

Cross Platform In Silico Design And Evaluation Of Small Interfering Rnas That Target The Expression Of Plasmodium Falciparum Heat Shock Protein 90 (pfhsp90) Gene

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Article ID: WMC001005

Article Type: Original Articles

Submitted on:16-Oct-2010, 09:42:16 AM GMT **Published on:** 16-Oct-2010, 01:07:49 PM GMT

Article URL: http://www.webmedcentral.com/article_view/1005

Subject Categories:BIOINFORMATICS

Keywords:siRNA, Target validation, Malaria, Plasmodia, Gene silencing, Bioinformatics

How to cite the article:Bello S . Cross Platform In Silico Design And Evaluation Of Small Interfering Rnas That Target The Expression Of Plasmodium Falciparum Heat Shock Protein 90 (pfhsp90) Gene . WebmedCentral BIOINFORMATICS 2010;1(10):WMC001005

Source(s) of Funding:

Self funded

Competing Interests:

No competing interest

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Abstract

It has been shown that it is both efficient and cost effective to run experiments in silico before actual laboratory work in real time. Plasmodium falciparum heat shock protein 90(pfHsp90) appears sufficiently different from the human type to serve as an appropriate drug target. This expectation will need to be evaluated by targeted silencing of the expression of this protein. It is considerably much cheaper and effective to conduct as much of the study in silico. This report presents the design and evaluation of small interfering RNAs that target the mRNA of pfHsp90.

Introduction

Plasmodium is still a major killer, especially in Africa, where current statistics suggest it kills 2 to 4 children every minute [1]. It is disheartening that for such a major killer, only few effective drugs currently exist. Only the Artemisinin and Quinine may be considered highly effective blood schizonticides, only Artemisinin is effective against trophozoites, only primaquine effectively targets hypnozoites and only Atovaquone-proguanil (Malarone) effectively targets both the primary hepatocyte and red cell stages [2]. Conventional argument is that prevention of infection by plasmodium is particularly difficult because the invading sporozoites spend only minutes in the blood before gaining residence within hepatocytes, thereby avoiding the immune system[3] Furthermore, intra-erythrocytic schizogony occurs in an immune sanctuary and post erythrocytic merozoites present rapidly changing immune targets[4]. Others have argued to the contrary and explain that the obligate multiple stages of plasmodiasis present targets-rich situations that should have being exploited [5]. For example, heat shock proteins and chaperonins have been shown to be critical to the survival and reproduction of all the stages of plasmodiasis [6]. It

has also been shown that although heat shock proteins are highly conserved evolutionally, the plasmodium falciparum heat shock protein 90(PfHsp90) is sufficiently unique to serve as a drug target [6]. Furthermore, heat shock protein docking molecules, like steroids, are already available and have well-defined binding profiles that could easily be used as scaffolds to design effective drugs. Yet, to the best of our knowledge, no attempts have been made to exploit PfHsp90 for malaria therapy. It appears that part of the hindrance to the development of new therapeutics is the acceptable market performance of current anti-malarial drugs matched against the expected little increase in profit of a new agent versus competing more profitable diseases [5]. Indeed, drug discovery and development is a very costly venture and current estimates suggest that \$800 million is required to get a drug through the discovery and development process [7] and it is reasonable to expect to recover the investment with good profits. Recent advances in molecular biology and bioinformatics present possible methods of reducing the cost of drug discovery because they allow efficient in-silico evaluation of hypothesis or concepts. This will reduce the quantity and cost of attritions where a large percentage of the cost of drug development is expended. The viability of PfHsp90 as a drug target could easily be confirmed by targeted gene suppression. One method of such gene suppression is using RNA interference pathways, especially small interfering RNAs (siRNA). This study was, therefore, conducted to design and evaluate, in silico, siRNAs that could silence the expression of PfHsp90 gene.

