US 20080097091A1

(19) United States

(12) Patent Application Publication

(10) Pub. No.: US 2008/0097091 A1

(43) Pub. Date: Apr. 24, 2008

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(54) SIRNA TARGETING TNFALPHA

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(21) Appl. No.: **11/975,902**

(22) Filed: **Oct. 22, 2007**

Related U.S. Application Data

(63) Continuation-in-part of application No. 10/940,892, filed on Sep. 14, 2004, which is a continuation of application No. PCT/US04/14885, filed on May 12, 2004.
Continuation-in-part of application No. 10/714,333, filed on Nov. 14, 2003.

(60) Provisional application No. 60/426,137, filed on Nov. 14, 2002. Provisional application No. 60/502,050, filed on Sep. 10, 2003.

Publication Classification

(51) **Int. Cl.**
C07H 21/04 (2006.01)

(52) **U.S. Cl.** **536/24.5**

(57) **ABSTRACT**

Efficient sequence specific gene silencing is possible through the use of siRNA technology. By selecting particular siRNAs by rational design, one can maximize the generation of an effective gene silencing reagent, as well as methods for silencing genes. Methods, compositions, and kits generated through rational design of siRNAs are disclosed including those directed to nucleotide sequences for TNFα.

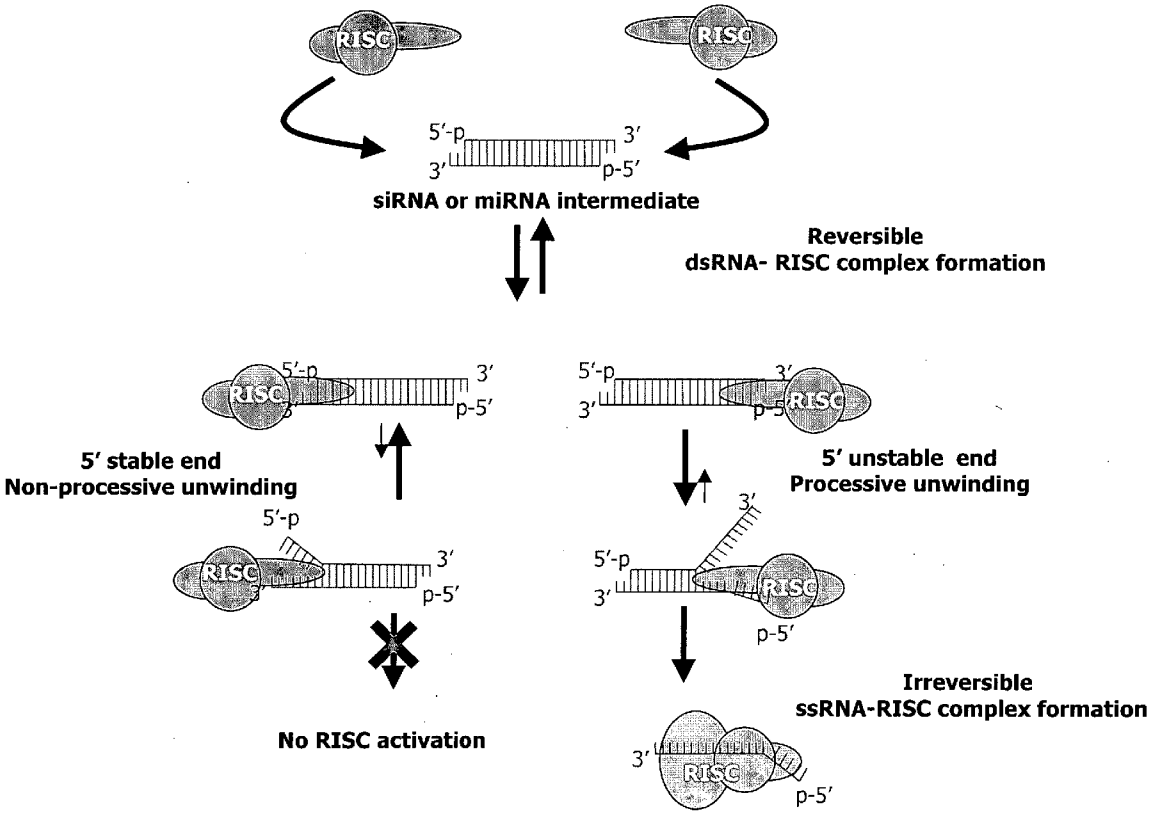


Figure 1

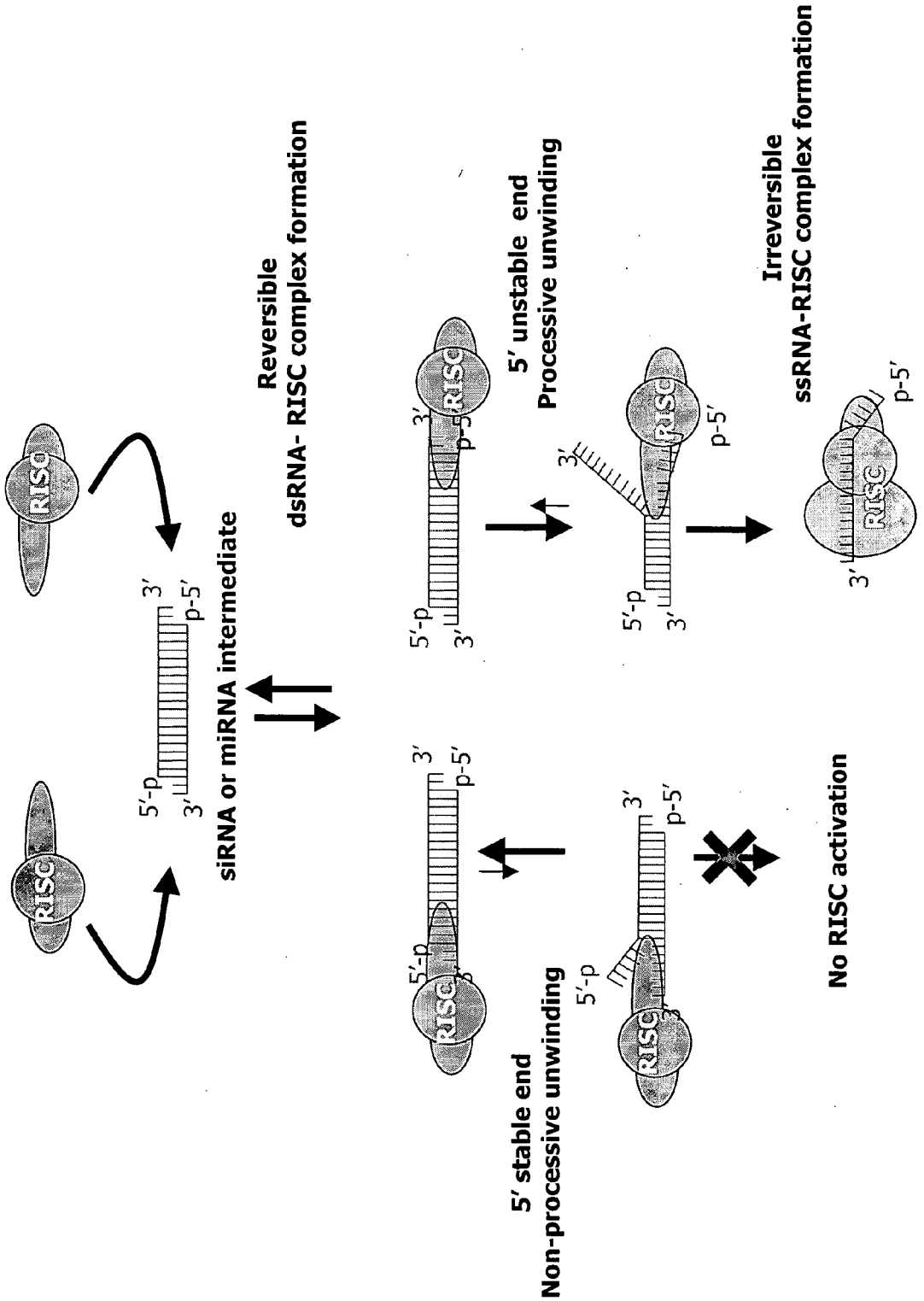

RISC
RISC
5′-p
3′
3′
p-5′
siRNA or miRNA intermediate
Reversible
dsRNA- RISC complex formation
5′-p
3′
3′
p-5′
RISC
5′ unstable end
Processive unwinding
3′
5′-p
3′
p-5′
RISC
Irreversible
ssRNA-RISC complex formation
3′
p-5′
RISC
5′-p
3′
3′
p-5′
RISC
5′ stable end
Non-processive unwinding
5′-p
3′
p-5′
RISC
No RISC activation

Figure 2

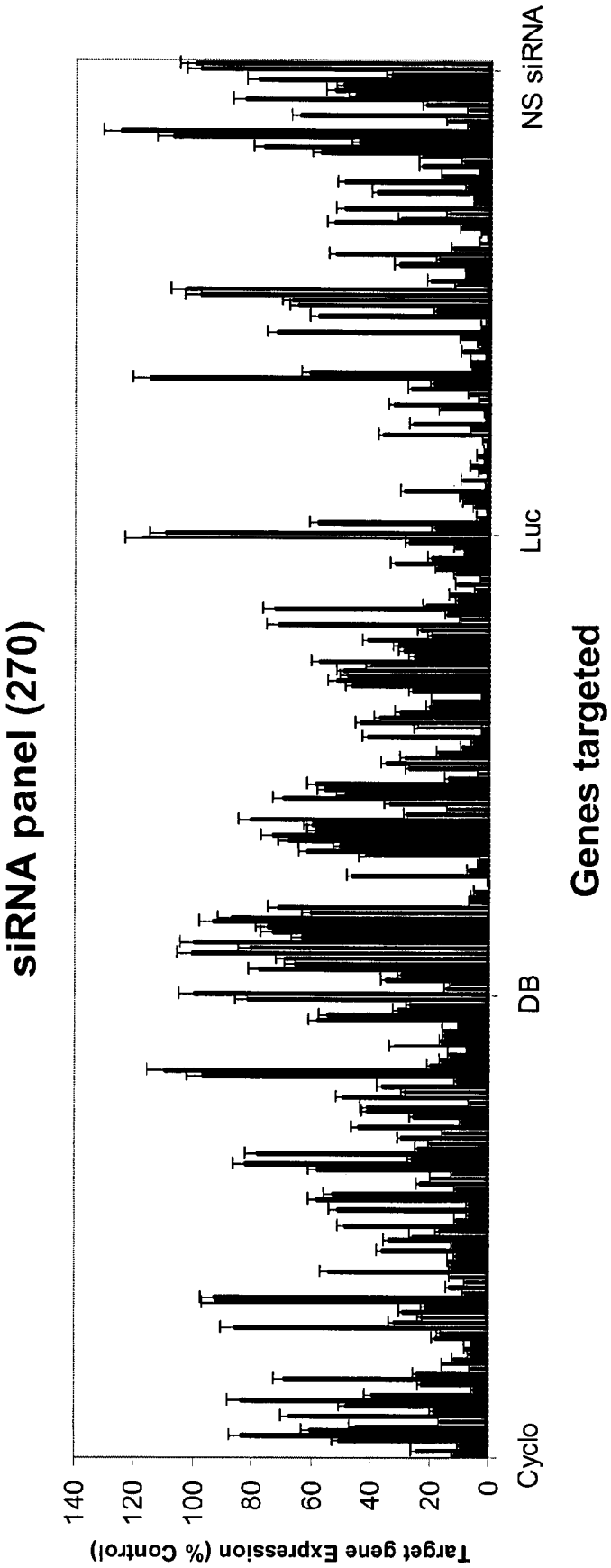
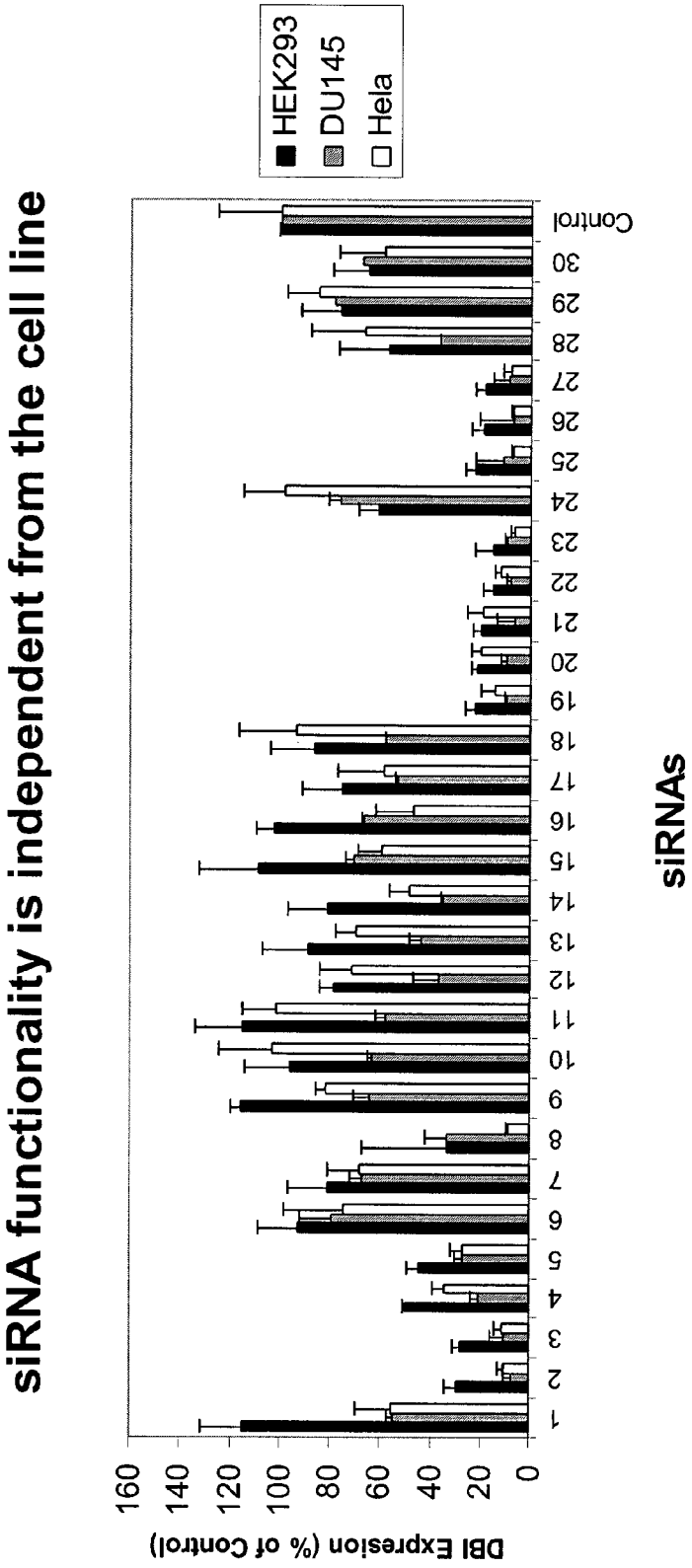

siRNA panel (270)
Target gene Expression (% Control)
140
120
100
80
60
40
20
0
Cyclo
DB
Luc
NS siRNA
Genes targeted

Figure 3A


siRNA functionality is independent from the cell line
HEK293
DU145
Hela
DBI Expression (% of Control)
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
Control
siRNAs

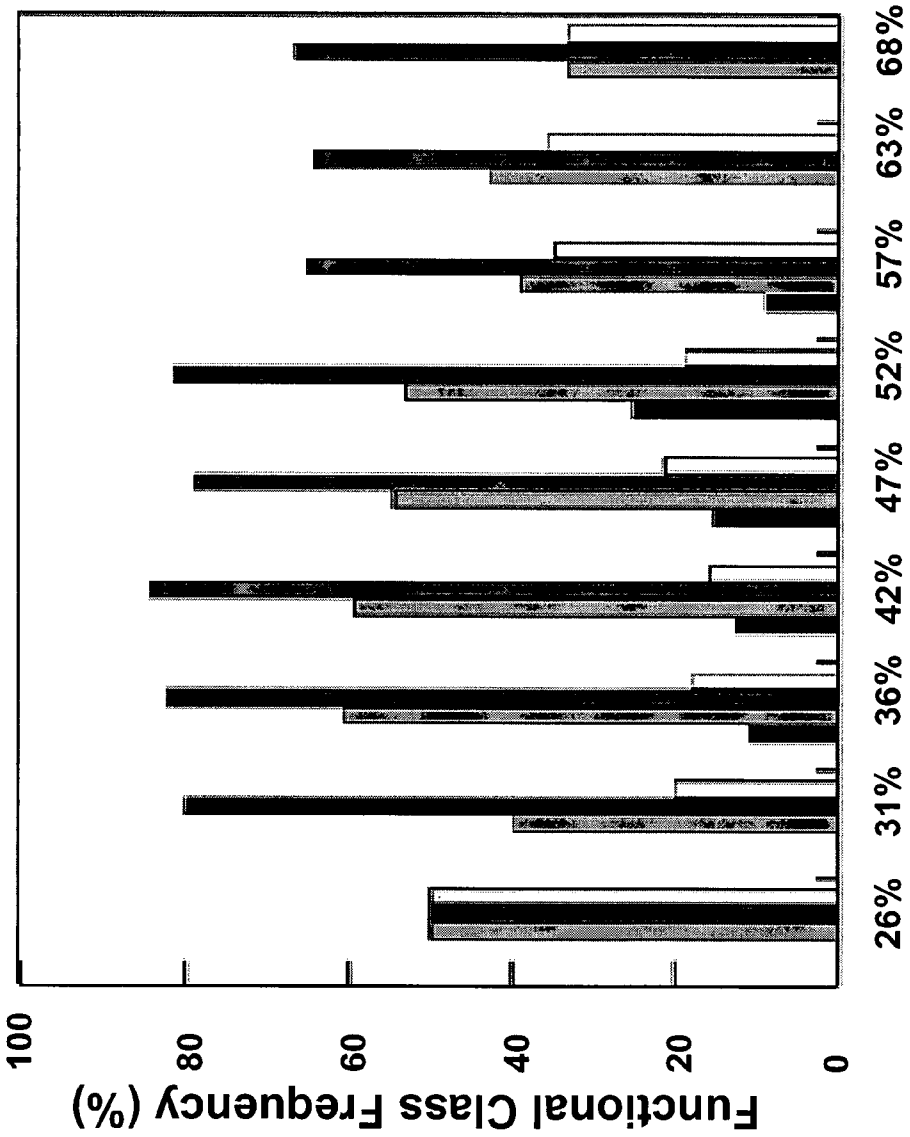

Functional Class Frequency (%)
100
80
60
40
20
0
26%
31%
36%
42%
47%
52%
57%
63%
68%

FIGURE 3B

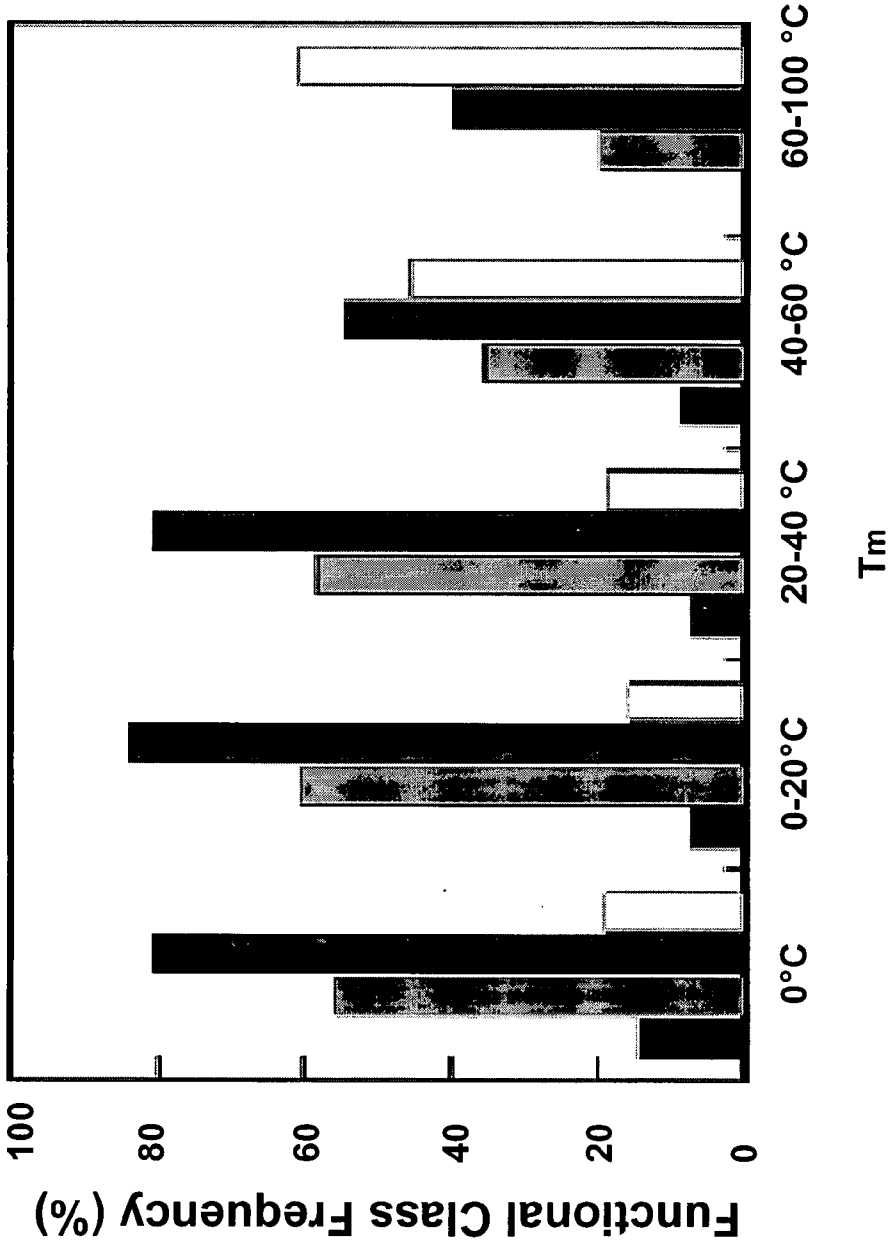

Functional Class Frequency (%)
100
80
60
40
20
0
0°C
0-20°C
20-40 °C
40-60 °C
60-100 °C
Tm

FIGURE 3C

Figure 4A

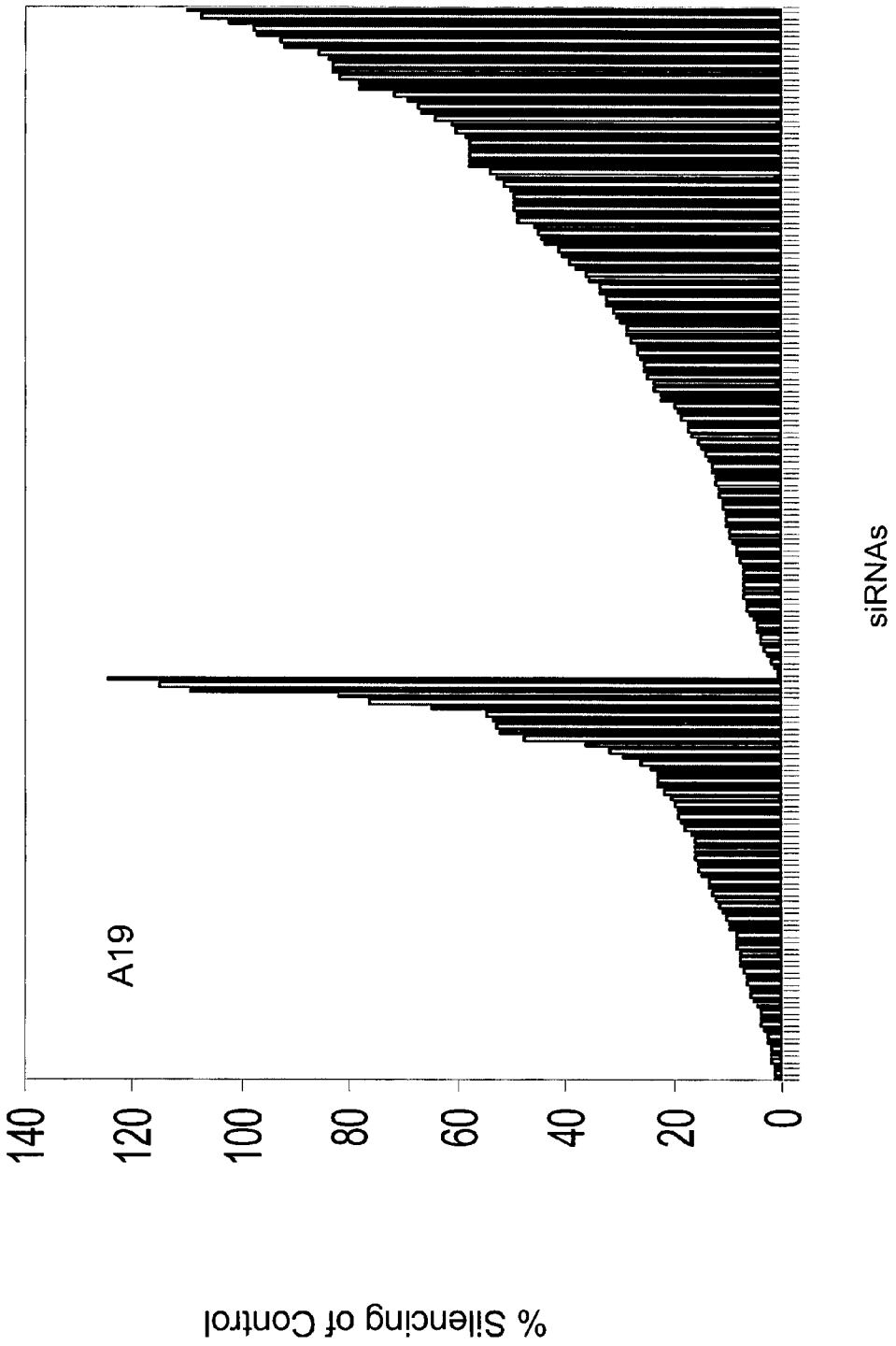


Figure 4B

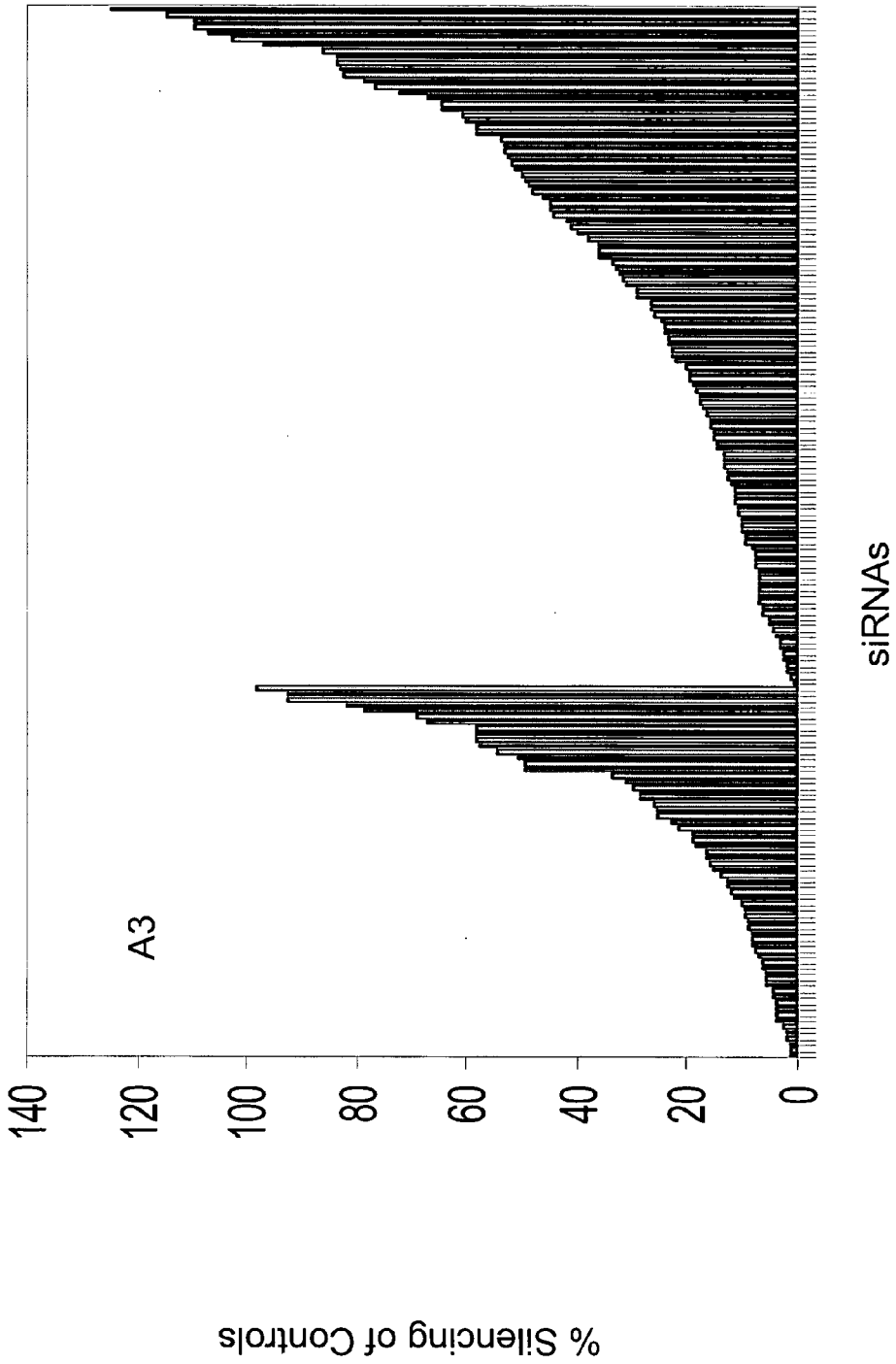


Figure 4C

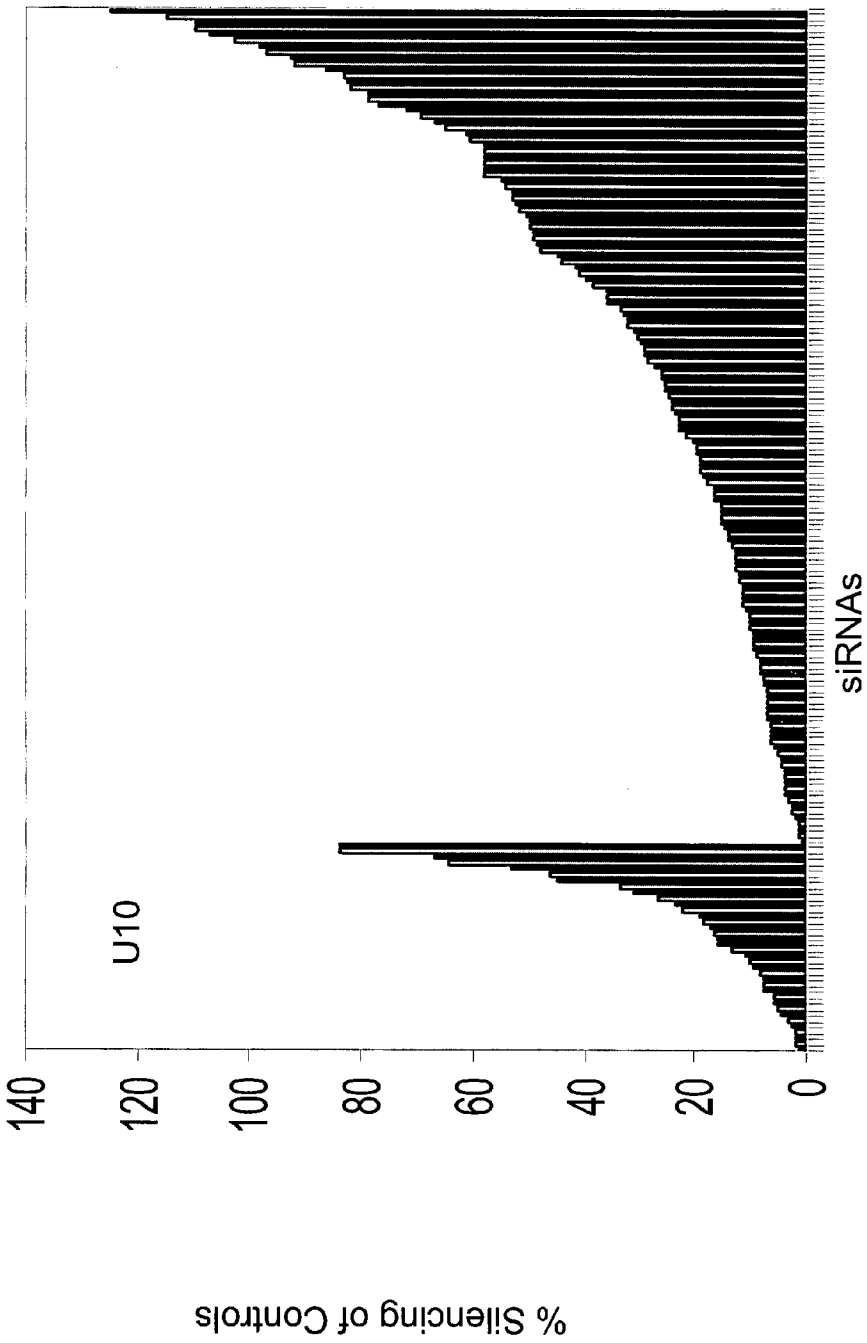


Figure 4D

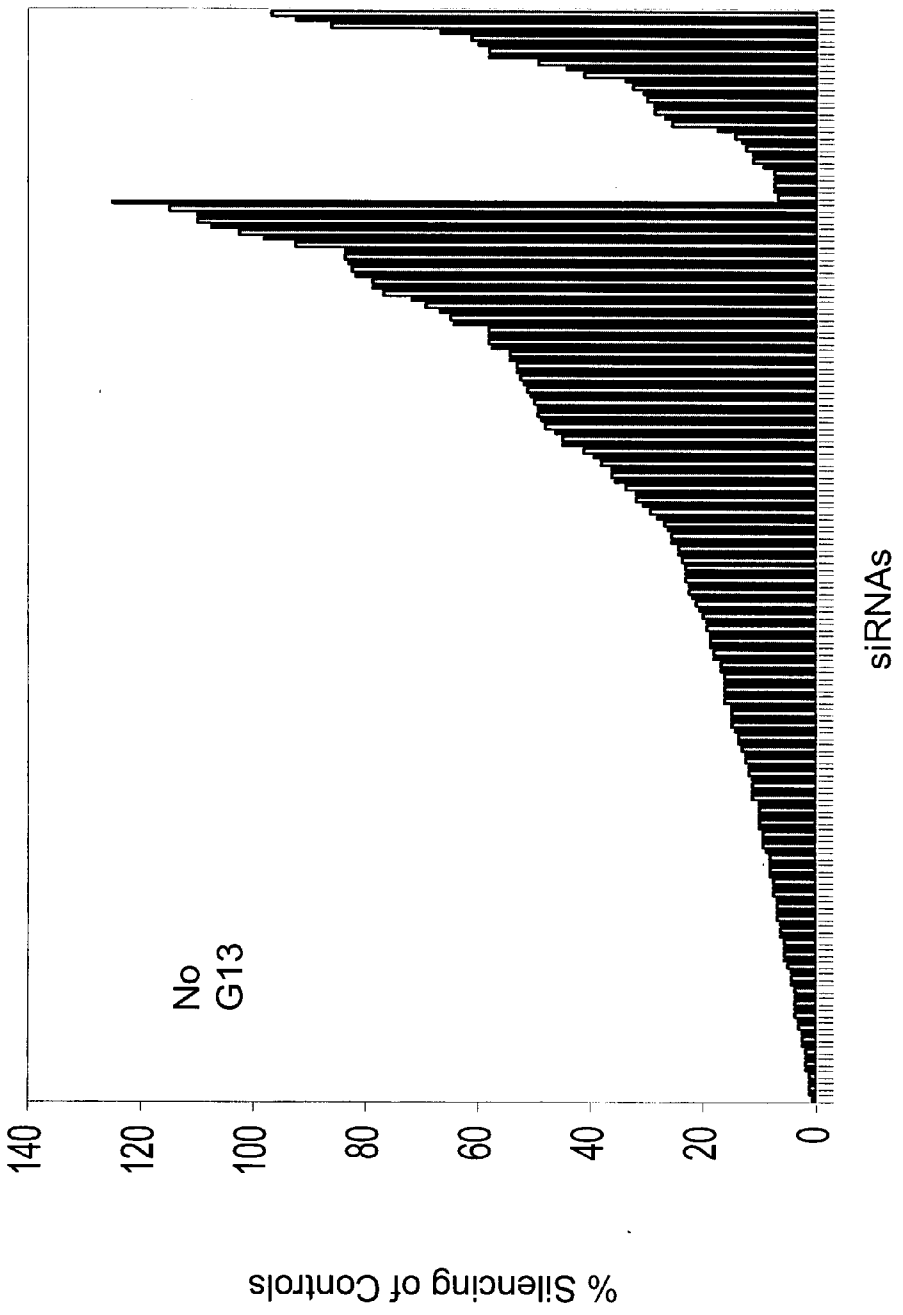


Figure 4E

Figure 5A

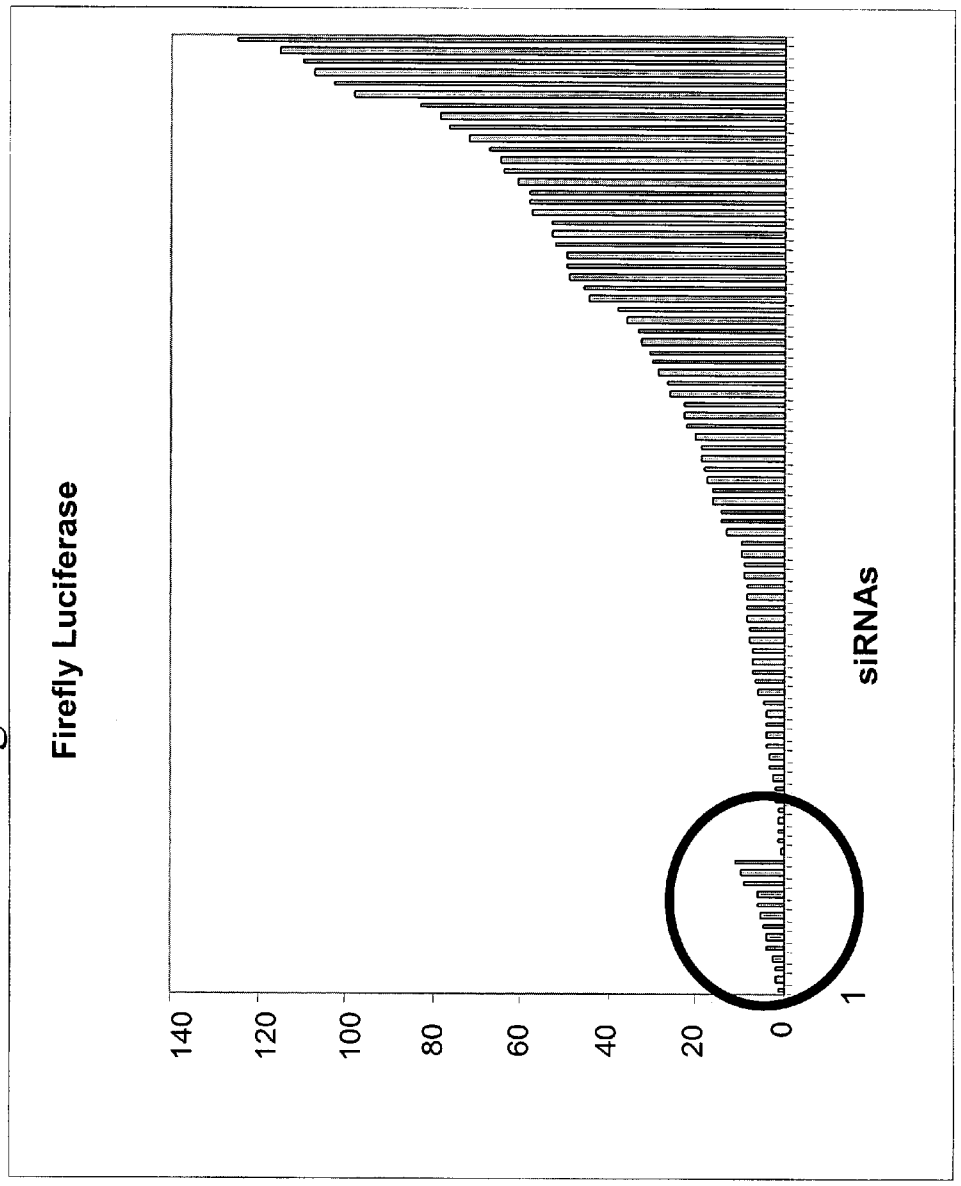

Firefly Luciferase
140
120
100
80
60
40
20
0
1
siRNAs
Expression (% Control)

