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(54) **Title**: ENHANCED RNAI MEDIATED GENE REGULATION

(57) **Abstract**: The present disclosure relates to nucleic acid agents for the simultaneous down-regulation of multiple gene targets. Compositions comprising the nucleic acid agents and methods for using the agent to target specific cell populations, such as those of the parasite in a host/parasite relationship, are also disclosed.

ENHANCED RNAi MEDIATED GENE REGULATION

TECHNICAL FIELD

The present invention relates to nucleic acid agents for the simultaneous down-regulation of multiple gene targets. Compositions comprising the nucleic acid agents and methods for using the agent to target specific cell populations, such as those of the parasite in a host/parasite relationship, are also disclosed.

BACKGROUND

RNA interference

RNAi is an RNA-dependent gene silencing process that is controlled by the RNA-induced silencing complex (RISC) and is initiated by short double-stranded RNA molecules in a cell's cytoplasm, where they interact with the catalytic RISC component argonaute.

When the dsRNA is exogenous (for example, coming from infection by a virus with an RNA genome), the RNA is imported directly into the cytoplasm and cleaved to short fragments by the argonaute enzyme.

The initiating dsRNA can also be endogenous (originating in the cell), as in pre-microRNAs expressed from RNA-coding genes in the genome. The primary transcripts from such genes are first processed to form the characteristic stem-loop structure of pre-miRNA in the nucleus, then exported to the cytoplasm to be cleaved by Dicer. Thus, the two dsRNA pathways, exogenous and endogenous, converge at the RISC complex.

dsRNA initiates RNAi by activating the ribonuclease protein Dicer, which binds and cleaves double-stranded RNAs (dsRNAs) to produce double-stranded fragments of 20–25 base pairs with a 2-nucleotide overhang at the 3' end. Bioinformatics studies on the genomes of multiple organisms suggest this length maximizes target-gene specificity and minimizes non-specific effects. These short double-stranded fragments are called small interfering RNAs (siRNAs). These siRNAs are then separated into two single-stranded (ss) ssRNAs, namely the passenger strand and the guide strand. The passenger strand is degraded, and the guide strand is incorporated into the RNA-induced silencing complex (RISC). After integration into the RISC, siRNAs base-pair to their target mRNA and induce cleavage of the mRNA, thereby preventing it from being used as a translation template. In some organisms, this process is known to spread systemically, despite the initially limited molar concentrations of siRNA.

A key feature required for the RNAi effect is a short stretch (~21 nucleotides) of duplex RNA having 100% sequence identity to the downregulated mRNA. Any nucleic acid which will be processed into, or lead to the generation of, an siRNA with this feature can lead to RNAi suppression of the target mRNA. Thus in addition to dsRNA (which is processed into siRNA by the activity of Dicer and the RISC complex), short hairpin RNAs (shRNAs) and some miRNAs may also initiate RNAi suppression.

As an investigative tool, RNAi is becoming an ever more powerful for determining the functional role of specific genes that may be potential targets for chemotherapeutic intervention. It is a particularly useful method since the RNAi gene silencing mechanism appears to be present in all eukaryotic organisms. Thus ubiquity combined with relative ease of application means that RNAi is not only an important tool in modern cell biology research but also has potential beyond the laboratory to the clinic and other applied areas.

One field in which RNAi shows particular promise is in targeting cells within a mixed population, wherein the targeted cells are distinguished from the general population of cells by the identity of the genes, or gene combination, they express.

Pest / Parasite control uses of RNAi

One use of RNAi's selectivity is in pest / parasite control, where the sequence specificity of RNAi coupled with its ability to suppress genes critical for pest survival allow the development of targeted pesticides able to kill the pest or parasite without adversely affecting non-target species or hosts.

Gene knockdown by long double-stranded (dsRNA) has been demonstrated in over 30 insect, tick and mite species (Aronstein et al., 2011), many parasitic worms (Geldhof et al., 2007), economically important copepods such as sea lice (Campbell et al. 2009) and medically important protozoa such as *Trypanosome* spp., *Entoamoeba histolytica*, *Giardia intestinalis* and *Toxoplasma gondii* (Kolev et al., 2011). The possibility of suppression of critical genes by RNAi in these pests and parasites, thus causing pest death without harming host and non-target species holds great potential. Delivery systems for dsRNAs to pests include spraying on plants, delivery in food, and engineering transgenic plants to produce the dsRNA.

The use of RNAi to target the *Varroa destructor* parasitic mite of *Apis mellifera* honey bees has been documented in Garbian et al., 2012. In the assays described in that reference, the

authors prepare two separate mixtures of dsRNA, with 'Mixture I' containing a mixture of dsRNAs corresponding to five different *V.destructor* gene sequences, and 'Mixture II' containing a mixture of dsRNAs corresponding to fourteen different *V.destructor* gene sequences. Upon feeding bees with sucrose solution containing one or other of these mixtures, the authors noted mortality in the *V.destructor* mites parasitizing the fed *A.mellifera* bees.

'Mixture II' is reported as being the most efficacious mixture, with the highest reported mortality recorded at the end of a 60-day trial period being 61%. The 60-day trial period allowed for two reproductive cycles of *V.destructor*, and the authors did not directly measure *V.destructor* mite mortality; thus, the 61% figure represents the combined effects of mortality and reduced fecundity over two generations of *V.destructor* mite.

Medical uses of RNAi

Within the medical field, major pharmaceutical companies initially invested several billion dollars in RNAi therapeutics, but the initial optimism failed to deliver the promise. However, recently there has been a renewed optimism and investment in RNAi therapeutics following refinements in RNAi targeting and delivery for liver-based diseases, viral infections, cancer and more (Bender 2014)

DISCLOSURE

The present inventors have developed an improved method dsRNA delivery technology for inducing the RNAi-mediated down-regulation of multiple genes. As compared to existing technology for delivering multiple dsRNAs, the improved technology described herein leads to a significant increase in the mortality rates of the target cell or organism population.

When using RNAi to target a cell or organism population it is desirable to maximise the mortality rate in the targeted population. Existing methods have used a number of strategies to increase the observed mortality. For example, the authors of Garbian et al. 2012 describe selecting *V.destructor* target genes involved in key cellular processes such as cell architecture (alpha tubulin) and DNA transcription (RNA polymerase), the silencing of which was expected to harm the Varroa mites. The authors of Garbian et al. 2012 also describe the importance of selecting target sequences within the *V.destructor* genes which do not correspond to any *A.melliifera* (i.e. the host) or human genes, so as to prevent any off-target gene silencing.

In addition to selecting target genes involved in key cellular processes, the authors of Garbian et al. 2012 also co-administered dsRNA corresponding to multiple target genes with the aim of increasing the total mortality levels through simultaneously inhibiting multiple cellular pathways. Consistent with this, Garbian et al. 2012 reported that a mixture of 14 dsRNAs ("Mixture II") caused a significant decrease in *Varroa destructor* mite number when fed to bees, but a mixture of 5 dsRNAs ("Mixture I") did not.

Notwithstanding the Garbain et al. data from *V.destructor*, studies in other experimental systems indicate that there is not a consistent or simple relationship between the number of simultaneously targeted genes and the observed level of mortality. For example, studies in both the soybean plant parasitic nematode *Heterodera glycines* (Bakhetia et al., 2008) and the red flour beetle *Tribolium castaneum* (Miller et al., 2012) have demonstrated that treatment with multiple dsRNAs can, in fact, reduce the efficacy of gene knockdown.

This potential for reduced efficiency of gene knockdown in combinatorial or multi-target dsRNA treatments has been documented in other studies (Aronstein et al., 2011; Bakhetia et al., 2008; Charlton et al., 2010). A possible explanation for this effect has been put forward in Miller et al. (2012) based on carefully controlled competition studies in *T.castaneum*; the authors interpreted the results of these experiments as indicating that the decreased gene knockdown efficiency in multiple dsRNAs studies was due to cellular uptake competition. That is, the oversaturation of both the cellular uptake process and the intracellular dsRNAi machinery by multiple dsRNAs delivered simultaneously reduced the overall efficiency of gene knockdown.

The effects of multi-target dsRNA treatments is also described in WO2006/046148, whose authors describe the effects on *C.elegans* of simultaneously delivering dsRNA targeting up to 2 different genes (see, for example, Example 5 and Figure 28 of WO2006/046148). The data in WO2006/046148 indicates that dsRNA mixtures lead to significantly higher progeny mortality than individually delivered dsRNAs, but do not clearly show any significant difference between mixtures and concatamers. No lethality on the treated target organisms is reported – only progeny of treated organisms are examined.

The present inventors have developed an improved delivery technology for down-regulating multiple genes using RNAi. The improved technology does not exhibit the reduced efficacy of gene knockdown observed with some of the dsRNA delivery technologies described in the art. Furthermore, dsRNA delivered by the improved technology has been demonstrated to

result in a significantly higher target mortality in the treated animals than an equivalent dose of dsRNA delivered by conventional means.

The improved technology is based on the insight that when targeting multiple genes higher target mortality can be achieved if the dsRNAs corresponding to each target gene are administered to the target as a concatemer, rather than as a mixture of separate dsRNAs. So, for example, if the genes 'A', 'B', and 'C' are to be targeted, higher target mortality can be achieved by administering the dsRNA[A-B-C] than administering the same total amount of dsRNA as a mixture of dsRNA[A] + dsRNA[B] + dsRNA[C].

In addition to allowing for higher target mortality, producing a single dsRNA concatemer is typically simpler and less expensive than producing an equivalent mixture of separate dsRNAs.

Without wishing to be bound by theory, it is believed that the higher target mortality is at least partially because the concatemer structure enforces simultaneous down-regulation of all the targeted genes within each target cell (or cell within a target organism) that takes up a dsRNA molecule. That is, a cell which takes up a dsRNA[A-B-C] molecule will experience simultaneous down-regulation of genes 'A', 'B', and 'C', with the cumulative damage arising from the inhibition of multiple cellular processes multiplying the likelihood of cell death.

In contrast, to achieve a similar multiple process inhibition using a mixture of dsRNA[A] + dsRNA[B] + dsRNA[C], a target cell (or cell within a target organism) must take up each of the separate dsRNA molecules. This is true for each target cell (or cell within a target organism). Thus, even if each dsRNA is present in the mixture at equal concentration, it is unlikely that each target cell will take up an equal proportion of each dsRNA. Differences between the transport and/or uptake of the different dsRNAs will further magnify this uneven distribution of the dsRNA within the target cells. It can be envisioned that the overall effect of this less even distribution of dsRNAs is a reduced level of cumulative damage in individual cells and, therefore, a lower proportion of cell death.

Again, without wishing to be bound by theory, it is believed that the concatemer structure places a lower burden on the cellular dsRNA transport system. That is, for example, only concatemer dsRNA molecule (i.e. dsRNA[A-B-C]) needs to be taken up to achieve down-regulation of genes 'A', 'B', and 'C'. To achieve the same effect with a mixture, three dsRNA molecules must be taken up (i.e. dsRNA[A] + dsRNA[B] + dsRNA[C]). Thus, at saturating concentrations the concatemer can be expected to lead to more effective gene knockdown.

Accordingly, in one aspect the present invention provides an isolated nucleic acid concatemer comprising at least a first nucleic acid sequence and a second nucleic acid sequence;

wherein the first nucleic acid sequence is capable of down-regulating the expression of a first gene of a target, and the second nucleic acid sequence is capable of down-regulating the expression of a second gene of the target. Preferably the first and second genes are different genes.

The term "isolated nucleic acid concatemer" is used herein to refer to a two or more nucleic acid sequences capable of down-regulating gene expression which have been joined ('concatenated') such that they form a single, contiguous nucleic acid molecule. In this sense the term "isolated nucleic acid concatemer" is intended to refer to concatenates not naturally occurring in nature. Preferably each of the constituent nucleic acid sequences of an "isolated nucleic acid concatemer" targets a different gene such that the concatemer is capable of down-regulating the expression of at least two different genes simultaneously.

Typically, the constituent nucleic acid sequences of an isolated nucleic acid concatemer are found sequentially on the nucleic acid molecule with only short, or no, intervening sequence ('spacer sequence') between the sequences capable of down-regulating gene expression. In some embodiments there is no more than 500 base pairs of spacer sequence between each sequence capable of down-regulating gene expression, such as no more than 400, 300, 200, 100, 50, 20, 10 or 5 base pairs of spacer sequence.

An example of an "isolated nucleic acid concatemer" is shown in Figure 10. In this Figure, an isolated nucleic acid concatemer according to the present description extends from the first base of MOA (base 1395) to the last base of AChE (base 2355) to give a total concatemer length of 960 bases. The concatemer is composed of three nucleic acid sequences capable of down-regulating the expression (marked MOA, ATP, AChE) which are arranged sequentially on the nucleic acid molecule with no spacer sequences. This isolated nucleic acid concatemer is capable of down-regulating three genes (MOA, vATPc, AChE) simultaneously (see Figure 9; in this example, the target is the *Varroa destructor* mite). Such a concatemer consisting of three nucleic acid sequences capable of down-regulating the expression genes is herein called a "tricatemer".

Thus, in one aspect the present invention provides an isolated nucleic acid concatemer comprising at least a first nucleic acid sequence, a second nucleic acid sequence, and a third nucleic acid sequence;

wherein the first nucleic acid sequence is capable of down-regulating the expression of a first gene of a target, the second nucleic acid sequence is capable of down-regulating the expression of a second gene of the target, and the third nucleic acid sequence is capable of down-regulating the expression of a third gene of the target. Preferably the first, second and third genes are different genes.

In a further aspect the present invention provides an isolated nucleic acid concatemer comprising at least a first nucleic acid sequence, a second nucleic acid sequence, a third nucleic acid sequence, and a fourth nucleic acid sequence;

wherein the first nucleic acid sequence is capable of down-regulating the expression of a first gene of a target, the second nucleic acid sequence is capable of down-regulating the expression of a second gene of the target, the third nucleic acid sequence is capable of down-regulating the expression of a third gene of the target, and the fourth nucleic acid sequence is capable of down-regulating the expression of a fourth gene of the target. Preferably the first, second, third and fourth genes are different genes.

In a yet further aspect the present invention provides an isolated nucleic acid concatemer comprising at least a first nucleic acid sequence, a second nucleic acid sequence, a third nucleic acid sequence, a fourth nucleic acid sequence, and a fifth nucleic acid sequence;

wherein the first nucleic acid sequence is capable of down-regulating the expression of a first gene of a target, the second nucleic acid sequence is capable of down-regulating the expression of a second gene of the target, the third nucleic acid sequence is capable of down-regulating the expression of a third gene of the target, the fourth nucleic acid sequence is capable of down-regulating the expression of a fourth gene of the target, and the fifth nucleic acid sequence is capable of down-regulating the expression of a fifth gene of the target. Preferably the first, second, third, fourth and fifth genes are different genes.

In a yet further aspect the present invention provides an isolated nucleic acid concatemer comprising at least a first nucleic acid sequence, a second nucleic acid sequence, a third nucleic acid sequence, a fourth nucleic acid sequence, a fifth nucleic acid sequence, and a sixth nucleic acid sequence;

wherein the first nucleic acid sequence is capable of down-regulating the expression of a first gene of a target, the second nucleic acid sequence is capable of down-regulating the expression of a second gene of the target, the third nucleic acid sequence is capable of down-regulating the expression of a third gene of the target, the fourth nucleic acid

sequence is capable of down-regulating the expression of a fourth gene of the target, the fifth nucleic acid sequence is capable of down-regulating the expression of a fifth gene of the target, and the sixth nucleic acid sequence is capable of down-regulating the expression of a sixth gene of the target. Preferably the first, second, third, fourth, fifth and sixth genes are different genes.

Further concatemers comprising seven, eight, nine, ten, fifteen, twenty or more than twenty nucleic acid sequences are envisaged (preferably capable of, respectively, down-regulating the expression of two, three, four, five, six, seven, eight, nine, ten, fifteen, twenty or more than twenty different genes of the target).

Target organisms for gene down-regulation

The present inventors have found that the concatemers described herein are consistently more effective than the equivalent dsRNAs delivered as a mixture. Furthermore, the increased effectiveness of the concatemer relative to the corresponding mixture appears to be independent of the identity of the specific genes, or the species of the target. This independence is consistent with the postulated theory underpinning the present invention, as well as the known presence of dsRNA-mediated gene silencing (RNAi) mechanisms in many eukaryotic organisms.

Accordingly, the 'target' whose gene expression is down regulated by the isolated nucleic acid concatemers described herein may be any cell or organism capable of dsRNA-mediated gene silencing.

In some embodiments the target is an organism. In some embodiments the target is a member of the *Acari* subclass. In some embodiments the target is a member of the *Arthropoda* phylum, for example a member of the *Insecta* class (such as a member of the order *Coleoptera*).

In some embodiments the target is not the *Varroa destructor* mite. In some embodiments the target is not the *Caenorhabditis elegans* nematode.

For example, the target may be a pest organism such as *Tribolium castaneum* or *Aedes aegypti*.

In some embodiments the target is a cell, or population of cells. For example, a human tumour cell. The target cell or population of cells may be *in vivo*, *ex vivo*, or *in vitro*.

In some embodiments the target is an organism (or a cell or population of cells derived therefrom) listed in any one of Tables 'A' to 'E'.

COMMON NAME	LATIN NAME
Cattle tick	*Rhipicephalus microplus*
Brown dog tick	*Rhipicephalus sanguineus*
Cat flea	*Ctenocephalides felis*
Common Bed bug	*Cimex lectularius*
Yellow fever mosquito	*Aedes aegypti*
Malaria mosquitoes	*Anopheles gambiae complex*
Sea louse	*Lepeophtheirus salmonis*
Sea louse	*Caligus rogercresse*

Table A

COMMON NAME	LATIN NAME
German cockroach	*Blattella germanica*
American cockroach	*Periplaneta americana*
Wasp	*Vespula Vulgaris*
Euopean Hornet	*Vespro crabro*
Asian giant hornet	*Vespa mandarinia*
Formosan subterranean termite	*Coptotermes formosanus*
Drywood termites	*Incisitermes snyderi*
Eastern Subterranean termites	*Reticulitermes flavipes*
Common furniture beetle	*Anobium punctatum*
House fly	*Musca domestica*
Common clothes moth	*Tineola bisselliella*

Table B

COMMON NAME	LATIN NAME
Honey bee mite	*Varroa destructor*
Greater wax moth	*Galleria mellonella*
Lesser Wax moth	*Achroia grisella*
Small hive beetle	*Aethina tumida*
Acarine (Tracheal) mites	
	Acarapis woodi
Tropilaelaps	*Tropilaelaps clareae*
Nosema	*Nosema apis*
Noseama	*Nosema ceranae*

Table C

COMMON NAME	LATIN NAME
Pea aphid	*Acyrthosiphon pisum*
Mealworm beetle	*Tenebrio molitor*
Red flour beetle	*Tribolium castaneum*
Confused flour beetle	*ribolium confusum*
Grain weevil	*Sitophilus granarius*
Cotton boll weevil	*Anthonomus grandis*
Diamondback moth	*Plutella xylostella*
Gypsy moth	*Lymantria dispar dispar*
Cotton bollworm	*Helicoverpa zea*
Snails	*e.g. Cornu aspersa*
Field slug	*Deroceras reticulatum*
Garden slug	*Arion hortensis*
Two-spotted spider mite	*Tetranychus urticae*

Table D

COMMON NAME	LATIN NAME
Sleeping disease	*Trypanosma brucei*
Chagas disease	*Trypanosma cruzi*
	Entamoeba histolytica
Toxoplasmosis	*Toxoplasma gondi*
Giardiasis	*Giardia intestinalis*

Table E

In some embodiments the target is not *Varroa destructor*, or a cell or population of cells derived therefrom.

More generally, in one aspect it is envisioned that any embodiment described herein in which the target is *Varroa destructor* is not encompassed by the present invention. Furthermore. In one aspect it is envisioned that any embodiment described herein in which or the first, second, third, and/or further genes are *Varroa destructor* genes is not encompassed by the present invention

Target genes for down-regulation

As noted above, the increased effectiveness of the concatemer relative to the corresponding mixture appears to be independent of the identity of the specific genes. Nonetheless, for applications where the aim is toxicity to the target, a typical strategy is to select genes with functions in key cellular processes such as cell architecture (for example, alpha tubulin), DNA transcription (for example, RNA polymerase), or energy generation / gradient maintenance (Pyruvate kinase, vacuolar ATPase). The silencing of a number of these genes within a target can be expected to harm the target.

Accordingly, in some embodiments the first and/or second gene and/or third gene (if present) and/or fourth gene (if present) and/or fifth gene (if present) and/or sixth gene (if present) are selected from the group consisting of the genes which encode: Na+/K+-ATPase (any of the subunits), Vacuolar ATPase (proton pump; any of the subunits), Plasma membrane Calcium ATPase (PMCA), Sarcoplasmic reticulum Ca2+ ATPase (SERCA), ADP/ATP- translocase, Sodium-glucose linked transporter, Trehalase, Pyruvate dehydrogenase, Pyruvate kinase, Pyruvate carboxylase, Tubulin, Monoamine oxidase, Acetylcholinesterase, Phosphodiesterase. In some embodiments all of the first and second gene (and third gene, if present) are selected from the above group.

In some embodiments the target is the *V.destructor* organism and the first and/or second gene (and/or third gene, if present) are selected from the group consisting of the genes which encode: Acetylcholinesterase (AChE; GenBank accession number ADDG01069748.1), Monoamine Oxidase (MOA; GenBank accession number ADDG01053234.1), and vATPase subunit C (vATPc; GenBank accession number ADDG01035752.1. Preferably all of the first and second gene (and third gene, if present) are selected from the above group.

In some embodiments the target is the *V.destructor* organism and the first and/or second nucleic acid sequence (and/or third nucleic acid sequence, if present) comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides (such as at least 21, 25, 30, 50, 100, 200, or 500 nucleotides) encoded by a sequence selected from the group consisting of SEQ ID NO.1, SEQ ID NO.2, and SEQ ID NO.3. Preferably the first or second nucleic acid sequence is SEQ ID NO.2. In some embodiments, all of the first and second nucleic acid sequence (and third nucleic acid sequence, if present) are selected from the above group.

In some embodiments the target is the *T.castaneum* organism and the first and/or second gene and/or third gene (if present) and/or fourth gene (if present) and/or fifth gene (if present) and/or sixth gene (if present) are selected from the group consisting of the genes which encode: Plasma membrane calcium-transporting ATPase 1 (TcPMCA; NCBI accession number XM_008201630.1), Na/K ATPase alpha (TcNaK; NCBI accession number XM_008198203.1), ADP/ATP translocase (TcADPt; NCBI accession number XM_968164.3) , vATPase subunit E (TcvATPe; NCBI accession number XM_965528.2), Calcium-transporting ATPase sarcoplasmic /endoplasmic reticulum type (TcSERCA; NCBI accession number XM_961690.3), a-tubulin 1 (TcaTUB; NCBI accession number XP_966492.1), and Heat shock protein 90 (TcHSP90; NCBI accession number NP_001094067.1). Preferably all of the first and second gene (and third gene, if present) are selected from the above group. In some embodiments the first, second, and third genes are selected from the combinations: (i)TcPMCA, TcNaK, TcADPt, (ii) TcPMCA, TcNaK, TcvATPe, (iii) TcaTUB, TcHSP90, TcADPt, and (iv) TcaTUB, TcHSP90, TcvATPe.

In some embodiments the target is the *T.castaneum* organism and the first and/or second nucleic acid sequence and/or third nucleic acid sequence (if present) and/or fourth nucleic acid sequence (if present) and/or fifth nucleic acid sequence (if present) and/or sixth nucleic acid sequence (if present) comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides (such as at least 21, 25, 30, 50, 100, 200, or

500 nucleotides) encoded by a sequence selected from the group consisting of SEQ ID NO.8, SEQ ID NO.9, SEQ ID NO.113, SEQ ID NO.10, SEQ ID NO.11, SEQ ID NO.12, SEQ ID NO.13, and SEQ ID NO.14. In some embodiments, all of the first and second nucleic acid sequence (and third nucleic acid sequence, if present) are selected from the above group. In some embodiments the first, second, and third genes are selected from the combinations: (i) SEQ ID NO.8, 9, and 10, (ii) SEQ ID NO.8, 9, and 11, (iii) SEQ ID NO.13, 14, and 10, (iv) SEQ ID NO.13, 14, and 11, (v) SEQ ID NO.8, 113, and 10, and (vi) SEQ ID NO.8, 113, and 11.

In some embodiments the target is the *A.aegypti* organism and the first and/or second gene (and/or third gene, if present) are selected from the group consisting of the genes which encode: Tubulin beta chain (AabTub; NCBI accession number XM_001662168.1), Na/K ATPase alpha subunit (AaNaK; NCBI accession number ADDG01053234.1), and ADP/ATP carrier protein (AaADPt; NCBI accession number XM_001649861.1). Preferably all of the first and second gene (and third gene, if present) are selected from the above group.

In some embodiments the target is the *A.aegypti* organism and the first and/or second nucleic acid sequence (and/or third nucleic acid sequence, if present) comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides (such as at least 21, 25, 30, 50, 100, 200, or 500 nucleotides) encoded by a sequence selected from the group consisting of SEQ ID NO.19, SEQ ID NO.20, and SEQ ID NO.21. In some embodiments, all of the first and second nucleic acid sequence (and third nucleic acid sequence, if present) are selected from the above group.

In some embodiments the target is the *L.salmonis* organism and the first and/or second gene (and/or third gene, if present) are selected from the group consisting of the genes which encode: ADP/ATP translocase 1 (LsADPt; NCBI accession number BT077972.1), V-type ATPase unit E (LsvATPe; NCBI accession number BT120776.1), and acetylcholinesterase (LsAChE; NCBI accession number KJ132369.1). Preferably all of the first and second gene (and third gene, if present) are selected from the above group.

In some embodiments the target is the *L.salmonis* organism and the first and/or second nucleic acid sequence (and/or third nucleic acid sequence, if present) comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides (such as at least 21, 25, 30, 50, 100, 200, or 500 nucleotides) encoded by a sequence selected from the group consisting of SEQ ID NO.23, SEQ ID NO.24, and SEQ ID NO.25. In some

embodiments, all of the first and second nucleic acid sequence (and third nucleic acid sequence, if present) are selected from the above group.

In some embodiments the target is the *C.elegans* organism and the first and/or second gene (and/or third gene, if present) are selected from the group consisting of the genes which encode: pat-10 (NCBI accession number NM_059100.6), bli-5 (NCBI accession number NM_067371.1), and egl-30 (NCBI accession number U56864.1). Preferably all of the first and second gene (and third gene, if present) are selected from the above group.

In some embodiments the target is the *C.elegans* organism and the first and/or second nucleic acid sequence (and/or third nucleic acid sequence, if present) comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides (such as at least 21, 25, 30, 50, 100, 200, or 500 nucleotides) encoded by a sequence selected from the group consisting of SEQ ID NO.27, SEQ ID NO.28, and SEQ ID NO.29. In some embodiments, all of the first and second nucleic acid sequence (and third nucleic acid sequence, if present) are selected from the above group.

Concatemers and constructs

Concatemers according to the present invention will be recombinant and may be provided isolated and/or purified, in substantially pure or homogeneous form, or free or substantially free of other nucleic acid. The term “isolated” encompasses all these possibilities.

Concatemers may be ribonucleic acids or deoxy ribonucleic acids. In some embodiments the concatemer is a dsRNA, such as siRNA, shRNA or miRNA. In other embodiments the concatemer is antisense RNA, or a ribozyme.

Since nucleic acid may be double stranded, where the concatemer (or nucleotide sequence) of the invention is referred to herein, use of the complement of that nucleic acid agent (or nucleotide sequence) will also be embraced by the invention. The 'complement' in each case is the same length as the reference, but is 100% complementary thereto whereby by each nucleotide is base paired to its counterpart i.e. G to C, and A to T or U.

In some embodiments the total length of the nucleic acid concatemer is less than 10,000 bases (or base pairs) long. For example, in some embodiments the nucleic acid concatemer is less than 5000 bases long, such as less than 4000, 3000, 2000, 1500, 1000, 500, 400,

300, 200 or less than 100 bases (or base pairs) long. In some embodiments the nucleic acid concatemer is less than 950 bases long, such as less than 900, 850, 800, 750, 700, 650, 600, 550, 500, 450, 400, 300, 250, 200, 150, 100 or less than 50 bases long.

In some embodiments the total length of the nucleic acid concatemer greater than 500 bases (or base pairs) long, such as greater than 600, 700, 800, 900, or greater than 1000 base pairs long.

In preferred embodiments the total length of the nucleic acid concatemer is 501 to 2000 bases (or base pairs) long, such as 600 to 1800, 700 to 1600, or 750 to 1500 bases.

(The "total length of the concatemer" as used herein is measured from the first base of the 5'-most sequence capable of down-regulating gene expression to the last base of the 3'-most sequence capable of down-regulating gene expression.)

The present invention also provides nucleic acid constructs (for example, DNA constructs) encoding concatemers according to the present invention. Such vectors may include, in addition to the sequence encoding the concatemer of the invention, a promoter, a terminator and/or other regulatory sequence such as to define an expression cassette comprising the sequence encoding the nucleic acid agent of the invention. The sequence of some vectors according to the present invention are shown in SEQ ID NOs. 4, 15, 22, and 26.

Generally speaking, in the light of the present disclosure, those skilled in the art will be able to construct vectors according to the present invention. For further details see, for example, Molecular Cloning: a Laboratory Manual: 2nd edition, Sambrook et al, l989, Cold Spring Harbor Laboratory Press. Many known techniques and protocols for manipulation of nucleic acid, for example in preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in Protocols in Molecular Biology, Second Edition, Ausubel et al. eds., John Wiley & Sons, 1992.

Genes and gene expression

"Gene of a/the target" is a term used to mean a coding sequence in the genome of the *target* organism or cell which is, or may be, expressed as a functional gene product. For example,

via transcription to mRNA and translation to a protein according to well established principles.

Expression of a gene is a term used to describe the process by which the information from, a gene is used to synthesise a gene product, such as an mRNA or polypeptide.

"Capable of downregulating the expression" is a term generally used to refer to the ability to reduce the levels of a gene product in response to the presence of the agent. Reduction is measured compared to an otherwise identical gene expression system which has not been exposed to the agent in question. The degree of reduction may be so as to totally abolish production of the encoded gene product, but may also be such that the abolition of expression is not complete, with some small degree of expression remaining. The term should not therefore be taken to require a complete absence of expression. It is used herein where convenient because those skilled in the art well understand this. Examples of downregulated expression are (i) reduced transcription of the gene, (ii) reduced mRNA amount, stability or translatability, and (iii) reduced amount of polypeptide product.

The ability to downregulate expression can be assayed, for example, via direct detection of gene transcripts (e.g. via PCR) or polypeptides (e.g. via Western blot), via polypeptide activity (e.g. enzyme activity) or via observation of target behaviour (e.g. via cell/organism mortality). Thus, whether a particular agent inhibits translation of mRNA, or induces degradation of mRNA, can be readily assayed using the above methods, or other methods well-known in the art. In some embodiments, translation of an mRNA is considered "inhibited" if the amount of expressed protein is at least 10% lower than in an otherwise identical system not exposed to the agent; for example, at least 20% lower, at least 30% lower, at least 40% lower, at least 50% lower, at least 60% lower, at least 70% lower, at least 80% lower, or at least 90% lower than in an otherwise identical system not exposed to the agent. Similarly, in some embodiments, degradation of mRNA is "induced" if the amount of mRNA (µg/µl) is at least 10% lower than in an otherwise identical system not exposed to the agent; for example, at least 20% lower, at least 30% lower, at least 40% lower, at least 50% lower, at least 60% lower, at least 70% lower, at least 80% lower, at least 90% lower, at least 95% lower, at least 98% lower, or at least 99% lower than in an otherwise identical system not exposed to the agent.

Thus, in some embodiments the mRNA levels of the targeted genes in treated target cells / organisms is at least 10% lower than in targets treated with a control agent (for example, GFP dsRNA). For example, mRNA levels (µg/µl) of the targeted genes may be at least 20%

lower, at least 30% lower, at least 40% lower, at least 50% lower, at least 60% lower, at least 70% lower, at least 80% lower, at least 90%, at least 95%, at least 98%, or at least 99% lower than in mites treated with a control agent (for example, GFP dsRNA).

In some embodiments the amount of protein or mRNA is measured 24 hours after the system is first exposed to the agent. In other embodiments the amount of protein or mRNA is measured 48 or 72 hours after the system is first exposed to the agent, composition or concatemer.

In preferred embodiments, the mRNA levels of the targeted genes in treated target cells / organisms is at least 95% lower than in mites treated with a control agent (for example, GFP dsRNA) 72 hours after exposure to the agent, composition or concatemer.

Target mortality

The effectiveness of the isolated nucleic acid concatemer disclosed herein, for example in methods of inhibiting the growth of, or reducing, a population of a target, may be assessed by monitoring the % mortality of the population of the target treated with the nucleic acid concatemer.

For example, in some embodiments the nucleic acid agent causes greater than 30% target mortality (= less than 70% target survival), as measured, for example, 108 hours after a 12 hour soaking of the target in a 1.25 µg/µl solution of the nucleic acid concatemer. In some embodiments the nucleic acid agent causes greater than 40% target mortality, such as greater than 50%, greater than 60%, greater than 70%, greater than 80%, or greater than 90% mortality as measured, for example, 108 hours after a 12 hour soaking of the target in a 1.25 µg/µl solution of the nucleic acid concatemer.

In preferred embodiments, the nucleic acid concatemer causes greater than 60% target mortality (= less than 40% target survival), as measured, for example, 108 hours after a 12 hour soaking of the target in a 1.25 µg/µl solution of the nucleic acid concatemer.

In preferred embodiments, mortality is observed in the target organisms contacted with the concatemer. In other embodiments, the mortality phenotype is not observed in the target organisms contacted with the concatemer and, if observed at all, is first observed in the progeny of the organisms contacted with the concatemer (or in subsequent generations).

Interaction with non-target cells / organisms

The mechanisms of gene down-regulation described above are widespread throughout a broad range of organisms. Thus, in situations where the nucleic acid agent will come into contact with more than one variety of cell / organism, it is preferable to ensure that only the target cell / organism (= cell or organism) is susceptible to expression downregulation by the agent. That is, gene expression in non-target cell / organism exposed to the nucleic acid agent should preferably remain unaltered.

In some applications the target cell / organism is a different species to non-target organisms; for example, in applications where the target is a parasitic organism such as *V.destructor*. In these applications it is preferable to ensure the nucleic acid concatemer down-regulates gene expression only in the target organism species. Such species-specific gene downregulation can be achieved by ensuring (i) that the concatemer selected do not possess sufficient sequence identity with any non-target cell/organism to induce repression of gene expression in those non-target cells/organisms, or (ii) that the nucleic acid concatemer is only expressed in the target cell/organism (by, for example, through using a construct having a target specific promoter).

In other applications the target may be a specific cell or populations of cells within an organism; that is, the target cells are in an environment where they are surrounded by non-target cells of the same species. For example, the target may be a population of tumour cells within an organism. In these applications, target-specific repression of gene expression can be achieved by ensuring that that the nucleic acid concatemer is only expressed in the target cells (by, for example, through using a construct having a promoter activated by a combination of factors only present in (or greatly enriched in) the target cell population).

Accordingly, in some embodiments the nucleic acid concatemer described herein is capable of specifically down-regulating genes within the target organism; that is, the concatemer does not down-regulate the expression of genes in any non-target organisms. In some embodiments the concatemer is capable of downregulating the targeted gene to a significantly greater extent a non-target orthologue (such as the human orthologue). For example, the nucleic acid concatemer may induce a reduction in the target gene product that is at least 2-fold greater than the reduction in a non-target orthologue (for example, if the nucleic acid concatemer causes a 70% reduction in target mRNA levels, there will be no more than a 35% reduction in non-target mRNA levels). In some embodiments the nucleic acid agent may induce a reduction in target gene product that is at least 3-fold, 4-, 5-, 6-, 8-,

10-, 20-, 50-, 100-, 200-, 500- or 1000-fold greater than the reduction in a non-target gene product.

In some embodiments the nucleic acid a concatemer of the present invention is capable of downregulating a *targeted* gene to a significantly greater extent than any gene in a non-targeted cell / organism (e.g. human) gene. For example, the nucleic acid agent may induce a reduction in the target gene product that is at least 2-fold greater than the reduction in any non-target gene product (for example, if the nucleic acid agent causes a 70% reduction in targeted gene mRNA levels, there will be no more than a 35% reduction in non-targeted gene mRNA level). In some embodiments the nucleic acid agent may induce a reduction in targeted gene product that is at least 3-fold, 4-, 5-, 6-, 8-, 10-, 20-, 50-, 100-, 200-, 500- or 1000-fold greater than the reduction in any non-targeted gene product.

In some embodiments, the nucleic acid concatemer according to the present invention does not comprise a nucleic acid sequence that has 100% sequence identity to at least 18 (for example, at least 21, at least 25, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100) contiguous nucleotides of the transcribed portions of the *Varroa destructor* genome. In some embodiments, the nucleic acid concatemer according to the present invention does not comprise a nucleic acid sequence that has 100% sequence identity to at least 18 (for example, at least 21, at least 25, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, or at least 100) contiguous nucleotides of the transcribed portions of the human genome.

The specificity of gene regulation may also be assayed through monitoring the mortality of non-target cells / organisms exposed to the isolated nucleic acid concatemer) For example, in some embodiments there is less than an additional 10% non-target mortality (relative to an untreated control), as measured 168 hours after the onset of treatment of the target population with the isolated nucleic acid concatemer. In some embodiments there is less than an additional 5%, 2%, 1%, 0.5%, 0.2%, 0.1% non-target mortality (relative to an untreated control), as measured 168 hours after the onset of treatment of the target population with the isolated nucleic acid concatemer. In preferred embodiments, there is no significant additional non-target mortality (relative to an untreated control, as measured 168 hours after the onset of treatment of the target population with the isolated nucleic acid agent).