Methods

Short interfering RNAs may be designed for direct cytoplasmic delivery (using transfection methods previously optimized for antisense oligonucleotide) or they may be designed for processing through a cell expression system. All these options were explored in this study. Also, siRNAs are 20-23 oligonucleotide

sequence double strands composed of a guide (mRNA binding) strand and complementary passenger strand. Given an mRNA sequence, stupendous combinations of 20-23mers could be conceived by visual scan or by using computer platforms, each of which have their points and counterpoints. In this study, therefore, multiple software platforms were used and the results were combined (cross-platform). First, *Plasmodium falciparum* heat shock protein 90 genomic sequence was queried in simple text format and identified at GenBank (Accession number: AE014187; Gene ID: 811999). The last updated sequence (5th August 2010) was downloaded and stored in a predefined local database. The sequence was carefully reviewed and therefrom several algorithms were defined for 21-nucleotide oligonucleotides with best probability of silencing the target gene. This was used to explore for best fit siRNA sequence using the web-based Bioinformatics & Research computing software (BaRC) for siRNA library searching (<http://jura.wi.mit.edu/siRNAext/>) at Whitehead Institute for Biomedical Research (Massachusetts Institute of Technology, Cambridge, MA). Search options used include , siRNAs with the pattern: N2[CG]N8[AUT]N8[AUT]N2 and restricted to GC content of 30-52%. Other filtering criteria were exclusion of siRNAs with sequences containing 4 or more T, A or Gs in a row but to include those with less than 7 consecutive GCs in a row and to end siRNAs with UU. All potential siRNAs identified were then blasted onto NCBI human and rat species sequence to identify alignments. Exploring alignment with rat genomic sequence was considered important because rats are common models for evaluating new anti-malarials. In order to further reduce the chance of off-target effects, siRNA with more than 15 matched bases of the antisense sequence were further exclude. Similarly, siRNAs were generated using platform recommended default algorithms with the software siDirect version 2.0 (<http://sirect2.rnai.jp/>), siRNA target Designer (<http://www.promega.com/siRNADesigner/>), QIAGEN-RNAi solution (<http://www.qiagen.com/>) and Genomatrix (<http://www.genomatix.de/>). All cross platform consensus siRNAs were extracted and stored into the local database in FASTA/Pearson format because this format accepts descriptors and multiple sequences. The sequence and predicted secondary structure of pfHsp90 mRNA was generated from the gene sequence using CLC RNA workbench 4.4 (CLC Bio, Aarhus, Denmark) and cross-checked with that obtained with the software, Vector NTI (advance) II.0 (Invitrogen Cooperation, USA). In the second phase of the study, the sequence of both the guide and

passenger strand of selected siRNAs were aligned against the sequence of pfHsp90 mRNA and the degree and location of alignments were defined. Also, the sequence and structure of Argonuate, the RNase component of RNA-Induced Silencing Complex (RISC), was downloaded from RSCP protein database and evaluated by JMOL viewer (www.jalview.org/). The Rachamandan plot of Argonuate was developed and studied. The amino acids that defined the binding site were identified by direct manual plotting on JMOL. Subsequently, the software RAMACHANDAN plot explorer was used to explore the likely effect of various mutations at/and remote from the binding site of Argonuate using computer assisted randomization and full factorial design to select amino acid candidates for mutation and their replacements. Selected siRNAs were then optimized for Argonuate docking by scrambling the first 3 nucleotides at 5' ends because lack of complementarity at the 5' end has been suggested as one of the criteria for choice of guide strands by Argonuate [8]. These siRNAs were then docked onto the Argonaut protein and the complex (RISC-siRNA) was evaluated. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco [9]. Studies have shown that RNAi is not operative in Plasmodia [10, 11] but siRNAs have been used effectively in plasmodia [12, 13]. It has been suggested that the demonstrated ability of siRNA to silence plasmodia genes might be due to general toxicity of siRNAs or specific antisense effect distinct from RNAi [14]. This controversy is still unsettled. For these reasons, we explored both deliveries by direct electroporation and by plasmids. For direct delivery, we first developed a model of the red cell membrane using the software VMD (<http://www.ks.uiuc.edu/Research/vmd/>) using membrane thickness of 55 and 78 Angstroms and length of 100 Angstroms [15]. This model was then interacted with human lipid carrier protein, flippase, previously downloaded from RCSP PDB (PDB ID: 2J8B). Phosphatidylethanolamine (PE); Phosphoinositol (PI), Phosphatidylserine, all lipids known to be transported to the Inner monolayer of red cell membrane[16], were loaded separately onto siRNA1 and the interaction with the membrane model was evaluated using standard protocols as defined by Humphrey et al[17]. Cholesterol, a pan membrane lipid, was also loaded onto siRNA1 and its interaction with membrane was similarly evaluated. To transfect plasmid into intra-erythrocytic plasmodia, we performed in silico evaluation of various techniques. The technique of Yimim et al [18] was modified and

found to be the best option. Promoters (T7) types were generated for selected siRNAs using siRNA Target designer (<http://www.promega.com/siRNADesigner/>). This was then used to develop a construct using appropriate Invitrogen vector.