Figure 5B

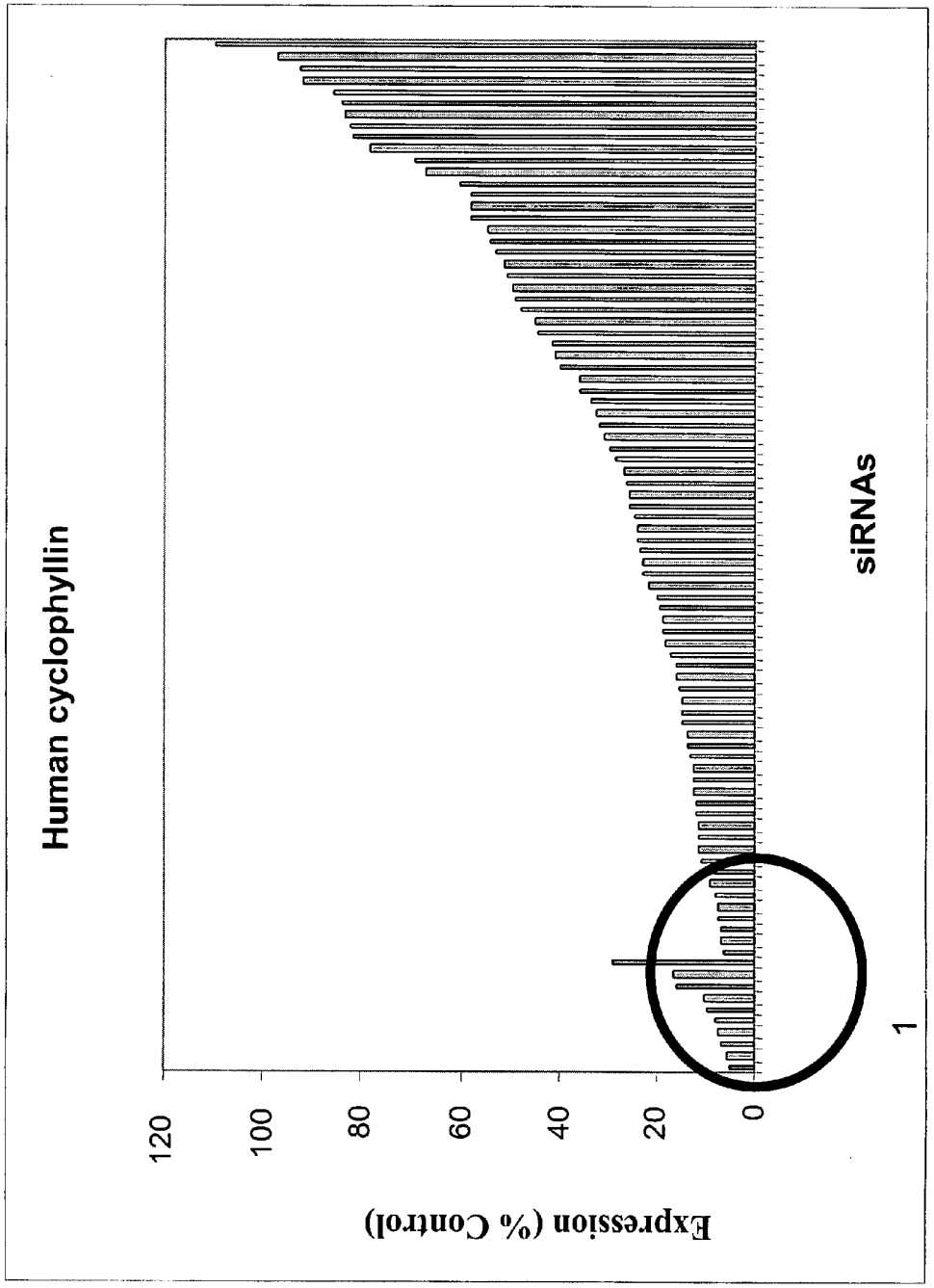

Human cyclophyllin
Expression (% Control)
120
100
80
60
40
20
0
1
siRNAs

Figure 6A

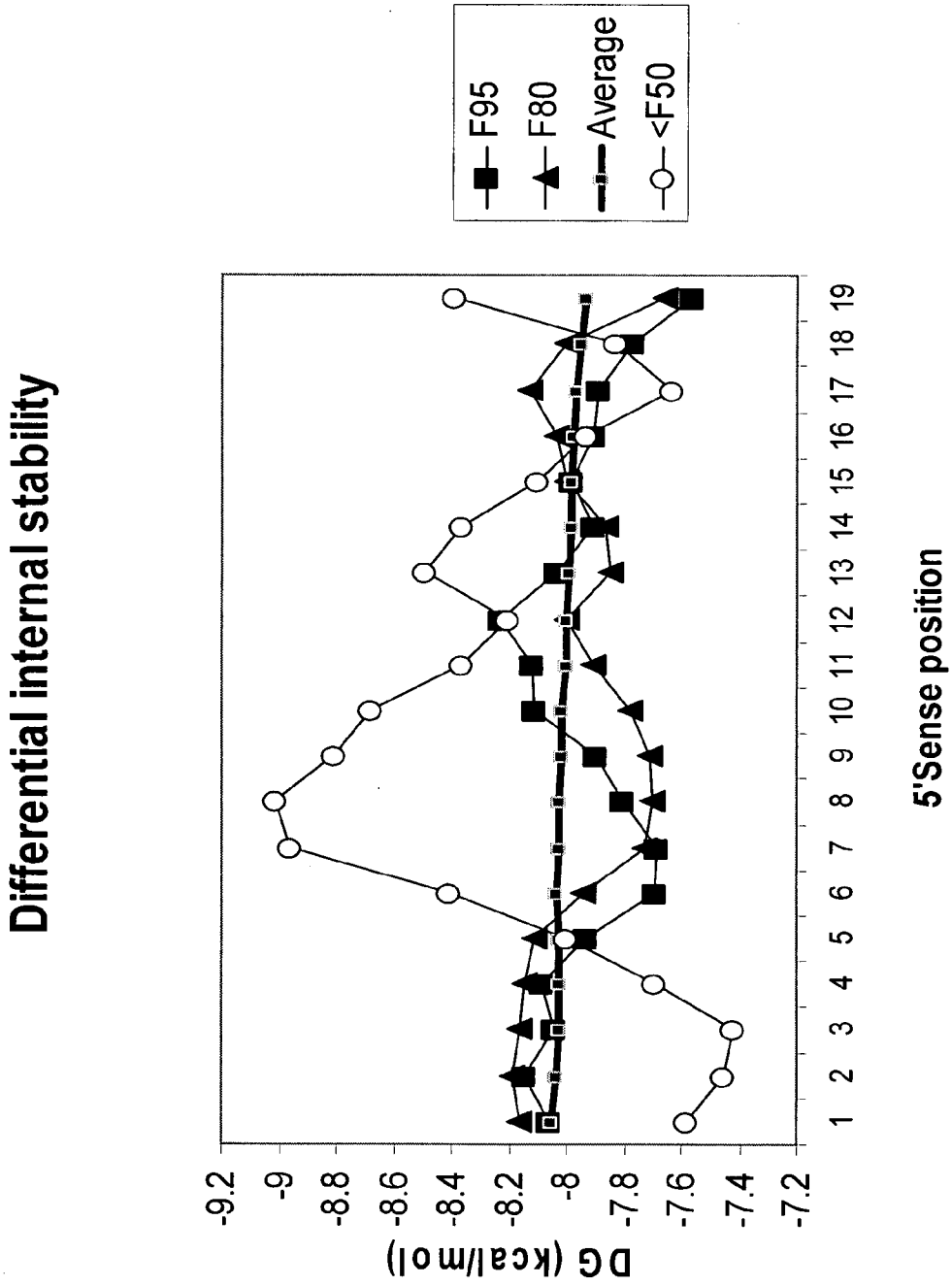
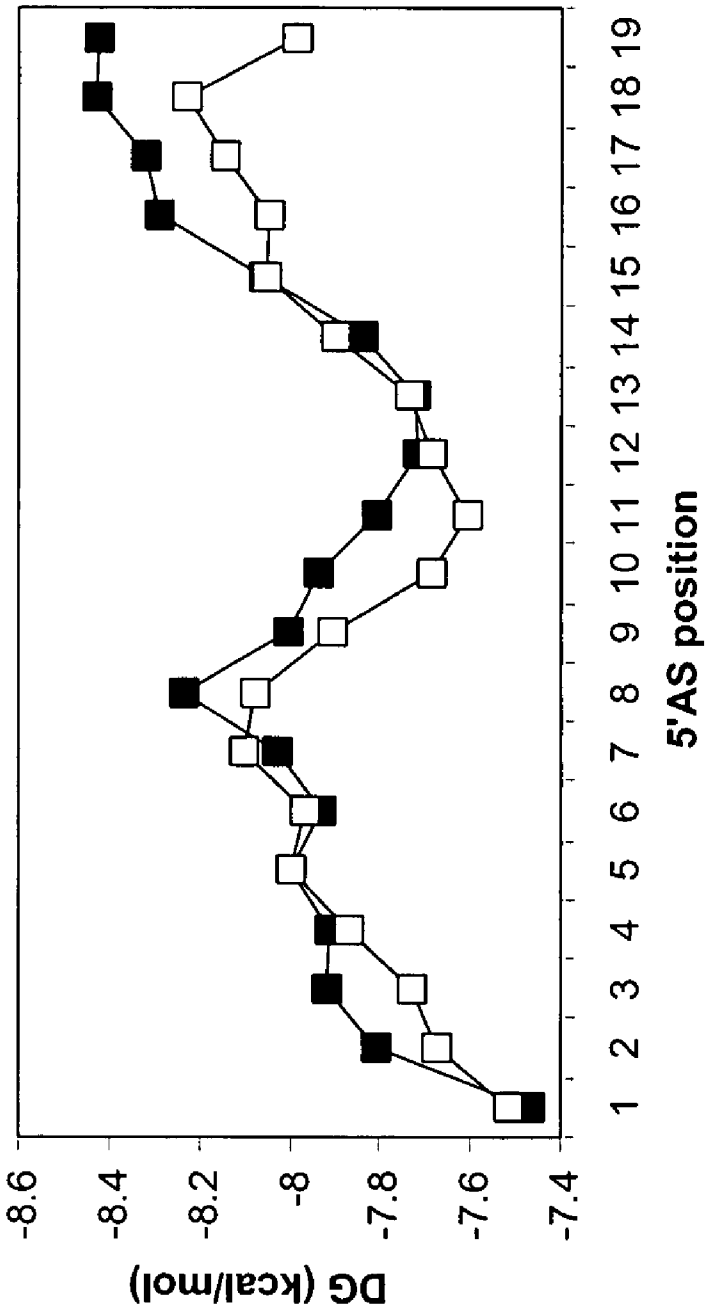
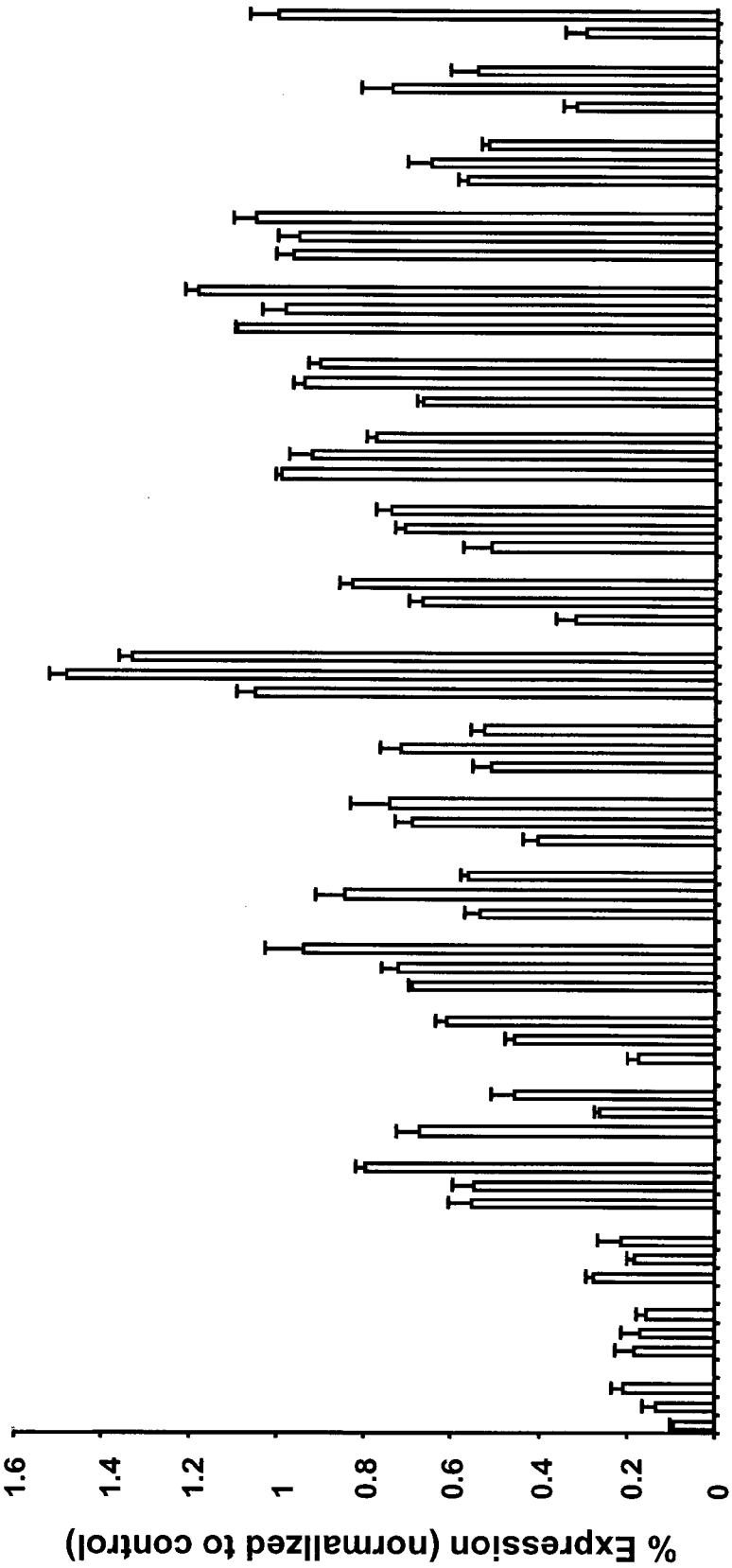

Differential internal stability
DG (kcal/mol)
-9.2
-9
-8.8
-8.6
-8.4
-8.2
-8
-7.8
-7.6
-7.4
-7.2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
5'Sense position
F95
F80
Average
<F50

Figure 6B


-8.6
-8.4
-8.2
-8
-7.8
-7.6
-7.4
DG (kcal/mol)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
5'AS position



siRNA 100 nM

FIGURE 7

Figure 8

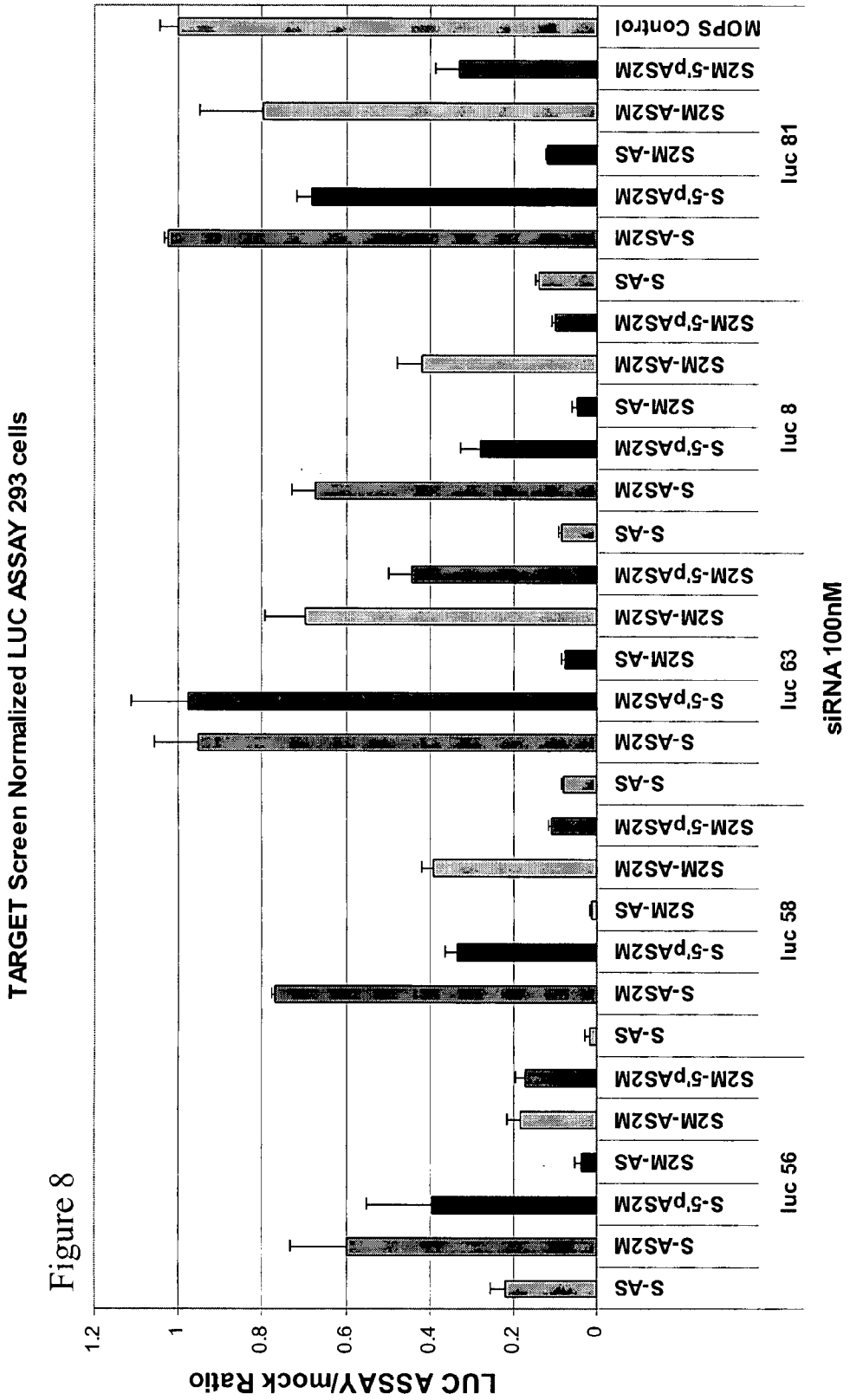

TARGET Screen Normalized LUC ASSAY 293 cells
LUC ASSAY/mock Ratio
1.2
1
0.8
0.6
0.4
0.2
0
S-AS
S-AS2M
S-5'pAS2M
S2M-AS
S2M-AS2M
S2M-5'pAS2M
luc 56
S-AS
S-AS2M
S-5'pAS2M
S2M-AS
S2M-AS2M
S2M-5'pAS2M
luc 58
S-AS
S-AS2M
S-5'pAS2M
S2M-AS
S2M-AS2M
S2M-5'pAS2M
luc 63
S-AS
S-AS2M
S-5'pAS2M
S2M-AS
S2M-AS2M
S2M-5'pAS2M
luc 8
S-AS
S-AS2M
S-5'pAS2M
S2M-AS
S2M-AS2M
S2M-5'pAS2M
luc 81
MOPS Control
siRNA 100nM

Figure 9

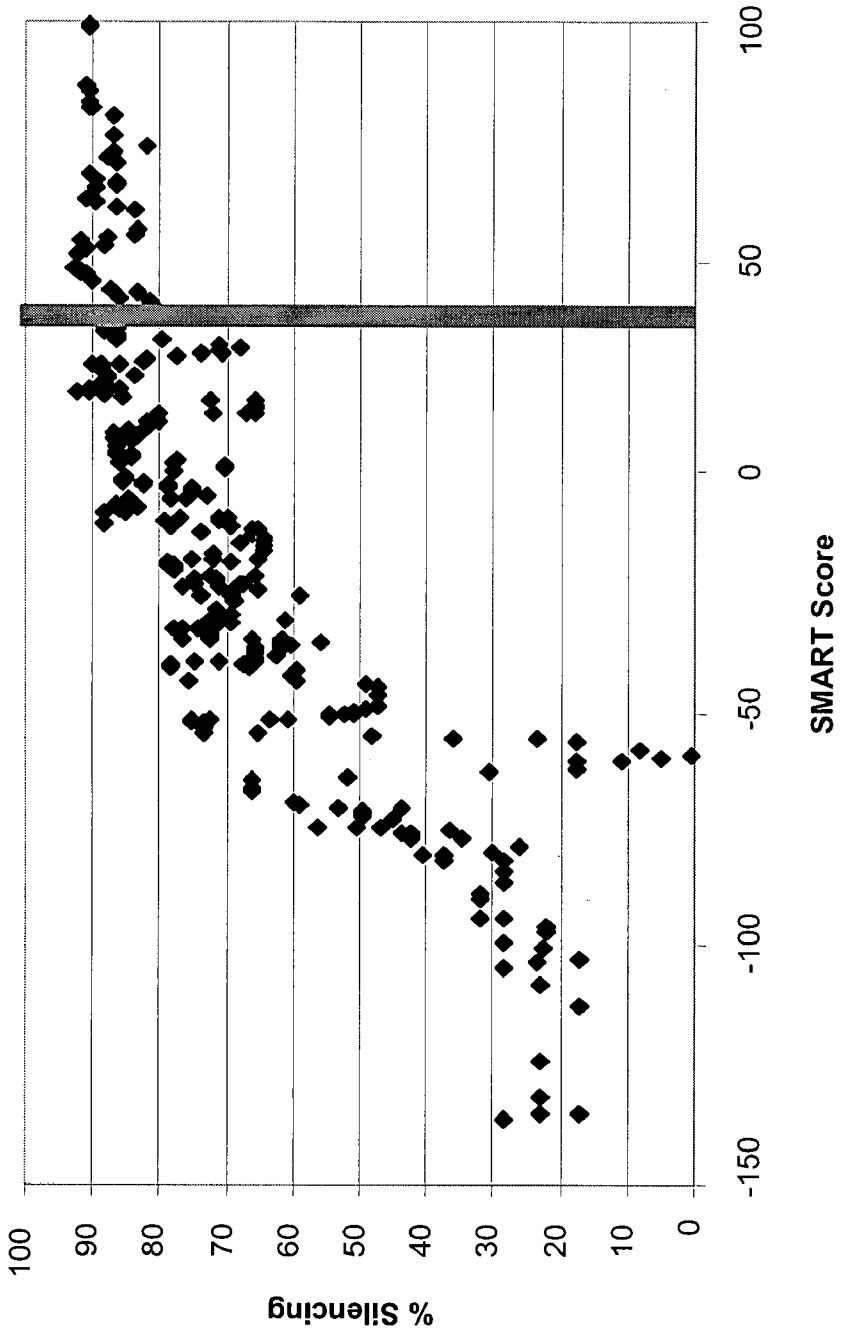

% Silencing
100
90
80
70
60
50
40
30
20
10
0
-150
-100
-50
0
50
100
SMART Score

Figure 10A

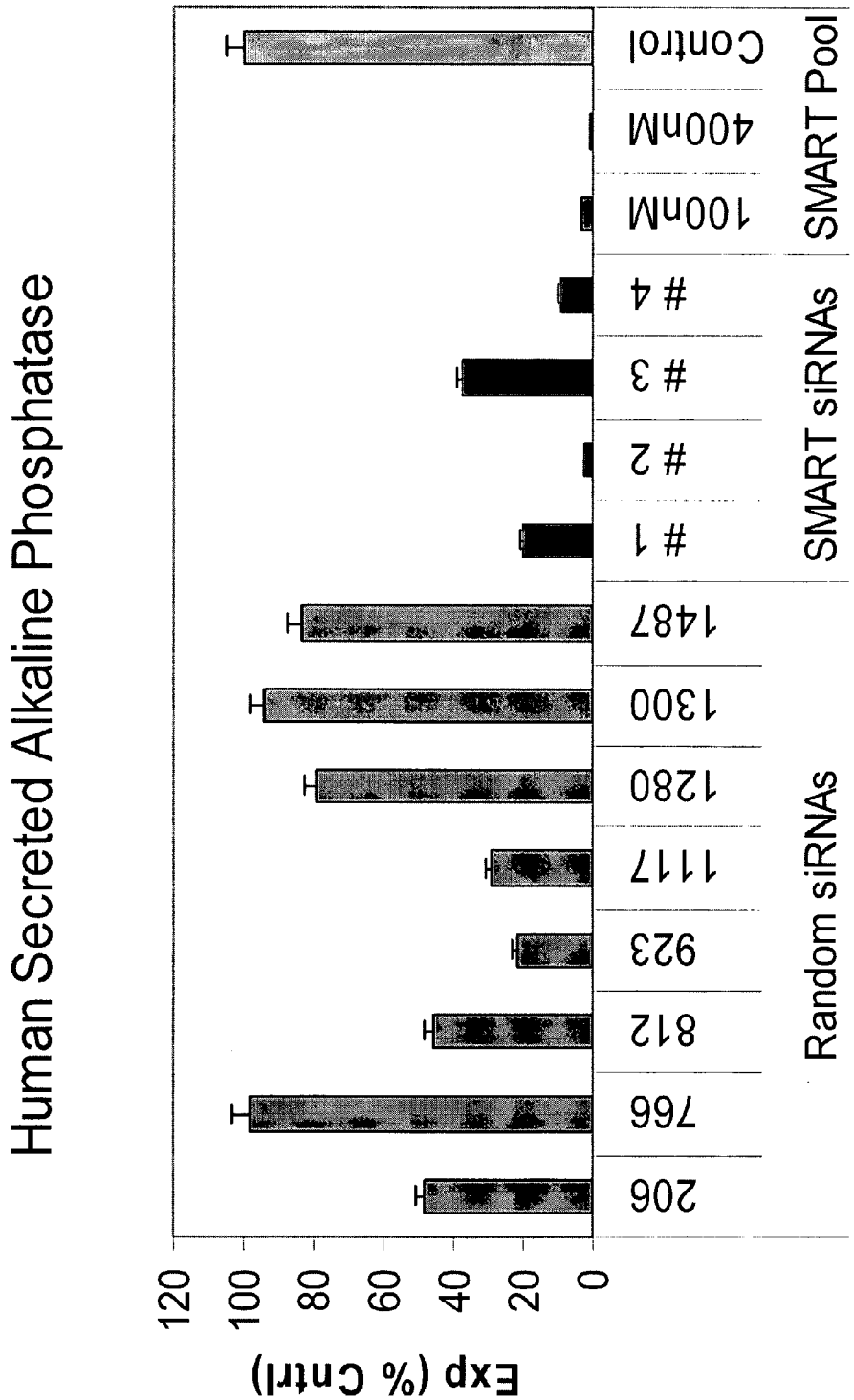

Human Secreted Alkaline Phosphatase
Exp (% Cntrl)
120
100
80
60
40
20
0
206
766
812
923
1117
1280
1300
1487
1
2
3
4
100nM
400nM
Control
Random siRNAs
SMART siRNAs
SMART Pool

Figure 10B

Homo sapiens Acyl-Coenzyme A binding protein

(DBI)

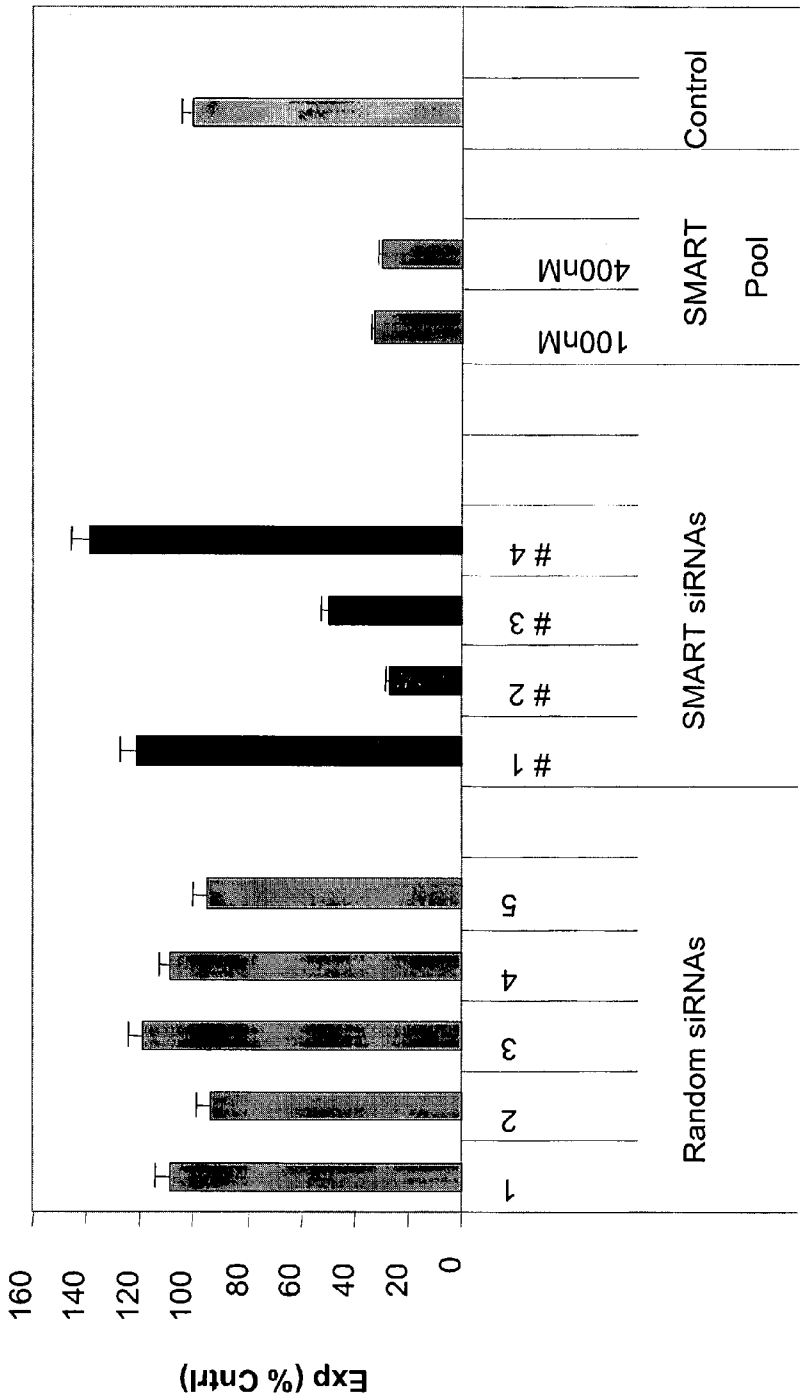


Figure 10C

Homo sapiens polo-like kinase (PLK)

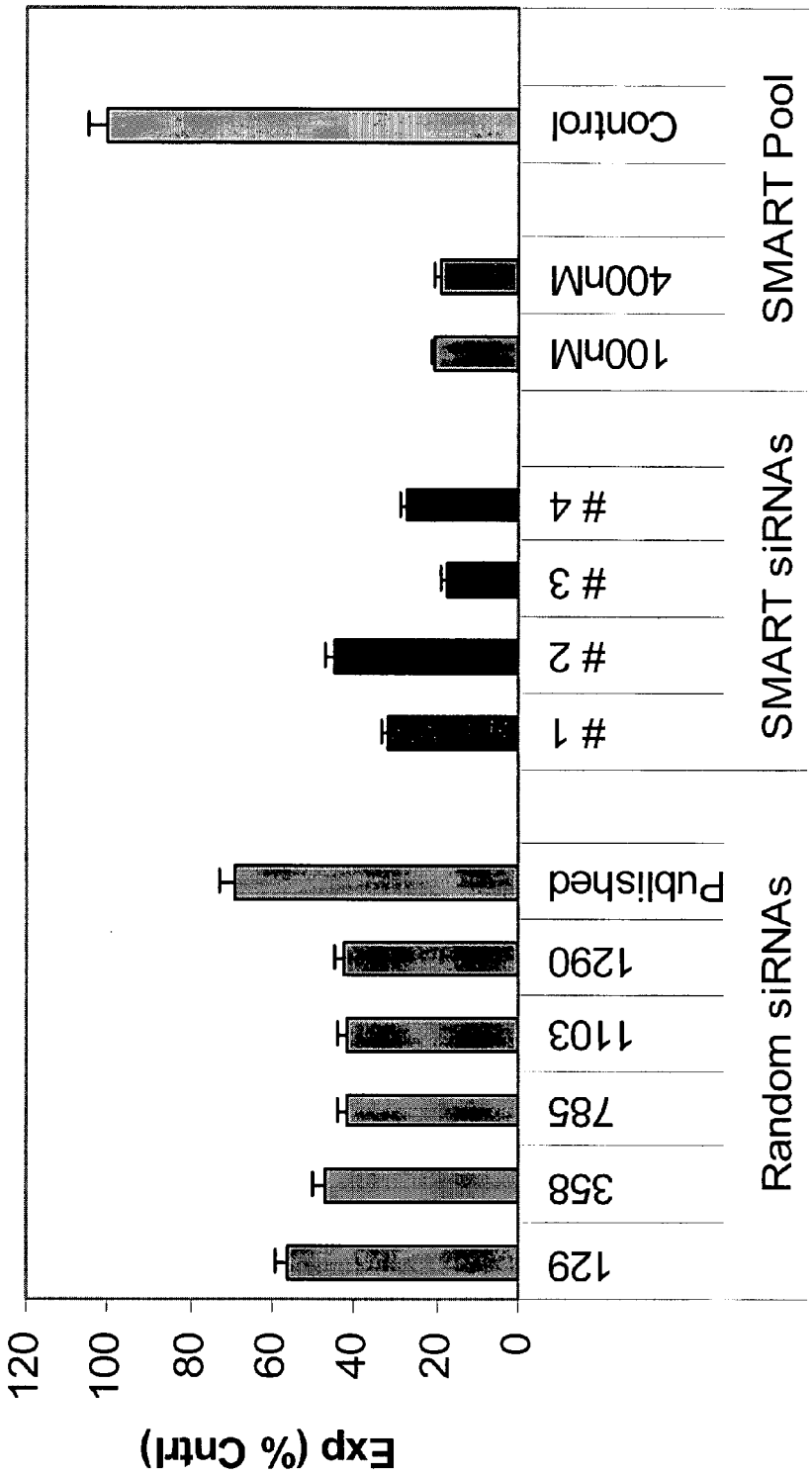


Figure 10D

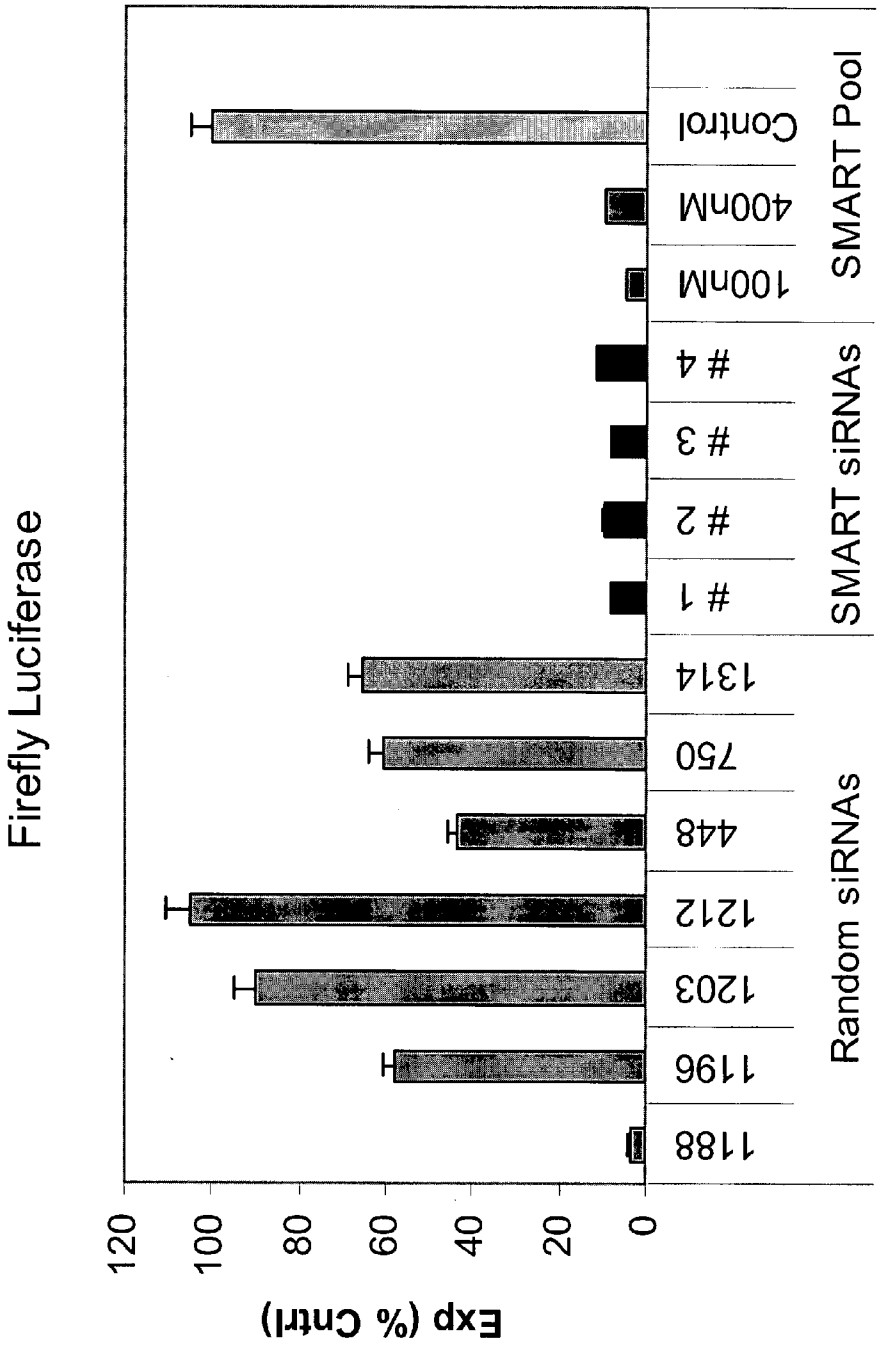

Firefly Luciferase
Exp (% Cntrl)
120
100
80
60
40
20
0
1188
1196
1203
1212
448
750
1314
1
2
3
4
100nM
400nM
Control
Random siRNAs
SMART siRNAs
SMART Pool

Figure 10E

Renila Luciferase

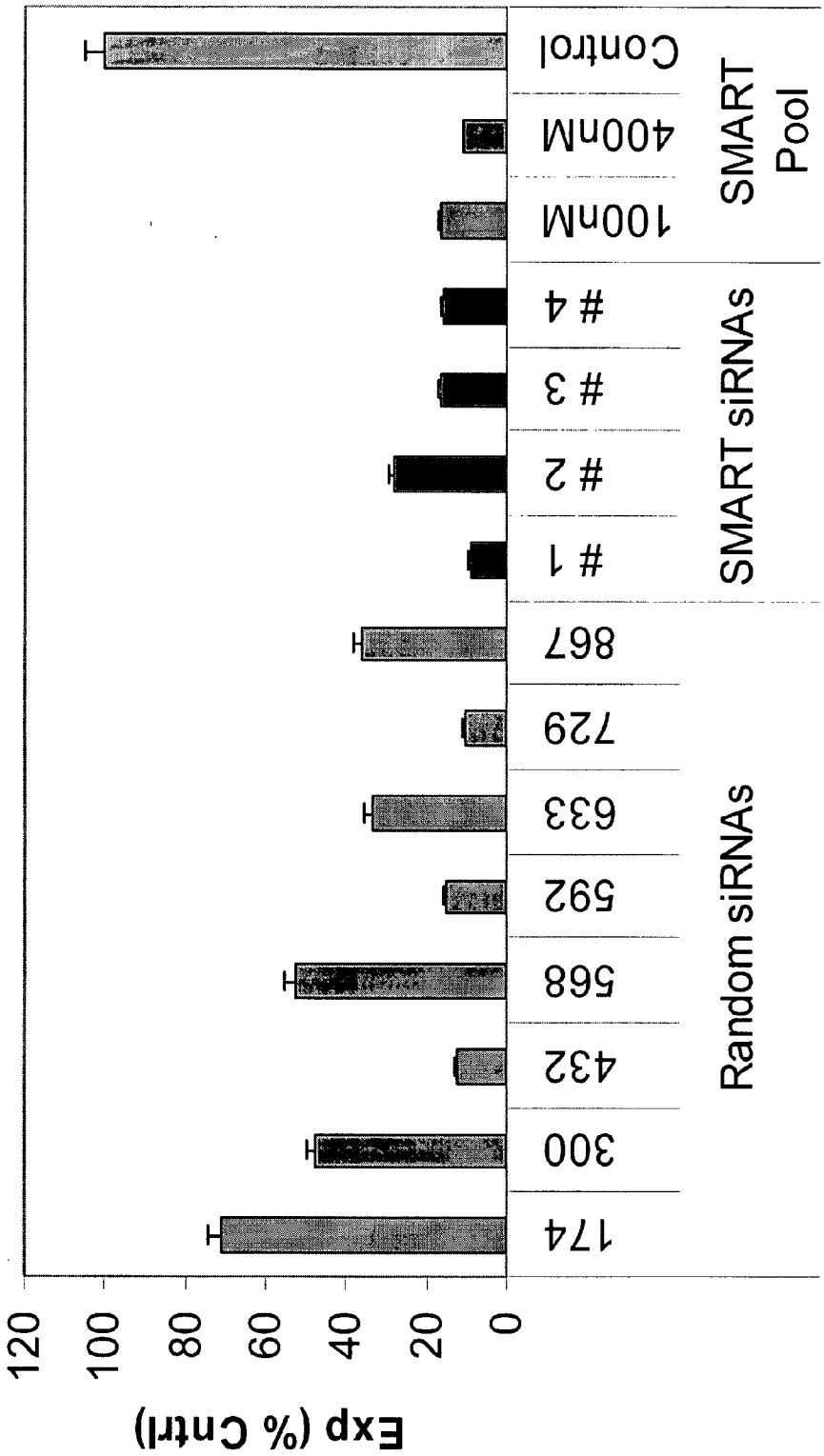


Figure 10F

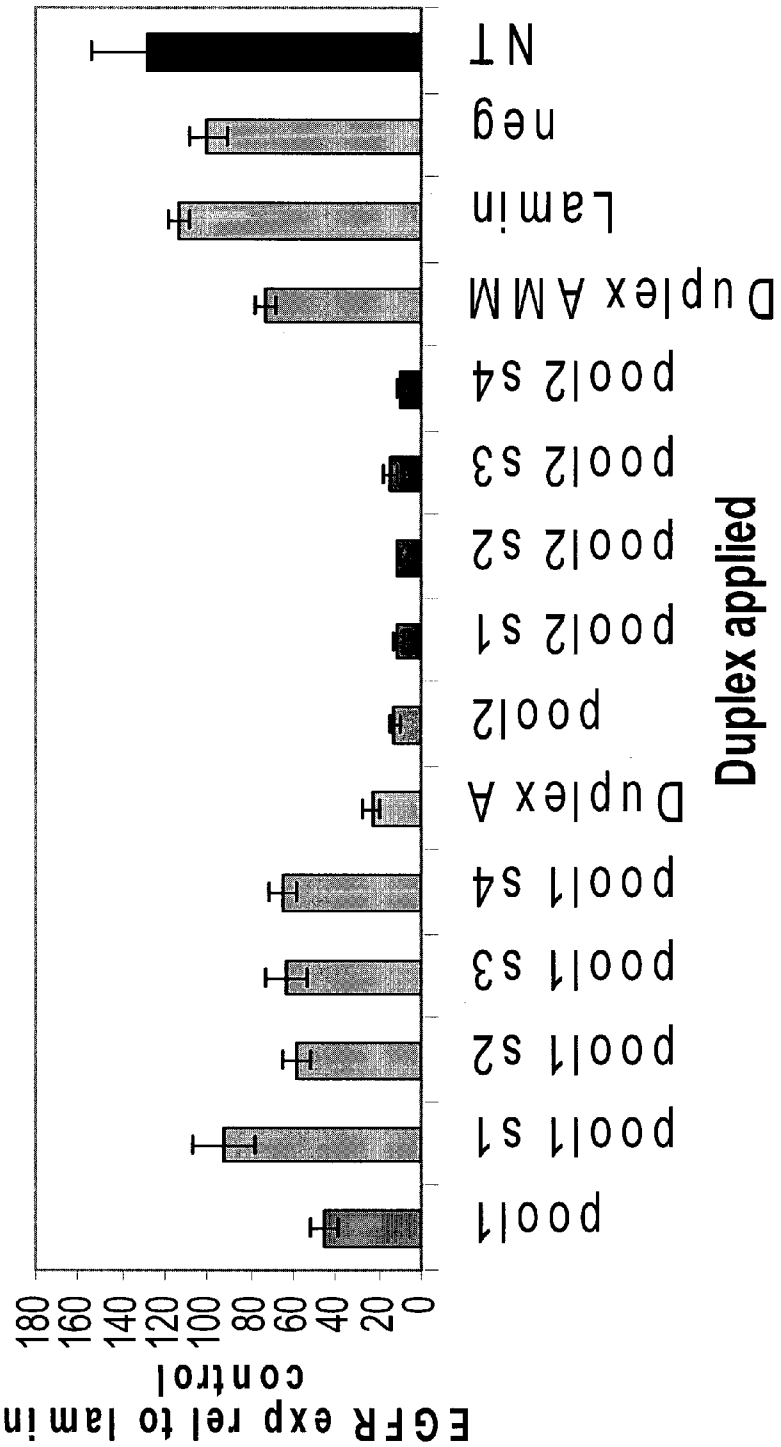

EGFR
EGFR exp rel to lamin control
180
160
140
120
100
80
60
40
20
0
pool1
pool1 s1
pool1 s2
pool1 s3
pool1 s4
Duplex A
pool2
pool2 s1
pool2 s2
pool2 s3
pool2 s4
Duplex AMM
Lamin
neg
NT
Duplex applied

Figure 11

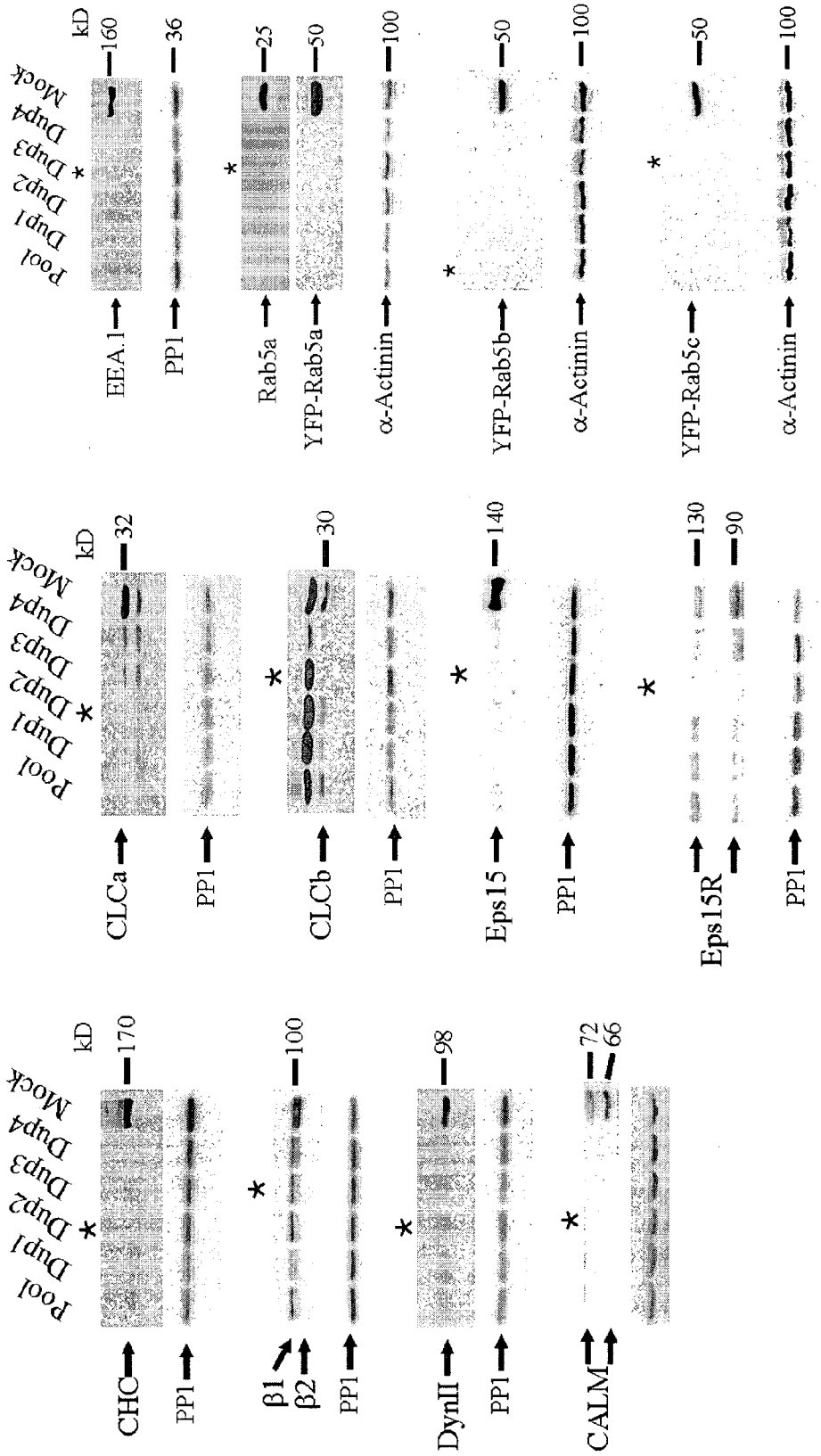

Pool
Dup1
Dup2
Dup3
Dup4
Mock
kD
CHC
170
PP1
β1
β2
100
DynII
98
CALM
72
66
CLCa
32
CLCb
30
Eps15
140
Eps15R
130
90
EEA.1
160
Rab5a
25
YFP-Rab5a
50
α-Actinin
100
YFP-Rab5b
YFP-Rab5c

