In Silico Designing, Validation and In-Vitro Synthesis of YPS1 Silencing siRNAs.

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Abstract : Gene silencing is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic modification. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell. Genes are regulated at either the transcriptional or post-transcriptional level. Fission yeast is a useful model for RNA interference because it has single-copy genes for components of the RNAi pathway such as argonaute, dicer and RNA-dependent RNA polymerase (RdRP) (Wood, V. et al., 2002). Schizosaccharomyces pombe has DCR and AGO but not in Saccharomyces cerevisiae (Verdel, A. et al. 2004). Studies till date have proved that RNAi machinery in fission yeast has only epigenetic effects as they help in histone modifications. Present study involved designing of siRNAs against fission yeast protease yps1. Critical design concerns in the selection of siRNA duplexes that are potent and specific.

IndexTerms **– Yps1, siRNA, Fission yeast, S. pombe..**

Introduction

Gene silencing is a general term describing epigenetic processes of gene regulation. The term gene silencing is generally used to describe the "switching off" of a gene by a mechanism other than genetic modification. That is, a gene which would be expressed (turned on) under normal circumstances is switched off by machinery in the cell. Genes are regulated at either the transcriptional or post-transcriptional level.

RNA silencing is an evolutionarily conserved gene regulatory mechanism with many species-specific variations. As it has become a powerful tool for genetic analyses and is likely to become a potent therapeutic approach for gene silencing. Two ribonucleases that are vital to this pathway are: Dicer, a multidomain RNase III family enzyme that initiates RNAi by generating small interfering RNAs (siRNA), and Argonaute or Slicer, an RNase H signature enzyme that affects cleavage of mRNA. The RNAi pathway can be divided into three major steps:

• First is the conversion of dsRNA input into 21-23 bp small fragments by the enzyme Dicer
• Secondly the loading of small RNAs into large multiprotein complex RISC
• Lastly the sequence specific silencing of the cognate gene by RISC that is guided by the small RNA fragment.

Many in silico studies has been carried out using biologically validated mRNA targets in *C. elegans* and *Drosophila* to predict additional mRNA targets that follow the same rules in a variety of species, including mammals (Lai et al., 2003).

Fission yeast is a useful model for RNA interference because it has single-copy genes for components of the RNAi pathway such as argonaute, dicer and RNA-dependent RNA polymerase (RdRP) (Wood, V. *et al*., 2002). *Schizosaccharomyces pombe* has DCR and AGO but not in *Saccharomyces cerevisiae* (Verdel, A. *et al*. 2004). Studies till date have proved that RNAi machinery in fission yeast has only epigenetic effects as they help in histone modifications. Present study involved designing of siRNAs against fission yeast protease yps1. Critical design concerns in the selection of siRNA duplexes that are potent and specific. There are two major considerations with regard to siRNA specificity:

•‘off-targeting’ due to silencing of genes sharing partial homology with the siRNA, and
•‘immune stimulation’ due to the engagement of components of the innate immune system by the siRNA duplex.

Whereas in case of fission yeast immune stimulation is not of major concern as it lacks immune system, so potency of the siRNAs and reduction in off target effects is the main criteria for designing effective siRNAs.

Materials and Methods

Materials

***S.pombe* Yps1 Gene Sequence:** From the website of NCBI (http://www.ncbi.nlm.nih.gov/), the following nucleotide sequence of YPS1 in FASTA format with the Gene Id: NM_001023027.1 was retrieved

REAGENTS USED: The following reagents were procured from various suppliers as mentioned below:- Na_2HPO_4, NaH_2PO_4, MgCl2, TritonX100, TBE, APES, TEMED, Sodium Carbonate, and SDS were procured from HIMEDIA. NaCl, Tris HCl, Acrylamide, Glycerol and Potassium Dichromate were procured from QUALIGENS. Nitric Acid, Formaldehyde and Silver Nitrate were procured from LOBA CHEMIE. Proteinase K and Taq Polymerase were procured from *Fermentas, US.* All reagents procured were of AR Grade. dNTP's and Oligo dT primer were synthesised and procured from Bangalore GENEI.

