Characterization of side reactions during the annealing of small interfering RNAs

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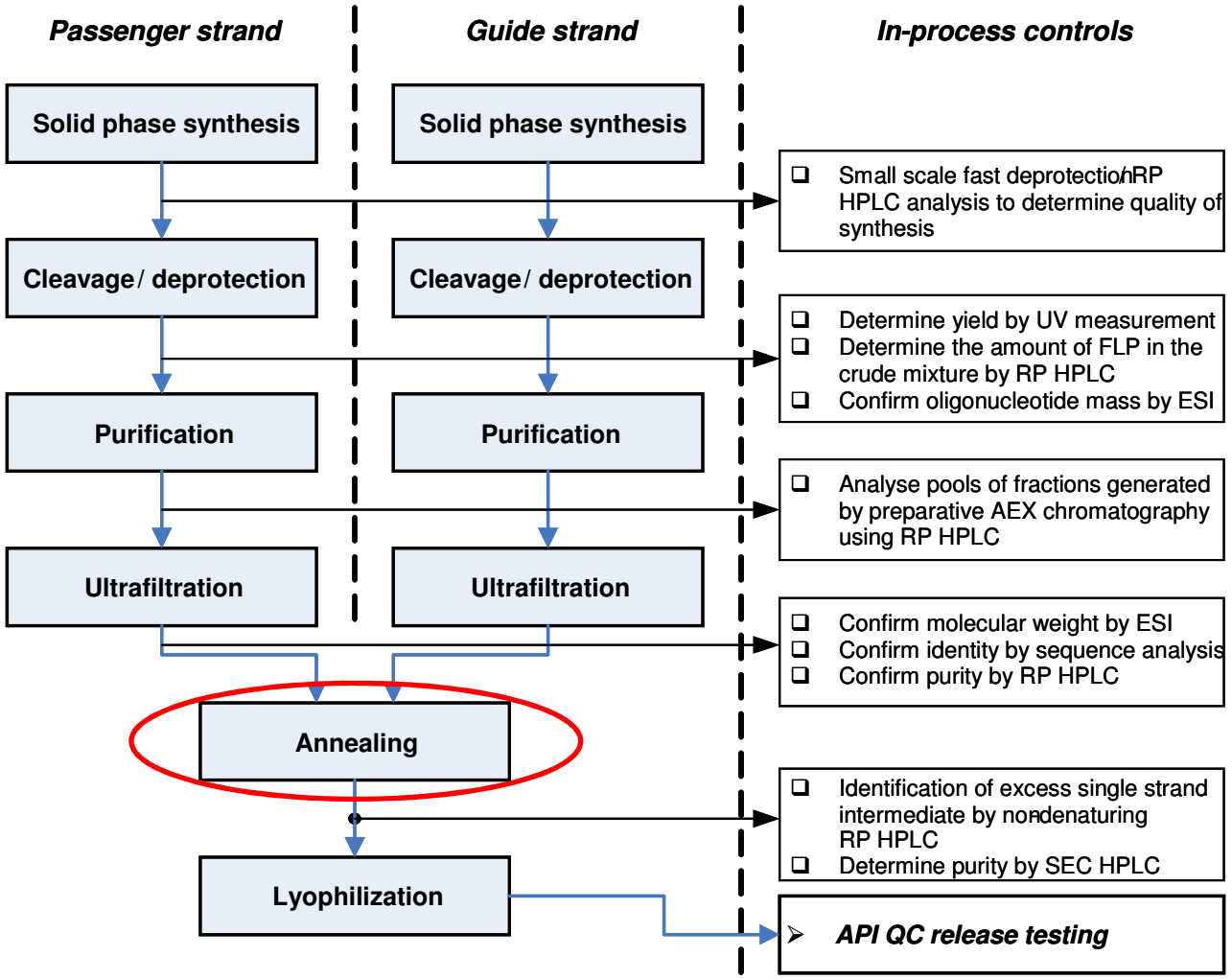
Eurotides, Berlin
15 – 16 November 2011

Introduction

- The impact of annealing temperature on siRNA-composition was investigated
- Non-denaturing IP-RP HPLC was optimized
- The impact of annealing temperature on strand-degradation was investigated for 2‘-OH RNA and 2‘-deoxy-2‘-Fluoro RNA
- Impact of buffer composition and strand concentration on siRNA T_m was investigated

Scheme of siRNA manufacturing



Sequence of siRNA-1

siRNA-1

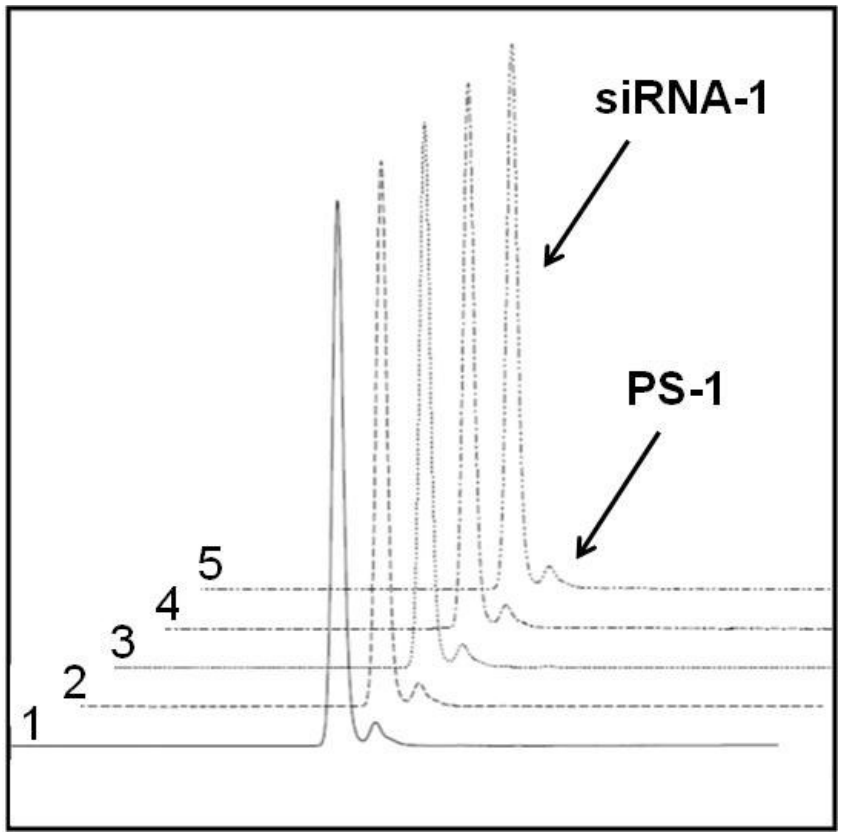
PS-1 5'-cuuAcGcuGAGuAcuucGATT-3'