Delivery of nucleic acids

In order to influence the expression of genes the nucleic acid concatemer of the present invention must be delivered to the target.

Delivery of the nucleic acid concatemers of the present invention to the target can be achieved in several ways. For example, the nucleic acid agents may be delivered to the target directly by contacting the target with a solution of the nucleic acid agents, for example by spraying a solution of the nucleic acid agents or concatemers directly onto the targets; on contact with the target, the nucleic acid concatemer can enter the target body via diffusion or transfer through orifices on the target body.

Accordingly, the present invention provides a concatemer of the invention (or a solution thereof) for use in a method of treatment; for example, a method of inhibiting the growth of, or reducing, a population of a target cell / organism. The present invention also provides for the use of a concatemer of the invention (or a solution thereof) in the manufacture of a medicament for, for example, inhibiting the growth of, or reducing, a population of a target cell / organism. The present invention further provides a method of inhibiting the growth of, or reducing, a population of a target cell / organism, the method comprising spraying, or otherwise contacting, the target cell/organism population with a solution comprising concatemer of the invention.

Encompassed within the above methods of inhibiting the growth of, or reducing, a population of a target cell / organism are:

- methods where the target is a parasitic / infectious organism (see, for example, Tables A and E above). Thus, the above methods of inhibiting the growth of, or reducing, a population of a target cell / organism encompass methods of treating the disorders caused by these organism.
- methods where the target is a pathogenic cell population (for example a cancerous tumour). Thus, the above methods of inhibiting the growth of, or reducing, a population of a target cell / organism encompass methods of treating disorders caused by pathogenic cell populations (for example, cancer).

Concatemers of the present invention may be delivered to the target indirectly via adding the concatemer nucleic acid to the target's feed.

According to another embodiment of the present invention, the nucleic acid concatemers of the present invention are delivered to the target organism indirectly via non-target organisms

parasitized by the target organism. The nucleic acid agents or concatemers of the present invention may be delivered to the non-target organism by, for example, spraying or otherwise contacting the non-target organism with a solution comprising a nucleic acid concatemer of the invention.

Thus, the nucleic acid concatemers of the present invention may be delivered to the target organism by feeding the concatemer to the non-target organism.

Following a similar principle, the nucleic acid concatemers of the present invention may be delivered to the target organism by providing the target organism with feed comprising the nucleic acid concatemers. For example, in embodiments where the target feeds on a plant, the nucleic acid concatemers of the present invention may be delivered to the target organism by providing a plant comprising the nucleic acid concatemers of the present invention.

Accordingly, the present disclosure provides a transgenic plant cell, plant, or part thereof, comprising a nucleic acid concatemer of the present invention, or a nucleic acid construct encoding a nucleic acid concatemer of the invention. For example, the present disclosure provides reproductive or propagation material for a transgenic plant, a transgenic tuber, stem, seed, and/or fruit comprising a nucleic acid concatemer as described herein, or a nucleic acid construct encoding a nucleic acid concatemer as described herein.

Similarly, the present disclosure provides a transgenic plant cell, plant, or part thereof, which expresses a nucleic acid concatemer of the present invention. For example, the present disclosure provides a transgenic tuber, stem, seed, and/or fruit comprising a nucleic acid concatemer as described herein.

The present disclosure also provides methods for producing a transgenic plant cell, plant, or part thereof, which expresses a nucleic acid concatemer of the present invention

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The present disclosure also provides a cell, e.g. a host cell, comprising any of the nucleic acid concatemers, nucleotide sequences or nucleic acid (e.g. DNA) constructs described herein. Such cells include prokaryotic cells (such as, but not limited to, gram-positive and gram-negative bacterial cells) and eukaryotic cells (such as, but not limited to, yeast cells or plant cells). Preferably said cell is a bacterial cell or a plant cell.

The present disclosure provides a composition comprising at least one comprising at least one nucleic acid concatemer or nucleic acid (e.g. DNA) constructs construct described herein, plus a physiological or agronomical acceptable carrier, excipient or diluent.

The composition may contain further components which serve to stabilise dsRNA and/or prevent degradation of dsRNA during prolonged storage of the composition.

The composition may still further contain components which enhance or promote uptake of dsRNA by the target organism. These may include, for example, chemical agents which generally promote the uptake of RNA into cells e.g. lipofectamin etc., and enzymes or chemical agents capable of digesting the fungal cell wall, e.g. a chitinase.

The composition may be in any suitable physical form for application to the target, to substrates, to cells (e.g. plant cells), or to organism infected by or susceptible to infection by a target species. It is contemplated that the "composition" of the disclosure may be supplied as a "kit-of-parts" comprising the nucleic acid concatemer in one container and a suitable diluent or carrier in a separate container.

The invention also relates to supply of the nucleic acid concatemer alone without any further components. In these embodiments the nucleic acid concatemer may be supplied in a concentrated form, such as a concentrated aqueous solution. It may even be supplied in frozen form or in freeze-dried or lyophilised form. The latter may be more stable for long term storage and may be de-frosted and/or reconstituted with a suitable diluent immediately prior to use.

The present invention relates to pesticidal compositions developed to be used in agriculture or horticulture. These pesticidal compositions may be prepared in a manner, known per se. For example, the active compounds can be converted into formulations, such as solutions, emulsions, wettable powders, water dispersible granules, suspensions, powders, dusting agents, foaming agents, pastes, soluble powders, granules, suspo-emulsion concentrates, microcapsules, fumigants, natural and synthetic materials impregnated with active compound and very fine capsules and polymeric substances.

Furthermore, the pesticidal compositions according to the present disclosure may comprise a synergist. The dsRNA or dsRNA constructs according to the invention, as such or in their formulations, can also be used in a mixture with known fungicides, bactericides, acaricides, nematicides or insecticides, to widen, for example, the activity spectrum or to prevent the development of resistance. In many cases, this results in synergistic effects, i.e. the activity of the mixture exceeds the activity of the individual components.

Additionally the active compounds according to the disclosure, as such or in their formulations or above-mentioned mixtures, can also be used in a mixture with other known active compounds, such as herbicides, fertilizers and/or growth regulators.

The present invention also relates to fibrous pesticide composition and its use as pesticide, wherein the fibrous composition comprises a non-woven fibre and an effective amount of at least one of the nucleic acid concatamers described herein, covalently attached or stably adsorbed to the fibre. The present invention also relates to surfactant-diatomaceous earth compositions for pesticidal use in the form of dry spreadable granules comprising at least one nucleic acid concatemer, or at least two nucleic acid concatamers as described herein. The present disclosure also provides solid, water-insoluble lipospheres and their use as pesticide, wherein said lipospheres are formed of a solid hydrophobic core having a layer of a phospholipid embedded on the surface of the core, containing at least nucleic acid concatemer as described herein in the core, in the phospholipid, adhered to the phospholipid, or a combination thereof. The invention further relates to pesticidal formulations in the form of microcapsules having a capsule wall made from a urea/dialdehyde precondensate and comprising at least one nucleic acid concatemer as described herein.

Contemplated combinations

In one aspect, the below combinations are encompasses by the present invention. In an alternative aspect, the below combinations are not encompassed by the present invention.

1. An isolated nucleic acid agent according to any one of paragraphs 4 to 13, a nucleic acid composition according to either one of paragraphs 15 or 16, or a composition according to paragraph 17 for use in a method of treating or preventing a Varroa destructor mite infestation of a beehive.

2. Use of an isolated nucleic acid agent according to any one of paragraphs 4 to 13, a nucleic acid composition according to either one of paragraphs 15 or 16, or a composition

according to paragraph 17 in the manufacture of a medicament for the treatment or prevention of a Varroa destructor mite infestation of a beehive.

3. A method of treating or preventing a Varroa destructor mite infestation of a beehive, the method comprising administering to a member of the beehive an isolated nucleic acid agent according to any one of paragraphs 4 to 13, a nucleic acid composition according to either one of paragraphs 15 or 16, or a composition according to paragraph 17.

4. An isolated nucleic acid agent comprising a nucleic acid sequence that is capable of downregulating the expression of a gene of the Varroa destructor mite, wherein the gene encodes Acetylcholinesterase (AChE; GenBank accession number ADDG01069748.1), Monoamine Oxidase (MOA; GenBank accession number ADDG01053234.1), vATPase subunit C (vATPc; GenBank accession number ADDG01035752.1, GABA-receptor alpha subunit (GABA-Rα; GenBank accession number ADDG01060981.1), Chitin Synthase 1 (CHS-1; GenBank accession number ADDG01037469.1), Pyruvate Kinase (PyK; GenBank accession number ADDG01095321.1), alpha Tubulin (αTUB; GenBank accession number ADDG01073340.1), Prothoracicostatic peptide precursor (PTTH; GenBank accession number ADDG01000788.1), Crustacean hyperglycaemic hormone (CHH;GenBank accession number ADDG01078386.1) or Glutathione transferase mu1 (GSTμ1; GenBank accession number ADDG01001667.1).

5. The isolated nucleic acid agent according to paragraph 4, wherein the nucleic acid agent comprises at least two or at least three nucleic acid sequences, wherein, optionally, the at least two or at least three nucleic acid sequences are capable of downregulating the expression of at least two or at least three different genes from Varroa destructor.

6. The isolated nucleic acid agent according to either one of paragraph 4 or paragraph 5, wherein the agent is less than 2000 bases long, or less than 1000 bases long, or less than 500 bases long.

7. The isolated nucleic acid agent according to any one of paragraph 4 to paragraph 6 wherein the or each nucleic acid sequence independently has at least 80% sequence identity to at least 18 contiguous nucleotides of an mRNA encoded by the gene of the Varroa destructor mite, and wherein the nucleic acid agent inhibits translation of the mRNA.

8. The isolated nucleic acid agent according to paragraph 7 wherein the or each nucleic acid sequence independently has at least 80% sequence identity to at least 18 contiguous

nucleotides encoded by SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, SEQ ID NO.5, SEQ ID NO.6, SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, SEQ ID NO.113, or SEQ ID NO.10.

9. The isolated nucleic acid agent according to any one of paragraph 4 to paragraph 6 wherein the or each nucleic acid sequence independently has 100% sequence identity to at least 18 contiguous nucleotides of an mRNA encoded by the gene of the Varroa destructor mite, and wherein the nucleic acid agent induces the degradation of the mRNA.

10. The isolated nucleic acid agent according to paragraph 9 wherein the or each nucleic acid sequence independently has 100% sequence identity to at least 18 contiguous nucleotides encoded by SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, SEQ ID NO.5, SEQ ID NO.6, SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, SEQ ID NO.113, or SEQ ID NO.10.

11. The isolated nucleic acid agent according to any one of paragraphs 4 to10 wherein the nucleic acid agent is a dsRNA, antisense RNA, or a ribozyme.

12. The isolated nucleic acid agent according to paragraph 11 wherein the dsRNA is an siRNA, shRNA or miRNA.

13. The isolated nucleic acid agent according to any one of paragraphs 7 to 12 wherein the at least 18 contiguous nucleotides is at least 21 contiguous nucleotides, at least 25 contiguous nucleotides, or at least 30 contiguous nucleotides.

14. A nucleic acid construct encoding the isolated nucleic acid agent of any one of paragraphs 4 to 13.

15. A nucleic acid composition comprising at least two isolated nucleic acid agents according to any one of paragraphs 4 to 13.

16. The nucleic acid composition according to paragraph 15 wherein the at least two isolated nucleic acid agents are capable of downregulating the expression of at least two of the genes of the Varroa destructor mite.

17. A composition for feeding to bees comprising an isolated nucleic acid agent according to any one of paragraphs 4 to 13 or a nucleic acid composition according to either one of paragraphs 15 or 16.

FIGURES

Figure 1. Assessing the effect of different dsRNA targets on *Tribolium* larvae mortality. Larvae were microinjected with 100 nl of solutions containing 60ng dsRNAs coding for one of five Tribolium target genes, or dsGFP serving as a negative control. Larvae were maintained in Petri dishes with food at 23°C and 80% RH. Mortality was determined daily. Each treatment consisted of 2 Petri dishes containing 7-12 larvae each (n = 2), except for the GFP treatment where n = 3.

Figure 2. Assessment of the level of the gene knockdown in Tribolium larvae 72 hours after injection with 100 nl (60 ng) dsRNA coding for Tribolium V-ATPase subunit E or 100 nl saline. For each treatment the RNA from 2 larvae were assessed for transcript abundance by qPCR using RP6 as the normalising gene. Data are presented (mean ± SEM, n = 2) as relative to the expression observed in larvae administered with saline

Figure 3. Effect of different dsRNA treatments on Tribolium larvae mortality in Trial #1. Larvae were microinjected with 100 nl of solutions containing different dsRNAs and maintained in Petri dishes with food at 23°C and 80% RH. Mortality was determined daily. Each treatment consisted of 3 Petri dishes containing 7-10 larvae each (n = 3). The 'Mix' treatments contained of a mixture of individual dsRNAs coding for PMCA, Na+/K+-ATPase subunit alpha and ADP/ATP-translocase. The 'Tricat' treatments contained dsRNAs coding for those three genes, but concatamerized together into a single dsRNA. '1x' treatments were doses of 60 ng $larva^{-1}$. '3x' treatments were doses of 180 ng $larvae^{-1}$.

Figure 4. Effect of different dsRNA treatment on *Tribolium* larvae mortality in Trial one. Experimental details are given in legend to Figure 3, above. Effect of treatments on larvae mortality at 216 hours post-treatment was assessed initially by oneway-ANOVA and pairwise comparisons determined by Fisher's LSD. Treatments that do not share a letter are significantly different ($P<0.15$).

Figure 5. Assessment of the level of the gene knockdown in Tribolium larvae 72-96 hours after injection with various dsRNA preparations, as described for Trial # 1 in legend to Figure

3, above. For each treatment the RNA from 4 larvae were assessed for transcript abundance by qPCR using the RP6 as the normalising gene. Data are presented (mean ± SEM, n = 4) as relative to the expression observed in larvae administered with dsGFP.

Figure 6. Effect of different dsRNA treatments on *Tribolium* larvae mortality in Trial #2. Larvae were microinjected with 100 nl of solutions containing different dsRNAs and maintained in Petri dishes with food at 23°C and 80% RH. Mortality was determined daily. Each treatment consisted of 3 Petri dishes containing 7-10 larvae each (n = 3). The 'Mix' treatments contained of a mixture of individual dsRNAs coding for PMCA, Na+/K+-ATPase subunit alpha and V-ATPase subunit E. The 'Tricat' treatments contained dsRNAs coding for those three genes, but concatamerized together into a single dsRNA. '1x' treatments were total doses of 60 ng $larva^{-1}$. '3x' treatments were total doses of 180 ng $larvae^{-1}$.

Figure 7. Effect of different dsRNA treatment on Tribolium larvae mortality in Trial #2. Experimental details are given in legend to Figure 6, above. Effect of treatments on larvae mortality at 216 hours post-treatment was assessed initially by oneway-ANOVA and pairwise comparisons determined by Fisher's LSD. Treatments that do not share a letter are significantly different ($P<0.15$).

Figure 8. Effect of different dsRNA treatment on Varroa mite mortality. In groups of 10, mites were soaked overnight at 4°C in 40 µl 0.9% saline containing various dsRNA treatments. Subsequently, mites were maintained on *Apis mellifera* larvae in Petri dishes at 30°C and 85% RH. Each treatment consisted of three petri dishes containing 10 mites (n = 3). Effect of treatments on mite mortality at 105 hours post-treatment was assessed initially by oneway-ANOVA and pairwise comparisons determined by Fisher's LSD. Treatments that do not share a letter are significantly different ($P<0.05$).

Figure 9. Assessment of the level of the gene knockdown in adult Varroa 72 hours after overnight immersion in dsRNA preparations coding for AChE, MOA and V-ATPase C-subunit either presented as a 3.75 µg $µl^{-1}$ mixture (Mix 3.75) or as a 1.2 or 5 3.75 µg $µl^{-1}$ tricatamer (Tri 1.25 or Tri 3.75, respectively) . For each treatment the RNA from 3 mites was assessed for transcript abundance by qPCR using R18s as the normalising gene. Data are presented (mean ± SEM, n = 4) as relative to the expression observed in mites administered with dsGFP.

Figure 10. L4440-MOA-V-ATPC-ACHE-Tricatemer plasmid map: MOA, vATPc, and AChE targets are indicated

Figure 11. L4440-PMCA-NAK-ADP Tricatamer 1 plasmid map

Figure 12. *L.salmonis* L4440-ADP-vATP-AChE plasmid map

Figure 13. Effect of different dsRNA treatments on Tribolium larvae mortality Example 6. Larvae were microinjected with 100 nl of solutions containing different dsRNAs and maintained in Petri dishes with food at 23°C and 80% RH. Mortality was determined daily. Each treatment consisted of 3 Petri dishes containing 7-10 larvae each (n = 3). The Mix treatments contained of a mixture of individual dsRNAs coding for alpha-tubulin, HSP90 and ADP/ATP-translocase. The tricatemer treatments contained dsRNAs coding for those three genes, but concatamerized together into a single dsRNA. 1X treatments were doses of 60 ng $larva^{-1}$. 3X treatments were doses of 180 ng $larvae^{-1}$.

Figure 14. Effect of different dsRNA treatment on Tribolium larvae mortality Example 6. The Mix treatments contained of a mixture of individual dsRNAs coding for alpha-tubulin, HSP90 and ADP/ATP-translocase. The Tricatemer treatments contained dsRNAs coding for those three genes, but concatamerized together into a single dsRNA. 1X treatments were doses of 60 ng $larva^{-1}$. 3X treatments were doses of 180 ng $larvae^{-1}$. Effect of treatments on larvae mortality at 216 hours post-treatment was assessed initially by oneway-ANOVA ($P<0.0001$) and pairwise comparisons determined by Fisher's LSD. Treatments that do not share a letter are significantly different ($P<0.05$).

Figure 15. Effect of different dsRNA treatments on Tribolium larvae mortality Example 5. Larvae were fed food administered with 10 µl of either 1.25 or 3.75 µg/µl of different dsRNAs and maintained in Petri dishes at 23°C and 80% RH. Mortality was determined daily. Each treatment consisted of 3 Petri dishes containing 8 larvae each (n = 3). The Mix treatments contained of a mixture of individual dsRNAs coding for PMCA, Na^+/K^+-ATPase-α, and ADP/ATPtranslocase. The tricatemer treatments contained dsRNAs coding for those three genes, but concatamerized together into a single dsRNA.

Figure 16. Effect of different dsRNA treatments on Tribolium larvae mortality in Example 5. Larvae were fed food administered with 10 µl of either 1.25 or 3.75 µg/µl of different dsRNAs and maintained in Petri dishes at 23°C and 80% RH. Mortality was determined daily. Each treatment consisted of 3 Petri dishes containing 8 larvae each (n = 3). The Mix treatments contained of a mixture of individual dsRNAs coding for PMCA, Na^+/K^+-ATPase-α, and ADP/ATPtranslocase. The tricatemer treatments contained dsRNAs coding for those three

genes, but concatamerized together into a single dsRNA. Effect of treatments on larvae mortality at 96 hours post-treatment was assessed initially by oneway-ANOVA ($P<0.005$) and pairwise comparisons determined by Fisher's LSD. Treatments that do not share a letter are significantly different ($P<0.06$).

Figure 17. Effect of different dsRNA treatments on *Aedes aegypti* larvae mortality in Example 9. Larvae (ca. 50 per tube, 3 tubes per treatment, n =3)) were soaked in 75 µl water containing either 1.25 or 3.75 µg/µl of different dsRNAs for 2hr at 21°C and then transferred to 48-well culture plates of water containing 5mg/ml rat diet. Mortality was monitored daily. The Mix treatments contained of a mixture of individual dsRNAs coding for β-tubulin, Na^+/K^+-ATPase alpha subunit and ADP/ATP translocase. The tricatemer treatments contained dsRNAs coding for those three genes, but concatamerized together into a single dsRNA.

Figure 18. Effect of different dsRNA treatments on *Aedes aegypti* larvae mortality in Example 9. Larvae (ca. 50 per tube, 3 tubes per treatment, n =3)) were soaked in 75 µl water containing either 1.25 or 3.75 µg/µl of different dsRNAs for 2hr at 21°C and then transferred to 48-well culture plates of water containing 5mg/ml rat diet. The Mix treatments contained of a mixture of individual dsRNAs coding for β-tubulin, Na^+/K^+-ATPase alpha subunit and ADP/ATP translocase. The tricatemer treatments contained dsRNAs coding for those three genes, but concatamerized together into a single dsRNA. Effect of treatments on larvae mortality at 144 hours post-treatment was assessed initially by oneway-ANOVA ($P<0.001$) and pairwise comparisons determined by Fisher's LSD. Treatments that do not share a letter are significantly different ($P<0.05$).

DEFINITIONS

Percentage Identity

As used herein, the term "percentage sequence identity" refers to identity as measure over the entire length of the SEQ ID in question.

For example, a polypeptide comprising a sequence having 70% sequence identity to SEQ ID NO:1 would contain a contiguous polypeptide where:

(Number of amino acids identical to SEQ ID NO 1) / Total number of amino acids in SEQ ID NO 1 = 0.7

The percent identity of two amino acid or two nucleic acid sequences can be determined by visual inspection and mathematical calculation, or more preferably, the comparison is done by comparing sequence information using a computer program. An exemplary, preferred computer program is the Genetics Computer Group (GCG; Madison, Wis.) Wisconsin package version 10.0 program, 'GAP' (Devereux et al., 1984, Nucl. Acids Res. 12: 387). The preferred default parameters for the 'GAP' program includes: (1) The GCG implementation of a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted amino acid comparison matrix of Gribskov and Burgess, Nucl. Acids Res. 14:6745,1986, as described by Schwartz and Dayhoff, eds., Atlas of Polypeptide Sequence and Structure, National Biomedical Research Foundation, pp. 353-358, 1979; or other comparable comparison matrices; (2) a penalty of 30 for each gap and an additional penalty of 1 for each symbol in each gap for amino acid sequences, or penalty of 50 for each gap and an additional penalty of 3 for each symbol in each gap for nucleotide sequences; (3) no penalty for end gaps; and (4) no maximum penalty for long gaps.

Independently

As used herein, the term "independently" is used with reference to nucleic acid sequences within a single nucleic acid agent to indicate that the features of each sequence should be considered independently of any other sequences in a particular agent.

Thus, for example, *"an isolated nucleic acid agent comprising at least two nucleic acid sequences wherein each nucleic acid sequence independently has at least 80% sequence identity to at least 18 contiguous nucleotides encoded by SEQ ID NO.1, SEQ ID NO.2, SEQ ID NO.3, SEQ ID NO.4, SEQ ID NO.5, SEQ ID NO.6, SEQ ID NO.7, SEQ ID NO.8, SEQ ID NO.9, SEQ ID NO.113, or SEQ ID NO.10"* encompasses an isolated nucleic acid agent wherein (for example) one nucleic acid sequence has identity to SEQ ID NO.1 and another

has identity to SEQ ID NO.2. That is, both sequences do not have to have identity to the same SEQ ID (since they are independent).

Statistical Significance

Unless stated otherwise, the significance of overall treatment effect is assessed by oneway-ANOVA and, if there a significant effect is detected, pairwise comparisons are performed by Fisher's least significant difference method. Statistical analysis is performed using Minitab Vers 16.0.

Unless stated otherwise, significance is assessed at the $P<0.05$ level

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Following a description of the experimental methods employed by the present inventors, some particular embodiments of the invention will be discussed.

MATERIALS AND METHODS

V.destructor mite collection and husbandry

Varroa destructor (adult female) mites were collected from capped brood cells frames from *Apis mellifera* hives in York, England that had purposefully been left untreated for Varroa control. Prior to harvesting mites the frames were maintained at 27ºC in a 80% relative humidity environment, 15.5h : 8.5h, light:dark regime. Mites were attached ventral side down on double sided tape attached to Petri dishes and approximately 50 were harvested for synganglion in phosphate buffered saline (PBS) before being washed in sterile ice-cold PBS and pooled together in a 1.5ml eppendorf tube containing 200µl RNA-later (Sigma, Poole, UK). Prior to RNA extraction, an additional 450µl dissection buffer was added to sample tubes and centrifuged at 14000rpm for 15 min. Supernatant was removed and the synganglion washed with fresh PBS before a final centrifuge again at 14000rpm for 15 min. Supernatant was again removed and 600µl ZR extraction buffer added to each tissue sample. Total RNA was extracted using a mini-RNA isolation II Kit (Zymo Research, Orange, California, USA), as per manufacturer's instructions and eluted in 50µl water. RNA was co-precipitated with 1.5µl glycogen blue (NEB Biolabs, Ipswich, UK) and 2µl 3M sodium acetate in 95% ethanol and resuspended in 5µl of DEPC-treated water.

Methods to brood *Varroa* by artificial *in vitro* feeding have been tested. "Feeding units" utilising parafilm and artificial liquid food containing blue dye have been successful in showing that adult Varroa will feed as measured by the presence / absence after 48h of blue excretions. Adult *Varroa* have successfully lived in these chambers for up to 14 days although mortality is still high compared with mites living on fresh bee larvae.

Generation of a *Varroa destructor* cDNA library

3.5µl (0.5µg) of total *Varroa destructor* RNA was used for first strand cDNA synthesis. The construction of cDNA libraries was done using the SMART cDNA library construction kit (Clontech, St-Germain-en-Laye, France) according to the protocol provided by manufacturer, with some modifications. To determine optimal number of cycles, two identical amplification reactions were prepared. After the 10th amplification cycle the first reaction was stored on the ice, while the second one was used for the PCR cycle number optimization by removing 3µl samples from the reaction every two cycles until cycle number 20. Samples were checked by visualization on a 1.1% agarose gel. The optimal number of cycles with visible and equally represented products, in this case 20 cycles, was used for primary amplification. cDNA was proteinase K treated, followed by phenol:chloroform extraction and resuspension

in water. After SfiI digestion and size fractionation with Chroma Spin-400 column, the fractions were checked using agarose gel and pooled into large or medium libraries. Pooled cDNA was ethanol precipitated and eluted in 4ul of water. 3ul from each fraction was ligated into the λTripleEx2 vector and packed into phage using the Gigapack III Gold Packaging extract (Stratagene). Each un-amplified library was mixed with E.coli XL1 blue cells and top agar supplemented with X-gal and IPTG before being plated onto LB MgSO4 agar plates in serial dilutions of 1, 1:10, 1:100 and 1:1000. The large library consisted of 6.23 x106 colony forming units (cfu)/ml and the medium library 1.07 x107 cfu/ml with recombination of 94.3 and 96.3% respectively.

EST sequencing and target selection

600 randomly selected recombinant plaques (white) were picked as agar plugs into plates of 96-wells, each well containing 100µl of SM buffer (0.58% NaCl, 0.2% MgSO4 • H2O 0.05M Tris-HCl, pH 7.5, 0.02% gelatin). Four plates were picked from the large fraction library, two from the medium fraction library and an additional 24 clones from the large fraction library for initial quality control. PCR with vector-specific primers was carried out using SM buffer / picked plaques as templates. PCR was carried out in 96-well plates containing 25ul 2xBiomix (Bioline), 5ul template, 1ul (10ng/ul) each of PT2F1 (5'-AAGTACTCTAGCAATTGTGAGC-3') and PT2R1 (5'- CTCTTCGCTATTACGCCAGCTG- 3') and 18ul water to give a 50ul final reaction volume. Cycling conditions were 94'C for 15min followed by 33 cycles of 94'C for 1 min, 49'C for 1 min and 72'C for 1 min 20s. PCR products were sent to GATC (Konstanz, Germany) for PCR reaction clean up and sequenced using primer PT2F3 (5' – CTCGGGAAGCGCGCCATTGT- 3'). PT2F3 is upstream from inserted cDNA and downstream from PT2F1 primer used in initial PCR reaction.

Following sequencing the Expressed sequence tags (ESTs) were modified in silico. ESTs were trimmed of primer and vector sequences, clusterized and checked for sequence quality using Lasergene Seqman (Lasergene v8.03, DNAstar, Madison, USA). BLASTn, BLASTx and tBLASTx programmes were used within the program BLAST2GO to compare the EST nucleotide sequences with the nonredundant (NR) databases of the NCBI and to the Gene Ontology (GO) database (www.blast2go.org). Following analysis of results, transcripts were primarily classified as novel sequences, putative identity or unknown function. Transcripts with a putative identity were further divided into functional categories by analysing GO identity and homology to known genes. Putative targets were chosen from the annotated sequences obtained in the EST library and were resequenced.

In addition, other putative targets were postulated based on their likelihood of having critical function in Acari and the likelihood of being fast–acting with little chance of having alternative rescue pathways. The whole genome shotgun database for V. destructor proved unsatisfactory to mine for targets due to the preliminary nature of the database and annotation. Such targets were obtained by designing primers around conserved regions in homologues in public databases of related species including *Ixodes scapularis*, *Dermacentor variabilis* ticks and the *Metaseiulus occidentalis* and *Tetranychus urticae* mites. Primers were designed and employed in anchored-PCR reactions with the pooled Varroa synganglia cDNA library as a template. Utilising the cDNA library as the template allowed anchored-PCR reactions to be employed, thus enhancing the chances of success when forward and reverse primers were not totally accurate. Further, using a cDNA library constructed from the synganglia ("brains") permitted greater success when searching for low-abundant neural targets. Resultant PCR products were then sequenced and specific Varroa primers designed. BLASTn was carried out against the Varroa whole genome shotgun database using the NCBI BLAST servers to obtain accession numbers.

Preparation of dsRNA

dsRNA was prepared using a BLOCK-iT RNAi TOPO transcription kit (Invitrogen), according to the manufacturer's instructions. LacZ-dsRNA was prepared and used as a negative control. Briefly, PCR was carried out as described above using adult female *V. destructor* cDNA in conjunction with specific primers, or with control LacZ-plasmid and LacZ specific primers (LacZ-F2, ACCAGAAGCGGTGCCGGAAA and LacZ-R2, CCACAGCGGTGGTTCGGAT).

Products were resolved on an agarose gel, excised and purified using a Qiagen gel extraction kit (Qiagen, Crawley, UK). TOPO-T7 linker was ligated to target and LacZ reactions before a secondary PCR was carried out to gain sense and antisense templates. T7-RNA polymerase was used in transcription reactions with target templates to generate sense and antisense RNA. Finally, RNA strands were annealed and the resultant dsRNA purified and quantified in a micro-spectrophotometer (Nanodrop Technology Ltd). dsRNA was ethanol precipitated and resuspended in DEPC-treated water to a working concentration of 2.5 µg/µl and stored at -80°C.

Protocol of dsRNA injection and soaking

Adult female *V. destructor* were removed from capped brood cells along with associated bee larvae. Microinjections were carried out using pulled glass capillary needles in conjunction with a Harvard micro-injector system. Mites were placed on double-sided tape ventral side

up, and injected with 20 nl (2.5 µg/µl) of either VdGST-mu1-dsRNA or LacZ-dsRNA in either the soft tissue proximal to the anal region and postcoxal plate, or in the coxa IV region, as indicated in Figure 7. Needles were left in each mite for 1 - 2 min to reduce the expulsion of fluid from the wound and withdrawn slowly. Mites were left for 1 - 2 min to allow the injection site to "seal" then returned to Petri dishes containing 1 bee larvae per 4 mites. Dead or unhealthy looking mites were removed after 1 hour and mortality was monitored over 72 h in LacZ-dsRNA, VdGSTmu1-dsRNA and non-injected mites.

To assess non-invasive techniques for dsRNA delivery, mites were either completely immersed in dsRNA or were exposed to a droplet of dsRNA on their ventral carapace. For soaking experiments, adult mites were removed from capped brood cells and placed in 500 µl microfuge tubes containing 20 µl VdGST-mu1-dsRNA or LacZ-dsRNA (2.5 µg/µl) supplemented with either nothing, 0.9% NaCl, 0.2% Triton-X100 or both. Mites were soaked at 4ºC overnight before being removed, dried and placed in Petri dishes at 27ºC, 95% relative humidity with bee larvae. Alternatively, a sample of mites was exposed to dsRNA by attaching them to double-sided tape and placing a 1 µl drop of VdGST-mu1-dsRNA or LacZ-dsRNA (2.5 µl/µg) supplemented with either nothing, 0.9% NaCl, 0.2% Triton-X100 or both on the ventral carapace. Mortality was monitored for 48 h prior to collection and validation of knockdown.

V.destructor L4440-MOA-V-ATPC-ACHE Tricatemer construction

MOA, vATPc and AChE targets were assembled into a single assembly using the Gibson Assembly cloning kit (New England Biolabs). Initial PCR reactions to add overlapping assembly regions were carried out using 25µl Biomix (Bioline), 23µl water, 1µl (1ng/µl) of PCR4.1 plasmids containing either MOA, AChE or vATPc dsRNA target sequences and 1µl (2mM) respective target primers containing target and L4440 overlapping regions (Table 1). The following cycling conditions were used: 1 cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C and 45 s at 72°C. Products were resolved on an agarose gel and visualised by UV light to check product size prior to assembly. Reaction was assembled on ice with the following 2µl MOA, 1.5µl ATP, 1µl AChE and 0.5µl L4440 plasmid, 10µl Gibson Assembly Master Mix and 5µl RNAse-free water. Samples were incubated at 50°C for 60 minutes.

1µl of GA reaction was transformed into 200µl ribonuclease-III deficient E. coli HT115(DE3), plated onto LB agar containing 12.5mg/ml tetracycline and 100mg/ml ampicillin and incubated at 37°C for 36 hours. Multiple colonies were picked, grown overnight in LB broth containing 100mg/ml ampicillin at 37°C. Plasmids were purified using Qiagen miniprep

columns and sequenced to verify tricatemer insertion (Fig. 13). Glycerol stocks of positive clones were kept at -80°C.

GIB-MOA-FWD : tggatccaccggttcgaacccactagccgaaatggac
GIB-MOA-REV: tcctttcgtgacctccacccttaatagaaacg
GIB-vATPc-FWD: ggaggtcacgaaaggagcattttgtgcttgg
GIB-vATPc-REV: gcaactaattctcgacaaagagacgcagtgc
GIB-AChE-FWD: ttgtcgagaattagttgctcgccacgatatcattg
GIB-AChE-REV: cgtcacgtggctagctggcaagaggacttcccataag

Table 1. Gibson assembly primers

Insertion of targets into L4440 plasmid and expression bacteria

PCR was carried out using 25µl Biomix (Bioline), 23µl water, 1µl (1ng/µl) of PCR4.1 plasmids containing either MOA, AChE, vATPc or the tricatemer dsRNA target sequences and 1µl (2mM) respective target primers containing restriction enzyme BglII sites at 5'ends (Table 2). The following cycling conditions were used: 1 cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 56°C and 45 s at 72°C. Products were resolved on an agarose gel and visualised by UV light. PCR products were purified using a Qiaquick PCR purification kit. Restriction digests were carried out on the purified PCR products, as well as dsRNA expression plasmid L4440, using BglII restriction enzymes (Promega). Digested PCR and plasmids were ligated using a quick ligation it (New England Biolabs).
1µl (100ng) purified L4440 plasmids, containing individual target inserts, were transformed into 200µl ribonuclease-III deficient E. coli HT115(DE3), plated onto LB agar containing 12.5mg/ml tetracycline and 100mg/ml ampicillin and incubated at 37°C for 36 hours. Multiple colonies were selected, grown overnight in LB broth containing 100mg/ml ampicillin at 37°C. Plasmids were purified using Qiagen miniprep columns and sequenced to verify target insertion. Glycerol stocks of positive clones were kept at -80°C.

Production of dsRNA by E. coli HT115 (DE3)

Single colony stocks were grown overnight at 37°C in 5ml LB broth containing 12.5mg/ml tetracycline and 100mg/ml ampicillin. Each starter culture was diluted 100-fold with 2xYT broth containing 100mg/ml ampicillin only and incubated at 37°C until OD600 reached 0.4. T7 RNA polymerase was then induced by the addition of 0.4mM IPTG and incubated again at 37°C until OD600 reached 1.0.

Cells were harvested by centrifugation at 6000xg for 5 min and supernatant was discarded prior to dsRNA extraction with TRI-reagent (Life technologies). 1ml Tri-reagent was used per 107 bacterial cells. Briefly, cells were disrupted in Tri-reagent by pipetting and allowed to stand for 10 minutes. 0.2ml chloroform was added per ml Tri-reagent and samples were shaken vigorously for 20s before incubating at room temperature for a further 10 minutes. Samples were centrifuged at 12000 x g for 15 minutes and aqueous layer retained. An additional chloroform extraction was performed and RNA isolated by the addition of 0.5ml isopropanol per ml Tri-reagent. Precipitated RNA was pelleted by centrifugation at 12000 x g for 15 minutes. RNA pellets were washed in 75% ethanol and air dried prior to re-suspension in RNAse-free water. RNA was treated with RNAse A to remove endogenous bacterial ssRNA. To assess the dsRNA quality, Tri-reagent extracted dsRNA was digested with RNAse A or RNase III which specifically digest either ssRNA or dsRNA, respectively. The resultant RNAs were visualised by agarose gel electrophoresis. dsRNA purity and quantity was analysed by both Nanodrop ND-1000 and by comparison with dsRNA markers.