Methods

3.1 siRNA Designing Procedure outline

RNA interference is the newly discovered phenomenon to inhibit post transcriptional gene expression. Two hurdles, which must be overcome in developing the specific siRNAs is correct target identification and minimization of potential off targets. The *in silico* design process of siRNAs against YPS1 protease of *S. pombe* is therefore, the first and most important step.

Following steps were undertaken for designing of siRNAs:

I. Retrieval of YPS1 sequence with gene ID (NM_001023027.1) from NCBI database in FASTA format.

II. Prediction of effective siRNA using the bioinformatics softwares. Four servers were used for the analysis:

SIDIRECT: (http://designRNA.jp/);
GENSCRIPT: (https://www.genscript.com/sslbin/app/rnai);
AMBION: (http://www.ambion.com/techlib/resources/siRNA/index.html);
MWG: (http://www.mwg-biotech.com/html/).

III. **Validation of predicted siRNAs**: Validation of predicted siRNAs based on Reynolds score was carried out manually. Following characteristics are used in siRNA validation to evaluate potential targeted sequences and assign scores to them, which makes it easier to select the effective siRNAs. Sequences with higher scores will have higher chance of success in knocking the gene down. The table 1 below lists the 8 criteria and the methods of score assignment.

Table 1: Showing criteria for calculating Reynolds Score

S No	Criteria	Description Score Yes /No
1	Moderate to low (30%-52%) GC Content	1 point
2	At least 3 A or U at positions 15-19	1 point per A or U
3	Lack of internal repeats (low Tm)	1 point
4	A at position 19	1 point
5	A at position 3	1 point
6	U at position 10	1 point
7	No G or C at position 19	-1 point
8	No G at position 13	-1 point

After calculating score, those with score greater than 5 were selected further process.

IV. In this, the secondary structure of shortlisted siRNAs in above step was checked by Contrafold (http://contra.stanford.edu/contrafold/). siRNAs which showed linear structure were taken further in the third category.

V. After short listing linear siRNAs different thermodynamic parameters were calculated using Oligowalk software from the package RNA structure 5.03 (http://rna.urmc.rochester.edu/rnastructure.html).

VI. The secondary structure prediction for targeted mRNA was carried out by mfold version 2 (http://bioweb.pasteur.fr/seqanal/interfaces/mfold-simple.html) the siRNAs selected in the last step were

superimposed on the targeted mRNA structure to check target accessibility. Only those candidates were selected which had easily accessible targets.

VII. siRNAs that showed higher negative value of $\Delta G^{overall}$ were further subjected to BLAST analysis for checking their off targets silencing potential.

3.2. DESIGNING OF SCRAMBLED siRNA

Scrambled siRNA is a negative control siRNA, designed by scrambling the nucleotide sequence of the gene-specific siRNA by Scramble siRNA tool (http://www.sirnawizard.com/scrambled.php) and conducting a BLAST search to make sure it lacks homology to any other gene. To check the self folding of the siRNA, the secondary structure prediction of siRNAs selected was done by Nucleic Acid Quikfold (http://dinamelt.bioinfo.rpi.edu/quikfold.php).

3.3 siRNA synthesis and validation

3.3.1 *In Vitro* Transcription Method

The designed siRNAs were synthesized by in vitro transcription method. Production of siRNAs by in vitro transcription is useful method that is simple, effective, and inexpensive. For in vitro transcription, a 39 nt DNA template oligonucleotides were designed to produce 21 nt siRNAs. The template and a 19nt T_7 promoter (GGT AAT ACG ACT CAC TATA) were synthesized and procured from IDT (table 2). Modified methodologies for IVT as given by Donze *et al.*, 2002 and Zhang *et al.*, 2004.