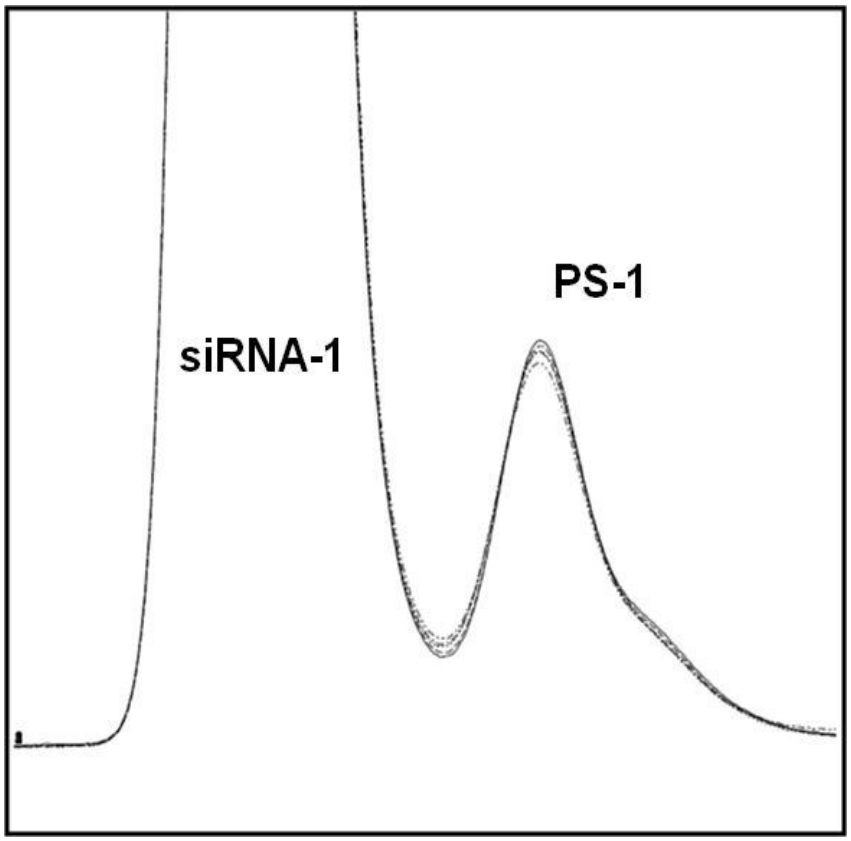
GS-1 3'-TTGAAuGCGAcUCAuGAAGCU-5'

Upper case = 2'-OH Lower case = 2'-*O*-Methyl-RNA TT = 2'-deoxy phosphorothioate

SEC analysis of siRNA-1

No difference in SEC profiles is detected at elevated annealing temperatures
Annealing: 10 minutes at target temperature, slow cooling to RT (~2-3 hours)

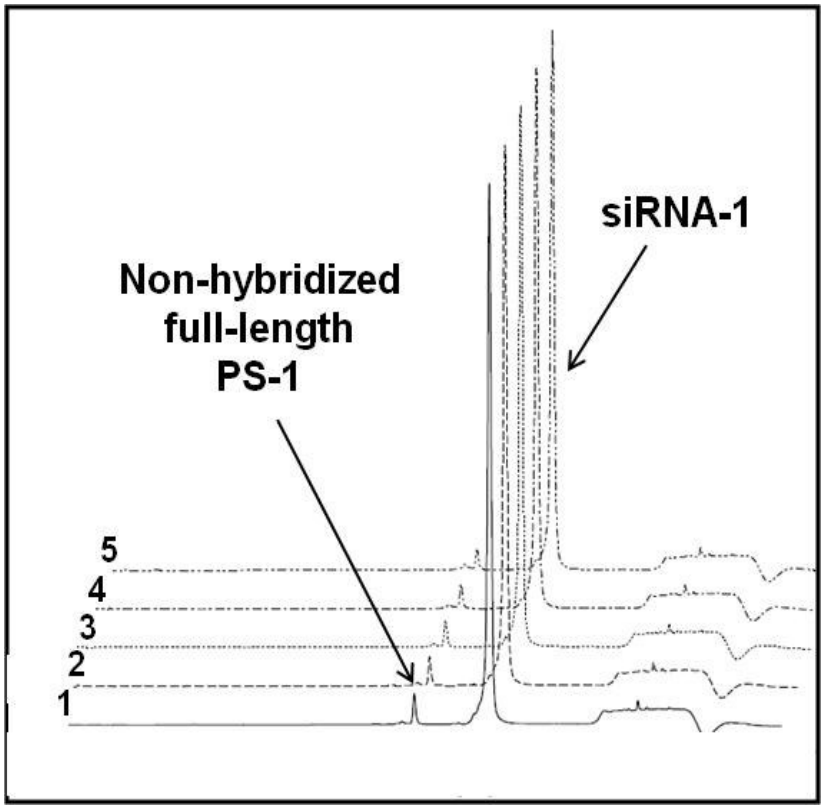


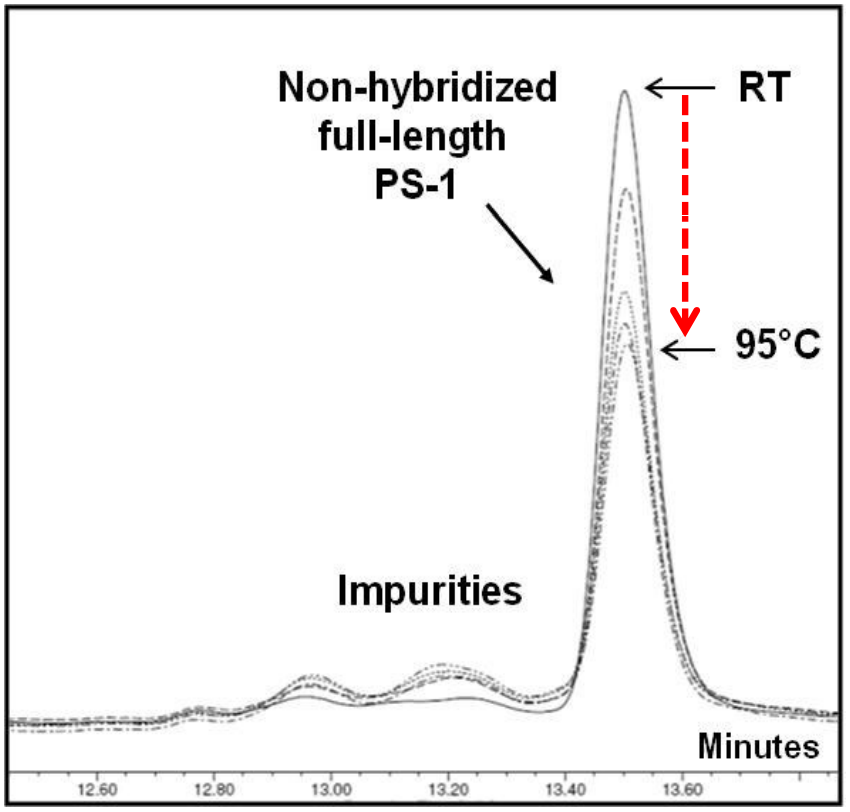


Annealing temperature: 1 = RT; 2 = 60°C; 3 = 70°C; 4 = 80°C; 5 = 95°C

Non-denaturing IP-RP analysis of siRNA-1

A decrease in **non-hybridized full-length strand** is observed at elevated annealing temperatures