MOA dsRNA BglII For primer: atagatctgaacccactagccgaaatg
MOA dsRNA BglII Rev primer:atagatcttgacctccacccttaatagaaac
vATPc dsRNA BglII For primer: atagatctcgaaaggagcattttgtgct
vATPc dsRNA BglII1 Rev primer: atagatctctcgacaaagagacgcagtg
ACE dsRNA BglII For primer: atagatctaattagttgctcgccacgat
ACE dsRNA BglII Rev primer: atagatcttggcaagaggacttcccata

Table 2. Target L4440 insertion primers

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Tribolium castaneum, the red flour beetle, is an economically important pest of stored products, including grains. T. castaneum is a model organism that is easy to rear in large numbers, has a well annotated genome and is used extensively in food safety research. Tribolium demonstrate a strong, systemic RNAi response elicited by intra-hemocoel injection with dsRNA.

cDNA isolation and PCR of *Tribolium castaneum* targets

T.castanuem were purchased from Blades Biologicals (Edinbridge, UK) and maintained in bran, flour and yeast medium in temperature / humidity controlled chambers at 23°C and 80%RH.

Larvae and adults were removed from culture and the RNA extracted by Tri-reagent (Life Technologies), according to manufacturer's instructions. After isolation, 1.5 µg total RNA was DNase treated with 1 µl (2U) RQ1-DNase (Promega, Southampton, UK) and 1 µlRQ1 buffer and incubated at 37°C for 30 min. DNase-treated total RNA was incubated at 70°C with 0.5µg of oligo d(T)15 (Promega) in a total volume of 10 µl for 5 min. Material was snap-chilled on ice for 5 min prior to the addition of 5 µl 5×RT buffer, 1 µl dNTPs (25 mM each), 0.5 µl Bioscript-reverse transcriptase and DEPC water to 25 µl. The reaction was incubated at 42°C for 1 hour.

Primers were designed for seven Tribolium gene transcript targets (see Table 3):

1) Plasma membrane Ca2+ ATPase (PMCA, XP_008199852.1)
Plasma membrane Ca2+ ATPase (PMCA) is a transport protein found in the plasma membrane of cells. Its major function is to export calcium (Ca2+) from the cell across membranes, facilitating a vital role in regulating the amount of Ca2+ within cells and, thus, maintaining the much lower intracellular Ca2+ levels (µM) relative to the extracellular (e.g. blood) levels (mM).

2) Na+/K+ ATPase subunit alpha (NaK, XM_008198203.1)
Na+/K+-ATPase is found in the plasma membrane of eukaryotic cells where it acts as an antiporter-like enzyme; pumping Na+ out of cells while simultaneously pumping K+ inwards. Na+/K+-ATPase is vital in regulating and maintaining cellular resting potential as well as volume. It also functions as signal transducer. Na+/K+-ATPase places high energy demands on cells and is responsible for around 20% of cellular energy expenditure.

3) ADP/ATP translocase (ADP, XM_968164.3)
ADP/ATP translocase is a transporter protein that enables ATP and ADP to traverse the inner mitochondrial membrane. ATP produced from oxidative phosphorylation in the mitochondria is transferred via ATP-ADP translocase from the mitochondrial matrix to the cytoplasm, with ADP moving in the opposite direction. ADP/ATP translocase is vital to cellular energy production.

4) V-ATPase subunit E (vATPe, XM_965528.2)
Vacuolar-type H+-ATPases are a class of enzyme that couple energy from ATP hydrolysis to facilitate proton transport across intracellular and plasma membranes of eukaryotic cells. They play a variety of roles crucial for the function of cells and

organelles pH homeostasis. V-ATPase complexes are made of 13 subunits including units A-H, all of which are necessary for regulation and function.

5) Tubulin alpha-1 chain (aTUB, XP_966492.1)

α-tubulin is a small globular protein that together with β-tubulin makes up cellular microtubules that are vital in cellular cytoskeletal architecture and perform essential and diverse transport and structural functions within the eukaryotic cell.

6) heat shock protein 90 (HSP90, NP_001094067)

Heat shock protein 90 (Hsp90) is a chaperone protein that folds proteins and stabilises them against heat stress. Hsp90 also aids in protein degradation and has roles in cell signalling. Previous investigations demonstrated that HSP90 was plays a crucial role in development, indeed silencing by dsRNA is lethal at all developmental stages.

7) Ca^{2+}-transporting ATPase sarco/endoplasmic reticulum (SERCA, XP_966783.1)

Primer name	**Sequence**
TcPMCA_fwd	CAGGAAGCCGAAATTAAGAA
TcPMCA_rev	ACTTGCCATTCAGATTGGAT
TcNaK_fwd	TCTTGGCATCGTCTTAGCTG
TcNaK_rev	CTCATCCTTGACAGGCGAAT
TcADP_fwd	ATTACGCTCGTACTCGTTTG
TcADP_rev	CACCGTTCCTCATCTCATTC
TcATP-E_ fwd	AGGGACGCCACTGGTAAAGACGTT
TcATP-E_ rev	CCAAACAAGGCCGTACGAATTTC
Tc-SERCA_ fwd	TCTCATTCTTATCGCCAATG
Tc-SERCA_ rev	GAATCGGTCTCAGTCATCAA
Tc-atub_ fwd	CAAGGAAATCGTCGACTTGG
Tc-atub_ rev	TGAAGGCACAGTCAGAATGC
Tc-HSP_ fwd	GTGAGCCGCATCAAGCCTAAC
Tc-HSP_ rev	TATCAGCCTCGGCCTTCTGTC

Table 3 Primers for HSP90 and RP6 qPCR primers were from (Law 2011). Primers for ATPase are from (Whyard et al. 2009).

PCR was carried out using 25µl Biomix (Bioline), 23µl water, 1µl cDNA and 1µl (2mM) respective target primers. The following cycling conditions were used: 1 cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C and 45 s at 72°C. Products were resolved on an agarose gel and visualised by UV light. PCR products were visible for all targets at expected sizes except SERCA.

PCR products were purified using Qiagen PCR purification kit and eluted in 20µl water. These purified PCR products were used in a secondary PCR using primers containing T7-Promoter sites for single target dsRNA constructs and with overlapping region primers between targets for use in Gibson Assembly.

Production of *T.castanuem* tricatemer templates into plasmid by Gibson Assembly

Four tricatemers were designed that incorporated six viable targets in combination into single assembly plasmids (Tables 4 and 5) using the Gibson Assembly cloning kit (New England Biolabs).

Initial PCR reactions to add overlapping assembly regions were carried out using 25µl Biomix (Bioline), 23µl water, 1µl (1ng/ul) of purified PCR products of target sequences and 1µl (2mM) respective target primers containing target and L4440 plasmid overlapping regions (Table. 3). The following cycling conditions were used: 1 cycle of 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C and 45 s at 72°C. Products were resolved on an agarose gel and visualised by UV light to check product size prior to assembly. Reaction was assembled on ice with the following; 1µl PMCA, 1.5µl NaK, 1µl ADP/ATP translocase and 0.5µl L4440 plasmid, 10µl Gibson Assembly Master Mix and 6µl RNAse-free water. Samples were incubated at 50°C for 60 minutes.

1µl Gibson Assembly reaction was transformed into 200µl DH5α E. coli, plated onto LB agar containing 100mg/ml ampicillin and incubated at 37°C for 16 hours. Multiple colonies were picked, grown overnight in LB broth containing 100mg/ml ampicillin at 37°C. Plasmids were purified using Qiagen miniprep columns. Glycerol stocks of positive clones were kept at -80°C. Tricatemer dsRNA templates were produced by PCR using tricatemer plasmid template with T7-adapted forward and reverse primers for the outer target sequence regions. PCR conditions are as described above. T7-tricatemer PCR product was purified by Qiagen PCR purification kit and eluted in 32µl water.

Tricatamer	Targeted genes
Tc1	PMCA + NaK + ADP
Tc2	PMCA + NaK + vATPe
Tc3	aTUB + HSP90 + ADP
Tc4	aTUB + HSP90 + vATPe

Table 4

Tricatamer	Primer name	Sequence
Tc1		
"	gaT7-PMCA_fwd	actagtggatccaccggttcTAATACGACTCACTATAGGGAGACAGG
"	gaT7-PMCA_rev	gatgccaagaATCCAATCTGAATGGCAAGTTTCGTC
"	gaNAK_fwd	cagattggatTCTTGGCATCGTCTTAGCTGC
"	gaNAK_rev	cgagcgtaatATTCGCCTGTCAAGGATGAGTTG
"	gaADP-T7_fwd	acaggcgaatATTACGCTCGTACTCGTTTGGG
"	gaADP-T7_rev	ccacgcgtcacgtggctagcTAATACGACTCACTATAGGGAGAGAATGAG
Tc2		
"	T7-PCMA_fwd	actagtggatccaccggttcTAATACGACTCACTATAGGGAGACAGG
"	T7-PCMA_rev	gatgccaagaATCCAATCTGAATGGCAAGTTTCGTC
"	NAK_fwd	cagattggatTCTTGGCATCGTCTTAGCTGC
"	gaNAK_rev2	tggcgtccctATTCGCCTGTCAAGGATGAGTTG
"	gaATPase_fwd	acaggcgaatAGGGACGCCACTGGTAAAG
"	gaATPase_rev	ccacgcgtcacgtggctagcTAATACGACTCACTATAGGGAGACCAAAC
Tc3		
"	t7_tubulin_fwd	actagtggatccaccggttcTAATACGACTCACTATAGGGAGACAAGG
"	t7_tubulin_rev	tgcggctcacTGAAGGCACAGTCAGAATGCTC
"	hsp_fwd	tgtgccttcaGTGAGCCGCATCAAGCCTAAC
"	gahsp_rev4	cgagcgtaatTATCAGCCTCGGCCTTCTG
"	gaADP T7 fwd4	gaggctgataATTACGCTCGTACTCGTTTGGG
"	ADP_T7_rev	ccacgcgtcacgtggctagcTAATACGACTCACTATAGGGAGAGAATGAG
Tc4		
"	gat7_tubulin_fwd	actagtggatccaccggttcTAATACGACTCACTATAGGGAGACAAGG
"	gat7_tubulin_rev	tgcggctcacTGAAGGCACAGTCAGAATGCTC
"	gahsp_fwd	tgtgccttcaGTGAGCCGCATCAAGCCTAAC
"	gahsp_rev	tggcgtccctTATCAGCCTCGGCCTTCTG
"	gaATPase_fwd3	gaggctgataAGGGACGCCACTGGTAAAG
"	ATPase_rev	ccacgcgtcacgtggctagcTAATACGACTCACTATAGGGAGACCAAAC

Table 5

dsRNA was produced using T7 Ribomax kit (Promega) in conjuction with either T7-single targets or, for tricatemer production, T7-tricatemer PCR template. The following reaction was

assembled at room temperature; 40µl T7-ribomax buffer, 32µl T7-adapted template (1.7µg) and 8µl Ribomax enzyme. The reaction was incubated at 37°C for 2 hours, subsequently treated with 4µl RNAse-A and 1µl RQ1 DNAse with a final incubation at 37°C for 30 minutes. dsRNA was precipitated by the addition of 8.5µl 3M Sodium Acetate and 90µl Isopropanol, pelleted by centrifugation and washed with 70% EtOH. dsRNA purity and quantity was analysed using a Nanodrop ND-1000 microspectrophotometer and by comparison with dsRNA markers.

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Production of *Aedes aegypti* tricatemer templates into plasmid by Gibson Assembly

A tricatemer has been designed which incorporates three *Aedes aegypti* targets into a single assembly plasmids using the Gibson Assembly cloning kit (New England Biolabs) in a similar manner to that described above for the *T.castanuem* tricatamer. The Gibson assembly primers are shown in Table 6 below.

Primer name	**Sequence**
AaB_TUB_fwd	actagtggatccaccggttcGGAAATCATCTCCGACGAACATGG
AaB_TUB_rev	tagacgaagcCACGGTACTGTTGCGATCC
AaNaK_fwd	cagtaccgtgGCTTCGTCTACCCACTCGAC
AaNaK_rev	cgaggaacacCAGCATTCCCTTGGCAGTATC
AaADPt_fwd	gggaatgctgGTGTTCCTCGGTGTGACCTTC
AaADPt_rev	ccacgcgtcacgtggctagcACATGTGGGCGACAGTCATAC

Table 6

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Production of *Lepeophtheirus salmonis* tricatemer templates into plasmid by Gibson Assembly

A tricatemer has been designed which incorporates three *Lepeophtheirus salmonis* targets into a single assembly plasmids using the Gibson Assembly cloning kit (New England Biolabs) in a similar manner to that described above for the *T.castanuem* tricatamer. The Gibson assembly primers are shown in Table 7 below.

Primer name	Sequence
LsADPt_fwd	actagtggatccaccggttcTAGCTCAAACTGTGGCAGCATG
LsADPt_rev	ctcaaagacaAACAAGGACCAAGGCACATCC
LsvATP_E_fwd	ggtccttgttTGTCTTTGAGCGATGCTGACG
LsvATP_E_rev	gagagccatcCAACGACTTCATCCACATGCTC
LsAChE_fwd	gaagtcgttgGATGGCTCTCCAATGGGTAAAGAAC
LsAChE_rev	ccacgcgtcacgtggctagcCTTCGACCACGACGGATACTTTC

Table 7

A plasmid map of the assembled tricatamer is shown in Figure 12.

EXAMPLES

Example 1: Assessment of efficacy of *T. castaneum* gene targets by administration of the corresponding individual dsRNAs

dsRNA for individual targets (vATPe, PMCA, NaK, aTUB, ADP, HSP90) were diluted with 0.9% NaCl to 0.6µg/µl concentration.

For single target dsRNA, *Tribolium* larvae of similar age and class, approximately 4mm in length, were removed from culture medium by sieves, chilled on ice for approximately 5 minutes and attached to injection platform using restraints. Larvae were then micro-injected between abdominal tergits VI – VII with 100nl (60 ng) dsRNA and placed in groups of 7 -10 in 85mm vented Petri dishes containing culture medium. Petri dishes were kept in humidity controlled environments at 23°C 80%RH.

Any injured or dead individuals from the injection trauma were removed within the first 24 hours and not included within the dataset. Larvae mortality was monitored daily. Live larvae, for example those injected with vATPe were removed after 72-96 hours and placed in Tri-reagent at -80°C to test for the degree of gene knockdown by qPCR.

As described above, *Tribolium* larvae were microinjected with 100 nl containing 60 ng of dsRNA coding for one of the following *Tribolium* genes: PMCA, Na+/K+-ATPase alpha subunit, alpha-tubulin, ADT/ATP-translocase, heat shock protein 90 or a similar amount of dsRNA GFP, acting as the negative control.

By 72 hours post-injection, increased larvae mortality was observed for all treatments with *Tribolium*-specific dsRNA as compared to the dsGFP (see Figure 1). This increased mortality rate (compared the dsGFP) became more apparent over the 6.5 day period, confirming the induction of an effective RNAi effect by the dsRNA constructs.

Previously studies have shown that dsRNA coding for *Tribolium* vATPAe was lethal to *Tribolium* larvae when administered by feeding (Whyard et al., 2009). When 60 ng dsRNA for the similar coding region was injected into Tribolium larvae, a significant ($P<0.0003$) reduction of 78% in gene expression of the V-ATPase-E was observed (as compared to larvae injected with saline; see Figure 2).

Example 2: Comparison of the mortality induced by mixed versus concatemerised PMCA, NaK and ADP dsRNAs

dsRNA mixtures were prepared with 0.9% NaCl to either 0.2µg/µl individual PMCA, NaK and ADP ("Mix 1X" → total concentration 0.6µg/ul), or 0.6µg/µl individual PMCA, NaK and ADP ("Mix 3X" → total concentration 1.8µg/ul).

Tricatemer "Tc1" (PMCA-NaK-ADP) dsRNA was prepared with 0.9% NaCl to either 0.6µg/µl ("Tc1 1X") or 1.8µg/µl ("Tc1 3X").

Control dsRNA was GFP at 0.6µg/µl or 1.8µg/ul (see summary in Table 8 below).

Treatment	**dsRNA Concentration ($\mu g\ \mu l^{-1}$)**	**dsRNA injected (ng / larvae)**
dsRNA-GFP	0.6	60
dsRNA-GFP	1.8	180
dsRNA "Mix 1X"	0.6	60
dsRNA "Mix 3X"	1.8	180
dsRNA "Tc1 1X"	0.6	60
dsRNA "Tc1 3X"	1.8	180

Table 8

Tribolium larvae of similar age class, approximately 8mm in length, were removed from culture medium by sieves, chilled on ice for approximately 5 minutes and attached to injection platform using restraints. Larvae were then micro-injected between abdominal tergits VI – VII with 100nl dsRNA and placed in groups of 7 -10 in 85mm vented Petri dishes containing culture medium. Petri dishes were kept in humidity controlled environments at 23°C 80%RH. Larvae were allowed to recover with any injured or dead individuals removed within the first 24 hours. Larvae were then monitored every 24 hours for mortality.

Additionally, some individuals injected were removed after 72-96 hours and placed in Tri-reagent at -80°C to test for knockdown by qPCR. Overall treatment effect was assessed by oneway-ANOVA and, if there was significant effect detected, then pairwise comparisons were performed by Fisher's least significant difference method. Statistical analysis was performed using Minitab Vers 16.0.

Over the entire 9 days post-treatment period, there was a steady increase in the number of *Tribolium* larvae dying with any of the treatments involving dsRNA designed against any *Tribolium* gene compared to larvae injected with the negative control dsRNA GFP (see Figure 3). This indicates that the higher mortality of larvae treated with *Tribolium* gene-targeted dsRNA was a specific effect.

Statistical analysis was performed on data at time point 9 days (216 hours) post-treatment. A significant effect was detected of treatment upon larvae mortality ($P<0.023$, $F = 3.11$, df 5/12).

All the *Tribolium* gene-specific dsRNA treatments, irrespective of dose or whether presented as a mixture or tricatemer, caused higher mortality than the dsGFP at either dose (60 and 180 ng /larvae) treatments (Fig. 4). However, neither of the "Mix" doses produced significantly ($P>0.15$) higher mortality than the dsGFP negative controls.

In contrast, both the tricatamer concentrations assayed resulted in significantly higher mortality ($P<0.02$) than either concentration of the dsGFP controls.

The data also shows that each tricatamer formulation was significantly more effective than the corresponding "Mix" formulation with an equivalent dsRNA concentration. That is, the "Tc1 1X" causes significantly more mortality than the "Mix 1x" (survival rates 55.5 ± 5.3% vs. 73.0 ± 2.4%, $P = 0.032$) and "Tc1 3X" causes significantly more mortality than the "Mix 3x" (44.4 ± 8.0% vs 63.7 ± 11.1%, $P =0.055$).

Thus, the data consistently shows that the dsRNAs coding for 3 target genes was significantly more effective when administered in a concatamerized form, as opposed to a mixture of separate dsRNAs (for a given total dsRNA dose).

Example 3: Assessing level of gene knockdown of target genes in Example 2 using qPCR

One possible explanation for the increased mortality observed in Example 2 with the tricatamer treatments was that the tricatamer led to enhanced suppression of the target genes. In order to test this hypothesis, the degree of target gene knockdown was assessed in larvae 72 – 96 hours post-injection with dsGFP (60 ng), "Mix 1x", "Mix 3x", and "Tc1 1x".

Larvae were treated as per Example 2, and sampled 72 hours after treatment with either dsRNA-GFP, dsRNA mixtures or dsRNA-tricatamer, placed in 1ml Tri-reagent and kept at -80°C until use. RNA was extracted by Tri-reagent (Life Technologies) according to manufacturer's instructions. 1.5 µg total RNA was DNAse treated with 1 µl (2U) RQ1-DNase (Promega, Southampton, UK) and 1 µlRQ1 buffer and incubated at 37°C for 30 min. DNase-treated total RNA was incubated at 70°C with 0.5µg of oligo d(T)15 (Promega) in a total volume of 10 µl for 5 min. Material was snap-chilled on ice for 5 min prior to the addition of 5 µl 5×RT buffer, 1 µl dNTPs (25 mM each), 0.5 µl Bioscript-reverse transcriptase and DEPC water to 25 µl. The reaction was incubated at 42°C for 1 hour. Resultant cDNA was measured by microspectrophotometry using a Nanodrop 100 and adjusted to 0.25µg/ul.

The normalising reference gene employed in this study was ribosomal protein 6 (RP6, Accession # NM_001172390.1), as described by Whyard et al. (2009). Relative expression qPCR was carried out on an CFX-96 platform (Biorad) by Sybr-Green detection using iTaq reaction mix of 10µl Itaq buffer (BioRad), 4µl water, 5µl (5ng/ul) of template cDNA and 1µl (2mM) respective target and control RP6 primers (Table 9). The following cycling conditions were used: 1 cycle of 10 min at 94°C, followed by 45 cycles of 30s at 94°C and 30s at 60°C. Melting curve analysis was performed to confirm specificity of reaction products. Ct values were extracted by automatic adjustment from sample reaction curves in the linear phase. Knockdown was assessed by Biorad gene expression software (Bio-Rad CFX manager 3.1) using the 2-ΔΔCT method.

Primer name	Sequence
TcqPCRRpS6F	ACGCAAGTCAGTTAGAGGGTGCAT
TcqPCRRpS6R	TCCTGTTCGCCTTTACGCACGATA
Tc-qPCR_ADPtrans_F	GGTGCGACATCACTTTGCTT
Tc-qPCR_ADPtrans_R	TCCGTCCGATTTCACTGTCT
Tc-qPCR_PMCA_F	CTCCCAAGCAGGTATCATCTTC
Tc-qPCR_PMCA_R	GTTTTCATCGTCAGCACCTG
Tc-qPCR_NaKATPase_F	TAGCACCGTGGAGGAACC
Tc-qPCR_NaKATPase_R	TTCGAACTCTTGCTTTCTTG

Table 9

All dsRNA treatments targeting *Tribolium* genes significantly ($P<0.05$) reduced the expression of all the target genes (PCMA, ADP/ATP-translocase and Na+/K+-ATPase α subunit) relative to the GFP control (see Figure 5).

No consistent or significant difference was observed in the degree of gene knock-down achieved by "Mix 1x", "Mix 3x", and "Tc1 1x". Indeed, though larval mortality with the tricatemer treatment (survival 55.5 ± 5.3%) was higher than either the 60 ng dose (73.0 ± 2.4%) or the 180 ng dose mixture treatment (63.7 ± 11.1%), this was not reflected in the level of knockdown of any of the three target genes.

Example 4: Comparison of the mortality induced by mixed versus concatemerised PMCA, NaK and vATPe dsRNAs

The protocol described in Example 2 was followed, but with "Tc2" used in place of "Tc1".

dsRNA mixtures were prepared with 0.9% NaCl to either 0.2µg/µl individual PMCA, NaK and V-ATPaseE ("Mix 1X" → total concentration 0.6µg/ul) or 0.6µg/µl individual PMCA, NaK and V-ATPase-E ("Mix 3X" → total concentration 1.8µg/ul).

Tricatemer 2 (PMCA-NAK-V-ATPase-E) dsRNA was prepared with 0.9% NaCl to either 0.6µg/µl ("Tc2 1X") or 1.8µg/µl ("Tc2 3X").

Control dsRNA was GFP at 0.6µg/µl or 1.8µg/ul. (See summary in Table 10 below).

Treatment	**dsRNA Concentration ($\mu g\ \mu l^{-1}$)**	**dsRNA injected (ng / larvae)**
dsRNA-GFP	0.6	60
dsRNA-GFP	1.8	180
dsRNA "Mix 1X"	0.6	60
dsRNA "Mix 3X"	1.8	180
dsRNA "Tc2 1X"	0.6	60
dsRNA "Tc2 3X"	1.8	180

Table 10

As observed in Example 2, the data in example 4 shows that over the 9-day period that there is greater mortality in *Tribolium* larvae treated with dsRNAs targeting Tribolium genes, as compared the control dsRNA GFP (see Figure 6). This indicates that the higher mortality of larvae treated with Tribolium gene-targeted dsRNA is a specific effect. Over the 4.5 days post-treatment period, there was a steady increase in the number of mites dying with any of the treatments involving dsRNA designed against any mite gene (see figure 6). In contrast, little mortality was observed over the 4.5 day period in mites treated with either 1.25 or 3.75 µg/µl dsGFP, indicating that the high mortality of mites treated with mite gene-targeted dsRNA was a specific effect brought about by careful selection of the targets.

Statistical analysis was performed on data at time point 8 days (192 hours) post-treatment. A significant effect was detected of treatment upon larvae mortality ($P<0.0001$, $F = 12.38$, df 5/12).

All the Tribolium gene-specific dsRNA treatments, irrespective of dose or whether presented as a mixture or tricatemer, caused significantly higher mortality than the dsGFP at either dose (60 and 180 ng /larvae) treatments (see Figure 7).

The tricatamer formulations were more efficacious than the corresponding mixtures at killing larvae at both the "1X" dose (survival rates 47.6 ± 4.7% vs 57.1 ± 8.2%, $P = 0.182$) and the "3X" dose (38.1 ± 8.3% vs 50.6 ± 11.3%, $P =0.02$). Thus, the results of example 4 are consistent with those from example 2, showing as they do that the dsRNAs coding for 3 target genes are significantly more effective when administered in a concatamerized form, as opposed to a mixture of separate dsRNAs (for a given total dsRNA dose).

Example 5: Comparisons of the mortality rates with dsRNA for PMCA, NaK and ADP/ATPt administered in diet.

L4440-tricatemer were purified from glycerol stocks and 1µl (100ng) transformed into 200µl ribonuclease-III deficient E. coli HT115(DE3), plated onto LB agar containing 12.5mg/ml tetracycline and 100mg/ml ampicillin and incubated at 37°C for 36 hours.

Multiple colonies were selected, grown overnight in LB broth containing 100mg/ml ampicillin at 37°C. Glycerol stocks of E. coli HT115(DE3)-L4440-tricatemer positive clones were kept at -80°C.

Tricatemer-L4440 plasmid HT115 glycerol stock picks were grown overnight at 37°C in 5ml LB broth containing 12.5mg/ml tetracycline and 100mg/ml ampicillin. Each starter culture

was diluted 100-fold with 2xYT broth containing 100mg/ml ampicillin only and incubated at 37°C until OD600 reached 0.4. T7 RNA polymerase, then induced by the addition of 0.4mM IPTG and incubated again at 37°C until OD600 reached 1.0.

Cells were harvested by centrifugation at 6000xg for 5 min and supernatant discarded prior to dsRNA extraction with TRI-reagent (Life technologies). 1ml Tri-reagent was used per 10^7 bacterial cells. Briefly, cells were disrupted in Tri-reagent by pipetting and allowed to stand for 10 minutes. 0.2ml chloroform was added per ml Tri-reagent and samples shaken vigorously for 20s before incubating at room temperature for a further 10 minutes. Samples were centrifuged at 12000 x g for 15 minutes and aqueous layer retained. An additional chloroform extraction was performed and RNA isolated by the addition of 0.5ml isopropanol per ml Tri-reagent. Precipitated RNA was pelleted by centrifugation at 12000 x g for 15 minutes. RNA pellets was washed in 75% ethanol and air dried prior to re-suspension in RNAse-free water. RNA was treated with RNAse A to remove endogenous bacterial ssRNA.

To assess the dsRNA quality, Tri-reagent extracted dsRNA was digested with RNAse A or RNase III which specifically digest either ssRNA or dsRNA, respectively. The resultant RNAs are visualised by agarose gel electrophoresis. dsRNA purity and quantity is analysed by both Nanodrop ND-1000 and by comparison with dsRNA markers.

Feeding of dsRNA-tricatemer was carried out as described in Whyard et al. (2009). 10 mg of tribolium diet to be placed in 48-well plates, and 10µl of either the prepared 5 mg/ml dsRNA-tricatemer or a mixture of the three individual dsRNAs (PMCA, Na+/K+-ATPase-α, and ADP/ATPt) at total concentration of 5 mg/ml to be pipetted onto the surface of the food. As a negative control, similar diets were set up with equal amounts of dsGFP.

Tribolium larvae of similar age class, approximately 6 mm in length, were removed from culture medium by sieves and larvae (6-8mm) placed in each well, which is then sealed with parafilm M (Pechiney, USA), with pin holes for respiration. Survival of larvae was monitored every 24h with samples removed after 72h for qPCR analysis of knockdown. Treatment effects were determined by ANOVA and, if significant, pair-wise comparisons investigated by Fisher's LSD.

Results

Over the 9-day period there was greater mortality in *Tribolium* larvae fed diets containing dsRNAs targeting *Tribolium* genes compared to larvae fed with the irrelevant dsRNA GFP

(Fig. 15). This indicated that the higher mortality of larvae treated with *Tribolium* gene-targeted dsRNA was a specific effect.

Statistical analysis was performed on data at time point 4 days (96 hours) post-treatment. A significant effect was detected of treatment upon larvae mortality ($P<0.04$, $F = 3.53$, df 5/12).

Although mortality in larvae fed the dsRNAs mixture at 1X dose was not significantly different from the dsGFP negative control treatment, the dsRNA fed as a tricatemer did result in significantly higher mortality ($P<0.05$), regardless of dose (Fig. 16).

The dietary dsRNAs presented in the single concatemerized form rather than as a mixture of 3 individual dsRNAs was significantly more effective in killing the *Tenebrio* larvae at the 1X dose (survival rates 62.5 ± 7.2% vs 81.5 ± 5.1%, $P <0.05$).

Example 6: Comparison of the mortality induced by mixed versus concatemerised aTUB, HSP90, and ADP dsRNAs

The protocol described in Example 2 was followed, but with "Tc3" used in place of "Tc1" (see Tables 4 & 5 above)

dsRNA mixtures were prepared with 0.9% NaCl to either 0.2µg/µl individual aTUB, HSP90, and ADP ("Mix 1X" → total concentration 0.6µg/ul) or 0.6µg/µl individual aTUB, HSP90, and ADP ("Mix 3X" → total concentration 1.8µg/ul).

Tricatemer 3 (aTUB-HSP90-ADP) dsRNA was prepared with 0.9% NaCl to either 0.6µg/µl ("Tc3 1X") or 1.8µg/µl ("Tc3 3X").

Control dsRNA was GFP at 0.6µg/µl or 1.8µg/ul. (See summary in Table 11 below).

Treatment	**dsRNA Concentration ($\mu g\ \mu l^{-1}$)**	**dsRNA injected (ng / larvae)**
dsRNA-GFP	0.6	60
dsRNA-GFP	1.8	180
dsRNA "Mix 1X"	0.6	60
dsRNA "Mix 3X"	1.8	180
dsRNA "Tc3 1X"	0.6	60
dsRNA "Tc3 3X"	1.8	180

Table 11

As observed in Examples 2 and 4, the data in example 6 shows that over the 9-day period that there is a steady increase in the mortality of *Tribolium* larvae treated with dsRNAs targeting Tribolium genes, as compared the control dsRNA GFP (see Figure 13). This indicates that the higher mortality of larvae treated with Tribolium gene-targeted dsRNA is a specific effect.

Statistical analysis was performed on data at time point 9 days (216 hours) post-treatment. A significant effect was detected of treatment upon larvae mortality ($P<0.0001$, $F = 22.87$, df 5/12). All the *Tribolium* gene-specific dsRNA treatments, irrespective of dose or whether presented as a mixture or tricatemer, caused higher more mortality than either dose of the dsGFP (60 and 180 ng /larvae) treatments (Fig. 14).

The tricatamer formulations were significantly more efficacious than mixtures at killing larvae at both (a) the 1X (60 ng $larvae^{-1}$) dose (survival rates 66.7 ± 4.7% vs 47.6 ± 2.4%, $P = 0.023$) and (b) the 3X (180 ng $larvae^{-1}$) dose (50.0 ± 7.2% vs 34.5 ± 3.0%, $P <0.05$). Thus, tha data confirms that the dsRNAs coding for 3 target genes was significantly more effective when presented in a single concatamerized form rather than as a mixture of 3 individual dsRNAs, even though the equimolar amount remains the same.

Significantly, this result is consisten with that found in Examples 2 and 4, despite there being no overlap in gene identity in the three tested genes making up the tricatamer. This indicates that the observed increase in mortality upon upon concaterisation is not a gene-specific effect.

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Example 6: Assessing tricatemer's ability to cause gene knockdown of all three targets in *V.destructor*

Treatment of mites:

21 adult Varroa mites were removed from capped brood cells, maintained in humidity and temperature controlled environmental boxes in Petri dishes and with bee larvae to assess health. Active mites (18) were randomly divided into two groups and placed in 1.5ml Eppendorf tubes containing either 40µl of 1.25µg/µl dsRNA-GFP control in 0.9% NaCl or 1.25µg/µl dsRNA-tricatemer in 0.9% saline. Mites were soaked at 4°C overnight before being removed, dried and placed in Petri dishes. Mites were fed on similar aged developing

bee larvae (replaced every 24h) and maintained at 30°C and 85% RH. Mites were harvested after 72h and stored in RNAlater at -80°C for qPCR analysis.

Measuring gene knockdown of dsRNA-tricatemer targets using qPCR:
Mites were sampled 72hours after treatment with either dsRNA-GFP or dsRNA-tricatemer, placed in 100µl RNAseLater and kept at -80°C until use. Mites removed from RNAse later, washed briefly in cold PBS and homogenised with plastic pestles under 800µl RNA lysis buffer. Samples were further homogenised by repeatedly passing debris and tissue through 23 gauge needles attached to 1ml syringes. Mites were then processed according to ZR Tissue & Insect RNA MicroPrep Kit (Zymogen), DNAse-treated with RQ1 (Promega) and eluted in 10µl RNAse-free water.

RNA concentration of targets was measured by Nanodrop ND-1000 and 0.25µg RNA for each sample was used in reverse transcription reactions with oligo-dt and Bioscript (Bioline). Resultant cDNA was again measured by Nanodrop-100.

Relative expression qPCR was carried out on an Opticon 2 Engine (Biorad) by Sybr-green detection using reaction mix of 12.5µl Immolase DNA polymerase (Bioline), 10.5µl water, 1µl (1ng/µl) of template cDNA and 1µl (2mM) of the respective target or actin, used as a normalising reference gene. Primers (Table 11) were designed to hybridise to sequences of the cDNA that were external to the region of the dsRNA, thereby amplifying cDNAs derived from varroa mRNA but not amplifying the dsRNA itself. The following cycling conditions were used: 1 cycle of 15 min at 94°C, followed by 35 cycles of 45s at 94°C, 45s at 56°C and 45 s at 72°C. Melting curve analysis was carried out to confirm specificity of the reaction products. Ct values were extracted by manual adjustment from sample reaction curves in the linear phase. Knockdown was assessed by the $2^{-\Delta\Delta CT}$ method (Livak et al. 2001).

MOA Exf1 : ggacgacttcccacacttct
MOA Exr1 : tgccacccttcatcttcatt
vATPc exf1 : tccttacttgtgcgcaatct
vATPc exr1 : ccggtagtccatagcgaagt
AChE exf1 : aattagttgctcgccacgat
AChE Exr2 : gaaaatagccctttggcaag
Actin qPCR f1: catcaccattggtaacgag
Actin qPCR r1:cgatccagacggaatactt

Table 11. qPCR primers for determining knockdown of targets

Results:

Compared to mites soaked in GFP dsRNA, the mites soaked l 1.25µg/µl tricatemer dsRNA demonstrated a dramatic decrease (>98%) in their content of amplicons of all three targets, namely MOA, vATPc, and AChE 72hours after treatment (Table 12). It was noteworthy, that very similar levels of knockdown was observed for all three targets. This indicates that equal absolute amounts or, at least equal efficacy amounts, of dsRNA were generated for each of the gene targets using the 5' and 3' T7-flanked construct within the L440 plasmid. This is most notable for vATPc which sits in the centre of the construct (5'-T7-MOA-vATPc-AChE-T7-3') and might have been expected to have been generated in lower amounts.

Gene knockdown vs dsRNA-GFP control	**% reduction**	**Upper limit**	**Lower limit**
vATPc	98.1	99.6	92.0
MOA	99.7	99.8	99.5
AChE	98.2	98.9	97.1

Table 12: Knockdown for each individual gene target by the dsRNA tricatemer compared with dsRNA-GFP controls

Example 7: Assessing tricatemer's ability to kill mites and its effectiveness relative to MOA, AChE and vATPc singly or in combination

300 adult Varroa mites were removed from capped brood cells and then maintained in Petri dishes within humidity and temperature controlled environmental boxes with bee larvae to assess health. Active mites (270) were randomly assigned into groups of 10 and placed in 1.5ml eppendorf tubes containing 40µl 0.9% NaCl and treatments, as detailed in Table 13, giving 3 replicates of 10 mites per treatment. Mites were soaked at 4°C overnight before being removed, dried and placed in Petri dishes. Mites were fed on similar aged developing bee larvae (replaced every 24h) and maintained at 30°C and 85% RH. Mites were monitored for mortality over the subsequent 5 days. Overall treatment effect was assessed by oneway-ANOVA and, if there was significant effect detected, then pairwise comparisons were performed by Fisher's least significant difference method. Statistical analysis was performed using Minitab Vers 16.0.

Treatment	dsRNA Concentration ($\mu g\ \mu l^{-1}$)
0.9% NaCl control	0
dsRNA-GFP (1.25µg/µl)	1.25
dsRNA-GFP (3.75µg/µl)	3.75
dsRNA MOA (1.25µg/µl)	1.25
dsRNA vATPc (1.25µg/µl)	1.25
dsRNA AChE (1.25µg/µl)	1.25
dsRNA MOA + vATPc + AChE (1.25µg/µl each)	3.75
dsRNA-tricatemer (1.25µg/µl)	1.25
dsRNA-tricatemer (3.75µg/µl)	3.75

Table 13

Results

Over the entire 4.5 days post-treatment period, there was a steady increase in the number of mites dying with any of the treatments involving dsRNA designed against any mite gene (Fig. 14). In contrast, little mortality was observed over the 4.5 day period in mites treated with either 1.25 or 3.75 µg/µl dsGFP, indicating that the high mortality of mites treated with mite gene-targeted dsRNA was a specific effect brought about by careful selection of the targets.

At time point 4.5 days post-treatment, a significant effect was detected of treatment upon mite mortality ($P<0.0001$, $F = 16.75$, df 8/18). All the mite gene-specific dsRNAs caused significantly ($P<0.05$) more mite mortality than either the saline or the dsGFP (1.25 and 3.75 µg/µl) treatments (Fig. 8).