Results

The gene sequence of pfHsp90 for 3D7 strain is as shown in illustration 1 and the secondary structure of its mRNA targeted for siRNA induced breakdown in this study was derived as shown in illustration 2. Twenty one siRNAs were obtained across platforms but only one was a cross platform consensus, labelled siRNA 1 in illustration 3, and this was chosen as the best candidate. The secondary structure of siRNA1 was predicted to be as shown in illustration 4 and has freely accessible ends. Scrambling this siRNA on siRNA target design platform revealed four additional siRNA candidates as shown in illustration 5. Promoter sequences for these siRNA were also obtained as shown in illustration 5. Both the guide and passenger strands of siRNA1 align significantly with pfHsp90 mRNA as shown in illustrations 6 and 7 respectively, suggesting significant binding. siRNA1 also docks to the binding site of argonuate as shown in illustration 8. siRNA1 and its promoter sequences were loaded onto Invitrogen plasmid construct as shown in illustration 9. The simulated lipid membrane is as shown in illustration 10. siRNA1 conjugated with cholesterol and inner membrane lipid docks on both human and prokaryotic flippase and loads within computer simulated lipid bilayer as shown in illustration 11 and 12. In contrast, Dicer did not dock on the membrane (negative control).

Discussion

Gene silencing is a robust way of target verification in drug discovery. Short interfering RNAs are highly efficient methods of gene silencing but designing an efficient siRNA is still confronted with failures. In this study, we have used multiple platforms to design and select siRNA. The advantage is that consensus siRNAs across platforms may optimize quality and reduce attritions. The secondary structure of the guide strand of siRNA1 fits the criteria of Patzel et al [19] for high siRNA efficiency. Also, in this study, we have used docking software to show that the selected siRNA docks onto the binding site of the Argonuate portions of RNA-induced silencing complex (RISC). To the best of our knowledge no other report has been

published of such in silico docking. It has been previously demonstrated that in-silico docking usually translates true on bench experiments. Its use is, therefore, expected to reduce the cost of design and validation of siRNAs. Furthermore, both the predicted guide and passenger siRNA strands show good alignment with internal sequences on pfHsp90 mRNA which are easily accessible as shown on the secondary structure of the mRNA. This is important because predicting the guide strand of siRNAs is still not a perfect science. In this study we have moved the model forward to include in silico membrane interactions to evaluate deliverability of siRNA. This is important because results of visual molecular dynamics have been shown to be consistent with experimental findings.

Conclusion(s)

Many attempts at generating siRNAs usually result in many (frequently 90s) candidate siRNAs for synthesis. This study has attempted to reduce this number and therefore the cost by using multiple software and platforms. Evaluation of binding to Argonuate, complementarity with the mRNA targeted for destruction and delivery across cell membrane in silico further improves the design. The selected siRNA will need to be validated on wet laboratory studies. Nevertheless, this paper showcase the ability to improve the design of siRNA and evaluate its potentials on validated in-silico platforms.

Authors Contribution(s)

Bello Shaibu Oricha conceived and conducted the study, Developed local database, performed computational analysis and wrote the manuscript

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- 5.