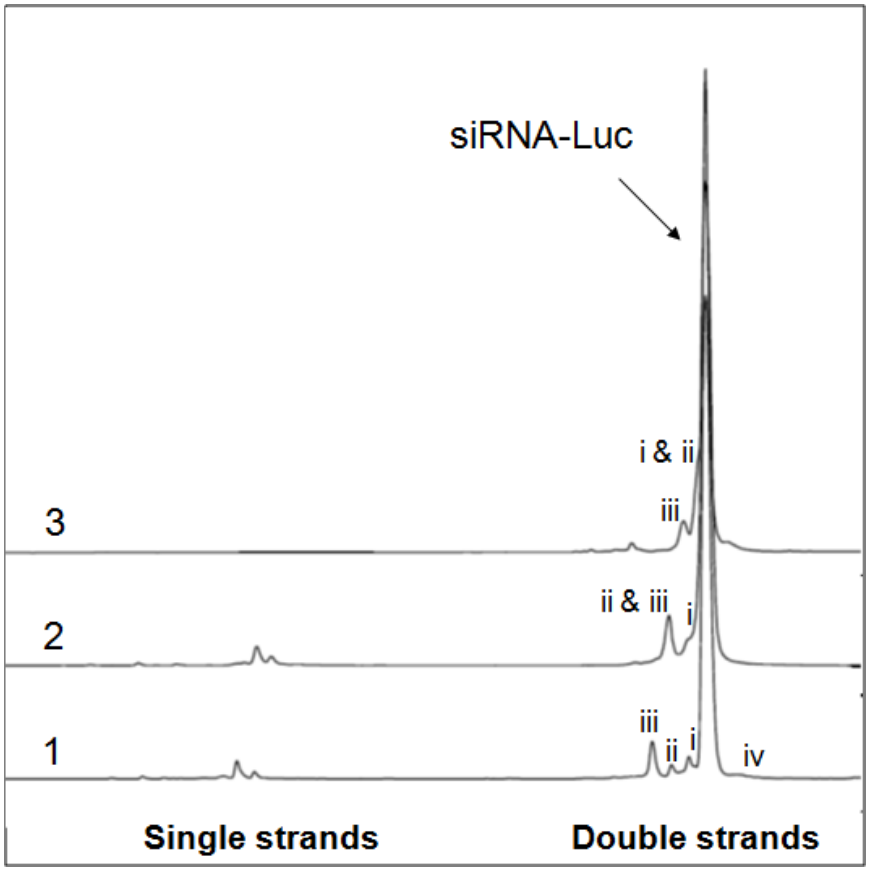




Annealing temperature: 1 = RT; 2 = 60°C; 3 = 70°C; 4 = 80°C; 5 = 95°C

Optimizing non-denaturing IP-RP HPLC

3 ion-pairing buffer systems were evaluated for duplex separation efficiency



Buffer composition:

Trace 1: 25 mM HA/100 mM HFIP

Trace 2: 25 mM HAA

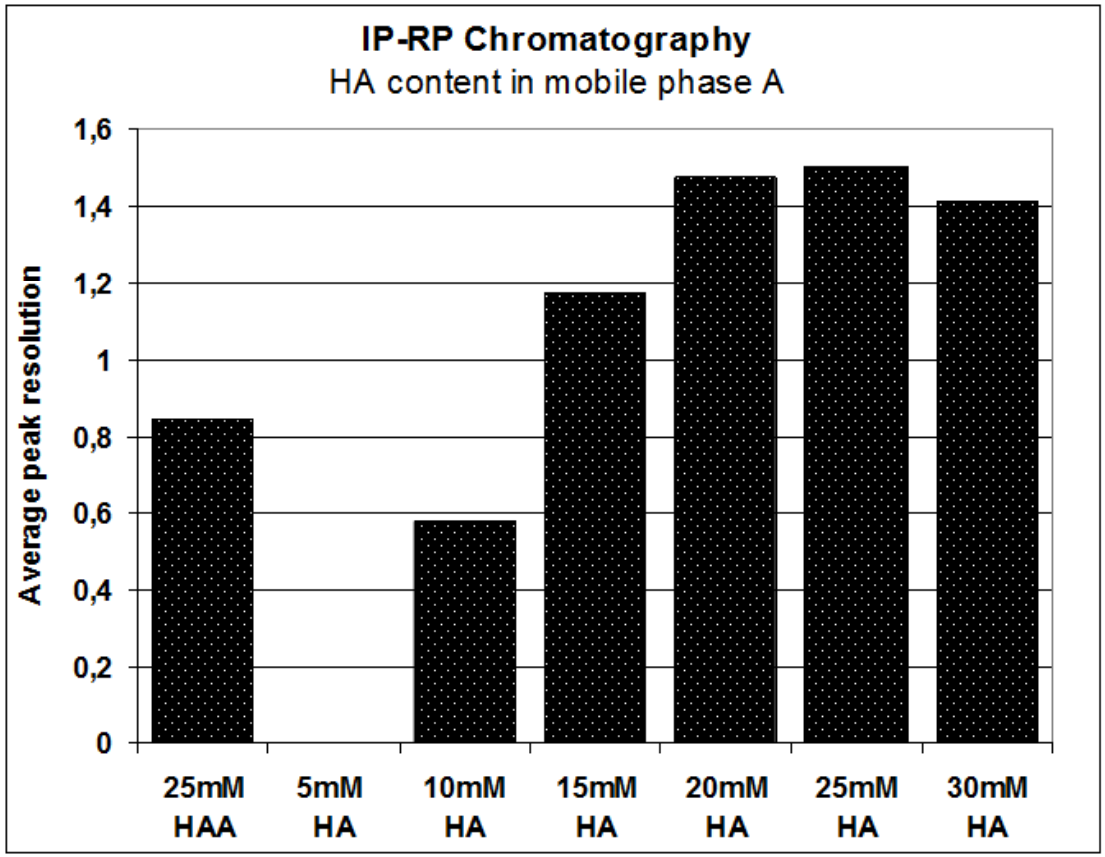
Trace 3: 16.5 mM TEA/100 mM HFIP

Peaks:

i = Variant 1 (N_P/N-1_G)
ii = Variant 2 (N_P/N-2_G)
iii = Variant 3 (N-1_P/N_G)
iv = late eluters

Optimizing non-denaturing IP-RP HPLC

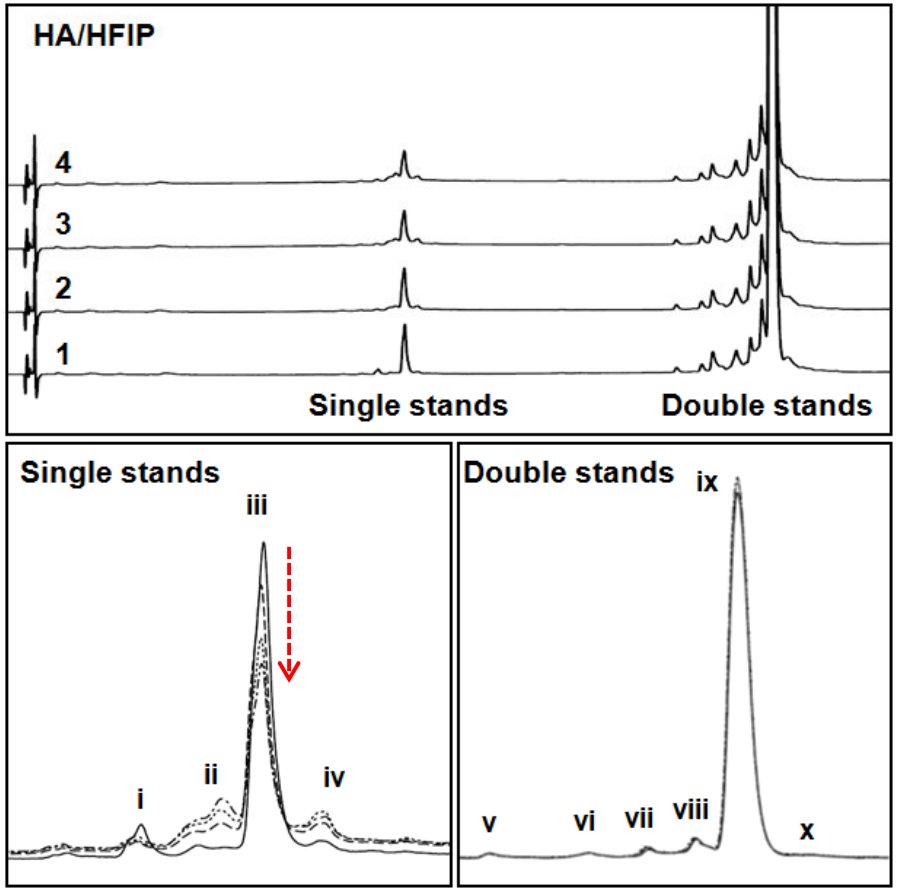
Mobile Phase A: 25 mM HA / 100 mM HFIP
Mobile Phase B: water / acetonitrile (20:80)