The tricatemer proved to be particularly effective at both 1.25 and 3.75 µg/µl concentrations. The tricatemer at 3.75 µg/µl resulted in very high mite mortality with low variation. Variation for the tricatemer at 1.25 µg/µl also showed very high mite mortality, but with much higher variation due to a restriction on the number of replicates which could be performed (limited mite numbers). It is anticipated that subsequent replicates will reduce the observed variation. Even without additional replicates, the tricatemer led to significant mite mortality, as described in more detail below.

At 3.75 µg/µl, the tricatemer was significantly more effective than the singly targeted AChE and vATPc dsRNAs (ds RNAs at1.25 µg/µl; $P<0.05$); at 3.75 µg/µl the tricatemer was also significantly more effective than the singly targeted MOA dsRNA (ds RNA at1.25 µg/µl; $P<0.07$).

Surprisingly, the tricatemer at 3.75 µg/µl was significantly more effective than the 3.75 µg/µl mixture of MOA + AChE + vATPc ($P<0.05$; Fig. 8). Consistent with the increased potency of the tricatemer versus a mixture of dsRNAs, the 3.75 µg/µl mixture of MOA + AChE + vATPc is not significantly better than the tricatemer at 1.25 µg/µl, despite having a three-fold higher dsRNA concentration. Indeed, the tricatemer at 1.25 µg/µl causes significantly more lethality than the 3.75 µg/µl mixture of MOA + AChE + vATPc ($P<0.125$).

Comparison to earlier V.destructor dsRNA studies

As noted in the introduction, previous studies of the transfer of dsRNA from *A.mellifera* hosts to *V.destructor* mites have been reported a decrease in mite population in tested mini-hives of up to 61% (Garbain et al. 2012).

The reported 61% reduction in mite population was recorded at the end of a 60-day trial period during which mites were exposed to a dsRNA mix containing 14 *V.destructor* sequences. The 60-day trial period allowed for two reproductive cycles of *V.destructor,* and the authors of Garbain et al. 2012 did not directly measure *V.destructor* mite mortality; thus, the 61% figure represents the combined effects of mortality and reduced fecundity over two generations of *V.destructor* mite.

In comparison, the results obtained using the nucleic acid agents of the present invention (see Figure 8) show that for each of the single gene dsRNA treatments of MOA, AChE, and vATPc a mite mortality of ~52% was recorded. This figure was directly recorded mortality (i.e. mite death) on a single mite generation. Repeated over two generations, this level of mite death would result in a reduction in mite numbers of at least $(1 - 0.48^2) = 77\%$.

For the MOA/AChE/vATPc tricatemer, a mortality of 71% was recorded. Repeated over two generations, this level of mite death would result in a reduction in mite numbers of $(1 - 0.29^2) = 92\%$ (Both this figure and the above figure of 77% considers only direct mite mortality: an even greater reduction would be seen if the likely reduction in mite fecundity was also accounted for).

In addition to increased potency, the ability to achieve high levels of mite mortality using a single, or a small number, of dsRNA sequences (as opposed to 14 different sequences) results in a range of handling and safety advantages. For example, fewer targets means a lower likelihood of "off target" gene silencing (that is, silencing genes other than the intended target(s)), and also reduces production costs and complexity.

Example 8: Comparing levels of gene knockdown achieved by the tricatemer relative to a mixture of MOA, AChE and vATPc singly or in combination

In order to assess if the enhanced kill rate of the tricatamer treatments was due to an increased knockdown of the target genes by this formulation, the level of gene expression in mites treated with dsGFP (1.25 µg/µl), the dsRNA mixture (3.75 µg/µl) and the dsRNA tricatamer (both 1.25 and 3.75 µg/µl) was determined.

As expected, all the treatments containing dsRNA targeting Varroa genes significantly reduced the expression of all the target genes (by 80 – 99%; see Figure 9). However, there was no significant or consistent difference between the levels of down-regulation observed with the different dsRNA formats or concentrations tested. In particular, it was there was no significant correlation between the level of mortality observed and the degree of knockdown of any of the targeted genes (AChE, MOA and V-ATPase; compare Figures 8 & 9).

This observation is what would be expected when the processes of dsRNA uptake and RNAi are not limiting the observed mortality.

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Example 9: *Aedes aegypti* soaking methods using the β-tubulin, Na+/K+-ATPase-α, and ADP/ATPt tricatamer

A further trial was conducted to see if the "conatemer effect" was effective in a dipteran (true fly). Trials were conducted on the yellow fever mosquito (*Aedes aegypti*) which is a medically important vector that transmits yellow fever, dengue fever and other important pathogens. The genes involved were β-tubulin, Na^+/K^+-ATPase alpha subunit and ADP/ATP translocase.

As described above, fragments of *Aedes aegypti* β-tubulin (XM_001655975.1), Na^+/K^+-ATPase alpha subunit (XM_001662168.1) and ADP/ATP translocase (XM_001649861.1)

were amplified from *A. aegypti* cDNA and then conjoined into a tricatemer by appropriate Gibson assembly primers. The resultant tricatmer was inserted into L4440 plasmid and then transformed into *E. coli* HT115(DE3). Tricatemer was produced by the *E. coli* HT115(DE3)-L4440, purified and quantified as described above and diluted to appropriate concentration in preparation for efficacy testing in mortality studies against *A. aegypti* larvae.

Treatment of animals:

Aedes aegypti were maintained in netted boxes in a secure insectary at 28°C, 40%RH, on a 16:8 L:D photoperiod. Females to be fed warmed sheep blood encased in stretched parafilm M (Pechiney, USA). Mosquito eggs to be allowed to develop to larval stage and then maintained on a ground liver powder and fish food.

Soaking:

Larvae were treated in groups of ~50 individuals in a final volume 75 µl with either (i) a mixture of three individual dsRNAs (β-tubulin, Na+/K+-ATPase-α, and ADP/ATPt, total = 1.25µg/µl), (ii) dsRNA-tricatemers as described above in material at 1.25µg/µl or 3.75 µg/µl in a 1.5 ml eppendorf tube, or (iii) dsRNA GFP at either 1.25 or 3.75 µg/µl..

Larvae were soaked in the dsRNA solutions for 2 hr at 21° C, transferred to 48-well tissue culture plates (also maintained at 21° C) and provided 5 mg/ml lab rat diet (Purina Mills, www.purinamills.com) suspended in water as a source of food on a daily basis. Survival and pupation rates of the larvae were monitored daily. Treatment effects were determined by ANOVA followed by pairwise comparisons by Fisher's LSD.

Results

Statistical analysis was performed on data at time point 6 days (144 hours) post-treatment (Fig. 17). A significant effect of treatment upon larvae mortality was detected ($P<0.0001$, $F = 17.69$, df 5/21). All treatments containing *A. aegypti* gene-specific dsRNA killed significantly ($P<0.05$) more mosquito larvae than the dsGFP negative control treatment (Fig. 18).

The dsRNA presented in the single concatemerized form rather than as a mixture of the 3 individual dsRNAs was significantly more effective at killing the mosquitoes at both the 1X dose (survival rates 43.7 ± 3.6% vs 59.5 ± 2.4%, $P <0.01$) and the 3X dose (survival rates 35.0 ± 4.7% vs 52.5 ± 4.7%, $P <0.03$).

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Example 10: *Lepeophtheirus salmonis* soaking methods using the ATPt, vATP, AChE tricatamer

Adult and pre-adult *Lepeophtheirus salmonis* lice to be collected from a commercial Atlantic salmon (*Salmo salar*) farm. Lice were maintained at 12ºC in sea water in 1 litre beakers with constant aeration. Water to be changed and dead lice removed daily. Egg strings to be carefully removed from any gravid female lice and kept in similar conditions to adults. After hatching, nauplius to be separated from the remaining eggs and allowed to develop into copepodid for dsRNA assays. All stages to be maintained in a 15.5h : 8.5h, light:dark regime.

Fresh copepodids (20 individuals) to be removed from aerated beakers and placed in 1ml eppendorf tubes in 75ul sterile sea water along with 10ul (1ug/ul) of either a mixture of three individual dsRNAs (PMCA, Na^+/K^+-ATPase-α, and ADP/ATPt, total = 1.0µg/µl) dsRNA-tricatemers at 1.0µg/µl and left at 4'C for 7h. Negative control larvae to be similarly treated with dsRNA-GFP. After 7h lice to be removed from dsRNA sea water and placed into 25ml seawater in Petri dishes. Mortality to be monitored every 24 hours. Lice to be removed at 72h to validate knockdown by qPCR. Treatment effects to be determined by ANOVA followed by pairwise comparisons by Fisher's LSD.

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Example 11: *Caenorhabditis elegans* feeding methods using the egl-30, pat-10, and bli-5 tricatamer

The *C.elegans* L4440-tricatemer described herein to be purified from glycerol stocks and 1µl (100ng) transformed into 200µl ribonuclease-III deficient E. coli HT115(DE3), plated onto LB agar containing 12.5mg/ml tetracycline and 100mg/ml ampicillin and incubated at 37°C for 36 hours. Multiple colonies to be selected, grown overnight in LB broth containing 100mg/ml ampicillin at 37°C. Glycerol stocks of E. coli HT115 (DE3)-L4440-tricatemer positive clones kept at -80°C.

Tricatemer-L4440 plasmid HT115 glycerol stock picks to be grown overnight at 37°C in 5ml LB broth containing 100mg/ml ampicillin. Bacterial cultures to be used to seed NGM/Amp/IPTG plates.

C.elegans to be put on each plate to feed, and their L4 or adult progeny scored for GFP expression in the intestine. Worms grown on plates to be monitored by microscopy at 100× magnification using the appropriate filter. OpenLab 3.1.7 software (Improvision) to be used for capturing images (100×) of live worms put on 2% agarose pads; the same exposure time (typically ~500 msec) is used for capturing images to be compared in the same experiment.

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SEQUENCES: target genes and constructs

GENE → *V.destructor* Acetylcholinesterase (AChE)
Database details → GenBank accession number ADDG01069748.1
Target sequence → SEQ ID NO.1

GGAATTAGTTGCTCGCCACGATATCATTGTGGTAATAATAAACTACCGCCTGTCTGTAATGGGTTTCC
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TAAAGGAGAATGCGCGAGCTTTAAATGGAGATCCAGATAAGTTCACCCTATGGGGCCAGTCTGCTGGG
CGTTTGCCGTCGGCTTCCTTATGGGAAGTCCTCTTGCCAAAGGGCTATTTTC

GENE → *V.destructor* Monoamine Oxidase (MOA)
Database details → GenBank accession number ADDG01053234.1
Target sequence → SEQ ID NO.2

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GCGAGTCAAT

GENE → *V.destructor* vATPase subunit C (vATPc)
Database details → GenBank accession number ADDG01035752.1
Target sequence → SEQ ID NO.3

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CONSTRUCT → L4440-MOA-V-ATPC-ACHE-Tricatemer (*V.destructor*)
Sequence identifier → SEQ ID NO.4
Notes → L4440 vector is shown in normal text
MOA target sequence is shown in BOLD text
V-ATPase target sequence is shown in ITALIC text
AChE target sequence is shown in UNDERLINED text

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Sequence identifier → SEQ ID NO.5
Notes → MOA target sequence

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Sequence identifier → SEQ ID NO.6
Notes → V-ATPase target sequence

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Sequence identifier → SEQ ID NO.7
Notes → AChE target sequence

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GENE → *T.castaneum* Plasma membrane calcium-transporting ATPase 1 (PMCA)
Database details → NCBI accession number XM_008201630.1
Target sequence → SEQ ID NO. 8

Target sequence is shown in BOLD UNDERLINED text

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CACCTGGCCCCCTCCTCCGCGAACAACTCGGATTTGCCCAAGCCCGTGCATGAGACTCGCATCTAGTG
CCGGTCCTGGCCGCAGAGCCGCGGCGCGCACCGCATGCATCATATCCGTTTCCGGTGTAGCTTGCCGT
TGCCGTTGCCGTTGCCGCCATAGCGTAGTCGCTCTGCCTCTGCTGCTCTGCTCTCTTGACCGCTCCAG
CGTCCCACCCCCGACACTTACCACCACCCCCACCAACCACTACCGCCCTGGATCCATCACCAATCAGA
CGATTTTATTATCTAGTAGTAGATGTTGCCTACCACTATTGCCATCCTTGTTATTATTATAACCCTAT
ACTACCCGACTATCCTTCTATCGTTTAGCTTAGCACTAAGTAGACTTTTTCTTACTTGACCAAATCTG
AGTATTTTGTATTATTTTGTACATAATTTCGTCGTTCACTGTAGACTAGCTGTATGTATAGCATTTTA
GATAGCCTTAGACCTCAGGTTAGTGGTAATGGAAACAAATATTTTATTTTTGAACTTGTTGAACGCAT
GCACACAACTGCGAGTCATCTGTATTGTGTGTCGACCGCCAACGACATGATCAGTGTTAATAATACAT
AAAAAAACAAGTCAAACGAAAAACGATCATGCGAAACGAATGATAAATTACATCAATTTTTATTTATT
ATTATTTTTGTATTCGGTCAGCAGGCCACATTACTTAATACTCAATTATCTAATTTATAAGATGTTAC
ATTTGTATATTGATGGTTAAAATTTCCTGTTCATTTTTTACGTCGAATATAACGTTTTTTTATACTA

GENE	→	***T.castaneum*** **Na/K ATPase alpha (NaK)**
Database details	→	**NCBI accession number XM_008198203.1**
Target sequence	→	**SEQ ID NO. 9**

Target sequence is shown in BOLD UNDERLINED text

ACTTTTAGTGGGTCCGCGCCCGTCGTCGCTGCTTCTAGTGCGATTTGTGTGCAGTGGTCGACATCACA
TGAAGTACAGTATTTAACACCACTCCCCGGGATATTATCACACAATCAGCATGGGGGAGTCACGGAGG
AAAAATAAGAAGGTCAGGAAAGCGGACGATTTAGATGATTTGAAACAAGAATTGGACATCGATTATCA
TAAAATCACCCCAGAAGAATTATATCAGAGATTCCAGACACATCCAGAAAATGGCCTCAGTCATGCGA
AAGCGAAAGAGAATTTGGAACGGGACGGACCCAATGCACTCACACCCCCAAAGACTACCCCCGAATGG
GTGAAATTTTGTAAAAATCTCTTCGGGGGTTTCGCTCTCTTATTGTGGATCGGCGCCATCCTCTGCTT
CATAGCCTATTCTATTCAGGCTAGCACCGTGGAGGAACCAGCCGATGATAATCTTTA**TCTTGGCATCG**
TCTTAGCTGCCGTTGTTATCGTTACAGxxGTATATTTTCTTATTATCAAGAAAGCAAGAGTTCGAAGA
TTATGGAGTCGTTCAAAAACATGGTCCCCCAATTCGCTACAGTGATCCGCGAGGGTGAAAAGCTGACC
CTCCGCGCGGAGGACCTGGTACTGGGCGACGTGGTCGAGGTGAAATTCGGTGACAGAATCCCAGCCGA
TATCCGAATCATCGAATCTCGCGGCTTCAAAGTAGACAACTCATCCTTGACAGGCGAATCCGAACCGC
AGTCCCGCAGTCCGGAGTTCACTCACGAGAACCCTCTCGAAACGAAAAACTTGGCGTTCTTCTCGACC
AACGCCGTCGAAGGCACTGCCAAAGGTGTTGTGATTAGTTGTGGTGACAATACCGTGATGGGTCGCAT
CGCCGGTCTCGCCTCCGGTCTGGACACCGGCGAGACGCCCATCGCCAAAGAAATCCATCATTTCATTC
ACCTCATTACTGGCGTGGCTGTTTTCCTCGGAGTTACCTTCTTCGTAATCGCCTTCATCCTCGGCTAC
CACTGGCTCGACGCTGTTATTTTCCTCATCGGTATTATCGTGGCGAACGTGCCCGAGGGGCTCCTCGC
CACCGTCACCGTGTGTCTCACCCTCACTGCTAAGAGGATGGCTTCCAAGAACTGCCTCGTGAAGAATC
TCGAGGCCGTAGAGACCCTCGGCTCCACAAGCACGATCTGCTCGGACAAGACCGGAACTTTGACCCAA
AACCGGATGACGGTAGCACACATGTGGTTCGACAATCAGATCATTGAAGCCGACACCACTGAAGACCA
GTCGGGAGTCCAATACGACCGCACAAGTCCAGGATTCAAAGCTTTGTCGCGCATTGCCACACTTTGCA
ACCGGGCTGAGTTCAAAGGGGGGCAGAACGACGTCCCGATCCTTAAACGCGAAGTCAACGGAGACGCC
TCTGAAGCCGCTCTCCTCAAATGCATGGAACTGGCTCTGGGCGACGTGATGTCCATCAGACGCAAGAA
CAAGAAAGTTTGCGAAATTCCCTTCAACTCGACCAACAAATACCAAGTTTCCATCCACGAGAACGAGG
ACGCGAGCGATCCTCGCCATATCCTTGTGATGAAGGGCGCTCCTGAACGAATCCTCGAACGCTGCAGC
ACGATCTTCATCTGCGGCAAGGAGAAAGTCCTGGATGAGGAAATGAAGGAAGCTTTCAATAACGCCTA
CTTGGAGTTGGGTGGTTTGGGCGAGCGTGTGCTCGGCTTCTGCGATTTTATGTTGCCCACTGATAAGT
ACCCAATTGGGTACAAATTCAATTGCGATGACCCCAACTTCCCGTTGGATGGTTTGAGATTTGTTGGC
TTGATGTCCATGATTGATCCTCCCAGAGCTGCAGTGCCTGACGCCGTTGCTAAATGCAGAAGTGCCGG
TATTAAGGTCATTATGGTGACGGGAGATCACCCGATTACGGCCAAGGCTATTGCAAAGTCGGTTGGGA
TTATTTCGGAGGGTAACGAAACGGTTGAAGATATTGCTCAACGGTTGAATATTCCTGTCTCGGAAGTC
AACCCGAGGGAAGCCAAAGCTGCCGTTGTTCACGGATCTGATCTCAGAGACCTATCTTCCGATCAATT
AGACGAAATTTTGAGATACCACACTGAAATTGTATTCGCTAGAACCTCGCCGCAACAGAAGTTGATCA
TCGTCGAGGGGTGCCAACGGATGGGCGCTATTGTCGCCGTGACAGGCGACGGCGTGAACGACTCGCCG
GCTTTGAAGAAGGCGGACATCGGTGTGGCCATGGGTATCGCGGGTTCGGATGTGTCCAAGCAAGCCGC
CGACATGATCCTGCTGGACGATAACTTCGCGTCGATCGTGACAGGAGTGGAGGAAGGCCGTTTGATCT

TCGATAACTTGAAGAAATCTATTGCCTACACCTTGACCTCAAACATTCCCGAAATCTCGCCTTTCCTT
GCTTTCATTTTGTGCGACATTCCTTTGCCTCTCGGTACCGTAACAATTCTGTGCATCGATCTTGGAAC
TGACATGGTGCCTGCTATTTCTCTGGCTTACGAAGCCCCGGAGTCCGACATAATGAAACGTCAGCCGC
GCGACCCCTATAGGGACAACCTGGTTAATCGCAGGTTGATTTCGATGGCATACGGCCAGATTGGTATG
ATTCAAGCAGCTGCTGGTTTCTTCGTGTACTTTGTCATCATGGCTGAGAACGGCTTCCGCCCGACTGA
CTTGTTCGGTATTCGAAAGCAATGGGACTCGAAAGCTGTCAATGATCTCACAGATTCGTACGGTCAGG
AATGGACTTATCGGGACAGGAAGACATTGGAATACACTTGCCACACTGCATTCTTCGTGTCCATCGTG
GTTGTCCAATGGGCCGATTTGATCATTTGTAAGACCCGTCGCAATTCGATCCTCCACCAGGGAATGCG
TAACTGGGCGCTCAACTTTGGTTTGGTTTTCGAAACTGCACTCGCAGCCTTCCTGTCGTACACTCCCG
GGATGGACAAGGGTCTGCGCATGTTCCCGCTCAAGTTTGTGTGGTGGTTGCCTGCAATTCCGTTTATG
TTGTCCATCTTCATTTACGACGAAACCCGTCGGTTTTATTTGCGTCGCAATCCAGGAGGTTGGCTGGA
ACAGGAGACCTACTATTAAGCGCATCACACCGTTTTGCGTACTGCCACCAGGTGGCACTGCGTCTGCT
GCCGCAGGTCACAAATAACAACAAACAAAACAAAACAAAAAATCACCACACTCGATTGGAAGGACTCC
GTTTCACCCGCCGTGACGGCGTTGTTCCTTATCGGTCGTTGTGTTGTACAAACAAATCCTGTTGCTAT
TTCTAGTAAAATTTGCTGCATGCTTACGCTGCAGTCCGTACATTTGTAATTCGAGAGTTTGGTCGTGT
GCGAGGGCGAGTGAATGGGTGATAGACCGAGAGCGAGGATGATTGCAAGCGGGGCCAGTTGGATTGGT
TGGTTGGTTGGTCTGCAAGAGCAGCAGTAGAGACGCAATAGTGTTTTCATACATTCCAAGTTGAAAAC
AGTTGCCTTAGAGCCGATGTCGGACTGTGTGCGAGTGGGTGGGGGCGGAGCCTTGACGGACGGCGGTT
GGTTGTTGCGCGGCCTTCGGGGCTCCGCCACTGAACGTCTTGAACGAGGTAGTATTATTTAGGCTTTC
AACACTTAGGTTAGCTAGATTGTAACTGTGTTGTTATATCGTGTTAAGTGTCATACCAGTCGTGCTCA
ATACACATGACTACCAACTACTATCACGATTGTAAACGTTTGACATTCGTTTCGTTTGTTGTGTATAT
AGTTTACGTAACGGGCGGGTAGCAGTTACACTGTGTAGGCAGCACGAGTCCATAATATTGTTTCTAAC
ATTGTTACGTTACGTGCGGTTAGTTGTTCAACGTTGGAGACACACTTCTGGAGGTCTGCTTCTGTAGA
GCCCTATTTTCTTATACTCTCTGATGCGCGCTGCGTTGGTCTGAGACCAGCCGACCACGGTATTGTTA
TCTTATTATTGTAAATATTTTTAAGTGATCACTTAATTATTTTTGCTTGTGTTCTTTTCACATTCATG
TTTTAGTATGTAACGAAGCTGTATAAATTTGGGTTTTAATAAAATGGATGATAGTATTACATTACAAA

GENE → *T.castaneum* ADP/ATP translocase (ADP)
Database details → NCBI accession number XM_968164.3
Target sequence → SEQ ID NO. 10
Target sequence is shown in BOLD UNDERLINED text

GCTCGTTCTGTCAAAACTGCCTCTTTCGTTCCCGAACAACCATCCGGTGTTAACGTGTACGAGTGTAA
AAACCTTCAAAATGCCCACTGATCCCATGAGTTTCGCCAAGGATTTCCTCGCTGGGGGCATCTCGGCG
GCTGTGTCCAAGACAGCGGTGGCCCCCATCGAGCGCGTGAAGCTCCTCCTCCAGGTCCAAGCCGCTAG

CAAGCAAATTGCCGCCGACAAACAGTATAAAGGGATCATCGATTGCCTGGTCCGTATCCCCAAAGAAC
AGGGATTCTTCAGTTTCTGGCGTGGAAATCTCGCCAACGTGATCCGTTATTTCCCAACCCAGGCATTG
AACTTCGCCTTCAAGGATGTCTACAAACAGATGTTCTTGGGCGGTGTTGACAAAAACACCCAATTTTG
GAGGTATTTCGCCGGTAATTTGGCGTCAGGTGGTGCCGCTGGTGCGACATCACTTTGCTTCGTCTACC
CTCTAG**ATTACGCTCGTACTCGTTTGGGCGCCGATGTCGGCAAAGGCAAGGGCGAAAGGCAGTACACC**
GGCCTTCTGGACTGCATTAAGAAGACAGTGAAATCGGACGGACCGATCGGTTTGTACCGAGGTTTCGT
TGTCTCAGTGCAAGGTATCATCATCTACCGTGCCTCCTACTTCGGCTTCTTCGATACTGCCAAGGGAA
TGTTGCCCGATCCCAAGAACACACCGTTCCTCATCTCATTCCTTATTGCACAGTGCGTAACGACAGTT
TCTGGAATTACGTCATATCCATTCGACACCGTCAGAAGGCGTATGATGATGCAGTCTGGACGCGCTAA
AGCTGATATTATGTACAAGAATACGTTGGATTGCTGGATCAAGATCGGCAAAACCGAAGGCCCAACTG
CCTTCTTCAAAGGAGCGTTCTCTAACGTTCTCCGTGGCACTGGCGGAGCTTTGGTTCTTGTACTATAC
GACGAGCTTAAAGCTTTGCTCTAAACAGAAATAGTAGAATTATTACGGTTTAAATTATTAATTGTCTC
ATAATTTATTTGTTTCATTCCGTGGTTGATGCATTTTTTAGGCCGACATTCCTTTTTTTAACACTATC
AGGCGCAGGAATTTACATTCCAGCAATTTTTTTTCGTTACACGGTTTTAATAATGGCATTGTAAGCTG
AAGTATTGATAGCCCTGTATTTTAAATCCTGTATATTTTAACAGCCGTTTACCAATAAACAGTTGTGA
TAAGTTACTTTA

GENE	→	***T.castaneum* vATPase subunit E (vATPe)**
Database details	→	**NCBI accession number XM_965528.2**
Target sequence	→	**SEQ ID NO. 11**
		Target sequence is shown in BOLD UNDERLINED text

GTCAAAATCATATGATCAATCAGTCTGTCACACTTTTGAGACAAGTTTTACTATAAAGGCGTTGTCTA
AGGTGTCCCGTGAATTCAACCAATTTATTTCATTTAAAAAGTGTTAAAAACAATCTCCAACAATGGCA
CTAAGCGATGTCGACGTCCAAAAACAAATCAAGCATATGATGGCTTTCATTGAGCAAGAAGCCAATGA
AAAAGCCGAAGAAATCGATGCGAAAGCTGAGGAGGAGTTTAACATTGAAAAAGGGCGCCTGGTCCAAC
AACAGCGCTTGAAGATCATGGAATATTACGAGAAGAAGGAGAAACAGGTGGAATTGCAGAAGAAAATT
CAGTCGTCAAACATGCTGAACCAAGCCCGTTTGAAAGTATTAAAAGTGCGTGAAGACCACGTCCACAA
TGTGCTGGATGACGCCCGCAAACGTCTGGGCGAAATCACCAATGACCAGGCGAGATATTCACAGCTTT
TGGAGTCTCTCATTCTCCAGAGTCTCTACCAGTTGTTTGAGAACAATATAGTGGTGAGAGTCAGGCAA
CAGGACAGGAGTATAATCCAGGGCATTCTCCCAGTTGTTGCGACGAAATAC**AGGGACGCCACTGGTAA**
AGACGTTCATCTTAAAATCGACGATGAGAGCCACTTGCCATCCGAAACCACCGGAGGAGTGGTTTTGT
ATGCGCAAAAGGGTAAAATCAAGATTGACAACACCTTGGAGGCTCGTTTGGATTTAATCGCACAGCAA
CTTGTGCCAGAAATTCGTACGGCCTTGTTTGGACGTAACGTCAACCGTAAATTCACCGATTAAATATT
ATCAAGACAATTTTTCATCTCGTTAAAAATAACATTTTTACTGTAATTCCAAGCATTTTTAATGCACC

ACCATAATGTAAAATAAAATTGTTGCTTACTGTACCAATGTTGTATATTAAATTATTTAGAATTGTAT
TAAGAAGTATTCCATTTTTTGTGTAGTTGCGTTTGTAGCTATTCAAGGTCGTGGTGGTTGGTAACCTC
ATGTAATCAAATGTACAACCCCATTTGTAAATAGATGGTTTTATGTTGAAACAATACATGTTACAAAT
TA

GENE → *T.castaneum* Calcium-transporting ATPase sarcoplasmic /endoplasmic reticulum type (SERCA)

Database details → NCBI accession number XM_961690.3

Target sequence → SEQ ID NO.12

Target sequence is shown in BOLD UNDERLINED text

GGACGTGCCGATGATAAACTGCATGTTATCTGCCTCCTTAAAATAAGTGCAAGGCATGTGCGTCTATT
TCGGTAATTTGGAAATTGCGGCGCGCGTTCAAATCGGCGAAAATGTAAAATGCGGGCGCTTAGGGGAG
GCCCGAAGGCCCGGGTTGGCACTTTGCCCATGGACAGCGTCCCCGACAGGTAGCTACCGAGCTTATAA
AAGCCCCGACAAACTTCGTCCGGCGCCCGTTCGTTTAGCAGCTTAGTACACGTTGCGCTCATCCATGG
GGGCCTAACCACTTCGTGTTAGTTTTTATTTATACAAAGCGTTATCGAGTGATACGACTGGGACCACA
ATTGTGATAGCGAAGTGACTGACAAACACCATGGAGGACGGACACACCAAAACGGTGGAAGAAGTATT
AAACTATTTCAATACCGACCCAGAACGGGGGCTCACCTTAGATCAAGTCAAAAGAAACCAAGAAAAAT
ATGGACCCAATGAACTTCCAGCGGAAGAAGGAAAGTCCATTTGGCAATTAGTTTTAGAACAGTTCGAT
GATCTACTAGTCAAGATTTTATTGTTGGCCGCCATTATTTCATTCGTTCTCGCTTTATTTGAAGAACA
CGATGGAGCTTTCACCGCTTTCGTAGAACCTTTCGTTATTCT**TCTCATTCTTATCGCCAATGCAGTCG**
TCGGTGTCTGGCAGGAACGAAATGCCGAATCGGCCATTGAAGCGCTCAAAGAGTACGAGCCCGAAATG
GGCAAAGTCCTCCGCGGCGACAAATCCGGCGTCCAGAAAATCCGGGCGAAAGAAATCGTCCCCGGAGA
CATCGTCGAGGTCTCCGTCGGCGACAAAATCCCCGCTGATATTCGTCTAACAAAAATCTTTTCGACAA
CTTTGCGCATTGATCAGTCGATTTTGACCGGAGAATCGGTCTCAGTCATCAAACACACCGACGCTATT
CCCGACCCACGTGCCGTCAACCAGGACAAGAAAAACATCCTCTTCTCGGGTACCAATGTAGCGGCTGG
CAAGGCACGTGGTGTTGTCGTTGGCACCGGCTTGAACACTGCGATCGGTAAGATTCGTACCGAAATGT
CCGAAACTGAGGAAATCAAAACGCCGCTGCAACAAAAACTTGACGAGTTTGGCGAGCAATTGTCGAAG
GTTATTTCTGTGATTTGTGTCGCTGTTTGGGCCATCAATATTGGGCATTTTAACGATCCGGCCCATGG
CGGGTCCTGGATCAAGGGTGCTGTCTATTACTTTAAAATTGCCGTTGCCCTGGCTGTGGCTGCGATTC
CCGAGGGCTTGCCTGCTGTTATTACGACTTGTCTGGCTTTGGGCACGCGCCGTATGGCCAAGAAGAAC
GCAATTGTTAGGTCACTACCGTCTGTTGAAACCCTGGGTTGCACTTCGGTCATCTGTTCGGACAAGAC
CGGCACTTTGACCACCAATCAAATGTCCGTTTCGCGCATGTTCGTGTTCGAGAAGGTTGAGGGTAGCG
ATAGCAGTTTCCATGAGTTTGAAATCACCGGTTCGACGTACGAACCAATCGGCGAGGTTTTCCTCAAA
GGCCAGAAGGTCAAGTGTTCTGAATACGAAGGTCTGCAAGAACTTGGCGTTATCTGCATTATGTGCAA

CGACTCTGCCATCGATTTCAATGAGTTTAAGCAAGCGTTCGAGAAGGTCGGTGAAGCTACCGAGACTG
CGCTGATTGTCCTGGCCGAGAAGATGAATCCGTTCCAAGTCACCAAGGCTGGTGATCGTCGCCAGACG
GCCATTTGCGTGCGCCAGGACATTGAGACCAAGTGGAAGAAGGAGTTCACGCTGGAGTTTTCGCGCGA
TCGCAAATCGATGTCTTCCTATTGTGTTCCTTTGAAGCCCTCGCGTCTGGGTAATGGTCCTAAGCTGT
TCGTTAAAGGTGCCCCTGAAGGTGTGCTCGAGCGGTGCACGCATGCCCGTGTTGGTACCCAGAAAGTT
CCTCTTACTAACACGCTCAAGAACCGGATTTTGGATTTGACGAAAGTTTACGGTACTGGACGGGACAC
TCTCCGTTGTCTTGCGCTTGCGACCGGCGATAACCCGATGAAGCCCGAAGAGATGGACTTGGGTGATT
CCACCAAATTCTACACTTATGAAGTTAATCTCACCTTTGTGGGTGTTGTGGGGATGTTGGATCCTCCA
CGTAAGGAAGTTATGGATTCGATTGCCAGGTGCCGGGCGGCTGGTATTCGGGTTATTGTTATCACTGG
TGATAATAAGGCTACTGCTGAGGCTATCTGCAGACGTATTGGTGTCTTTACGGAAGATGAGGATACAA
CTGGAAAATCTTTCTCTGGAAGGGAATTTGACGATTTGAGTCCGGCTGAACAAAAGGCCGCCTGTGCC
AAAGCCAGGCTGTTCTCACGTGTGGAGCCCGCTCACAAATCCAAGATTGTTGAATATTTGCAAAGCAT
GAACGAAATTTCCGCTATGACTGGTGATGGTGTCAACGACGCCCCAGCCTTGAAGAAGGCCGAGATTG
GCATTGCCATGGGTTCTGGAACGGCCGTCGCTAAATCAGCCTCTGAGATGGTCTTGGCCGACGATAAC
TTCTCGTCCATTGTAGCAGCGGTTGAAGAAGGTCGCGCCATTTACAACAACATGAAACAGTTCATCCG
TTACCTGATTTCCTCGAACATCGGTGAAGTCGTATCAATTTTCTTGACGGCTGCTCTTGGTCTTCCCG
AAGCTTTGATCCCCGTACAACTTTTGTGGGTCAATTTGGTAACTGACGGTCTCCCCGCTACTGCATTA
GGTTTCAATCCACCCGACTTGGACATCATGTCAAAACCGCCCAGAAAAGCCGACGAATCGCTCATTTC
CGGCTGGTTGTTCTTCAGGTATCTCGCAATTGGTGGCTATGTCGGTGCTGCAACTGTTGGTGCTGCCG
CCTGGTGGTTTATGTACTCGCCTGAAGGCCCACAAATGAATTATTACCAATTGACTCATCACTTGCAA
TGCATCAGCGGTGGGCCTGAATTCAAAGGTATCGACTGCAAGGTCTTCAACGATCCTCATCCCATGAC
CATGGCTCTCTCTGTACTCGTAACTATTGAAATGCTGAACGCTATGAACAGCTTGTCTGAGAACCAGT
CGTTGATTGTCATGCCCCCATGGTCCAACTGGTGGTTGATGGGCTCGATGGCTCTGTCCTTCACCCTT
CATTTTGTTATTCTTTACATTGATGTCTTATCCGTTGTGTTCCAAGTGTGTCCATTGACCGGAGACGA
GTGGTTAACTGTAATGAAATTCTCAATTCCAGTAGTATTACTTGATGAAACGCTCAAATTCGTCGCAA
GAAAGATCACAGATGGTGAGAGTCCAATTTACACTGTGCATTGGATTGTACTAATGTGGGCCGTTTTC
TTTGGTTTACTGTGTGTGAGCCCCATCTAAGTGTGACTGCCTCCTTGGTCGAGGACAGTGTTAGTGCG
TAAACGGGTGTCAATAGTAGGACCAACTTTTTTTCACTTAATTTGTTTATATCAAGTGCCCTTGATTG
ATTGATTATCAAAAAAAAGTAATAAAAAGTGCAGTTTTAAATTAGATTTTTAATAATGGGATGGGTTA
GGTTAACGCTTTGAATACTGAATCTCGTTTACTCACTAACTGTGCTACATAACTCTGCTAGCGCCCCC
TAGTACCCTAAGAACAGGCGTCTTCCAGCACCTCGTTCAAGGCATGCCTCATGCCTGTGATCAATAAC
AAGTGCATGCCTTGAACGCGATCGACAAGAACAGGCAAGTTGGTAAACCTCAAGTGTTGTGATCATAG
ACAAAAGCGAATAAAAGTGCATGGTTCAATAAAACGCAACAAGACTGACAGTTTTGACCAGCAACGAA
TGTTACAGACCATACGATATGGCGTAAATTAGTGAAATTAATACAAGCGAGAGCATGATGTTTGTTTG
TTGATCAGAAGTAGTTTCAAATTTATGGCGCGTTTCGTAGAGATTTCGGTGATATTTTTAATAGTGCT
CGTCCATTTTACTTAGAAACATAGCCATTCGTGTACTTATGCAGTAAATTTGAAATTATCACTGGTTC
ACGTCTCTTACCCCTTGAGTTGAAGCGATTTTCGTAGATTCAATTTGTATAACAATTACAAAACAATT

TTGTGATTTACTAGTTTATTTATTGGCGCGTCTTCGACGCGCTCGTCGGTGTTTCGGCAAATAGTTTT
CATTTTCACAAGTATAACACTGGAGGAAAGTCTTTTTATTCATTGGGGGAGGATACATGTCTTTCTTC
CCCTGGTGTACAAATAGCCACTGTGTGCAGTTTGATTGAGATTTCTAAATAGGTTTTTCTTTGGAAAC
TGTTTGTCTTACACTATGTAAACTGTTACAAAAATGTACATATTTGTAATGGGCAGTGTTATTGAAAT
AATAAAGTTATAAACGTTTTCAGTGTGTTTGCCATTAACTGTTTTGCA