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Illustrations

Illustration 1

Gene sequence of PfHsp90

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PfHsp90 gene sequence  GTCACTCTTTGCAGCCCCAAAGTGGTTCTAAAGGCTACCACAGGAAACTGGACT
                        20      40      60      80      100
PfHsp90 gene sequence  TCTAAATTCTTCAGTGACCTCAATCCCAGGTTGGGGGAATGTTTTTATAGATC
                        60      80      100      120
PfHsp90 gene sequence  ATCACCGACCGACCTGGCTTTCATGATGAGAGTGCCATCTACCCCGTGGGCTAT
                        140      160      180      200
PfHsp90 gene sequence  TGCAGTACTCGAATATATGCCAGCATGAAGTGCCAGACCAAGAGTGTCTATAT
                        220      240      260      280
PfHsp90 gene sequence  ACCTGTCAGATCAAGGATGGTGGTGTGCCAGCCTCAGTTTGAAATTGTTCTCTGAA
                        300      320      340      360
PfHsp90 gene sequence  GATGACCCCCAGGAATGCCATTGTGAGCTTCTTCTGGCAGATGCTTGTCATGCA
                        380      400      420      440
PfHsp90 gene sequence  GAACTGCTCAGGACTATAAGGCAGTACTATGGGGGAAACTAATGGGCT
                        460      480      500

```

Illustration 2

Predicted secondary structure of the mRNA of PfHsp90



Illustration 3

siRNA1: The cross platform consensus sequence

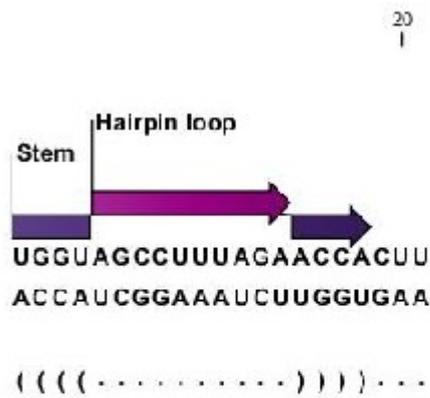


Illustration 4

Predicted secondary structure of the guide strand of siRNA1

Secondary structure: $\Delta G = -1.6\text{kcal/mol}$