Modified from: Noll et al., Journal of Chromatography A (2011) 1218(33), 5609-17

Non-denaturing IP-RP analysis of siRNA-1

With temperature, strands distribute between single and duplex fractions



IP-RP Buffer:
25 mM HA/100 mM HFIP

Single strand peaks:

i =	$N\text{-}2_G$
ii =	$N\text{-}1_G$
iii =	N_G
iv =	$N+A_G$

Duplex peaks:

v =	$(N\text{-}4_P/N_G)$
vi =	$(N\text{-}1_P/N_G)$
vii =	$(N_P/N\text{-}2_G)$
viii =	$(N_P/N\text{-}1_G)$
ix =	(N_P/N_G)
x =	$(N_P/N+G_G)$

Annealing temperature: 1 = RT; 2 = 60°C; 3 = 70°C; 4 = 80°C; 5 = 95°C

Effect of annealing temperature on siRNA

Annealing temperature: Decrease in non-hybridized full-length strand

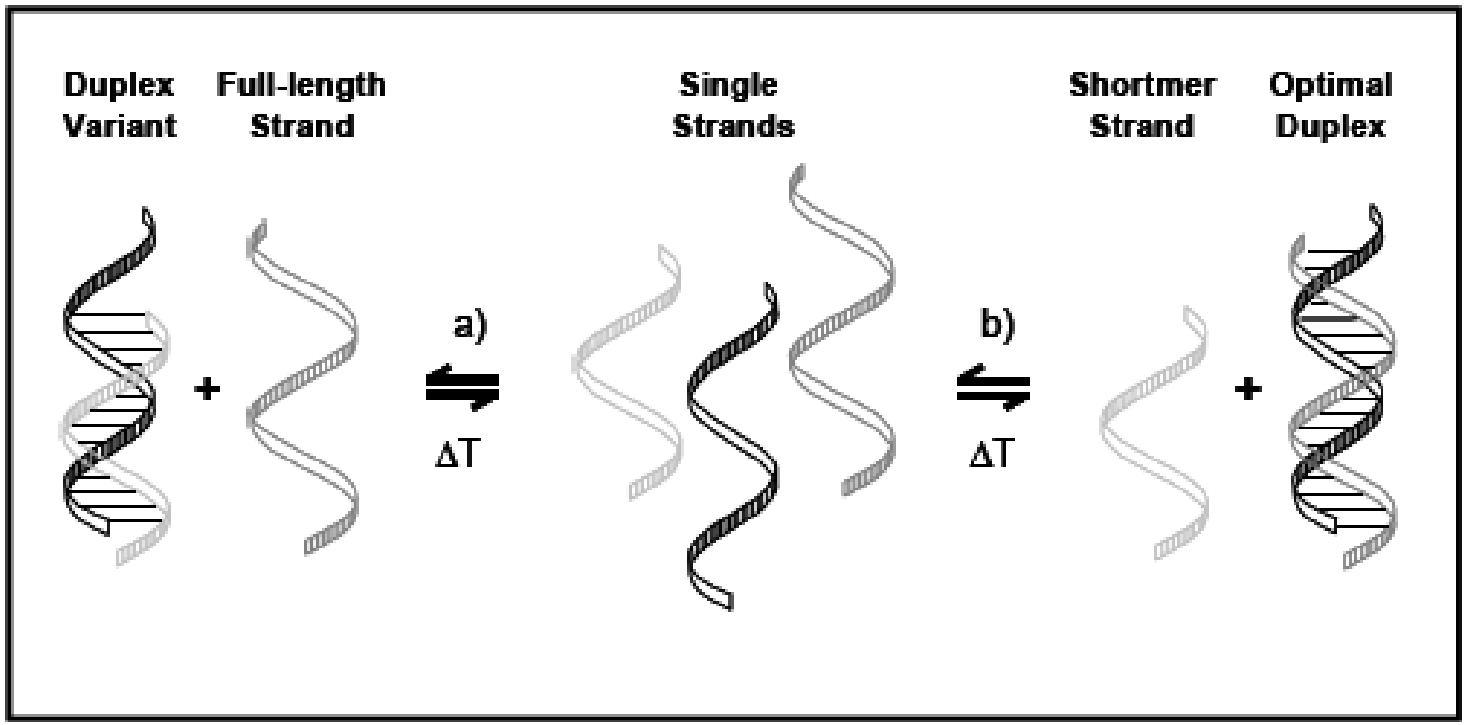
siRNA-1	Duplex [Peak Area %]		Total Single Strand [Peak Area %]	PS-1 FLP [Peak Area %]
	SEC	IP-RP	SEC	IP-RP
RT	94,2	96,5	5,8	2,6
60°C	94,3	96,5	5,7	2,4
70°C	94,3	96,7	5,7	2,1
85°C	94,3	96,7	5,7	1,9
95°C	94,4	96,7	5,6	1,8

siRNA-2	Duplex [Peak Area %]		Total Single Strand [Peak Area %]	PS-2 FLP [Peak Area %]
	SEC	IP-RP	SEC	IP-RP
RT	94,2	95,4	6,6	3,6
60°C	94,3	95,4	6,6	3,5
70°C	94,3	95,5	6,7	3,3
85°C	94,3	95,5	6,7	3,1
95°C	94,4	95,5	6,7	2,6

- Total duplex peak area remains constant
- Total single-strand peak area remains constant
- Non-hybridized full-length single strand peak area decreases

Effect of annealing temperature on siRNA

- Changing the annealing temperature leads to re-distribution of strands between single-strand and duplex fraction
- Thermodynamic control over kinetic control



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Effect of annealing temperature on siRNA

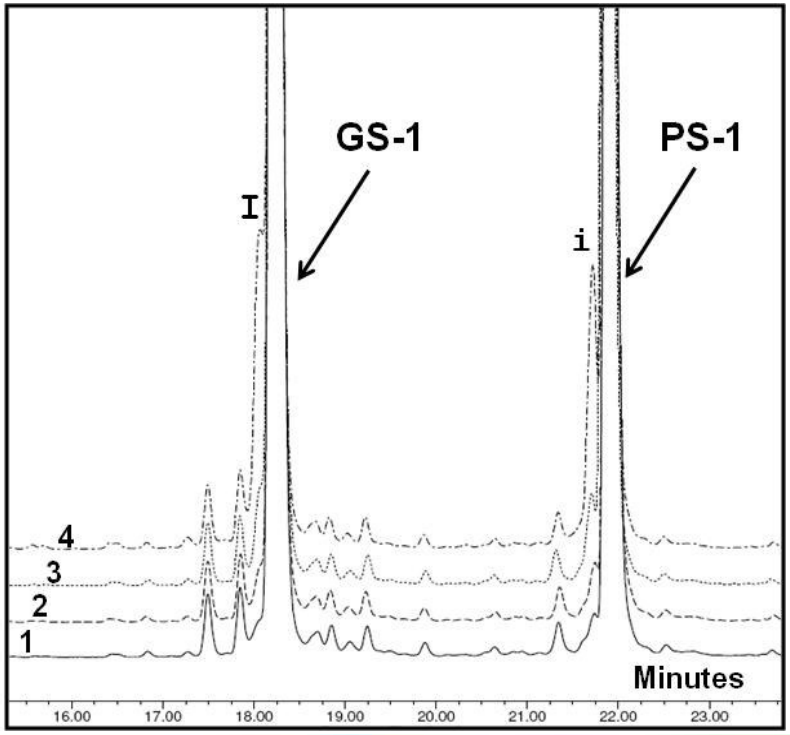
T_m of a duplex depends on buffer conditions and strand concentration