GENE	→	***T.castaneum* a-tubulin 1 (aTUB)**
Database details	→	**NCBI accession number XP_966492.1**
Target sequence	→	**SEQ ID NO.13**
		Target sequence is shown in BOLD UNDERLINED text

ACGACAGTTGAAAATCGAATCAAAGTCGTTTGGAAAAAGCCAGAGCTTGTATTTCCGAAGCGTACTCC
CGTTTTTCTGCTCTTTTGTGGTGTAATTTGTAAAACTCAACTACCAAAATGCGTGAATGTATCTCAGT
TCATGTCGGCCAAGCCGGAGTCCAGATCGGCAACGCCTGTTGGGAATTGTACTGTTTGGAACATGGAA
TCCAACCCGACGGCCAAATGCCCTCTGATAAAACTGTCGGGGGCGGTGACGACAGTTTCAACACCTTC
TTCAGCGAGACCGGTGCTGGAAAGCACGTCCCGAGGGCCGTCTTCGTCGACTTGGAACCCACTGTCGT
CGATGAGGTCCGCACCGGGACTTACCGCCAGTTGTTCCACCCCGAACAATTGATCACTGGCAAAGAAG
ACGCCGCCAATAACTACGCCAGAGGCCACTACACCATTGG**CAAGGAAATCGTCGACTTGGTTTTGGAC**
CGCATCCGTAAATTGGCCGATCAATGCACGGGGCTCCAAGGTTTCTTGATTTTCCACTCGTTCGGTGG
AGGCACCGGCTCAGGGTTCACTTCCTTGTTGATGGAAAGATTGTCGGTTGATTACGGCAAAAAATCGA
AATTAGAATTCGCTATTTACCCCGCACCTCAGGTTTCTACAGCCGTTGTGGAGCCGTACAACTCGATC
TTGACCACTCACACCACTTTGGAGCATTCTGACTGTGCCTTCATGGTCGACAATGAGGCGATCTACGA
TATTTGTCGCCGAAACTTGGACATCGAACGCCCGACTTACACCAACTTGAACAGATTGATCGGCCAAA
TTGTCTCCTCAATCACCGCTTCGTTGCGATTCGATGGGGCTTTGAACGTTGACTTGACCGAATTCCAG
ACCAACTTGGTACCTTACCCACGTATCCACTTCCCTCTAGTCACCTACGCCCCAGTCATTTCCGCCGA
GAAGGCCTACCATGAACAATTATCCGTTGCGGAAATCACCAACGCCTGCTTCGAGCCCGCCAACCAGA
TGGTCAAATGCGACCCACGTCATGGTAAATACATGGCTTGCTGCATGTTGTACCGTGGAGATGTTGTC
CCCAAGGATGTCAACGCGGCTATTGCCACCATCAAGACCAAACGTACCATTCAATTCGTTGACTGGTG
TCCAACTGGGTTCAAAGTTGGTATCAACTACCAACCACCCACCGTCGTGCCAGGAGGTGACTTGGCCA
AGGTACAACGTGCTGTTTGCATGTTGTCCAATACCACTGCTATCGCCGAAGCTTGGGCTCGTTTGGAT
CATAAATTCGACTTGATGTACGCCAAACGTGCTTTCGTCCACTGGTATGTGGGTGAAGGTATGGAAGA
AGGTGAATTCTCTGAAGCTCGTGAAGATTTGGCCGCTTTGGAAAAGGATTACGAAGAGGTCGGCATGG
ACTCCGGAGAAGGAGAAGGCGAAGGTGGCGAAGAGTATTAAACGACAACTCGTTTAAAAAATTTCGAA
ATTTTCCGATTTTTTTTACGAAACTTTTGAGTCTGATTTATTTCAATACATTTTTCCAACTCTGT

GENE	→	***T.castaneum* Heat shock protein 90 (HSP90)**
Database details	→	**NCBI accession number NP_001094067.1**
Target sequence	→	**SEQ ID NO.14**
		Target sequence is shown in BOLD UNDERLINED text

GCAACCGAACAAACACGAATCGCTCAGAGTTGAAAAAGCAAGCGCTAAGTGAAGAGCTAAAAAGCGAC
AAATTTCCCAAGTGATAATTTTCCCAAAGCAATTTTCAAGTGATTTGTGCGTGTGTGCATTAATTTAA
GCAAGGTAGGTGCAAATTTTCCTATTTTCCGGCCGTTTTCAGTCAAGGTTATGTCAAGCCGGTGTTTG
ACCCCCAGGTCTGGGAAATCGTACGTTTTTCGCTAGGTTGCGGTTATTTGAGACGAGTTTATTAATCC
TTTGACTATTTTAACGAATTTGAAGACCGCTTTACAGTTTTCCCGTTTTTTGTGTGGTTTTCTGCCAA
GGTTATTCAGAGCGGTCTTTGACATTGCTACGGAATTTAGTCAAAATTTCCGGACTTTTCCACGTTGT
TCAGTGTCCGATTAGTCATTTTTTAGTGATTCATGAGTTCGGTGAAATTTCAAGGTCGAATTTAATTG
CAGATGCCGGAAGAAAACCAAAATGGAGATGTGGAAACCTTCGCCTTCCAGGCGGAAATCGCCCAGTT
GATGAGTCTGATCATCAACACCTTCTACTCGAACAAGGAAATTTTCCTTCGGGAGTTGATTTCCAATT
CGTCAGATGCGTTGGATAAAATCCGTTACGAGTCCTTGACCAACCCTTCCAGACTCGATTCAGGCAAA
GAACTCTACATCAAGATCATCCCTAACAAGAATGACGGGACCTTGACCATTATTGACACCGGTATCGG
GATGACTAAAGCCGATTTGGTCCATAACTTGGGCACCATCGCCAAGTCCGGCACCAAGGCCTTCATGG
AGGCCCTCCAAGCTGGGGCTGACATCAGCATGATCGGTCAATTCGGTGTCGGTTTCTACTCGGCTTAC
TTGGTAGCCGACAAGGTCACAGTCGTTTCGAAGAACAACGATGATGAGCAATACGTTTGGGAGTCGTC
AGCTGGTGGTAGCTTCACTGTAACACAAGACCGTGGCGAGCCTTTGGGCCGTGGCACCAAGATTGTCC
TTCACATGAAAGAGGACCAAACCGAATTTTTGGAAGAACACAAAATTAAAGAAATTGTAAAGAAACAC
TCGCAGTTCATTGGCTATCCCATCAAATTGGTCGTGGAGAAGGAACGCGAGAAGGAGTTGAGCGACGA
TGAGGCCGAAGAAGAGAAGAAGGAGGAAGAAGGCGAAGACAAGGATAAAGATAAGCCAAAGATTGAGG
ATGTAGGCGAGGACGAAGATGAAGACACGAAGAAGGAAGATAAGAAAAAGAAGAAGACTATTAAGGAG
AAATACACAGAAGATGAAGAATTGAACAAAACCAAGCCGATTTGGACAAGGAACGCTGACGATATCAG
TCAGGAAGAATACGGAGAGTTTTACAAATCGTTGACTAATGATTGGGAGGACCATTTGGCCGTCAAAC
ACTTTAGTGTTGAGGGTCAATTGGAGTTCCGTGCCCTCCTCTTTGTCCCACGTCGCGTTCCATTCGAT
CTTTTCGAAAATAAGAAGCGCAAGAATAATATTAAATTATACGTGAGGAGGGTCTTCATTATGGACAA
CTGCGAAGAACTCATCCCCGAATATTTGAACTTTATCAAGGGTGTCGTCGATTCGGAAGACTTGCCTT
TGAACATTTCCCGTGAGATGTTGCAACAAAATAAGATCTTGAAGGTCATTCGTAAGAATTTGGTCAAG
AAATGCCTAGAGTTGTTCGAGGAGTTGGCCGAGGATAAGGACGGCTACAAGAAATTCTACGAACAGTT
CTCGAAGAATATTAAATTGGGTATTCATGAAGACTCGCAAAACCGGGCCAAATTGTCCGAATTGCTCC
GTTATCACACTTCTGCAAGTGGCGATGAGGCTTGCTCTTTGAAGGACTAC**GTGAGCCGCATCAAGCCT**
AACCAGAAACACATTTATTACATTACTGGCGAAAGCAAGGAGCAAGTGGCGAATTCGTCGTTCGTTGA
GAGGGTCAAGAAGCGCGGTTTCGAGGTCGTTTACATGACTGAGCCCATTGATGAATACGTCGTACAAC

AAATGAAAGAATTCGACGGCAAAACTCTCGTTTCGGTCACAAAGGAAGGTCTCGAATTGCCTGAAGAC
GAAGAAGAGAAGAAGAAGCGCGAAGAAGACAAAGCCAAATTCGAGGGACTTTGCAAGGTTATGAAGAG
CATCCTCGATAATAAGGTTGAGAAGGTCGTGGTATCGAACCGTCTAGTCGAATCTCCCTGCTGTATTA
CGATGCGCAGGTATGGCTGGACCGCCAACATGGAACGTATCATGAAAGCACAAGCTTTGAGAGACACC
TCCACTATGGGCTACATGGCAGCCAAAAAGCACCTCGAAATCAACCCCGACCCTTCCATCCTTAAAAA
TTTGAGACAGAAGGCCGAGGCTGATAAGAACGACAAGGCTGTTAAAGACTTGGTTATTCTTTTGTTCG
AAACCGCTTTACTCAGCTCTGGGTTCACCTTGGATGAGCCTCAAGTCCACGCATCCAGGATCTACAGG
ATGATCAAGCTGGGTCTGGGTATTGATGAGGAGGAAGCCATGATCACCGAAGATGCACAAGGAGGCGA
TGCACCCTCTGCTGATGCCGCCGAGTCCGAGGACGCGTCGAGGATGGAGGAAGTTGATTAAGTGTTCG
GATGTTAGGACATGTGTTCTAGTATGTTCTAATTGTCATTCCTAGTGTTTTTTATATTTCTTAATTAT
TTTTATAAAAAATGAAGTATTATTTTGGCGGCCTACCGCCGCCGTGTTAACACCTGTAAAAGTCTGAG
TTCGTTTTTTGTGAATAAAATTGATTTAATGAATTTCTTGGTTTTATTTAAACGCCTTTAGCAAATAA
TTGTTGAAAAGCAAAAAAATCGATCACAAATTAGGCTTGGCTTCGCAAAATCAAGTCACGTGACACAT
TTGACAGATGTTTGAGTGAATCTTAACCTTAAAAATGGAACCGGACGAAAAAAGCAAGAGCCAAAAGC
CGGCCCCTACAGGTAACTCGCTCCCAAAACGGGACTTTTAGTCCAATTTCGCGTTTCTAGACGATCCG
GAATGCATTTATCGCATTTACACGTCTCTCTTACTCATCGTCTCGAATCACATCTGGTAGATCGAAAC
CTGGAGTCATCAGGAGTTCGATCCTCTGGGCAATGCGATACAGGGTGCT

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CONSTRUCT → L4440-PMCA-NAK-ADP Tricatamer
Sequence identifier → SEQ ID NO.15
Notes → L4440 vector is shown in normal text
T7 sites are shown in BOLD & UNDERLINED text
PMCA target sequence is shown in BOLD text
NaK target sequence is shown in ITALIC text
ADP target sequence is shown in UNDERLINED text

GAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACT
TACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGC
GCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGT
ATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCA
GGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAAC
TGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATC
TAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGC
GTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCT
TGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTT

CCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGG
CCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTG
CTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAG
CGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAG
ATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGG
TAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTAT
AGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAG
CCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACA
TGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACC
GCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCAACCTGGCTTATCGAAAT
TAATACGACTCACTATAGGGAGACCGGCAGATCTGATATCATCGATGAATTCGAGCTCCACCGCGGTG
GCGGCCGCTCTAGAACTAGTGGATCCACCGGTTC**TAATACGACTCACTATAGGG**AGACAGGAAGCCGA
AATTAAGAAAATGAAGAAAGAAGCTAAAAAGCAGCGGAAGAAGAAAAGTCTAACAGGTGCTGACGATG
AAAACGTAACTGGTAACAGTCATATGAATTCTCCCGCTCCGGTTCCAAATAAGCTTAACGAGAGTAAA
CAAGAATCCAAAGAAAATCACGTATCGTCACCACCGGCGTCGGCGGAAAGTCACAAGAAAGAAAAGTC
GGTTCTTCAAGCAAAATTGACGAAACTTGCCATTCAGATTGGT*TCTTGGCATCGTCTTAGCTGCCGTT*
GTTATCGTTACAGGTATATTTTCTTATTATCAAGAAAGCAAGAGTTCGAAGATTATGGAGTCGTTCAA
AAACATGGTCCCCCAATTCGCTACAGTGATCCGCGAGGGTGAAAAGCTGACCCTCCGCGCGGAGGACC
TGGTACTGGGCGACGTGGTCGAGGTGAAATTCGGTGACAGAATCCCAGCCGATATCCGAATCATCGAA
TCTCGCGGCTTCAAAGTAGACAACTCATCCTTGACAGGCGAAT<u>*ATTACGCTCGTACTCGTTTGGGCGC*
CGATGTCGGCAAAGGCAAGGGCGAAAGGCAGTACACCGGCCTTCTGGACTGCATTAAGAAGACAGTGA
AATCGGACGGACCGATCGGTTTGTACCGAGGTTTCGTTGTCTCAGTGCAAGGTATCATCATCTACCGT
GCCTCCTACTTCGGCTTCTTCGATACTGCCAAGGGAATGTTGCCCGATCCCAAGAACACACCGTTCCT
*CATCTCATTC***TCTCCCTATAGTGAGTCGTATTA**</u>GCTAGCCACGTGACGCGTGGATCCCCCGGGCTGCA
GGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTAT
AGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGT
TACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCA
CCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTA
AGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCC
TTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGC
TCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGT
TCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAA
TAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAG
GGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTT
AACAAAATATTAACGCTTACAATTTAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGT
TTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATA
ATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCAT

TTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGT
GCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGA
ACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCG
GGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACA
GAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAA
CACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACA
TGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGAC

Sequence identifier → **SEQ ID NO.16**
Notes → **PMCA target sequence**

AGACAGGAAGCCGAAATTAAGAAAATGAAGAAAGAAGCTAAAAAGCAGCGGAAGAAGAAAAGTCTAAC
AGGTGCTGACGATGAAAACGTAACTGGTAACAGTCATATGAATTCTCCCGCTCCGGTTCCAAATAAGC
TTAACGAGAGTAAACAAGAATCCAAAGAAAATCACGTATCGTCACCACCGGCGTCGGCGGAAAGTCAC
AAGAAAGAAAAGTCGGTTCTTCAAGCAAAATTGACGAAACTTGCCATTCAGATTGGT

Sequence identifier → **SEQ ID NO.17**
Notes → **NaK target sequence**

TCTTGGCATCGTCTTAGCTGCCGTTGTTATCGTTACAGGTATATTTTCTTATTATCAAGAAAGCAAGA
GTTCGAAGATTATGGAGTCGTTCAAAAACATGGTCCCCCAATTCGCTACAGTGATCCGCGAGGGTGAA
AAGCTGACCCTCCGCGCGGAGGACCTGGTACTGGGCGACGTGGTCGAGGTGAAATTCGGTGACAGAAT
CCCAGCCGATATCCGAATCATCGAATCTCGCGGCTTCAAAGTAGACAACTCATCCTTGACAGGCGAAT

Sequence identifier → **SEQ ID NO.18**
Notes → **ADP target sequence**

ATTACGCTCGTACTCGTTTGGGCGCCGATGTCGGCAAAGGCAAGGGCGAAAGGCAGTACACCGGCCTT
CTGGACTGCATTAAGAAGACAGTGAAATCGGACGGACCGATCGGTTTGTACCGAGGTTTCGTTGTCTC
AGTGCAAGGTATCATCATCTACCGTGCCTCCTACTTCGGCTTCTTCGATACTGCCAAGGGAATGTTGC
CCGATCCCAAGAACACACCGTTCCTCATCTCATTC

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GENE	→	***Aedes aegypti* Tubulin beta chain (bTub)**
Database details	→	**NCBI accession number XM_001655975.1**
Target sequence	→	**SEQ ID NO.19**
		Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED

ACTAGTCCCCTTCAGAGCAGCTCATTTAGTGTGTGATCGTCAAGGAGTACAGTCTCGGCGTTCTTTGATT
GTTGCCCGTTTGTGTCTTCTTTCTGTGCTTTGAGGAGAGAAAAGCAGCAGAAGAACAGAAAAAAAGGAGT
GAAGTGTGAAAAACAGACAAGTTTTAAAACTCGAATTTAAGAGCAGCCCTCGCCAAAGGCTACGCCGAAG
TTTCTCTGTTAATTTGTTAAGAAACAAAAAAAAACCTTTCACCATGAGAGAAATCGTCCACATCCAAGCC
GGTCAGTGCGGAAACCAAATTGGAGCTAAGTTTTGGGAAATCATCTCCGACGAAC**ATGGAATCGACGCCA**
CCGGAGCCTACCATGGTGACTCAGACCTGCAGCTGGAACGCATCAACGTGTACTACAATGAAGCCTCCGG
CGGCAAATACGTGCCACGTGCCGTGCTAGTCGATCTGGAACCCGGTACCATGGACTCCGTCCGCTCGGGG
CCATTCGGACAGATCTTCCGCCCGGACAACTTCGTCTTCGGACAGTCCGGTGCCGGTAACAACTGGGCCA
AGGGACACTACACCGAGGGTGCCGAACTGGTCGATTCAGTGTTGGACGTTGTCCGCAAAGAAGCCGAATC
GTGCGACTGCCTGCAAGGATTCCAGCTGACCCACTCGCTCGGAGGTGGTACCGGCTCCGGTATGGGCACA
CTGTTGATCTCGAAAATCCGCGAAGAATATCCCGACAGAATCATGAACACATACTCAGTTGTCCCCTCGC
CAAAAGTATCAGACACCGTCGTAGAACCGTACAACGCCACCCTCTCAGTGCACCAGCTGGTCGAAAACAC
CGACGAGACGTACTGTATCGACAATGAAGCCCTGTATGATATCTGCTTCCGCACCCTGAAGCTCACAACC
CCAACCTACGGTGATCTGAACCATCTCGTGTCACTGACCATGTCCGGAGTTACCACCTGCCTGCGTTTCC
CTGGTCAATTGAATGCTGATCTCCGAAAACTGGCTGTCAACATGGTTCCATTCCCACGTCTGCACTTCTT
CATGCCTGGATTTGCCCCACTCACCTCCCGCGGATCGCAACAGTACCGTGCCCTCACCGTCCCAGAACTG
ACCCAACAGATGTTCGATGCCAAGAACATGATGGCCGCCTGCGACCCACGACATGGACGTTACCTGACAG
TTGCCGCCGTTTTCCGAGGACGCATGTCGATGAAGGAAGTCGATGAACAGATGCTGAACATCCAAAACAA
GAACAGCAGCTACTTCGTTGAATGGATCCCCAACAACGTTAAGACCGCCGTCTGTGATATTCCTCCACGA
GGACTGAAGATGTCTGCCACCTTCATCGGTAACTCGACCGCCATCCAGGAACTGTTCAAGCGTATCTCCG
AACAATTCACTGCTATGTTCCGTCGTAAGGCTTTCTTGCATTGGTACACTGGCGAGGGTATGGATGAGAT
GGAATTCACTGAAGCCGAAAGCAACATGAACGATCTGGTGTCCGAATATCAGCAATACCAGGAAGCCACC
GCCGACGAGGATGCTGAATTCGACGAAGAACAGGAAGCTGAAGTTGACGAAAACTAAACTAATTGAGCTC
TCACTCACACACACGAACCTGCCTCCCCTTCTATACAAATCTCCCCATCCCCCTCAAAGGGAAACTCTAC
TCTCTCATTCCAAAAAAAAAAAAAACTTTCCTCTATCTGCGCCACTTCTACTACTAATCTCAAAAAGTACC
AATTCAGAGAGAATGCAACGTTCTTTTTTCGAAAAGAAAAACGAAAAAGTATCGATCAGGAGAGAATACA
ACATCATCAAGCGAAAACCACAAAACAACAGCAGAAATGTGAAGAAAAAAACGCAGCAGCAGTAACACCA
ACAAAACAGCCAGCGCAGCAAAAAAAAATCCTACAAAACAACTAAAAAAGAGTCGAAAAATAGCAAGAGA
AAAGTCGCAAAATTAGTAACCACTGCCAGCTCAGCAAAAAAGAAAAGAATAAAAGTGAAGTAATTTAAAA
AAAAACGGAAAACAAACTAAAATCAATTTCCTCTTGTTTGATTTTATTCTTTAGTGCACTTTTTTGCTTC
AAAAACCCCCCAACAAGAGAAACTGCCATTTCGTTCGTTAGGTTTGTTCGGAGATCCCATCATTCCACAC
CGCCTATCCAAGCGAACTCTCTTCTCTGATTTGTGTTGATTTCGTGTTTTGCATATTTCTTCCCACTTCT
CTCTTCCCACACTTTCGTATTCGTCTCTCTTCACAGGCACGTGTGCAAAAGAGATGTAAAATCGTTATAT
CGTAGCAGAAAGTACATTACTTTTCTCTTATAATTATTGATCAGCTAATTTTCTTCATTACTAATT

GENE	→	***Aedes aegypti* Na/K ATPase alpha subunit (AaNaK)**
Database details	→	**NCBI accession number XM_001662168.1**

Target sequence → SEQ ID NO.20

Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED

ATGCCACCAAAGAAGAAAGGAGATAACTTGGATGATCTGAAACAGGAGTTGGACATCGATTATCACAAAA
TCACACCGGAAGAATTGTACCAGCGACTTCAGACACATCCAGAGAATGGTCTCAGCCACGCGAAGGCGAA
GGAGAACCTAGAACGAGATGGACCAAACGCACTTACCCCACCTAAACAGACGCCCGAATGGGTCAAGTTC
TGTAAGAATCTCTTCGGTGGCTTCGCTCTGCTGCTGTGGATCGGTGCTATCCTGTGTTTCATTGCCTACT
CGATCCTGGCCAGTACCGTCGAGGAACCGGCCGACGATAACCTGTACCTCGGCATCGTGCTGACCGCCGT
CGTGATAGTTACCGGTATTTTCTCGTATTATCAGGAATCGAAAAGTTCGAAGATTATGGAATCGTTCAAG
AACATGGTGCCCCAGTTTGCGACCGTACTGCGTGAGGGCGAGAAGCTGACCCTGCGCGCCGAAGATCTGG
TCATCGGTGACGTCGTGGAGGTCAAGTTTGGCGACAGGTTACCGGCCGATATTCGCATCATCGAAGCCCG
AAACTTCAAGGTCGACAACTCTTCCCTGACCGGAGAGTCGGAGCCGCAGTCCCGTGGACCGGATTTCACC
CATGAGAACCCCCTGGAAACCAAGAATCTGGCCTTCTTCTCGACCAATGCCGTCGAAGGTACCGCCAAGG
GTGTCGTCATCAGCTGCGGTGATCACACCGTGATGGGTCGTATCGCTGGTCTCGCTTCCGGTCTGGACAC
CGGTGAAACTCCGATCGCCAAGGAAATCCACCATTTCATCCATCTGATTACCGGCGTGGCTGTGTTCCTC
GGTGTGACCTT**CTTCGTGATTGCCTTCATCCTCGGCTACCACTGGCTGGACGCCGTTATCTTCCTGATCG**
GTATCATTGTCGCCAACGTGCCGGAAGGTCTGCTCGCCACCGTTACCGTCTGTTTGACCCTGACTGCCAA
GCGTATGGCCTCGAAGAACTGTTTGGTCAAGAATTTGGAAGCCGTCGAAACCCTCGGATCGACCTCGACC
ATCTGCTCGGATAAGACCGGTACACTGACCCAGAACCGTATGACTGTCGCCCACATGTGGTTCGACAACC
AGATCATCGAAGCCGACACCACTGAGGATCAGAGCGGTGTTCAGTACGACCGTACCAGCCCTGGATTCAA
GGCCCTGTCCCGCATCGCTACCCTGTGCAACCGTGCTGAATTCAAGGGAGGTCAAGAAGGTGTCCCAATT
CTGAAGAAGGAAGTCAGTGGTGATGCTTCGGAAGCTGCTTTGCTCAAATGTATGGAACTGGCTCTCGGTG
ATGTCCTGAGCATCCGCAAGCGCAACAAGAAGGTCTGCGAAATTCCATTCAACTCCACCAACAAGTACCA
GGTTTCCATCCACGAAACTGAAGATCCCAGCGACCCACGTTATCTGCTGGTCATGAAGGGTGCCCCCGAA
CGTATTCTGGAACGCTGCTCGACCATCTTCATCAACGGCAAGGAGAAGCTGATGGACGAAGAGATGAAGG
AAGCCTTCAACAATGCCTACCTGGAGCTCGGAGGTCTCGGTGAACGTGTGCTCGGATTCTGCGACTTCAT
GCTGCCATCGGACAAATTCCCCGCTGGATTCAAGTTCAACTCGGATGAAGTGAACTTCCCGTGCGAGAAC
CTGCGCTTCGTCGGCCTCATGTCCATGATTGACCCTCCCCGCGCGGCTGTACCCGATGCCGTCGCCAAGT
GCCGCTCCGCCGGTATTAAAGTTATCATGGTTACCGGTGATCACCCGATCACTGCCAAGGCCATTGCCAA
GTCTGTTGGTATCATCTCGGAGGGCAACGAAACCGTCGAAGACATCGCCCAGCGTCTGAACATTCCGGTT
TCGGAGGTTAATCCTCGTGAGGCTAAGGCCGCCGTTGTGCACGGTTCGGAACTGCGCGACCTGTCCACCG
ATCAGATCGACGAAATTCTGCGCTACCACACGGAGATCGTGTTCGCTCGTACCTCGCCGCAGCAGAAGCT
GATCATCGTGGAGGGTTGCCAGCGGATGGGAGCCATCGTGGCCGTCACCGGTGACGGTGTCAACGATTCG
CCTGCCCTGAAGAAGGCTGACATTGGTGTTGCCATGGGTATCGCCGGGTCCGATGTGTCCAAGCAGGCCG
CTGACATGATCCTGCTCGATGACAACTTCGCTTCGATCGTTACCGGAGTCGAGGAGGGCCGTCTCATTTT
CGACAACCTGAAGAAGTCGATCGCGTACACGCTGACATCCAACATTCCGGAGATCTCGCCCTTCTTGGCG
TTCATCCTGTGCGACATTCCGCTCCCGCTCGGAACCGTCACCATTCTGTGCATCGATCTGGGAACTGACA
TGGTACCGGCCATTTCTCTTGCCTACGAAGCCGCCGAGAGCGACATTATGAAGCGCCGCCCGAGAGATCC
GTACCGTGACAATCTGGTCAACCGCAGACTTATCTCGATGGCCTACGGACAGATCGGTATGATCCAGGCC
GCGGCCGGTTTCTTCGTCTACTTCGTCATCATGGCTGAAAACGGATTCTTGCCGCTGCACCTGTTTGGCC
TCCGCAAGGGCTGGGACTCAAAGGCCGTCAACGATCTGACCGACTCGTACGGACAGGAATGGACCTACCG
TGACCGCAAGACGCTCGAGTTCACCTGCCACACCGCGTTCTTCGTCTCGATCGTCGTCGTCCAGTGGGCC
GATTTGATCATTTGCAAGACCCGTCGTAACTCGATTTTCCACCAGGGCATGAGAAACTGGGCGCTCAACT
TCGGTCTCGTGTTCGAGACGATACTGGCTGCGATCCTCTCGTATACGCCCGGCATGGACAAGGGCCTGCG
CATGTTCCCACTCAAATTCGTTTGGTGGCTGCCAGCCTTGCCGTTCAGCTTGTCCATCTTCGTGTACGAC
GAAATTCGTCGTTTCTACTTGAGACGCAACCCAGGTGGCTGGTTAGAGCAAGAAACGTACTATTAGGTCA
ACAAATTTATATTTAAAAAGATTAATTAAAAAGAAGAAGGAAAAACTGAAAAAACTGGAGATAAAAAAGA
ACCGTAAAAAATAACAAAAAATGAGCATAAAAAAACACACGAAAAAAGATGACGAAGAAGAAGAAGAAAC
ACACAGACACACGAAGATGAAAAATACTAGCGGAAAAAATCGTGAAGAAGATAAGAAGCAGTATCTCCCG
TCCTCATCGAACTCAATTCCCCCTTGTGCACATTAACATTCACAACCGACAAGTCTCCCCTTCGCATCTA
TATAATTATCTCTCACGCACACAGTCACACACGTTTCCCGCTCACGCGCGTATGGATGTTGCCATGGAAA
CTTTGTGTAAACGGAGAAAAAAGTCGTACAAACACAAGCATACAGCAGCAACAGCAGTAGCGGCGTGCCT

ACAATACAACGCGATCGACTCCGGTTGAACCAACACATTCGCACCCAGCCCACCACCCGTCGTCGTCGTC
GTACACTTACACACTCACACACACACACATGATAACATCCGCATACACACATACAAAATCACTCACATAC
GCCAGTTGCGCATTTTCGGAGCAGCTAATCGTTATTGAAGAAAAATCCCGCGTTCGTGTTAGTTTTTAGC
CACAGGCAGAGACTCTATAGCAGCCTAAAAGCCGAAACGGGAAGAGCGAGAGAGGACATTTTACTAAATT
TGAGAAATAAATAATAAACAAGCGAAACAAGTAATCT

GENE	→	***Aedes aegypti* ADP/ATP carrier protein (AaADP)**
Database details	→	**NCBI accession number XM_001649861.1**
Target sequence	→	**SEQ ID NO.21** **Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED**

ACTCGCAACTCGGCTACAACTAGCGCAGCCATCCGGCGTTCATTCTCTTTGGATTGTGCCCTCCGTTCGG
TTAGGAAGCTCGCTGCCTTTGATTGCGCTCTGCTGCGTGTGTGTGTGTTGCAAGACTAGAAAGAATCCCC
GCAGAAAATGTCTGGAAAGAAGGCTGATCCCTATGGCTTCGCCAAGGATTTCCTGGCTGGTGGAATCTCC
GCCGCCGTTTCCAAGACTGCCGTGGCCCCAATTGAGCGCGTCAAGCTGCTGCTCCAGGTTCAGGCTGCCT
CCAAGCAGATCGCCGCCGACAAGCAGTACAAAGGTATCGTCGATTGCTTTGTTCGCATCCCCAAGGAACA
GGGCTTCGGAGCTTTCTGGAGAGGTAACCTTGCCAACGTGATCCGGTACTTCCCAACCCAGGCGCTGAAC
TTCGCCTTCAAGGATGTCTACAAACAGATCTTCTTGGGTGGCGTCGACAAGAACACACAGTTCTGGCGCT
ACTTCATGGGTAACTTGGGATCCGGCGGTGCCGCTGGTGCCACCTCGCTGTGCTTCGTCTACCCACTCGA
CTTTGCCCGTACCCGTCTGGGCGCCGATGTTGGCCGTGCCGGAGCCGAGCGCGAGTACAACGGTCTGATC
GACTGCCTGAAGAAGACCGTCAAGTCCGATGGTCTGATCGGTCTGTACCGTGGATTCAACGTGTCGGTCC
AGGGTATCATCATCTATCGTGCTGCCTACTTTGGTTGCTTCGATACTGCCAAGGGAATGCTGCCCGACCC
GAAGAACACCTCGATCTTCGTCTCGTGGGCCATCGCTCAGGTTGTAACGACGGCCTCCGGCGTTATCTCC
TATCCATTCGATACCGTCAGAAGACGTATGATGATGCAGTCCGGCCGTGCCAAGTCGGAAATCATGTACA
AGAACACCCTCGACTGCTGGGTCAAGATCGGCAAGACGGAAGGTTCGTCGGCCTTCTTCAAGGGCGCCTT
CTCCAACGTTCTGCGTGGTACTGGTGGCGCTCTTGTGCTCGTGTTCTACGATGAAGTGAAGGCTCTGATG
GGTTAGATTTAAGTTTAGCGAAAAAAAAATTAAACTAAAAAAAGTTACAAAAACCAGCTAAAGTAAACTA
AACCAATTAAGGTGGAAAATCAGCAGTAAAACCAGTGATCTATTTATTTGACGCTAGAAAGAAATTCACA
AGCAAGCACATCTTGCGCGTTATTTGTGTAGAAAAAACTAAAATACCACTCAAAACTGAAATGATGTTCT
TACTACCAACCAGGACAAAATTGTAATCAGCCATACGAAAATAAAAGTGTTCTCACGAATCGAACGAAAC
AACAGACGAAAACCATTCGTATTTGAGTTGAAAATAGGAAAGACATGTGCACATTCCGTAATATTTCCAT
CCGACTCGCAACCCGAATGCTGCAATGTTGTTGCACTCGAGAAATCTCACCAATTTGCCGTGCGAAGTCA
TTTCAGAGACCGATTACAAGACAGTAGCTGTGAAATAAAAAAAAAAAAAAAAAAAAACGAGCAAACACTCT
TCAAGAGTTCCCAAAGACCAGTATAACAATTTGCATGACATGCGATCAAGCCGGGCTTGACTTAACCGAA
AGGGCACGATAAGGAAAAAAAGAAGCTTCTGTTTTATGATGCACCTTAACAGACGTTAATTTGCTGAGGA
GAATCGAATTGCGAATAATAGTTACAACCTGAGTAAAAAAAAA

CONSTRUCT → *A.aegypti* L4440- bTUB-AaNaK-AaADP Tricatamer

Sequence identifier → SEQ ID NO.22

Notes → bTUB target sequence is shown in BOLD text

AaNaK target sequence is shown in ITALIC text

AaADP target sequence is shown in UNDERLINED text

GAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTA
GCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCG
GCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCA
GATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAG
ATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATT
GATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCT
TAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTT
CTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTA
CCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAG
TTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCT
GCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGC
TGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAG
CTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGA
GAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTT
GAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGG
TTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATT
ACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCA
ACCTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCGGCAGATCTGATATCATCGATGAATTCGAGCTC
CACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCACCGGTTC**GGAAATCATCTCCGACGAACATGGAATCGAC**
GCCACCGGAGCCTACCATGGTGACTCAGACCTGCAGCTGGAACGCATCAACGTGTACTACAATGAAGCCTCCGGC
GGCAAATACGTGCCACGTGCCGTGCTAGTCGATCTGGAACCCGGTACCATGGACTCCGTCCGCTCGGGGCCATTC
GGACAGATCTTCCGCCCGGACAACTTCGTCTTCGGACAGTCCGGTGCCGGTAACAACTGGGCCAAGGGACACTAC
ACCGAGGGTGCCGAACTGGTCGATTCAGTGTTGGACGTTGTCCGCAAAGAAGCCGAATCGTGCGACTGCCTGCAA
GGATTCCAGCTGACCCACTCGCTCGGAGGTGGTACCGGCTCCGGTATGGGCACACTGTTGATCTCGAAAATCCGC
GAAGAATATCCCGACAGAATCATGAACACATACTCAGTTGTCCCCTCGCCAAAAGTATCAGACACCGTCGTAGAA
CCGTACAACGCCACCCTCTCAGTGCACCAGCTGGTCGAAAACACCGACGAGACGTACTGTATCGACAATGAAGCC
CTGTATGATATCTGCTTCCGCACCCTGAAGCTCACAACCCCAACCTACGGTGATCTGAACCATCTCGTGTCACTG
ACCATGTCCGGAGTTACCACCTGCCTGCGTTTCCCTGGTCAATTGAATGCTGATCTCCGAAAACTGGCTGTCAAC
ATGGTTCCATTCCCACGTCTGCACTTCTTCATGCCTGGATTTGCCCCACTCACCTCCCGCGGATCGCAACAGTAC
CGTG*GCTTCGTCTACCCACTCGACTTTGCCCGTACCCGTCTGGGCGCCGATGTTGGCCGTGCCGGAGCCGAGCGC*
GAGTACAACGGTCTGATCGACTGCCTGAAGAAGACCGTCAAGTCCGATGGTCTGATCGGTCTGTACCGTGGATTC
*AACGTGTCGGTCCAGGGTATCATCATCTATCGTGCTGCCTACTTTGGTTGCTTCGATACTGCCAAGGGAA*TGCTG
GTGTTCCTCGGTGTGACCTTCTTCGTGATTGCCTTCATCCTCGGCTACCACTGGCTGGACGCCGTTATCTTCCTG
ATCGGTATCATTGTCGCCAACGTGCCGGAAGGTCTGCTCGCCACCGTTACCGTCTGTTTGACCCTGACTGCCAAG
CGTATGGCCTCGAAGAACTGTTTGGTCAAGAATTTGGAAGCCGTCGAAACCCTCGGATCGACCTCGACCATCTGC
TCGGATAAGACCGGTACACTGACCCAGAACCGTATGACTGTCGCCCACATGTGCTAGCCACGTGACGCGTGGATC
CCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGC
CCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTA
CCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCC
CTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGG
TGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTC
TCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTAC
GGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTC
GCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCT
CGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAA
AATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTAGGTGGCACTTTTCGGGGAAATGTGCGCGGAAC
CCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCA
ATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTG
CCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGG
TTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAG
CACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCAT

ACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAG
AGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACC
GAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAA
TGAAGCCATACCAAACGAC

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GENE	→	***Lepeophtheirus salmonis* ADP/ATP translocase 1 (LsADP)**
Database details	→	**NCBI accession number BT077972.1**
Target sequence	→	**SEQ ID NO.23**
		Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED

GGGGGACAGTAGTTTGTAAATTTACAGGGGAACTCATACCTCTTCACCGTCGTCGTTTAAGAACAGTTTG
AAATAATGAGCAAGGACTTTGTTTTGGATCTTGTCGCAGGTGGGGTGTCTGCCGCGATATCCAAGACCAT
TGTTGCTCCATTGGAACGAATCAAAATTCTCCTCCAAATACAAGATGCTTCCAAGTATATTCCTAAAGAT
CAACGCTACACTGGTCTCGTTGACTGTTTTCGTCGAGTGAATGCAGAGCAGGGAACCCTGTCCTTTTGGC
GTGGAAACGTTGTGAATGTGGTTCGATACTTCCCCACTCAAGCCTTTAATTTTGCATTTAAGGATAAATA
TCAAAAGATATTTTTAGATGGAGTGGATAAAAAGGACTTTTGGAGATTTTTTGCTGGAAATTTAGCTTCT
GGCGGTGCTGCTGGAGCAACTTCACTTTGTATTGTATATCCCTTGGATTTTGCACGTACTCGCCTTGGTG
CAGACGTTGGGAAGGCTGCAGCGGATAGGGAGTTCAAAGGACTTTTCGACTGCATCGGTAAATGCTACAA
AGCTGATGGTCTTGTGCGTGGACTGTATCCTGGTTTCCTCTCCTCTGTACAAGGAATCATTGTTTATCGA
GCTATTTATTTCGGTGCCTATGATACTTGCAAACAAATGATAGATAAACCCACATTCGGTACTAAATTTG
CCATAGCTCAAACTGTGGCAGCA**TGCTCCGTCTCAATTGCCTATCCCTTTGACACCGTTCGTCGTCGATT**
GATGATGATGTCTGGGGAAGGTGAGAAAATGTACAGTGGCACTGTGGATTGTTGGAAAAAAATCGTTAAG
GAAGAAGGATCCAGAGCTCTATTCAAAGGCAATTTTACCAATGTTCTCAGGTCTGTCGGATGTGCCTTGG
TCCTTGTTCTCTATGATGAAATCATTGTTGTTCTTAAAAGTGCAACATAATTTTTGTACTATGTCATAAA
GTCAATGTAGTCTGGCATTTACAATATCGTCATAATGAAAATAATTGTGATATATTCCTGTAATAATTAT
TTATGTAATTAAAAAAAAAAAAAAGATATATCATGTTGTCAATCCTAATCGCCAATTACAACTTTCTTCC
TACATCAATCATTTATTAATATAATGC

GENE	→	***Lepeophtheirus salmonis* V-type ATPase unit E (LsvATPe)**
Database details	→	**NCBI accession number BT120776.1**
Target sequence	→	**SEQ ID NO.24**
		Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED

TTCACATATTCTACATTGTCATAATAAGATACACAAGTGTTGGAGATCCTCCTACCTTTCCGTTACTTTG
GTTGAGAAATTTCATCTACTCCAACATCCAAGATGTCTTTGAGCGATGCTGAC**GTTAGCAAGCAGATTAG**
CCACATGACGGCTTTTATCGAGCAAGAAGCGAATGAAAAAGCTGAGGAAATCGATGCAAAGGCTGAAGAG
GAATTCAATATAGAGAAAGGGCGTCTCGTTCAACAACAAAGACTCAAAATCATGGAATACTATGACCGTA
AAGAGAAGCAAGTTGAATTGCAAAAGAAAATTCAATCTTCCAACATGCTCAATCAGGCGCGTCTCAAAGT
ACTTAAGGCTCGGGATGAGCATGTGGATGAAGTCGTTGAAGAATCACGTAAAAAGCTGGTCCTTATTACG
AAGGATAAATCCAAATATTCTAAAATCATAGAGGGTCTAATAGCTCAGGGTTTATGCCAATTGTTAGAGT

CAAATGTCACGATTCGTTGCCATCAAAATGATCTCTCTCTGGTGGAGCAAGCCATTTCCGTAGCAGTTAA
AAATGTCAAAGATAAAATAAAGAAGGATATAGTTGTTAAAGTTGATAAAGAAAATTTTTTACCACAAGAT
AGCTCTGGTGGCATTGAATTATACGCTCAAAGAGGAAGGATAAAGGTGGATAACACCCTTGAGGCTCGCT
TGCATTTGATTGCCCAAAATATGATGCCACAGATTCGCACTTCCTTATTCGGCGCTAATCCTAATAGAAA
ATTTGATGATTAAAAATATAATTCCTTTTTTTTCTAATTGTAAGATGGCATTTAAAAAAAACAACAACT
CTGATTTGATCGATCAACGGCTTTGTTATTTTGAATAATTAATCATCATATTTATTTGTAGTCAATCATC
TTTTTATTCTTTGTTTCGCTGATGAGATCAACCAAGGAGTCGTTTTTTTTAAAATTTAATTTAAAATGTA
CTCGTCTTTATCAACAACATTCCTTTTTATGTTTGTCACCCTTTAAAATGATTATAATTTATTTGTTCCA
TTATATCGTTATTTATAAATTTAATATTCTTAATAATACATAAATACCAAAATCATAG

GENE	→	***Lepeophtheirus salmonis* acetylcholinesterase (LsAChE)**
Database details	→	**NCBI accession number KJ132369.1**
Target sequence	→	**SEQ ID NO.25** **Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED**

GAGTGCCTCCCATTAAGTTAAACAGTCGACGTTATTTATAGGTCTATTAAATATTGTTTTAGATGTGGAT
TCAAGTCCGAAAACACAACTTAGGTCTTTCCTTTGAAAGGATATTAGTGTATTTACTCACATTGTCATGG
AGTCTGGGATCCATCGTACAAGAAGATTTGGTGATCACCACAAGAAAAGGAAAGATCCGAGGTGTTACTC
TGAAATCTGCAACAAATAAGGAAGTAGATGCATGGTATGGGATACCATACGCACAACCTCCCGTGGGTAA
TCTTCGATTTCGTCACCCCAAAGACATTAATGCCTGGGATGGGATGAAAGAAACGACCAAACATCCAAAT
TCTTGTATTCAAGTAGTTGATACATTTTTTCCGGGCTTTGAAGGCTCAGAGATGTGGAATACAAATACTG
AGCAAAGCGAGGACTGCCTTTACTTAAGTGTTCATGCCCCTAAACCCCGTCCTACAAAATCAGCTGTTCT
GGTATGGATCTACGGTGGAGGATTTTATTCTGGAACTTCAACTCTGGAACTCTATGATCCACGAGTTCTT
GTGTCAGAAGAAAACATAATCTTCGTCGGCATACAATATCGTGTTGCAAGTTTAGGATTCTTATTCTTTG
ATACGGAGGATGTTCCTGGAAATGCGGGATTGTATGATCAAATGATGGCTCTCCAATGGGTAA**AGAACAA**
TATAGAGGCATTTGGTGGTGATCCTGATAAAATCACCATTTTTGGAGAGTCCGCCGGTGGTTGCTCCGTA
GCCCTTCATCTCCTTTCTCCACTCTCAAGGAACCTATTCTCTCAAGCCATTATGCAAAGTTCTTCAGCTC
TTGTTCCATGGGGAGTCATATCAAAAAAAGAAAGTATCCGTCGTGGTCGAAGACTTGCAGAAGAGATGCG
TTGTCCTTATGGTGAAAATAATACCAATGCTATGATTGAATGCCTGCTGCAAAAGGACGCAACAGAGTTG
GTCAACCAGGAGTGGAGTGGTACCGTCTTTGGGATTTCAGAGTTCCCATTTGTTCCAATTGTGGATGGAA
AATTCATGGATAAAACCCCTGAAAAATCTCTTAAAGAAAAGGACTATAAAAAAACCAACATTTTAATGGG
AGTCAATAAGGACGAAGGGAACTTTTTCATCATGTATTATCTTCCAGAACTCTTCAAAAAAAACGAAAAC
GTTTATATTAACCGAACAGATTTCATCCGCAGTGTTTCAGATTTGAACATCTATGTGAACAATGCAGGAA
GAGAGGCAATAACTTTTGAATACACAGATTGGCTCAATCCAAACGATCCCATAAAAAATAGAGAAGCAAT
TGATCGCATGGTCGGTGACTATCAATTCATTTGCCCAACTGCTGACTTTGCTCGTATTTATGCTAGTACA
GGAAATAATATATACATGTACTATTTCACTGAGCGATCTTCCACTAGCCCATGGCCAACATGGTCTGGTG
TACTTCATGGCGATGAAATTGCTTTTGTTTTTGGAGAGCCCCTAAATACGTCAAAAAATTATGATGATTC
AGAAATTGCCTTATCAAAAAGAATAATGAGCTATTGGGCTAATTTTGCAAAAACTGGGAACCCGAATGTT
TTGGCTAATGGAAACTACAGCAACAAAATCTGGCCCTTACATACACCAATAAAACAGGAGGTACTTGAAC
TAAATGCAAATTATTCTCGAGTTTTTGAGGGGCTTCGAGTTAGAAAATGTGCTTTTTGGAAAACATATCT
TCCTAAGCTTTTATCATTAACTTCAAACAATACAAAGTCTGAAGTTGTAACCAATCCGTCATAAAGATTG
AAAAACGTCAACTACATTACTGGAGATACACATTCAATGGTATAAATAAATAAATTTAACACTAGCACAA
TGATAGACTATAATTAAATATGTTCATACGTACGTACCATCCATCGTATAATAGTTTGTTTTGTTGATAC
CAAAAAGACAAAAAGGCTAAATATGTATACCAACTGCAATAAAGATTATTCTCTATAAAAAAAAAAAAAA
AAAAAAAAAAAAAA

CONSTRUCT → *L.salmonis* L4440- LsADP-LsvATPe-LsAChETricatamer

Sequence identifier → SEQ ID NO.26

Notes → LsADP target sequence is shown in BOLD text

LsvATPe target sequence is shown in ITALIC text

LsAChE target sequence is shown in UNDERLINED text

GAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTA
GCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCG
GCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCA
GATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAG
ATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATT
GATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCT
TAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTT
CTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTA
CCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAG
TTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCT
GCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGC
TGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAG
CTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGA
GAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTT
GAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGG
TTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATT
ACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCA
ACCTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCGGCAGATCTGATATCATCGATGAATTCGAGCTC
CACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCACCGGTTC**TAGCTCAAACTGTGGCAGCATGCTCCGTCTC**
AATTGCCTATCCCTTTGACACCGTTCGTCGTCGATTGATGATGATGTCTGGGGAAGGTGAGAAAATGTACAGTGG
CACTGTGGATTGTTGGAAAAAAATCGTTAAGGAAGAAGGATCCAGAGCTCTATTCAAAGGCAATTTTACCAATGT
TCTCAGGTCTGTCGGATGTGCCTTGGTCCTTGTT*TGTCTTTGAGCGATGCTGACGTTAGCAAGCAGATTAGCCAC*
ATGACGGCTTTTATCGAGCAAGAAGCGAATGAAAAAGCTGAGGAAATCGATGCAAAGGCTGAAGAGGAATTCAAT
ATAGAGAAAGGGCGTCTCGTTCAACAACAAAGACTCAAAATCATGGAATACTATGACCGTAAAGAGAAGCAAGTT
GAATTGCAAAAGAAAATTCAATCTTCCAACATGCTCAATCAGGCGCGTCTCAAAGTACTTAAGGCTCGGGATGAG
*CATGTGGATGAAGTCGTTG*GATGGCTCTCCAATGGGTAAAGAACAATATAGAGGCATTTGGTGGTGATCCTGATA
AAATCACCATTTTTGGAGAGTCCGCCGGTGGTTGCTCCGTAGCCCTTCATCTCCTTTCTCCACTCTCAAGGAACC
TATTCTCTCAAGCCATTATGCAAAGTTCTTCAGCTCTTGTTCCATGGGGAGTCATATCAAAAAAAGAAAGTATCC
GTCGTGGTCGAAGGCTAGCCACGTGACGCGTGGATCCCCCGGGCTGCAGGAATTCGATATCAAGCTTATCGATAC
CGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACGCGCGCTCACTGGCCGTCG
TTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCA
GCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGGACG
CGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCGCCC
TAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATC
GGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTT
CACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGAC
TCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTT
CGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAA
TTTAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTA
TCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTT
CCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGT
AAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGA
GAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCG
TATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGT
CACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACAC
TGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCA
TGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGAC

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GENE → *Caenorhabditis elegans* egl-30

Database details → NCBI accession number U56864.1

Target sequence → SEQ ID NO.27

Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED

GTACACACACACCCGCCACCACCACATTTCCACCAACAGAGAGGCATCCCTGTGCGTTGTTGTGTTGTTG
TTTTTTTGTGATGTTTATAACTTGACGCCCTCAATCGTCCCACCGAAATACAAAAATTGCATCGAACTTC
TATCCTCGCTCTAGCGTGTTCTTCTTGTTCTATTCGCTGGCTTCATCTGCGGCCTTGGTGGCACCTTTTC
GGCCGCCATGGCCTGCTGTTTATCCGAAGAGGCTCGCGAGCAGAAGCGAATAAATCAAGAAATTGAGAAG
CAGCTTCAGCGTGACAAAAGAAATGCTCGACGAGAACTCAAACTTCTTTTATTGGGGACTGGAGAGTCCG
GCAAGTCAACGTTCATCAAGCAGATGCGAATTATCCACGGTCAGGGATATTCGGAAGAGGACAAGCGAGC
ACACATTCGACTTGTCTATCAGAACGTGTTTATGGCCATACAGTCTATGATACGAGCGATGGACACATTA
GATATAAAGTTTGGTAACGAATCAGAGGAGCTGCAGGAGAAGGCGGCTGTGGTGCGGGAAGTGGATTTCG
AGTCGGTGACGTCTTTCGAGGAACCCTACGTGTC**GTATATCAAAGAGCTATGGGAGGATTCTGGTATTCA**
GGAATGTTATGATAGGAGGCGAGAATATCAGCTCACCGATTCAGCCAAATACTATCTCTCCGATCTCCGA
CGGCTGGCGGTGCCAGACTATCTGCCAACCGAGCAGGACATTCTGCGTGTTCGTGTGCCAACCACTGGTA
TCATTGAATATCCATTTGATTTGGAGCAGATCATCTTTCGAATGGTGGACGTCGGAGGTCAGCGATCAGA
AAGGCGGAAGTGGATCCACTGTTTCGAAAATGTCACCTCAATCATGTTCCTGGTGGCGCTTTCCGAGTAT
GATCAGGTGTTGGTCGAGTGTGACAACGAGAACCGAATGGAAGAATCGAAAGCTCTGTTCCGAACGATCA
TCACGTACCCATGGTTCACCAACTCATCGGTCATTCTATTCCTGAACAAGAAGGATCTGCTCGAGGAGAA
GATTCTGTACTCGCATCTCGCTGACTACTTTCCCGAATATGACGGACCCCCACGCGATCCGATCGCCGCC
CGCGAGTTTATTCTCAAAATGTTTGTCGACTTGAATCCGGACGCCGACAAGATTATCTACTCTCATTTTA
CGTGCGCGACTGATACGGAAAACATTCGGTTCGTGTTCGCCGCCGTCAAAGACACAATTCTACAGCATAA
TCTGAAGGAGTACAACTTGGTGTAAGAAGAAAGTCGCATGTCGGATTGGATGATGATGATGATGATCCAT
CTCTCTCTCTCTCTCTCTCTCACTGGGTCGAGTGAGACACCACCACCTAAACCTAGGAAACATTTTCTTG
TACTCCTTCTAATTTTTGTTTTTTTTTTGCAAAAAACTTTCTCTCTCTGTCTGTCTCTCTCTCCATCTCT
TCCTTATTTTCTTATTTTCTCATTTTCCTCCCTAAAACAAATGCTCCTCCCGAATATTCTTTCCATATAA
GCACTTTTTTCTTCTTTTTTTGGATGTGCTTTCTGATATAGCTAATGCAAAAAAAAAAAACGG

GENE → *Caenorhabditis elegans* pat-10

Database details → NCBI accession number NM_059100.6

Target sequence → SEQ ID NO.28

Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED

ATGGCTGAGGATATCGAAGAGATTCTTGCTGAAATCGACGGATCCCAAATTGAGGAATACCAAAAGTTCT
TCGATGCCTTCGACAGAGGAAAGCAAGGATACATCATGGCCACTCAGATCGGTCAAAT**CATGCATGGAAT**
GGAACAGGATTTCGATGAGAAGACCCTTCGTAAACTGATCCGCAAGTTCGACGCTGACGGTTCCGGAAAG
CTCGAGTTTGACGAGTTCTGCGCTCTCGTGTACACCGTTGCAAACACTGTCGACAAGGAAACATTGGAGA
AAGAACTTCGTGAAGCTTTCCGTCTTTTCGACAAGGAGGGTAACGGATATATTTCTCGACCAACTCTGAA
GGCTCTTCTCAAAGAAATCGCCGATGACCTCACCGATCAACAACTCGAGGAGGCTGTCGACGAGATTGAC
GAGGACGGTTCCGGAAAGATTGAGTTCGAGGAGTTCTGGGAGTTGATGGCTGGAGAGTCTGATTAA

GENE	→	*Caenorhabditis elegans* bli-5
Database details	→	NCBI accession number NM_067371.1
Target sequence	→	SEQ ID NO.29
		Target sequence is shown in BOLD text and flanked by primer sequences, which are UNDERLINED

ATGGTATCTATCCATAATTCATTCATCTTATTGATGTTAATGATATCAATTTGTTTTTGTGAGAAATGCC
TGACCAATGAAGAATGCGATTTGAAATGGCCAGACGCAATATGTGTTCGTGGAAGATGCCGTTGTTCT**GA**
GAATACAATTCGAAAGAAAAGTGCATCAAGAGAATGGGTTTGTTTGGCAACTAATGATGCAACCGGCAAC
TCGGGTCCTCCATTGACATGTCCAACTCCGGAAGGAGCTGGATACCAAGTAATGTACCGAAAAGATGGAG
AACCGGTGAAATGTTCGAGTAAAAAGAAGCCAGATACGTGTCCAGAAGGATTTGAATGTATTCAGGGATT
ATCAATTCTTGGAGCATTGGATGGAGTTTGTTGTCCTGATAGAGCCAAAACATGCGTCCACCCAATATTC
GATCATCCGGATGATGGATATCTGTCTAGATGGGGATTCGATGGTGAACAATGTATTGAATTCAAATGGA
ATCCCGAAAGGCCGTCATCGGCAAACAATTTCAAAACTCGCGCACATTGTGAGGATTACTGTATCGGTTC
GATAAATGGAATTACTAATTATCATCAGTCCAACTTTCATCTTTTCTGA

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GENE	→	*T.castaneum* Na/K ATPase alpha (NaK), [preferred]
Database details	→	NCBI accession number XM_008198203.1
Target sequence	→	SEQ ID NO. 113
		Target sequence is shown in BOLD UNDERLINED text

ACTTTTAGTGGGTCCGCGCCCGTCGTCGCTGCTTCTAGTGCGATTTGTGTGCAGTGGTCGACATCACA
TGAAGTACAGTATTTAACACCACTCCCCGGGATATTATCACACAATCAGCATGGGGGAGTCACGGAGG
AAAAATAAGAAGGTCAGGAAAGCGGACGATTTAGATGATTTGAAACAAGAATTGGACATCGATTATCA
TAAAATCACCCCAGAAGAATTATATCAGAGATTCCAGACACATCCAGAAAATGGCCTCAGTCATGCGA
AAGCGAAAGAGAATTTGGAACGGGACGGACCCAATGCACTCACACCCCCAAAGACTACCCCCGAATGG
GTGAAATTTTGTAAAAATCTCTTCGGGGGTTTCGCTCTCTTATTGTGGATCGGCGCCATCCTCTGCTT
CATAGCCTATTCTATTCAGGCTAGCACCGTGGAGGAACCAGCCGATGATAATCTTTA**TCTTGGCATCG**
TCTTAGCTGCCGTTGTTATCGTTACAGGTATATTTTCTTATTATCAAGAAAGCAAGAGTTCGAAGATT
ATGGAGTCGTTCAAAAACATGGTCCCCCAATTCGCTACAGTGATCCGCGAGGGTGAAAAGCTGACCCT
CCGCGCGGAGGACCTGGTACTGGGCGACGTGGTCGAGGTGAAATTCGGTGACAGAATCCCAGCCGATA
TCCGAATCATCGAATCTCGCGGCTTCAAAGTAGACAACTCATCCTTGACAGGCGAATCCGAACCGCAG
TCCCGCAGTCCGGAGTTCACTCACGAGAACCCTCTCGAAACGAAAAACTTGGCGTTCTTCTCGACCAA
CGCCGTCGAAGGCACTGCCAAAGGTGTTGTGATTAGTTGTGGTGACAATACCGTGATGGGTCGCATCG
CCGGTCTCGCCTCCGGTCTGGACACCGGCGAGACGCCCATCGCCAAAGAAATCCATCATTTCATTCAC
CTCATTACTGGCGTGGCTGTTTTCCTCGGAGTTACCTTCTTCGTAATCGCCTTCATCCTCGGCTACCA
CTGGCTCGACGCTGTTATTTTCCTCATCGGTATTATCGTGGCGAACGTGCCCGAGGGGCTCCTCGCCA
CCGTCACCGTGTGTCTCACCCTCACTGCTAAGAGGATGGCTTCCAAGAACTGCCTCGTGAAGAATCTC

GAGGCCGTAGAGACCCTCGGCTCCACAAGCACGATCTGCTCGGACAAGACCGGAACTTTGACCCAAAA
CCGGATGACGGTAGCACACATGTGGTTCGACAATCAGATCATTGAAGCCGACACCACTGAAGACCAGT
CGGGAGTCCAATACGACCGCACAAGTCCAGGATTCAAAGCTTTGTCGCGCATTGCCACACTTTGCAAC
CGGGCTGAGTTCAAAGGGGGGCAGAACGACGTCCCGATCCTTAAACGCGAAGTCAACGGAGACGCCTC
TGAAGCCGCTCTCCTCAAATGCATGGAACTGGCTCTGGGCGACGTGATGTCCATCAGACGCAAGAACA
AGAAAGTTTGCGAAATTCCCTTCAACTCGACCAACAAATACCAAGTTTCCATCCACGAGAACGAGGAC
GCGAGCGATCCTCGCCATATCCTTGTGATGAAGGGCGCTCCTGAACGAATCCTCGAACGCTGCAGCAC
GATCTTCATCTGCGGCAAGGAGAAAGTCCTGGATGAGGAAATGAAGGAAGCTTTCAATAACGCCTACT
TGGAGTTGGGTGGTTTGGGCGAGCGTGTGCTCGGCTTCTGCGATTTTATGTTGCCCACTGATAAGTAC
CCAATTGGGTACAAATTCAATTGCGATGACCCCAACTTCCCGTTGGATGGTTTGAGATTTGTTGGCTT
GATGTCCATGATTGATCCTCCCAGAGCTGCAGTGCCTGACGCCGTTGCTAAATGCAGAAGTGCCGGTA
TTAAGGTCATTATGGTGACGGGAGATCACCCGATTACGGCCAAGGCTATTGCAAAGTCGGTTGGGATT
ATTTCGGAGGGTAACGAAACGGTTGAAGATATTGCTCAACGGTTGAATATTCCTGTCTCGGAAGTCAA
CCCGAGGGAAGCCAAAGCTGCCGTTGTTCACGGATCTGATCTCAGAGACCTATCTTCCGATCAATTAG
ACGAAATTTTGAGATACCACACTGAAATTGTATTCGCTAGAACCTCGCCGCAACAGAAGTTGATCATC
GTCGAGGGGTGCCAACGGATGGGCGCTATTGTCGCCGTGACAGGCGACGGCGTGAACGACTCGCCGGC
TTTGAAGAAGGCGGACATCGGTGTGGCCATGGGTATCGCGGGTTCGGATGTGTCCAAGCAAGCCGCCG
ACATGATCCTGCTGGACGATAACTTCGCGTCGATCGTGACAGGAGTGGAGGAAGGCCGTTTGATCTTC
GATAACTTGAAGAAATCTATTGCCTACACCTTGACCTCAAACATTCCCGAAATCTCGCCTTTCCTTGC
TTTCATTTTGTGCGACATTCCTTTGCCTCTCGGTACCGTAACAATTCTGTGCATCGATCTTGGAACTG
ACATGGTGCCTGCTATTTCTCTGGCTTACGAAGCCCCGGAGTCCGACATAATGAAACGTCAGCCGCGC
GACCCCTATAGGGACAACCTGGTTAATCGCAGGTTGATTTCGATGGCATACGGCCAGATTGGTATGAT
TCAAGCAGCTGCTGGTTTCTTCGTGTACTTTGTCATCATGGCTGAGAACGGCTTCCGCCCGACTGACT
TGTTCGGTATTCGAAAGCAATGGGACTCGAAAGCTGTCAATGATCTCACAGATTCGTACGGTCAGGAA
TGGACTTATCGGGACAGGAAGACATTGGAATACACTTGCCACACTGCATTCTTCGTGTCCATCGTGGT
TGTCCAATGGGCCGATTTGATCATTTGTAAGACCCGTCGCAATTCGATCCTCCACCAGGGAATGCGTA
ACTGGGCGCTCAACTTTGGTTTGGTTTTCGAAACTGCACTCGCAGCCTTCCTGTCGTACACTCCCGGG
ATGGACAAGGGTCTGCGCATGTTCCCGCTCAAGTTTGTGTGGTGGTTGCCTGCAATTCCGTTTATGTT
GTCCATCTTCATTTACGACGAAACCCGTCGGTTTTATTTGCGTCGCAATCCAGGAGGTTGGCTGGAAC
AGGAGACCTACTATTAAGCGCATCACACCGTTTTGCGTACTGCCACCAGGTGGCACTGCGTCTGCTGC
CGCAGGTCACAAATAACAACAAACAAAACAAAACAAAAAATCACCACACTCGATTGGAAGGACTCCGT
TTCACCCGCCGTGACGGCGTTGTTCCTTATCGGTCGTTGTGTTGTACAAACAAATCCTGTTGCTATTT
CTAGTAAAATTTGCTGCATGCTTACGCTGCAGTCCGTACATTTGTAATTCGAGAGTTTGGTCGTGTGC
GAGGGCGAGTGAATGGGTGATAGACCGAGAGCGAGGATGATTGCAAGCGGGGCCAGTTGGATTGGTTG
GTTGGTTGGTCTGCAAGAGCAGCAGTAGAGACGCAATAGTGTTTTCATACATTCCAAGTTGAAAACAG
TTGCCTTAGAGCCGATGTCGGACTGTGTGCGAGTGGGTGGGGGCGGAGCCTTGACGGACGGCGGTTGG
TTGTTGCGCGGCCTTCGGGGCTCCGCCACTGAACGTCTTGAACGAGGTAGTATTATTTAGGCTTTCAA

CACTTAGGTTAGCTAGATTGTAACTGTGTTGTTATATCGTGTTAAGTGTCATACCAGTCGTGCTCAAT
ACACATGACTACCAACTACTATCACGATTGTAAACGTTTGACATTCGTTTCGTTTGTTGTGTATATAG
TTTACGTAACGGGCGGGTAGCAGTTACACTGTGTAGGCAGCACGAGTCCATAATATTGTTTCTAACAT
TGTTACGTTACGTGCGGTTAGTTGTTCAACGTTGGAGACACACTTCTGGAGGTCTGCTTCTGTAGAGC
CCTATTTTCTTATACTCTCTGATGCGCGCTGCGTTGGTCTGAGACCAGCCGACCACGGTATTGTTATC
TTATTATTGTAAATATTTTTAAGTGATCACTTAATTATTTTTGCTTGTGTTCTTTTCACATTCATGTT
TTAGTATGTAACGAAGCTGTATAAATTTGGGTTTTAATAAAATGGATGATAGTATTACATTACAAA

Claims

1. An isolated nucleic acid concatemer comprising at least a first nucleic acid sequence and a second nucleic acid sequence;

wherein the first nucleic acid sequence is capable of down-regulating the expression of a first gene of a target, and the second nucleic acid sequence is capable of down-regulating the expression of a second gene of the target.

2. The isolated nucleic acid concatemer according to claim 1, further comprising a third nucleic acid sequence, wherein the third nucleic acid sequence is capable of down-regulating the expression of a third gene of the target;

optionally further comprising a fourth nucleic acid sequence, wherein the fourth nucleic acid sequence is capable of down-regulating the expression of a fourth gene of the target;

optionally further comprising a fifth nucleic acid sequence, wherein the fifth nucleic acid sequence is capable of down-regulating the expression of a fifth gene of the target; and

optionally further comprising a sixth nucleic acid sequence, wherein the sixth nucleic acid sequence is capable of down-regulating the expression of a sixth gene of the target.

3. The isolated nucleic acid concatemer according to either one of claims 1 or 2 wherein the first and/or second nucleic acid sequence and/or third nucleic acid sequence (if present) and/or fourth nucleic acid sequence (if present) and/or fifth nucleic acid sequence (if present) and/or sixth nucleic acid sequence (if present) comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides of the corresponding gene of the target

4. The isolated nucleic acid concatemer according to any one of claims 1 to 3, wherein the target is an organism, or a cell or population of cells derived therefrom, selected from the group comprising:

a member of the *Acari* subclass, a member of the *Arthropoda* phylum, a member of the *Insecta* class, a member of the order *Coleoptera),* an organism which is not *Varroa destructor,* an organism which is not *Caenorhabditis elegans, Rhipicephalus microplus, Rhipicephalus sanguineus, Ctenocephalides felis, Cimex lectularius, Aedes aegypti, Anopheles gambiae complex, Lepeophtheirus salmonis, Caligus rogercresse, Blattella germanica, Periplaneta Americana, Vespula Vulgaris, Vespro crabro, Vespa mandarinia, Coptotermes formosanus, Incisitermes snyderi, Reticulitermes flavipes, Anobium punctatum, Musca domestica, Tineola bisselliella, Varroa destructor, Galleria mellonella, Achroia*

grisella, Aethina tumida, Acarapis woodi, Tropilaelaps clareae, Nosema apis, Nosema ceranae, Acyrthosiphon pisum, Tenebrio molitor, Tribolium castaneum, Tribolium confusum, Sitophilus granaries, Anthonomus grandis, Plutella xylostella, Lymantria dispar dispar, Helicoverpa zea, Cornu aspersa, Deroceras reticulatum, Arion hortensis, Tetranychus urticae, Trypanosma brucei, Trypanosma cruzi, Entamoeba histolytica, Toxoplasma gondi, and *Giardia intestinalis.*

5. The isolated nucleic acid concatemer according to any one of claims 1 to 4, wherein the first, second, third, fourth, fifth and/or sixth gene, if present, is selected from the group consisting of the genes which encode:

a Na+/K+-ATPase subunits, a Vacuolar ATPase subunit, Plasma membrane Calcium ATPase, Sarcoplasmic reticulum Ca2+ ATPase, ADP/ATP- translocase, Sodium-glucose linked transporter, Trehalase, Pyruvate dehydrogenase, Pyruvate kinase, Pyruvate carboxylase, Tubulin, Monoamine oxidase, Acetylcholinesterase, and Phosphodiesterase.

6. The isolated nucleic acid concatemer according to any preceding claim, wherein the target is the *V.destructor* organism and the first and/or second gene and/or third gene, if present are selected from the group consisting of the genes which encode: Acetylcholinesterase (AChE; GenBank accession number ADDG01069748.1), Monoamine Oxidase (MOA; GenBank accession number ADDG01053234.1), andvATPase subunit C (vATPc; GenBank accession number ADDG01035752.1).

7. The isolated nucleic acid concatemer according to claim 6, wherein the target is the *V.destructor* organism and the first and/or second nucleic acid sequence and/or third nucleic acid sequence, if present, comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides encoded by a sequence selected from the group consisting of SEQ ID NO.1, SEQ ID NO.2, and SEQ ID NO.3.

8. The isolated nucleic acid concatemer according to any one of claims 1 to 5, wherein the target is the *T.castaneum* organism and the first and/or second gene and/or third and/or fourth and/or fifth and/or sixth gene, if present, are selected from the group consisting of the genes which encode: Plasma membrane calcium-transporting ATPase 1 (TcPMCA; NCBI accession number XM_008201630.1), Na/K ATPase alpha (TcNaK; NCBI accession number XM_008198203.1), ADP/ATP translocase (TcADPt; NCBI accession number XM_968164.3) , vATPase subunit E (TcvATPe; NCBI accession number XM_965528.2), Calcium-transporting ATPase sarcoplasmic /endoplasmic reticulum type (TcSERCA; NCBI accession number XM_961690.3), a-tubulin 1 (TcaTUB; NCBI accession number

XP_966492.1), and Heat shock protein 90 (TcHSP90; NCBI accession number NP_001094067.1).

9. The isolated nucleic acid concatemer according to claim 8, wherein the target is the *T.castaneum* organism and the first and/or second nucleic acid sequence and/or third nucleic acid sequence and/or fourth nucleic acid sequence and/or fifth nucleic acid sequence and/or sixth nucleic acid sequence, if present, comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides encoded by a sequence selected from the group consisting of SEQ ID NO.8, SEQ ID NO.9, SEQ ID NO.113, SEQ ID NO.10, SEQ ID NO.11, SEQ ID NO.12, SEQ ID NO.13, and SEQ ID NO.14.

10. The isolated nucleic acid concatemer according to any one of claims 1 to 5, wherein the target is the *A.aegypti* organism and the first and/or second gene and/or third gene, if present, are selected from the group consisting of the genes which encode: Tubulin beta chain (AabTub; NCBI accession number XM_001662168.1), Na/K ATPase alpha subunit (AaNaK; NCBI accession number ADDG01053234.1), and ADP/ATP carrier protein (AaADPt; NCBI accession number XM_001649861.1).

11. The isolated nucleic acid concatemer according to claim 10, wherein the target is the *A.aegypti* organism and the first and/or second nucleic acid sequence and/or third nucleic acid sequence, if present, comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides encoded by a sequence selected from the group consisting of SEQ ID NO.19, SEQ ID NO.20, and SEQ ID NO.21.

12. The isolated nucleic acid concatemer according to any one of claims 1 to 5, wherein the target is the *L.salmonis* organism and the first and/or second gene and/or third gene, if present, are selected from the group consisting of the genes which encode: ADP/ATP translocase 1 (LsADPt; NCBI accession number BT077972.1), V-type ATPase unit E (LsvATPe; NCBI accession number BT120776.1), and acetylcholinesterase (LsAChE; NCBI accession number KJ132369.1).

13. The isolated nucleic acid concatemer according to claim 12, wherein the target is the *L.salmonis* organism and the first and/or second nucleic acid sequence and/or third nucleic acid sequence, if present, comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides encoded by a sequence selected from the group consisting of SEQ ID NO.23, SEQ ID NO.24, and SEQ ID NO.25.

14. The isolated nucleic acid concatemer according to any one of claims 1 to 5, wherein the target is the *C.elegans* organism and the first and/or second gene and/or third gene, if present, are selected from the group consisting of the genes which encode: pat-10 (NCBI accession number NM_059100.6), bli-5 (NCBI accession number NM_067371.1), and egl-30 (NCBI accession number U56864.1).

15. The isolated nucleic acid concatemer according to claim 14, wherein the target is the *C.elegans* organism and the first and/or second nucleic acid sequence and/or third nucleic acid sequence, if present, comprises a nucleic acid sequence that has 100% sequence identity to at least 18 contiguous nucleotides encoded by a sequence selected from the group consisting of SEQ ID NO.27, SEQ ID NO.28, and SEQ ID NO.29.

16. The isolated nucleic acid concatemer according to any preceding claim, wherein the total length of the concatemer is less than 2000 bases.

17. The isolated nucleic acid concatemer according to any preceding claim, wherein the total length of the concatemer is greater than 500 bases.

18. The isolated nucleic acid concatemer according to any preceding claim, wherein the total length of the concatemer is between 750 and 1500 bases.

19. The isolated nucleic acid concatemer according to any preceding claim, wherein mRNA levels of the targeted genes in the treated target cells or organisms are 40% lower 72 hours after exposure to the concatemer.

20. The isolated nucleic acid concatemer according to any preceding claim, wherein mRNA levels of the targeted genes in the treated target cells or organisms are 80% lower 72 hours after exposure to the concatemer.

21. The isolated nucleic acid concatemer according to any preceding claim, wherein the concatemer causes greater than 30% target mortality, as measured 108 hours after a 12 hour soaking of the mite in a 1.25 µg/µl solution of the concatemer.

22. The isolated nucleic acid concatemer according to any preceding claim, wherein the concatemer causes greater than 60% target mortality, as measured 108 hours after a 12 hour soaking of the mite in a 1.25 µg/µl solution of the concatemer.

23. The isolated nucleic acid concatemer according to either one of claims 21 or 22, wherein the mortality is observed in the organisms contacted with the concatamer.

24. The isolated nucleic acid concatemer according to any preceding claim, wherein the nucleic acid concatemer is a dsRNA, antisense RNA, or a ribozyme.

25. The isolated nucleic acid concatemer according to claim 24 wherein the dsRNA is an siRNA, shRNA or miRNA.

26. A nucleic acid construct encoding the isolated nucleic acid concatemer according to any preceding claim.

27. A nucleic acid construct according to claim 26, wherein the nucleic acid construct is a deoxyribonucleic acid encoding a dsRNA nucleic acid concatemer.

28. The nucleic acid construct of either one of claims 26 or claim 27 having the sequence set out in SEQ ID NOs. 4, 15, 22, or 26.

29. A host cell comprising a nucleic acid concatemer according to any one of claims 1 to 25, or the nucleic acid construct according to any one of claims 26 to 28.

30. A composition comprising at least one nucleic acid concatemer according to any one of claims 1 to 25, and/or at least one nucleic acid construct according to any one of claims 26 to 28, in combination with a physiologically or agronomically acceptable excipient, carrier, or diluent.