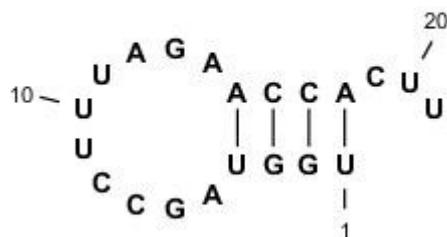


Illustration 5

Scrambled siRNA1 and Promoter sequences

T7 Promoter sequence	Target sequence	U Overhang
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UGGUAGCCUUUAGAACCACUU
UUACCAUCGAAAUCUUGGUG

5' - GGATCCTAATACGACTCACTATA TGGTAGCCTTTAGAACCAC -3'
3' - CCTAGGATTATGCTGAGTGATAT ACCATCGGAAATCTTGGTG AA -5

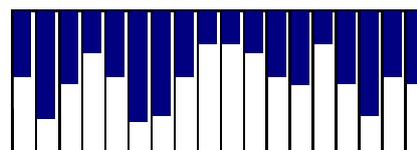
5' - GGATCCTAATACGACTCACTATA GTGGTTCTAAAGGCTACCA -3'
3' - CCTAGGATTATGCTGAGTGATAT CACCAAGATTCCGATGGT AA -5

Oligos written 5' to 3' for easy ordering.

5'-GGATCCTAATACGACTCACTATATGGTAGCCTTTAGAACCAC -3'
5'-AAGTGGTTCTAAAGGCTACCATATAGTGAGTCGTATTAGGATCC -3'
5'-GGATCCTAATACGACTCACTATAGTGGTTCTAAAGGCTACCA -3'
5'-AATGGTAGCCTTTAGAACCCTATAGTGAGTCGTATTAGGATCC -3'

- Length: **19 bases**
- GC content: **47%**
- Average free energy of the 5' 5' bases of the antisense: **-2.156kcal/mol**
- siRNA Type **Type II**

ΔG value of each base



Scrambled Sequence Number 1

GCGCACUGUCUUAAAAGAGCUU
UUCGCGUGACAGAAUUUCUG

5' - GGATCCTAATACGACTCACTATA GCGCACTGTCTTAAAGAGC -3'
3' - CCTAGGATTATGCTGAGTGATAT CGCGTGACAGAATTTCTCG AA -5

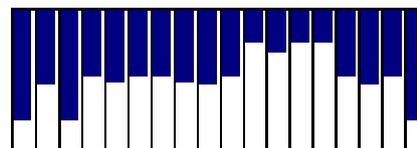
5' - GGATCCTAATACGACTCACTATA GCTCTTTAAGACAGTGCGC -3'
3' - CCTAGGATTATGCTGAGTGATAT CGAGAAATTTCTGTCACGCG AA -5

Oligos written 5' to 3' for easy ordering.

5'-GGATCCTAATACGACTCACTATAGCGCACTGTCTTAAAGAGC -3'
5'-AAGCTCTTTAAGACAGTGCCTATAGTGAGTCGTATTAGGATCC -3'