Figure 12

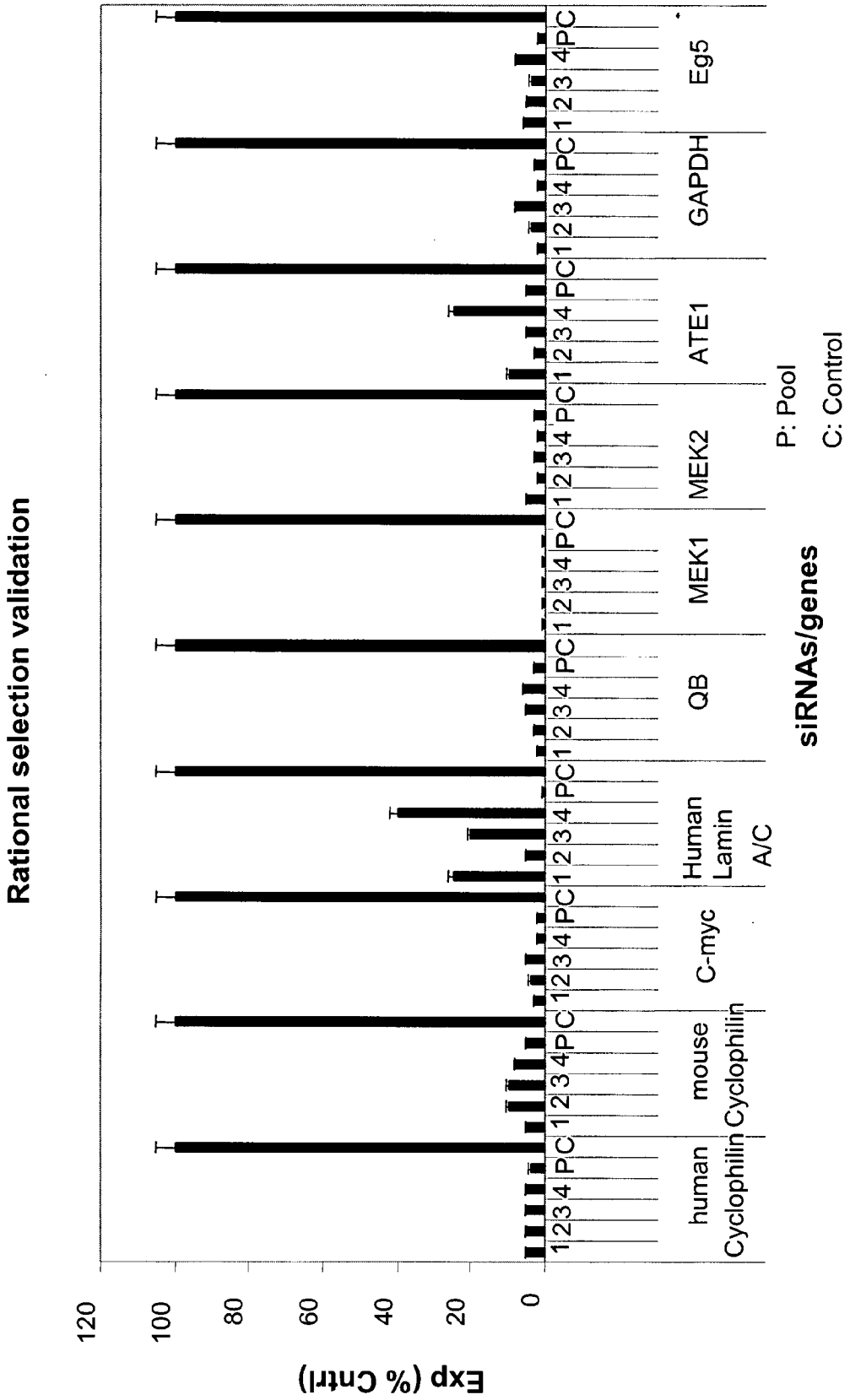
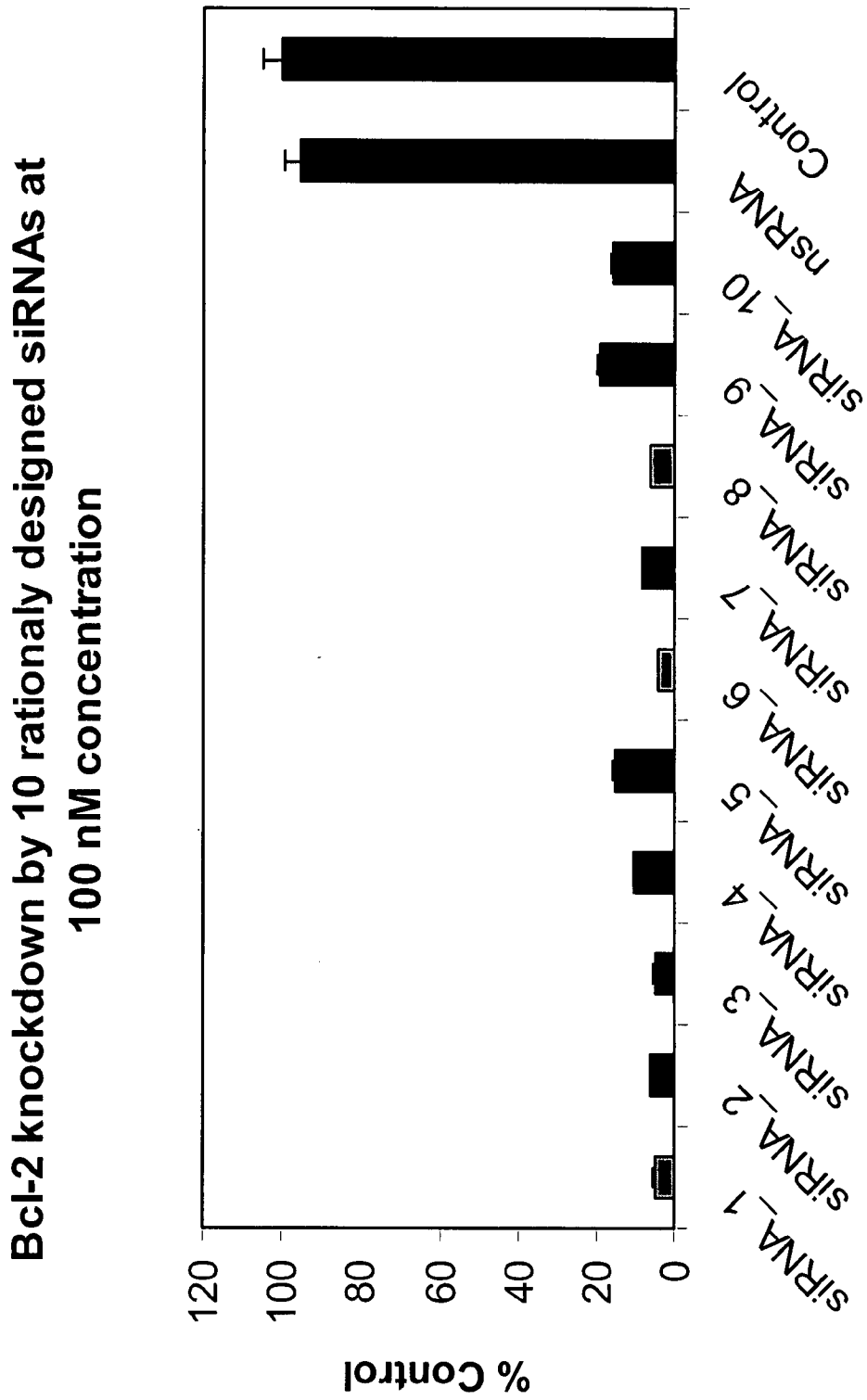

Rational selection validation
Exp (% Cntrl)
120
100
80
60
40
20
0
1 2 3 4 PC
human Cyclophilin
mouse Cyclophilin
C-myc
Human Lamin A/C
QB
MEK1
MEK2
ATE1
GAPDH
Eg5
siRNAs/genes
P: Pool
C: Control

Figure 13 Sequences of top Bcl2

siRNA 1 GGGAGAUAGUGAUGAAGUA (SEQ. ID NO. 302)
siRNA 2 GAAGUACAUCCAUUAUAAG (SEQ. ID NO. 303)
siRNA 3 GUACGACAACCGGGAGAUA (SEQ. ID NO. 304)
siRNA 4 AGAUAGUGAUGAAGUACAU (SEQ. ID NO. 305)
siRNA 5 UGAAGACUCUGCUCAGUUU (SEQ. ID NO. 306)
siRNA 6 GCAUGCGGCCUCUGUUUGA (SEQ. ID NO. 307)
siRNA 7 UGCGGCCUCUGUUUGAUUU (SEQ. ID NO. 308)
siRNA 8 GAGAUAGUGAUGAAGUACA (SEQ. ID NO. 309)
siRNA 9 GGAGAUAGUGAUGAAGUAC (SEQ. ID NO. 310)
siRNA 10 GAAGACUCUGCUCAGUUUG (SEQ. ID NO. 311)

Figure 14


Bcl-2 knockdown by 10 rationaly designed siRNAs at 100 nM concentration
% Control
120
100
80
60
40
20
0
siRNA_1
siRNA_2
siRNA_3
siRNA_4
siRNA_5
siRNA_6
siRNA_7
siRNA_8
siRNA_9
siRNA_10
nsRNA
Control

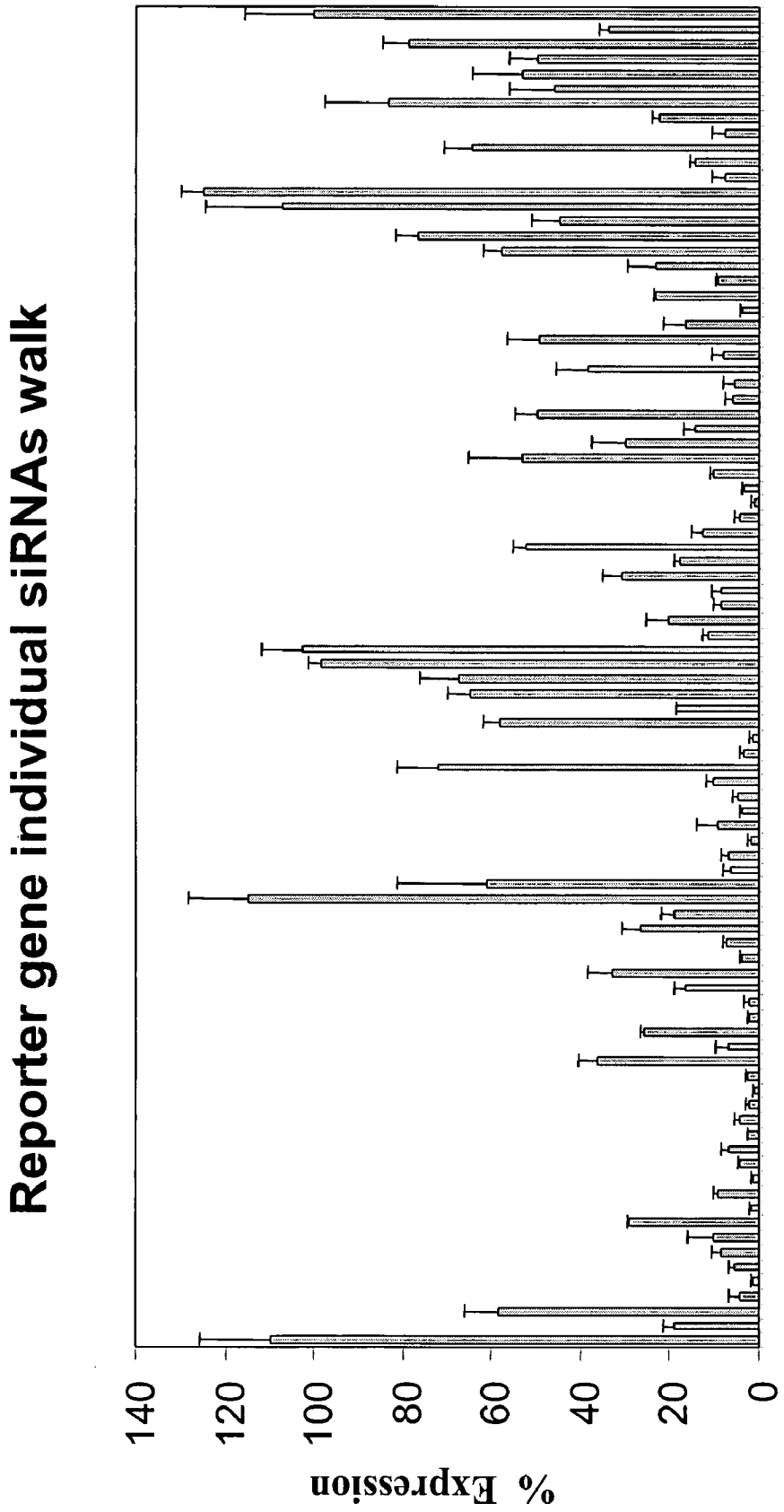

Reporter gene individual siRNAs walk
% Expression
140
120
100
80
60
40
20
0

Figure 15

Figure 16 A

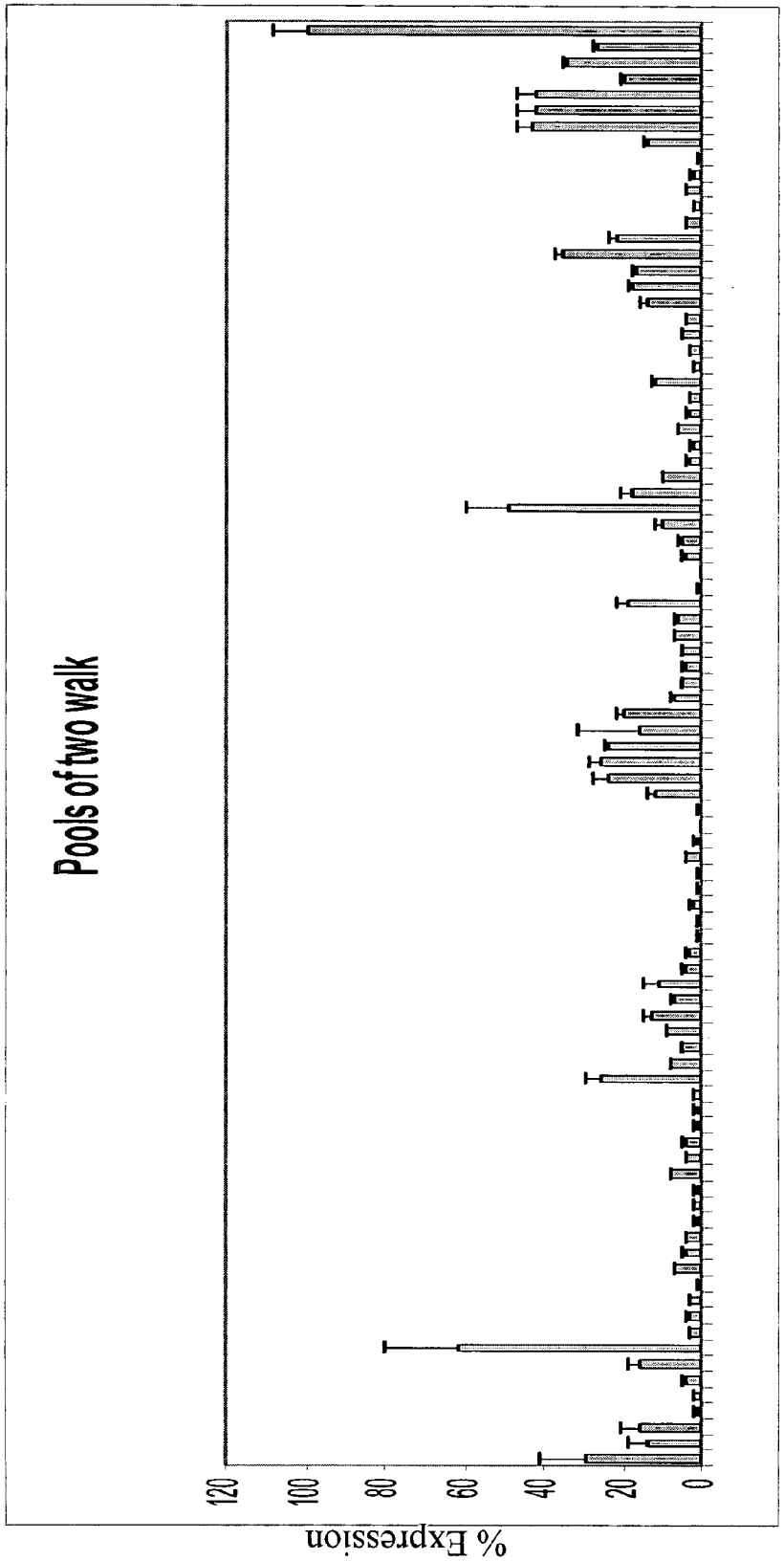


Figure 16 B

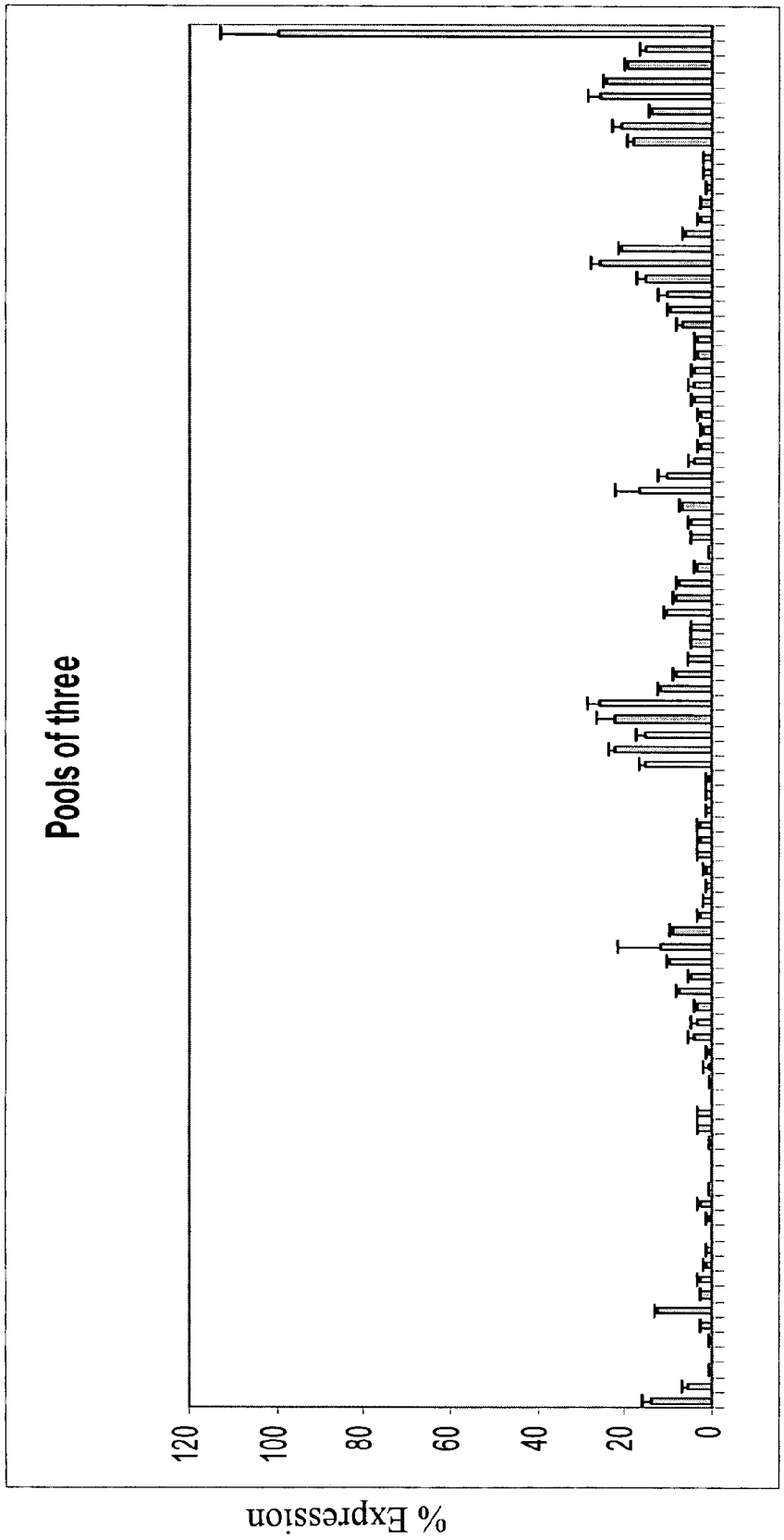


Figure 17 A

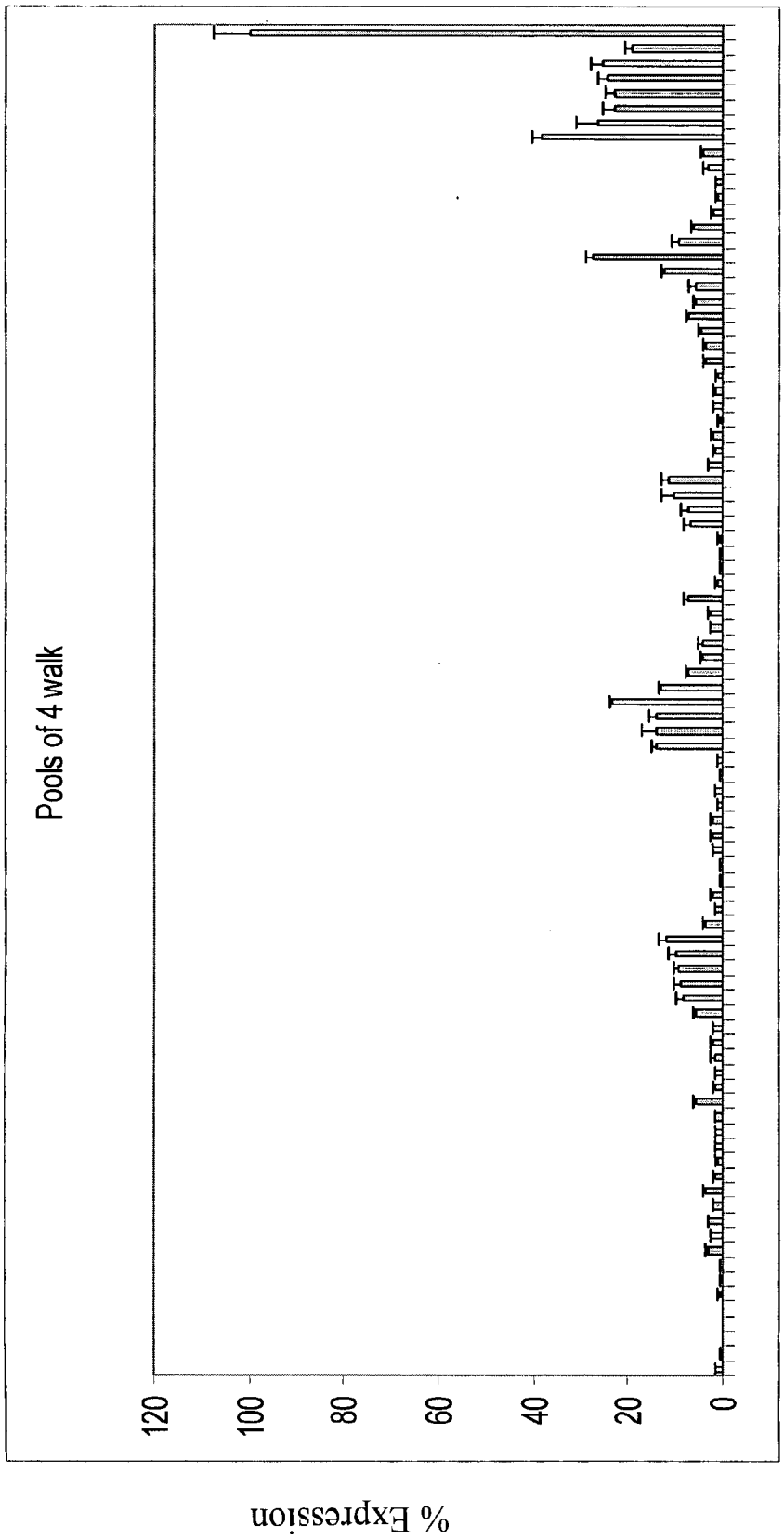


Figure 17 B

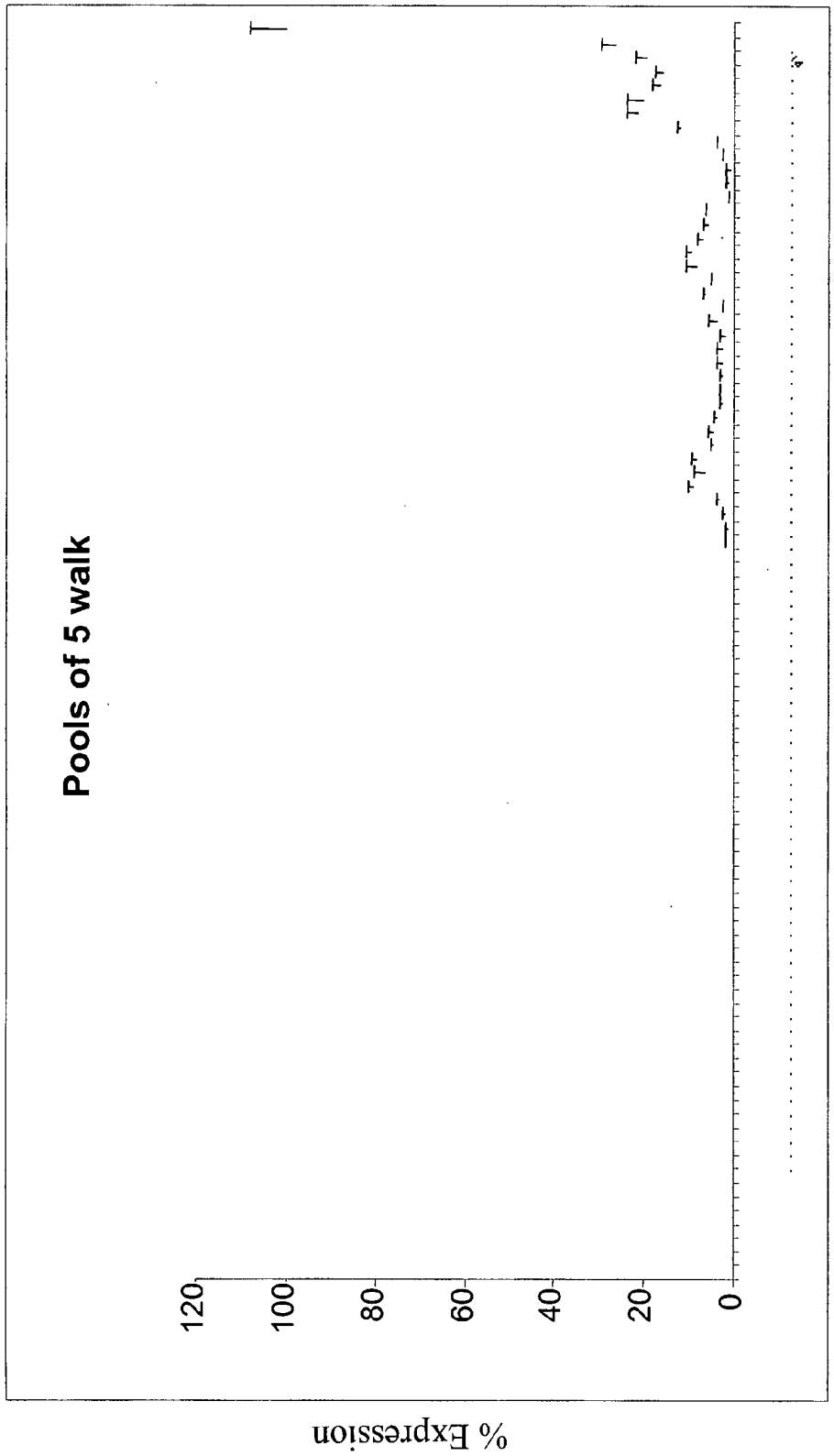


Figure 18 A

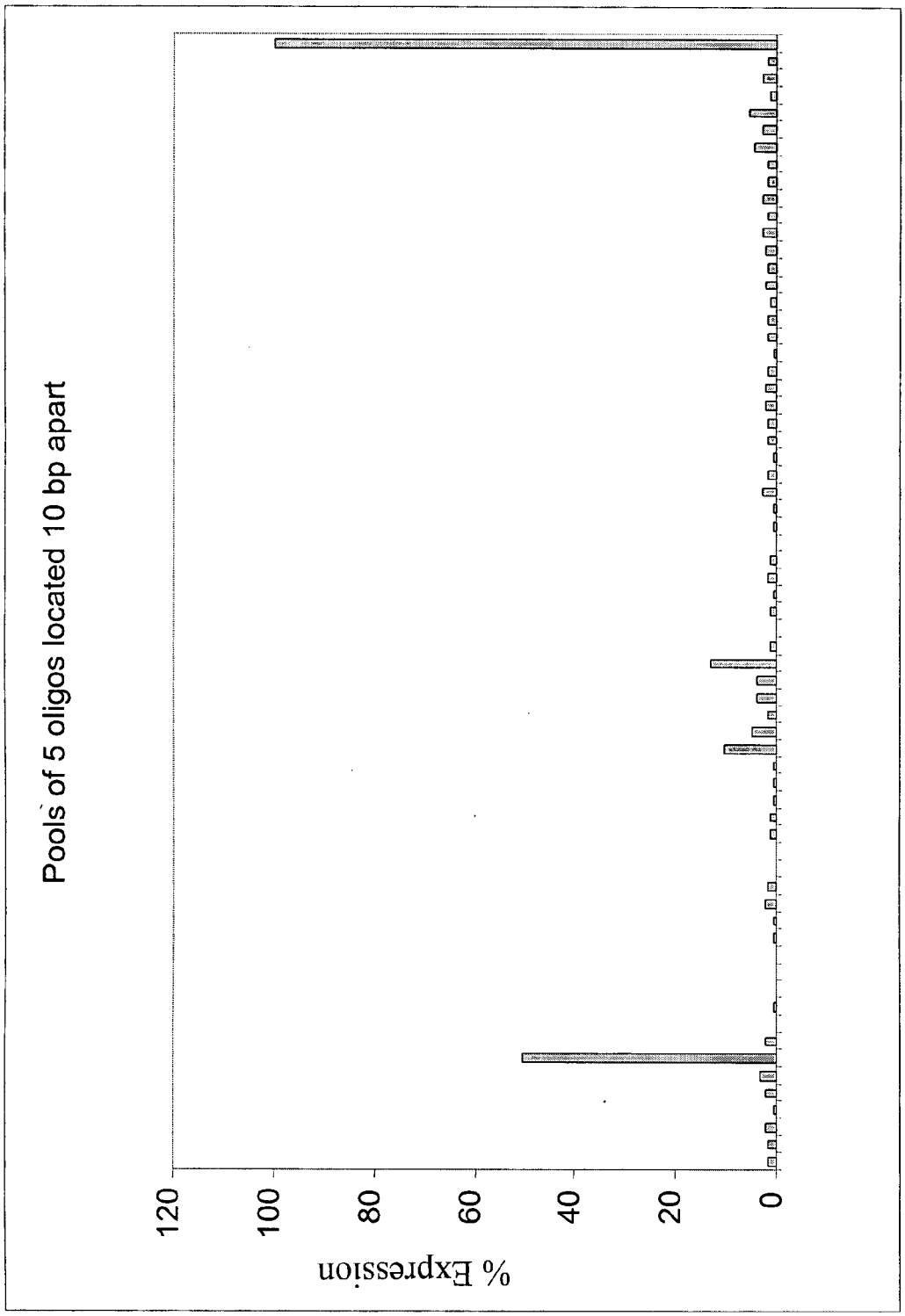
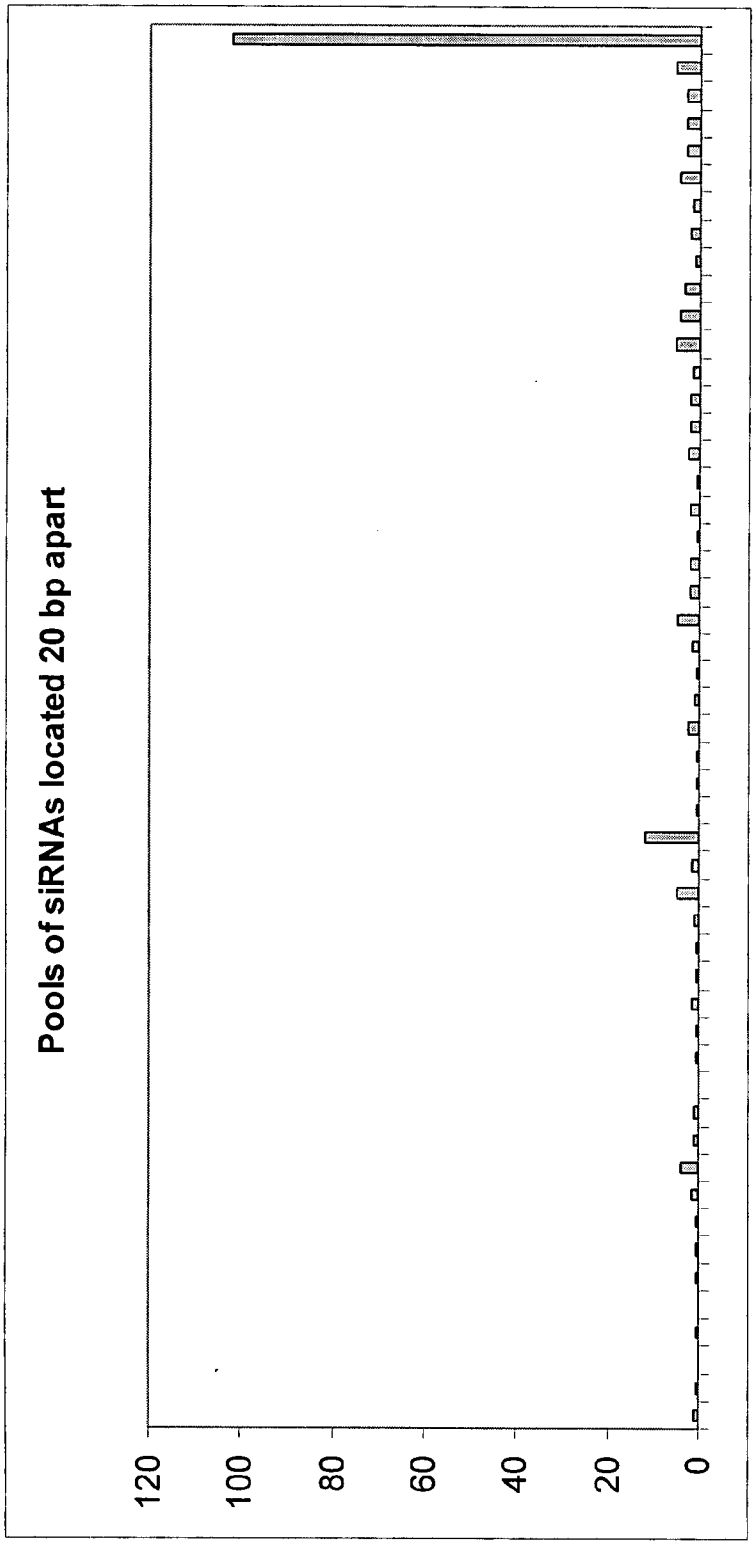

Pools of 5 oligos located 10 bp apart
% Expression
120
100
80
60
40
20
0

Figure 18 B


Pools of siRNAs located 20 bp apart
120
100
80
60
40
20
0
% Expression

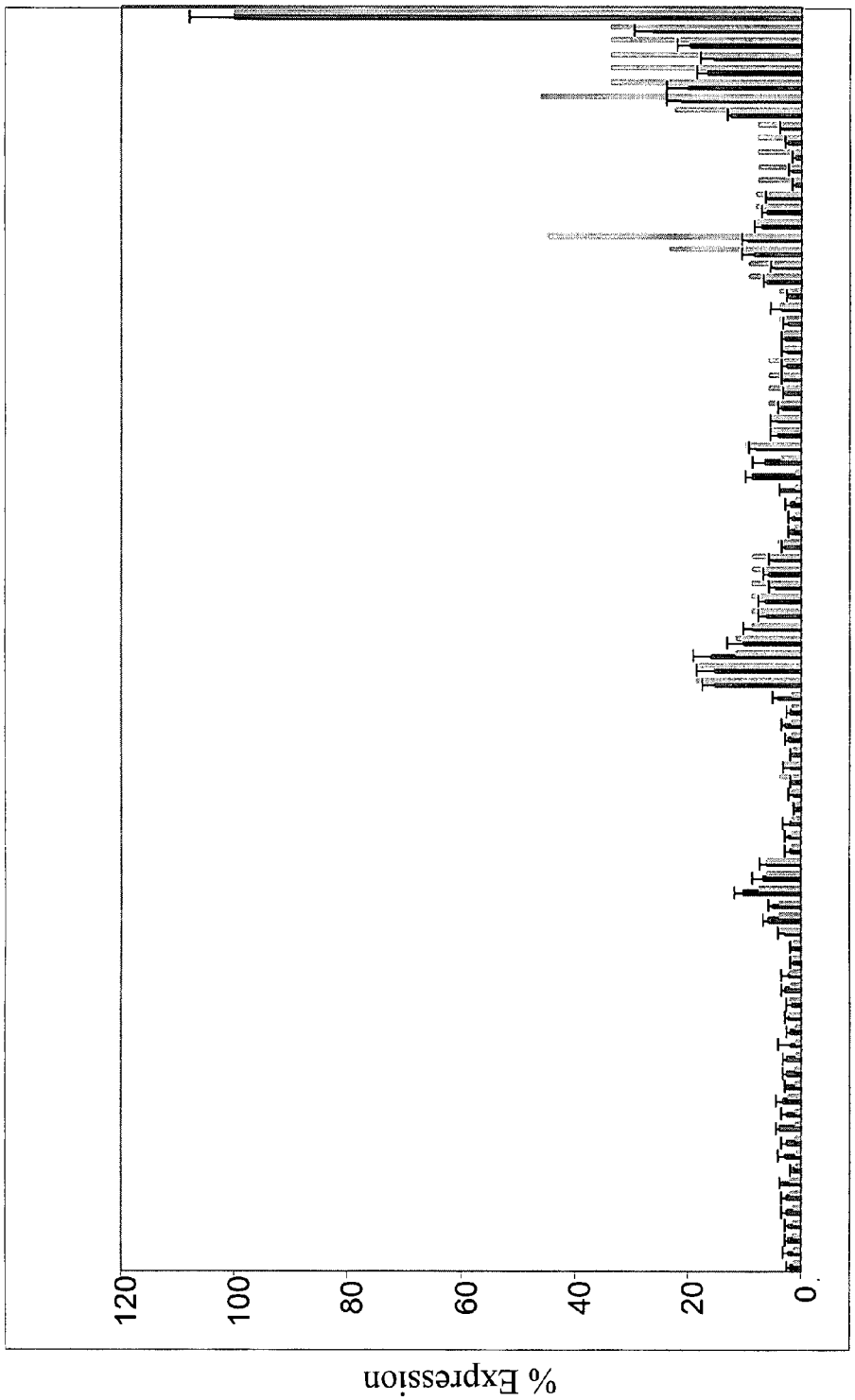


Figure 19

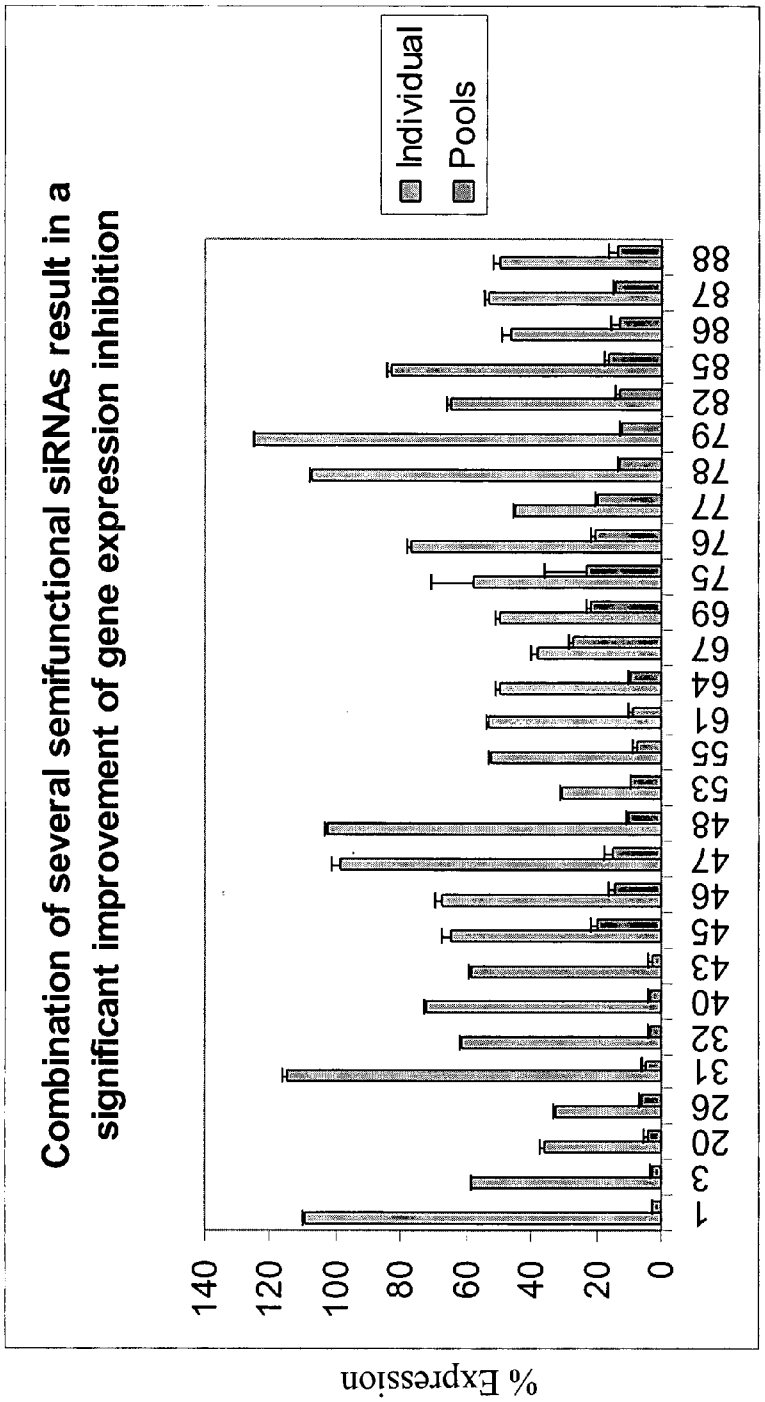

Combination of several semifunctional siRNAs result in a significant improvement of gene expression inhibition
Individual
Pools
% Expression
140
120
100
80
60
40
20
0
1
3
20
26
31
32
40
43
45
46
47
48
53
55
61
64
67
69
75
76
77
78
79
82
85
86
87
88

Figure 20

Figure 21A

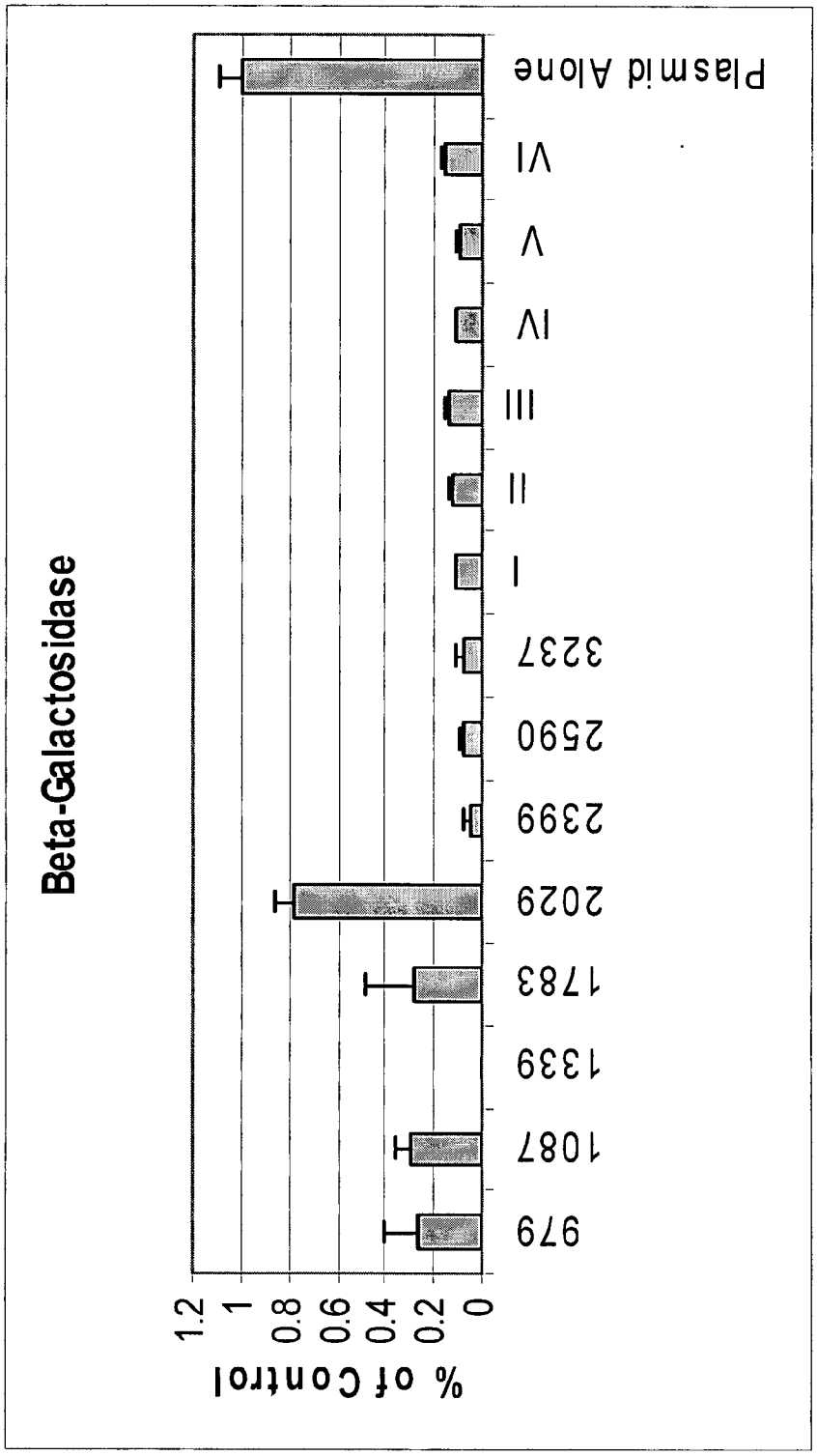

Beta-Galactosidase
% of Control
1.2
1
0.8
0.6
0.4
0.2
0
979
1087
1339
1783
2029
2399
2590
3237
I
II
III
IV
V
VI
Plasmid Alone

Figure 21B

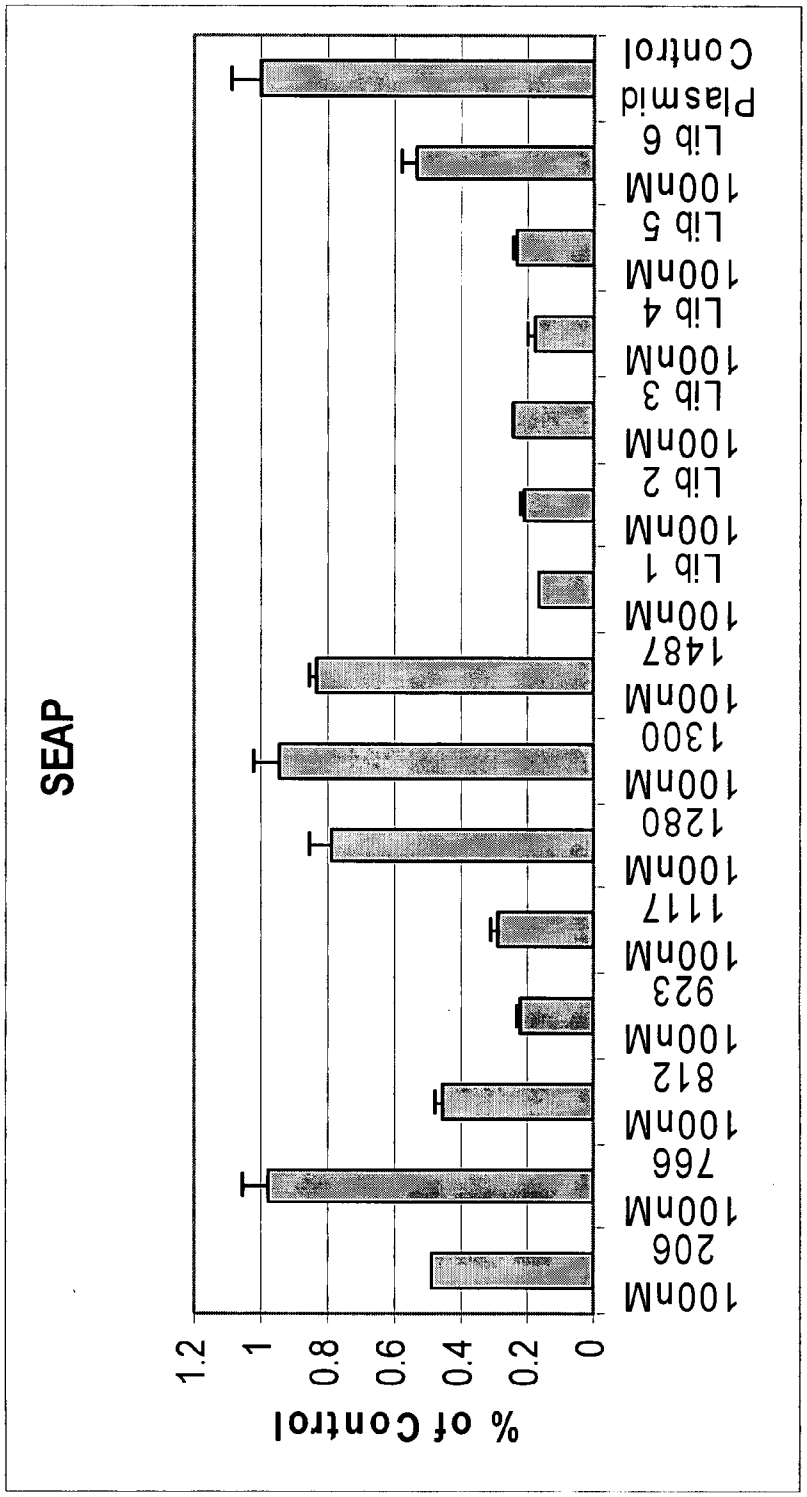

SEAP
% of Control
1.2
1
0.8
0.6
0.4
0.2
0
100nM
206
100nM
766
100nM
812
100nM
923
100nM
1117
100nM
1280
100nM
1300
100nM
1487
100nM
Lib 1
100nM
Lib 2
100nM
Lib 3
100nM
Lib 4
100nM
Lib 5
100nM
Lib 6
Plasmid
Control