Table 2: DNA templates for IVT

S.No	siRNAs	DNA template for IVT
1.	siRNA 1	S: 5' TTTAATTGATTTTCCTGGTTGCTATAGTGAGTCGTATTA 3' AS: 5' GGCAACCAGGAAAATCAATTCCTATAGTGAGTCGTATTA 3'
2.	siRNA 2	S: 5' TTTATAGGAATAAACGACGCTCTATAGTGAGTCGTATTA 3' AS: 5' AAAGCGTCGTTTATTCCTATACTATAGTGAGTCGTATTA 3'
3.	siRNA 3	S: 5' TTTGATGATAAAATGTGTAGGCTATAGTGAGTCGTATTA 3' AS: 5' CGCCTACACATTTTATCATCACTATAGTGAGTCGTATTA 3'
4.	siRNA 4	S: 5' TTATGATAAAATGTGTAGGCGCTATAGTGAGTCGTATTA 3' AS: 5' AACGCCTACACATTTTATCATCTATAGTGAGTCGTATTA 3'
5.	siRNA 5	S: 5' TTGAATTGGAAGTCCGGTAAAGCCTATAGTGAGTCGTATTA 3' AS: 5' AAGCTTTACCGGACTTCCAATTCCTATAGTGAGTCGTATTA 3'
6.	Scramble siRNA 1S	S: 5' TTTAGTATGTTGGTTATTTCCCTATAGTGAGTCGTATTA 3' AS: 5' AAGGAAATAACCAACATACTACTATAGTGAGTCGTATTA 3'

3.3.2 Quantitative Analysis of RNA

Quality of isolated RNA was checked spectrophotometrically by taking ratio between absorbance at 260nm to that at 280nm. Quantity of RNA was calculated using the formulae:

Concentration of RNA = **OD_{260} X 40 X DF**

OD = 1 at 260nm pertains to concentration of 40µg/ml RNA (DF = Dilution Factor).

4.1Results for siRNA designing experiment

4.1.1 The siDirect predicted 26, Genscript predicted 6, MWG predicted 43 and Ambion predicted 49 potent sites.

Out of 26 siRNAs predicted 17 had score above 5 and were selected for further analysis. Genscript predicted six siRNA which were at different positions from those predicted by siDirect. Five of these had

score above 5 and were taken for further analysis. MWG predicted 43 siRNA which were at different positions from those predicted by Genscript. Nineteen of these had score above 5 and were taken for further analysis. Ambion predicted 49 siRNA which were at different positions from those predicted by other tools. Twenty six of these had score above 5 and were taken for further analysis.

4.1.2 Reynolds score was calculated for the above siRNAs and those having score more than 5 were selected for further process.

4.1.3. siRNA selected showed linear structure checked by CONTRAFOLD (Do *et. al.*, 2006) for each siRNA pair sequence. Contrafold is a novel secondary structure prediction method based on conditional log-linear models (CLLMs), a flexible class of probabilistic model which generalize upon SCFGs (stochastic context-free grammers) by using discriminative training and feature rich scoring. This method receives the highest single sequence prediction accuracies to date, outperforming currently available probabilistic and physics-based techniques'.

4.1.4. Oligowalk results: At the end free energy was calculated by oligowalk is shown in table no. 11. The table also contains the column for BLAST hits