31. An isolated nucleic acid concatemer according to any one of claims 1 to 25, or composition according to claim 30, for use in a method of:

(i) inhibiting the growth of, or reducing, a population of a target cell / organism;

(ii) treating a disorder associated with a parasitic or infectious target cell / organism; or

(iii) treating a disorder associated with a pathogenic cell population (for example, cancer).

32. Use of an isolated nucleic acid concatemer according to any one of claims 1 to 25, or composition according to claim 30, in the manufacture of a medicament for:

(i) inhibiting the growth of, or reducing, a population of a target cell / organism;

(ii) treating a disorder associated with a parasitic or infectious target cell / organism; or

(iii) treating a disorder associated with a pathogenic cell population (for example, cancer).

33. A method of:

(i) inhibiting the growth of, or reducing, a population of a target cell / organism;

(ii) treating a disorder associated with a parasitic or infectious target cell / organism; or

(iii) treating a disorder associated with a pathogenic cell population (for example, cancer).

the method comprising exposing a target cell or organism administering to an isolated nucleic acid concatemer according to any one of claims 1 to 25, or composition according to claim 30.

34. A transgenic plant cell, plant, or part thereof, which expresses a nucleic acid concatemer nucleic acid concatemer according to any one of claims 1 to 25, or which contains a nucleic acid construct according to any one of claims 26 to 28.

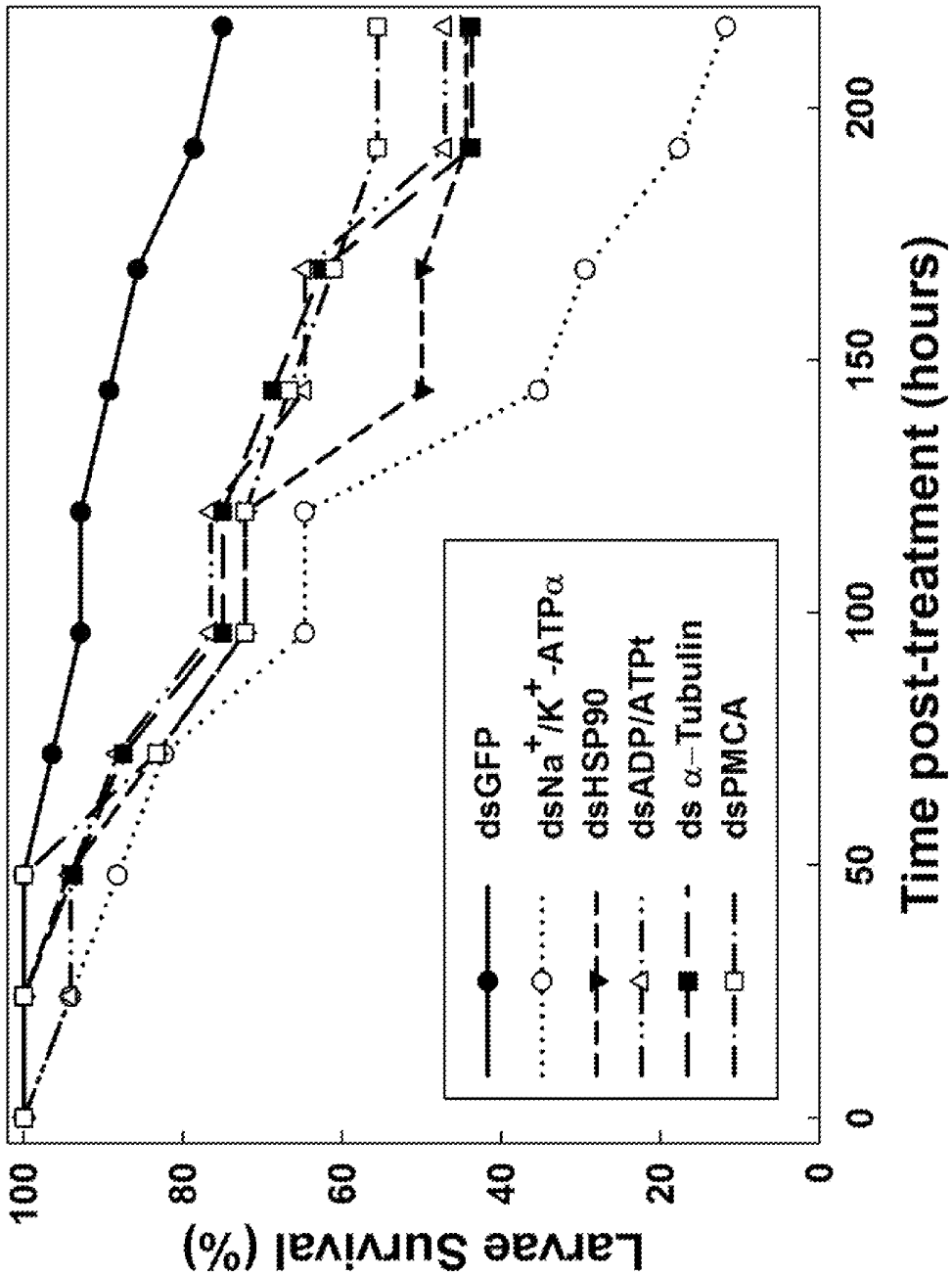
Larvae Survival (%)
0
20
40
60
80
100
0
50
100
150
200
Time post-treatment (hours)
dsGFP
dsNa+/K+-ATPα
dsHSP90
dsADP/ATPt
ds α–Tubulin
dsPMCA

Figure 1

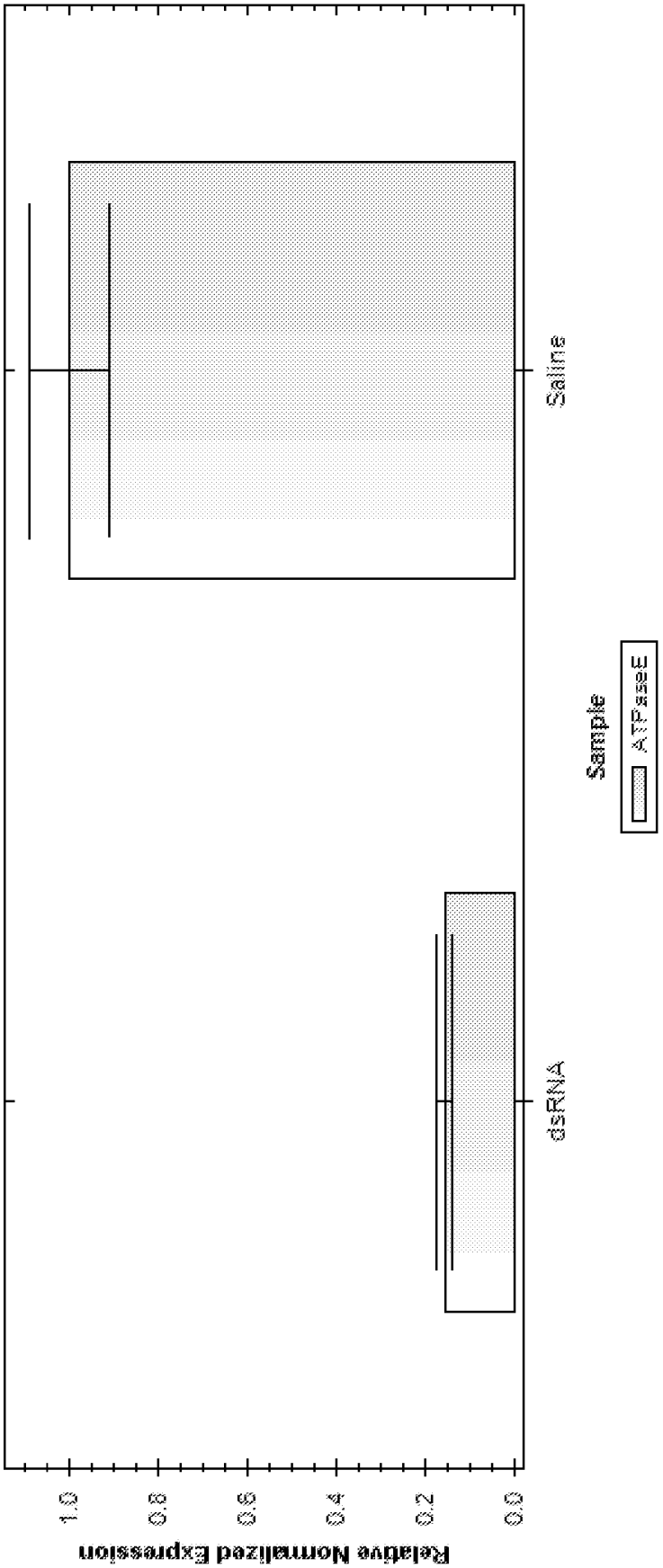
Relative Normalized Expression
1.0
0.8
0.6
0.4
0.2
0.0
dsRNA
Saline
Sample
ATPaseE

Figure 2

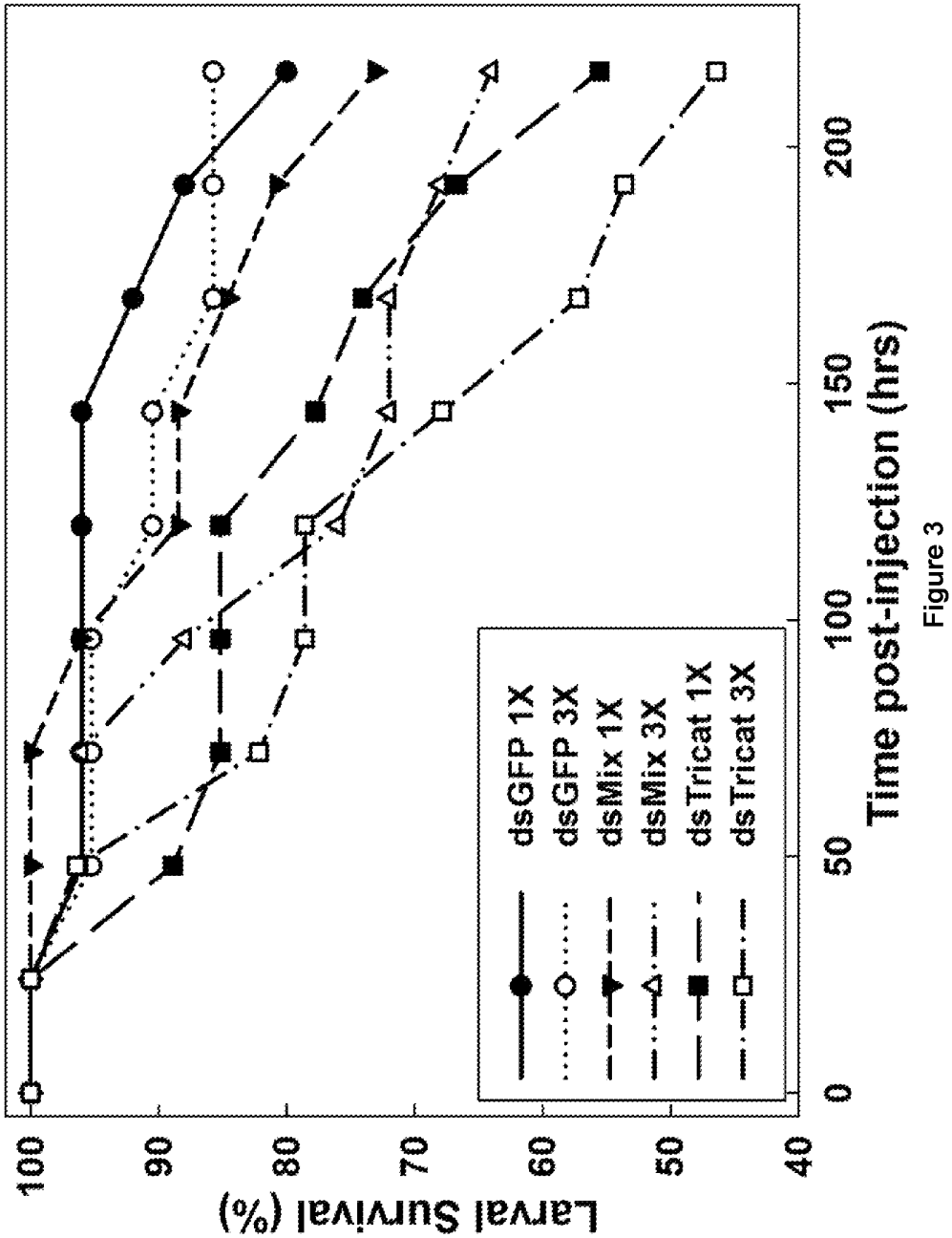
Larval Survival (%)
100
90
80
70
60
50
40
0
50
100
150
200
Time post-injection (hrs)
dsGFP 1X
dsGFP 3X
dsMix 1X
dsMix 3X
dsTricat 1X
dsTricat 3X

Figure 3

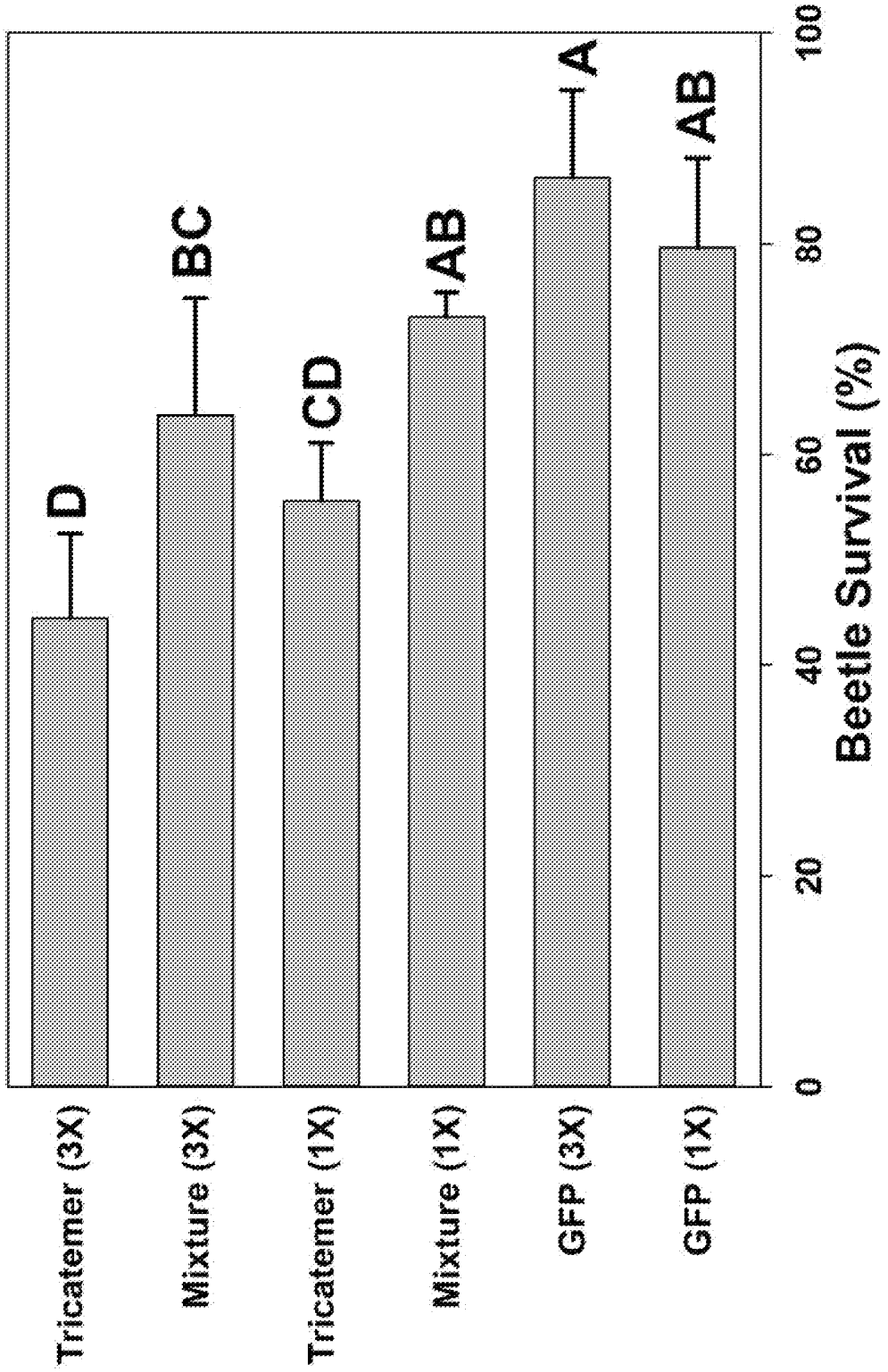
D
BC
CD
AB
A
AB
Tricatemer (3X)
Mixture (3X)
Tricatemer (1X)
Mixture (1X)
GFP (3X)
GFP (1X)
0
20
40
60
80
100
Beetle Survival (%)

Figure 4

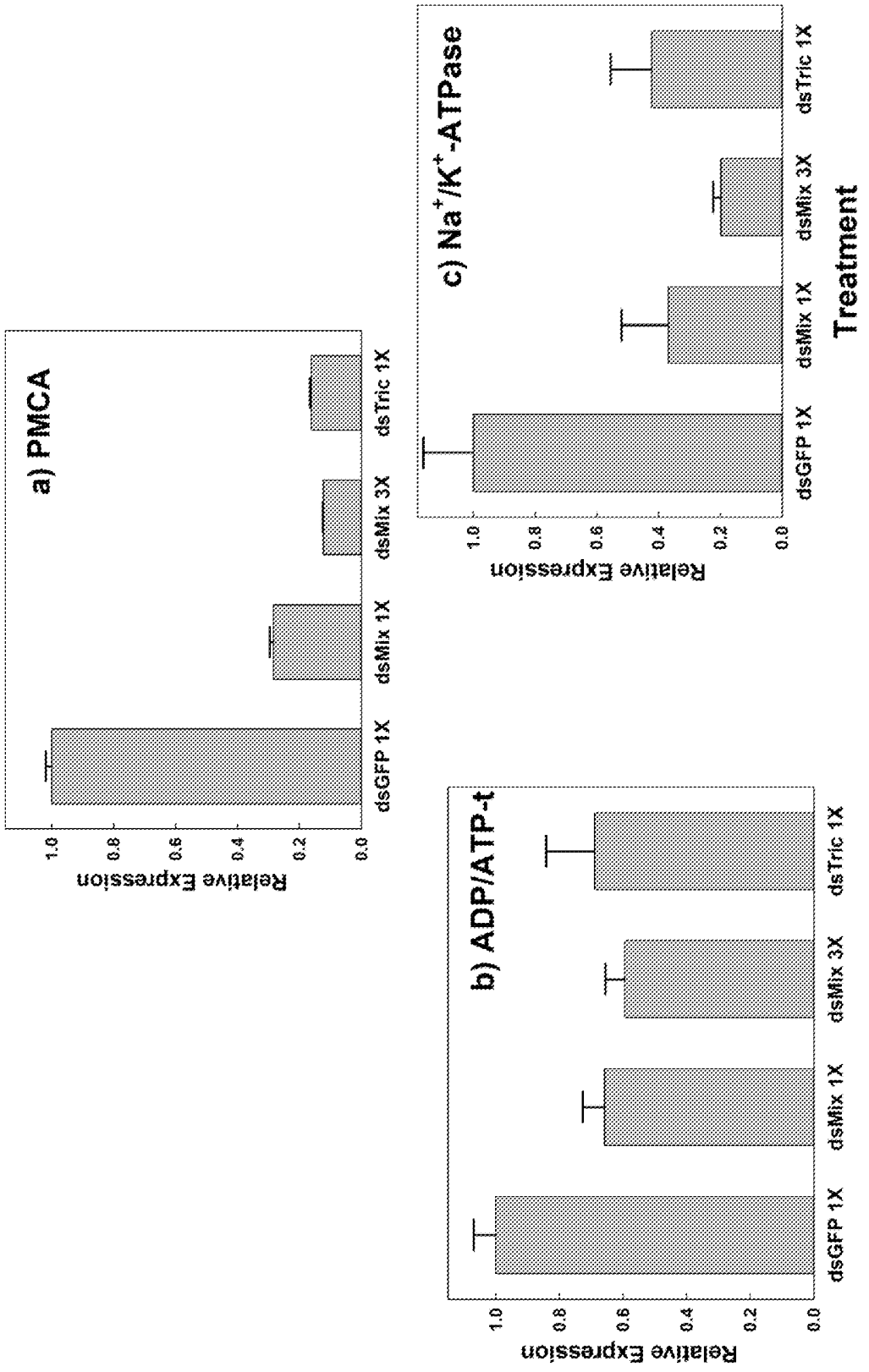
a) PMCA
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
dsGFP 1X
dsMix 1X
dsMix 3X
dsTric 1X
b) ADP/ATP-t
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
dsGFP 1X
dsMix 1X
dsMix 3X
dsTric 1X
c) Na+/K+-ATPase
Relative Expression
1.0
0.8
0.6
0.4
0.2
0.0
dsGFP 1X
dsMix 1X
dsMix 3X
dsTric 1X
Treatment

Figure 5

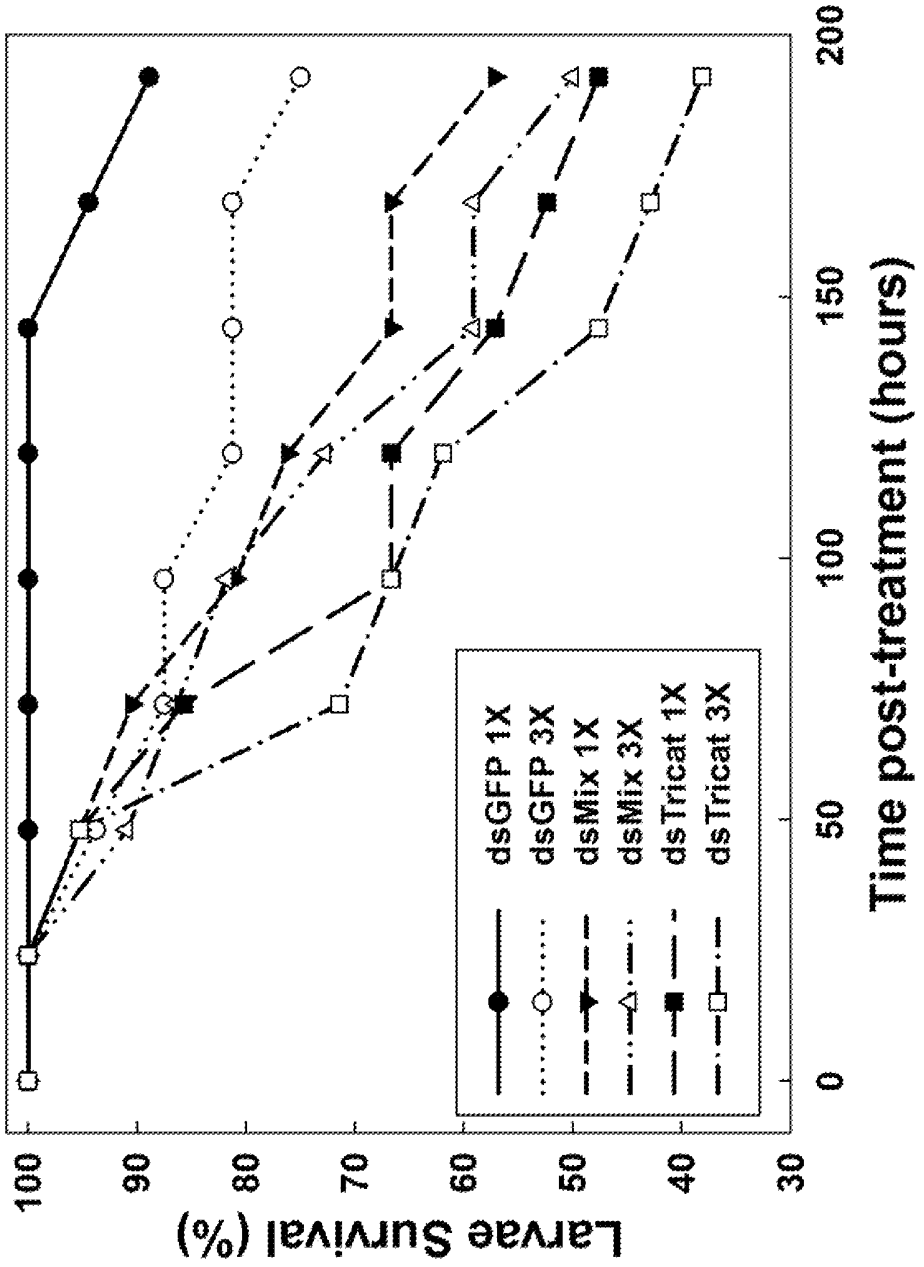
Larvae Survival (%)
100
90
80
70
60
50
40
30
0
50
100
150
200
Time post-treatment (hours)
dsGFP 1X
dsGFP 3X
dsMix 1X
dsMix 3X
dsTricat 1X
dsTricat 3X

Figure 6

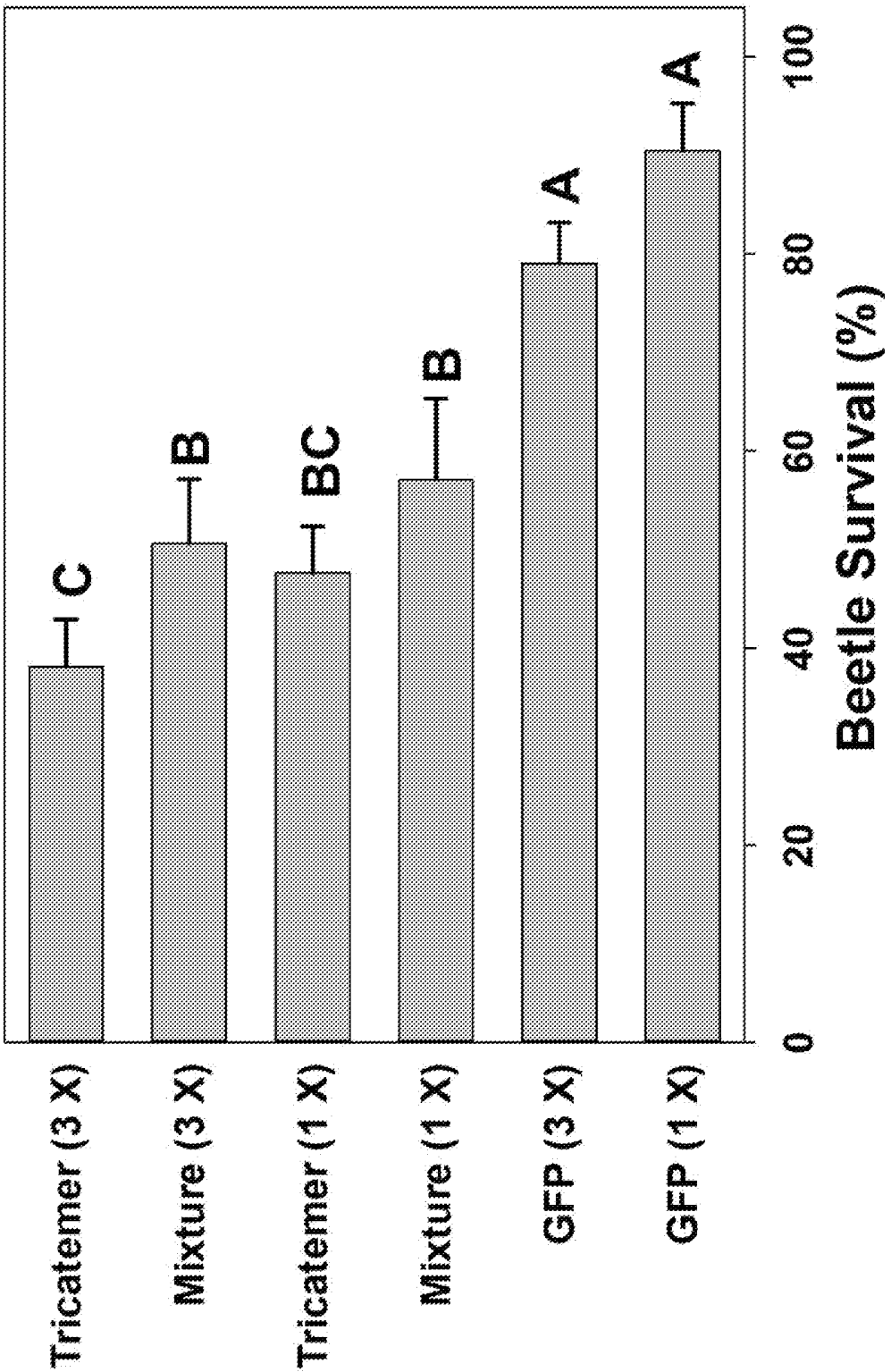
Tricatemer (3 X)
Mixture (3 X)
Tricatemer (1 X)
Mixture (1 X)
GFP (3 X)
GFP (1 X)
C
B
BC
B
A
A
0
20
40
60
80
100
Beetle Survival (%)

Figure 7

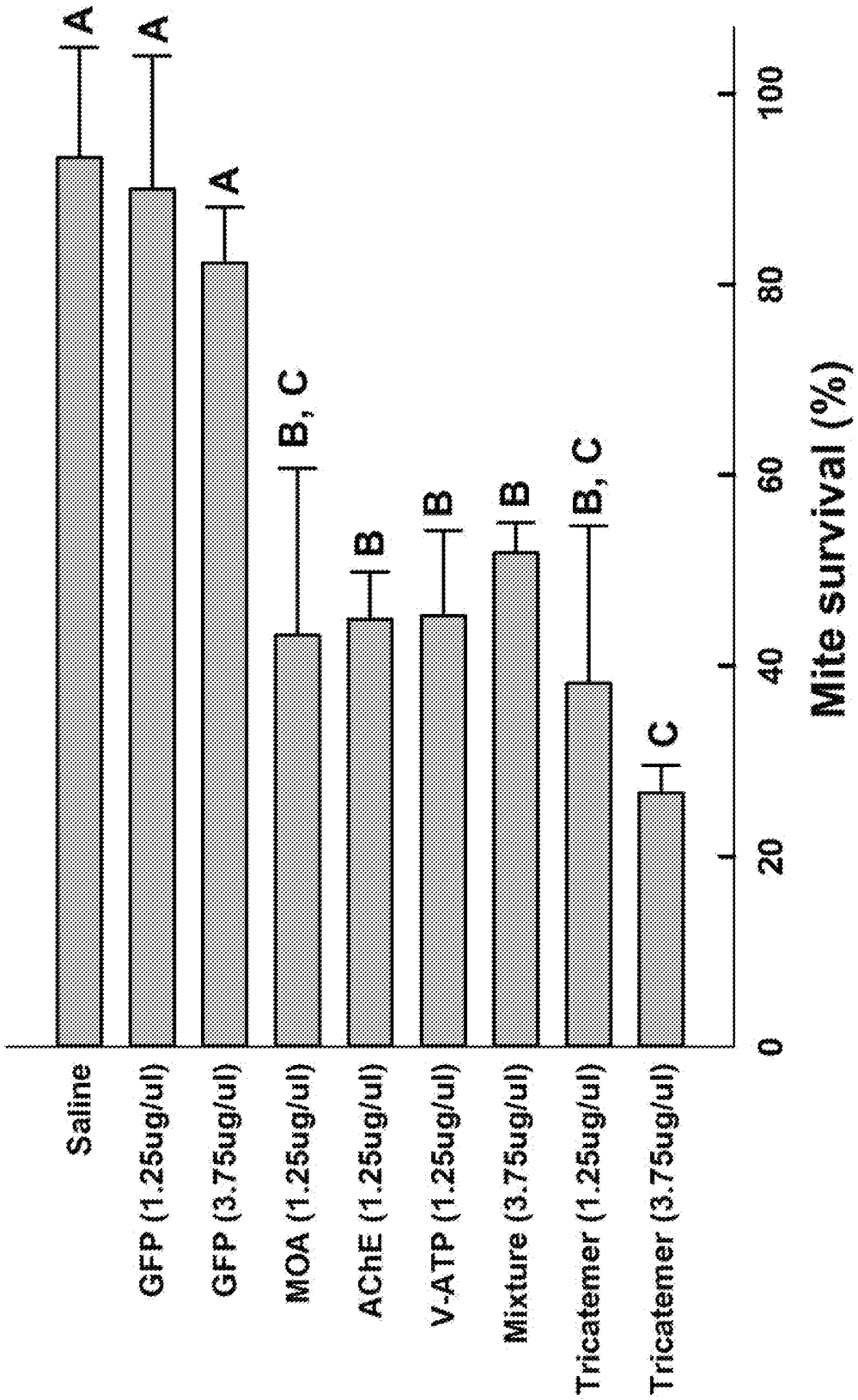
A
A
A
B, C
B
B
B
B, C
C
Saline
GFP (1.25ug/ul)
GFP (3.75ug/ul)
MOA (1.25ug/ul)
AChE (1.25ug/ul)
V-ATP (1.25ug/ul)
Mixture (3.75ug/ul)
Tricatemer (1.25ug/ul)
Tricatemer (3.75ug/ul)
0
20
40
60
80
100
Mite survival (%)

Figure 8

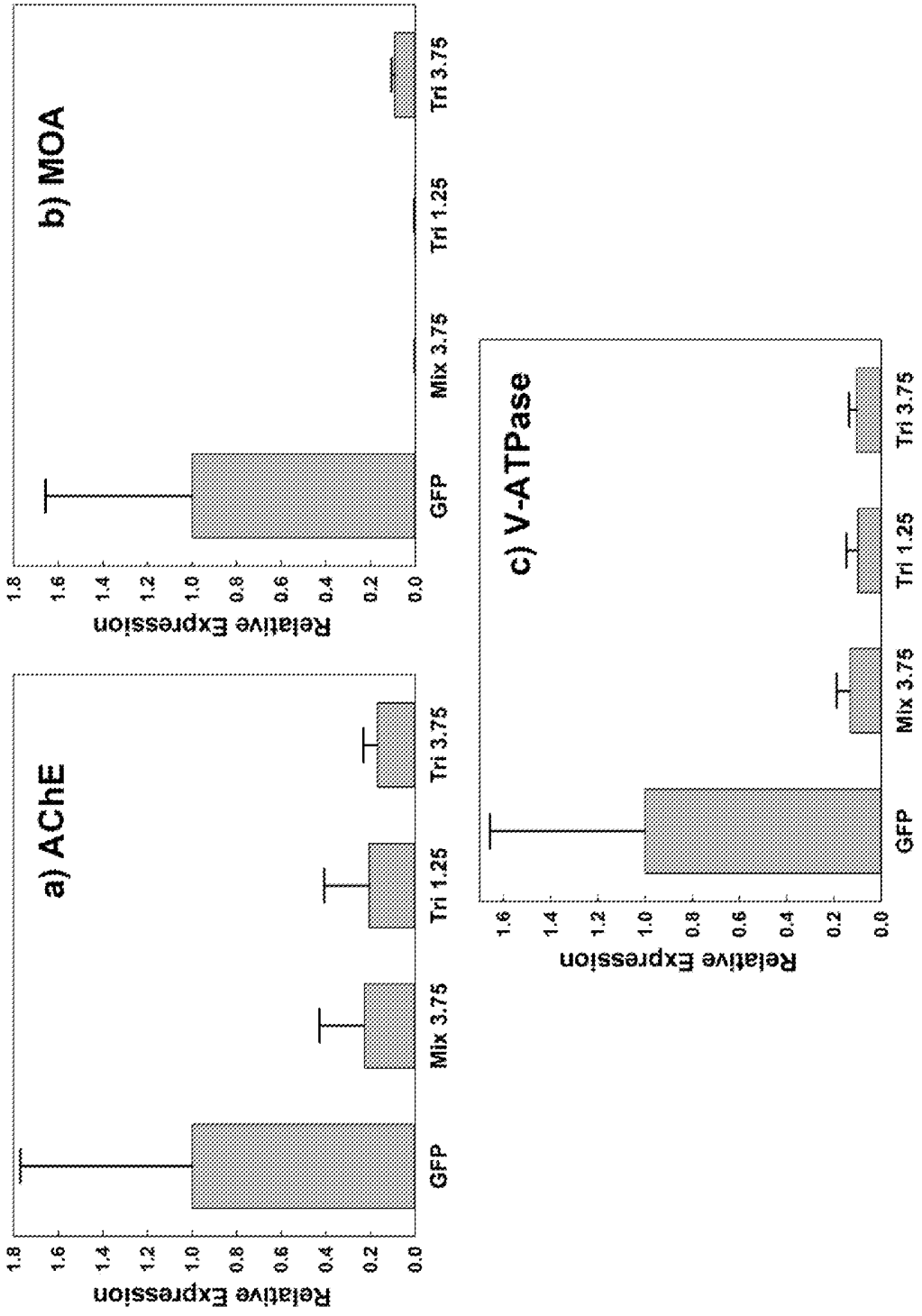
a) AChE
b) MOA
c) V-ATPase
Relative Expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GFP
Mix 3.75
Tri 1.25
Tri 3.75

