

Supplementary Figures

Supplementary Figure 1. The number of mutant sequences that can be produced using various mutation-generation strategies.

1a)

$$\begin{aligned} \text{Total Mutant Sequences} &= \left[\sum_{i=2}^N \binom{N}{i} (n-1)^i \right]^x \\ &= \left[\sum_{i=2}^6 \binom{6}{i} (4-1)^i \right]^3 \\ &= \left[\binom{6}{2} 3^2 + \binom{6}{3} 3^3 + \binom{6}{4} 3^4 + \binom{6}{5} 3^5 + \binom{6}{6} 3^6 \right]^3 \\ &\approx 6.8 \times 10^{10} \end{aligned}$$

1b)

$$\begin{aligned} \text{Total Mutant Sequences} &= \left[\binom{N-1}{1} (n-1)^2 \right]^x \\ &= [(6-1)(4-1)^2]^3 \\ &\approx 9.1 \times 10^4 \end{aligned}$$

1c)

$$\text{Total Mutant Sequences} = \left[\left[\binom{N-1}{1} (n-1)^2 \right]^x \right]^y$$

$$= \left[[(6-1)(4-1)^2]^3 \right]^2$$

$$\approx 8.3 \times 10^9$$

1d)

$$\text{Total Mutant Sequences} = \left[\left[\binom{N-1}{1} (n-1)^2 \right]^x \right] \times y$$

$$= \left[[(6-1)(4-1)^2]^3 \right] \times 2$$

$$\approx 1.5 \times 10^5$$

Supplementary Figure 1. The number of mutant sequences that can be produced using various mutation-generation strategies.

"N" represents the number of nucleotide positions to mutate per predicted target site. ImiRP introduces mutations only into the 6mer core seed region of each predicted target site. The number of the four nucleotide bases to use for mutagenesis is represented by "n". "x" and "y" designate the number of specified target sites per independent region and the number of independent regions, respectively. **a)** The number of possible mutant sequences produced when at least two nucleotide changes are introduced per target site using any permutation of the four nucleotide bases. In this example, three non-overlapping target sites are present in a single independent region, and the total possible mutants are greater than 60 billion. **b)** When exactly two adjacent nucleotide changes are introduced per site, using any of the four nucleotide bases to generate mutations, the number of possible mutants for a single independent region containing three non-overlapping sites is reduced to approximately 90,000. **c)** If two independent regions containing three target sites each are mutated simultaneously by introducing two adjacent nucleotide changes using any of the four nucleotide bases, over 8 billion mutant sequences are possible. **d)** However, mutating predicted target sites spaced at a distance of seven or more nucleotides apart independent of one another significantly reduces the number of redundant mutations generated. When two independent regions containing three non-overlapping target sites each are mutated in isolation, the number of mutant sequences that need to be processed is reduced to 180,000

```
do:  
  copy input_sequence to mutant  
  for each site in mutationites:  
    for each position in site:  
      set nuc to select_random(allowable_nucleotides)  
      overwrite mutant[position] to nuc  
    end  
  end  
  if mutant acceptable:  
    output mutant to callback  
  end  
while(further mutations are needed)
```

Supplementary Figure 2.

Pseudocode for ImiRP's mutation generation algorithm.

```
for each five_prime in mirbase:
  do:
    set sequence_chunk to sequence[window_start, window_end]
    set match_result to check_conditions(five_prime, sequence_chunk)
    increment window_start
    increment window_end
  while(window_end < sequence_end)
end

function check_conditions(five_prime, sequence_chunk):

end
```

Supplementary Figure 3.

Pseudocode for ImiRP's microRNA target site prediction algorithm.

1a) 150 nucleotide segment of human *PAX6* 3'UTR

GTTTTGAAGTTGTTCACTACATATCAAATTAAACCATTGTTGATTGT
 AAAAAACCATGCCAAAGCCTTTGTATTTCTTTATTATACAGTTTTCTTT
 TTAACCTTATAGTGTGGTGTACAAATTTTATTTCCATGTTAGATCAACA

Start Position	miRNA	Site Type	Predicted by
22	hsa-miR-190a/b	8mer	TS
35	hsa-miR-140-5p	7mer-m8+G:U	PITA
60	hsa-miR-96/182/507/ 1271	7mer-A1	TS