Table 11: Table showing free energy and BLAST hits

S.No	Sense/ antisense	Source	Start post.	dGS	dGAS	dG overall	ΔG duplex	ΔG breakT	ΔG interO	ΔG intraO	Blast hits
1.	**CAACCAGGAAAAUCAAUUA/ GUUGGUCCUUUUAGUUAAU**	**Sidirect**	**108**	**0.1**	**1.1**	**-21.2**	**-26.3**	**-5.1**	**-3.5**	**0**	**0**
2.	GAAACGCCUACACAUUUUA/ CUUUGCGGAUGUGUAAAAU	SiDirect	1176	0.5	-2.9	-2.4	-28	-1.1	-13.2	-2.8	2
3.	ACUCUACGUUCUUUUCUAA/ UGAGAUGCAAGAAAAGAUU	MWG	867	0.7	-0.2	-21.6	-26	-4.4	-3.7	0	2
4.	**AGCGUCGUUUAUUCCUAUA/ UCGCAGCAAAUAAGGAUAU**	**Ambion**	**1390**	**1.1**	**1.1**	**-22.2**	**-28.6**	**-6.4**	**-2.1**	**0**	**0**
5.	GAUUCCAUUUCAAGUUUUU/ CUAAGGUAAAGUUCAAAAA	SiDirect	648	0.02	0.6	-14.8	-23.8	-9	0	0	1
6.	GCGUGACUAUACUUAUUCU/ CGCACUGAUAUGAAUAAGA	MWG	130	-0.3	0.2	-15.6	-28.9	-13.3	-1.8	0	1
7.	ACGCCUACACAUUUUAUCA/ UGCGGAUGUGUAAAAUAGU	Ambion	1181	2.5	-1	-28.1	-28.9	0	-8.8	-0.6	2
8.	CAUUACAGUUGCUGGAAUU/ GUAAUGUCAAC	Ambion	471	-1.2	-2.5	-23.1	-28.6	-5	-5.1	-0.2	2

	GACCUUAA										
9.	UUAGAGAAGCG UGACUAUA/ AAUCUCUUCGC ACUGAUAU	MWG	122	0.6	-0.6	-22.5	-29.2	-6.7	-3.3	0	2
10.	UCAGGGUAAUG GAAGUUUA/ AGUCCCAUUAC CUUCAAAU	Ambion	741	-1.3	1.1	-12.8	-29.7	-16.9	0	0	1
11.	CAUCUGGUAUC ACUUCAUU/ GUAGACCAUAG UGAAGUAA	SiDirect	152	-0.8	0.7	-5.3	-29.3	-2.4	-2.4	0	1
12.	CUGCGAUACUU UCUUUGUA/ GACGCUAUGAA AGAAACAU	Ambion	358	1.5	0.4	-17.2	-28.2	-1.1	-2.7	0	2
13.	CCCUGCUUUUA GUAUGUAU/ GGGACGAAAAU GAUACAUA	SiDirect	714	1.2	0.4	-11.2	-29.1	-17.9	-2.7	0	2
14.	GGGCAAGUUUC AAACAUAU/ CCCGUUCAAAG UUUGUAUA	SiDirect	1081	-0.8	-0.2	-13.6	-27	-1.5	-3.6	0	2
15.	**CCUACACAUUU UAUCAUCA/ GGAUGUGUAA AAUAGUAGU**	**SiDirect**	**1184**	**2.5**	**-0.2**	**-26.6**	**-28.3**	**0**	**0**	**0**	**0**
16.	**CGCCUACACA UUUUAUCAU/ GCGGAUGUGU AAAAUAGUA**	**MWG**	**1182**	**2.5**	**-0.3**	**-27.9**	**-33.9**	**0**	**-7**	**0**	**0**
17.	UCCAGCGUAUC UUGGCAUA/ AGGUCGCAUAG AACCGUAU	MWG	304	-1.7	-0.6	-14.1	-25.9	-18.1	-5.8	-1.7	2
18.	UGUAAUGGCGU UUAAUCCA/ ACAUUACCGCA AAU UAGGU	MWG	289	-1	-2.2	-7.4	-32.2	-18.5	-1.5	0	2
19.	UGCAUCUUCUA GCUUCCAU/ ACGUAGAAGAU CGAAGGUA	SiDirect	408	-0.9	-0.1	-23.7	-30.8	-6.8	-4.5	-1.7	3
20.	GCCGCUCUGAU AUUUCAAU/ CGGCGAGACUA UAAAGUUA	Ambion	1031	-0.9	-2.4	-28.4	-26.8	-1.9	-7.7	-0.1	1
21.	CAGGAAAAUCA AUUAGAGA/ GUCCUUUUAGU	SiDirect	112	-0.4	1.5	-17.6	-29	-9.2	-2.2	0	1