Figure 9

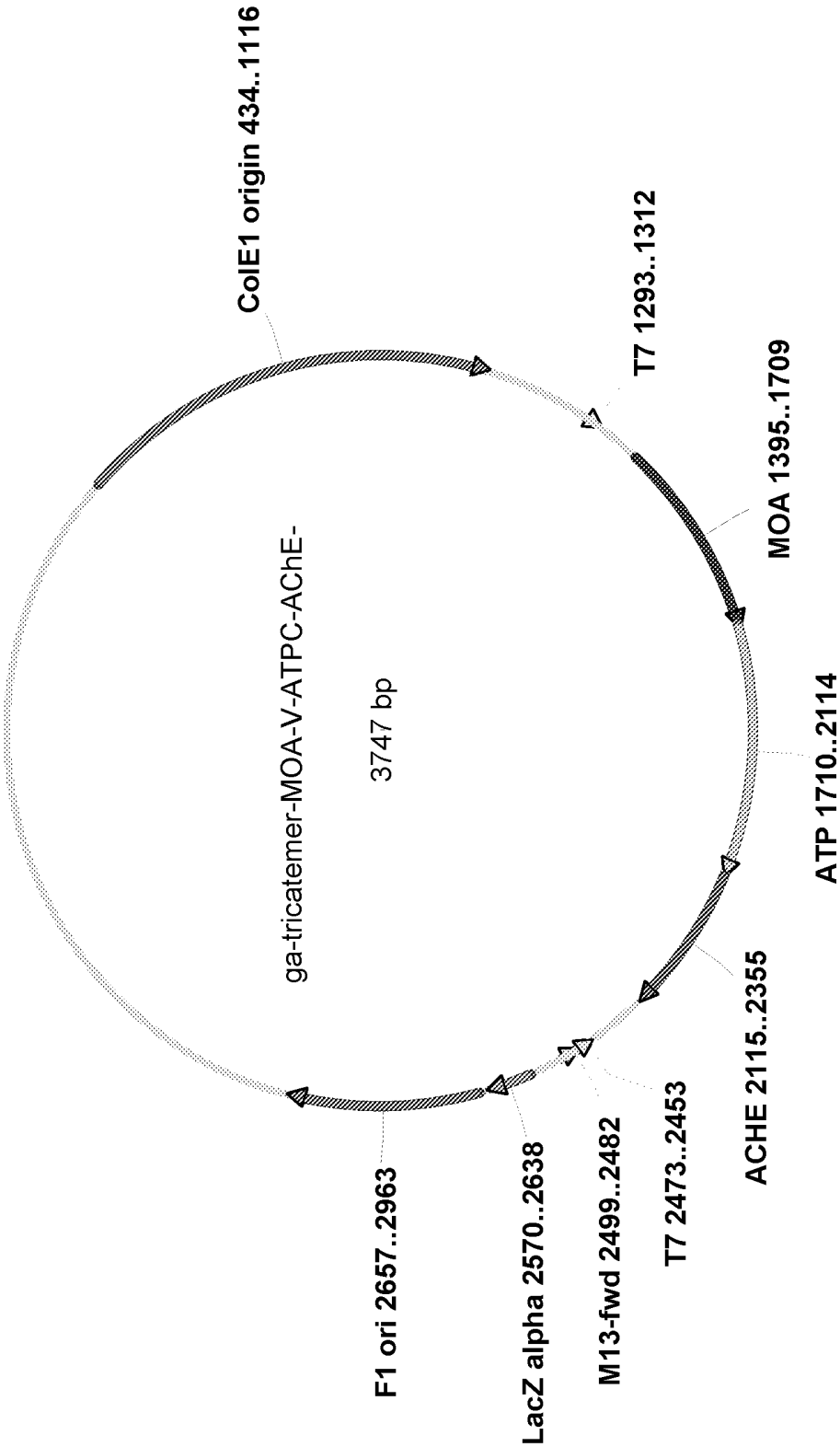
ColE1 origin 434..1116
T7 1293..1312
MOA 1395..1709
ATP 1710..2114
ACHE 2115..2355
T7 2473..2453
M13-fwd 2499..2482
LacZ alpha 2570..2638
F1 ori 2657..2963
ga-tricatemer-MOA-V-ATPC-AChE-
3747 bp

Figure 10

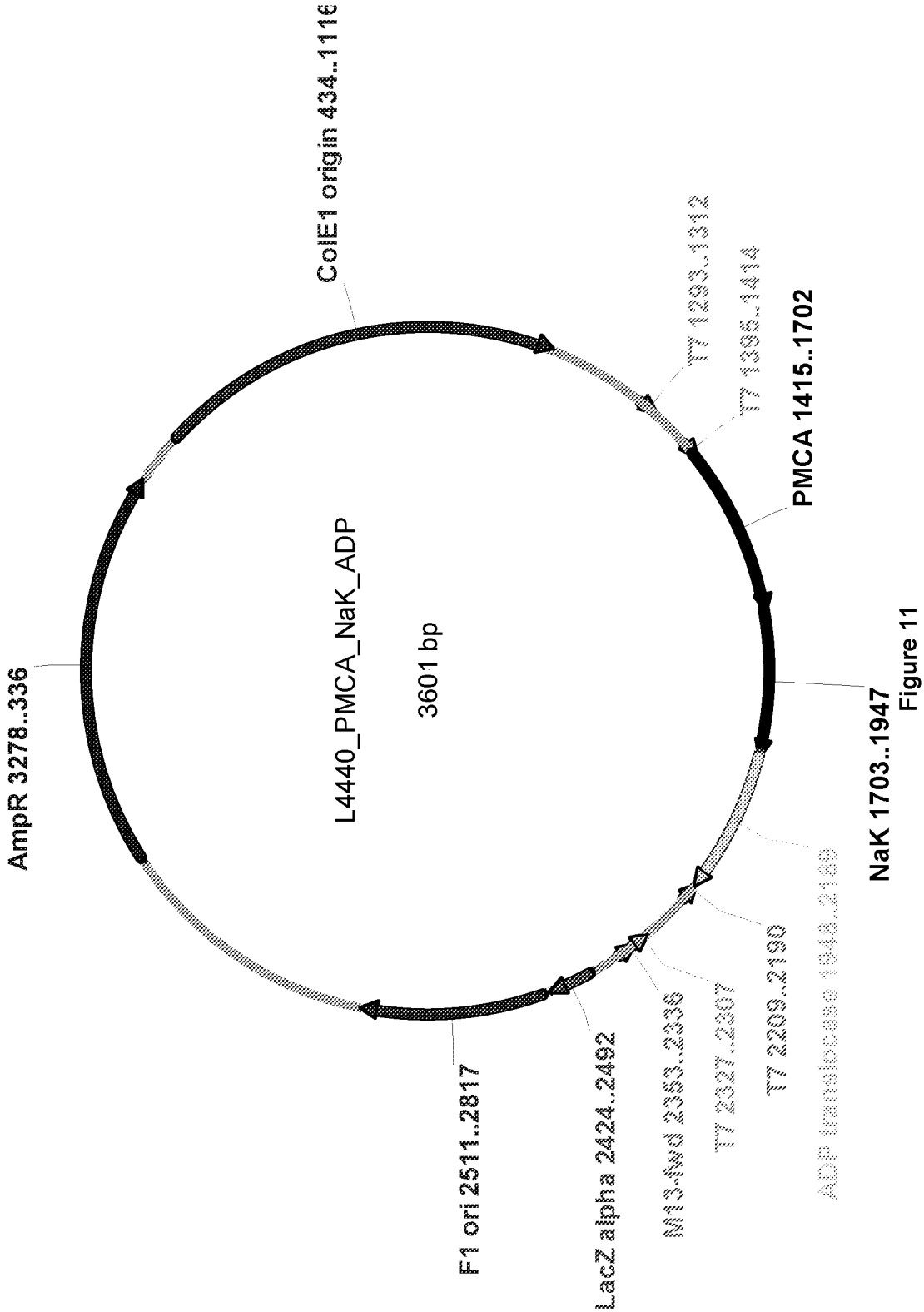
L4440_PMCA_NaK_ADP
3601 bp
ColE1 origin 434..1116
T7 1203..1312
T7 1395..1414
PMCA 1415..1702
NaK 1703..1947
ADP translocase 1948..2180
T7 2209..2190
T7 2327..2307
M13-fwd 2353..2336
LacZ alpha 2424..2492
F1 ori 2511..2817
AmpR 3278..336

Figure 11

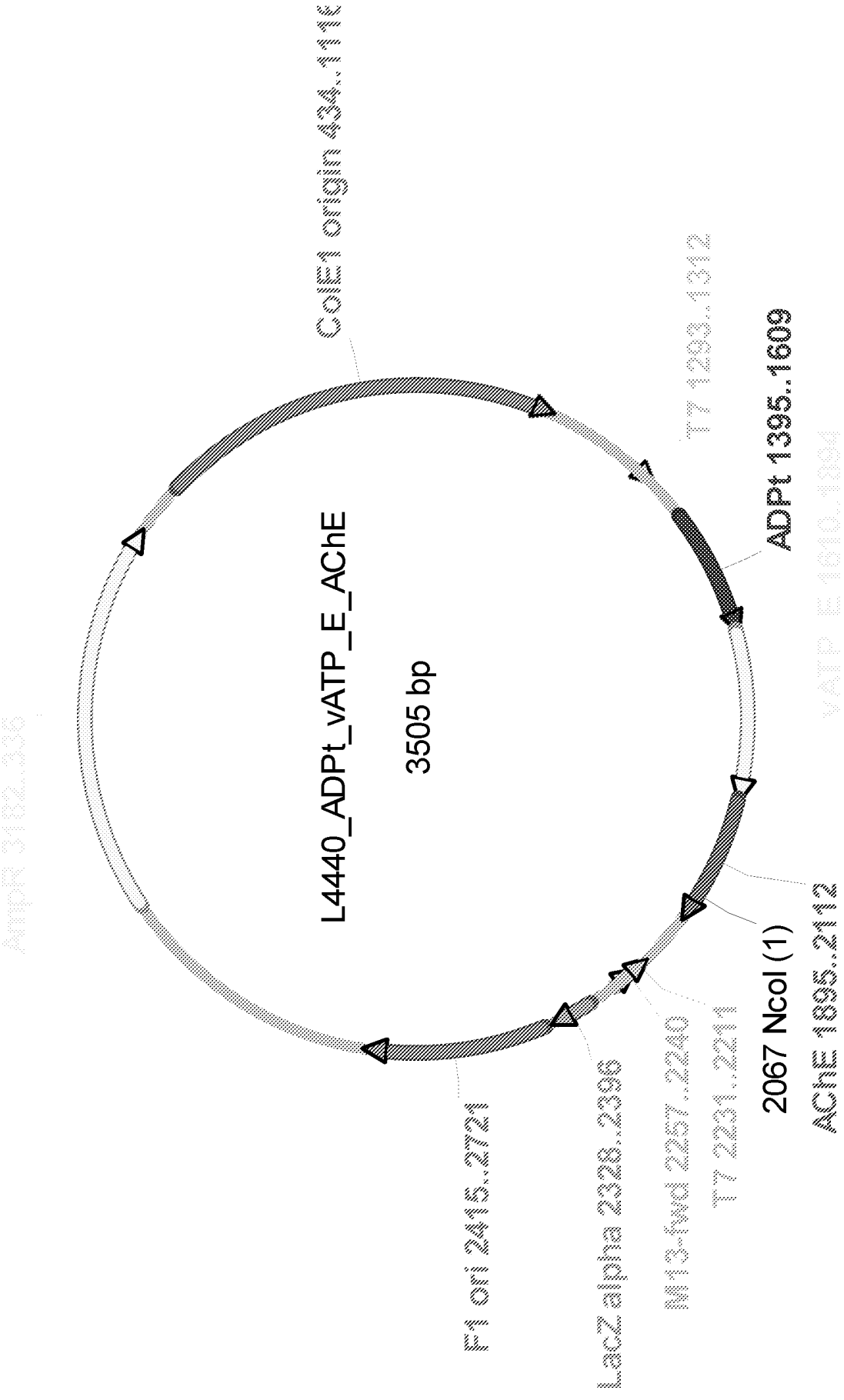
ColE1 origin 434..1114
T7 1293..1312
ADPt 1395..1609
L4440_ADPt_vATP_E_AChE
3505 bp
2067 NcoI (1)
AChE 1895..2112
T7 2231..2211
M13-fwd 2257..2240
LacZ alpha 2328..2396
F1 ori 2415..2721

Figure 12

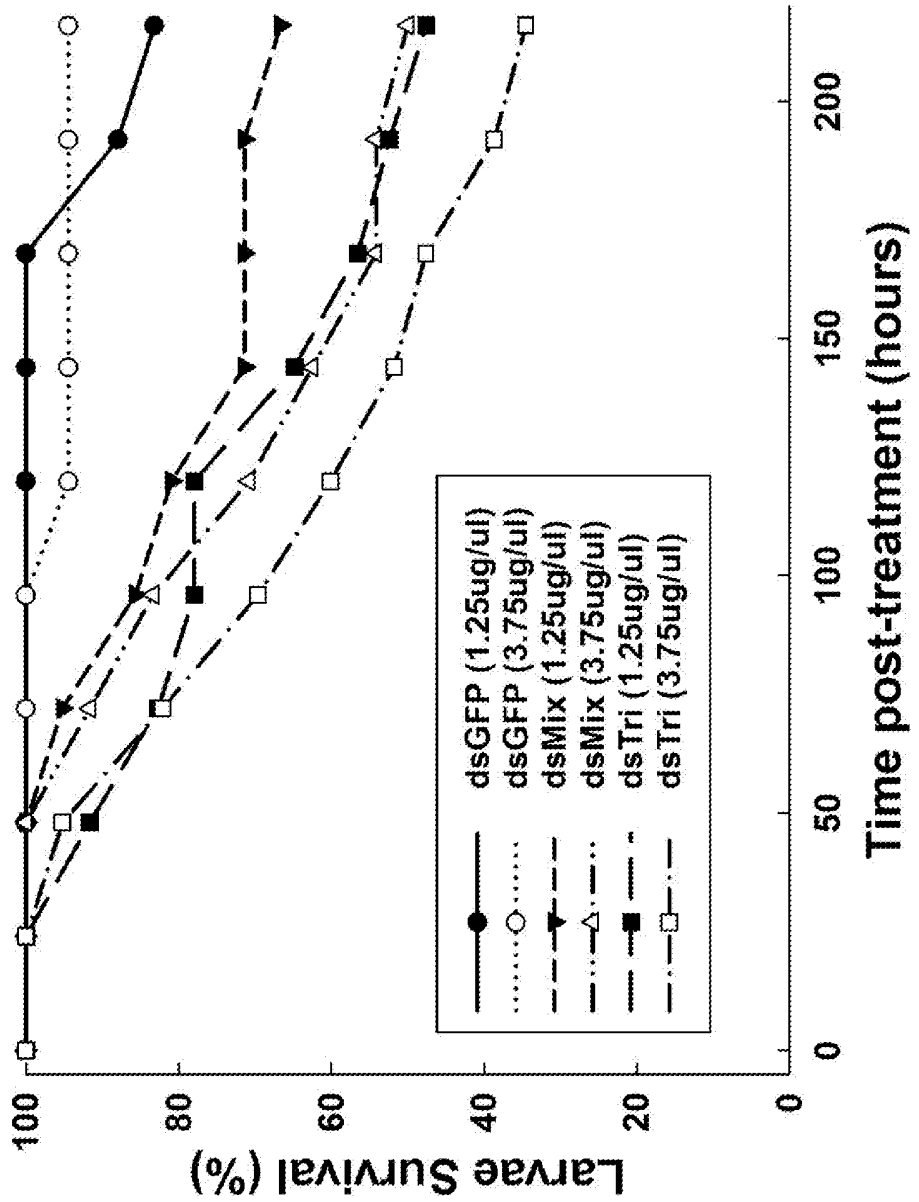
Larvae Survival (%)
100
80
60
40
20
0
0
50
100
150
200
Time post-treatment (hours)
dsGFP (1.25ug/ul)
dsGFP (3.75ug/ul)
dsMix (1.25ug/ul)
dsMix (3.75ug/ul)
dsTri (1.25ug/ul)
dsTri (3.75ug/ul)

Figure 13

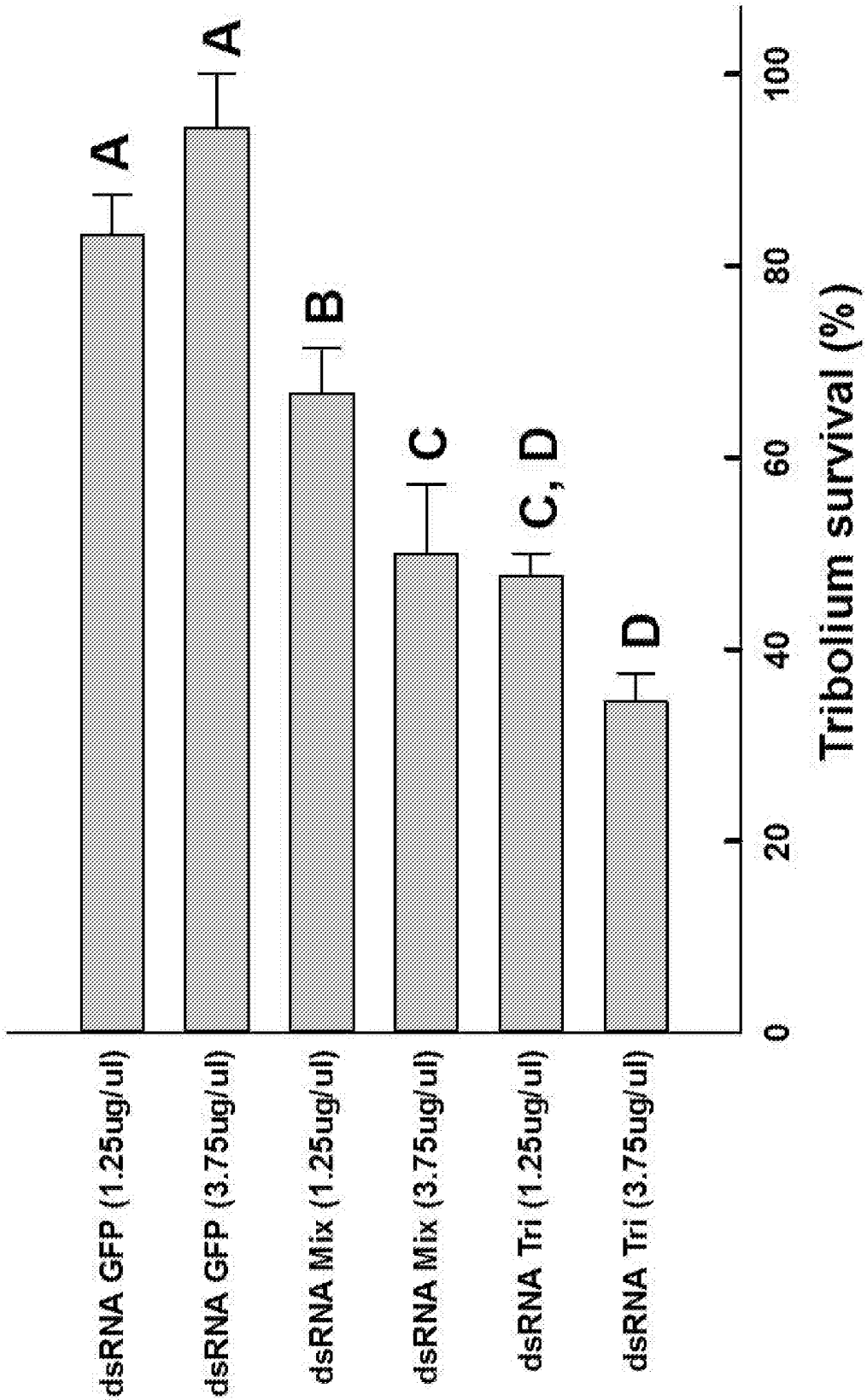
A
A
B
C
C, D
D
0
20
40
60
80
100
Tribolium survival (%)
dsRNA GFP (1.25ug/ul)
dsRNA GFP (3.75ug/ul)
dsRNA Mix (1.25ug/ul)
dsRNA Mix (3.75ug/ul)
dsRNA Tri (1.25ug/ul)
dsRNA Tri (3.75ug/ul)

Figure 14

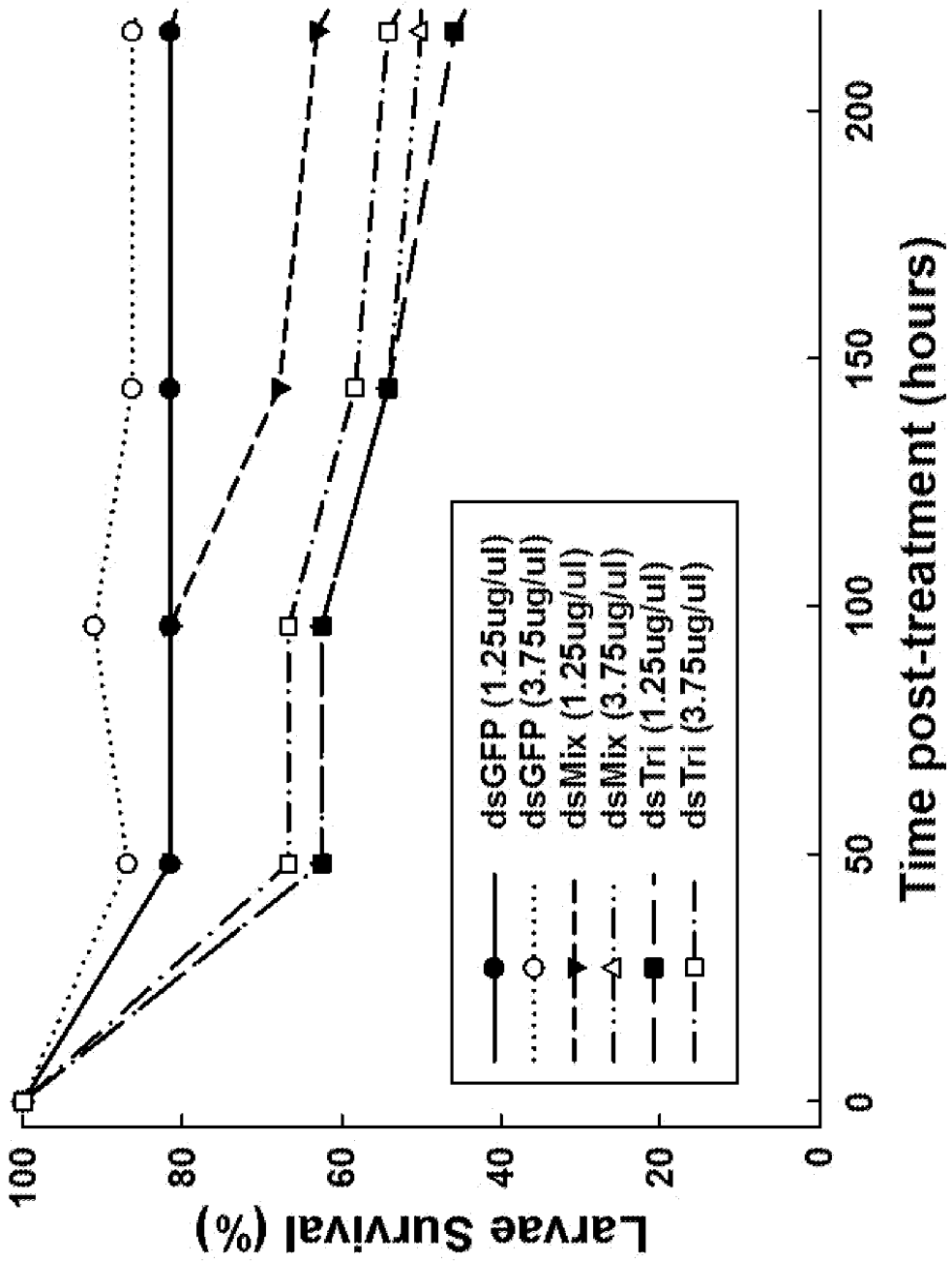
Larvae Survival (%)
100
80
60
40
20
0
0
50
100
150
200
Time post-treatment (hours)
dsGFP (1.25ug/ul)
dsGFP (3.75ug/ul)
dsMix (1.25ug/ul)
dsMix (3.75ug/ul)
dsTri (1.25ug/ul)
dsTri (3.75ug/ul)

Figure 15

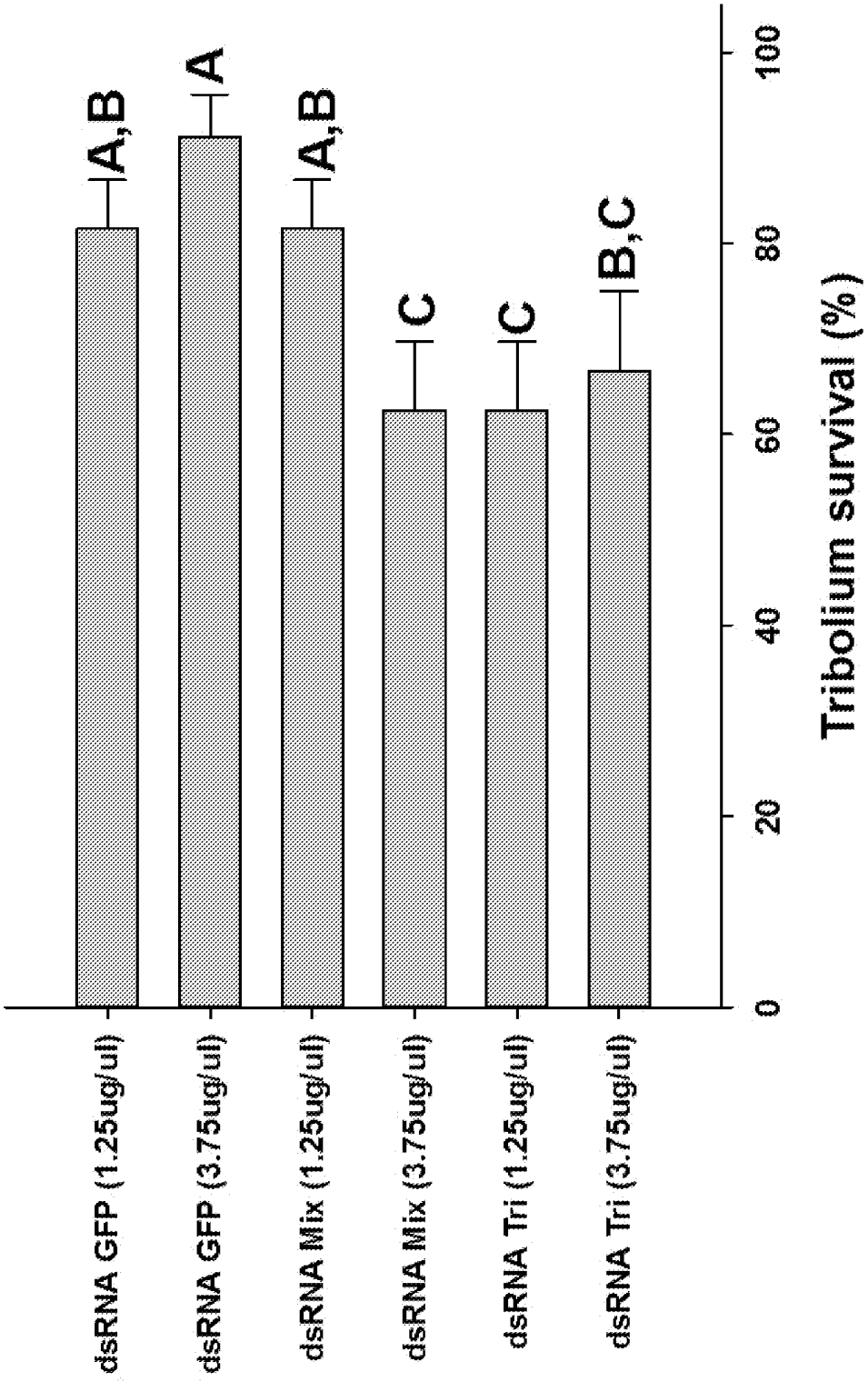
A,B
A
A,B
C
C
B,C
0
20
40
60
80
100
Tribolium survival (%)
dsRNA GFP (1.25ug/ul)
dsRNA GFP (3.75ug/ul)
dsRNA Mix (1.25ug/ul)
dsRNA Mix (3.75ug/ul)
dsRNA Tri (1.25ug/ul)
dsRNA Tri (3.75ug/ul)

Figure 16

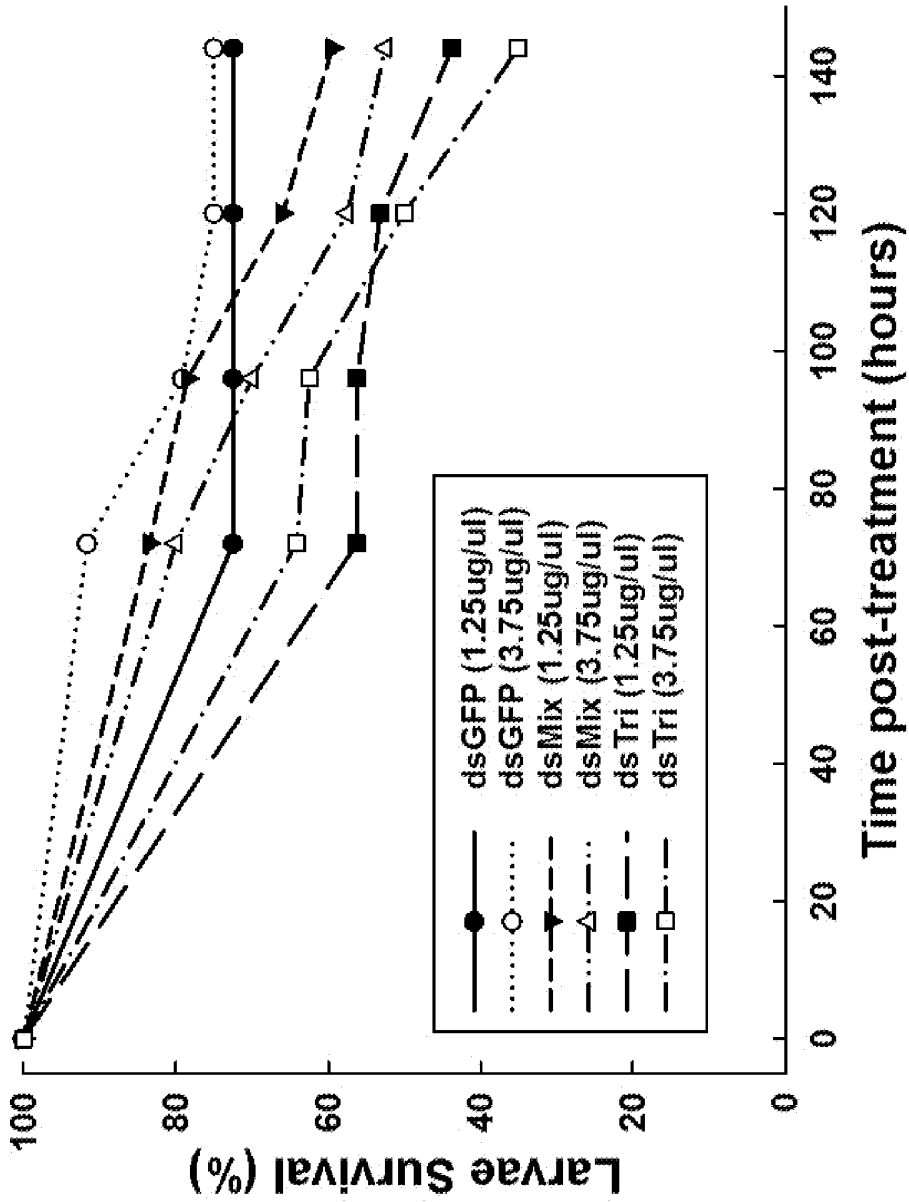
Larvae Survival (%)
0
20
40
60
80
100
Time post-treatment (hours)
0
20
40
60
80
100
120
140
dsGFP (1.25ug/ul)
dsGFP (3.75ug/ul)
dsMix (1.25ug/ul)
dsMix (3.75ug/ul)
dsTri (1.25ug/ul)
dsTri (3.75ug/ul)

Figure 17

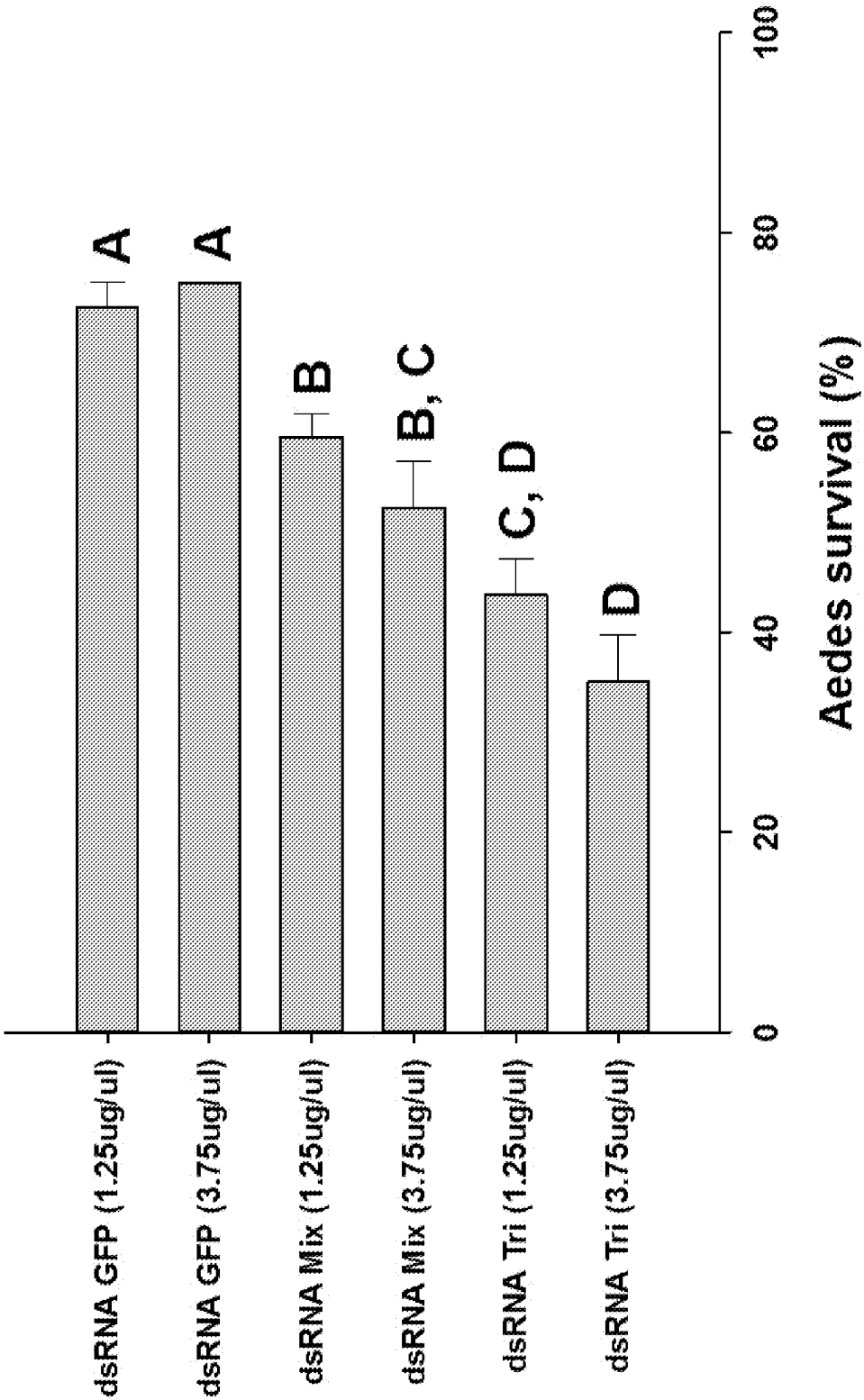
Aedes survival (%)
0
20
40
60
80
100
A
A
B
B, C
C, D
D
dsRNA GFP (1.25ug/ul)
dsRNA GFP (3.75ug/ul)
dsRNA Mix (1.25ug/ul)
dsRNA Mix (3.75ug/ul)
dsRNA Tri (1.25ug/ul)
dsRNA Tri (3.75ug/ul)

Figure 18

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/050015

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 A61K31/7088
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2015/001336 A2 (UNIV ABERDEEN [GB]; SEC DEP FOR ENVIRONMENT FOOD AND RURAL AFFAIRS ACT) 8 January 2015 (2015-01-08) cited in the application the whole document	1-7, 16-33
X	WO 2006/046148 A2 (DEVGEN NV [BE]) 4 May 2006 (2006-05-04) cited in the application	1-5, 16-27, 29-34
Y	the whole document	5
X	WO 2006/128739 A1 (POLYPLUS-TRANSFECTION SA [FR]) 7 December 2006 (2006-12-07) the whole document	1,3,16, 19,20, 24-27, 29,30

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 March 2016	21/03/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Macchia, Giovanni

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2011/045796 A1 (YISSUM RES DEV CO [IL]; BEEOLOGICS LLC [US]; SELA ILAN [IL]; SHAFIR SH) 21 April 2011 (2011-04-21) the whole document	5
A	WHYARD S. ET AL.: "Ingested double-stranded RNAs can act as species-specific insecticides", INSECT BIOCHEMISTRY AND MOLECULAR BIOLOGY, ELSEVIER SCIENCE LTD, GB, vol. 39, no. 11, 1 November 2009 (2009-11-01), pages 824-832, XP026777046, ISSN: 0965-1748, DOI: 10.1016/J.IBMB.2009.09.007 [retrieved on 2009-10-06] cited in the application the whole document	8,9
A	-& "Supplementary Material: Tables", , 1 November 2009 (2009-11-01), XP055255693, Retrieved from the Internet: URL:http://www.sciencedirect.com/science/MiamiMultiMediaURL/1-s2.0-S0965174809001374/1-s2.0-S0965174809001374-mmc1.doc/271245/html/S0965174809001374/c4ea6edf0d284f57f463ff779a54507d/mmc1.doc [retrieved on 2016-03-07] the whole document	8,9
A	-& "supplementary figure S1", , November 2009 (2009-11), XP002755109, Retrieved from the Internet: URL:http://www.sciencedirect.com/science/article/pii/S0965174809001374 [retrieved on 2016-03-07] the whole document	8,9
A	EP 2 431 378 A2 (KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY [KR]) 21 March 2012 (2012-03-21) the whole document	1-34

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2016/050015

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