Tm [°C]	siRNA-1		siRNA-2	
	Water	NaCl	Water	NaCl
4µM	31,8	80,6	40,1	84,1
20µM	43,1	82,5	50,7	85,5
100µM	52,2	84,2	59,3	87,7
500µM	67,1	86,9	75,3	90,6

Effect of elevated temperature on siRNA

- Elevated temperatures cause side reactions in nucleic acids
- Additional peaks observed in IP-RP HPLC and AEX HPLC
- Molecular mass of the new peaks identical to full-length strands

Incubation time: 4 hours in water



I = 2‘,5‘-isomers of GS-1
(Molecular mass = $M_{r(GS-1)}$)

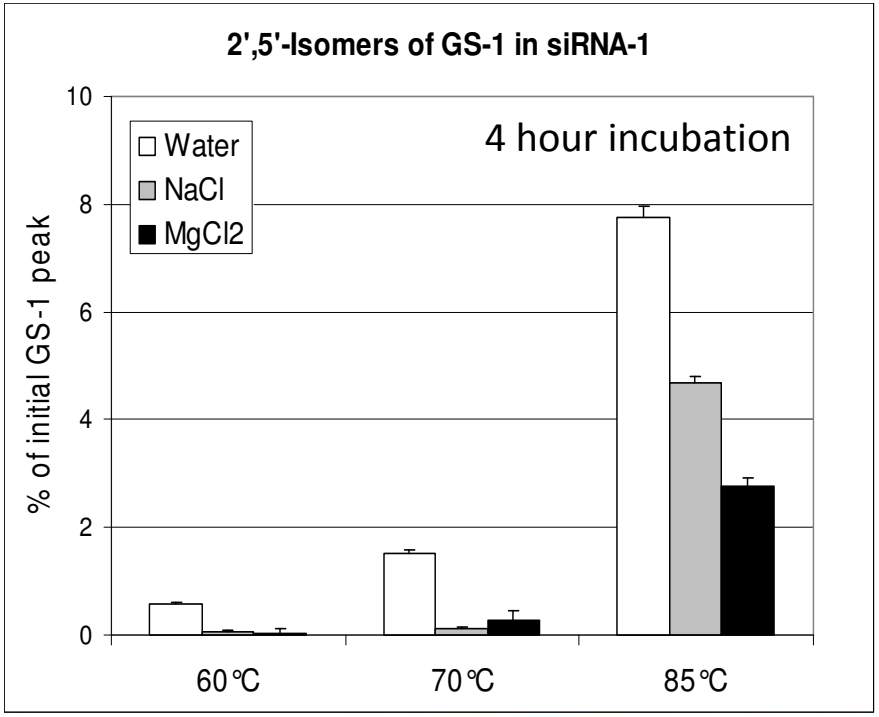
i = 2‘,5‘-isomers of PS-1
(Molecular mass = $M_{r(PS-1)}$)

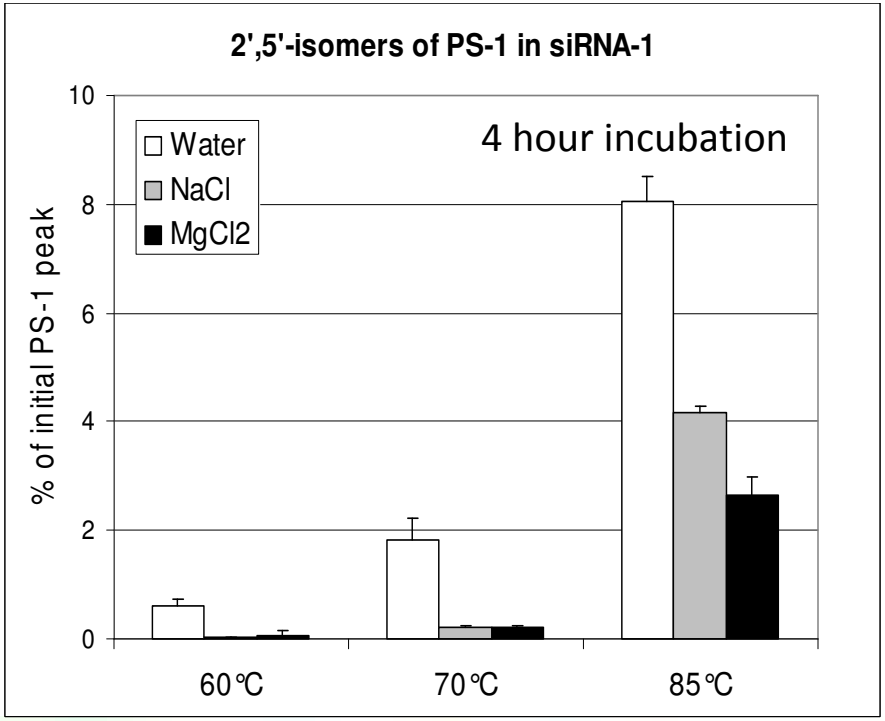
Incubation Temperature:

1 = RT
2 = 60°C
3 = 70°C
4 = 80°C

Side reactions of 2‘-OH RNA

- 2‘,3‘-isomerization is the major side reaction in 2‘OH RNA
- Side reactions are inhibited by duplex formation
- Duplex stability depends on buffer composition



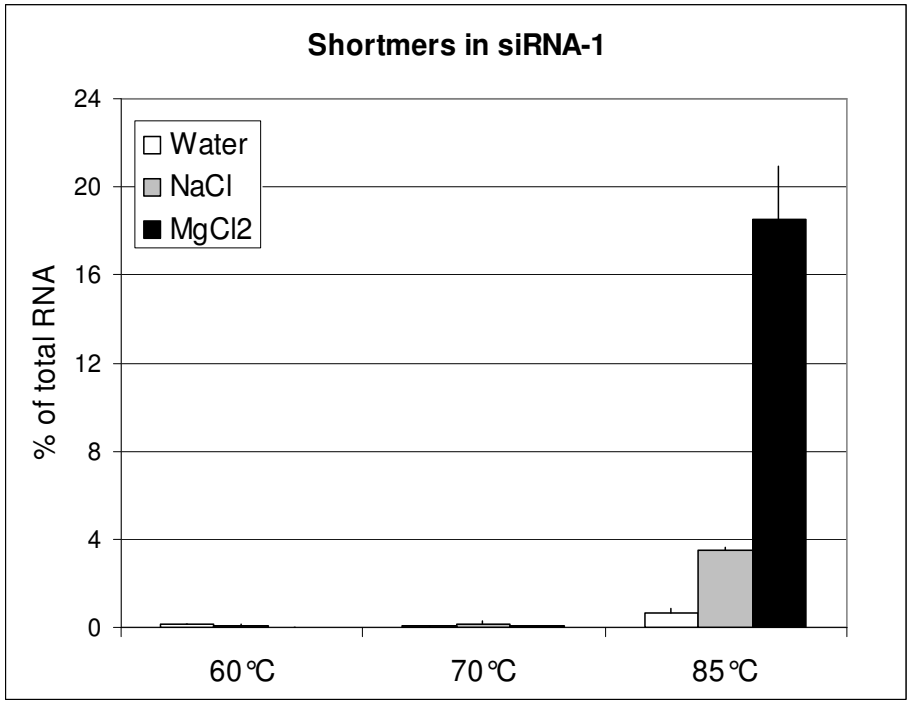


Modified from: Seiffert et al., Analytical Biochemistry (2011), 414(1), 47-57

Side reactions of 2‘-OH RNA

- Strand scission is observed in the presence of divalent cations
- Strand scission occurs on all 2‘-OH positions (Detected by LC-MS)
- Strand scission is inhibited by duplex formation

