGGTCATCTCGTGGACTCCCATGCCCGAAATCGAGCACAATGCCC
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2234 CCAATTTCCATTATTATGTTAGCTGGAAACGCGATATTCCTGCCGCTGCGTGGGAAAACAATAACATATTCGACTGG
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2388 GGGTGAGTCCAATGTGGCCGCCGAGGAGGTGGTTGGCTACTCTGGCGAAGATCGTCCCCTGGATGCGCCCACCAACT
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2465 TCACAATGAGGCAAATCACATCATCGACCAGTGGCTACATGGCCTGGACGCCGGTAAGTGAGGAATCGGTGCGCGGA
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2542 CACTTCAAGGGCTACAAAATCCAAACGTGGACGGAGAACGAGGGCGAGGAGGGTCTGCGGGAGATCCATGTGAAGGG
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2696 GCTTCAATGGCCCACCCAGTGCCGTCATCGACTTCGATACTCCGGAGGGTGTACCATCGCCGGTTCAGGGACTGGAT
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2773 GCCTATCCTCTGGGCTCCTCGGCCTTCATGCTCCACTGGAAGAAGCCGCTGTATCCCAATGGCAAGCTCACTGGCTA
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2850 CAAGATCTACTACGAGGAGGTTAAGGAGAGCTATGTGGGCGAGCGACGCGAATACGATCCACACATCACCGATCCCA
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3004 GGCGAGGGATCTGAACACTATATCGAAAAGACCACGCTCAAGGATGCCGTCAATGTGGCCCCTGCCACGCCATCTTT
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3158 CAGGCACTCACTTCTTTACGATGCACAGGATCAAGGGCGAAACCCAATGGATACGCGAGAATGAGGAAAAGAACTCC
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3389 ATGCCGGATGGTTCATTGGCATGATGCTGGCCCTGGCCTTCATCATCATCCTCTTCATCATCATCTGCATTATCCGA
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