	UAAUCUCU										
22.	CAUCUUCUAGC UUCCAUUU/ GUAGAAGAUCG AAGGUAAA	SiDirec t	410	1.9	-0.1	- 19.2	-29	-9.8	-4.5	0	3

On the basis of BLAST hits 5 siRNAs were selected. These siRNAs do not show presence of any secondary structure as they all have linear structures. It can be concluded that both thermodynamics properties of the siRNA and target mRNA structure, contribute significantly towards the efficiency of siRNA. The local mRNA structure at the target site has been described as the main cause for positional effect of different siRNAs. The target site accessibility was calculated on the basis of reliable parameter i.e. "hydrogen bond index". Hydrogen bond index represents the average no of hydrogen bonds formed between nucleotide in the target region and rest of mRNA. Moreover it is related inversely with the gene silencing efficiency (Luo& Chang 2004). Among the 5 siRNA selected along with the scrambled sequence which is used as negative control the number of hydrogen bonds were very less as compared to others which will further help in increasing the silencing effect. The bioinformatics analysis of the selected siRNA result into lower free energies. This is in accordance with the established fact that a linear correlation exists between the local free energy and the siRNA binding region and silencing of the targeted region (Kurrecket *et. al.*, 2006).

Final selected siRNAs

S. No.	DNA Template for siRNA synthesis by in vitro transcription	position
si1 sense	5' TTTAATTGATTTTCCTGGTTGCTATAGTGAGTCGTATTA 3'	108
si1Antisense	5' GGCAACCAGGAAAATCAATTCCTATAGTGAGTCGTATTA 3'	
si2 sense	5' TTTATAGGAATAAACGACGCTCTATAGTGAGTCGTATTA 3'	1390
si2Antisense	5' AAAGCGTCGTTTATTCCTATACTATAGTGAGTCGTATTA 3'	
si3 sense	5' TTTGATGATAAAATGTGTAGGCTATAGTGAGTCGTATTA 3'	1184
si3Antisense	5' CGCCTACACATTTTATCATCACTATAGTGAGTCGTATTA 3'	
si4 sense	5' TTATGATAAAATGTGTAGGCGCTATAGTGAGTCGTATTA 3'	1182
si4Antisense	5' AACGCCTACACATTTTATCATCTATAGTGAGTCGTATTA 3'	
si5 sense	5'TTGAATTGGAAGTCCGGTAAAGCCTATAGTGAGTCGTATTA3'	526
si5Antisense	5'AAGCTTTACCGGACTTCCAATTCCTATAGTGAGTCGTATTA3'	

Position	S. No.	DNA Template for scramble siRNA by in vitro transcription
108	si1 sense	5' TTTAGTATGTTGGTTATTTCCCTATAGTGAGTCGTATTA 3'
	si1Antisense	5' AAGGAAATAACCAACATACTACTATAGTGAGTCGTATTA 3'
1390	si2 sense	5' TTAAATAGTAGGACGACATTCCTATAGTGAGTCGTATTA 3'
	si2Antisense	5' AAGAATGTCGTCCTACTATTTCTATAGTGAGTCGTATTA 3'
1184	si3 sense	5' TTGTTAAGGTATAGTAGGAATCTATAGTGAGTCGTATTA 3'
	si3Antisense	5' AAATTCCTACTATACCTTAACCTATAGTGAGTCGTATTA 3'
1182	si4 sense	5' TTAATTAGAGGGTTGGAACCTATAGTGAGTCGTATTA 3'
	si4Antisense	5' AAGTTCCAACCCTATCTAATTCTATAGTGAGTCGTATTA 3'

526	si5 sense	5' TTACAATTCGGTGCAAGGAGGATCTATAGTGAGTCGTATTA 3'
	si5Antisense	5' AAATCCTCCTTGCACCGAATCGTCTATAGTGAGTCGTATTA 3'

The 5 siRNAs along with their binding sites are shown below. The positions of target sequences of the motifs are given below as predicted by mfold for YPS1.