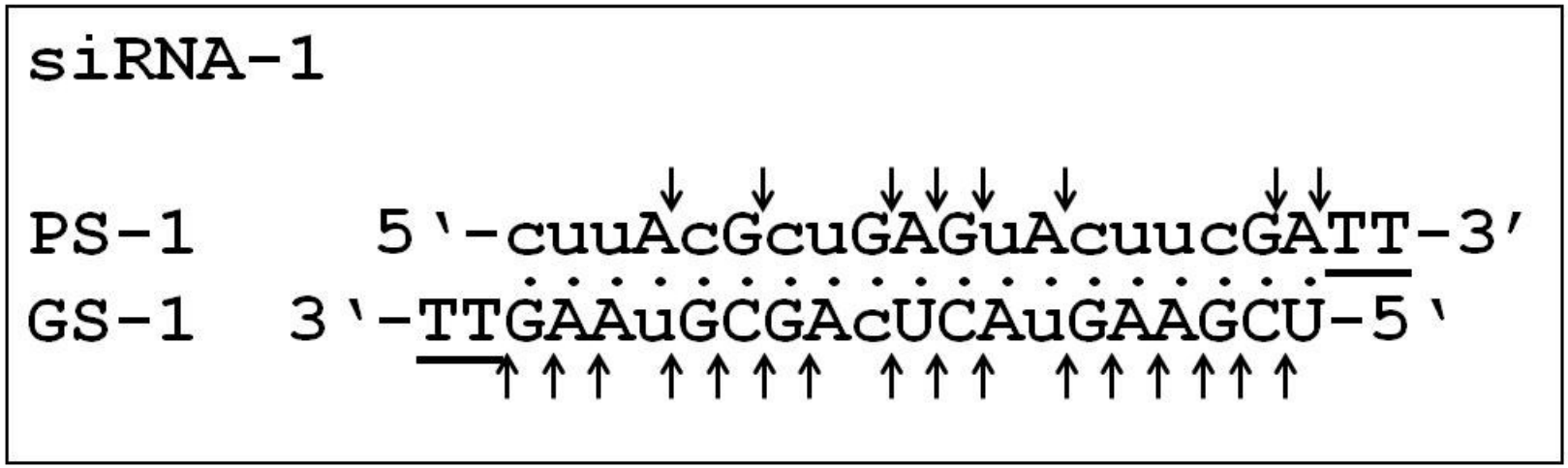
Incubation time: 4 hours



Strand ID	5'-Sequence-3'	3' Mod	M_r calculated	M_r detected
GS-1	UCGAAGuACUcAGCGuAAGTT	OH	6751,3	6751,9
5'N-1|3'N-0	CGAAGuACUcAGCGuAAGTT	OH	6445,1	6447,7
5'N-2|3'N-0	GAAGuACUcAGCGuAAGTT	OH	6139,9	
5'N-3|3'N-0	AAGuACUcAGCGuAAGTT	OH	5794,7	5795,9
5'N-4|3'N-0	AGuACUcAGCGuAAGTT	OH	5465,5	5466,0
5'N-5|3'N-0	GuACUcAGCGuAAGTT	OH	5136,3	5136,1
5'N-6|3'N-0	uACUcAGCGuAAGTT	OH	4791,1	4790,9
5'N-8|3'N-0	CUcAGCGuAAGTT	OH	4141,7	4142,9
5'N-9|3'N-0	UcAGCGuAAGTT	OH	3836,5	3836,9
5'N-10|3'N-0	cAGCGuAAGTT	OH	3530,3	3530,1
5'N-12|3'N-0	GCGuAAGTT	OH	2881,9	2881,9
5'N-0|3'N-3	UCGAAGuACUcAGCGuAA	PO4	5861,6	5861,9
5'N-0|3'N-4	UCGAAGuACUcAGCGuA	PO4	5532,4	5532,1
5'N-0|3'N-4	UCGAAGuACUcAGCGuA	cP	5514,4	5513,9
5'N-0|3'N-6	UCGAAGuACUcAGCG	PO4	4883,0	4884,0
5'N-0|3'N-6	UCGAAGuACUcAGCG	cP	4865,0	4865,9
5'N-0|3'N-7	UCGAAGuACUcAGC	PO4	4537,8	4538,9
5'N-0|3'N-7	UCGAAGuACUcAGC	cP	4519,8	4520,9
5'N-0|3'N-8	UCGAAGuACUcAG	PO4	4232,6	4232,9
5'N-0|3'N-8	UCGAAGuACUcAG	cP	4214,6	4215,0
5'N-0|3'N-9	UCGAAGuACUcA	PO4	3887,4	3888,0
5'N-0|3'N-9	UCGAAGuACUcA	cP	3869,4	3870,0

Strand ID	5'-Sequence-3'	3' Mod	M_r calculated	M_r detected
PS-1	cuuAcGcuGAGuAcuucGATT	OH	6777,4	6779,9
5'N-4|3'N-0	cGcuGAGuAcuucGATT	OH	5488,6	5489,9
5'N-6|3'N-0	cuGAGuAcuucGATT	OH	4824,2	4823,9
5'N-9|3'N-0	AGuAcuucGATT	OH	3839,5	3840,0
5'N-10|3'N-0	GuAcuucGATT	OH	3510,3	3510,1
5'N-11|3'N-0	uAcuucGATT	OH	3165,1	3165,9
5'N-13|3'N-0	cuucGATT	OH	2515,7	2516,0
5'N-0|3'N-2	cuuAcGcuGAGuAcuucGA	cP	6214,9	6215,8
5'N-0|3'N-3	cuuAcGcuGAGuAcuucG	PO4	5903,7	5903,9
5'N-0|3'N-3	cuuAcGcuGAGuAcuucG	cP	5885,7	5885,8
5'N-0|3'N-8	cuuAcGcuGAGuA	PO4	4279,7	4280,8
5'N-0|3'N-8	cuuAcGcuGAGuA	cP	4261,7	4262,9
5'N-0|3'N-10	cuuAcGcuGAG	PO4	3630,3	3630,0
5'N-0|3'N-10	cuuAcGcuGAG	cP	3612,3	3612,0
5'N-0|3'N-12	cuuAcGcuG	cP	2937,9	2937,9
5'N-0|3'N-15	cuuAcG	cP	1953,3	1954,0