Figure 21C

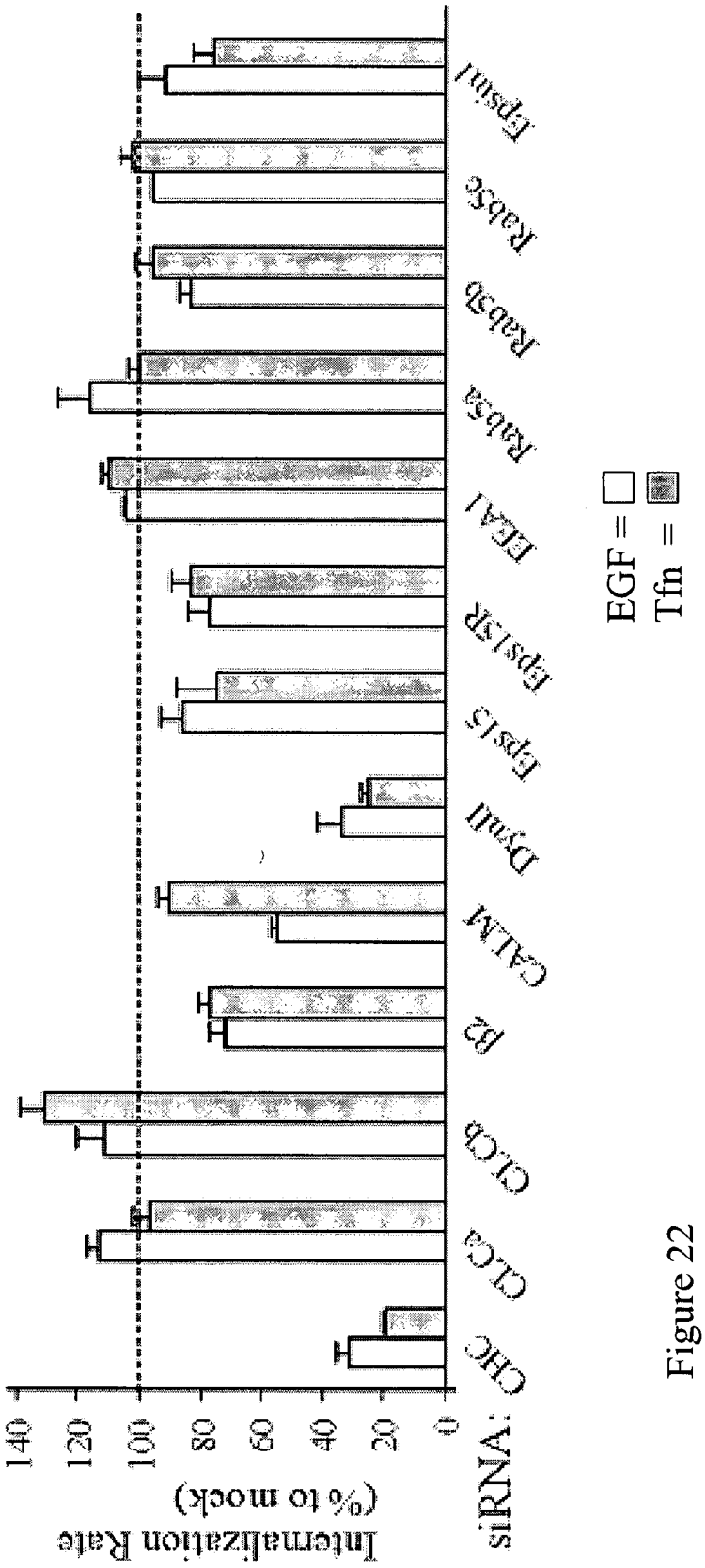

Internalization Rate
(% to mock)
140
120
100
80
60
40
20
0
siRNA:
CHC
CLCa
CLCb
B2
CALM
Dynll
Eps15
Eps15R
EEA1
Rab5a
Rab5b
Rab5c
Epsin1
EGF =
Tfn =

Figure 22

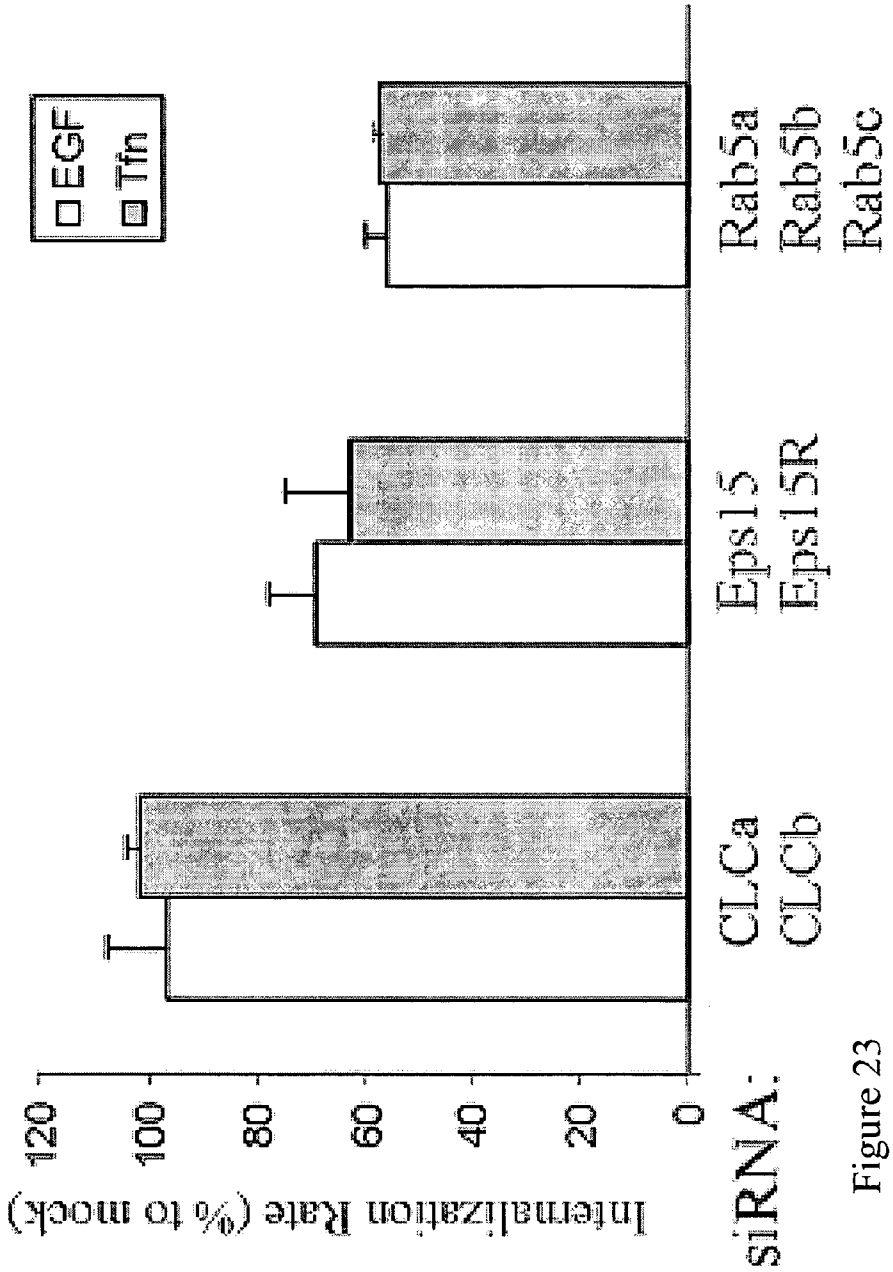

EGF
Tfn
Internalization Rate (% to mock)
120
100
80
60
40
20
0
siRNA:
CLCa
CLCb
Eps15
Eps15R
Rab5a
Rab5b
Rab5c

Figure 23

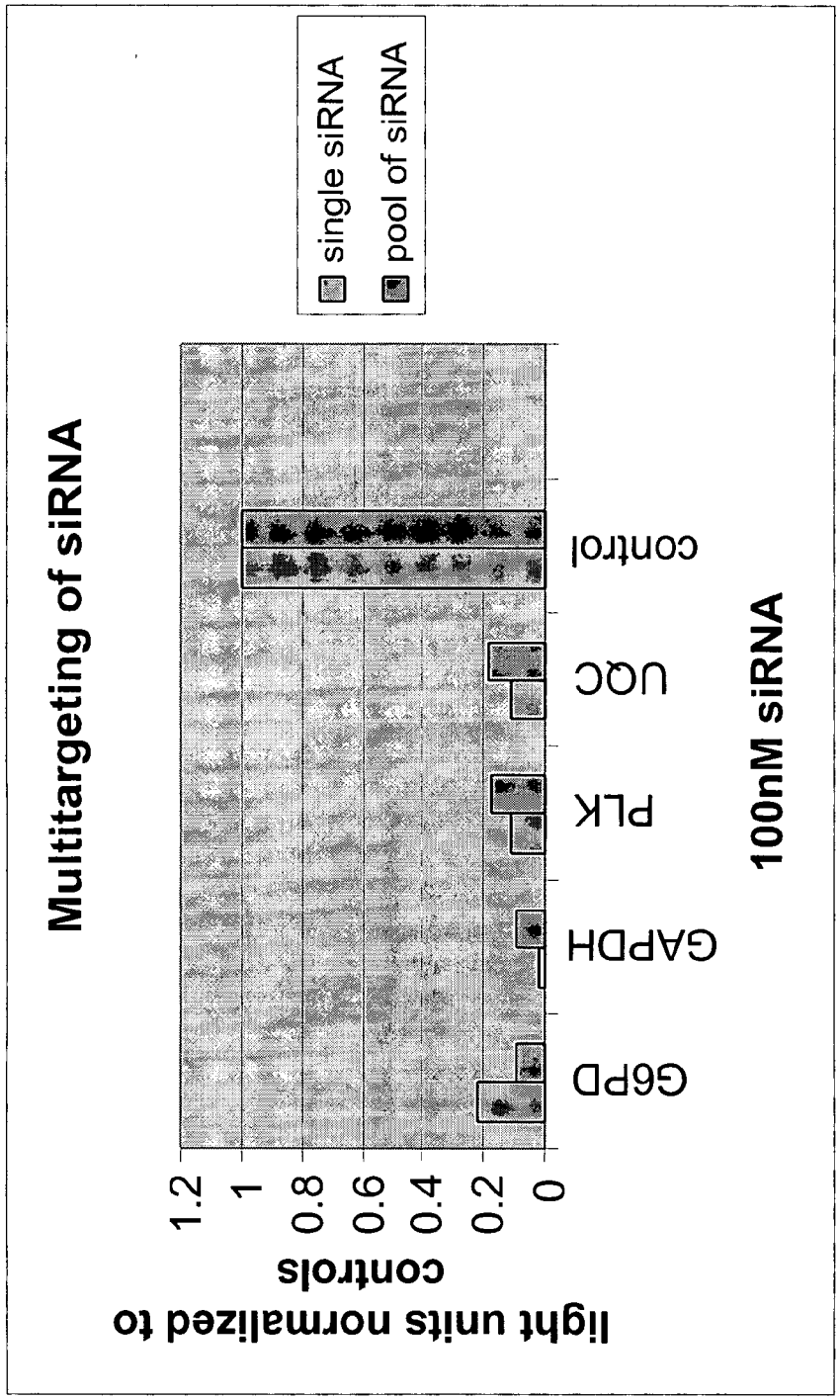
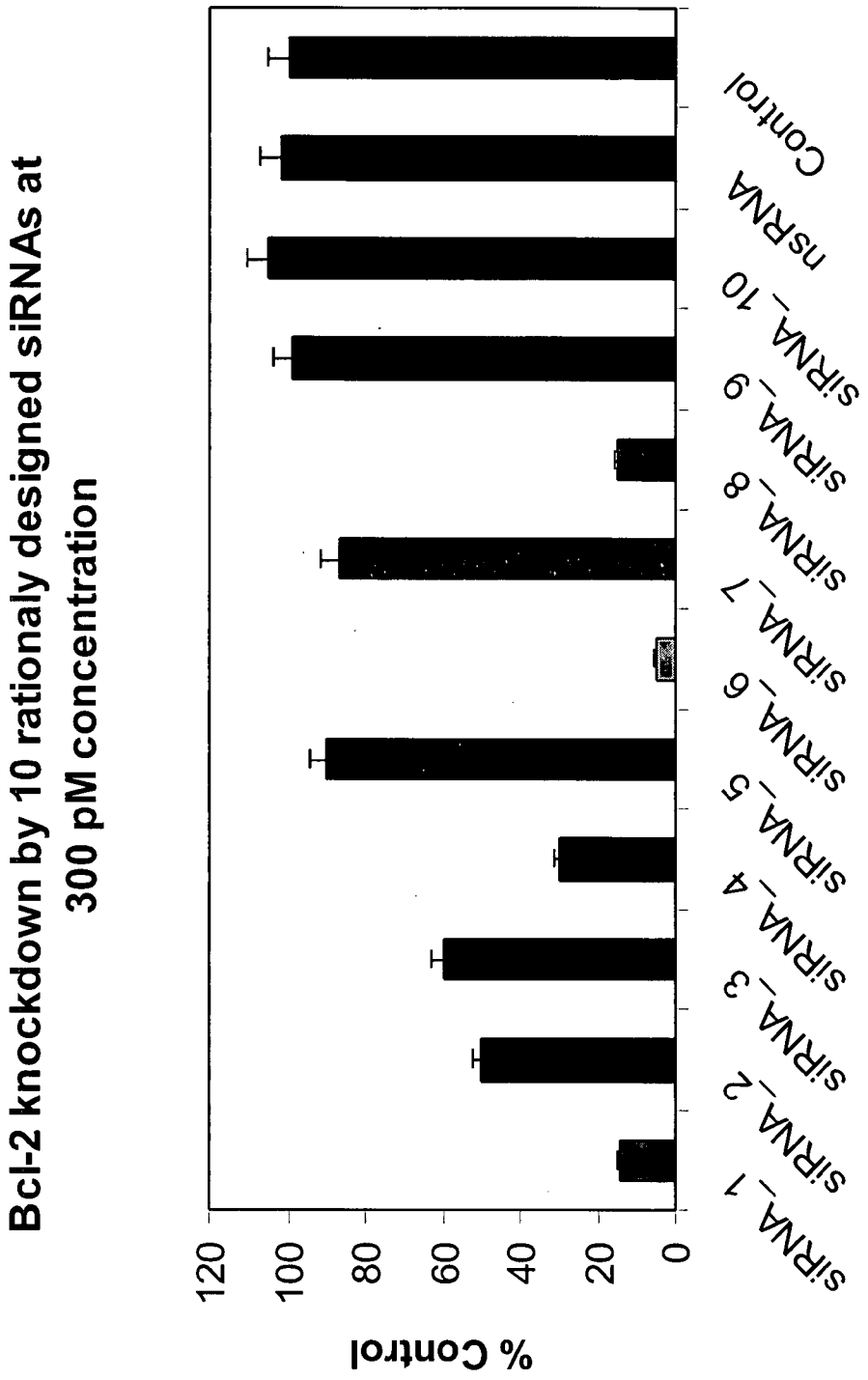

Multitargeting of siRNA
single siRNA
pool of siRNA
light units normalized to controls
1.2
1
0.8
0.6
0.4
0.2
0
G6PD
GAPDH
PLK
UQC
control
100nM siRNA

Figure 24

Figure 25


Bcl-2 knockdown by 10 rationaly designed siRNAs at 300 pM concentration
% Control
120
100
80
60
40
20
0
siRNA_1
siRNA_2
siRNA_3
siRNA_4
siRNA_5
siRNA_6
siRNA_7
siRNA_8
siRNA_9
siRNA_10
nsRNA
Control

SIRNA TARGETING TNFALPHA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of U.S. Ser. No. 10/714,333, filed Nov. 14, 2003, which claims the benefit of U.S. Provisional Application No. 60/426,137, filed Nov. 14, 2002, and also claims the benefit of U.S. Provisional Application No. 60/502,050, filed Sep. 10, 2003; this application is also a continuation-in-part of U.S. Ser. No. 10/940,892, filed Sep. 14, 2004, which is a continuation of PCT Application No. PCT/US04/14885, international filing date May 12, 2004. The disclosures of the priority applications, including the sequence listings and tables submitted in electronic form in lieu of paper, are incorporated by reference into the instant specification.

SEQUENCE LISTING

[0002] The sequence listing for this application has been submitted in accordance with 37 CFR §1.52(e) and 37 CFR §1.821 on CD-ROM in lieu of paper on a disk containing the sequence listing file entitled "DHARMA_2100-US73_CRF.txt" created Sep. 27, 2007, 92 kb. Applicants hereby incorporate by reference the sequence listing provided on CD-ROM in lieu of paper into the instant specification.

FIELD OF INVENTION

[0003] The present invention relates to RNA interference ("RNAi").

BACKGROUND OF THE INVENTION

[0004] Relatively recently, researchers observed that double stranded RNA ("dsRNA") could be used to inhibit protein expression. This ability to silence a gene has broad potential for treating human diseases, and many researchers and commercial entities are currently investing considerable resources in developing therapies based on this technology.

[0005] Double stranded RNA induced gene silencing can occur on at least three different levels: (i) transcription inactivation, which refers to RNA guided DNA or histone methylation; (ii) siRNA induced mRNA degradation; and (iii) mRNA induced transcriptional attenuation.

[0006] It is generally considered that the major mechanism of RNA induced silencing (RNA interference, or RNAi) in mammalian cells is mRNA degradation. Initial attempts to use RNAi in mammalian cells focused on the use of long strands of dsRNA. However, these attempts to induce RNAi met with limited success, due in part to the induction of the interferon response, which results in a general, as opposed to a target-specific, inhibition of protein synthesis. Thus, long dsRNA is not a viable option for RNAi in mammalian systems.

[0007] More recently it has been shown that when short (18-30 bp) RNA duplexes are introduced into mammalian cells in culture, sequence-specific inhibition of target mRNA can be realized without inducing an interferon response. Certain of these short dsRNAs, referred to as small inhibitory RNAs ("siRNAs"), can act catalytically at sub-molar concentrations to cleave greater than 95% of the target mRNA in the cell. A description of the mechanisms for siRNA activity, as well as some of its applications are described in Provost et al. (2002) Ribonuclease Activity and RNA Binding of Recombinant Human Dicer, *EMBO J.* 21(21): 5864-5874; Tabara et al. (2002) The dsRNA Binding Protein RDE-4 Interacts with RDE-1, DCR-1 and a DexH-box Helicase to Direct RNAi in *C. elegans, Cell* 109(7):861-71; Ketting et al. (2002) Dicer Functions in RNA Interference and in Synthesis of Small RNA Involved in Developmental Timing in *C. elegans*; Martinez et al., Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi, *Cell* 110(5):563; Hutvagner & Zamore (2002) A microRNA in a multiple-turnover RNAi enzyme complex, *Science* 297:2056.

[0008] From a mechanistic perspective, introduction of long double stranded RNA into plants and invertebrate cells is broken down into siRNA by a Type II endonuclease known as Dicer. Sharp, *RNA interference*—2001, Genes Dev. 2001, 15:485. Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs. Bernstein, Caudy, Hammond, & Hannon (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference, *Nature* 409:363. The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition. Nykanen, Haley, & Zamore (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway, *Cell* 107:309. Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleaves the target to induce silencing. Elbashir, Lendeckel, & Tuschl (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs, *Genes Dev.* 15:188, FIG. **1**.

[0009] The interference effect can be long lasting and may be detectable after many cell divisions. Moreover, RNAi exhibits sequence specificity. Kisielow, M. et al., (2002) Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA, *J. Biochem.* 363: 1-5. Thus, the RNAi machinery can specifically knock down one type of transcript, while not affecting closely related mRNA. These properties make siRNA a potentially valuable tool for inhibiting gene expression and studying gene function and drug target validation. Moreover, siRNAs are potentially useful as therapeutic agents against: (1) diseases that are caused by over-expression or misexpression of genes; and (2) diseases brought about by expression of genes that contain mutations.

[0010] Successful siRNA-dependent gene silencing depends on a number of factors. One of the most contentious issues in RNAi is the question of the necessity of siRNA design, i.e., considering the sequence of the siRNA used. Early work in *C. elegans* and plants circumvented the issue of design by introducing long dsRNA (see, for instance, Fire, A. et al. (1998) *Nature* 391:806-811). In this primitive organism, long dsRNA molecules are cleaved into siRNA by Dicer, thus generating a diverse population of duplexes that can potentially cover the entire transcript. While some fraction of these molecules are non-functional (i.e., induce little or no silencing) one or more have the potential to be highly functional, thereby silencing the gene of interest and alleviating the need for siRNA design. Unfortunately, due to the interferon response, this same approach is unavailable for mammalian systems. While this effect can be circum-

vented by bypassing the Dicer cleavage step and directly introducing siRNA, this tactic carries with it the risk that the chosen siRNA sequence may be non-functional or semi-functional.

[0011] A number of researches have expressed the view that siRNA design is not a crucial element of RNAi. On the other hand, others in the field have begun to explore the possibility that RNAi can be made more efficient by paying attention to the design of the siRNA. Unfortunately, none of the reported methods have provided a satisfactory scheme for reliably selecting siRNA with acceptable levels of functionality. Accordingly, there is a need to develop rational criteria by which to select siRNA with an acceptable level of functionality, and to identify siRNA that have this improved level of functionality, as well as to identify siRNAs that are hyperfunctional.

SUMMARY OF THE INVENTION

[0012] The present invention is directed to increasing the efficiency of RNAi, particularly in mammalian systems. Accordingly, the present invention provides kits, siRNAs and methods for increasing siRNA efficacy.

[0013] According to a first embodiment, the present invention provides a kit for gene silencing, wherein said kit is comprised of a pool of at least two siRNA duplexes, each of which is comprised of a sequence that is complementary to a portion of the sequence of one or more target messenger RNA, and each of which is selected using non-target specific criteria.

[0014] According to a second embodiment, the present invention provides a method for selecting an siRNA, said method comprising applying selection criteria to a set of potential siRNA that comprise 18-30 base pairs, wherein said selection criteria are non-target specific criteria, and said set comprises at least two siRNAs and each of said at least two siRNAs contains a sequence that is at least substantially complementary to a target gene; and determining the relative functionality of the at least two siRNAs.

[0015] According to a third embodiment, the present invention also provides a method for selecting an siRNA wherein said selection criteria are embodied in a formula comprising:

$$(-14)*G_{13}-13*A_1-12*U_7-11*U_2-10*A_{11}-10*U_4-10*C_3-10*C_5-10*C_6-9*A_{10}-9*U_9-9*C_{18}-8*G_{10}-7*U_1-7*U_{16}-7*C_{17}-7*C_{19}+7*U_{17}+8*A_2+8*A_4+8*A_5+8*C_4+9*G_8+10*A_7+10*U_{18}+11*A_{19}+11*C_9+15*G_1+18*A_3+19*U_{10}-Tm-3*(GC_{total})-6*(GC_{15\text{-}19})-30*X; \text{ or} \qquad \text{Formula VIII}$$

$$(-8)*A1+(-1)*A2+(12)*A3+(7)*A4+(18)*A5+(12)*A6+(19)*A7+(6)*A8+(-4)*A9+(-5)*A10+(-2)*A11+(-5)*A12+(17)*A13+(-3)*A14+(4)*A15+(2)*A16+(8)*A17+(11)*A18+(30)*A19+(-13)*U1+(-10)*U2+(2)*U3+(-2)*U4+(-5)*U5+(5)*U6+(-2)*U7+(-10)*U8+(-5)*U9+(15)*U10+(-1)*U11+(0)*U12+(10)*U13+(-9)*U14+(-13)*U15+(-10)*U16+(3)*U17+(9)*U18+(9)*U19+(7)*C1+(3)*C2+(-21)*C3+(5)*C4+(-9)*C5+(-20)*C6+(-18)*C7+(-5)*C8+(5)*C9+(1)*C10+(2)*C11+(-5)*C12+(-3)*C13+(-6)*C14+(-2)*C15+(-5)*C16+(-3)*C17+(-12)*C18+(-18)*C19+(14)*G1+(8)*G2+(7)*G3+(-10)*G4+(-4)*G5+(2)*G6+(1)*G7+(9)*G8+(5)*G9+(-11)*G10+(1)*G11+(9)*G12+(-24)*G13+(18)*G14+(11)*G15+(13)*G16+(-7)*G17+(-9)*G18+(-22)*G19+6*(\text{number of A+U in position 15-19})-3*(\text{number of G+C in whole siRNA}), \qquad \text{Formula X}$$

wherein position numbering begins at the 5'-most position of a sense strand, and

A_1=1 if A is the base at position 1 of the sense strand, otherwise its value: is 0;

A_2=1 if A is the base at position 2 of the sense strand, otherwise its value: is 0;

A_3=1 if A is the base at position 3 of the sense strand, otherwise its value: is 0;

A_4=1 if A is the base at position 4 of the sense strand, otherwise its value is 0;

A_5=1 if A is the base at position 5 of the sense strand, otherwise its value is 0;

A_6=1 if A is the base at position 6 of the sense strand, otherwise its value is 0;

A_7=1 if A is the base at position 7 of the sense strand, otherwise its value is 0;

A_{10}=1 if A is the base at position 10 of the sense strand, otherwise its value is 0;

A_{11}=1 if A is the base at position 11 of the sense strand, otherwise its value is 0;

A_{13}=1 if A is the base at position 13 of the sense strand, otherwise its value is 0;

A_{19}=1 if A is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

C_3=1 if C is the base at position 3 of the sense strand, otherwise its value is 0;

C_4=1 if C is the base at position 4 of the sense strand, otherwise its value is 0;

C_5=1 if C is the base at position 5 of the sense strand, otherwise its value is 0;

C_6=1 if C is the base at position 6 of the sense strand, otherwise its value is 0;

C_7=1 if C is the base at position 7 of the sense strand, otherwise its value: is 0;

C_9=1 if C is the base at position 9 of the sense strand, otherwise its value is 0;

C_{17}=1 if C is the base at position 17 of the sense strand, otherwise its value is 0;

C_{18}=1 if C is the base at position 18 of the sense strand, otherwise its value is 0;

C_{19}=1 if C is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

G_1=1 if G is the base at position 1 on the sense strand, otherwise its value is 0;

G_2=1 if G is the base at position 2 of the sense strand, otherwise its value is 0;

G_8=1 if G is the base at position 8 on the sense strand, otherwise its value is 0;

G_{10}=1 if G is the base at position 10 on the sense strand, otherwise its value is 0;

G_{13}=1 if G is the base at position 13 on the sense strand, otherwise its value is 0;

G_{19}=1 if G is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

U_1=1 if U is the base at position 1 on the sense strand, otherwise its value is 0;

U_2=1 if U is the base at position 2 on the sense strand, otherwise its value is 0;

U_3=1 if U is the base at position 3 on the sense strand, otherwise its value is 0;

U_4=1 if U is the base at position 4 on the sense strand, otherwise its value is 0;

U_7=1 if U is the base at position 7 on the sense strand, otherwise its value is 0;

U_9=1 if U is the base at position 9 on the sense strand, otherwise its value is 0;

U_{10}=1 if U is the base at position 10 on the sense strand, otherwise its value is 0;

U_{15}=1 if U is the base at position 15 on the sense strand, otherwise its value is 0;

U_{16}=1 if U is the base at position 16 on the sense strand, otherwise its value is 0;

U_{17}=1 if U is the base at position 17 on the sense strand, otherwise its value is 0;

U_{18}=1 if U is the base at position 18 on the sense strand, otherwise its value is 0.

$GC_{15\text{-}19}$=the number of G and C bases within positions 15-19 of the sense strand, or within positions 15-18 if the sense strand is only 18 base pairs in length;

GC_{total}=the number of G and C bases in the sense strand;

Tm=100 if the si RNA oligo has the internal repeat longer then 4 base pairs, otherwise its value is 0; and

X=the number of times that the same nucleotide repeats four or more times in a row.

[0016] According to a fourth embodiment, the invention provides a method for developing an algorithm for selecting siRNA, said method comprising: (a) selecting a set of siRNA; (b) measuring gene silencing ability of each siRNA from said set; (c) determining relative functionality of each siRNA; (d) determining improved functionality by the presence or absence of at least one variable selected from the group consisting of the presence or absence of a particular nucleotide at a particular position, the total number of As and Us in positions 15-19, the number of times that the same nucleotide repeats within a given sequence, and the total number of Gs and Cs; and (e) developing an algorithm using the information of step (d).

[0017] According to a fifth embodiment, the present invention provides a kit, wherein said kit is comprised of at least two siRNAs, wherein said at least two siRNAs comprise a first optimized siRNA and a second optimized siRNA, wherein said first optimized siRNA and said second optimized siRNA are optimized according a formula comprising Formula X.

[0018] The present invention also provides a method for identifying a hyperfunctional siRNA, comprising applying selection criteria to a set of potential siRNA that comprise 18-30 base pairs, wherein said selection criteria are non-target specific criteria, and said set comprises at least two siRNAs and each of said at least two siRNAs contains a sequence that is at least substantially complementary to a target gene; determining the relative functionality of the at least two siRNAs and assigning each of the at least two siRNAs a functionality score; and selecting siRNAs from the at least two siRNAs that have a functionality score that reflects greater than 80 percent silencing at a concentration in the picomolar range, wherein said greater than 80 percent silencing endures for greater than 120 hours.

[0019] According to a sixth embodiment, the present invention provides a hyperfunctional siRNA that is capable of silencing Bcl2.

[0020] According to a seventh embodiment, the present invention provides a method for developing an siRNA algorithm for selecting functional and hyperfunctional siRNAs for a given sequence. The method comprises:

[0021] (a) selecting a set of siRNAs;

[0022] (b) measuring the gene silencing ability of each siRNA from said set;

[0023] (c) determining the relative functionality of each siRNA;

[0024] (d) determining the amount of improved functionality by the presence or absence of at least one variable selected from the group consisting of the total GC content, melting temperature of the siRNA, GC content at positions 15-19, the presence or absence of a particular nucleotide at a particular position, relative thermodynamic stability at particular positions in a duplex, and the number of times that the same nucleotide repeats within a given sequence; and

[0025] (e) developing an algorithm using the information of step (d).

[0026] According to this embodiment, preferably the set of siRNAs comprises at least 90 siRNAs from at least one gene, more preferably at least 180 siRNAs from at least two different genes, and most preferably at least 270 and 360 siRNAs from at least three and four different genes, respectively. Additionally, in step (d) the determination is made with preferably at least two, more preferably at least three, even more preferably at least four, and most preferably all of the variables. The resulting algorithm is not target sequence specific.

[0027] In another embodiment, the present invention provides rationally designed siRNAs identified using the formulas above.

[0028] In yet another embodiment, the present invention is directed to hyperfunctional siRNA.

[0029] The ability to use the above algorithms, which are not sequence or species specific, allows for the cost-effective selection of optimized siRNAs for specific target sequences. Accordingly, there will be both greater efficiency and reliability in the use of siRNA technologies.

[0030] In various embodiments, siRNAs that target nucleotide sequences for transient receptor potential cation channel, subfamily V, member 1 (TNFα) are provided. In various embodiments, the siRNAs are rationally designed. In various embodiments, the siRNAs are functional or hyperfunctional.

[0031] In various embodiments, an siRNA that targets TNFα is provided, wherein the siRNA is selected from the group consisting of various siRNA sequences targeting TNFα that are disclosed herein. In various embodiments, the siRNA sequence is selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513.

[0032] In various embodiments, siRNA comprising a sense region and an antisense region are provided, said sense region and said antisense region together form a duplex region comprising 18-30 base pairs, and said sense region comprises a sequence that is at least 90% similar to a sequence selected from the group consisting of siRNA sequences targeting TNFα that are disclosed herein. In various embodiments, the siRNA sequence is selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513.

[0033] In various embodiments, an siRNA comprising a sense region and an antisense region is provided, said sense region and said antisense region together form a duplex region comprising 18-30 base pairs, and said sense region comprises a sequence that is identical to a contiguous stretch of at least 18 bases of a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513. In various embodiments, the duplex region is 19-30 base pairs, and the sense region comprises a sequence that is identical to a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513.

[0034] In various embodiments, a pool of at least two siRNAs is provided, wherein said pool comprises a first siRNA and a second siRNA, said first siRNA comprising a duplex region of length 18-30 base pairs that has a first sense region that is at least 90% similar to 18 bases of a first sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513, and said second siRNA comprises a duplex region of length 18-30 base pairs that has a second sense region that is at least 90% similar to 18 bases of a second sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513, wherein said first sense region and said second sense region are not identical.

[0035] In various embodiments, the first sense region comprises a sequence that is identical to at least 18 bases of a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513, and said second sense region comprises a sequence that is identical to at least 18 bases of a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513. In various embodiments, the duplex of said first siRNA is 19-30 base pairs, and said first sense region comprises a sequence that is at least 90% similar to a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513, and said duplex of said second siRNA is 19-30 base pairs and comprises a sequence that is at least 90% similar to a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513.

[0036] In various embodiments, the duplex of said first siRNA is 19-30 base pairs and said first sense region comprises a sequence that is identical to at least 18 bases of a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513, and said duplex of said second siRNA is 19-30 base pairs and said second region comprises a sequence that is identical to a sequence selected from the group consisting of SEQ ID NO. 438 to SEQ ID NO. 513.

[0037] For a better understanding of the present invention together with other and further advantages and embodiments, reference is made to the following description taken in conjunction with the examples, the scope of which is set forth in the appended claims.

BRIEF DESCRIPTION OF THE FIGURES

[0038] FIG. **1** shows a model for siRNA-RISC interactions. RISC has the ability to interact with either end of the siRNA or miRNA molecule. Following binding, the duplex is unwound, and the relevant target is identified, cleaved, and released.

[0039] FIG. **2** is a representation of the functionality of two hundred and seventy siRNA duplexes that were generated to target human cyclophilin, human diazepam-binding inhibitor (DB), and firefly luciferase.

[0040] FIG. **3***a* is a representation of the silencing effect of 30 siRNAs in three different cells lines, HEK293, DU145, and Hela. FIG. **3***b* shows the frequency of different functional groups (>95% silencing (black), >80% silencing (gray), >50% silencing (dark gray), and <50% silencing (white)) based on GC content. In cases where a given bar is absent from a particular GC percentage, no siRNA were identified for that particular group. FIG. **3***c* shows the frequency of different functional groups based on melting temperature (Tm).

[0041] FIG. **4** is a representation of a statistical analysis that revealed correlations between silencing and five sequence-related properties of siRNA: (A) an A at position 19 of the sense strand, (B) an A at position 3 of the sense strand, (C) a U at position 10 of the sense strand, (D) a base other than G at position 13 of the sense strand, and (E) a base other than C at position 19 of the sense strand. All variables were correlated with siRNA silencing of firefly luciferase and human cyclophilin. siRNAs satisfying the criterion are grouped on the left (Selected) while those that do not, are grouped on the right (Eliminated). Y-axis is "% Silencing of Control." Each position on the X-axis represents a unique siRNA.

[0042] FIGS. **5**A and **5**B are representations of firefly luciferase and cyclophilin siRNA panels sorted according to functionality and predicted values using Formula VIII. The siRNA found within the circle represent those that have

Formula VIII values (SMARTSCORES™, or siRNA rank) above zero. siRNA outside the indicated area have calculated Formula VIII values that are below zero. Y-axis is "Expression (% Control)." Each position on the X-axis represents a unique siRNA.

[0043] FIG. **6**A is a representation of the average internal stability profile (AISP) derived from 270 siRNAs taken from three separate genes (cyclophilin B, DBI and firefly luciferase). Graphs represent AISP values of highly functional, functional, and non-functional siRNA. FIG. **6**B is a comparison between the AISP of naturally derived GFP siRNA (filled squares) and the AISP of siRNA from cyclophilin B, DBI, and luciferase having >90% silencing properties (no fill) for the antisense strand. "DG" is the symbol for ΔG, free energy.

[0044] FIG. **7** is a histogram showing the differences in duplex functionality upon introduction of base pair mismatches. The X-axis shows the mismatch introduced in the siRNA and the position it is introduced (e.g., 8C>A reveals that position 8 (which normally has a C) has been changed to an A). The Y-axis is "% Silencing (Normalized to Control)." The samples on the X-axis represent siRNAs at 100 nM and are, reading from left to right: 1A to C, 1A to G, 1A to U; 2A to C, 2A to G, 2A to U; 3A to C, 3A to G, 3A to U; 4G to A, 4G to C; 4G to U; 5U to A, 5U to C, 5U to G; 6U to A, 6U to C, 6U to G; 7G to A, 7G to C, 7G to U; 8C to A, 8C to G, 8C to U; 9G to A, 9G to C, 9G to U; 10C to A, 10C to G, 10C to U; 11G to A, 11G to C, 11G to U; 12G to A, 12G to C, 12G to U; 13A to C, 13A to G, 13A to U; 14G to A, 14G to C, 14G to U; 15G to A, 15G to C, 15G to U; 16A to C, 16A to G, 16A to U; 17G to A, 17G to C, 17G to U; 18U to A, 18U to C, 18U to G; 19U to A, 19U to C, 19U to G; 20 wt; Control.

[0045] FIG. **8** is histogram that shows the effects of 5' sense and antisense strand modification with 2'-O-methylation on functionality.

[0046] FIG. **9** shows a graph of SMARTSCORES™, or siRNA rank, versus RNAi silencing values for more than 360 siRNA directed against 30 different genes. SiRNA to the right of the vertical bar represent those siRNA that have desirable SMARTSCORES™, or siRNA rank.

[0047] FIGS. **10**A-E compare the RNAi of five different genes (SEAP, DBI, PLK, Firefly Luciferase, and Renilla Luciferase) by varying numbers of randomly selected siRNA and four rationally designed (SMART-selected) siRNA chosen using the algorithm described in Formula VIII. In addition, RNAi induced by a pool of the four SMART-selected siRNA is reported at two different concentrations (100 and 400 nM). **10**F is a comparison between a pool of randomly selected EGFR siRNA (Pool 1) and a pool of SMART-selected EGFR siRNA (Pool 2). Pool 1, S1-S4 and Pool 2 S1-S4 represent the individual members that made up each respective pool. Note that numbers for random siRNAs represent the position of the 5' end of the sense strand of the duplex. The Y-axis represents the % expression of the control(s). The X-axis is the percent expression of the control.

[0048] FIG. **11** shows the Western blot results from cells treated with siRNA directed against twelve different genes involved in the clathrin-dependent endocytosis pathway (CHC, DynII, CALM, CLCa, CLCb, Eps15, Eps15R, Rab5a, Rab5b, Rab5c, β2 subunit of AP-2 and EEA.1). siRNA were selected using Formula VIII. "Pool" represents a mixture of duplexes 1-4. Total concentration of each siRNA in the pool is 25 nM. Total concentration=4×25=100 nM.

[0049] FIG. **12** is a representation of the gene silencing capabilities of rationally-selected siRNA directed against ten different genes (human and mouse cyclophilin, C-myc, human lamin A/C, QB (ubiquinol-cytochrome c reductase core protein 1), MEK1 and MEK2, ATE1 (arginyl-tRNA protein transferase), GAPDH, and Eg5). The Y-axis is the percent expression of the control. Numbers 1, 2, 3 and 4 represent individual rationally selected siRNA. "Pool" represents a mixture of the four individual siRNA.

[0050] FIG. **13** is the sequence of the top ten Bcl2 siRNAs as determined by Formula VIII. Sequences are listed 5' to 3'.

[0051] FIG. **14** is the knockdown by the top ten Bcl2 siRNAs at 100 nM concentrations. The Y-axis represents the amount of expression relative to the non-specific (ns) and transfection mixture control.

[0052] FIG. **15** represents a functional walk where siRNA beginning on every other base pair of a region of the luciferase gene are tested for the ability to silence the luciferase gene. The Y-axis represents the percent expression relative to a control. The X-axis represents the position of each individual siRNA. Reading from left to right across the X-axis, the position designations are 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, and Plasmid.

[0053] FIGS. **16**A and **16**B are histograms demonstrating the inhibition of target gene expression by pools of 2 (**16**A) and 3 (**16**B) siRNA duplexes taken from the walk described in FIG. **15**. The Y-axis in each represents the percent expression relative to control. The X-axis in each represents the position of the first siRNA in paired pools, or trios of siRNAs. For instance, the first paired pool contains siRNAs 1 and 3. The second paired pool contains siRNAs 3 and 5. Pool 3 (of paired pools) contains siRNAs 5 and 7, and so on. For each of **16**A and **16**B, the X-axis from left to right reads 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, and Plasmid.

[0054] FIGS. **17**A and **17**B are histograms demonstrating the inhibition of target gene expression by pools of 4 (**17**A) and 5 (**17**B) siRNA duplexes. The Y-axis in each represents the percent expression relative to control. The X-axis in each represents the position of the first siRNA in each pool. For each of **17**A and **17**B, the X-axis from left to right reads 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, and Plasmid.

[0055] FIGS. **18**A and **18**B are histograms demonstrating the inhibition of target gene expression by siRNAs that are ten (**18**A) and twenty (**18**B) base pairs base pairs apart. The Y-axis represents the percent expression relative to a control. The X-axis represents the position of the first siRNA in each pool. For each of **18**A and **18**B, the X-axis from left to right reads 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, and Plasmid.

[0056] FIG. **19** shows that pools of siRNAs (dark gray bar) work as well (or better) than the best siRNA in the pool (light gray bar). The Y-axis represents the percent expression relative to a control. The X-axis represents the position of the first siRNA in each pool. The X-axis from left to right reads 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, and Plasmid.

[0057] FIG. **20** shows that the combination of several semifunctional siRNAs (dark gray) result in a significant improvement of gene expression inhibition over individual (semi-functional; light gray) siRNA. The Y-axis represents the percent expression relative to a control.

[0058] FIGS. **21**A, **21**B and **21**C show both pools (Library, Lib) and individual siRNAs in inhibition of gene expression of Beta-Galactosidase, Renilla Luciferase and SEAP (alkaline phosphatase). Numbers on the X-axis indicate the position of the 5'-most nucleotide of the sense strand of the duplex. The Y-axis represents the percent expression of each gene relative to a control. Libraries contain 19 nucleotide long siRNAs (not including overhangs) that begin at the following nucleotides: SEAP: Lib 1: 206, 766, 812, 923, Lib 2: 1117, 1280, 1300, 1487, Lib 3: 206, 766, 812, 923, 1117, 1280, 1300, 1487, Lib 4: 206, 812, 1117, 1300, Lib 5: 766, 923, 1280, 1487, Lib 6: 206, 1487; Bgal: Lib 1: 979, 1339, 2029, 2590, Lib 2: 1087, 1783, 2399, 3257, Lib 3: 979, 1783, 2590, 3257, Lib 4: 979, 1087, 1339, 1783, 2029, 2399, 2590, 3257, Lib 5: 979, 1087, 1339, 1783, Lib 6: 2029, 2399, 2590, 3257; Renilla: Lib 1: 174, 300, 432, 568, Lib 2: 592, 633, 729, 867, Lib 3: 174, 300, 432, 568, 592, 633, 729, 867, Lib 4: 174, 432, 592, 729, Lib 5: 300, 568, 633, 867, Lib 6: 592, 568.

[0059] FIG. **22** shows the results of an EGFR and TfnR internalization assay when single gene knockdowns are performed. The Y-axis represents percent internalization relative to control.

[0060] FIG. **23** shows the results of an EGFR and TfnR internalization assay when multiple genes are knocked down (e.g., Rab5a, b, c). The Y-axis represents the percent internalization relative to control.

[0061] FIG. **24** shows the simultaneous knockdown of four different genes. siRNAs directed against G6PD, GAPDH, PLK, and UQC were simultaneously introduced into cells. Twenty-four hours later, cultures were harvested and assayed for mRNA target levels for each of the four genes. A comparison is made between cells transfected with individual siRNAs vs. a pool of siRNAs directed against all four genes.

[0062] FIG. **25** shows the functionality of ten siRNAs at 0.3 nM concentrations.

DETAILED DESCRIPTION

Definitions

[0063] Unless stated otherwise, the following terms and phrases have the meanings provided below:

Complementary

[0064] The term "complementary" refers to the ability of polynucleotides to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands. Complementary polynucleotide strands can base pair in the Watson-Crick manner (e.g., A to T, A to U, C to G), or in any other manner that allows for the formation of duplexes. As persons skilled in the art are aware, when using RNA as opposed to DNA, uracil rather than thymine is the base that is considered to be complementary to adenosine. However, when a U is denoted in the context of the present invention, the ability to substitute a T is implied, unless otherwise stated.

[0065] Perfect complementarity or 100% complementarity refers to the situation in which each nucleotide unit of one polynucleotide strand can hydrogen bond with a nucleotide unit of a second polynucleotide strand. Less than perfect complementarity refers to the situation in which some, but not all, nucleotide units of two strands can hydrogen bond with each other. For example, for two 20-mers, if only two base pairs on each strand can hydrogen bond with each other, the polynucleotide strands exhibit 10% complementarity. In the same example, if 18 base pairs on each strand can hydrogen bond with each other, the polynucleotide strands exhibit 90% complementarity.

Deoxynucleotide

[0066] The term "deoxynucleotide" refers to a nucleotide or polynucleotide lacking a hydroxyl group (OH group) at the 2' and/or 3' position of a sugar moiety. Instead, it has a hydrogen bonded to the 2' and/or 3' carbon. Within an RNA molecule that comprises one or more deoxynucleotides, "deoxynucleotide" refers to the lack of an OH group at the 2' position of the sugar moiety, having instead a hydrogen bonded directly to the 2' carbon.