Fig 14: siRNA1

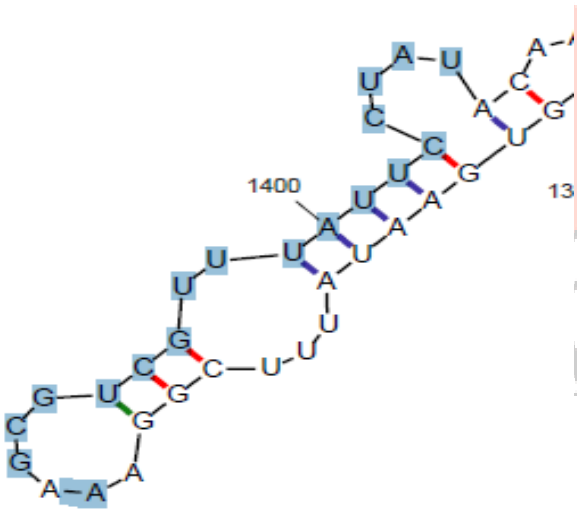

Fig 15: siRNA2

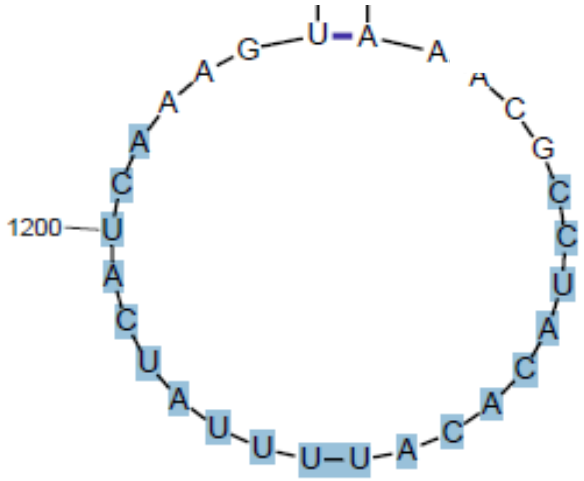

Fig 16: siRNA3

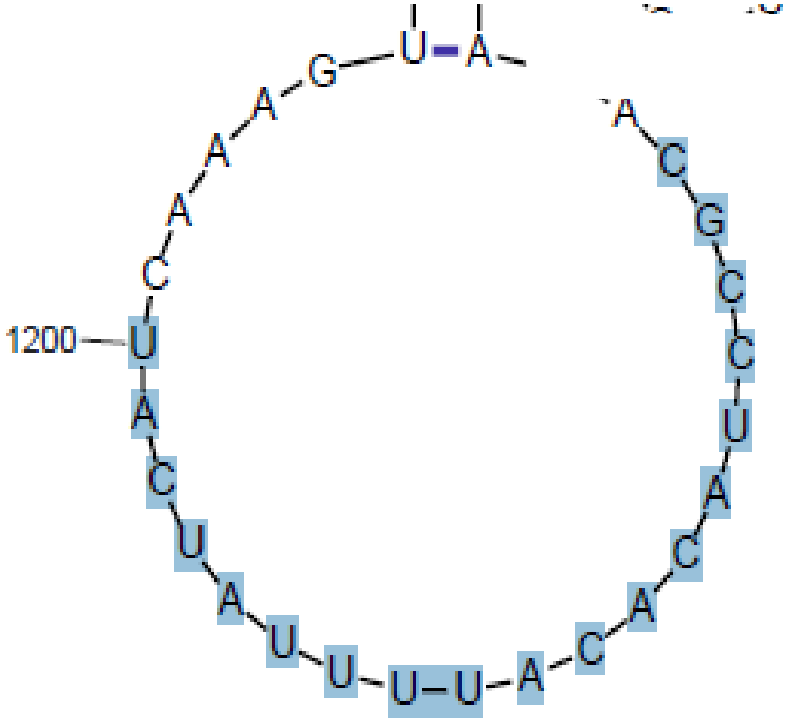

Fig 17: siRNA4

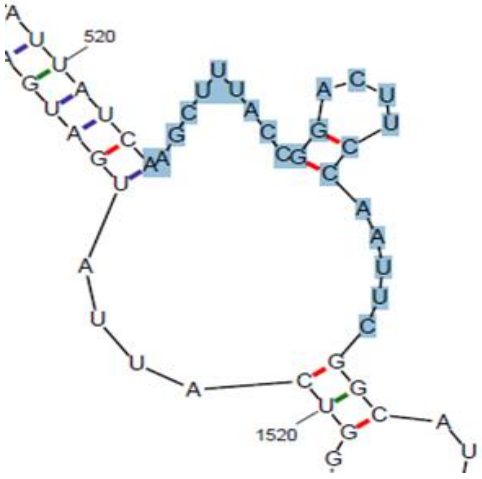

Fig 18: siRNA5

4.2 QUANTITATIVE ANALYSIS OF RNA

Using in vitro transcription method of siRNA synthesis, six double stranded siRNAs were prepared that spanned the length of target mRNA. For quantitative analysis of siRNAs ratio between λ260 and λ280 was calculated. Table 17 shows results of quantitative analysis of siRNAs.

Table 17:Results of quantitative analysis of RNA

S.No.	SiRNA	λ260	λ280	λ260/λ280	Concentration (µg/ml)
1.	SiRNA	0.506	0.289	1.750	2024µg/ml
2.	SiRNA	0.249	0.145	1.719	996µg/ml
3.	SiRNA	0.536	0.279	1.819	2144µg/ml