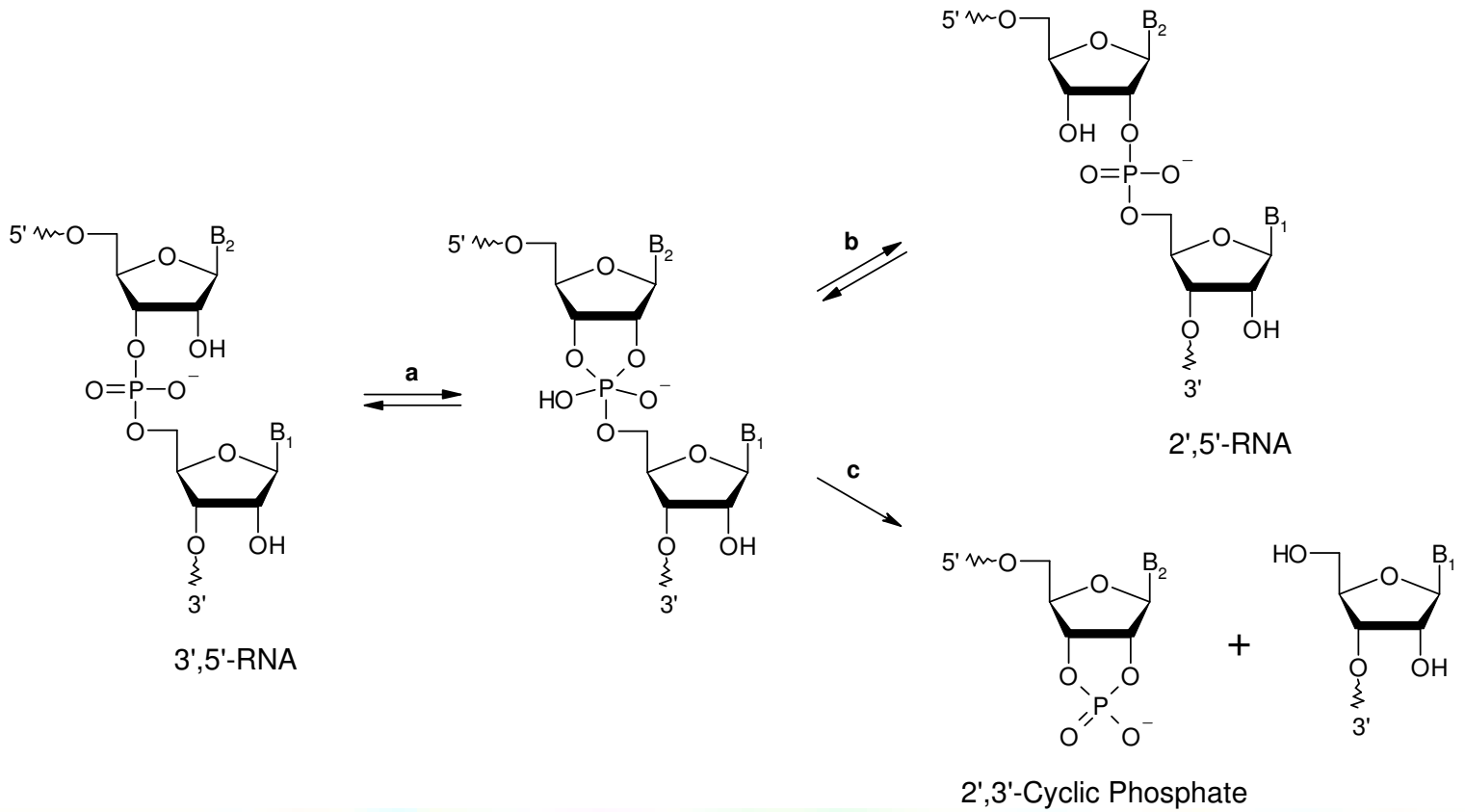
Effect of elevated temperature on siRNA



Upper case = 2'-OH Lower case = 2'-OMe-RNA TT = 2'-deoxy phosphorothioate

Side reactions of 2‘-OH RNA

- Absence of divalent cations leads to 2‘,3‘-isomerization (pathways a + b)
- Presence of divalent cations leads to strand scission (pathway c)



Modified from: Seiffert et al., Analytical Biochemistry (2011), 414(1), 47-57

Sequence of siRNA-2

siRNA-2

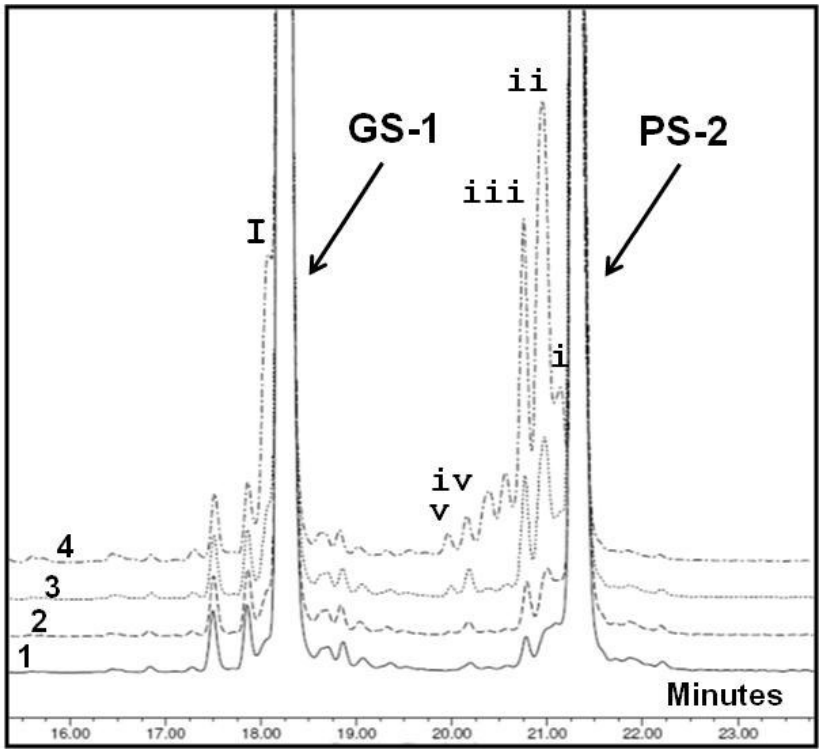
PS-2 5'-*cuu*A*c*G*cu*GAG*u*A*cuuc*GATT-3'

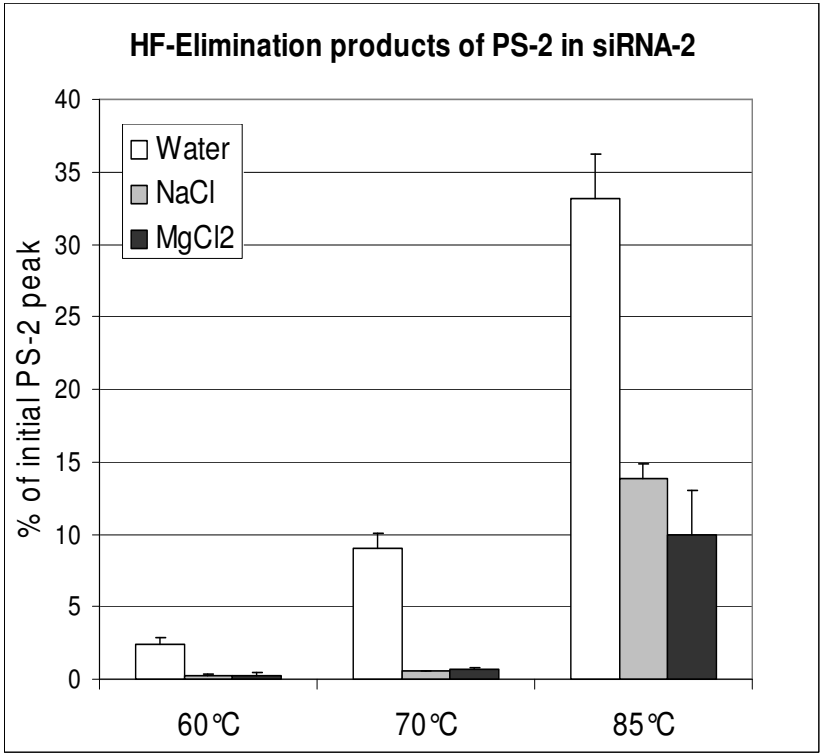
GS-1 3'-TTGAAuGCGAcUCAuGAAGCU-5'

