Cloning of artificial microRNAs

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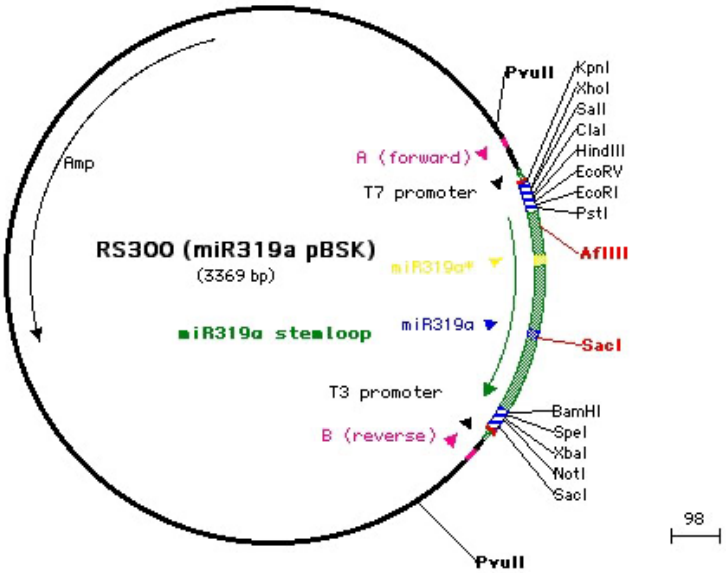
The artificial microRNA designer WMD delivers 4 oligonucleotide sequences (I to IV), which are used to engineer your artificial microRNA into the endogenous miR319a precursor by site-directed mutagenesis.
As a template for the PCRs, you need the plasmid pRS300, which contains the miR319a precursor in pBSK (cloned via SmaI site).
You can request this plasmid from Addgene (https://www.addgene.org/22846/).

map of pRS300:



sequence of pRS300 (notice: do not copy the sequence from Acrobat, it will lead to “deletion” of bases!):

GGAAATTGTAAACGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGAGATAGGGTTGAGTGTTGTTCCAG
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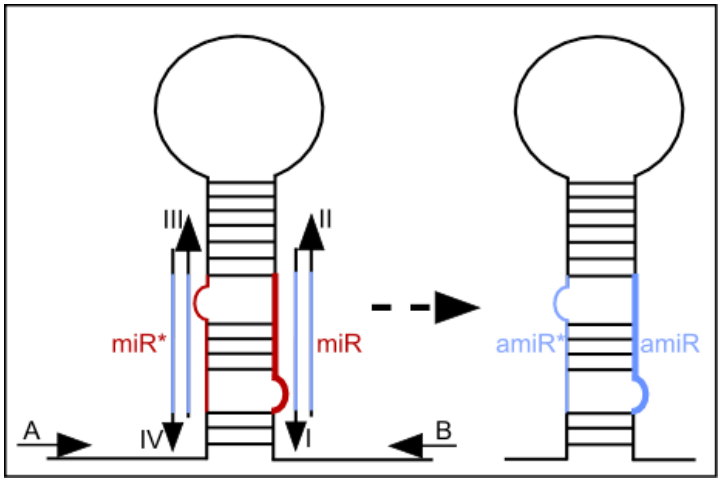
AGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTG

T7 SP6 amp resistance oligo A oligo B miRNA miRNA*

Cloning strategy:



I: microRNA forward
II: microRNA reverse
III: microRNA* forward
IV: microRNA* reverse

	forward oligo	reverse oligo	template
(a)	A	IV	pRS300
(b)	III	II	pRS300
(c)	I	B	pRS300
(d)	A	B	(a)+(b)+(c)

The amiRNA containing precursor is generated by overlapping PCR. A first round amplifies fragments (a) to (c), which are listed in the table above. These are subsequently fused in PCR (d).

Oligonucleotides A and B are based on the template plasmid sequence. They are located outside of the multiple cloning site of pBSK to generate bigger PCR products.

Their sequences:

A 5' CTG CAA GGC GAT TAA GTT GGG TAA C 3'
B 5' GCG GAT AAC AAT TTC ACA CAG GAA ACA G 3'

Cloning protocol:

PCR reactions (a), (b), (c):

5µl 10xPCR buffer (with Mg^{++})	95°C 2’
5µl dNTPs @ 2mM	95°C 30”
2µl each oligo @10µM	55°C 30” -> lower temp. for (b)
2µl plasmid DNA (1:100)	72°C 40”
0.5µl Pfu	-> 24 cycles
33.5µl water	72°C 7’

-> 50µl	

run on 2% gel -> cut bands -> elute in 20µl water

PCR reaction (d):

5µl 10xPCR buffer (with Mg^{++})	
5µl dNTPs @ 2mM	95°C 2’
2µl oligo A @ 10µM	95°C 30”
2µl oligo B @ 10µM	55°C 30”
0.5µl PCR (a)	72°C 1’30”
0.5µl PCR (b)	-> 24 cycles
0.5µl PCR (c)	72°C 7’
0.5µl Pfu	
34.5µl water	

-> 50µl	

run on 1% gel -> cut bands -> elute in 20µl water

Cloning of PCR products:
Any vector possible, but make sure that you choose the right sites to sequence. T3 and T7 are already part of the PCR fragment.
A-tailing of PCR products and cloning in to pGEM-T-easy works reliably, sequencing can be carried out with oligonucleotides A and B.

To release the amiRNA precursor fragment, any sites of the pBSK multiple cloning site can be used, since they are part of the PCR fragment. We normally use the EcoRI and BamHI sites. EcoRI is at the 5', BamHI at the 3' end of the precursor.

Alternative PCR strategy:

	forward oligo	reverse oligo	template
(a)	A	II	pRS300
(b)	I	B	pRS300
(c)	A	B	(a)+(b)
(d)	A	IV	(c)
(e)	III	B	(c)
(f)	A	B	(d)+(e)