Deoxyribonucleotide

[0067] The terms "deoxyribonucleotide" and "DNA" refer to a nucleotide or polynucleotide comprising at least one sugar moiety that has an H, rather than an OH, at its 2' and/or 3'position.

Duplex Region

[0068] The phrase "duplex region" refers to the region in two complementary or substantially complementary polynucleotides that form base pairs with one another, either by Watson-Crick base pairing or any other manner that allows for a stabilized duplex between polynucleotide strands that are complementary or substantially complementary. For example, a polynucleotide strand having 21 nucleotide units can base pair with another polynucleotide of 21 nucleotide units, yet only 19 bases on each strand are complementary or substantially complementary, such that the "duplex region" has 19 base pairs. The remaining bases may, for example, exist as 5' and 3' overhangs. Further, within the duplex region, 100% complementarity is not required; substantial complementarity is allowable within a duplex region. Substantial complementarity refers to 79% or greater complementarity. For example, a mismatch in a duplex region consisting of 19 base pairs results in 94.7% complementarity, rendering the duplex region substantially complementary.

Filters

[0069] The term "filter" refers to one or more procedures that are performed on sequences that are identified by the algorithm. In some instances, filtering includes in silico

procedures where sequences identified by the algorithm can be screened to identify duplexes carrying desirable or undesirable motifs. Sequences carrying such motifs can be selected for, or selected against, to obtain a final set with the preferred properties. In other instances, filtering includes wet lab experiments. For instance, sequences identified by one or more versions of the algorithm can be screened using any one of a number of procedures to identify duplexes that have hyperfunctional traits (e.g., they exhibit a high degree of silencing at subnanomolar concentrations and/or exhibit high degrees of silencing longevity).

Gene Silencing

[0070] The phrase "gene silencing" refers to a process by which the expression of a specific gene product is lessened or attenuated. Gene silencing can take place by a variety of pathways. Unless specified otherwise, as used herein, gene silencing refers to decreases in gene product expression that results from RNA interference (RNAi), a defined, though partially characterized pathway whereby small inhibitory RNA (siRNA) act in concert with host proteins (e.g., the RNA induced silencing complex, RISC) to degrade messenger RNA (mRNA) in a sequence-dependent fashion. The level of gene silencing can be measured by a variety of means, including, but not limited to, measurement of transcript levels by Northern Blot Analysis, B-DNA techniques, transcription-sensitive reporter constructs, expression profiling (e.g., DNA chips), and related technologies. Alternatively, the level of silencing can be measured by assessing the level of the protein encoded by a specific gene. This can be accomplished by performing a number of studies including Western Analysis, measuring the levels of expression of a reporter protein that has e.g., fluorescent properties (e.g., GFP) or enzymatic activity (e.g., alkaline phosphatases), or several other procedures.

miRNA

[0071] The term "miRNA" refers to microRNA.

Nucleotide

[0072] The term "nucleotide" refers to a ribonucleotide or a deoxyribonucleotide or modified form thereof, as well as an analog thereof. Nucleotides include species that comprise purines, e.g., adenine, hypoxanthine, guanine, and their derivatives and analogs, as well as pyrimidines, e.g., cytosine, uracil, thymine, and their derivatives and analogs.

[0073] Nucleotide analogs include nucleotides having modifications in the chemical structure of the base, sugar and/or phosphate, including, but not limited to, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, and substitution of 5-bromo-uracil; and 2'-position sugar modifications, including but not limited to, sugar-modified ribonucleotides in which the 2'-OH is replaced by a group such as an H, OR, R, halo, SH, SR, NH_2, NHR, NR_2, or CN, wherein R is an alkyl moiety. Nucleotide analogs are also meant to include nucleotides with bases such as inosine, queuosine, xanthine, sugars such as 2'-methyl ribose, non-natural phosphodiester linkages such as methylphosphonates, phosphorothioates and peptides.

[0074] Modified bases refer to nucleotide bases such as, for example, adenine, guanine, cytosine, thymine, uracil, xanthine, inosine, and queuosine that have been modified by the replacement or addition of one or more atoms or groups. Some examples of types of modifications that can comprise nucleotides that are modified with respect to the base moieties include but are not limited to, alkylated, halogenated, thiolated, aminated, amidated, or acetylated bases, individually or in combination. More specific examples include, for example, 5-propynyluridine, 5-propynylcytidine, 6-methyladenine, 6-methylguanine, N,N,-dimethyladenine, 2-propyladenine, 2-propylguanine, 2-aminoadenine, 1-methylinosine, 3-methyluridine, 5-methylcytidine, 5-methyluridine and other nucleotides having a modification at the 5 position, 5-(2-amino)propyl uridine, 5-halocytidine, 5-halouridine, 4-acetylcytidine, 1-methyladenosine, 2-methyladenosine, 3-methylcytidine, 6-methyluridine, 2-methylguanosine, 7-methylguanosine, 2,2-dimethylguanosine, 5-methylaminoethyluridine, 5-methyloxyuridine, deazanucleotides such as 7-deaza-adenosine, 6-azouridine, 6-azocytidine, 6-azothymidine, 5-methyl-2-thiouridine, other thio bases such as 2-thiouridine and 4-thiouridine and 2-thiocytidine, dihydrouridine, pseudouridine, queuosine, archaeosine, naphthyl and substituted naphthyl groups, any O- and N-alkylated purines and pyrimidines such as N6-methyladenosine, 5-methylcarbonylmethyluridine, uridine 5-oxyacetic acid, pyridine-4-one, pyridine-2-one, phenyl and modified phenyl groups such as aminophenol or 2,4,6-trimethoxy benzene, modified cytosines that act as G-clamp nucleotides, 8-substituted adenines and guanines, 5-substituted uracils and thymines, azapyrimidines, carboxyhydroxyalkyl nucleotides, carboxyalkylaminoalkyl nucleotides, and alkylcarbonylalkylated nucleotides. Modified nucleotides also include those nucleotides that are modified with respect to the sugar moiety, as well as nucleotides having sugars or analogs thereof that are not ribosyl. For example, the sugar moieties may be, or be based on, mannoses, arabinoses, glucopyranoses, galactopyranoses, 4'-thioribose, and other sugars, heterocycles, or carbocycles.

[0075] The term nucleotide is also meant to include what are known in the art as universal bases. By way of example, universal bases include but are not limited to 3-nitropyrrole, 5-nitroindole, or nebularine. The term "nucleotide" is also meant to include the N3' to P5' phosphoramidate, resulting from the substitution of a ribosyl 3' oxygen with an amine group.

[0076] Further, the term nucleotide also includes those species that have a detectable label, such as for example a radioactive or fluorescent moiety, or mass label attached to the nucleotide.

Off-Target Silencing and Off-Target Interference

[0077] The phrases "off-target silencing" and "off-target interference" are defined as degradation of mRNA other than the intended target mRNA due to overlapping and/or partial homology with secondary mRNA messages.

Polynucleotide

[0078] The term "polynucleotide" refers to polymers of nucleotides, and includes but is not limited to DNA, RNA, DNA/RNA hybrids including polynucleotide chains of regularly and/or irregularly alternating deoxyribosyl moieties and ribosyl moieties (i.e., wherein alternate nucleotide units have an —OH, then and —H, then an —OH, then an —H, and so on at the 2' position of a sugar moiety), and modifications of these kinds of polynucleotides, wherein the

attachment of various entities or moieties to the nucleotide units at any position are included.

Polyribonucleotide

[0079] The term "polyribonucleotide" refers to a polynucleotide comprising two or more modified or unmodified ribonucleotides and/or their analogs. The term "polyribonucleotide" is used interchangeably with the term "oligoribonucleotide."

Ribonucleotide and Ribonucleic Acid

[0080] The term "ribonucleotide" and the phrase "ribonucleic acid" (RNA), refer to a modified or unmodified nucleotide or polynucleotide comprising at least one ribonucleotide unit. A ribonucleotide unit comprises an hydroxyl group attached to the 2' position of a ribosyl moiety that has a nitrogenous base attached in N-glycosidic linkage at the 1' position of a ribosyl moiety, and a moiety that either allows for linkage to another nucleotide or precludes linkage.

siRNA

[0081] The term "siRNA" refers to small inhibitory RNA duplexes that induce the RNA interference (RNAi) pathway. These molecules can vary in length (generally 18-30 base pairs) and contain varying degrees of complementarity to their target mRNA in the antisense strand. Some, but not all, siRNA have unpaired overhanging bases on the 5' or 3' end of the sense strand and/or the antisense strand. The term "siRNA" includes duplexes of two separate strands, as well as single strands that can form hairpin structures comprising a duplex region.

[0082] siRNA may be divided into five (5) groups (non-functional, semi-functional, functional, highly functional, and hyper-functional) based on the level or degree of silencing that they induce in cultured cell lines. As used herein, these definitions are based on a set of conditions where the siRNA is transfected into said cell line at a concentration of 100 nM and the level of silencing is tested at a time of roughly 24 hours after transfection, and not exceeding 72 hours after transfection. In this context, "non-functional siRNA" are defined as those siRNA that induce less than 50% (<50%) target silencing. "Semi-functional siRNA" induce 50-79% target silencing. "Functional siRNA" are molecules that induce 80-95% gene silencing. "Highly-functional siRNA" are molecules that induce greater than 95% gene silencing. "Hyperfunctional siRNA" are a special class of molecules. For purposes of this document, hyper-functional siRNA are defined as those molecules that: (1) induce greater than 95% silencing of a specific target when they are transfected at subnanomolar concentrations (i.e., less than one nanomolar); and/or (2) induce functional (or better) levels of silencing for greater than 96 hours. These relative functionalities (though not intended to be absolutes) may be used to compare siRNAs to a particular target for applications such as functional genomics, target identification and therapeutics.

SMARTSCORE™, or siRNA Rank

[0083] The term "SMARTSCORE™", or "siRNA rank" refers to a number determined by applying any of the formulas to a given siRNA sequence. The term "SMART-selected" or "rationally selected" or "rational selection" refers to siRNA that have been selected on the basis of their SMARTSCORES™, or siRNA ranking.

Substantially Similar

[0084] The phrase "substantially similar" refers to a similarity of at least 90% with respect to the identity of the bases of the sequence.

Target

[0085] The term "target" is used in a variety of different forms throughout this document and is defined by the context in which it is used. "Target mRNA" refers to a messenger RNA to which a given siRNA can be directed against. "Target sequence" and "target site" refer to a sequence within the mRNA to which the sense strand of an siRNA shows varying degrees of homology and the anti-sense strand exhibits varying degrees of complementarity. The phrase "siRNA target" can refer to the gene, mRNA, or protein against which an siRNA is directed. Similarly, "target silencing" can refer to the state of a gene, or the corresponding mRNA or protein.

Transfection

[0086] The term "transfection" refers to a process by which agents are introduced into a cell. The list of agents that can be transfected is large and includes, but is not limited to, siRNA, sense and/or anti-sense sequences, DNA encoding one or more genes and organized into an expression plasmid, proteins, protein fragments, and more. There are multiple methods for transfecting agents into a cell including, but not limited to, electroporation, calcium phosphate-based transfections, DEAE-dextran-based transfections, lipid-based transfections, molecular conjugate-based transfections (e.g., polylysine-DNA conjugates), microinjection and others.

[0087] The present invention is directed to improving the efficiency of gene silencing by siRNA. Through the inclusion of multiple siRNA sequences that are targeted to a particular gene and/or selecting an siRNA sequence based on certain defined criteria, improved efficiency may be achieved.

[0088] The present invention will now be described in connection with preferred embodiments. These embodiments are presented in order to aid in an understanding of the present invention and are not intended, and should not be construed, to limit the invention in any way. All alternatives, modifications and equivalents that may become apparent to those of ordinary skill upon reading this disclosure are included within the spirit and scope of the present invention.

[0089] Furthermore, this disclosure is not a primer on RNA interference. Basic concepts known to persons skilled in the art have not been set forth in detail.

[0090] The present invention is directed to increasing the efficiency of RNAi, particularly in mammalian systems. Accordingly, the present invention provides kits, siRNAs and methods for increasing siRNA efficacy.

[0091] According to a first embodiment, the present invention provides a kit for gene silencing, wherein said kit is comprised of a pool of at least two siRNA duplexes, each of which is comprised of a sequence that is complementary to a portion of the sequence of one or more target messenger RNA, and each of which is selected using non-target specific criteria. Each of the at least two siRNA duplexes of the kit

complementary to a portion of the sequence of one or more target mRNAs is preferably selected using Formula X.

[0092] According to a second embodiment, the present invention provides a method for selecting an siRNA, said method comprising applying selection criteria to a set of potential siRNA that comprise 18-30 base pairs, wherein said selection criteria are non-target specific criteria, and said set comprises at least two siRNAs and each of said at least two siRNAs contains a sequence that is at least substantially complementary to a target gene; and determining the relative functionality of the at least two siRNAs.

[0093] In one embodiment, the present invention also provides a method wherein said selection criteria are embodied in a formula comprising:

$$(-14)*G_{13}-13*A_1-12*U_7-11*U_2-10*A_{11}-10*U_4-10*C_3-10*C_5-10*C_6-9*A_{10}-9*U_9-9*C_{18}-8*G_{10}-7*U_1-7*U_{16}-7*C_{17}-7*C_{19}+7*U_{17}+8*A_2+8*A_4+8*A_5+8*C_4+9*G_8+10*A_7+10*U_{18}+11*A_{19}+11*C_9+15*G_1+18*A_3+19*U_{10}-Tm-3*(GC_{total})-6*(GC_{15\text{-}19})-30*X; \text{ or} \qquad \text{Formula VIII}$$

$$(-8)*A1+(-1)*A2+(12)*A3+(7)*A4+(18)*A5+(12)*A6+(19)*A7+(6)*A8+(-4)*A9+(-5)*A10+(-2)*A11+(-5)*A12+(17)*A13+(-3)*A14+(4)*A15+(2)*A16+(8)*A17+(11)*A18+(30)*A19+(-13)*U1+(-10)*U2+(2)*U3+(-2)*U4+(-5)*U5+(5)*U6+(-2)*U7+(-10)*U8+(-5)*U9+(15)*U10+(-1)*U11+(0)*U12+(10)*U13+(-9)*U14+(-13)*U15+(-10)*U16+(3)*U17+(9)*U18+(9)*U19+(7)*C1+(3)*C2+(-21)*C3+(5)*C4+(-9)*C5+(-20)*C6+(-18)*C7+(-5)*C8+(5)*C9+(1)*C10+(2)*C11+(-5)*C12+(-3)*C13+(-6)*C14+(-2)*C15+(-5)*C16+(-3)*C17+(-12)*C18+(-18)*C19+(14)*G1+(8)*G2+(7)*G3+(-10)*G4+(-4)*G5+(2)*G6+(1)*G7+(9)*G8+(5)*G9+(-11)*G10+(1)*G11+(9)*G12+(-24)*G13+(18)*G14+(11)*G15+(13)*G16+(-7)*G17+(-9)*G18+(-22)*G19+6*(\text{number of A+U in position 15-19})-3*(\text{number of G+C in whole siRNA}), \qquad \text{Formula X}$$

[0094] wherein position numbering begins at the 5'-most position of a sense strand, and

[0095] A_1=1 if A is the base at position 1 of the sense strand, otherwise its value is 0;

[0096] A_2=1 if A is the base at position 2 of the sense strand, otherwise its value is 0;

[0097] A_3=1 if A is the base at position 3 of the sense strand, otherwise its value is 0;

[0098] A_4=1 if A is the base at position 4 of the sense strand, otherwise its value is 0;

[0099] A_5=1 if A is the base at position 5 of the sense strand, otherwise its value is 0;

[0100] A_6=1 if A is the base at position 6 of the sense strand, otherwise its value is 0;

[0101] A_7=1 if A is the base at position 7 of the sense strand, otherwise its value is 0;

[0102] A_{10}=1 if A is the base at position 10 of the sense strand, otherwise its value is 0;

[0103] A_{11}=1 if A is the base at position 11 of the sense strand, otherwise its value is 0;

[0104] A_{13}=1 if A is the base at position 13 of the sense strand, otherwise its value is 0;

[0105] A_{19}=1 if A is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

[0106] C_3=1 if C is the base at position 3 of the sense strand, otherwise its value is 0;

[0107] C_4=1 if C is the base at position 4 of the sense strand, otherwise its value is 0;

[0108] C_5=1 if C is the base at position 5 of the sense strand, otherwise its value is 0;

[0109] C_6=1 if C is the base at position 6 of the sense strand, otherwise its value is 0;

[0110] C_7=1 if C is the base at position 7 of the sense strand, otherwise its value is 0;

[0111] C_9=1 if C is the base at position 9 of the sense strand, otherwise its value is 0;

[0112] C_{17}=1 if C is the base at position 17 of the sense strand, otherwise its value is 0;

[0113] C_{18}=1 if C is the base at position 18 of the sense strand, otherwise its value is 0;

[0114] C_{19}=1 if C is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

[0115] G_1=1 if G is the base at position 1 on the sense strand, otherwise its value is 0;

[0116] G_2=1 if G is the base at position 2 of the sense strand, otherwise its value is 0;

[0117] G_8=1 if G is the base at position 8 on the sense strand, otherwise its value is 0;

[0118] G_{10}=1 if G is the base at position 10 on the sense strand, otherwise its value is 0;

[0119] G_{13}=1 if G is the base at position 13 on the sense strand, otherwise its value is 0;

[0120] G_{19}=1 if G is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

[0121] U_1=1 if U is the base at position 1 on the sense strand, otherwise its value is 0;

[0122] U_2=1 if U is the base at position 2 on the sense strand, otherwise its value is 0;

[0123] U_3=1 if U is the base at position 3 on the sense strand, otherwise its value is 0;

[0124] U_4=1 if U is the base at position 4 on the sense strand, otherwise its value is 0;

[0125] U_7=1 if U is the base at position 7 on the sense strand, otherwise its value is 0;

[0126] U_9=1 if U is the base at position 9 on the sense strand, otherwise its value is 0;

[0127] U_{10}=1 if U is the base at position 10 on the sense strand, otherwise its value is 0;

[0128] U_{15}=1 if U is the base at position 15 on the sense strand, otherwise its value is 0;

[0129] U_{16}=1 if U is the base at position 16 on the sense strand, otherwise its value is 0;

[0130] U_{17}=1 if U is the base at position 17 on the sense strand, otherwise its value is 0;

[0131] U_{18}=1 if U is the base at position 18 on the sense strand, otherwise its value is 0.

[0132] $GC_{15\text{-}19}$=the number of G and C bases within positions 15-19 of the sense strand, or within positions 15-18 if the sense strand is only 18 base pairs in length;

[0133] GC_{total}=the number of G and C bases in the sense strand;

[0134] Tm=100 if the siRNA oligo has the internal repeat longer then 4 base pairs, otherwise its value is 0; and

[0135] X=the number of times that the same nucleotide repeats four or more times in a row.

[0136] Any of the methods of selecting siRNA in accordance with the invention can further comprise comparing the internal stability profiles of the siRNAs to be selected, and selecting those siRNAs with the most favorable internal stability profiles. Any of the methods of selecting siRNA can further comprise selecting either for or against sequences that contain motifs that induce cellular stress. Such motifs include, for example, toxicity motifs. Any of the methods of selecting siRNA can further comprise either selecting for or selecting against sequences that comprise stability motifs.

[0137] In another embodiment, the present invention provides a method of gene silencing, comprising introducing into a cell at least one siRNA selected according to any of the methods of the present invention. The siRNA can be introduced by allowing passive uptake of siRNA, or through the use of a vector.

[0138] According to a third embodiment, the invention provides a method for developing an algorithm for selecting siRNA, said method comprising: (a) selecting a set of siRNA; (b) measuring gene silencing ability of each siRNA from said set; (c) determining relative functionality of each siRNA; (d) determining improved functionality by the presence or absence of at least one variable selected from the group consisting of the presence or absence of a particular nucleotide at a particular position, the total number of As and Us in positions 15-19, the number of times that the same nucleotide repeats within a given sequence, and the total number of Gs and Cs; and (e) developing an algorithm using the information of step (d).

[0139] In another embodiment, the invention provides a method for selecting an siRNA with improved functionality, comprising using the above-mentioned algorithm to identify an siRNA of improved functionality.

[0140] According to a fourth embodiment, the present invention provides a kit, wherein said kit is comprised of at least two siRNAs, wherein said at least two siRNAs comprise a first optimized siRNA and a second optimized siRNA, wherein said first optimized siRNA and said second optimized siRNA are optimized according a formula comprising Formula X.

[0141] According to a fifth embodiment, the present invention provides a method for identifying a hyperfunctional siRNA, comprising applying selection criteria to a set of potential siRNA that comprise 18-30 base pairs, wherein said selection criteria are non-target specific criteria, and said set comprises at least two siRNAs and each of said at least two siRNAs contains a sequence that is at least substantially complementary to a target gene; determining the relative functionality of the at least two siRNAs and assigning each of the at least two siRNAs a functionality score; and selecting siRNAs from the at least two siRNAs that have a functionality score that reflects greater than 80 percent silencing at a concentration in the picomolar range, wherein said greater than 80 percent silencing endures for greater than 120 hours.

[0142] In other embodiments, the invention provides kits and/or methods wherein the siRNA are comprised of two separate polynucleotide strands; wherein the siRNA are comprised of a single contiguous molecule such as, for example, a unimolecular siRNA (comprising, for example, either a nucleotide or non-nucleotide loop); wherein the siRNA are expressed from one or more vectors; and wherein two or more genes are silenced by a single administration of siRNA.

[0143] According to a sixth embodiment, the present invention provides a hyperfunctional siRNA that is capable of silencing Bcl2.

[0144] According to a seventh embodiment, the present invention provides a method for developing an siRNA algorithm for selecting functional and hyperfunctional siRNAs for a given sequence. The method comprises:

[0145] (a) selecting a set of siRNAs;

[0146] (b) measuring the gene silencing ability of each siRNA from said set;

[0147] (c) determining the relative functionality of each siRNA;

[0148] (d) determining the amount of improved functionality by the presence or absence of at least one variable selected from the group consisting of the total GC content, melting temperature of the siRNA, GC content at positions 15-19, the presence or absence of a particular nucleotide at a particular position, relative thermodynamic stability at particular positions in a duplex, and the number of times that the same nucleotide repeats within a given sequence; and

[0149] (e) developing an algorithm using the information of step (d).

[0150] According to this embodiment, preferably the set of siRNAs comprises at least 90 siRNAs from at least one gene, more preferably at least 180 siRNAs from at least two different genes, and most preferably at least 270 and 360 siRNAs from at least three and four different genes, respectively. Additionally, in step (d) the determination is made

with preferably at least two, more preferably at least three, even more preferably at least four, and most preferably all of the variables. The resulting algorithm is not target sequence specific.

[0151] In another embodiment, the present invention provides rationally designed siRNAs identified using the formulas above.

[0152] In yet another embodiment, the present invention is directed to hyperfunctional siRNA.

[0153] The ability to use the above algorithms, which are not sequence or species specific, allows for the cost-effective selection of optimized siRNAs for specific target sequences. Accordingly, there will be both greater efficiency and reliability in the use of siRNA technologies.

[0154] The methods disclosed herein can be used in conjunction with comparing internal stability profiles of selected siRNAs, and designing an siRNA with a desirable internal stability profile; and/or in conjunction with a selection either for or against sequences that contain motifs that induce cellular stress, for example, cellular toxicity.

[0155] Any of the methods disclosed herein can be used to silence one or more genes by introducing an siRNA selected, or designed, in accordance with any of the methods disclosed herein. The siRNA(s) can be introduced into the cell by any method known in the art, including passive uptake or through the use of one or more vectors.

[0156] Any of the methods and kits disclosed herein can employ either unimolecular siRNAs, siRNAs comprised of two separate polynucleotide strands, or combinations thereof. Any of the methods disclosed herein can be used in gene silencing, where two or more genes are silenced by a single administration of siRNA(s). The siRNA(s) can be directed against two or more target genes, and administered in a single dose or single transfection, as the case may be.

Optimizing siRNA

[0157] According to one embodiment, the present invention provides a method for improving the effectiveness of gene silencing for use to silence a particular gene through the selection of an optimal siRNA. An siRNA selected according to this method may be used individually, or in conjunction with the first embodiment, i.e., with one or more other siRNAs, each of which may or may not be selected by this criteria in order to maximize their efficiency.

[0158] The degree to which it is possible to select an siRNA for a given mRNA that maximizes these criteria will depend on the sequence of the mRNA itself. However, the selection criteria will be independent of the target sequence. According to this method, an siRNA is selected for a given gene by using a rational design. That said, rational design can be described in a variety of ways. Rational design is, in simplest terms, the application of a proven set of criteria that enhance the probability of identifying a functional or hyperfunctional siRNA. In one method, rationally designed siRNA can be identified by maximizing one or more of the following criteria:

[0159] (1) A low GC content, preferably between about 30-52%.

[0160] (2) At least 2, preferably at least 3 A or U bases at positions 15-19 of the siRNA on the sense strand.

[0161] (3) An A base at position 19 of the sense strand.

[0162] (4) An A base at position 3 of the sense strand.

[0163] (5) A U base at position 10 of the sense strand.

[0164] (6) An A base at position 14 of the sense strand.

[0165] (7) A base other than C at position 19 of the sense strand.

[0166] (8) A base other than G at position 13 of the sense strand.

[0167] (9) A Tm, which refers to the character of the internal repeat that results in inter- or intramolecular structures for one strand of the duplex, that is preferably not stable at greater than 50° C., more preferably not stable at greater than 37° C., even more preferably not stable at greater than 30° C. and most preferably not stable at greater than 20° C.

[0168] (10) A base other than U at position 5 of the sense strand.

[0169] (11) A base other than A at position 11 of the sense strand.

[0170] (12) A base other than an A at position 1 of the sense strand.

[0171] (13) A base other than an A at position 2 of the sense strand.

[0172] (14) An A base at position 4 of the sense strand.

[0173] (15) An A base at position 5 of the sense strand.

[0174] (16) An A base at position 6 of the sense strand.

[0175] (17) An A base at position 7 of the sense strand.

[0176] (18) An A base at position 8 of the sense strand.

[0177] (19) A base other than an A at position 9 of the sense strand.

[0178] (20) A base other than an A at position 10 of the sense strand.

[0179] (21) A base other than an A at position 11 of the sense strand.

[0180] (22) A base other than an A at position 12 of the sense strand.

[0181] (23) An A base at position 13 of the sense strand.

[0182] (24) A base other than an A at position 14 of the sense strand.

[0183] (25) An A base at position 15 of the sense strand

[0184] (26) An A base at position 16 of the sense strand.

[0185] (27) An A base at position 17 of the sense strand.

[0186] (28) An A base at position 18 of the sense strand.

[0187] (29) A base other than a U at position 1 of the sense strand.

[0188] (30) A base other than a U at position 2 of the sense strand.

[0189] (31) A U base at position 3 of the sense strand.

[0190] (32) A base other than a U at position 4 of the sense strand.

[0191] (33) A base other than a U at position 5 of the sense strand.

[0192] (34) A U base at position 6 of the sense strand.

[0193] (35) A base other than a U at position 7 of the sense strand.

[0194] (36) A base other than a U at position 8 of the sense strand.

[0195] (37) A base other than a U at position 9 of the sense strand.

[0196] (38) A base other than a U at position 1 of the sense strand.

[0197] (39) A U base at position 13 of the sense strand.

[0198] (40) A base other than a U at position 14 of the sense strand.

[0199] (41) A base other than a U at position 15 of the sense strand.

[0200] (42) A base other than a U at position 16 of the sense strand.

[0201] (43) A U base at position 17 of the sense strand.

[0202] (44) A U base at position 18 of the sense strand.

[0203] (45) A U base at position 19 of the sense strand.

[0204] (46) A C base at position 1 of the sense strand.

[0205] (47) A C base at position 2 of the sense strand.

[0206] (48) A base other than a C at position 3 of the sense strand.

[0207] (49) A C base at position 4 of the sense strand.

[0208] (50) A base other than a C at position 5 of the sense strand.

[0209] (51) A base other than a C at position 6 of the sense strand.

[0210] (52) A base other than a C at position 7 of the sense strand.

[0211] (53) A base other than a C at position 8 of the sense strand.

[0212] (54) A C base at position 9 of the sense strand.

[0213] (55) A C base at position 10 of the sense strand.

[0214] (56) A C base at position 11 of the sense strand.

[0215] (57) A base other than a C at position 12 of the sense strand.

[0216] (58) A base other than a C at position 13 of the sense strand.

[0217] (59) A base other than a C at position 14 of the sense strand.

[0218] (60) A base other than a C at position 15 of the sense strand.

[0219] (61) A base other than a C at position 16 of the sense strand.

[0220] (62) A base other than a C at position 17 of the sense strand.

[0221] (63) A base other than a C at position 18 of the sense strand.

[0222] (64) A G base at position 1 of the sense strand.

[0223] (65) A G base at position 2 of the sense strand.

[0224] (66) A G base at position 3 of the sense strand.

[0225] (67) A base other than a G at position 4 of the sense strand.

[0226] (68) A base other than a G at position 5 of the sense strand.

[0227] (69) A G base at position 6 of the sense strand.

[0228] (70) A G base at position 7 of the sense strand.

[0229] (71) A G base at position 8 of the sense strand.

[0230] (72) A G base at position 9 of the sense strand.

[0231] (73) A base other than a G at position 10 of the sense strand.

[0232] (74) A G base at position 11 of the sense strand.

[0233] (75) A G base at position 12 of the sense strand.

[0234] (76) A G base at position 14 of the sense strand.

[0235] (77) A G base at position 15 of the sense strand.

[0236] (78) A G base at position 16 of the sense strand.

[0237] (79) A base other than a G at position 17 of the sense strand.

[0238] (80) A base other than a G at position 18 of the sense strand.

[0239] (81) A base other than a G at position 19 of the sense strand.

[0240] The importance of various criteria can vary greatly. For instance, a C base at position 10 of the sense strand makes a minor contribution to duplex functionality. In contrast, the absence of a C at position 3 of the sense strand is very important. Accordingly, preferably an siRNA will satisfy as many of the aforementioned criteria as possible.

[0241] With respect to the criteria, GC content, as well as a high number of AU in positions 15-19 of the sense strand, may be important for easement of the unwinding of double stranded siRNA duplex. Duplex unwinding has been shown to be crucial for siRNA functionality in vivo.

[0242] With respect to criterion 9, the internal structure is measured in terms of the melting temperature of the single strand of siRNA, which is the temperature at which 50% of the molecules will become denatured. With respect to criteria 2-8 and 10-11, the positions refer to sequence positions on the sense strand, which is the strand that is identical to the mRNA.

[0243] In one preferred embodiment, at least criteria 1 and 8 are satisfied. In another preferred embodiment, at least criteria 7 and 8 are satisfied. In still another preferred embodiment, at least criteria 1, 8 and 9 are satisfied.

[0244] It should be noted that all of the aforementioned criteria regarding sequence position specifics are with respect to the 5' end of the sense strand. Reference is made to the sense strand, because most databases contain information that describes the information of the mRNA.

Because according to the present invention a chain can be from 18 to 30 bases in length, and the aforementioned criteria assumes a chain 19 base pairs in length, it is important to keep the aforementioned criteria applicable to the correct bases.

[0245] When there are only 18 bases, the base pair that is not present is the base pair that is located at the 3' of the sense strand. When there are twenty to thirty bases present, then additional bases are added at the 5' end of the sense chain and occupy positions ⁻1 to ⁻11. Accordingly, with respect to SEQ. ID NO. 0001 NNANANNNNUCNAANNNNA and SEQ. ID NO. 0028 GUCNNANANNNNUCNAANNNNA, both would have A at position 3, A at position 5, U at position 10, C at position 11, A and position 13, A and position 14 and A at position 19. However, SEQ. ID NO. 0028 would also have C at position −1, U at position −2 and G at position −3.

[0246] For a 19 base pair siRNA, an optimal sequence of one of the strands may be represented below, where N is any base, A, C, G, or U:

SEQ. ID NO. 0001.	NNANANNNNUCNAANNNNA
SEQ. ID NO. 0002.	NNANANNNNUGNAANNNNA
SEQ. ID NO. 0003.	NNANANNNNUUNAANNNNA
SEQ. ID NO. 0004.	NNANANNNNUCNCANNNNA
SEQ. ID NO. 0005.	NNANANNNNUGNCANNNNA
SEQ. ID NO. 0006.	NNANANNNNUUNCANNNNA
SEQ. ID NO. 0007.	NNANANNNNUCNUANNNNA
SEQ. ID NO. 0008.	NNANANNNNUGNUANNNNA
SEQ. ID NO. 0009.	NNANANNNNUUNUANNNNA
SEQ. ID NO. 0010.	NNANCNNNNUCNAANNNNA
SEQ. ID NO. 0011.	NNANCNNNNUGNAANNNNA
SEQ. ID NO. 0012.	NNANCNNNNUUNAANNNNA
SEQ. ID NO. 0013.	NNANCNNNNUCNCANNNNA
SEQ. ID NO. 0014.	NNANCNNNNUGNCANNNNA
SEQ. ID NO. 0015.	NNANCNNNNUUNCANNNNA
SEQ. ID NO. 0016.	NNANCNNNNUCNUANNNNA
SEQ. ID NO. 0017.	NNANCNNNNUGNUANNNNA
SEQ. ID NO. 0018.	NNANCNNNNUUNUANNNNA
SEQ. ID NO. 0019.	NNANGNNNNUCNAANNNNA
SEQ. ID NO. 0020.	NNANGNNNNUGNAANNNNA
SEQ. ID NO. 0021.	NNANGNNNNUUNAANNNNA
SEQ. ID NO. 0022.	NNANGNNNNUCNCANNNNA
SEQ. ID NO. 0023.	NNANGNNNNUGNCANNNNA
SEQ. ID NO. 0024.	NNANGNNNNUUNCANNNNA
SEQ. ID NO. 0025.	NNANGNNNNUCNUANNNNA

-continued

SEQ. ID NO. 0026.	NNANGNNNNUGNUANNNNA
SEQ. ID NO. 0027.	NNANGNNNNUNUANNNNA

[0247] In one embodiment, the sequence used as an siRNA is selected by choosing the siRNA that score highest according to one of the following seven algorithms that are represented by Formulas I-VI:

Relative functionality of siRNA $=-(GC/3)+(AU_{15\text{-}19})-(Tm_{20°C})*3-(G_{13})*3-(C_{19})+(A_{19})*2+(A_3)+(U_{10})+(A_{14})-(U_5)-(A_{11})$ Formula I

Relative functionality of siRNA $=-(GC/3)-(AU_{15\text{-}19})*3-(G_{13})*3-(C_{19})+(A_{19})*2+(A_3)$ Formula II

Relative functionality of siRNA $=-(GC/3)+(AU_{15\text{-}19})-(Tm_{20°C})*3$ Formula III

Relative functionality of siRNA $=-GC/2+(AU_{15\text{-}19})/2-(Tm_{20°C})*2-(G_{13})*3-(C_{19})+(A_{19})*2+(A_3)+(U_{10})+(A_{14})-(U_5)-(A_{11})$ Formula IV

Relative functionality of siRNA $=-(G_{13})*3-(C_{19})+(A_{19})*2+(A_3)+(U_{10})+(A_{14})-(U_5)-(A_{11})$ Formula V

Relative functionality of siRNA $=-(G_{13})*3-(C_{19})+(A_{19})*2+(A_3)$ Formula VI

Relative functionality of siRNA $=-(GC/2)+(AU_{15\text{-}19})/2-(Tm_{20°C})*1-(G_{13})*3-(C_{19})+(A_{19})*3+(A_3)*3+(U_{10})/2+(A_{14})/2-(U_5)/2-(A_{11})/2$ Formula VII

[0248] In Formulas I-VII:

[0249] wherein A_{19}=1 if A is the base at position 19 on the sense strand, otherwise its value is 0,

[0250] $AU_{15\text{-}19}$=0-5 depending on the number of A or U bases on the sense strand at positions 15-19;

[0251] G_{13}=1 if G is the base at position 13 on the sense strand, otherwise its value is 0;

[0252] C_{19}=1 if C is the base at position 19 of the sense strand, otherwise its value is 0;

[0253] GC=the number of G and C bases in the entire sense strand;

[0254] $Tm_{20°C}$=1 if the Tm is greater than 20° C.;

[0255] A_3=1 if A is the base at position 3 on the sense strand, otherwise its value is 0;

[0256] U_{10}=1 if U is the base at position 10 on the sense strand, otherwise its value is 0;

[0257] A_{14}=1 if A is the base at position 14 on the sense strand, otherwise its value is 0;

[0258] U_5=1 if U is the base at position 5 on the sense strand, otherwise its value is 0; and

[0259] A_{11}=1 if A is the base at position 11 of the sense strand, otherwise its value is 0.

[0260] Formulas I-VII provide relative information regarding functionality. When the values for two sequences are compared for a given formula, the relative functionality is ascertained; a higher positive number indicates a greater functionality. For example, in many applications a value of 5 or greater is beneficial.

[0261] Additionally, in many applications, more than one of these formulas would provide useful information as to the relative functionality of potential siRNA sequences. However, it is beneficial to have more than one type of formula,

because not every formula will be able to help to differentiate among potential siRNA sequences. For example, in particularly high GC mRNAs, formulas that take that parameter into account would not be useful and application of formulas that lack GC elements (e.g., formulas V and VI) might provide greater insights into duplex functionality. Similarly, formula II might by used in situations where hairpin structures are not observed in duplexes, and formula IV might be applicable for sequences that have higher AU content. Thus, one may consider a particular sequence in light of more than one or even all of these algorithms to obtain the best differentiation among sequences. In some instances, application of a given algorithm may identify an unusually large number of potential siRNA sequences, and in those cases, it may be appropriate to re-analyze that sequence with a second algorithm that is, for instance, more stringent. Alternatively, it is conceivable that analysis of a sequence with a given formula yields no acceptable siRNA sequences (i.e. low SMARTSCORES™, or siRNA ranking). In this instance, it may be appropriate to re-analyze that sequences with a second algorithm that is, for instance, less stringent. In still other instances, analysis of a single sequence with two separate formulas may give rise to conflicting results (i.e. one formula generates a set of siRNA with high SMARTSCORES™, or siRNA ranking, while the other formula identifies a set of siRNA with low SMARTSCORES™, or siRNA ranking). In these instances, it may be necessary to determine which weighted factor(s) (e.g. GC content) are contributing to the discrepancy and assessing the sequence to decide whether these factors should or should not be included. Alternatively, the sequence could be analyzed by a third, fourth, or fifth algorithm to identify a set of rationally designed siRNA.

[0262] The above-referenced criteria are particularly advantageous when used in combination with pooling techniques as depicted in Table I:

TABLE I

	FUNCTIONAL PROBABILITY					
	OLIGOS			POOLS		
CRITERIA	>95%	>80%	<70%	>95%	>80%	<70%
CURRENT	33.0	50.0	23.0	79.5	97.3	0.3
NEW	50.0	88.5	8.0	93.8	99.98	0.005
(GC)	28.0	58.9	36.0	72.8	97.1	1.6

[0263] The term "current" used in Table I refers to Tuschl's conventional siRNA parameters (Elbashir, S. M. et al. (2002) "Analysis of gene function in somatic mammalian cells using small interfering RNAs" Methods 26: 199-213). "New" refers to the design parameters described in Formulas I-VII. "GC" refers to criteria that select siRNA solely on the basis of GC content.

[0264] As Table I indicates, when more functional siRNA duplexes are chosen, siRNAs that produce <70% silencing drops from 23% to 8% and the number of siRNA duplexes that produce >80% silencing rises from 50% to 88.5%. Further, of the siRNA duplexes with >80% silencing, a larger portion of these siRNAs actually silence >95% of the target expression (the new criteria increases the portion from 33% to 50%). Using this new criteria in pooled siRNAs, shows that, with pooling, the amount of silencing >95% increases from 79.5% to 93.8% and essentially eliminates any siRNA pool from silencing less than 70%.

[0265] Table II similarly shows the particularly beneficial results of pooling in combination with the aforementioned criteria. However, Table II, which takes into account each of the aforementioned variables, demonstrates even a greater degree of improvement in functionality.

TABLE II

	FUNCTIONAL PROBABILITY					
	OLIGOS			POOLS		
	FUNCTIONAL	AVERAGE	NON-FUNCTIONAL	FUNCTIONAL	AVERAGE	NON-FUNCTIONAL
RANDOM	20	40	50	67	97	3
CRITERIA 1	52	99	0.1	97	93	0.0040
CRITERIA 4	89	99	0.1	99	99	0.0000

[0266] The terms "functional,""Average," and "Non-functional" used in Table II, refer to siRNA that exhibit >80%, >50%, and <50% functionality, respectively. Criteria 1 and 4 refer to specific criteria described above.

[0267] The above-described algorithms may be used with or without a computer program that allows for the inputting of the sequence of the mRNA and automatically outputs the optimal siRNA. The computer program may, for example, be accessible from a local terminal or personal computer, over an internal network or over the Internet.

[0268] In addition to the formulas above, more detailed algorithms, may be used for selecting siRNA. Preferably, at least one RNA duplex of 18-30 base pairs is selected such that it is optimized according a formula selected from:

$$(-14)*G_{13}-13*A_1-12*U_7-11*U_2-10*A_{11}-10*U_4-10*C_3-10*C_5-10*C_6-9*A_{10}-$$

$$9*U_9-9*C_{18}-8*G_{10}-7*U_1-7*U_{16}-7*C_{17}-7*C_{19}+7*U_{17}+8*A_2+8*A_4+8*A_5+8*C_4$$

$$+9*G_8+10*A_7+10*U_{18}+11*A_{19}+11*C_9+15*G_1+18*A_3+19*U_{10}-Tm-3*(GC_{total})$$

$$-6*(GC_{15\text{-}19})-30*X;\text{ and} \qquad \text{Formula VIII}$$

$$(14.1)*A_3+(14.9)*A_6+(17.6)*A_{13}+(24.7)*A_{19}+(14.2)*U_{10}+(10.5)*$$

$$C_9+(23.9)*G_1+(16.3)*G_2+(-12.3)*A_{11}+(-19.3)*U_1+(-12.1)*U_2+(-$$

$$11)*U_3+(-15.2)*U_{15}+(-11.3)*U_{16}+(-11.8)*C_3+(-17.4)*C_6+(-10.5)*C_7+$$

$(-13.7)*G_{13}+(-25.9)*G_{19}-Tm-3*(GC_{total})-6*(GC_{15-19})-30*X$; and Formula IX

$(-8)*A1+(-1)*A2+(12)*A3+(7)*A4+(18)*A5+(12)*A6+$

$(19)*A7+(6)*A8+(-4)*A9+(-5)*A10+(-2)*A11+(-5)*A12+(17)*A13+(-$

$3)*A14+(4)*A15+(2)*A16+(8)*A17+(11)*A18+(30)*A19+(-13)*U1+(-$

$10)*U2+(2)*U3+(-2)*U4+(-5)*U5+(5)*U6+(-2)*U7+(-10)*U8+(-$

$5)*U9+(15)*U10+(-1)*U11+(0)*U12+(10)*U13+(-9)*U14+(-13)*U15+(-$

$10)*U16+(3)*U17+(9)*U18+(9)*U19+(7)*C1+(3)*C2+(-21)*C3+(5)*C4+(-$

$9)*C5+(-20)*C6+(-18)*C7+(-5)*C8+(5)*C9+(1)*C10+(2)*C11+(-$

$5)*C12+(-3)*C13+(-6)*C14+(-2)*C15+(-5)*C16+(-3)*C17+(-12)*C18+(-$

$18)*C19+(14)*G1+(8)*G2+(7)*G3+(-10)*G4+(-$

$4)*G5+(2)*G6+(1)*G7+(9)*G8+(5)*G9+(-11)*G10+(1)*G11+(9)*G12+(-$

$24)*G13+(18)*G14+(11)*G15+(13)*G16+(-7)*G17+(-9)*G18+(-22)*G19+$

6*(number of A+U in position 15-19)−3*(number of G+C in whole

siRNA). Formula X

[0269] wherein

[0270] A_1=1 if A is the base at position 1 of the sense strand, otherwise its value is 0;

[0271] A_2=1 if A is the base at position 2 of the sense strand, otherwise its value is 0;

[0272] A_3=1 if A is the base at position 3 of the sense strand, otherwise its value is 0;

[0273] A_4=1 if A is the base at position 4 of the sense strand, otherwise its value is 0;

[0274] A_5=1 if A is the base at position 5 of the sense strand, otherwise its value is 0;

[0275] A_6=1 if A is the base at position 6 of the sense strand, otherwise its value is 0;

[0276] A_7=1 if A is the base at position 7 of the sense strand, otherwise its value is 0;

[0277] A_{10}=1 if A is the base at position 10 of the sense strand, otherwise its value is 0;

[0278] A_{11}=1 if A is the base at position 11 of the sense strand, otherwise its value is 0;

[0279] A_{13}=1 if A is the base at position 13 of the sense strand, otherwise its value is 0;

[0280] A_{19}=1 if A is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

[0281] C_3=1 if C is the base at position 3 of the sense strand, otherwise its value is 0;

[0282] C_4=1 if C is the base at position 4 of the sense strand, otherwise its value is 0;

[0283] C_5=1 if C is the base at position 5 of the sense strand, otherwise its value is 0;

[0284] C_6=1 if C is the base at position 6 of the sense strand, otherwise its value is 0;

[0285] C_7=1 if C is the base at position 7 of the sense strand, otherwise its value is 0;

[0286] C_9=1 if C is the base at position 9 of the sense strand, otherwise its value is 0;

[0287] C_{17}=1 if C is the base at position 17 of the sense strand, otherwise its value is 0;

[0288] C_{18}=1 if C is the base at position 18 of the sense strand, otherwise its value is 0;

[0289] C_{19}=1 if C is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

[0290] G_1=1 if G is the base at position 1 on the sense strand, otherwise its value is 0;

[0291] G_2=1 if G is the base at position 2 of the sense strand, otherwise its value is 0;

[0292] G_8=1 if G is the base at position 8 on the sense strand, otherwise its value is 0;

[0293] G_{10}=1 if G is the base at position 10 on the sense strand, otherwise its value is 0;

[0294] G_{13}=1 if G is the base at position 13 on the sense strand, otherwise its value is 0;

[0295] G_{19}=1 if G is the base at position 19 of the sense strand, otherwise if another base is present or the sense strand is only 18 base pairs in length, its value is 0;

[0296] U_1=1 if U is the base at position 1 on the sense strand, otherwise its value is 0;

[0297] U_2=1 if U is the base at position 2 on the sense strand, otherwise its value is 0;

[0298] U_3=1 if U is the base at position 3 on the sense strand, otherwise its value is 0;

[0299] U_4=1 if U is the base at position 4 on the sense strand, otherwise its value is 0;

[0300] U_7=1 if U is the base at position 7 on the sense strand, otherwise its value is 0;

[0301] U_9=1 if U is the base at position 9 on the sense strand, otherwise its value is 0;

[0302] U_{10}=1 if U is the base at position 10 on the sense strand, otherwise its value is 0;

[0303] U_{15}=1 if U is the base at position 15 on the sense strand, otherwise its value is 0;

[0304] U_{16}=1 if U is the base at position 16 on the sense strand, otherwise its value is 0;

[0305] U_{17}=1 if U is the base at position 17 on the sense strand, otherwise its value is 0;

[0306] U_{18}=1 if U is the base at position 18 on the sense strand, otherwise its value is 0;

[0307] GC_{15-19}=the number of G and C bases within positions 15-19 of the sense strand, or within positions 15-18 if the sense strand is only 18 base pairs in length;

[0308] GC_{total}=the number of G and C bases in the sense strand;

[0309] Tm=100 if the siRNA oligo has the internal repeat longer then 4 base pairs, otherwise its value is 0; and

[0310] X=the number of times that the same nucleotide repeats four or more times in a row.