Upper case = 2'-OH Lower case italic = 2'-fluoro-RNA TT = 2'-deoxy phosphorothioate

Side reactions of 2‘-fluoro-RNA

- 2‘,3‘-isomerization is reduced in 2‘-F RNA
- Additional degradation products are observed
- Side reactions are inhibited by duplex formation

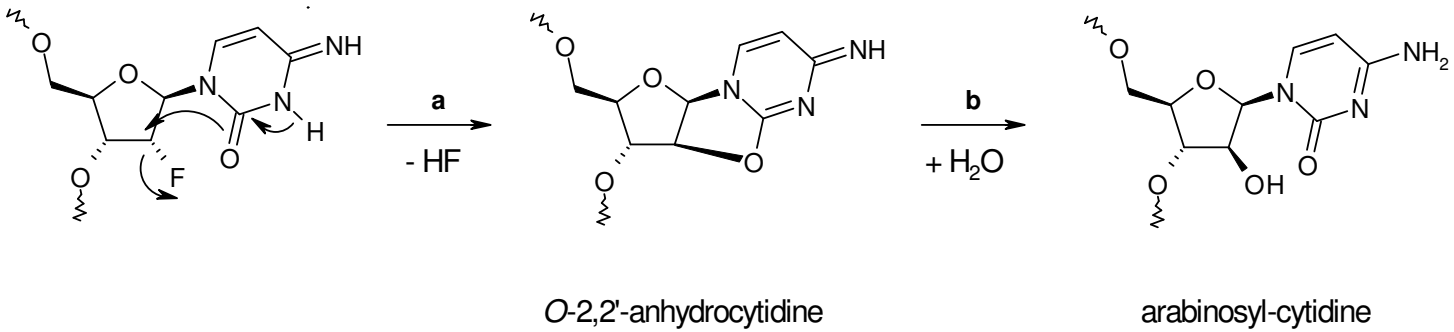




i = 2‘,5‘-isomers ii & iii = HF-elimination products $M_{r(parent)}$-2, $M_{r(parent)}$-4 iv & v = HF-elimin. products $M_{r(parent)}$-20

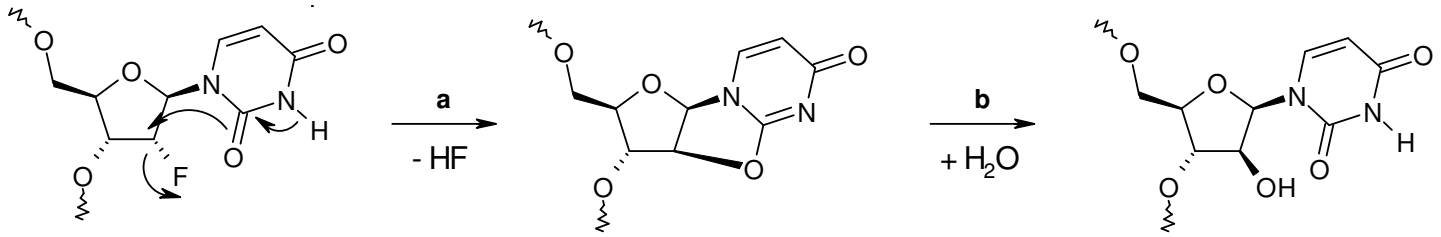
Side reactions of 2‘-fluoro-RNA

- HF-elimination leads to formation of *O*-2,2′-anhydro-nucleotides (pathway a)
- Hydrolysis leads to formation of arabinosyl-nucleotides (pathway b)



O-2,2'-anhydrocytidine

arabinosyl-cytidine

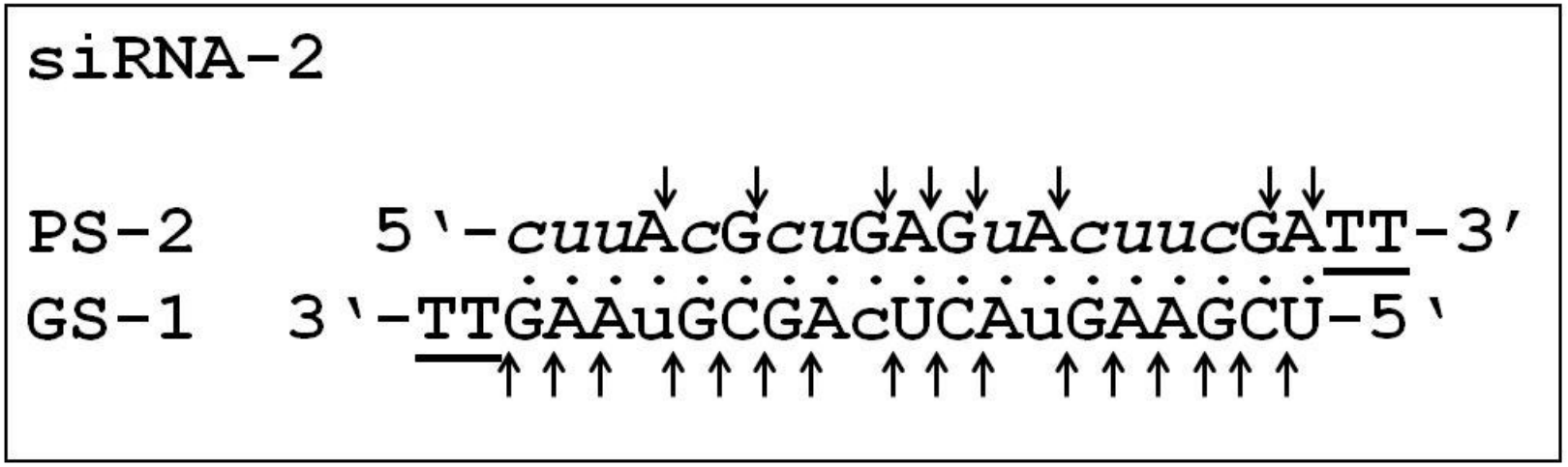


O-2,2'-anhydrouridine

arabinosyl-uridine

Doerr & Fox, J.Org.Chem 32 (1967), 1462
Krug et al., Nucleosides & Nucleotides 8 (1989) 1473

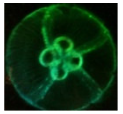
Side reactions of siRNA-2



Upper case = 2'-OH Lower case italic = 2'-fluoro-RNA TT = 2'-deoxy phosphorothioate

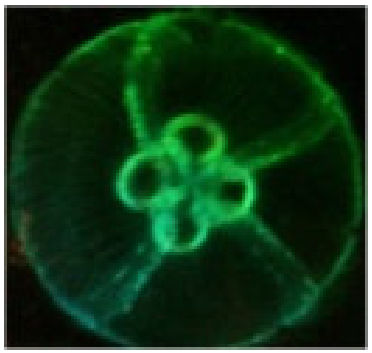
Summary

- Major side reactions during manufacturing of therapeutic siRNA are 2‘,3‘-isomerization, strand scission and HF-elimination
- Strand scission is strongly promoted by cations (divalent)
- Elevated temperatures promote side reactions
- Side reactions are inhibited in the duplex
- Annealing promotes formation of optimal duplex
- Annealing should be performed at the lowest possible temperature
- Optimal annealing temperature is close to the T_m of the duplex
- T_m is dependent on strand concentration and buffer composition



Acknowledgements

- Stephan Seiffert
- Mario Klobedanz
- Ingo Röhl
- Frank Hertel
- Josef Nerlich
- Harry Debelak
- Philipp Hadwiger
- Hans-Peter Vornlocher



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