5'-GGATCCTAATACGACTCACTATAGCTCTTTAAGACAGTGCGC -3'
5'-AAGCGCACTGTCTTAAAGAGCTATAGTGAGTCGTATTAGGATCC -3'

- Length: **19 bases**
- GC content: **53%**
- Average free energy of the 5' 5' bases of the antisense: **-2.172kcal/mol**
- siRNA Type **Type II**

ΔG value of each base



Scrambled Sequence Number 2

GUUCGCGAACAUUGAACGUCUU
UUCCAAGCGCUUGUACUUGCAG

5'- GGATCCTAATACGACTCACTATA GTTCGCGAACATGAACGTC -3'
 3'- CCTAGGATTATGCTGAGTGATAT CAAGCGCTTGTACTTGCAG AA -5

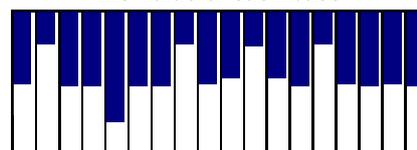
5'- GGATCCTAATACGACTCACTATA GACGTTTCATGTTTCGCGAAC -3'
 3'- CCTAGGATTATGCTGAGTGATAT CTGCAAGTACAAGCGCTTG AA -5

Oligos written 5' to 3' for easy ordering.

5'-GGATCCTAATACGACTCACTATAGTTCGCGAACATGAACGTC -3'
 5'-AAGACGTTTCATGTTTCGCGAACTATAGTGAGTCGTATTAGGATCC -3'
 5'-GGATCCTAATACGACTCACTATAGACGTTTCATGTTTCGCGAAC -3'
 5'-AAGTTCGCGAACATGAACGTTCTATAGTGAGTCGTATTAGGATCC -3'

- Length: **19 bases**
- GC content: **53%**
- Average free energy of the 5' bases of the antisense: **-2.024kcal/mol**
- **siRNA Type Type II**

ΔG value of each base

**Scrambled Sequence Number 3**

GAAUGAAAAGCCUGCGCUUCUU
UUCUUACUUUCGGACGCGAAG

5'- GGATCCTAATACGACTCACTATA GAATGAAAGCCTGCGCTTC -3'
 3'- CCTAGGATTATGCTGAGTGATAT CTTACTTTTCGGACGCGAAG AA -5

5'- GGATCCTAATACGACTCACTATA GAAGCGCAGGCTTTCATTC -3'
 3'- CCTAGGATTATGCTGAGTGATAT CTTTCGCGTCCGAAAGTAAG AA -5

Oligos written 5' to 3' for easy ordering.