[0311] The above formulas VIII, IX, and X, as well as formulas I-VII, provide methods for selecting siRNA in order to increase the efficiency of gene silencing. A subset of variables of any of the formulas may be used, though when fewer variables are used, the optimization hierarchy becomes less reliable.

[0312] With respect to the variables of the above-referenced formulas, a single letter of A or C or G or U followed by a subscript refers to a binary condition. The binary condition is that either the particular base is present at that particular position (wherein the value is "1") or the base is not present (wherein the value is "0"). Because position 19 is optional, i.e., there might be only 18 base pairs, when there are only 18 base pairs, any base with a subscript of 19 in the formulas above would have a zero value for that parameter. Before or after each variable is a number followed by *, which indicates that the value of the variable is to be multiplied or weighed by that number.

[0313] The numbers preceding the variables A, or G, or C, or U in Formulas VIII, IX, and X (or after the variables in Formula I-VII) were determined by comparing the difference in the frequency of individual bases at different positions in functional siRNA and total siRNA. Specifically, the frequency in which a given base was observed at a particular position in functional groups was compared with the frequency that that same base was observed in the total, randomly selected siRNA set. If the absolute value of the difference between the functional and total values was found to be greater than 6%, that parameter was included in the equation. Thus, for instance, if the frequency of finding a "G" at position 13 (G_{13}) is found to be 6% in a given functional group, and the frequency of G_{13} in the total population of siRNAs is 20%, the difference between the two values is 6%-20%-14%. As the absolute value is greater than six (6), this factor (−14) is included in the equation. Thus, in Formula VIII, in cases where the siRNA under study has a G in position 13, the accrued value is (−14)*(1)=−14. In contrast, when a base other than G is found at position 13, the accrued value is (−14)*(0)=0.

[0314] When developing a means to optimize siRNAs, the inventors observed that a bias toward low internal thermodynamic stability of the duplex at the 5'-antisense (AS) end is characteristic of naturally occurring miRNA precursors. The inventors extended this observation to siRNAs for which functionality had been assessed in tissue culture.

[0315] With respect to the parameter GC_{15-19}, a value of 0-5 will be ascribed depending on the number of G or C bases at positions 15 to 19. If there are only 18 base pairs, the value is between 0 and 4.

[0316] With respect to the criterion GC_{total} content, a number from 0-30 will be ascribed, which correlates to the total number of G and C nucleotides on the sense strand, excluding overhangs. Without wishing to be bound by any one theory, it is postulated that the significance of the GC content (as well as AU content at positions 15-19, which is a parameter for formulas III-VII) relates to the easement of the unwinding of a double-stranded siRNA duplex. Duplex unwinding is believed to be crucial for siRNA functionality in vivo and overall low internal stability, especially low internal stability of the first unwound base pair is believed to be important to maintain sufficient processivity of RISC complex-induced duplex unwinding. If the duplex has 19 base pairs, those at positions 15-19 on the sense strand will unwind first if the molecule exhibits a sufficiently low internal stability at that position. As persons skilled in the art are aware, RISC is a complex of approximately twelve proteins; Dicer is one, but not the only, helicase within this complex. Accordingly, although the GC parameters are believed to relate to activity with Dicer, they are also important for activity with other RISC proteins.

[0317] The value of the parameter Tm is 0 when there are no internal repeats longer than (or equal to) four base pairs present in the siRNA duplex; otherwise the value is 1. Thus for example, if the sequence ACGUACGU, or any other four nucleotide (or more) palindrome exists within the structure, the value will be one (1). Alternatively if the structure ACGGACG, or any other 3 nucleotide (or less) palindrome exists, the value will be zero (0).

[0318] The variable "X" refers to the number of times that the same nucleotide occurs contiguously in a stretch of four or more units. If there are, for example, four contiguous As in one part of the sequence and elsewhere in the sequence four contiguous Cs, X=2. Further, if there are two separate contiguous stretches of four of the same nucleotides or eight or more of the same nucleotides in a row, then X=2. However, X does not increase for five, six or seven contiguous nucleotides.

[0319] Again, when applying Formula VIII, Formula IX, or Formula X, to a given mRNA, (the "target RNA" or "target molecule"), one may use a computer program to evaluate the criteria for every sequence of 18-30 base pairs or only sequences of a fixed length, e.g., 19 base pairs. Preferably the computer program is designed such that it provides a report ranking of all of the potential siRNAs 18-30 base pairs, ranked according to which sequences generate the highest value. A higher value refers to a more efficient siRNA for a particular target gene. The computer program that may be used may be developed in any computer language that is known to be useful for scoring nucleotide sequences, or it may be developed with the assistance of commercially available product such as Microsoft's PRODUCT.NET. Additionally, rather than run every sequence through one and/or another formula, one may compare a subset of the sequences, which may be desirable if for example only a subset are available. For instance, it may be desirable to first perform a BLAST (Basic Local Alignment Search Tool) search and to identify sequences that have no homology to other targets. Alternatively, it may be desirable to scan the sequence and to identify regions of moderate GC context, then perform relevant calculations using one of the above-described formulas on these regions. These calculations can be done manually or with the aid of a computer.

[0320] As with Formulas I-VII, either Formula VIII, Formula IX, or Formula X may be used for a given mRNA target sequence. However, it is possible that according to

one or the other formula more than one siRNA will have the same value. Accordingly, it is beneficial to have a second formula by which to differentiate sequences. Formulas IX and X were derived in a similar fashion as Formula VIII, yet used a larger data set and thus yields sequences with higher statistical correlations to highly functional duplexes. The sequence that has the highest value ascribed to it may be referred to as a "first optimized duplex." The sequence that has the second highest value ascribed to it may be referred to as a "second optimized duplex." Similarly, the sequences that have the third and fourth highest values ascribed to them may be referred to as a third optimized duplex and a fourth optimized duplex, respectively. When more than one sequence has the same value, each of them may, for example, be referred to as first optimized duplex sequences or co-first optimized duplexes. Formula X is similar to Formula IX, yet uses a greater numbers of variables and for that reason, identifies sequences on the basis of slightly different criteria.

[0321] It should also be noted that the output of a particular algorithm will depend on several of variables including: (1) the size of the data base(s) being analyzed by the algorithm, and (2) the number and stringency of the parameters being applied to screen each sequence. Thus, for example, in U.S. patent application Ser. No. 10/714,333, entitled "Functional and Hyperfunctional siRNA," filed Nov. 14, 2003, Formula VIII was applied to the known human genome (NCBI REFSEQ database) through ENTREZ (EFETCH). As a result of these procedures, roughly 1.6 million siRNA sequences were identified. Application of Formula VIII to the same database in March of 2004 yielded roughly 2.2 million sequences, a difference of approximately 600,000 sequences resulting from the growth of the database over the course of the months that span this period of time. Application of other formulas (e.g., Formula X) that change the emphasis of, include, or eliminate different variables can yield unequal numbers of siRNAs. Alternatively, in cases where application of one formula to one or more genes fails to yield sufficient numbers of siRNAs with scores that would be indicative of strong silencing, said genes can be reassessed with a second algorithm that is, for instance, less stringent.

[0322] siRNA sequences identified using Formula VIII and Formula X (minus sequences generated by Formula VIII) are contained within the sequence listing. The data included in the sequence listing is described more fully below. The sequences identified by Formula VIII and Formula X that are disclosed in the sequence listing may be used in gene silencing applications.

[0323] It should be noted that for Formulas VIII, IX, and X all of the aforementioned criteria are identified as positions on the sense strand when oriented in the 5' to 3' direction as they are identified in connection with Formulas I-VII unless otherwise specified.

[0324] Formulas I-X, may be used to select or to evaluate one, or more than one, siRNA in order to optimize silencing. Preferably, at least two optimized siRNAs that have been selected according to at least one of these formulas are used to silence a gene, more preferably at least three and most preferably at least four. The siRNAs may be used individually or together in a pool or kit. Further, they may be applied to a cell simultaneously or separately. Preferably, the at least two siRNAs are applied simultaneously. Pools are particularly beneficial for many research applications. However, for therapeutics, it may be more desirable to employ a single hyperfunctional siRNA as described elsewhere in this application.

[0325] When planning to conduct gene silencing, and it is necessary to choose between two or more siRNAs, one should do so by comparing the relative values when the siRNA are subjected to one of the formulas above. In general a higher scored siRNA should be used.

[0326] Useful applications include, but are not limited to, target validation, gene functional analysis, research and drug discovery, gene therapy and therapeutics. Methods for using siRNA in these applications are well known to persons of skill in the art.

[0327] Because the ability of siRNA to function is dependent on the sequence of the RNA and not the species into which it is introduced, the present invention is applicable across a broad range of species, including but not limited to all mammalian species, such as humans, dogs, horses, cats, cows, mice, hamsters, chimpanzees and gorillas, as well as other species and organisms such as bacteria, viruses, insects, plants and *C. elegans*.

[0328] The present invention is also applicable for use for silencing a broad range of genes, including but not limited to the roughly 45,000 genes of a human genome, and has particular relevance in cases where those genes are associated with diseases such as diabetes, Alzheimer's, cancer, as well as all genes in the genomes of the aforementioned organisms.

[0329] The siRNA selected according to the aforementioned criteria or one of the aforementioned algorithms are also, for example, useful in the simultaneous screening and functional analysis of multiple genes and gene families using high throughput strategies, as well as in direct gene suppression or silencing.

Development of the Algorithms

[0330] To identify siRNA sequence features that promote functionality and to quantify the importance of certain currently accepted conventional factors—such as G/C content and target site accessibility—the inventors synthesized an siRNA panel consisting of 270 siRNAs targeting three genes, Human Cyclophilin, Firefly Luciferase, and Human DBI. In all three cases, siRNAs were directed against specific regions of each gene. For Human Cyclophilin and Firefly Luciferase, ninety siRNAs were directed against a 199 bp segment of each respective mRNA. For DBI, 90 siRNAs were directed against a smaller, 109 base pair region of the mRNA. The sequences to which the siRNAs were directed are provided below.

[0331] It should be noted that in certain sequences, "t" is present. This is because many databases contain information in this manner. However, the t denotes a uracil residue in in RNA and siRNA. Any algorithm will, unless otherwise specified, process at in a sequence as a u.

[0332] Human Cyclophilin: 193-390m M60857

```
SEQ. ID NO. 29:
gttccaaaaa cagtggataa ttttgtggcc ttagctacag

gagagaaagg atttggctac aaaaacagca aattccatcg

tgtaatcaag gacttcatga tccagggcgg agacttcacc

aggggagatg gcacaggagg aaagagcatc tacggtgagc

gcttccccga tgagaacttc aaactgaagc actacgggcc

tggctggg
```

[0333] Firefly Luciferase: 1434-1631, U47298 (pGL3, Promega)

```
SEQ. ID NO. 30:
tgaacttccc gccgccgttg ttgttttgga gcacggaaag

acgatgacgg aaaaagagat cgtggattac gtcgccagtc

aagtaacaac cgcgaaaaag ttgcgcggag gagttgtgtt

tgtggacgaa gtaccgaaag gtcttaccgg aaaactcgac

gcaagaaaaa tcagagagat cctcataaag gccaagaagg
```

[0334] DBI, NM_020548 (202-310) (Every Position)

```
SEQ. ID NO. 0031:
acgggcaagg ccaagtggga tgcctggaat gagctgaaag

ggacttccaa ggaagatgcc atgaaagctt acatcaacaa

agtagaagag ctaaagaaaa aatacggg
```

[0335] A list of the siRNAs appears in Table III (see Examples Section, Example II)

[0336] The set of duplexes was analyzed to identify correlations between siRNA functionality and other biophysical or thermodynamic properties. When the siRNA panel was analyzed in functional and non-functional subgroups, certain nucleotides were much more abundant at certain positions in functional or non-functional groups. More specifically, the frequency of each nucleotide at each position in highly functional siRNA duplexes was compared with that of nonfunctional duplexes in order to assess the preference for or against any given nucleotide at every position. These analyses were used to determine important criteria to be included in the siRNA algorithms (Formulas VIII, IX, and X).

[0337] The data set was also analyzed for distinguishing biophysical properties of siRNAs in the functional group, such as optimal percent of GC content, propensity for internal structures and regional thermodynamic stability. Of the presented criteria, several are involved in duplex recognition, RISC activation/duplex unwinding, and target cleavage catalysis.

[0338] The original data set that was the source of the statistically derived criteria is shown in FIG. **2**. Additionally, this figure shows that random selection yields siRNA duplexes with unpredictable and widely varying silencing potencies as measured in tissue culture using HEK293 cells. In the figure, duplexes are plotted such that each x-axis tick-mark represents an individual siRNA, with each subsequent siRNA differing in target position by two nucleotides for Human Cyclophilin B and Firefly Luciferase, and by one nucleotide for Human DBI. Furthermore, the y-axis denotes the level of target expression remaining after transfection of the duplex into cells and subsequent silencing of the target.

[0339] siRNA identified and optimized in this document work equally well in a wide range of cell types. FIG. **3***a* shows the evaluation of thirty siRNAs targeting the DBI gene in three cell lines derived from different tissues. Each DBI siRNA displays very similar functionality in HEK293 (ATCC, CRL-1573, human embryonic kidney), HeLa (ATCC, CCL-2, cervical epithelial adenocarcinoma) and DU145 (HTB-81, prostate) cells as determined by the B-DNA assay. Thus, siRNA functionality is determined by the primary sequence of the siRNA and not by the intracellular environment. Additionally, it should be noted that although the present invention provides for a determination of the functionality of siRNA for a given target, the same siRNA may silence more than one gene. For example, the complementary sequence of the silencing siRNA may be present in more than one gene. Accordingly, in these circumstances, it may be desirable not to use the siRNA with highest SMARTSCORE™, or siRNA ranking. In such circumstances, it may be desirable to use the siRNA with the next highest SMARTSCORE™, or siRNA ranking.

[0340] To determine the relevance of G/C content in siRNA function, the G/C content of each duplex in the panel was calculated and the functional classes of siRNAs (<F50, ≧F50, ≧F80, ≧F95 where F refers to the percent gene silencing) were sorted accordingly. The majority of the highly-functional siRNAs (≧F95) fell within the G/C content range of 36%-52% (FIG. **3**B). Twice as many non-functional (<F50) duplexes fell within the high G/C content groups (>57% GC content) compared to the 36%-52% group. The group with extremely low GC content (26% or less) contained a higher proportion of non-functional siRNAs and no highly-functional siRNAs. The G/C content range of 30%-52% was therefore selected as Criterion I for siRNA functionality, consistent with the observation that a G/C range 30%-70% promotes efficient RNAi targeting. Application of this criterion alone provided only a marginal increase in the probability of selecting functional siRNAs from the panel: selection of F50 and F95 siRNAs was improved by 3.6% and 2.2%, respectively. The siRNA panel presented here permitted a more systematic analysis and quantification of the importance of this criterion than that used previously.

[0341] A relative measure of local internal stability is the A/U base pair (bp) content; therefore, the frequency of A/U bp was determined for each of the five terminal positions of the duplex (5' sense (S)/5' antisense (AS)) of all siRNAs in the panel. Duplexes were then categorized by the number of A/U bp in positions 1-5 and 15-19 of the sense strand. The thermodynamic flexibility of the duplex 5'-end (positions 1-5; S) did not appear to correlate appreciably with silencing potency, while that of the 3'-end (positions 15-19; S) correlated with efficient silencing. No duplexes lacking A/U bp in positions 15-19 were functional. The presence of one A/U bp in this region conferred some degree of functionality, but the presence of three or more A/Us was preferable and

therefore defined as Criterion II. When applied to the test panel, only a marginal increase in the probability of functional siRNA selection was achieved: a 1.8% and 2.3% increase for F50 and F95 duplexes, respectively (Table IV).

[0342] The complementary strands of siRNAs that contain internal repeats or palindromes may form internal fold-back structures. These hairpin-like structures exist in equilibrium with the duplexed form effectively reducing the concentration of functional duplexes. The propensity to form internal hairpins and their relative stability can be estimated by predicted melting temperatures. High Tm reflects a tendency to form hairpin structures. Lower Tm values indicate a lesser tendency to form hairpins. When the functional classes of siRNAs were sorted by T_m (FIG. **3***c*), the following trends were identified: duplexes lacking stable internal repeats were the most potent silencers (no F95 duplex with predicted hairpin structure T_m>60° C.). In contrast, about 60% of the duplexes in the groups having internal hairpins with calculated T_m values less than 20° C. were F80. Thus, the stability of internal repeats is inversely proportional to the silencing effect and defines Criterion III (predicted hairpin structure $T_m \leqq 20°$ C.).

Sequence-Based Determinants of siRNA Functionality

[0343] When the siRNA panel was sorted into functional and non-functional groups, the frequency of a specific nucleotide at each position in a functional siRNA duplex was compared with that of a nonfunctional duplex in order to assess the preference for or against a certain nucleotide. FIG. **4** shows the results of these queries and the subsequent resorting of the data set (from FIG. **2**). The data is separated into two sets: those duplexes that meet the criteria, a specific nucleotide in a certain position—grouped on the left (Selected) and those that do not—grouped on the right (Eliminated). The duplexes are further sorted from most functional to least functional with the y-axis of FIG. **4***a-e* representing the % expression i.e., the amount of silencing that is elicited by the duplex (Note: each position on the X-axis represents a different duplex). Statistical analysis revealed correlations between silencing and several sequence-related properties of siRNAs. FIG. **4** and Table IV show quantitative analysis for the following five sequence-related properties of siRNA: (A) an A at position 19 of the sense strand; (B) an A at position 3 of the sense strand; (C) a U at position 10 of the sense strand; (D) a base other than G at position 13 of the sense strand; and (E) a base other than C at position 19 of the sense strand.

[0344] When the siRNAs in the panel were evaluated for the presence of an A at position 19 of the sense strand, the percentage of non-functional duplexes decreased from 20% to 11.8%, and the percentage of F95 duplexes increased from 21.7% to 29.4% (Table IV). Thus, the presence of an A in this position defined Criterion IV.

[0345] Another sequence-related property correlated with silencing was the presence of an A in position 3 of the sense strand (FIG. **4***b*). Of the siRNAs with A3, 34.4% were F95, compared with 21.7% randomly selected siRNAs. The presence of a U base in position 10 of the sense strand exhibited an even greater impact (FIG. **4***c*). Of the duplexes in this group, 41.7% were F95. These properties became criteria V and VI, respectively.

[0346] Two negative sequence-related criteria that were identified also appear on FIG. **4**. The absence of a G at position 13 of the sense strand, conferred a marginal increase in selecting functional duplexes (FIG. **4***d*). Similarly, lack of a C at position 19 of the sense strand also correlated with functionality (FIG. **4***e*). Thus, among functional duplexes, position 19 was most likely occupied by A, and rarely occupied by C. These rules were defined as criteria VII and VIII, respectively.

[0347] Application of each criterion individually provided marginal but statistically significant increases in the probability of selecting a potent siRNA. Although the results were informative, the inventors sought to maximize potency and therefore consider multiple criteria or parameters. Optimization is particularly important when developing therapeutics. Interestingly, the probability of selecting a functional siRNA based on each thermodynamic criteria was 2%-4% higher than random, but 4%-8% higher for the sequence-related determinates. Presumably, these sequence-related increases reflect the complexity of the RNAi mechanism and the multitude of protein-RNA interactions that are involved in RNAi-mediated silencing.

TABLE IV

CRITERION		PERCENT FUNCTIONAL	IMPROVEMENT OVER RANDOM (%)
I. 30%-52% G/C Content	<F50	16.4	−3.6
	≧F50	83.6	3.6
	≧F80	60.4	4.3
	≧F95	23.9	2.2
II. At least 3 A/U bases at positions 15-19 of the sense strand	<F50	18.2	−1.8
	≧F50	81.8	1.8
	≧F80	59.7	3.6
	≧F95	24.0	2.3
III. Absence of internal repeats, as measured by Tm of secondary structure ≦20° C.	<F50	16.7	−3.3
	≧F50	83.3	3.3
	≧F80	61.1	5.0
	≧F95	24.6	2.9
IV. An A base at position 19 of the sense strand	<F50	11.8	−8.2
	≧F50	88.2	8.2
	≧F80	75.0	18.9
	≧F95	29.4	7.7
V. An A base at position 3 of the sense strand	<F50	17.2	−2.8
	≧F50	82.8	2.8
	≧F80	62.5	6.4
	≧F95	34.4	12.7
VI. A U base at position 10 of the sense strand	<F50	13.9	−6.1
	≧F50	86.1	6.1
	≧F80	69.4	13.3
	≧F95	41.7	20
VII. A base other than C at position 19 of the sense strand	<F50	18.8	−1.2
	≧F50	81.2	1.2
	≧F80	59.7	3.6
	≧F95	24.2	2.5
VIII. A base other than G at position 13 of the sense strand	<F50	15.2	−4.8
	≧F50	84.8	4.8
	≧F80	61.4	5.3
	≧F95	26.5	4.8

The siRNA Selection Algorithm

[0348] In an effort to improve selection further, all identified criteria, including but not limited to those listed in Table IV were combined into the algorithms embodied in Formula VIII, Formula IX, and Formula X. Each siRNA was then assigned a score (referred to as a SMARTSCORE™, or siRNA ranking) according to the values derived from the formulas. Duplexes that scored higher than 0 or −20 (unadjusted), for Formulas VIII and IX, respectively, effectively selected a set of functional siRNAs and excluded all non-

functional siRNAs. Conversely, all duplexes scoring lower than 0 and −20 (minus 20) according to formulas VIII and IX, respectively, contained some functional siRNAs but included all non-functional siRNAs. A graphical representation of this selection is shown in FIG. **5**. It should be noted that the scores derived from the algorithm can also be provided as "adjusted" scores. To convert Formula VIII unadjusted scores into adjusted scores it is necessary to use the following equation:

(160+unadjusted score)/2.25

[0349] When this takes place, an unadjusted score of "0" (zero) is converted to 75. Similarly, unadjusted scores for Formula X can be converted to adjusted scores. In this instance, the following equation is applied:

(228+unadjusted score)/3.56

[0350] When these manipulations take place, an unadjusted score of 38 is converted to an adjusted score of 75.

[0351] The methods for obtaining the seven criteria embodied in Table IV are illustrative of the results of the process used to develop the information for Formulas VIII, IX, and X. Thus similar techniques were used to establish the other variables and their multipliers. As described above, basic statistical methods were use to determine the relative values for these multipliers.

[0352] To determine the value for "Improvement over Random" the difference in the frequency of a given attribute (e.g., GC content, base preference) at a particular position is determined between individual functional groups (e.g., <F50) and the total siRNA population studied (e.g., 270 siRNA molecules selected randomly). Thus, for instance, in Criterion I (30%-52% GC content) members of the <F50 group were observed to have GC contents between 30-52% in 16.4% of the cases. In contrast, the total group of 270 siRNAs had GC contents in this range, 20% of the time. Thus for this particular attribute, there is a small negative correlation between 30%-52% GC content and this functional group (i.e., 16.4%-20%=−3.6%). Similarly, for Criterion VI, (a "U" at position 10 of the sense strand), the >F95 group contained a "U" at this position 41.7% of the time. In contrast, the total group of 270 siRNAs had a "U" at this position 21.7% of the time, thus the improvement over random is calculated to be 20% (or 41.7%-21.7%).

Identifying the Average Internal Stability Profile of Strong siRNA

[0353] In order to identify an internal stability profile that is characteristic of strong siRNA, 270 different siRNAs derived from the cyclophilin B, the diazepam binding inhibitor (DBI), and the luciferase gene were individually transfected into HEK293 cells and tested for their ability to induce RNAi of the respective gene. Based on their performance in the in vivo assay, the sequences were then subdivided into three groups, (i) >95% silencing; (ii) 80-95% silencing; and (iii) less than 50% silencing. Sequences exhibiting 51-84% silencing were eliminated from further consideration to reduce the difficulties in identifying relevant thermodynamic patterns.

[0354] Following the division of siRNA into three groups, a statistical analysis was performed on each member of each group to determine the average internal stability profile (AISP) of the siRNA. To accomplish this the Oligo 5.0 Primer Analysis Software and other related statistical packages (e.g., Excel) were exploited to determine the internal stability of pentamers using the nearest neighbor method described by Freier et al., (1986) Improved free-energy parameters for predictions of RNA duplex stability, *Proc Natl. Acad. Sci. USA* 83(24): 9373-7. Values for each group at each position were then averaged, and the resulting data were graphed on a linear coordinate system with the Y-axis expressing the ΔG (free energy) values in kcal/mole and the X-axis identifying the position of the base relative to the 5' end.

[0355] The results of the analysis identified multiple key regions in siRNA molecules that were critical for successful gene silencing. At the 3'-most end of the sense strand (5'antisense), highly functional siRNA (>95% gene silencing, see FIG. **6***a*, >F95) have a low internal stability (AISP of position 19=~−7.6 kcal/mol). In contrast low-efficiency siRNA (i.e., those exhibiting less than 50% silencing, <F50) display a distinctly different profile, having high ΔG values (~−8.4 kcal/mol) for the same position. Moving in a 5' (sense strand) direction, the internal stability of highly efficient siRNA rises (position 12=~−8.3 kcal/mole) and then drops again (position 7=~−7.7 kcal/mol) before leveling off at a value of approximately −8.1 kcal/mol for the 5' terminus. siRNA with poor silencing capabilities show a distinctly different profile. While the AISP value at position 12 is nearly identical with that of strong siRNAs, the values at positions 7 and 8 rise considerably, peaking at a high of ~−9.0 kcal/mol. In addition, at the 5' end of the molecule the AISP profile of strong and weak siRNA differ dramatically. Unlike the relatively strong values exhibited by siRNA in the >95% silencing group, siRNAs that exhibit poor silencing activity have weak AISP values (−7.6, −7.5, and −7.5 kcal/mol for positions 1, 2 and 3 respectively).

[0356] Overall the profiles of both strong and weak siRNAs form distinct sinusoidal shapes that are roughly 180° out-of-phase with each other. While these thermodynamic descriptions define the archetypal profile of a strong siRNA, it will likely be the case that neither the ΔG values given for key positions in the profile or the absolute position of the profile along the Y-axis (i.e., the ΔG-axis) are absolutes. Profiles that are shifted upward or downward (i.e., having on an average, higher or lower values at every position) but retain the relative shape and position of the profile along the X-axis can be foreseen as being equally effective as the model profile described here. Moreover, it is likely that siRNA that have strong or even stronger gene-specific silencing effects might have exaggerated ΔG values (either higher or lower) at key positions. Thus, for instance, it is possible that the 5'-most position of the sense strand (position 19) could have ΔG values of 7.4 kcal/mol or lower and still be a strong siRNA if, for instance, a G-C→G-T/U mismatch were substituted at position 19 and altered duplex stability. Similarly, position 12 and position 7 could have values above 8.3 kcal/mol and below 7.7 kcal/mole, respectively, without abating the silencing effectiveness of the molecule. Thus, for instance, at position 12, a stabilizing chemical modification (e.g., a chemical modification of the 2' position of the sugar backbone) could be added that increases the average internal stability at that position. Similarly, at position 7, mismatches similar to those described previously could be introduced that would lower the ΔG values at that position.

[0357] Lastly, it is important to note that while functional and non-functional siRNA were originally defined as those molecules having specific silencing properties, both broader or more limiting parameters can be used to define these molecules. As used herein, unless otherwise specified, "non-functional siRNA" are defined as those siRNA that induce less than 50% (<50%) target silencing, "semi-functional siRNA" induce 50-79% target silencing, "functional siRNA" are molecules that induce 80-95% gene silencing, and "highly-functional siRNA" are molecules that induce great than 95% gene silencing. These definitions are not intended to be rigid and can vary depending upon the design and needs of the application. For instance, it is possible that a researcher attempting to map a gene to a chromosome using a functional assay, may identify an siRNA that reduces gene activity by only 30%. While this level of gene silencing may be "non-functional" for, e.g., therapeutic needs, it is sufficient for gene mapping purposes and is, under these uses and conditions, "functional." For these reasons, functional siRNA can be defined as those molecules having greater than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% silencing capabilities at 100 nM transfection conditions. Similarly, depending upon the needs of the study and/or application, non-functional and semi-functional siRNA can be defined as having different parameters. For instance, semi-functional siRNA can be defined as being those molecules that induce 20%, 30%, 40%, 50%, 60%, or 70% silencing at 100 nM transfection conditions. Similarly, non-functional siRNA can be defined as being those molecules that silence gene expression by less than 70%, 60%, 50%, 40%, 30%, or less. Nonetheless, unless otherwise stated, the descriptions stated in the "Definitions" section of this text should be applied.

[0358] Functional attributes can be assigned to each of the key positions in the AISP of strong siRNA. The low 5' (sense strand) AISP values of strong siRNAs may be necessary for determining which end of the molecule enters the RISC complex. In contrast, the high and low AISP values observed in the central regions of the molecule may be critical for siRNA-target mRNA interactions and product release, respectively.

[0359] If the AISP values described above accurately define the thermodynamic parameters of strong siRNA, it would be expected that similar patterns would be observed in strong siRNA isolated from nature. Natural siRNAs exist in a harsh, RNase-rich environment and it can be hypothesized that only those siRNA that exhibit heightened affinity for RISC (i.e., siRNA that exhibit an average internal stability profile similar to those observed in strong siRNA) would survive in an intracellular environment. This hypothesis was tested using GFP-specific siRNA isolated from *N. benthamiana*. Llave et al. (2002) Endogenous and Silencing-Associated Small RNAs in Plants, *The Plant Cell* 14, 1605-1619, introduced long double-stranded GFP-encoding RNA into plants and subsequently re-isolated GFP-specific siRNA from the tissues. The AISP of fifty-nine of these GFP-siRNA were determined, averaged, and subsequently plotted alongside the AISP profile obtained from the cyclophilin B/DBI/luciferase siRNA having >90% silencing properties (FIG. **6***b*). Comparison of the two groups show that profiles are nearly identical. This finding validates the information provided by the internal stability profiles and demonstrates that: (1) the profile identified by analysis of the cyclophilin B/DBI/luciferase siRNAs are not gene specific; and (2) AISP values can be used to search for strong siRNAs in a variety of species.

[0360] Both chemical modifications and base-pair mismatches can be incorporated into siRNA to alter the duplex's AISP and functionality. For instance, introduction of mismatches at positions 1 or 2 of the sense strand destabilized the 5' end of the sense strand and increases the functionality of the molecule (see Luc, FIG. **7**). Similarly, addition of 2'-O-methyl groups to positions 1 and 2 of the sense strand can also alter the AISP and (as a result) increase both the functionality of the molecule and eliminate off-target effects that results from sense strand homology with the unrelated targets (FIG. **8**).

Rationale for Criteria in a Biological Context

[0361] The fate of siRNA in the RNAi pathway may be described in 5 major steps: (1) duplex recognition and pre-RISC complex formation; (2) ATP-dependent duplex unwinding/strand selection and RISC activation; (3) mRNA target identification; (4) mRNA cleavage, and (5) product release (FIG. **1**). Given the level of nucleic acid-protein interactions at each step, siRNA functionality is likely influenced by specific biophysical and molecular properties that promote efficient interactions within the context of the multi-component complexes. Indeed, the systematic analysis of the siRNA test set identified multiple factors that correlate well with functionality. When combined into a single algorithm, they proved to be very effective in selecting active siRNAs.

[0362] The factors described here may also be predictive of key functional associations important for each step in RNAi. For example, the potential formation of internal hairpin structures correlated negatively with siRNA functionality. Complementary strands with stable internal repeats are more likely to exist as stable hairpins thus decreasing the effective concentration of the functional duplex form. This suggests that the duplex is the preferred conformation for initial pre-RISC association. Indeed, although single complementary strands can induce gene silencing, the effective concentration required is at least two orders of magnitude higher than that of the duplex form.

[0363] siRNA-pre-RISC complex formation is followed by an ATP-dependent duplex unwinding step and "activation" of the RISC. The siRNA functionality was shown to correlate with overall low internal stability of the duplex and low internal stability of the 3' sense end (or differential internal stability of the 3' sense compare to the 5' sense strand), which may reflect strand selection and entry into the RISC. Overall duplex stability and low internal stability at the 3' end of the sense strand were also correlated with siRNA functionality. Interestingly, siRNAs with very high and very low overall stability profiles correlate strongly with non-functional duplexes. One interpretation is that high internal stability prevents efficient unwinding while very low stability reduces siRNA target affinity and subsequent mRNA cleavage by the RISC.

[0364] Several criteria describe base preferences at specific positions of the sense strand and are even more intriguing when considering their potential mechanistic roles in target recognition and mRNA cleavage. Base preferences for A at position 19 of the sense strand but not C, are particularly

interesting because they reflect the same base preferences observed for naturally occurring miRNA precursors. That is, among the reported miRNA precursor sequences 75% contain a U at position 1 which corresponds to an A in position 19 of the sense strand of siRNAs, while G was under-represented in this same position for miRNA precursors. These observations support the hypothesis that both miRNA precursors and siRNA duplexes are processed by very similar if not identical protein machinery. The functional interpretation of the predominance of a U/A base pair is that it promotes flexibility at the 5'antisense ends of both siRNA duplexes and miRNA precursors and facilitates efficient unwinding and selective strand entrance into an activated RISC.

[0365] Among the criteria associated with base preferences that are likely to influence mRNA cleavage or possibly product release, the preference for U at position 10 of the sense strand exhibited the greatest impact, enhancing the probability of selecting an F80 sequence by 13.3%. Activated RISC preferentially cleaves target mRNA between nucleotides 10 and 11 relative to the 5' end of the complementary targeting strand. Therefore, it may be that U, the preferred base for most endoribonucleases, at this position supports more efficient cleavage. Alternatively, a U/A bp between the targeting siRNA strand and its cognate target in RNA may create an optimal conformation for the RISC-associated "slicing" activity.

Post Algorithm Filters

[0366] According to another embodiment, the output of any one of the formulas previously listed can be filtered to remove or select for siRNAs containing undesirable or desirable motifs or properties, respectively. In one example, sequences identified by any of the formulas can be filtered to remove any and all sequences that induce toxicity or cellular stress. Introduction of an siRNA containing a toxic motif into a cell can induce cellular stress and/or cell death (apoptosis) which in turn can mislead researchers into associating a particular (e.g., nonessential) gene with, e.g., an essential function. Alternatively, sequences generated by any of the before mentioned formulas can be filtered to identify and retain duplexes that contain toxic motifs. Such duplexes may be valuable from a variety of perspectives including, for instance, uses as therapeutic molecules. A variety of toxic motifs exist and can exert their influence on the cell through RNAi and non-RNAi pathways. Examples of toxic motifs are explained more fully in commonly assigned U.S. Provisional Patent Application Ser. No. 60/538,874, entitled "Identification of Toxic Sequences," filed Jan. 23, 2004. Briefly, toxic: motifs include A/G UUU A/G/U, G/C AAA G/C, and GCCA, or a complement of any of the foregoing.

[0367] In another instance, sequences identified by any of the before mentioned formulas can be filtered to identify duplexes that contain motifs (or general properties) that provide serum stability or induce serum instability. In one envisioned application of siRNA as therapeutic molecules, duplexes targeting disease-associated genes will be introduced into patients intravenously. As the half-life of single and double stranded RNA in serum is short, post-algorithm filters designed to select molecules that contain motifs that enhance duplex stability in the presence of serum and/or (conversely) eliminate duplexes that contain motifs that destabilize siRNA in the presence of serum, would be beneficial.

[0368] In another instance, sequences identified by any of the before mentioned formulas can be filtered to identify duplexes that are hyperfunctional. Hyperfunctional sequences are defined as those sequences that (1) induce greater than 95% silencing of a specific target when they are transfected at subnanomolar concentrations (i.e., less than one nanomolar); and/or (2) induce functional (or better) levels of silencing for greater than 96 hours. Filters that identify hyperfunctional molecules can vary widely. In one example, the top ten, twenty, thirty, or forty siRNA can be assessed for the ability to silence a given target at, e.g., concentrations of 1 nM and 0.5 nM to identify hyperfunctional molecules.

Pooling

[0369] According to another embodiment, the present invention provides a pool of at least two siRNAs, preferably in the form of a kit or therapeutic reagent, wherein one strand of each of the siRNAs, the sense strand comprises a sequence that is substantially similar to a sequence within a target mRNA. The opposite strand, the antisense strand, will preferably comprise a sequence that is substantially complementary to that of the target mRNA. More preferably, one strand of each siRNA will comprise a sequence that is identical to a sequence that is contained in the target mRNA. Most preferably, each siRNA will be 19 base pairs in length, and one strand of each of the siRNAs will be 100% complementary to a portion of the target mRNA.

[0370] By increasing the number of siRNAs directed to a particular target using a pool or kit, one is able both to increase the likelihood that at least one siRNA with satisfactory functionality will be included, as well as to benefit from additive or synergistic effects. Further, when two or more siRNAs directed against a single gene do not have satisfactory levels of functionality alone, if combined, they may satisfactorily promote degradation of the target messenger RNA and successfully inhibit translation. By including multiple siRNAs in the system, not only is the probability of silencing increased, but the economics of operation are also improved when compared to adding different siRNAs sequentially. This effect is contrary to the conventional wisdom that the concurrent use of multiple siRNA will negatively impact gene silencing (e.g., Holen, T. et al. (2003) Similar behavior of single strand and double strand siRNAs suggests they act through a common RNAi pathway. *NAR* 31: 2401-21407).

[0371] In fact, when two siRNAs were pooled together, 54% of the pools of two siRNAs induced more than 95% gene silencing. Thus, a 2.5-fold increase in the percentage of functionality was achieved by randomly combining two siRNAs. Further, over 84% of pools containing two siRNAs induced more than 80% gene silencing.

[0372] More preferably, the kit is comprised of at least three siRNAs, wherein one strand of each siRNA comprises a sequence that is substantially similar to a sequence of the target mRNA and the other strand comprises a sequence that is substantially complementary to the region of the target mRNA. As with the kit that comprises at least two siRNAs, more preferably one strand will comprise a sequence that is

identical to a sequence that is contained in the mRNA and another strand that is 100% complementary to a sequence that is contained in the mRNA. During experiments, when three siRNAs were combined together, 60% of the pools induced more than 95% gene silencing and 92% of the pools induced more than 80% gene silencing.

[0373] Further, even more preferably, the kit is comprised of at least four siRNAs, wherein one strand of each siRNA comprises a sequence that is substantially similar to a region of the sequence of the target mRNA, and the other strand comprises a sequence that is substantially complementary to the region of the target mRNA. As with the kit or pool that comprises at least two siRNAs, more preferably one strand of each of the siRNA duplexes will comprise a sequence that is identical to a sequence that is contained in the mRNA, and another strand that is 100% complementary to a sequence that is contained in the mRNA.

[0374] Additionally, kits and pools with at least five, at least six, and at least seven siRNAs may also be useful with the present invention. For example, pools of five siRNA induced 95% gene silencing with 77% probability and 80% silencing with 98.8% probability. Thus, pooling of siRNAs together can result in the creation of a target-specific silencing reagent with almost a 99% probability of being functional. The fact that such high levels of success are achievable using such pools of siRNA, enables one to dispense with costly and time-consuming target-specific validation procedures.

[0375] For this embodiment, as well as the other aforementioned embodiments, each of the siRNAs within a pool will preferably comprise 18-30 base pairs, more preferably 18-25 base pairs, and most preferably 19 base pairs. Within each siRNA, preferably at least 18 contiguous bases of the antisense strand will be 100% complementary to the target mRNA. More preferably, at least 19 contiguous bases of the antisense strand will be 100% complementary to the target mRNA. Additionally, there may be overhangs on either the sense strand or the antisense strand, and these overhangs may be at either the 5' end or the 3' end of either of the strands, for example there may be one or more overhangs of 1-6 bases. When overhangs are present, they are not included in the calculation of the number of base pairs. The two nucleotide 3' overhangs mimic natural siRNAs and are commonly used but are not essential. Preferably, the overhangs should consist of two nucleotides, most often dTdT or UU at the 3' end of the sense and antisense strand that are not complementary to the target sequence. The siRNAs may be produced by any method that is now known or that comes to be known for synthesizing double stranded RNA that one skilled in the art would appreciate would be useful in the present invention. Preferably, the siRNAs will be produced by Dharmacon's proprietary ACE® technology. However, other methods for synthesizing siRNAs are well known to persons skilled in the art and include, but are not limited to, any chemical synthesis of RNA oligonucleotides, ligation of shorter oligonucleotides, in vitro transcription of RNA oligonucleotides, the use of vectors for expression within cells, recombinant Dicer products and PCR products.

[0376] The siRNA duplexes within the aforementioned pools of siRNAs may correspond to overlapping sequences within a particular mRNA, or non-overlapping sequences of the mRNA. However, preferably they correspond to non-overlapping sequences. Further, each siRNA may be selected randomly, or one or more of the siRNA may be selected according to the criteria discussed above for maximizing the effectiveness of siRNA.

[0377] Included in the definition of siRNAs are siRNAs that contain substituted and/or labeled nucleotides that may, for example, be labeled by radioactivity, fluorescence or mass. The most common substitutions are at the 2' position of the ribose sugar, where moieties such as H (hydrogen) F, NH_3, OCH_3 and other O— alkyl, alkenyl, alkynyl, and orthoesters, may be substituted, or in the phosphorous backbone, where sulfur, amines or hydrocarbons may be substituted for the bridging of non-bridging atoms in the phosphodiester bond. Examples of modified siRNAs are explained more fully in commonly assigned U.S. patent application Ser. No. 10/613,077, filed Jul. 1, 2003.

[0378] Additionally, as noted above, the cell type into which the siRNA is introduced may affect the ability of the siRNA to enter the cell; however, it does not appear to affect the ability of the siRNA to function once it enters the cell. Methods for introducing double-stranded RNA into various cell types are well known to persons skilled in the art.

[0379] As persons skilled in the art are aware, in certain species, the presence of proteins such as RdRP, the RNA-dependent RNA polymerase, may catalytically enhance the activity of the siRNA. For example, RdRP propagates the RNAi effect in *C. elegans* and other non-mammalian organisms. In fact, in organisms that contain these proteins, the siRNA may be inherited. Two other proteins that are well studied and known to be a part of the machinery are members of the Argonaute family and Dicer, as well as their homologues. There is also initial evidence that the RISC complex might be associated with the ribosome so the more efficiently translated mRNAs will be more susceptible to silencing than others.

[0380] Another very important factor in the efficacy of siRNA is mRNA localization. In general, only cytoplasmic in RNAs are considered to be accessible to RNAi to any appreciable degree. However, appropriately designed siRNAs, for example, siRNAs modified with internucleotide linkages or 2'-O-methyl groups, may be able to cause silencing by acting in the nucleus. Examples of these types of modifications are described in commonly assigned U.S. patent application Ser. Nos. 10/431,027 and 10/613,077.

[0381] As described above, even when one selects at least two siRNAs at random, the effectiveness of the two may be greater than one would predict based on the effectiveness of two individual siRNAs. This additive or synergistic effect is particularly noticeable as one increases to at least three siRNAs, and even more noticeable as one moves to at least four siRNAs. Surprisingly, the pooling of the non-functional and semi-functional siRNAs, particularly more than five siRNAs, can lead to a silencing mixture that is as effective if not more effective than any one particular functional siRNA.

[0382] Within the kits of the present invention, preferably each siRNA will be present in a concentration of between 0.001 and 200 μM, more preferably between 0.01 and 200 nM, and most preferably between 0.1 and 10 nM.

[0383] In addition to preferably comprising at least four or five siRNAs, the kits of the present invention will also

preferably comprise a buffer to keep the siRNA duplex stable. Persons skilled in the art are aware of buffers suitable for keeping siRNA stable. For example, the buffer may be comprised of 100 mM KCl, 30 mM HEPES-pH 7.5, and 1 mM $MgCl_2$. Alternatively, kits might contain complementary strands that contain any one of a number of chemical modifications (e.g., a 2'-O-ACE) that protect the agents from degradation by nucleases. In this instance, the user may (or may not) remove the modifying protective group (e.g., deprotect) before annealing the two complementary strands together.

[0384] By way of example, the kits may be organized such that pools of siRNA duplexes are provided on an array or microarray of wells or drops for a particular gene set or for unrelated genes. The array may, for example, be in 96 wells, 384 wells or 1284 wells arrayed in a plastic plate or on a glass slide using techniques now known or that come to be known to persons skilled in the art. Within an array, preferably there will be controls such as functional anti-lamin A/C, cyclophilin and two siRNA duplexes that are not specific to the gene of interest.

[0385] In order to ensure stability of the siRNA pools prior to usage, they may be retained in lyophilized form at minus twenty degrees (−20° C.) until they are ready for use. Prior to usage, they should be resuspended; however, even once resuspended, for example, in the aforementioned buffer, they should be kept at minus twenty degrees, (−20° C.) until used. The aforementioned buffer, prior to use, may be stored at approximately 4° C. or room temperature. Effective temperatures at which to conduct transfections are well known to persons skilled in the art and include for example, room temperature.

[0386] The kits may be applied either in vivo or in vitro. Preferably, the siRNA of the pools or kits is applied to a cell through transfection, employing standard transfection protocols. These methods are well known to persons skilled in the art and include the use of lipid-based carriers, electroporation, cationic carriers, and microinjection. Further, one could apply the present invention by synthesizing equivalent DNA sequences (either as two separate, complementary strands, or as hairpin molecules) instead of siRNA sequences and introducing them into cells through vectors. Once in the cells, the cloned DNA could be transcribed, thereby forcing the cells to generate the siRNA. Examples of vectors suitable for use with the present application include but are not limited to the standard transient expression vectors, adenoviruses, retroviruses, lentivirus-based vectors, as well as other traditional expression vectors. Any vector that has an adequate siRNA expression and procession module may be used. Furthermore, certain chemical modifications to siRNAs, including but not limited to conjugations to other molecules, may be used to facilitate delivery. For certain applications it may be preferable to deliver molecules without transfection by simply formulating in a physiological acceptable solution.

[0387] This embodiment may be used in connection with any of the aforementioned embodiments. Accordingly, the sequences within any pool may be selected by rational design.