Summary of ImiRP predictions for wild type human *PAX6* sequence:

position	mirna	type	gu
2	>hsa-miR-616-5p MIMAT0003284 Homo sapiens	OS-6mer	no
6	>hsa-miR-513c-3p MIMAT0022728 Homo sapiens	OS-6mer	yes
14	>hsa-miR-758-3p MIMAT0003879 Homo sapiens	6mer	no
16	>hsa-miR-382-5p MIMAT0000737 Homo sapiens	OS-6mer	no
16	>hsa-miR-494-5p MIMAT0026607 Homo sapiens	7mer-A1	yes
17	>hsa-miR-758-5p MIMAT0022929 Homo sapiens	OS-6mer	yes
18	>hsa-miR-203a-5p MIMAT0031890 Homo sapiens	7mer-A1	yes
18	>hsa-miR-140-5p MIMAT0000431 Homo sapiens	OS-6mer	yes
18	>hsa-miR-876-3p MIMAT0004925 Homo sapiens	OS-6mer	yes
19	>hsa-miR-20b-3p MIMAT0004752 Homo sapiens	OS-6mer	no
20	>hsa-miR-299-3p MIMAT0000687 Homo sapiens	7mer-A1	yes
22	>hsa-miR-190a-5p MIMAT0000458 Homo sapiens	8mer	no
23	>hsa-miR-576-3p MIMAT0004796 Homo sapiens	OS-6mer	yes
26	>hsa-miR-9-5p MIMAT0000441 Homo sapiens	OS-6mer	yes
28	>hsa-miR-19a-5p MIMAT0004490 Homo sapiens	6mer	yes
28	>hsa-miR-19b-1-5p MIMAT0004491 Homo sapiens	6mer	yes
28	>hsa-miR-19b-2-5p MIMAT0004492 Homo sapiens	6mer	yes
29	>hsa-miR-590-3p MIMAT0004801 Homo sapiens	7mer-A1	no
30	>hsa-miR-29a-5p MIMAT0004503 Homo sapiens	OS-6mer	yes
34	>hsa-miR-487b-5p MIMAT0026614 Homo sapiens	6mer	no
34	>hsa-miR-758-5p MIMAT0022929 Homo sapiens	7mer-m8	yes
35	>hsa-miR-203a-5p MIMAT0031890 Homo sapiens	6mer	yes
35	>hsa-miR-140-5p MIMAT0000431 Homo sapiens	7mer-m8	yes
35	>hsa-miR-584-5p MIMAT0003249 Homo sapiens	OS-6mer	no
35	>hsa-miR-876-3p MIMAT0004925 Homo sapiens	OS-6mer	yes
37	>hsa-miR-194-3p MIMAT0004671 Homo sapiens	6mer	yes
38	>hsa-miR-106a-3p MIMAT0004517 Homo sapiens	OS-6mer	yes