5'-GGATCCTAATACGACTCACTATAGAATGAAAGCCTGCGCTTC -3'
 5'-AAGAAGCGCAGGCTTTCATTCCTATAGTGAGTCGTATTAGGATCC -3'
 5'-GGATCCTAATACGACTCACTATAGAAGCGCAGGCTTTCATTC -3'
 5'-AAGAATGAAAGCCTGCGCTTCTATAGTGAGTCGTATTAGGATCC -3'

- Length: **19 bases**
- GC content: **53%**
- Average free energy of the 5' bases of the antisense: **-2.228kcal/mol**
- **siRNA Type Type II**

ΔG value of each base

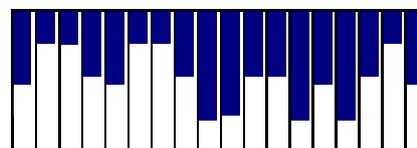


Illustration 6

Alignment of the guide strand of siRNA1 with mRNA of PfHsp90

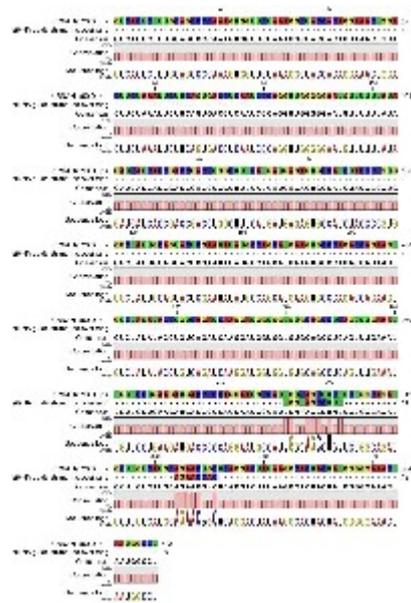


Illustration 7

Alignment of the passenger strand of siRNA1 with mRNA of PfHsp90

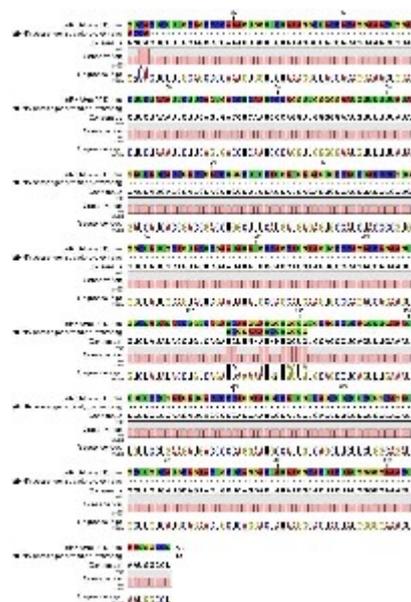


Illustration 8

siRNA1 docks specifically and strongly to the active site of Argonuate

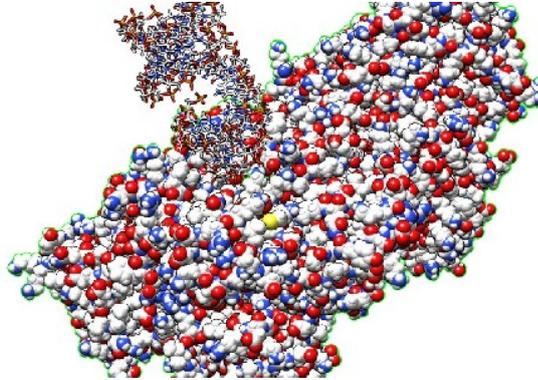


Illustration 9

Plasmid construct carrying siRNA1

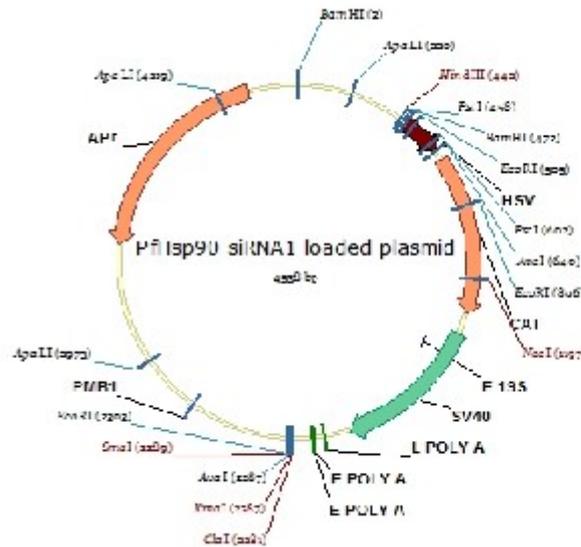


Illustration 10

Simulated red blood cell membrane before docking

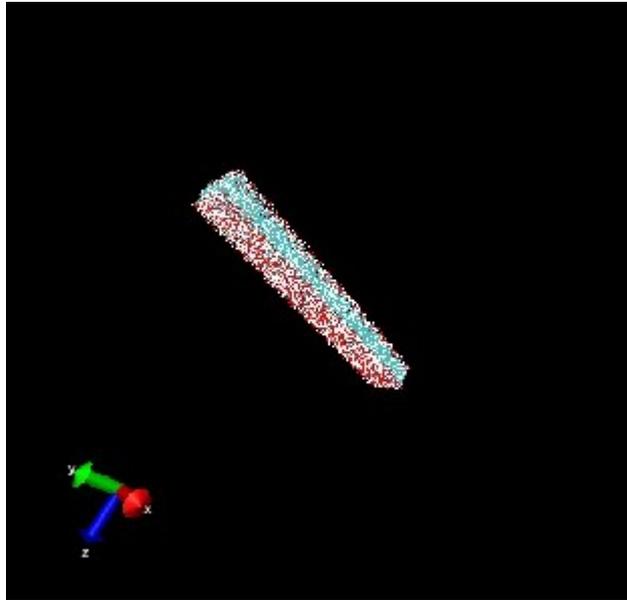


Illustration 11

Loaded Human (Red arrow) and Staphylococcal (Brown arrow) flipases and human Dicers (Yellow Arrows)

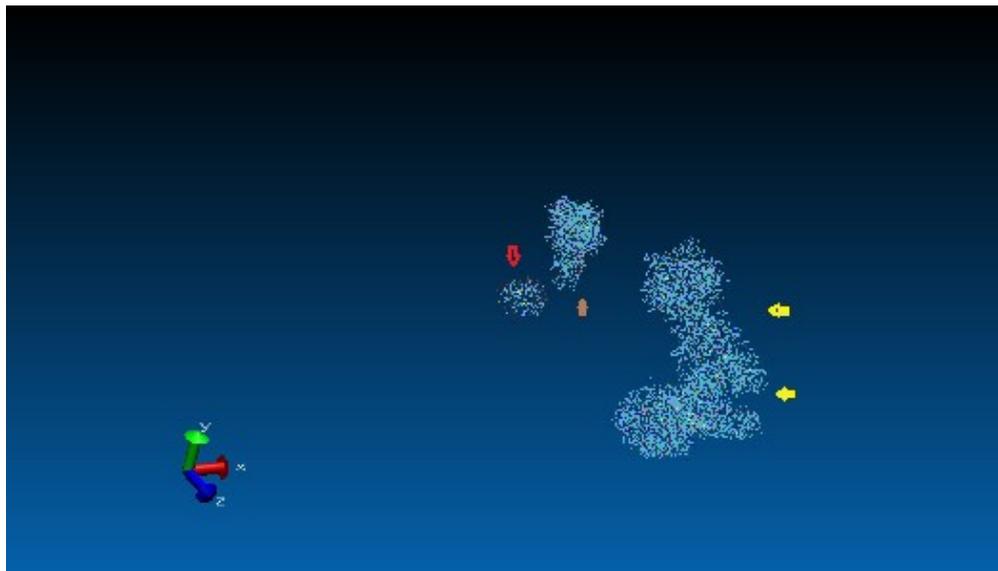
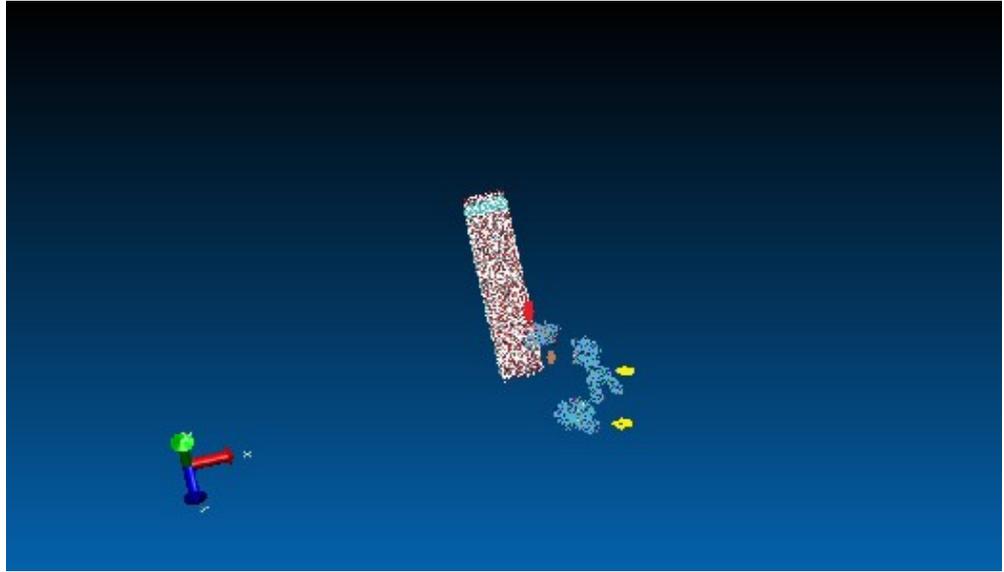


Illustration 12

Flipasses, Dicers docked onto the membrane-only human-flipasse siRNA1 complex docks within membrane



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