Multigene Silencing

[0388] In addition to developing kits that contain multiple siRNA directed against a single gene, another embodiment includes the use of multiple siRNA targeting multiple genes. Multiple genes may be targeted through the use of high- or hyper-functional siRNA. High- or hyper-functional siRNA that exhibit increased potency, require lower concentrations to induce desired phenotypic (and thus therapeutic) effects. This circumvents RISC saturation. It therefore reasons that if lower concentrations of a single siRNA are needed for knockout or knockdown expression of one gene, then the remaining (uncomplexed) RISC will be free and available to interact with siRNA directed against two, three, four, or more, genes. Thus in this embodiment, the authors describe the use of highly functional or hyper-functional siRNA to knock out three separate genes. More preferably, such reagents could be combined to knockout four distinct genes. Even more preferably, highly functional or hyperfunctional siRNA could be used to knock out five distinct genes. Most preferably, siRNA of this type could be used to knockout or knockdown the expression of six or more genes.

Hyperfunctional siRNA

[0389] The term hyperfunctional siRNA (hf-siRNA) describes a subset of the siRNA population that induces RNAi in cells at low- or sub-nanomolar concentrations for extended periods of time. These traits, heightened potency and extended longevity of the RNAi phenotype, are highly attractive from a therapeutic standpoint. Agents having higher potency require lesser amounts of the molecule to achieve the desired physiological response, thus reducing the probability of side effects due to "off-target" interference. In addition to the potential therapeutic benefits associated with hyperfunctional siRNA, hf-siRNA are also desirable from an economic perspective. Hyperfunctional siRNA may cost less on a per-treatment basis, thus reducing overall expenditures to both the manufacturer and the consumer.

[0390] Identification of hyperfunctional siRNA involves multiple steps that are designed to examine an individual siRNA agent's concentration- and/or longevity-profiles. In one non-limiting example, a population of siRNA directed against a single gene are first analyzed using the previously described algorithm (Formula VIII). Individual siRNA are then introduced into a test cell line and assessed for the ability to degrade the target mRNA. It is important to note that when performing this step it is not necessary to test all of the siRNA. Instead, it is sufficient to test only those siRNA having the highest SMARTSCORES™, or siRNA ranking (i.e., SMARTSCORES™M, or siRNA ranking >−10). Subsequently, the gene silencing data is plotted against the SMARTSCORES™, or siRNA rankings (see FIG. **9**). siRNA that (1) induce a high degree of gene silencing (i.e., they induce greater than 80% gene knockdown) and (2) have superior SMARTSCORES™ (i.e., a SMARTSCORE™, or siRNA ranking, of >−10, suggesting a desirable average internal stability profile) are selected for further investigations designed to better understand the molecule's potency and longevity. In one, non-limiting study dedicated to understanding a molecule's potency, an siRNA is introduced into one (or more) cell types in increasingly diminishing concentrations (e.g., 3.0→0.3 nM). Subsequently, the level of gene silencing induced by each concentration is examined and siRNA that exhibit hyperfunctional potency (i.e., those that induce 80% silencing or greater at, e.g., picomolar concentrations) are identified. In a second study, the longevity profiles of siRNA having high (>−10) SMARTSCORES™, or siRNA rankings and greater

than 80% silencing are examined. In one non-limiting example of how this is achieved, siRNA are introduced into a test cell line and the levels of RNAi are measured over an extended period of time (e.g., 24-168 hrs). siRNAs that exhibit strong RNA interference patterns (i.e., >80% interference) for periods of time greater than, e.g., 120 hours, are thus identified. Studies similar to those described above can be performed on any and all of the $>10^6$ siRNA included in this document to further define the most functional molecule for any given gene. Molecules possessing one or both properties (extended longevity and heightened potency) are labeled "hyperfunctional siRNA," and earmarked as candidates for future therapeutic studies.

[0391] While the example(s) given above describe one means by which hyperfunctional siRNA can be isolated, neither the assays themselves nor the selection parameters used are rigid and can vary with each family of siRNA. Families of siRNA include siRNAs directed against a single gene, or directed against a related family of genes.

[0392] The highest quality siRNA achievable for any given gene may vary considerably. Thus, for example, in the case of one gene (gene X), rigorous studies such as those described above may enable the identification of an siRNA that, at picomolar concentrations, induces 99^{+}% silencing for a period of 10 days. Yet identical studies of a second gene (gene Y) may yield an siRNA that at high nanomolar concentrations (e.g., 100 nM) induces only 75% silencing for a period of 2 days. Both molecules represent the very optimum siRNA for their respective gene targets and therefore are designated "hyperfunctional." Yet due to a variety of factors including but not limited to target concentration, siRNA stability, cell type, off-target interference, and others, equivalent levels of potency and longevity are not achievable. Thus, for these reasons, the parameters described in the before mentioned assays can vary. While the initial screen selected siRNA that had SMARTSCORES™ above −10 and a gene silencing capability of greater than 80%, selections that have stronger (or weaker) parameters can be implemented. Similarly, in the subsequent studies designed to identify molecules with high potency and longevity, the desired cutoff criteria (i.e., the lowest concentration that induces a desirable level of interference, or the longest period of time that interference can be observed) can vary. The experimentation subsequent to application of the rational criteria of this application is significantly reduced where one is trying to obtain a suitable hyperfunctional siRNA for, for example, therapeutic use. When, for example, the additional experimentation of the type described herein is applied by one skilled in the art with this disclosure in hand, a hyperfunctional siRNA is readily identified.

[0393] The siRNA may be introduced into a cell by any method that is now known or that comes to be known and that from reading this disclosure, persons skilled in the art would determine would be useful in connection with the present invention in enabling siRNA to cross the cellular membrane. These methods include, but are not limited to, any manner of transfection, such as, for example, transfection employing DEAE-Dextran, calcium phosphate, cationic lipids/liposomes, micelles, manipulation of pressure, microinjection, electroporation, immunoporation, use of vectors such as viruses, plasmids, cosmids, bacteriophages, cell fusions, and coupling of the polynucleotides to specific conjugates or ligands such as antibodies, antigens, or receptors, passive introduction, adding moieties to the siRNA that facilitate its uptake, and the like.

[0394] Having described the invention with a degree of particularity, examples will now be provided. These examples are not intended to and should not be construed to limit the scope of the claims in any way.

EXAMPLES

General Techniques and Nomenclatures

[0395] siRNA nomenclature. All siRNA duplexes are referred to by sense strand. The first nucleotide of the 5'-end of the sense strand is position 1, which corresponds to position 19 of the antisense strand for a 19-mer. In most cases, to compare results from different experiments, silencing was determined by measuring specific transcript mRNA levels or enzymatic activity associated with specific transcript levels, 24 hours post-transfection, with siRNA concentrations held constant at 100 nM. For all experiments, unless otherwise specified, transfection efficiency was ensured to be over 95%, and no detectable cellular toxicity was observed. The following system of nomenclature was used to compare and report siRNA-silencing functionality: "F" followed by the degree of minimal knockdown. For example, F50 signifies at least 50% knockdown, F80 means at least 80%, and so forth. For this study, all sub-F50 siRNAs were considered non-functional.

[0396] Cell culture and transfection. 96-well plates are coated with 50 μl of 50 mg/ml poly-L-lysine (Sigma) for 1 hr, and then washed 3× with distilled water before being dried for 20 min. HEK293 cells or HEK293Lucs or any other cell type of interest are released from their solid support by trypsinization, diluted to 3.5×10^5 cells/ml, followed by the addition of 100 μL of cells/well. Plates are then incubated overnight at 37° C., 5% CO_2. Transfection procedures can vary widely depending on the cell type and transfection reagents. In one non-limiting example, a transfection mixture consisting of 2 mL Opti-MEM 1 (Gibco-BRL), 80 μl Lipofectamine 2000 (Invitrogen), 15 μL SUPERNasin at 20 U/μl (Ambion), and 1.5 μl of reporter gene plasmid at 1 μg/μl is prepared in 5-ml polystyrene round bottom tubes. One hundred μl of transfection reagent is then combined with 100 μl of siRNAs in polystyrene deep-well titer plates (Beckman) and incubated for 20 to 30 min at room temperature. Five hundred and fifty microliters of Opti-MEM is then added to each well to bring the final siRNA concentration to 100 nM. Plates are then sealed with parafilm and mixed. Media is removed from HEK293 cells and replaced with 95 μl of transfection mixture. Cells are incubated overnight at 37° C., 5% CO_2.

[0397] Quantification of gene knockdown. A variety of quantification procedures can be used to measure the level of silencing induced by siRNA or siRNA pools. In one non-limiting example: to measure mRNA levels 24 hrs post-transfection, QuantiGene branched-DNA (bDNA) kits (Bayer) (Wang, et al, *Regulation of insulin preRNA splicing by glucose*. Proc. Natl. Acad. Sci. USA 1997, 94:4360.) are used according to manufacturer instructions. To measure luciferase activity, media is removed from HEK293 cells 24 hrs post-transfection, and 50 μl of Steady-GLO reagent (Promega) is added. After 5 minutes, plates are analyzed on a plate reader.

Example I

Sequences Used to Develop the Algorithm

[0398] Anti-Firefly and anti-Cyclophilin siRNAs panels (FIG. **5***a, b*) sorted according to using Formula VIII predicted values. All siRNAs scoring more than 0 (formula VIII) and more then 20 (formula IX) are fully functional. All ninety sequences for each gene (and DBI) appear below in Table III.

TABLE III

Cyclo	1	SEQ. ID 0032	GUUCCAAAAACAGUGGAUA
Cyclo	2	SEQ. ID 0033	UCCAAAAACAGUGGAUAAU
Cyclo	3	SEQ. ID 0034	CAAAAACAGUGGAUAAUUU
Cyclo	4	SEQ. ID 0035	AAAACAGUGGAUAAUUUUG
Cyclo	5	SEQ. ID 0036	AACAGUGGAUAAUUUUGUG
Cyclo	6	SEQ. ID 0037	CAGUGGAUAAUUUUGUGGC
Cyclo	7	SEQ. ID 0038	GUGGAUAAUUUUGUGGCCU
Cyclo	8	SEQ. ID 0039	GGAUAAUUUUGUGGCCUUA
Cyclo	9	SEQ. ID 0040	AUAAUUUUGUGGCCUUAGC
Cyclo	10	SEQ. ID 0041	AAUUUUGUGGCCUUAGCUA
Cyclo	11	SEQ. ID 0042	UUUUGUGGCCUUAGCUACA
Cyclo	12	SEQ. ID 0043	UUGUGGCCUUAGCUACAGG
Cyclo	13	SEQ. ID 0044	GUGGCCUUAGCUACAGGAG
Cyclo	14	SEQ. ID 0045	GGCCUUAGCUACAGGAGAG
Cyclo	15	SEQ. ID 0046	CCUUAGCUACAGGAGAGAA
Cyclo	16	SEQ. ID 0047	UUAGCUACAGGAGAGAAAG
Cyclo	17	SEQ. ID 0048	AGCUACAGGAGAGAAAGGA
Cyclo	18	SEQ. ID 0049	CUACAGGAGAGAAAGGAUU
Cyclo	19	SEQ. ID 0050	ACAGGAGAGAAAGGAUUUG
Cyclo	20	SEQ. ID 0051	AGGAGAGAAAGGAUUUGGC
Cyclo	21	SEQ. ID 0052	GAGAGAAAGGAUUUGGCUA
Cyclo	22	SEQ. ID 0053	GAGAAAGGAUUUGGCUACA
Cyclo	23	SEQ. ID 0054	GAAAGGAUUUGGCUACAAA
Cyclo	24	SEQ. ID 0055	AAGGAUUUGGCUACAAAAA
Cyclo	25	SEQ. ID 0056	GCAUUUGGCUACAAAAACA
Cyclo	26	SEQ. ID 0057	AUUUGGCUACAAAAACAGC
Cyclo	27	SEQ. ID 0058	UUGGCUACAAAAACAGCAA
Cyclo	28	SEQ. ID 0059	GGCUACAAAAACAGCAAAU
Cyclo	29	SEQ. ID 0060	CUACAAAAACAGCAAAUUC
Cyclo	30	SEQ. ID 0061	ACAAAAACAGCAAAUUCCA
Cyclo	31	SEQ. ID 0062	AAAAACAGCAAAUUCCAUC
Cyclo	32	SEQ. ID 0063	AAACAGCAAAUUCCAUCGU

TABLE III-continued

Cyclo	33	SEQ. ID 0064	ACAGCAAAUUCCAUCGUGU
Cyclo	34	SEQ. ID 0065	AGCAAAUUCCAUCGUGUAA
Cyclo	35	SEQ. ID 0066	CAAAUUCCAUCGUGUAAUC
Cyclo	36	SEQ. ID 0067	AAUUCCAUCGUGUAAUCAA
Cyclo	37	SEQ. ID 0068	UUCCAUCGUGUAAUCAAGG
Cyclo	38	SEQ. ID 0069	CCAUCGUGUAAUCAAGGAC
Cyclo	39	SEQ. ID 0070	AUCGUGUAAUCAAGGACUU
Cyclo	40	SEQ. ID 0071	CGUGUAAUCAAGGACUUCA
Cyclo	41	SEQ. ID 0072	UGUAAUCAAGGACUUCAUG
Cyclo	42	SEQ. ID 0073	UAAUCAAGGACUUCAUGAU
Cyclo	43	SEQ. ID 0074	AUCAAGGACUUCAUGAUCC
Cyclo	44	SEQ. ID 0075	CAAGGACUUCAUGAUCCAG
Cyclo	45	SEQ. ID 0076	AGGACUUCAUGAUCCAGGG
Cyclo	46	SEQ. ID 0077	GACUUCAUGAUCCAGGGCG
Cyclo	47	SEQ. ID 0078	CUUCAUGAUCCAGGGCGGA
Cyclo	48	SEQ. ID 0079	UCAUGAUCCAGGGCGGAGA
Cyclo	49	SEQ. ID 0080	AUGAUCCAGGGCGGAGACU
Cyclo	50	SEQ. ID 0081	GAUCCAGGGCGGAGACUUC
Cyclo	51	SEQ. ID 0082	UCCAGGGCGGAGACUUCAC
Cyclo	52	SEQ. ID 0083	CAGGGCGGAGACUUCACCA
Cyclo	53	SEQ. ID 0084	GGGCGGAGACUUCACCAGG
Cyclo	54	SEQ. ID 0085	GCGGAGACUUCACCAGGGG
Cyclo	55	SEQ. ID 0086	GGAGACUUCACCAGGGGAG
Cyclo	56	SEQ. ID 0087	AGACUUCACCAGGGGAGAU
Cyclo	57	SEQ. ID 0088	ACUUCACCAGGGGAGAUGG
Cyclo	58	SEQ. ID 0089	UUCACCAGGGGAGAUGGCA
Cyclo	59	SEQ. ID 0090	CACCAGGGGAGAUGGCACA
Cyclo	60	SEQ. ID 0091	CCAGGGGAGAUGGCACAGG
Cyclo	61	SEQ. ID 0092	AGGGGAGAUGGCACAGGAG
Cyclo	62	SEQ. ID 0093	GGGAGAUGGCACAGGAGGA
Cyclo	63	SEQ. ID 0094	GAGAUGGCACAGGAGGAAA
Cyclo	64	SEQ. ID 0095	GAUGGCACAGGAGGAAAGA
Cyclo	65	SEQ. ID 0096	UGGCACAGGAGGAAAGAGC
Cyclo	66	SEQ. ID 0097	GCACAGGAGGAAAGAGCAU
Cyclo	67	SEQ. ID 0098	ACAGGAGGAAAGAGCAUCU
Cyclo	68	SEQ. ID 0099	AGGAGGAAAGAGCAUCUAC
Cyclo	69	SEQ. ID 0100	GAGGAAAGAGCAUCUACGG
Cyclo	70	SEQ. ID 0101	GGAAAGAGCAUCUACGGUG

TABLE III-continued

Cyclo	71	SEQ. ID 0102	AAAGAGCAUCUACGGUGAG
Cyclo	72	SEQ. ID 0103	AGAGCAUCUACGGUGAGCG
Cyclo	73	SEQ. ID 0104	AGCAUCUACGGUGAGCGCU
Cyclo	74	SEQ. ID 0105	CAUCUACGGUGAGCGCUUC
Cyclo	75	SEQ. ID 0106	UCUACGGUGAGCGCUUCCC
Cyclo	76	SEQ. ID 0107	UACGGUGAGCGCUUCCCCG
Cyclo	77	SEQ. ID 0108	CGGUGAGCGCUUCCCCGAU
Cyclo	78	SEQ. ID 0109	GUGAGCGCUUCCCCGAUGA
Cyclo	79	SEQ. ID 0110	GAGCGCUUCCCCGAUGAGA
Cyclo	80	SEQ. ID 0111	GCGCUUCCCCGAUGAGAAC
Cyclo	81	SEQ. ID 0112	GCUUCCCCGAUGAGAACUU
Cyclo	82	SEQ. ID 0113	UUCCCCGAUGAGAACUUCA
Cyclo	83	SEQ. ID 0114	CCCCGAUGAGAACUUCAAA
Cyclo	84	SEQ. ID 0115	CCGAUGAGAACUUCAAACU
Cyclo	85	SEQ. ID 0116	GAUGAGAACUUCAAACUGA
Cyclo	86	SEQ. ID 0117	UGAGAACUUCAAACUGAAG
Cyclo	87	SEQ. ID 0118	AGAACUUCAAACUGAAGCA
Cyclo	88	SEQ. ID 0119	AACUUCAAACUGAAGCACU
Cyclo	89	SEQ. ID 0120	CUUCAAACUGAAGCACUAC
Cyclo	90	SEQ. ID 0121	UCAAACUGAAGCACUACGG
DB	1	SEQ. ID 0122	ACGGGCAAGGCCAAGUGGG
DB	2	SEQ. ID 0123	CGGGCAAGGCCAAGUGGGA
DB	3	SEQ. ID 0124	GGGCAAGGCCAAGUGGGAU
DB	4	SEQ. ID 0125	GGCAAGGCCAAGUGGGAUG
DB	5	SEQ. ID 0126	GCAAGGCCAAGUGGGAUGC
DB	6	SEQ. ID 0127	CAAGGCCAAGUGGGAUGCC
DB	7	SEQ. ID 0128	AAGGCCAAGUGGGAUGCCU
DB	8	SEQ. ID 0129	AGGCCAAGUGGGAUGCCUG
DB	9	SEQ. ID 0130	GGCCAAGUGGGAUGCCUGG
DB	10	SEQ. ID 0131	GCCAAGUGGGAUGCCUGGA
DB	11	SEQ. ID 0132	CCAAGUGGGAUGCCUGGAA
DB	12	SEQ. ID 0133	CAAGUGGGAUGCCUGGAAU
DB	13	SEQ. ID 0134	AAGUGGGAUGCCUGGAAUG
DB	14	SEQ. ID 0135	AGUGGGAUGCCUGGAAUGA
DB	15	SEQ. ID 0136	GUGGGAUGCCUGGAAUGAG
DB	16	SEQ. ID 0137	UGGGAUGCCUGGAAUGAGC
DB	17	SEQ. ID 0138	GGGAUGCCUGGAAUGAGCU
DB	18	SEQ. ID 0139	GGAUGCCUGGAAUGAGCUG

TABLE III-continued

DB	19	SEQ. ID 0140	GAUGCCUGGAAUGAGCUGA
DB	20	SEQ. ID 0141	AUGCCUGGAAUGAGCUGAA
DB	21	SEQ. ID 0142	UGCCUGGAAUGAGCUGAAA
DB	22	SEQ. ID 0143	GCCUGGAAUGAGCUGAAAG
DB	23	SEQ. ID 0144	CCUGGAAUGAGCUGAAAGG
DB	24	SEQ. ID 0145	CUGGAAUGAGCUGAAAGGG
DB	25	SEQ. ID 0146	UGGAAUGAGCUGAAAGGGA
DB	26	SEQ. ID 0147	GGAAUGAGCUGAAAGGGAC
DB	27	SEQ. ID 0148	GAAUGAGCUGAAAGGGACU
DB	28	SEQ. ID 0149	AAUGAGCUGAAAGGGACUU
DB	29	SEQ. ID 0150	AUGAGCUGAAAGGGACUUC
DB	30	SEQ. ID 0151	UGAGCUGAAAGGGACUUCC
DB	31	SEQ. ID 0152	GAGCUGAAAGGGACUUCCA
DB	32	SEQ. ID 0153	AGCUGAAAGGGACUUCCAA
DB	33	SEQ. ID 0154	GCUGAAAGGGACUUCCAAG
DB	34	SEQ. ID 0155	CUGAAAGGGACUUCCAAGG
DB	35	SEQ. ID 0156	UGAAAGGGACUUCCAAGGA
DB	36	SEQ. ID 0157	GAAAGGGACUUCCAAGGAA
DB	37	SEQ. ID 0158	AAAGGGACUUCCAAGGAAG
DB	38	SEQ. ID 0159	AAGGGACUUCCAAGGAAGA
DB	39	SEQ. ID 0160	AGGGACUUCCAAGGAAGAU
DB	40	SEQ. ID 0161	GGGACUUCCAAGGAAGAUG
DB	41	SEQ. ID 0162	GGACUUCCAAGGAAGAUGC
DB	42	SEQ. ID 0163	GACUUCCAAGGAAGAUGCC
DB	43	SEQ. ID 0164	ACUUCCAAGGAAGAUGCCA
DB	44	SEQ. ID 0165	CUUCCAAGGAAGAUGCCAU
DB	45	SEQ. ID 0166	UUCCAAGGAAGAUGCCAUG
DB	46	SEQ. ID 0167	UCCAAGGAAGAUGCCAUGA
DB	47	SEQ. ID 0168	CCAAGGAAGAUGCCAUGAA
DB	48	SEQ. ID 0169	CAAGGAAGAUGCCAUGAAA
DB	49	SEQ. ID 0170	AAGGAAGAUGCCAUGAAAG
DB	50	SEQ. ID 0171	AGGAAGAUGCCAUGAAAGC
DB	51	SEQ. ID 0172	GGAAGAUGCCAUGAAAGCU
DB	52	SEQ. ID 0173	GAAGAUGCCAUGAAAGCUU
DB	53	SEQ. ID 0174	AAGAUGCCAUGAAAGCUUA
DB	54	SEQ. ID 0175	AGAUGCCAUGAAAGCUUAC
DB	55	SEQ. ID 0176	GAUGCCAUGAAAGCUUACA
DB	56	SEQ. ID 0177	AUGCCAUGAAAGCUUACAU

TABLE III-continued

DB	57	SEQ. ID 0178	UGCCAUGAAAGCUUACAUC
DB	58	SEQ. ID 0179	GCCAUGAAAGCUUACAUCA
DB	59	SEQ. ID 0180	CCAUGAAAGCUUACAUCAA
DB	60	SEQ. ID 0181	CAUGAAAGCUUACAUCAAC
DB	61	SEQ. ID 0182	AUGAAAGCUUACAUCAACA
DB	62	SEQ. ID 0183	UGAAAGCUUACAUCAACAA
DB	63	SEQ. ID 0184	GAAAGCUUACAUCAACAAA
DB	64	SEQ. ID 0185	AAAGCUUACAUCAACAAAG
DB	65	SEQ. ID 0186	AAGCUUACAUCAACAAAGU
DB	66	SEQ. ID 0187	AGCUUACAUCAACAAAGUA
DB	67	SEQ. ID 0188	GCUUACAUCAACAAAGUAG
DB	68	SEQ. ID 0189	CUUACAUCAACAAAGUAGA
DB	69	SEQ. ID 0190	UUACAUCAACAAAGUAGAA
DB	70	SEQ. ID 0191	UACAUCAACAAAGUAGAAG
DB	71	SEQ. ID 0192	ACAUCAACAAAGUAGAAGA
DB	72	SEQ. ID 0193	CAUCAACAAAGUAGAAGAG
DB	73	SEQ. ID 0194	AUCAACAAAGUAGAAGAGC
DB	74	SEQ. ID 0195	UCAACAAAGUAGAAGAGCU
DB	75	SEQ. ID 0196	CAACAAAGUAGAAGAGCUA
DB	76	SEQ. ID 0197	AACAAAGUAGAAGAGCUAA
DB	77	SEQ. ID 0198	ACAAAGUAGAAGAGCUAAA
DB	78	SEQ. ID 0199	CAAAGUAGAAGAGCUAAAG
DB	79	SEQ. ID 0200	AAAGUAGAAGAGCUAAAGA
DB	80	SEQ. ID 0201	AAGUAGAAGAGCUAAAGAA
DB	81	SEQ. ID 0202	AGUAGAAGAGCUAAAGAAA
DB	82	SEQ. ID 0203	GUAGAAGAGCUAAAGAAAA
DB	83	SEQ. ID 0204	UAGAAGAGCUAAAGAAAAA
DB	84	SEQ. ID 0205	AGAAGAGCUAAAGAAAAAA
DB	85	SEQ. ID 0206	GAAGAGCUAAAGAAAAAAU
DB	86	SEQ. ID 0207	AAGAGCUAAAGAAAAAAUA
DB	87	SEQ. ID 0208	AGAGCUAAAGAAAAAAUAC
DB	88	SEQ. ID 0209	GAGCUAAAGAAAAAAUACG
DB	89	SEQ. ID 0210	AGCUAAAGAAAAAAUACGG
DB	90	SEQ. ID 0211	GCUAAAGAAAAAAUACGGG
Luc	1	SEQ. ID 0212	AUCCUCAUAAAGGCCAAGA
Luc	2	SEQ. ID 0213	AGAUCCUCAUAAAGGCCAA
Luc	3	SEQ. ID 0214	AGAGAUCCUCAUAAAGGCC
Luc	4	SEQ. ID 0215	AGAGAGAUCCUCAUAAAGG

TABLE III-continued

Luc	5	SEQ. ID 0216	UCAGAGAGAUCCUCAUAAA
Luc	6	SEQ. ID 0217	AAUCAGAGAGAUCCUCAUA
Luc	7	SEQ. ID 0218	AAAAUCAGAGAGAUCCUCA
Luc	8	SEQ. ID 0219	GAAAAAUCAGAGAGAUCCU
Luc	9	SEQ. ID 0220	AAGAAAAAUCAGAGAGAUC
Luc	10	SEQ. ID 0221	GCAAGAAAAAUCAGAGAGA
Luc	11	SEQ. ID 0222	ACGCAAGAAAAAUCAGAGA
Luc	12	SEQ. ID 0223	CGACGCAAGAAAAAUCAGA
Luc	13	SEQ. ID 0224	CUCGACGCAAGAAAAAUCA
Luc	14	SEQ. ID 0225	AACUCGACGCAAGAAAAAU
Luc	15	SEQ. ID 0226	AAAACUCGACGCAAGAAAA
Luc	16	SEQ. ID 0227	GGAAAACUCGACGCAAGAA
Luc	17	SEQ. ID 0228	CCGGAAAACUCGACGCAAG
Luc	18	SEQ. ID 0229	UACCGGAAAACUCGACGCA
Luc	19	SEQ. ID 0230	CUUACCGGAAAACUCGACG
Luc	20	SEQ. ID 0231	GUCUUACCGGAAAACUCGA
Luc	21	SEQ. ID 0232	AGGUCUUACCGGAAAACUC
Luc	22	SEQ. ID 0233	AAAGGUCUUACCGGAAAAC
Luc	23	SEQ. ID 0234	CGAAAGGUCUUACCGGAAA
Luc	24	SEQ. ID 0235	ACCGAAAGGUCUUACCGGA
Luc	25	SEQ. ID 0236	GUACCGAAAGGUCUUACCG
Luc	26	SEQ. ID 0237	AAGUACCGAAAGGUCUUAC
Luc	27	SEQ. ID 0238	CGAAGUACCGAAAGGUCUU
Luc	28	SEQ. ID 0239	GACGAAGUACCGAAAGGUC
Luc	29	SEQ. ID 0240	UGGACGAAGUACCGAAAGG
Luc	30	SEQ. ID 0241	UGUGGACGAAGUACCGAAA
Luc	31	SEQ. ID 0242	UUUGUGGACGAAGUACCGA
Luc	32	SEQ. ID 0243	UGUUUGUGGACGAAGUACC
Luc	33	SEQ. ID 0244	UGUGUUUGUGGACGAAGUA
Luc	34	SEQ. ID 0245	GUUGUGUUUGUGGACGAAG
Luc	35	SEQ. ID 0246	GAGUUGUGUUUGUGGACGA
Luc	36	SEQ. ID 0247	AGGAGUUGUGUUUGUGGAC
Luc	37	SEQ. ID 0248	GGAGGAGUUGUGUUUGUGG
Luc	38	SEQ. ID 0249	GCGGAGGAGUUGUGUUUGU
Luc	39	SEQ. ID 0250	GCGCGGAGGAGUUGUGUUU
Luc	40	SEQ. ID 0251	UUGCGCGGAGGAGUUGUGU
Luc	41	SEQ. ID 0252	AGUUGCGCGGAGGAGUUGU
Luc	42	SEQ. ID 0253	AAAGUUGCGCGGAGGAGUU

TABLE III-continued

Luc	43	SEQ. ID 0254	AAAAAGUUGCGCGGAGGAG
Luc	44	SEQ. ID 0255	CGAAAAAGUUGCGCGGAGG
Luc	45	SEQ. ID 0256	CGCGAAAAAGUUGCGCGGA
Luc	46	SEQ. ID 0257	ACCGCGAAAAAGUUGCGCG
Luc	47	SEQ. ID 0258	CAACCGCGAAAAAGUUGCG
Luc	48	SEQ. ID 0259	AACAACCGCGAAAAAGUUG
Luc	49	SEQ. ID 0260	GUAACAACCGCGAAAAAGU
Luc	50	SEQ. ID 0261	AAGUAACAACCGCGAAAAA
Luc	51	SEQ. ID 0262	UCAAGUAACAACCGCGAAA
Luc	52	SEQ. ID 0263	AGUCAAGUAACAACCGCGA
Luc	53	SEQ. ID 0264	CCAGUCAAGUAACAACCGC
Luc	54	SEQ. ID 0265	CGCCAGUCAAGUAACAACC
Luc	55	SEQ. ID 0266	GUCGCCAGUCAAGUAACAA
Luc	56	SEQ. ID 0267	ACGUCGCCAGUCAAGUAAC
Luc	57	SEQ. ID 0268	UUACGUCGCCAGUCAAGUA
Luc	58	SEQ. ID 0269	GAUUACGUCGCCAGUCAAG
Luc	59	SEQ. ID 0270	UGGAUUACGUCGCCAGUCA
Luc	60	SEQ. ID 0271	CGUGGAUUACGUCGCCAGU
Luc	61	SEQ. ID 0272	AUCGUGGAUUACGUCGCCA
Luc	62	SEQ. ID 0273	AGAUCGUGGAUUACGUCGC
Luc	63	SEQ. ID 0274	AGAGAUCGUGGAUUACGUC
Luc	64	SEQ. ID 0275	AAAGAGAUCGUGGAUUACG
Luc	65	SEQ. ID 0276	AAAAAGAGAUCGUGGAUUA
Luc	66	SEQ. ID 0277	GGAAAAAGAGAUCGUGGAU
Luc	67	SEQ. ID 0278	ACGGAAAAAGAGAUCGUGG
Luc	68	SEQ. ID 0279	UGACGGAAAAAGAGAUCGU
Luc	69	SEQ. ID 0280	GAUGACGGAAAAAGAGAUC
Luc	70	SEQ. ID 0281	ACGAUGACGGAAAAAGAGA
Luc	71	SEQ. ID 0282	AGACGAUGACGGAAAAAGA
Luc	72	SEQ. ID 0283	AAAGACGAUGACGGAAAAA
Luc	73	SEQ. ID 0284	GGAAAGACGAUGACGGAAA
Luc	74	SEQ. ID 0285	ACGGAAAGACGAUGACGGA
Luc	75	SEQ. ID 0286	GCACGGAAAGACGAUGACG
Luc	76	SEQ. ID 0287	GAGCACGGAAAGACGAUGA
Luc	77	SEQ. ID 0288	UGGAGCACGGAAAGACGAU
Luc	78	SEQ. ID 0289	UUUGGAGCACGGAAAGACG
Luc	79	SEQ. ID 0290	GUUUUGGAGCACGGAAAGA
Luc	80	SEQ. ID 0291	UUGUUUUGGAGCACGGAAA

TABLE III-continued

Luc	81	SEQ. ID 0292	UGUUGUUUUGGAGCACGGA
Luc	82	SEQ. ID 0293	GUUGUUGUUUUGGAGCACG
Luc	83	SEQ. ID 0294	CCGUUGUUGUUUUGGAGCA
Luc	84	SEQ. ID 0295	CGCCGUUGUUGUUUUGGAG
Luc	85	SEQ. ID 0296	GCCGCCGUUGUUGUUUUGG
Luc	86	SEQ. ID 0297	CCGCCGCCGUUGUUGUUUU
Luc	87	SEQ. ID 0298	UCCCGCCGCCGUUGUUGUU
Luc	88	SEQ. ID 0299	CUUCCCGCCGCCGUUGUUG
Luc	89	SEQ. ID 0300	AACUUCCCGCCGCCGUUGU
Luc	90	SEQ. ID 0301	UGAACUUCCCGCCGCCGUU

Example II

Validation of the Algorithm Using DBI, Luciferase, PLK, EGFR, and SEAP

[0399] The algorithm (Formula VIII) identified siRNAs for five genes, human DBI, firefly luciferase (fLuc), renilla luciferase (rLuc), human PLK, and human secreted alkaline phosphatase (SEAP). Four individual siRNAs were selected on the basis of their SMARTSCORES™ derived by analysis of their sequence using Formula VIII (all of the siRNAs would be selected with Formula IX as well) and analyzed for their ability to silence their targets expression. In addition to the scoring, a BLAST search was conducted for each siRNA. To minimize the potential for off-target silencing effects, only those target sequences with more than three mismatches against un-related sequences were selected. Semizarov, et al. (2003) Specificity of short interfering RNA determined through gene expression signatures, *Proc. Natl. Acad. Sci. USA,* 100:6347. These duplexes were analyzed individually and in pools of 4 and compared with several siRNAs that were randomly selected. The functionality was measured as a percentage of targeted gene knockdown as compared to controls. All siRNAs were transfected as described by the methods above at 100 nM concentration into HEK293 using Lipofectamine 2000. The level of the targeted gene expression was evaluated by B-DNA as described above and normalized to the non-specific control. FIG. **10** shows that the siRNAs selected by the algorithm disclosed herein were significantly more potent than randomly selected siRNAs. The algorithm increased the chances of identifying an F50 siRNA from 48% to 91%, and an F80 siRNA from 13% to 57%. In addition, pools of SMART siRNA silence the selected target better than randomly selected pools (see FIG. **10**F).

Example III

Validation of the Algorithm Using Genes Involved in Clathrin-Dependent Endocytosis

[0400] Components of clathrin-mediated endocytosis pathway are key to modulating intracellular signaling and play important roles in disease. Chromosomal rearrangements that result in fusion transcripts between the Mixed-

Lineage Leukemia gene (MLL) and CALM (clathrin assembly lymphoid myeloid leukemia gene) are believed to play a role in leukemogenesis. Similarly, disruptions in Rab7 and Rab9, as well as HIP1 (Huntingtin-interacting protein), genes that are believed to be involved in endocytosis, are potentially responsible for ailments resulting in lipid storage, and neuronal diseases, respectively. For these reasons, siRNA directed against clathrin and other genes involved in the clathrin-mediated endocytotic pathway are potentially important research and therapeutic tools.

[0401] siRNAs directed against genes involved in the clathrin-mediated endocytosis pathways were selected using Formula VIII. The targeted genes were clathrin heavy chain (CHC, accession # NM_004859), clathrin light chain A (CLCa. NM_001833), clathrin light chain B (CLCb, NM_001834), CALM (U45976), β2 subunit of AP-2 (β2, NM_001282), Eps15 (NM_001981), Eps15R (NM_021235), dynamin II (DYNII, NM_004945), Rab5a (BC001267), Rab5b (NM_002868), Rab5c (AF141304), and EEA.1 (XM_018197).

[0402] For each gene, four siRNAs duplexes with the highest scores were selected and a BLAST search was conducted for each of them using the Human EST database. In order to minimize the potential for off-target silencing effects, only those sequences with more than three mismatches against un-related sequences were used. All duplexes were synthesized at Dharmacon, Inc. as 21-mers with 3'-UU overhangs using a modified method of 2'-ACE chemistry, Scaringe (2000) Advanced 5'-silyl-2'-orthoester approach to RNA oligonucleotide synthesis, *Methods Enzymol.* 317:3, and the antisense strand was chemically phosphorylated to insure maximized activity.

[0403] HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, antibiotics and glutamine. siRNA duplexes were resuspended in 1×siRNA Universal buffer (Dharmacon, Inc.) to 20 μM prior to transfection. HeLa cells in 12-well plates were transfected twice with 4 μl of 20 μM siRNA duplex in 3 μl Lipofectamine 2000 reagent (Invitrogen, Carlsbad, Calif., USA) at 24-hour intervals. For the transfections in which 2 or 3 siRNA duplexes were included, the amount of each duplex was decreased, so that the total amount was the same as in transfections with single siRNAs. Cells were plated into normal culture medium 12 hours prior to experiments, and protein levels were measured 2 or 4 days after the first transfection.

[0404] Equal amounts of lysates were resolved by electrophoresis, blotted, and stained with the antibody specific to targeted protein, as well as antibodies specific to unrelated proteins, PP1 phosphatase and Tsg101 (not shown). The cells were lysed in Triton X-100/glycerol solubilization buffer as described previously. Tebar, Bohlander, & Sorkin (1999) Clathrin Assembly Lymphoid Myeloid Leukemia (CALM) Protein: Localization in Endocytic-coated Pits, Interactions with Clathrin, and the Impact of Overexpression on Clathrin-mediated Traffic, *Mol. Biol. Cell,* 10:2687. Cell lysates were electrophoresed, transferred to nitrocellulose membranes, and Western blotting was performed with several antibodies followed by detection using enhanced chemiluminescence system (Pierce, Inc). Several x-ray films were analyzed to determine the linear range of the chemiluminescence signals, and the quantifications were performed using densitometry and AlphaImager v5.5 software (Alpha Innotech Corporation). In experiments with Eps15R-targeted siRNAs, cell lysates were subjected to immunoprecipitation with Ab860, and Eps15R was detected in immunoprecipitates by Western blotting as described above.

[0405] The antibodies to assess the levels of each protein by Western blot were obtained from the following sources: monoclonal antibody to clathrin heavy chain (TD.1) was obtained from American Type Culture Collection (Rockville, Md., USA); polyclonal antibody to dynamin II was obtained from Affinity Bioreagents, Inc. (Golden, Colo., USA); monoclonal antibodies to EEA.1 and Rab5a were purchased from BD Transduction Laboratories (Los Angeles, Calif., USA); the monoclonal antibody to Tsg101 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, Calif., USA); the monoclonal antibody to GFP was from ZYMED Laboratories Inc. (South San Francisco, Calif., USA); the rabbit polyclonal antibodies Ab32 specific to α-adaptins and Ab20 to CALM were described previously (Sorkin et al. (1995) Stoichiometric Interaction of the Epidermal Growth Factor Receptor with the Clathrin-associated Protein Complex AP-2, *J. Biol. Chem.,* 270:619), the polyclonal antibodies to clathrin light chains A and B were kindly provided by Dr. F. Brodsky (UCSF); monoclonal antibodies to PP1 (BD Transduction Laboratories) and α-Actinin (Chemicon) were kindly provided by Dr. M. Dell'Acqua (University of Colorado); Eps15 Ab577 and Eps15R Ab860 were kindly provided by Dr. P. P. Di Fiore (European Cancer Institute).

[0406] FIG. **11** demonstrates the in vivo functionality of 48 individual siRNAs, selected using Formula VIII (most of them will meet the criteria incorporated by Formula IX as well) targeting 12 genes. Various cell lines were transfected with siRNA duplexes (Dup1-4) or pools of siRNA duplexes (Pool), and the cells were lysed 3 days after transfection with the exception of CALM (2 days) and β2 (4 days).

[0407] Note a β1-adaptin band (part of AP-1 Golgi adaptor complex) that runs slightly slower than β2 adaptin. CALM has two splice variants, 66 and 72 kD. The full-length Eps15R (a doublet of 130 kD) and several truncated spliced forms of ~100 kD and ~70 kD were detected in Eps15R immunoprecipitates (shown by arrows). The cells were lysed 3 days after transfection. Equal amounts of lysates were resolved by electrophoresis and blotted with the antibody specific to a targeted protein (GFP antibody for YFP fusion proteins) and the antibody specific to unrelated proteins PP1 phosphatase or α-actinin, and TSG101. The amount of protein in each specific band was normalized to the amount of non-specific proteins in each lane of the gel. Nearly all of them appear to be functional, which establishes that Formula VIII and IX can be used to predict siRNAs' functionality in general in a genome wide manner.

[0408] To generate the fusion of yellow fluorescent protein (YFP) with Rab5b or Rab5c (YFP-Rab5b or YFP-Rab5c), a DNA fragment encoding the full-length human Rab5b or Rab5c was obtained by PCR using Pfu polymerase (Stratagene) with a SacI restriction site introduced into the 5' end and a KpnI site into the 3' end and cloned into pEYFP-C1 vector (CLONTECH, Palo Alto, Calif., USA). GFP-CALM and YFP-Rab5a were described previously (Tebar, Bohlander, & Sorkin (1999) Clathrin Assembly Lymphoid Myeloid Leukemia (CALM) Protein: Localization in

Endocytic-coated Pits, Interactions with Clathrin, and the Impact of Overexpression on Clathrin-mediated Traffic, *Mol. Biol. Cell* 10:2687).

Example IV

Validation of the Algorithm Using Eg5, GADPH, ATE1, MEK2, MEK1, QB, Lamina/C, C-MYC, Human Cyclophilin, and Mouse Cyclophilin

[0409] A number of genes have been identified as playing potentially important roles in disease etiology. Expression profiles of normal and diseased kidneys has implicated Edg5 in immunoglobulin A neuropathy, a common renal glomerular disease. Myc1, MEK1/2 and other related kinases have been associated with one or more cancers, while lamins have been implicated in muscular dystrophy and other diseases. For these reasons, siRNA directed against the genes encoding these classes of molecules would be important research and therapeutic tools.

[0410] FIG. **12** illustrates four siRNAs targeting 10 different genes (Table V for sequence and accession number information) that were selected according to the Formula VIII and assayed as individuals and pools in HEK293 cells. The level of siRNA induced silencing was measured using the B-DNA assay. These studies demonstrated that thirty-six out of the forty individual SMART-selected siRNA tested are functional (90%) and all 10 pools are fully functional.

Example V

Validation of the Algorithm Using Bcl2

[0411] Bcl-2 is a ~25 kD, 205-239 amino acid, anti-apoptotic protein that contains considerable homology with other members of the BCL family including BCLX, MCL1, BAX, BAD, and BIK. The protein exists in at least two forms (Bcl2a, which has a hydrophobic tail for membrane anchorage, and Bcl2b, which lacks the hydrophobic tail) and is predominantly localized to the mitochondrial membrane. While Bcl2 expression is widely distributed, particular interest has focused on the expression of this molecule in B and T cells. Bcl2 expression is down-regulated in normal germinal center B cells yet in a high percentage of follicular lymphomas, Bcl2 expression has been observed to be elevated. Cytological studies have identified a common translocation ((14;18)(q32;q32)) amongst a high percentage (>70%) of these lymphomas. This genetic lesion places the Bcl2 gene in juxtaposition to immunoglobulin heavy chain gene (IgH) encoding sequences and is believed to enforce inappropriate levels of gene expression, and resistance to programmed cell death in the follicle center B cells. In other cases, hypomethylation of the Bcl2 promoter leads to enhanced expression and again, inhibition of apoptosis. In addition to cancer, dysregulated expression of Bcl-2 has been correlated with multiple sclerosis and various neurological diseases.

[0412] The correlation between Bcl-2 translocation and cancer makes this gene an attractive target for RNAi. Identification of siRNA directed against the bcl2 transcript (or Bcl2-IgH fusions) would further our understanding Bcl2 gene function and possibly provide a future therapeutic agent to battle diseases that result from altered expression or function of this gene.

In Silico Identification of Functional siRNA

[0413] To identify functional and hyperfunctional siRNA against the Bcl2 gene, the sequence for Bcl-2 was downloaded from the NCBI Unigene database and analyzed using the Formula VIII algorithm. As a result of these procedures, both the sequence and SMARTSCORES™, or siRNA rankings of the Bcl2 siRNA were obtained and ranked according to their functionality. Subsequently, these sequences were BLAST'ed (database) to insure that the selected sequences were specific and contained minimal overlap with unrelated genes. The SMARTSCORES™, or siRNA rankings for the top 10 Bcl-2 siRNA are identified in FIG. **13**.

In Vivo Testing of Bcl-2 SiRNA

[0414] Bcl-2 siRNAs having the top ten SMARTSCORES™, or siRNA rankings were selected and tested in a functional assay to determine silencing efficiency. To accomplish this, each of the ten duplexes were synthesized using 2'-O-ACE chemistry and transfected at 100 nM concentrations into cells. Twenty-four hours later assays were performed on cell extracts to assess the degree of target silencing. Controls used in these experiments included mock transfected cells, and cells that were transfected with a non-specific siRNA duplex.

[0415] The results of these experiments are presented below (and in FIG. **14**) and show that all ten of the selected siRNA induce 80% or better silencing of the Bcl2 message at 100 nM concentrations. These data verify that the algorithm successfully identified functional Bcl2 siRNA and provide a set of functional agents that can be used in experimental and therapeutic environments.

siRNA 1	GGGAGAUAGUGAUGAAGUA	SEQ. ID NO. 302
siRNA 2	GAAGUACAUCCAUUAUAAG	SEQ. ID NO. 303
siRNA 3	GUACGACAACCGGGAGAUA	SEQ. ID NO. 304
siRNA 4	AGAUAGUGAUGAAGUACAU	SEQ. ID NO. 305
siRNA 5	UGAAGACUCUGCUCAGUUU	SEQ. ID NO. 306
siRNA 6	GCAUGCGGCCUCUGUUUGA	SEQ. ID NO. 307
siRNA 7	UGCGGCCUCUGUUUGAUUU	SEQ. ID NO. 308
siRNA 8	GAGAUAGUGAUGAAGUACA	SEQ. ID NO. 309
siRNA 9	GGAGAUAGUGAUGAAGUAC	SEQ. ID NO. 310
siRNA 10	GAAGACUCUGCUCAGUUUG	SEQ. ID NO. 311

Bcl2 siRNA: Sense Strand, 5'→3'

Example VI

Sequences Selected by the Algorithm

[0416] Sequences of the siRNAs selected using Formulas (Algorithms) VIII and IX with their corresponding ranking, which have been evaluated for the silencing activity in vivo in the present study (Formula VIII and IX, respectively) are shown in Table V. It should be noted that the "t" residues in Table V, and elsewhere, when referring to siRNA, should be replaced by "u" residues.