4.	SiRNA	0.473	0.259	1.828	1892µg/ml
5.	SiRNA	0.393	0.218	1.805	1572µg/ml
6.	Scramble siRNA	0.520	0.299	1.766	2080µg/ml

4.3 Synthesis of dsRNA by In Vitro Trancription

Using in vitro transcription method of siRNA synthesis, five double stranded siRNAs were prepared that spanned the length of target mRNA. Production of siRNA by in vitro transcription is simple, effective and inexpensive. For qualitative analysis of siRNAs 18% PAGE was prepared. Gel showing results of 18% PAGE are given below.

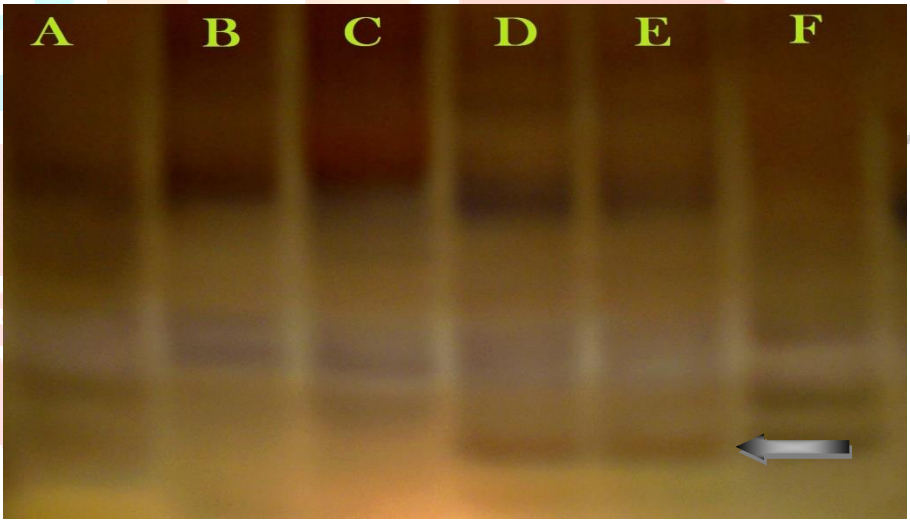

Fig:19 lane A:siRNA1, lane B:siRNA2, lane C:siRNA3, lane D:siRNA4, lane E:siRNA5, lane F:Scramble siRNAere visualized by adding sodium carbonate solution (developer).

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