38	>hsa-miR-128-3p	MIMAT0000424	Homo sapiens	OS-6mer	yes
39	>hsa-miR-132-3p	MIMAT0000426	Homo sapiens	6mer	yes
40	>hsa-miR-545-3p	MIMAT0003165	Homo sapiens	7mer-A1	yes
40	>hsa-miR-3065-5p	MIMAT0015066	Homo sapiens	7mer-A1	no
41	>hsa-miR-302c-5p	MIMAT0000716	Homo sapiens	6mer	yes
41	>hsa-miR-505-3p	MIMAT0002876	Homo sapiens	7mer-m8	yes
42	>hsa-miR-34a-3p	MIMAT0004557	Homo sapiens	7mer-m8	yes
42	>hsa-miR-369-5p	MIMAT0001621	Homo sapiens	OS-6mer	yes
43	>hsa-miR-551b-5p	MIMAT0004794	Homo sapiens	OS-6mer	no
45	>hsa-miR-132-3p	MIMAT0000426	Homo sapiens	OS-6mer	yes
46	>hsa-miR-106a-3p	MIMAT0004517	Homo sapiens	7mer-A1	yes
46	>hsa-miR-139-5p	MIMAT0000250	Homo sapiens	OS-6mer	yes
46	>hsa-miR-374a-5p	MIMAT0000727	Homo sapiens	7mer-A1	yes
46	>hsa-miR-374b-5p	MIMAT0004955	Homo sapiens	7mer-A1	yes
47	>hsa-miR-450b-5p	MIMAT0004909	Homo sapiens	8mer	yes
49	>hsa-miR-19a-5p	MIMAT0004490	Homo sapiens	OS-6mer	yes
49	>hsa-miR-19b-1-5p	MIMAT0004491	Homo sapiens	OS-6mer	yes
49	>hsa-miR-19b-2-5p	MIMAT0004492	Homo sapiens	OS-6mer	yes
49	>hsa-miR-129-5p	MIMAT0000242	Homo sapiens	8mer	yes
53	>hsa-miR-329-5p	MIMAT0026555	Homo sapiens	OS-6mer	no
54	>hsa-miR-29b-1-5p	MIMAT0004514	Homo sapiens	OS-6mer	no
55	>hsa-miR-203a-5p	MIMAT0031890	Homo sapiens	6mer	yes
55	>hsa-miR-140-5p	MIMAT0000431	Homo sapiens	OS-6mer	yes
55	>hsa-miR-584-5p	MIMAT0003249	Homo sapiens	7mer-m8	yes
55	>hsa-miR-758-5p	MIMAT0022929	Homo sapiens	6mer	no
55	>hsa-miR-876-3p	MIMAT0004925	Homo sapiens	OS-6mer	yes
60	>hsa-miR-96-5p	MIMAT0000095	Homo sapiens	7mer-A1	no
60	>hsa-miR-182-5p	MIMAT0000259	Homo sapiens	7mer-A1	no
60	>hsa-miR-1271-5p	MIMAT0005796	Homo sapiens	7mer-A1	no
61	>hsa-miR-9-5p	MIMAT0000441	Homo sapiens	7mer-m8	yes
63	>hsa-miR-19a-5p	MIMAT0004490	Homo sapiens	6mer	yes
63	>hsa-miR-19b-1-5p	MIMAT0004491	Homo sapiens	6mer	yes
63	>hsa-miR-19b-2-5p	MIMAT0004492	Homo sapiens	6mer	yes
64	>hsa-miR-329-5p	MIMAT0026555	Homo sapiens	7mer-m8	yes
65	>hsa-miR-27a-5p	MIMAT0004501	Homo sapiens	6mer	yes
65	>hsa-miR-3158-3p	MIMAT0015032	Homo sapiens	7mer-m8	yes
66	>hsa-miR-485-5p	MIMAT0002175	Homo sapiens	6mer	yes
69	>hsa-miR-340-5p	MIMAT0004692	Homo sapiens	7mer-m8	yes
69	>hsa-miR-624-3p	MIMAT0004807	Homo sapiens	7mer-A1	yes
70	>hsa-miR-19a-3p	MIMAT0000073	Homo sapiens	OS-6mer	yes
70	>hsa-miR-19b-3p	MIMAT0000074	Homo sapiens	OS-6mer	yes

a) 150 nucleotide segment of mouse *Pax6* 3'UTR

AAAACTATCTGTTGGTTTTCCAAAGGTTGTTAACAGATGAAGTTTATGT
 GCAAAAAGGGTAAGATATGAATTC AAGGAGAAGTTGATAGCTAAAAGGT
 AGAGTGTGTCTTCGATATAATAACAATTTGTTTTATGTCAAAATGTAAGTA

Start Position	miRNA	Site Type	Predicted by
16	mmu-miR-7a/b	8mer+G:U	PITA
21	mmu-miR-9	6mer	PITA
44	mmu-miR-376c	7mer-m8+G:U	PITA
50	mmu-miR-450b-5p	7mer-A1	PITA
51	mmu-miR-129-5p	8mer	TS, PITA
104	mmu-miR-329	6mer	PITA

Summary of ImiRP predictions for wild type mouse *Pax6* sequence:

position	mirna	type	gu
2	>mmu-miR-19a-5p MIMAT0004660 Mus musculus	7mer-A1	no
3	>mmu-miR-29b-1-5p MIMAT0004523 Mus musculus	OS-6mer	yes
3	>mmu-miR-29b-2-5p MIMAT0017063 Mus musculus	6mer	yes
4	>mmu-miR-218-2-3p MIMAT0005444 Mus musculus	6mer	yes
5	>mmu-miR-196a-5p MIMAT0000518 Mus musculus	7mer-m8	yes
5	>mmu-miR-196b-5p MIMAT0001081 Mus musculus	7mer-m8	yes
6	>mmu-let-7g-5p MIMAT0000121 Mus musculus	OS-6mer	yes
6	>mmu-let-7i-5p MIMAT0000122 Mus musculus	OS-6mer	yes
6	>mmu-let-7d-5p MIMAT0000383 Mus musculus	OS-6mer	yes
6	>mmu-let-7a-5p MIMAT0000521 Mus musculus	OS-6mer	yes
6	>mmu-let-7b-5p MIMAT0000522 Mus musculus	OS-6mer	yes
6	>mmu-let-7c-5p MIMAT0000523 Mus musculus	OS-6mer	yes
6	>mmu-let-7e-5p MIMAT0000524 Mus musculus	OS-6mer	yes
6	>mmu-let-7f-5p MIMAT0000525 Mus musculus	OS-6mer	yes
6	>mmu-miR-98-5p MIMAT0000545 Mus musculus	OS-6mer	yes
6	>mmu-miR-1839-5p MIMAT0009456 Mus musculus	6mer	yes
7	>mmu-miR-488-5p MIMAT0003449 Mus musculus	OS-6mer	no
9	>mmu-miR-191-5p MIMAT0000221 Mus musculus	6mer	yes
10	>mmu-miR-194-5p MIMAT0000224 Mus musculus	OS-6mer	yes
10	>mmu-miR-196b-3p MIMAT0017170 Mus musculus	6mer	yes
10	>mmu-miR-421-3p MIMAT0004869 Mus musculus	7mer-m8	yes
11	>mmu-miR-16-2-3p MIMAT0017018 Mus musculus	6mer	yes
11	>mmu-miR-338-3p MIMAT0000582 Mus musculus	6mer	yes
11	>mmu-miR-505-3p MIMAT0003513 Mus musculus	OS-6mer	yes

11	>mmu-miR-3098-5p MIMAT0014917 Mus musculus	OS-6mer	yes
14	>mmu-miR-497a-3p MIMAT0017247 Mus musculus	6mer	no
14	>mmu-miR-742-3p MIMAT0004237 Mus musculus	7mer-m8	yes
16	>mmu-miR-7a-5p MIMAT0000677 Mus musculus	8mer	yes
17	>mmu-miR-1958 MIMAT0009431 Mus musculus	OS-6mer	yes
18	>mmu-miR-204-3p MIMAT0017002 Mus musculus	OS-6mer	yes
18	>mmu-miR-450a-1-3p MIMAT0017182 Mus musculus	8mer	yes
18	>mmu-miR-450b-3p MIMAT0003512 Mus musculus	8mer	yes
19	>mmu-miR-3057-5p MIMAT0014822 Mus musculus	7mer-A1	yes
21	>mmu-miR-9-5p MIMAT0000142 Mus musculus	6mer	no
23	>mmu-miR-511-5p MIMAT0004940 Mus musculus	OS-6mer	yes
23	>mmu-miR-3061-3p MIMAT0014829 Mus musculus	OS-6mer	no
24	>mmu-miR-1264-3p MIMAT0014803 Mus musculus	OS-6mer	yes
27	>mmu-miR-196a-1-3p MIMAT0017013 Mus musculus	7mer-A1	yes
27	>mmu-miR-21a-3p MIMAT0004628 Mus musculus	7mer-A1	yes
28	>mmu-miR-194-5p MIMAT0000224 Mus musculus	OS-6mer	yes
30	>mmu-miR-758-5p MIMAT0017235 Mus musculus	OS-6mer	yes
32	>mmu-miR-544-5p MIMAT0017282 Mus musculus	OS-6mer	yes
38	>mmu-miR-205-5p MIMAT0000238 Mus musculus	OS-6mer	no
38	>mmu-miR-335-3p MIMAT0004704 Mus musculus	OS-6mer	yes
39	>mmu-miR-205-3p MIMAT0017003 Mus musculus	6mer	yes
40	>mmu-miR-3099-5p MIMAT0014815 Mus musculus	OS-6mer	yes
40	>mmu-miR-1964-3p MIMAT0009437 Mus musculus	OS-6mer	yes
41	>mmu-miR-100-3p MIMAT0017051 Mus musculus	OS-6mer	yes
42	>mmu-miR-151-3p MIMAT0000161 Mus musculus	6mer	yes
42	>mmu-miR-345-3p MIMAT0004656 Mus musculus	OS-6mer	yes
43	>mmu-miR-30a-5p MIMAT0000128 Mus musculus	6mer	yes
43	>mmu-miR-30b-5p MIMAT0000130 Mus musculus	6mer	yes
43	>mmu-miR-30e-5p MIMAT0000248 Mus musculus	6mer	yes
43	>mmu-miR-30c-5p MIMAT0000514 Mus musculus	6mer	yes
43	>mmu-miR-30d-5p MIMAT0000515 Mus musculus	6mer	yes
43	>mmu-miR-384-5p MIMAT0004745 Mus musculus	6mer	yes
43	>mmu-miR-463-3p MIMAT0004758 Mus musculus	OS-6mer	yes
44	>mmu-miR-340-5p MIMAT0004651 Mus musculus	6mer	yes
44	>mmu-miR-376b-3p MIMAT0001092 Mus musculus	OS-6mer	yes
44	>mmu-miR-376c-3p MIMAT0003183 Mus musculus	7mer-m8	yes
45	>mmu-miR-322-3p MIMAT0000549 Mus musculus	OS-6mer	yes
45	>mmu-miR-410-3p MIMAT0001091 Mus musculus	6mer	yes
45	>mmu-miR-344d-3p MIMAT0014808 Mus musculus	6mer	yes
46	>mmu-miR-466b-3p MIMAT0004876 Mus musculus	6mer	yes
46	>mmu-miR-466c-3p MIMAT0004878 Mus musculus	6mer	yes