TABLE V

GENE	Name	SEQ. ID No.	FTLLSEQTENCE	FORMULA VIII	FORMULA IX
CLTC	NM_004859	0312	GAAAGAATCTGTAGAGAAA	76	94.2
CLTC	NM_004859	0313	GCAATGAGCTGTTTGAAGA	65	39.9
CLTC	NM_004859	0314	TGACAAAGGTGGATAAATT	57	38.2
CLTC	NM_004859	0315	GGAAATGGATCTCTTTGAA	54	49.4
CLTA	NM_001833	0316	GGAAAGTAATGGTCCAACA	22	55.5
CLTA	NM_001833	0317	AGACAGTTATGCAGCTATT	4	22.9
CLTA	NM_001833	0318	CCAATTCTCGGAAGCAAGA	1	17
CLTA	NM_001833	0319	GAAAGTAATGGTCCAACAG	-1	-13
CLTB	NM_001834	0320	GCGCCAGAGTGAACAAGTA	17	57.5
CLTB	NM_001834	0321	GAAGGTGGCCCAGCTATGT	15	-8.6
CLTB	NM_001834	0322	GGAACCAGCGCCAGAGTGA	13	40.5
CLTB	NM_001834	0323	GAGCGAGATTGCAGGCATA	20	61.7
CALM	U45976	0324	GTTAGTATCTGATGACTTG	36	-34.6
CALM	U45976	0325	GAAATGGAACCACTAAGAA	33	46.1
CALM	U45976	0326	GGAAATGGAACCACTAAGA	30	61.2
CALM	U45976	0327	CAACTACACTTTCCAATGC	28	6.8
EPS15	NM_001981	0328	CCACCAAGATTTCATGATA	48	25.2
EPS15	NM_001981	0329	GATCGGAACTCCAACAAGA	43	49.3
EPS15	NM_001981	0330	AAACGGAGCTACAGATTAT	39	11.5
EPS15	NM_001981	0331	CCACACAGCATTCTTGTAA	33	-23.6
EPS15R	NM_021235	0332	GAAGTTACCTTGAGCAATC	48	33
EPS15R	NM_021235	0333	GGACTTGGCCGATCCAGAA	27	33
EPS15R	NM_021235	0334	GCACTTGGATCGAGATGAG	20	1.3
EPS15R	NM_021235	0335	CAAAGACCAATTCGCGTTA	17	27.7
DNM2	NM_004945	0336	CCGAATCAATCGCATCTTC	6	-29.6
DNM2	NM_004945	0337	GACATGATCCTGCAGTTCA	5	-14
DNM2	NM_004945	0338	GAGCGAATCGTCACCACTT	5	24
DNM2	NM_004945	0339	CCTCCGAGCTGGCGTCTAC	-4	-63.6
ARF6	AF93885	0340	TCACATGGTTAACCTCTAA	27	-21.1
ARF6	AF93885	0341	GATGAGGGACGCCATAATC	7	-38.4
ARF6	AF93885	0342	CCTCTAACTACAAATCTTA	4	16.9
ARF6	AF93885	0343	GGAAGGTGCTATCCAAAAT	4	11.5
RAB5A	BC001267	0344	GCAAGCAAGTCCTAACATT	40	25.1
RAB5A	BC001267	0345	GGAAGAGGAGTAGACCTTA	17	50.1
RAB5A	BC001267	0346	AGGAATCAGTGTTGTAGTA	16	11.5
RAB5A	BC001267	0347	GAAGAGGAGTAGACCTTAC	12	7
RAB5B	NM_002868	0348	GAAAGTCAAGCCTGGTATT	14	18.1

TABLE V-continued

GENE	Name	SEQ. ID No.	FTLLSEQTENCE	FORMULA VIII	FORMULA IX
RAB5B	NM_002868	0349	AAAGTCAAGCCTGGTATTA	6	-17.8
RAB5B	NM_002868	0350	GCTATGAACGTGAATGATC	3	-21.1
RAB5B	NM_002868	0351	CAAGCCTGGTATTACGTTT	-7	-37.5
RAB5C	AF141304	0352	GGAACAAGATCTGTCAATT	38	51.9
RAB5C	AF141304	0353	GCAATGAACGTGAACGAAA	29	43.7
RAB5C	AF141304	0354	CAATGAACGTGAACGAAAT	18	43.3
RAB5C	AF141304	0355	GGACAGGAGCGGTATCACA	6	18.2
EEA1	XM_018197	0356	AGACAGAGCTTGAGAATAA	67	64.1
EEA1	XM_018197	0357	GAGAAGATCTTTATGCAAA	60	48.7
EEA1	XM_018197	0358	GAAGAGAAATCAGCAGATA	58	45.7
EEA1	XM_018197	0359	GCAAGTAACTCAACTAACA	56	72.3
AP2B1	NM_001282	0360	GAGCTAATCTGCCACATTG	49	-12.4
AP2B1	NM_001282	0361	GCAGATGAGTTACTAGAAA	44	48.9
AP2B1	NM_001282	0362	CAACTTAATTGTCCAGAAA	41	28.2
AP2B1	NM_001282	0363	CAACACAGGATTCTGATAA	33	-5.8
PLK	NM_005030	0364	AGATTGTGCCTAAGTCTCT	-35	-3.4
PLK	NM_005030	0365	ATGAAGATCTGGAGGTGAA	0	-4.3
PLK	NM_005030	0366	TTTGAGACTTCTTGCCTAA	-5	-27.7
PLK	NM_005030	0367	AGATCACCCTCCTTAAATA	15	72.3
GAPDH	NM_002046	0368	CAACGGATTTGGTCGTATT	27	-2.8
GAPDH	NM_002046	0369	GAAATCCCATCACCATCTT	24	3.9
GAPDH	NM_002046	0370	GACCTCAACTACATGGTTT	22	-22.9
GAPDH	NM_002046	0371	TGGTTTACATGTTCCAATA	9	9.8
c-Myc		0372	GAAGAAATCGATGTTGTTT	31	-11.7
c-Myc		0373	ACACAAACTTGAACAGCTA	22	51.3
c-Myc		0374	GGAAGAAATCGATGTTGTT	18	26
c-Myc		0375	GAAACGACGAGAACAGTTG	18	-8.9
MAP2K1	NM_002755	0376	GCACATGGATGGAGGTTCT	26	16
MAP2K1	NM_002755	0377	GCAGAGAGAGCAGATTTGA	16	0.4
MAP2K1	NM_002755	0378	GAGGTTCTCTGGATCAAGT	14	15.5
MAP2K1	NM_002755	0379	GAGCAGATTTGAAGCAACT	14	18.5
MAP2K2	NM_030662	0380	CAAAGACGATGACTTCGAA	37	26.4
MAP2K2	NM_030662	0381	GATCAGCATTTGCATGGAA	24	-0.7
MAP2K2	NM_030662	0382	TCCAGGAGTTTGTCAATAA	17	-4.5
MAP2K2	NM_030662	0383	GGAAGCTGATCCACCTTGA	16	59.2
KNSL1 (EG5)	NM_004523	0384	GCAGAAATCTAAGGATATA	53	35.8

TABLE V-continued

GENE	Name	SEQ. ID No.	FTLLSEQTENCE	FORMULA VIII	FORMULA IX
KNSL1 (EG5)	NM_004523	0385	CAACAAGGATGAAGTCTAT	50	18.3
KNSL1 (EG5)	NM_004523	0386	CAGCAGAAATCTAAGGATA	41	32.7
KNSL1 (EG5)	NM_004523	0387	CTAGATGGCTTTCTCAGTA	39	3.9
CyclophilinA	NM_021130	0388	AGACAAGGTCCCAAAGACA	-16	58.1
CyclophilinA	NM_021130	0389	GGAATGGCAAGACCAGCAA	-6	36
CyclophilinA	NM_021130	0390	AGAATTATTCCAGGGTTTA	-3	16.1
CyclophilinA	NM_021130	0391	GCAGACAAGGTCCCAAAGA	8	8.9
LAMIN A/C	NM_170707	0392	AGAAGCAGCTTCAGGATGA	31	38.8
LAMIN A/C	NM_170707	0393	GAGCTTGACTTCCAGAAGA	33	22.4
LAMIN A/C	NM_170707	0394	CCACCGAAGTTCACCCTAA	21	27.5
LAMIN A/C	NM_170707	0395	GAGAAGAGCTCCTCCATCA	55	30.1
CyclophilinB	M60857	0396	GAAAGAGCATCTACGGTGA	41	83.9
CyclophilinB	M60857	0397	GAAAGGATTTGGCTACAAA	53	59.1
CyclophilinB	M60857	0398	ACAGCAAATTCCATCGTGT	-20	28.8
CyclophllinB	M60857	0399	GGAAAGACTGTTCCAAAAA	2	27
DBI1	NM_020548	0400	CAACACGCCTCATCCTCTA	27	-7.6
DBI2	NM_020548	0401	CATGAAAGCTTACATCAAC	25	-30.8
DBI3	NM_020548	0402	AAGATGCCATGAAAGCTTA	17	22
DBI4	NM_020548	0403	GCACATACCGCCTGAGTCT	15	3.9
rLUC1		0404	GATCAAATCTGAAGAAGGA	57	49.2
rLUC2		0405	GCCAAGAAGTTTCCTAATA	50	13.7
rLUC3		0406	CAGCATATCTTGAACCATT	41	-2.2
rLUC4		0407	GAACAAAGGAAACGGATGA	39	29.2
SeAP1	NM_031313	0408	CGGAAACGGTCCAGGCTAT	6	26.9
SeAP2	NM_031313	0409	GCTTCGAGCAGACATGATA	4	-11.2
SeAP3	NM_031313	0410	CCTACACGGTCCTCCTATA	4	4.9
SeAP4	NM_031313	0411	GCCAAGAACCTCATCATCT	1	-9.9
fLUC1		0412	GATATGGGCTGAATACAAA	54	40.4
fLUC2		0413	GCACTCTGATTGACAAATA	47	54.7
fLUC3		0414	TGAAGTCTCTGATTAAGTA	46	34.5
fLUC4		0415	TCAGAGAGATCCTCATAAA	40	11.4
mCyclo_1	NM_008907	0416	GCAAGAAGATCACCATTTC	52	46.4
mCyclo_2	NM_008907	0417	GAGAGAAATTTGAGGATGA	36	70.7
mCyclo_3	NM_008907	0418	GAAAGGATTTGGCTATAAG	35	-1.5
mCyclo_4	NM_008907	0419	GAAAGAAGGCATGAACATT	27	10.3
BCL2_1	NM_000633	0420	GGGAGATAGTGATGAAGTA	21	72
BCL2_2	NM_000633	0421	GAAGTACATCCATTATAAG	1	3.3

TABLE V-continued

GENE	Name	SEQ. ID No.	FTLLSEQTENCE	FORMULA VIII	FORMULA IX
BCL2_3	NM_000633	0422	GTACGACAACCGGGAGATA	1	35.9
BCL2_4	NM_000633	0423	AGATAGTGATGAAGTACAT	-12	22.1
BCL2_5	NM_000633	0424	TGAAGACTCTGCTCAGTTT	36	19.1
BCL2_6	NM_000633	0425	GCATGCGGCCTCTGTTTGA	5	-9.7
QB1	NM_003365.1	0426	GCACACAGCUUACUACAUC	52	-4.8
QB2	NM_003365.1	0427	GAAAUGCCCUGGUAUCUCA	49	22.1
QB3	NM_003365.1	0428	GAAGGAACGUGAUGUGAUC	34	22.9
QB4	NM_003365.1	0429	GCACUACUCCUGUGUGUGA	28	20.4
ATE1-1	NM_007041	0430	GAACCCAGCUGGAGAACUU	45	15.5
ATE1-2	NM_007041	0431	GAUAUACAGUGUGAUCUUA	40	12.2
ATE1-3	NM_007041	0432	GUACUACGAUCCUGAUUAU	37	32.9
ATE1-4	NM_007041	0433	GUGCCGACCUUUACAAUUU	35	18.2
EGFR-1	NM_005228	0434	GAAGGAAACTGAATTCAAA	68	79.4
EGFR-1	NM_005228	0435	GGAAATATGTACTACGAAA	49	49.5
EGFR-1	NM_005228	0436	CCACAAAGCAGTGAATTTA	41	7.6
EGFR-1	NM_005228	0437	GTAACAAGCTCACGCAGTT	40	25.9

[0417] Many of the genes to which the described siRNA are directed play critical roles in disease etiology. For this reason, the siRNAs listed in the sequence listing may potentially act as therapeutic agents. A number of prophetic examples follow and should be understood in view of the siRNA that are identified in the sequence listing. To isolate these siRNAs, the appropriate message sequence for each gene is analyzed using one of the before mentioned formulas (preferably formula VIII) to identify potential siRNA targets. Subsequently these targets are BLAST'ed to eliminate homology with potential off-targets.

Example VII

Evidence for the Benefits of Pooling

[0418] Evidence for the benefits of pooling have been demonstrated using the reporter gene, luciferase. Ninety siRNA duplexes were synthesized using Dharmacon proprietary ACE® chemistry against one of the standard reporter genes: firefly luciferase. The duplexes were designed to start two base pairs apart and to cover approximately 180 base pairs of the luciferase gene (see sequences in Table III). Subsequently, the siRNA duplexes were co-transfected with a luciferase expression reporter plasmid into HEK293 cells using standard transfection protocols and luciferase activity was assayed at 24 and 48 hours.

[0419] Transfection of individual siRNAs showed standard distribution of inhibitory effect. Some duplexes were active, while others were not. FIG. **15** represents a typical screen of ninety siRNA duplexes (SEQ. ID NO. 0032-0120) positioned two base pairs apart. As the figure suggests, the functionality of the siRNA duplex is determined more by a particular sequence of the oligonucleotide than by the relative oligonucleotide position within a gene or excessively sensitive part of the mRNA, which is important for traditional anti-sense technology.

[0420] When two continuous oligonucleotides were pooled together, a significant increase in gene silencing activity was observed (see FIGS. **16**A and B). A gradual increase in efficacy and the frequency of pools functionality was observed when the number of siRNAs increased to 3 and 4 (FIGS. **16**A, **16**B, **17**A, and **17**B). Further, the relative positioning of the oligonucleotides within a pool did not determine whether a particular pool was functional (see FIGS. **18**A and **18**B, in which 100% of pools of oligonucleotides distanced by 2, 10 and 20 base pairs were functional).

[0421] However, relative positioning may nonetheless have an impact. An increased functionality may exist when the siRNA are positioned continuously head to toe (5' end of one directly adjacent to the 3' end of the others).

[0422] Additionally, siRNA pools that were tested performed at least as well as the best oligonucleotide in the pool, under the experimental conditions whose results are depicted in FIG. **19**. Moreover, when previously identified non-functional and marginally (semi) functional siRNA duplexes were pooled together in groups of five at a time, a significant functional cooperative action was observed (see FIG. **20**). In fact, pools of semi-active oligonucleotides were 5 to 25 times more functional than the most potent oligonucleotide in the pool. Therefore, pooling several siRNA

duplexes together does not interfere with the functionality of the most potent siRNAs within a pool, and pooling provides an unexpected significant increase in overall functionality

Example VIII

Additional Evidence of the Benefits of Pooling

[0423] Experiments were performed on the following genes: β-galactosidase, Renilla luciferase, and Secreted alkaline phosphatase, which demonstrates the benefits of pooling. (see FIGS. **21**A, **21**B and **21**C). Individual and pools of siRNA (described in Figure legends **21**A-C) were transfected into cells and tested for silencing efficiency. Approximately 50% of individual siRNAs designed to silence the above-specified genes were functional, while 100% of the pools that contain the same siRNA duplexes were functional.

Example IX

Highly Functional siRNA

[0424] Pools of five siRNAs in which each two siRNAs overlap to 10-90% resulted in 98% functional entities (>80% silencing). Pools of siRNAs distributed throughout the mRNA that were evenly spaced, covering an approximate 20-2000 base pair range, were also functional. When the pools of siRNA were positioned continuously head to tail relative to in RNA sequences and mimicked the natural products of Dicer cleaved long double stranded RNA, 98% of the pools evidenced highly functional activity (>95% silencing).

Example X

Human Cyclophilin B

[0425] Table III above lists the siRNA sequences for the human cyclophilin B protein. A particularly functional siRNA may be selected by applying these sequences to any of Formula I to VII above.

[0426] Alternatively, one could pool 2, 3, 4, 5 or more of these sequences to create a kit for silencing a gene. Preferably, within the kit there would be at least one sequence that has a relatively high predicted functionality when any of Formulas I-VII is applied.

Example XI

Sample Pools of siRNAs and their Application to Human Disease

[0427] The genetic basis behind human disease is well documented and siRNA may be used as both research or diagnostic tools and therapeutic agents, either individually or in pools. Genes involved in signal transduction, the immune response, apoptosis, DNA repair, cell cycle control, and a variety of other physiological functions have clinical relevance and therapeutic agents that can modulate expression of these genes may alleviate some or all of the associated symptoms. In some instances, these genes can be described as a member of a family or class of genes and siRNA (randomly, conventionally, or rationally designed) can be directed against one or multiple members of the family to induce a desired result.

[0428] To identify rationally designed siRNA to each gene, the sequence was analyzed using Formula VIII or Formula X to identify rationally designed siRNA. To confirm the activity of these sequences, the siRNA are introduced into a cell type of choice (e.g., HeLa cells, HEK293 cells) and the levels of the appropriate message are analyzed using one of several art proven techniques. siRNA having heightened levels of potency can be identified by testing each of the before mentioned duplexes at increasingly limiting concentrations. Similarly, siRNA having increased levels of longevity can be identified by introducing each duplex into cells and testing functionality at 24, 48, 72, 96, 120, 144, 168, and 192 hours after transfection. Agents that induce >95% silencing at sub-nanomolar concentrations and/or induce functional levels of silencing for >96 hours are considered hyperfunctional.

Example XII

Validation of Multigene Knockout Using Rab5 and Eps

[0429] Two or more genes having similar, overlapping functions often leads to genetic redundancy. Mutations that knockout only one of, e.g., a pair of such genes (also referred to as homologs) results in little or no phenotype due to the fact that the remaining intact gene is capable of fulfilling the role of the disrupted counterpart. To fully understand the function of such genes in cellular physiology, it is often necessary to knockout or knockdown both homologs simultaneously. Unfortunately, concomitant knockdown of two or more genes is frequently difficult to achieve in higher organisms (e.g., mice) thus it is necessary to introduce new technologies dissect gene function. One such approach to knocking down multiple genes simultaneously is by using siRNA. For example, FIG. **11** showed that rationally designed siRNA directed against a number of genes involved in the clathrin-mediated endocytosis pathway resulted in significant levels of protein reduction (e.g., >80%). To determine the effects of gene knockdown on clathrin-related endocytosis, internalization assays were performed using epidermal growth factor and transferrin. Specifically, mouse receptor-grade EGF (Collaborative Research Inc.) and iron-saturated human transferrin (Sigma) were iodinated as described previously (Jiang, X., Huang, F., Marusyk, A. & Sorkin, A. (2003) *Mol Biol Cell* 14, 858-70). HeLa cells grown in 12-well dishes were incubated with ^{125}I-EGF (1 ng/ml) or ^{125}I-transferrin (1 μg/ml) in binding medium (DM EM, 0.1% bovine serum albumin) at 37° C., and the ratio of internalized and surface radioactivity was determined during 5-min time course to calculate specific internalization rate constant k_e as described previously (Jiang, X et al.). The measurements of the uptakes of radiolabeled transferrin and EGF were performed using short time-course assays to avoid influence of the recycling on the uptake kinetics, and using low ligand concentration to avoid saturation of the clathrin-dependent pathway (for EGF Lund, K. A., Opresko, L. K., Strarbuck, C., Walsh, B. J. & Wiley, H. S. (1990) *J. Biol. Chem.* 265, 15713-13723).

[0430] The effects of knocking down Rab5a, 5b, 5c, Eps, or Eps 15R (individually) are shown in FIG. **22** and demonstrate that disruption of single genes has little or no effect on EGF or Tfn internalization. In contrast, simultaneous knock down of Rab5a, 5b, and 5c, or Eps and Eps 15R, leads

to a distinct phenotype (note: total concentration of siRNA in these experiments remained constant with that in experiments in which a single siRNA was introduced, see FIG. **23**). These experiments demonstrate the effectiveness of using rationally designed siRNA to knockdown multiple genes and validates the utility of these reagents to override genetic redundancy.

Example XIII

Validation of Multigene Targeting Using G6PD, GAPDH, PLK, and UQC

[**0431**] Further demonstration of the ability to knock down expression of multiple genes using rationally designed siRNA was performed using pools of siRNA directed against four separate genes. To achieve this, siRNA were transfected into cells (total siRNA concentration of 100 nM) and assayed twenty-four hours later by B-DNA. Results shown in FIG. **24** show that pools of rationally designed molecules are capable of simultaneously silencing four different genes.

Example XIV

Validation of Multigene Knockouts as Demonstrated by Gene Expression Profiling, a Prophetic Example

[**0432**] To further demonstrate the ability to concomitantly knockdown the expression of multiple gene targets, single siRNA or siRNA pools directed against a collection of genes (e.g., 4, 8, 16, or 23 different targets) are simultaneously transfected into cells and cultured for twenty-four hours. Subsequently, mRNA is harvested from treated (and untreated) cells and labeled with one of two fluorescent probes dyes (e.g., a red fluorescent probe for the treated cells, a green fluorescent probe for the control cells.). Equivalent amounts of labeled RNA from each sample is then mixed together and hybridized to sequences that have been linked to a solid support (e.g., a slide, "DNA CHIP"). Following hybridization, the slides are washed and analyzed to assess changes in the levels of target genes induced by siRNA.

Example XV

Identifying Hyperfunctional siRNA

Identification of Hyperfunctional Bcl-2 siRNA

[**0433**] The ten rationally designed Bcl2 siRNA (identified in FIG. **13**, **14**) were tested to identify hyperpotent reagents. To accomplish this, each of the ten Bcl-2 siRNA were individually transfected into cells at a 300 pM (0.3 nM) concentrations. Twenty-four hours later, transcript levels were assessed by B-DNA assays and compared with relevant controls. As shown in FIG. **25**, while the majority of Bcl-2 siRNA failed to induce functional levels of silencing at this concentration, siRNA 1 and 8 induced >80% silencing, and siRNA 6 exhibited greater than 90% silencing at this sub-nanomolar concentration.

[**0434**] By way of prophetic examples, similar assays could be performed with any of the groups of rationally designed genes described in the Examples. Thus for instance, rationally designed siRNA sequences directed against a gene of interest could be introduced into cells at increasingly limiting concentrations to determine whether any of the duplexes are hyperfunctional.

Example XVI

Gene Silencing

Prophetic Example

[**0435**] Below is an example of how one might transfect a cell.

[**0436**] Select a cell line. The selection of a cell line is usually determined by the desired application. The most important feature to RNAi is the level of expression of the gene of interest. It is highly recommended to use cell lines for which siRNA transfection conditions have been specified and validated.

[**0437**] Plate the cells. Approximately 24 hours prior to transfection, plate the cells at the appropriate density so that they will be approximately 70-90% confluent, or approximately 1×10^5 cells/ml at the time of transfection. Cell densities that are too low may lead to toxicity due to excess exposure and uptake of transfection reagent-siRNA complexes. Cell densities that are too high may lead to low transfection efficiencies and little or no silencing. Incubate the cells overnight. Standard incubation conditions for mammalian cells are 37° C. in 5% CO_2. Other cell types, such as insect cells, require different temperatures and CO_2 concentrations that are readily ascertainable by persons skilled in the art. Use conditions appropriate for the cell type of interest.

[**0438**] siRNA re-suspension. Add 20 μl siRNA universal buffer to each siRNA to generate a final concentration of 50 μM.

[**0439**] siRNA-lipid complex formation. Use RNase-free solutions and tubes. Using the following table, Table XI:

TABLE XI

	96-WELL	24-WELL
MIXTURE 1 (TRANSIT-TKO-PLASMID DILUTION MIXTURE)		
Opti-MEM	9.3 μl	46.5 μl
TransIT-TKO (1 μg/μl)	0.5 μl	2.5 μl
MIXTURE 1 FINAL VOLUME	10.0 μl	50.0 μl
MIXTURE 2 (SIRNA DILUTION MIXTURE)		
Opti-MEM	9.0 μl	45.0 μl
siRNA (1 μM)	1.0 μl	5.0 μl
MIXTURE 2 FINAL VOLUME	10.0 μl	50.0 μl
MIXTURE 3 (SIRNA-TRANSFECTION REAGENT MIXTURE)		
Mixture 1	10 μl	50 μl
Mixture 2	10 μl	50 μl
MIXTURE 3 FINAL VOLUME	20 μl	100 μl

Incubate 20 minutes at room temperature

TABLE XI-continued

	96-WELL	24-WELL
MIXTURE 4 (MEDIA-SIRNA/TRANSFECTION REAGENT MIXTURE)		
Mixture 3	20 μl	100 μl
Complete media	80 μl	400 μl
MIXTURE 4 FINAL VOLUME	100 μl	500 μl
Incubate 48 hours at 37° C.		

[0440] Transfection. Create a Mixture 1 by combining the specified amounts of OPTI-MEM serum free media and transfection reagent in a sterile polystyrene tube. Create a Mixture 2 by combining specified amounts of each siRNA with OPTI-MEM media in sterile 1 ml tubes. Create a Mixture 3 by combining specified amounts of Mixture I and Mixture 2. Mix gently (do not vortex) and incubate at room temperature for 20 minutes. Create a Mixture 4 by combining specified amounts of Mixture 3 to complete media. Add appropriate volume to each cell culture well. Incubate cells with transfection reagent mixture for 24-72 hours at 37° C. This incubation time is flexible. The ratio of silencing will remain consistent at any point in the time period. Assay for gene silencing using an appropriate detection method such as RT-PCR, Western blot analysis, immunohistochemistry, phenotypic analysis, mass spectrometry, fluorescence, radioactive decay, or any other method that is now known or that comes to be known to persons skilled in the art and that from reading this disclosure would useful with the present invention. The optimal window for observing a knockdown phenotype is related to the mRNA turnover of the gene of interest, although 24-72 hours is standard. Final Volume reflects amount needed in each well for the desired cell culture format. When adjusting volumes for a Stock Mix, an additional 10% should be used to accommodate variability in pipetting, etc. Duplicate or triplicate assays should be carried out when possible.

Example XVII

siRNAs that Target TNFα

[0441] siRNAs that target TNFα[NCBI accession number NM_000594] having sequences generated in silico by the algorithms herein, are provided. In various embodiments, the siRNAs are rationally designed. In various embodiments, the siRNAs are functional or hyperfunctional. These siRNA that have been generated by the algorithms of the present invention include:

GCCUUCCUCUCUCCAGAUG (SEQ ID NO 438)

CCUCCUCUCUGCCAUCAAG (SEQ ID NO 439)

AGACCUCACCUAGAAAUUG (SEQ ID NO 440)

GGAGUUGUGUCUGUAAUCG (SEQ ID NO 441)

UGCCUUGGCUCAGACAUGU (SEQ ID NO 442)

CCUCUUCUCCUUCCUGAUC (SEQ ID NO 443)

-continued

GAUGUUUCCAGACUUCCUU (SEQ ID NO 444)

UGGCCUCUGUGCCUUCUUU (SEQ ID NO 445)

CCAGCUCCCUCUAUUUAUG (SEQ ID NO 446)

GACCAACUGUCACUCAUUG (SEQ ID NO 447)

ACAAGACCACCACUUCGAA (SEQ ID NO 448)

GCCUGUACCUCAUCUACUC (SEQ ID NO 449)

GGCAACCACUAAGAAUUCA (SEQ ID NO 450)

GUAAUCGCCCUACUAUUCA (SEQ ID NO 451)

UCUGUAAUCGCCCUACUAU (SEQ ID NO 452)

AAUAGGCUGUUCCCAUGUA (SEQ ID NO 453)

AGGGACCUCUCUCUAAUCA (SEQ ID NO 454)

CAUCCAACCUUCCCAAACG (SEQ ID NO 455)

GGUCUACUUUGGGAUCAUU (SEQ ID NO 456)

UAAACAAUGCUGAUUUGGU (SEQ ID NO 457)

AGCCAGCUCCCUCUAUUUA (SEQ ID NO 458)

CCGACUAUCUCGACUUUGC (SEQ ID NO 459)

GACAGCAGAGGACCAGCUA (SEQ ID NO 460)

UGGGAUCAUUGCCCUGUGA (SEQ ID NO 461)

AACCCAAGCUUAGAACUUU (SEQ ID NO 462)

GAACUUUAAGCAACAAGAC (SEQ ID NO 463)

GCAGAGGACCAGCUAAGAG (SEQ ID NO 464)

UGUAAUCGCCCUACUAUUC (SEQ ID NO 465)

UUUCCAGACUUCCUUGAGA (SEQ ID NO 466)

CCCAGGGACCUCUCUCUAA (SEQ ID NO 467)

GCCCGACUAUCUCGACUUU (SEQ ID NO 468)

GGCGAGAAAUAAAGUUUGC (SEQ ID NO 469)

GUUCUCUUCCUCUCACAUA (SEQ ID NO 470)

CUUCCUCUCUCCAGAUGUU (SEQ ID NO 471)

GAGGAGGACGAACAUCCAA (SEQ ID NO 472)

CAACCACUAAGAAUUCAAA (SEQ ID NO 473)

GAAGACCUCACCUAGAAAU (SEQ ID NO 474)

CCACUAAGAAUUCAAACUG (SEQ ID NO 475)

GACACCAUGAGCACUGAAA (SEQ ID NO 476)

GCAACAAGACCACCACUUC (SEQ ID NO 477)

CAGCUUUGAUCCCUGACAU (SEQ ID NO 478)

UCUAAACAAUGCUGAUUUG (SEQ ID NO 479)

UGGCAACCACUAAGAAUUC (SEQ ID NO 480)

CAAGACCACCACUUCGAAA (SEQ ID NO 481)

CCAGGGACCUCUCUCUAAU (SEQ ID NO 482)

-continued

GACCUCACCUAGAAAUUGA	(SEQ ID NO 483)
AGGCCAAGCCCUGGUAUGA	(SEQ ID NO 484)
GGUAUGAGCCCAUCUAUCU	(SEQ ID NO 485)
UGACACAAGUGGACCUUAG	(SEQ ID NO 486)
CCAGAUGUUUCCAGACUUC	(SEQ ID NO 487)
GUCUAAACAAUGCUGAUUU	(SEQ ID NO 488)
GAGAAGGGUGACCGACUCA	(SEQ ID NO 489)
GGUCGGAACCCAAGCUUAG	(SEQ ID NO 490)
AAACGGAGCUGAACAAUAG	(SEQ ID NO 491)
CCAGGGAGCCUUUGGUUCU	(SEQ ID NO 492)
CCUAGAAAUUGACACAAGU	(SEQ ID NO 493)
GCCUGUAGCCCAUGUUGUA	(SEQ ID NO 494)
GCUGGCAACCACUAAGAAU	(SEQ ID NO 495)
UAGCCCAUGUUGUAGCAAA	(SEQ ID NO 496)
CUGAUUAAGUUGUCUAAAC	(SEQ ID NO 497)
GUGGCGAGAAAUAAAGUUU	(SEQ ID NO 498)
ACCCAAGCUUAGAACUUUA	(SEQ ID NO 499)
CAGCAGAGGACCAGCUAAG	(SEQ ID NO 500)
CCAAUGCCCUCCUGGCCAA	(SEQ ID NO 501)
CGACAAAUAAAGUUUGCUU	(SEQ ID NO 502)
GGAGAGAAGCAACUACAGA	(SEQ ID NO 503)
CUGCAGGACUUGAGAAGAC	(SEQ ID NO 504)
CAGCUAAGAGGGAGAGAAG	(SEQ ID NO 505)
GAACCCAAGCUUAGAACUU	(SEQ ID NO 506)
GCGUGGAGCUGAGAGAUAA	(SEQ ID NO 507)
UGACAAGCCUGUAGCCCAU	(SEQ ID NO 508)
GAGAAGACCUCACCUAGAA	(SEQ ID NO 509)
GGCGUGGAGCUGAGAGAUA	(SEQ ID NO 510)
ACUAUUCAGUGGCGAGAAA	(SEQ ID NO 511)
CCAGAAUGCUGCAGGACUU	(SEQ ID NO 512)
GCUAAGAGGGAGAGAAGCA	(SEQ ID NO 513)

[0442] Thus, consistent with Example XVII, the present invention provides an siRNA that targets TNFα, wherein the siRNA is selected from the group consisting of SEQ. ID NOs. 438-513.

[0443] In another embodiment, an siRNA is provided, said siRNA comprising a sense region and an antisense region, wherein said sense region and said antisense region are at least 90% complementary, said sense region and said antisense region together form a duplex region comprising 18-30 base pairs, and said sense region comprises a sequence that is at least 90% similar to a sequence selected from the group consisting of: SEQ. ID NOs 438-513.

[0444] In another embodiment, an siRNA is provided wherein the siRNA comprises a sense region and an antisense region, wherein said sense region and said antisense region are at least 90% complementary, said sense region and said antisense region together form a duplex region comprising 18-30 base pairs, and said sense region comprises a sequence that is identical to a contiguous stretch of at least 18 bases of a sequence selected from the group consisting of: SEQ. ID NOs 438-513.

[0445] In another embodiment, an siRNA is provided wherein the siRNA comprises a sense region and an antisense region, wherein said sense region and said antisense region are at least 90% complementary, said sense region and said antisense region together form a duplex region comprising 19-30 base pairs, and said sense region comprises a sequence that is identical to a contiguous stretch of at least 18 bases of a sequence selected from the group consisting of: SEQ. ID NOs 438-513.

[0446] In another embodiment, a pool of at least two siRNAs is provided, wherein said pool comprises a first siRNA and a second siRNA, said first siRNA comprises a duplex region of length 18-30 base pairs that has a first sense region that is at least 90% similar to 18 bases of a first sequence selected from the group consisting of: SEQ. ID NOs 438-513 and said second siRNA comprises a duplex region of length 18-30 base pairs that has a second sense region that is at least 90% similar to 18 bases of a second sequence selected from the group consisting of: SEQ. ID NOs 438-513 and wherein said first sense region and said second sense region are not identical.

[0447] In another embodiment, a pool of at least two siRNAs is provided, wherein said pool comprises a first siRNA and a second siRNA, said first siRNA comprises a duplex region of length 18-30 base pairs that has a first sense region that is identical to at least 18 bases of a sequence selected from the group consisting of: SEQ. ID NOs 438-513 and wherein the second siRNA comprises a second sense region that comprises a sequence that is identical to at least 18 bases of a sequence selected from the group consisting of: SEQ. ID NOs 438-513.

[0448] In another embodiment, a pool of at least two siRNAs is provided, wherein said pool comprises a first siRNA and a second siRNA, said first siRNA comprises a duplex region of length 19-30 base pairs and has a first sense region comprising a sequence that is at least 90% similar to a sequence selected from the group consisting of: SEQ. ID NOs 438-513, and said duplex of said second siRNA is 19-30 base pairs and comprises a second sense region that comprises a sequence that is at least 90% similar to a sequence selected from the group consisting of: SEQ. ID NOs 438-513.

[0449] In another embodiment, a pool of at least two siRNAs is provided, wherein said pool comprises a first siRNA and a second siRNA, said first siRNA comprises a duplex region of length 19-30 base pairs and has a first sense region comprising a sequence that is identical to at least 18 bases of a sequence selected the group consisting of: SEQ. ID NOs 438-513 and said duplex of said second siRNA is 19-30 base pairs and comprises a second sense region

comprising a sequence that is identical to a sequence selected from the group consisting of: SEQ. ID NOs 438-513.

[0450] In each of the aforementioned embodiments, preferably the antisense region is at least 90% complementary to a contiguous stretch of bases of one of the NCBI sequences NM_00594, the sequences of which is incorporated by reference as if set forth fully herein. In some embodiments, the antisense region is 100% complementary to a contiguous stretch of bases of one of the NCBI sequences NM_00594.

[0451] Further, in some embodiments that are directed to siRNA duplexes in which the antisense region is 20-30 bases in length, preferably there is a stretch of 19 bases that is at least 90%, more preferably 100% complementary to the recited sequence id number and the entire antisense region is at least 90% and more preferably 100% complementary to a contiguous stretch of bases of one of the NCBI sequences NM_00594.

[0452] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departure from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth and as follows in the scope of the appended claims.

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nnanannnnu cncannnna 19

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nnanannnnu uncannnna 19

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nnanannnnu cnuannnna 19

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nnancnnnnu uncannnna 19

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nnancnnnnu cnuannnna 19

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nnangnnnnu gncannnna 19

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nnangnnnnu uncannnna 19

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nnangnnnnu gnuannnna 19

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nnangnnnnu unuannnna 19

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<223> OTHER INFORMATION: Synthetic

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caguggauaa uuuuguggc 19

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guggauaauu uuguggccu 19

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ggauaauuuu guggccuua 19

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uuuuguggcc uuagcuaca 19

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guggccuuag cuacaggag 19

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ccuuagcuac aggagagaa 19

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uuagcuacag gagagaaag 19

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agcuacagga gagaaagga 19

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cuacaggaga gaaaggauu 19

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gagagaaagg auuuggcua 19

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ggauuuggcu acaaaaaca 19

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uuggcuacaa aaacagcaa 19

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aaaaacagca aauuccauc 19

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aaacagcaaa uuccaucgu 19

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caaauuccau cguguaauc 19

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aauuccaucg uguaaucaa 19

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uuccaucgug uaaucaagg 19

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ccaucgugua aucaaggac 19

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cguguaauca aggacuuca 19

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uaaucaagga cuucaugau 19

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aucaaggacu ucaugaucc 19

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caaggacuuc augauccag 19

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aggacuucau gauccaggg 19

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gacuucauga uccagggcg 19

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cuucaugauc cagggcgga 19

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cagggcggag acuucacca 19

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gggcggagac uucaccagg 19

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gcggagacuu caccagggg 19

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gauggcacag gaggaaaga 19

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uggcacagga ggaaagagc 19

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gcacaggagg aaagagcau 19

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ugagaacuuc aaacugaag 19

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ggccaagugg gaugccugg 19

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ugggaugccu ggaaugagc 19

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gaugccugga augagcuga 19

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agcugaaagg gacuuccaa 19

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gcugaaaggg acuuccaag 19

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aagggacuuc caaggaaga 19

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agggacuucc aaggaagau 19

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gggacuucca aggaagaug 19

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gacuuccaag gaagaugcc 19

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aggaagaugc caugaaagc 19

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ggaagaugcc augaaagcu 19

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gaagaugcca ugaaagcuu 19

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agaugccaug aaagcuuac 19

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gaugccauga aagcuuaca 19

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augaaagcuu acaucaaca 19

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ugaaagcuua caucaacaa 19

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gaaagcuuac aucaacaaa 19

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agcuuacauc acaaaguag 19

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cuuacaucaa caaaguaga 19

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uuacaucaac aaaguagaa 19

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uacaucaaca aaguagaag 19

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acaucaacaa aguagaaga 19

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caucaacaaa guagaagag 19

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aucaacaaag uagaagagc 19

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caacaaagua gaagagcua 19

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aacaaaguag aagagcuaa 19

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gaagagcuaa agaaaaaau 19

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ucagagagau ccucauaaa 19

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aaucagagag auccucaua 19

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gucuuaccgg aaaacucga 19

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cgaaaggucu uaccggaaa 19

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accgaaaggu cuuaccgga 19

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gucgccaguc aaguaacaa 19

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agaucgugga uuacgucgc 19

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agauagugau gaaguacau 19

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ugaagacucu gcucaguuu 19

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gcaugcggcc ucuguuuga 19

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gagauaguga ugaaguaca 19

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gaagacucug cucaguuug 19

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gaaagaatct gtagagaaa 19

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gcaatgagct gtttgaaga 19

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tgacaaaggt ggataaatt 19

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agacagttat gcagctatt 19

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gcgccagagt gaacaagta 19

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gagcgagatt gcaggcata 19

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ggaaatggaa ccactaaga 19

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ccacacagca ttcttgtaa 19

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ggacttggcc gatccagaa 19

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gcacttggat cgagatgag 19

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ccgaatcaat cgcatcttc 19

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gacatgatcc tgcagttca 19

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gagcgaatcg tcaccactt 19

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cctccgagct ggcgtctac 19

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tcacatggtt aacctctaa 19

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gatgagggac gccataatc 19

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cctctaacta caaatctta 19

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ggaaggtgct atccaaaat 19

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gcaagcaagt cctaacatt 19

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ggaagaggag tagacctta 19

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aggaatcagt gttgtagta 19

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gaagaggagt agaccttac 19

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aaagtcaagc ctggtatta 19

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gctatgaacg tgaatgatc 19

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caagcctggt attacgttt 19

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ggaacaagat ctgtcaatt 19

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gcaatgaacg tgaacgaaa 19

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caatgaacgt gaacgaaat 19

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ggacaggagc ggtatcaca 19

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agacagagct tgagaataa 19

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gagaagatct ttatgcaaa 19

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gaagagaaat cagcagata 19

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gcaagtaact caactaaca 19

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gagctaatct gccacattg 19

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gcagatgagt tactagaaa 19

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caacttaatt gtccagaaa 19

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caacacagga ttctgataa 19

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agattgtgcc taagtctct 19

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atgaagatct ggaggtgaa 19

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tttgagactt cttgcctaa 19

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agatcaccct ccttaaata 19

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caacggattt ggtcgtatt 19

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gaaatcccat caccatctt 19

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gacctcaact acatggttt 19

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tggtttacat gttccaata 19

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gaagaaatcg atgttgttt 19

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acacaaactt gaacagcta 19

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ggaagaaatc gatgttgtt 19

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gaaacgacga gaacagttg 19

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gcacatggat ggaggttct 19

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gcagagagag cagatttga 19

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gaggttctct ggatcaagt 19

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gagcagattt gaagcaact 19

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caaagacgat gacttcgaa 19

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gatcagcatt tgcatggaa 19

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tccaggagtt tgtcaataa 19

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ggaagctgat ccaccttga 19

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gcagaaatct aaggatata 19

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caacaaggat gaagtctat 19

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cagcagaaat ctaaggata 19

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ctagatggct ttctcagta 19

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agacaaggtc ccaaagaca 19

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ggaatggcaa gaccagcaa 19

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agaattattc cagggttta 19

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gcagacaagg tcccaaaga 19

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agaagcagct tcaggatga 19

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gagcttgact tccagaaga 19

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ccaccgaagt tcaccctaa 19

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gagaagagct cctccatca 19

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gaaagagcat ctacggtga 19

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gaaaggattt ggctacaaa 19

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acagcaaatt ccatcgtgt 19

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ggaaagactg ttccaaaaa 19

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caacacgcct catcctcta 19

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catgaaagct tacatcaac 19

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gatcaaatct gaagaagga 19

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gccaagaagt ttcctaata 19

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cagcatatct tgaaccatt 19

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gaacaaagga aacggatga 19

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cggaaacggt ccaggctat 19

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gcttcgagca gacatgata 19

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cctacacggt cctcctata 19

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gccaagaacc tcatcatct 19

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gatatgggct gaatacaaa 19

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gcactctgat tgacaaata 19

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tgaagtctct gattaagta 19

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tcagagagat cctcataaa 19

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gcaagaagat caccatttc 19

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gagagaaatt tgaggatga 19

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gaaaggattt ggctataag 19

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gaaagaaggc atgaacatt 19

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gggagatagt gatgaagta 19

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gaagtacatc cattataag 19

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gtacgacaac cgggagata 19

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agatagtgat gaagtacat 19

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tgaagactct gctcagttt 19

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gcatgcggcc tctgtttga 19

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gcacacagcu uacuacauc 19

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gaaaugcccu gguaucuca 19

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gaaggaacgu gaugugauc 19

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gcacuacucc uguguguga 19

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gaacccagcu ggagaacuu 19

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gauauacagu gugaucuua 19

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guacuacgau ccugauuau 19

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gugccgaccu uuacaauuu 19

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gaaggaaact gaattcaaa 19

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aauaggcugu ucccaugua 19

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uaaacaaugc ugauuuggu 19

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gaacuuuaag caacaagac 19

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gcagaggacc agcuaagag 19

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uuuccagacu uccuugaga 19

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gaagaccuca ccuagaaau 19

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ucuaaacaau gcugauuug 19

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caagaccacc acuucgaaa 19

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aaacggagcu gaacaauag 19

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gccuguagcc cauguugua 19

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gagaagaccu caccuagaa 19

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acuauucagu ggcgagaaa 19

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We claim:

1. An siRNA comprising a sense region and an antisense region, wherein said sense region and said antisense region together form a duplex region, said antisense region and said sense region are each 18-30 nucleotides in length and said antisense region comprises a sequence that is at least 90% complementary to a sequence selected from the group consisting of SEQ. ID NOs. 438-513.

2. An siRNA comprising a sense region and an antisense region, wherein said sense region and said antisense region together form a duplex region and said sense region and said antisense region are each 18-30 nucleotides in length, and said antisense region comprises a sequence that is 100% complementary to a contiguous stretch of at least 18 bases of a sequence selected from the group consisting of SEQ. ID NOs. 438-513.

3. The siRNA of claim 2, wherein each of said antisense region and said sense region are 19-30 nucleotides in length, and said antisense region comprises a sequence that is 100% complementary to said sequence selected from the group consisting of: SEQ. ID NOs. 438-513.

4. A pool of at least two siRNAs, wherein said pool comprises a first siRNA and a second siRNA, said first siRNA comprises a first antisense region and a first sense region that together form a first duplex region and each of said first antisense region and said first sense region are 18-30 nucleotides in length and said first antisense region is at least 90% complementary to 18 bases of a first sequence selected from the group consisting of: SEQ. ID NOs. 438-513 and said second siRNA comprises a second antisense region and a second sense region that together form a second duplex region and each of said second antisense region and said second sense region are 18-30 nucleotides in length and said second antisense region is at least 90% complementary to 18 bases of a second sequence selected from the group consisting of: SEQ. ID NOs. 438-513, wherein said first antisense region and said second antisense region are not identical.

5. The pool of claim 4, wherein said first antisense region comprises a sequence that is 100% complementary to at least 18 bases of said first sequence, and said second antisense region comprises a sequence that is 100% complementary to at least 18 bases of said second sequence.

6. The pool of claim 4, wherein said first siRNA is 19-30 nucleotides in length and said first antisense region comprises a sequence that is at least 90% complementary to said first sequence, and second siRNA is 19-30 nucleotides in length and said second antisense region comprises a sequence that is at least 90% complementary to said second sequence.

7. The pool of claim 4, wherein said first antisense region is 19-30 nucleotides in length and said first antisense region comprises a sequence that is 100% complementary to at least 18 bases of said first sequence, and said second antisense region is 19-30 nucleotides in length and said second antisense region comprises a sequence that is 100% complementary to said second sequence.

8. The siRNA of claim 1, wherein said antisense region and said sense region are each 19-25 nucleotides in length.

9. The siRNA of claim 4, wherein said first antisense region, said first sense region, said second sense region and said second antisense region are each 19-25 nucleotides in length.

* * * * *