46	>mmu-miR-466e-3p MIMAT0004880 Mus musculus	OS-6mer	yes
46	>mmu-miR-669d-3p MIMAT0017324 Mus musculus	OS-6mer	yes
47	>mmu-miR-448-3p MIMAT0001533 Mus musculus	8mer	yes
48	>mmu-let-7g-3p MIMAT0004519 Mus musculus	OS-6mer	yes
48	>mmu-let-7i-3p MIMAT0004520 Mus musculus	7mer-A1	yes
48	>mmu-let-7a-2-3p MIMAT0017015 Mus musculus	7mer-A1	yes
48	>mmu-let-7c-1-3p MIMAT0004622 Mus musculus	7mer-A1	yes
49	>mmu-miR-92a-3p MIMAT0000539 Mus musculus	OS-6mer	no
49	>mmu-miR-25-3p MIMAT0000652 Mus musculus	OS-6mer	no
49	>mmu-miR-32-5p MIMAT0000654 Mus musculus	OS-6mer	no
49	>mmu-miR-363-3p MIMAT0000708 Mus musculus	OS-6mer	no
49	>mmu-miR-92b-3p MIMAT0004899 Mus musculus	OS-6mer	no
50	>mmu-miR-450a-5p MIMAT0001546 Mus musculus	7mer-A1	yes
50	>mmu-miR-452-5p MIMAT0001637 Mus musculus	OS-6mer	no
50	>mmu-miR-450b-5p MIMAT0003511 Mus musculus	7mer-A1	no
51	>mmu-miR-129-5p MIMAT0000209 Mus musculus	8mer	no
51	>mmu-miR-19b-1-5p MIMAT0017065 Mus musculus	OS-6mer	no
51	>mmu-miR-669b-5p MIMAT0003476 Mus musculus	OS-6mer	yes
55	>mmu-miR-130a-5p MIMAT0016983 Mus musculus	7mer-m8	yes
56	>mmu-miR-204-5p MIMAT0000237 Mus musculus	OS-6mer	no
56	>mmu-miR-130b-5p MIMAT0004583 Mus musculus	6mer	yes
56	>mmu-miR-211-5p MIMAT0000668 Mus musculus	OS-6mer	no
57	>mmu-miR-129-1-3p MIMAT0016994 Mus musculus	OS-6mer	yes
57	>mmu-miR-129-2-3p MIMAT0000544 Mus musculus	OS-6mer	yes
57	>mmu-miR-362-5p MIMAT0000706 Mus musculus	7mer-A1	yes
57	>mmu-miR-1249-3p MIMAT0010560 Mus musculus	OS-6mer	yes
57	>mmu-miR-500-5p MIMAT0017258 Mus musculus	7mer-A1	yes
57	>mmu-miR-3061-3p MIMAT0014829 Mus musculus	8mer	yes
58	>mmu-miR-18a-3p MIMAT0004626 Mus musculus	OS-6mer	yes
58	>mmu-miR-874-3p MIMAT0004853 Mus musculus	7mer-A1	yes
60	>mmu-miR-429-5p MIMAT0017178 Mus musculus	7mer-m8	no
61	>mmu-miR-200b-5p MIMAT0004545 Mus musculus	8mer	no
61	>mmu-miR-200a-5p MIMAT0004619 Mus musculus	8mer	no
61	>mmu-miR-200c-5p MIMAT0004663 Mus musculus	8mer	yes
61	>mmu-miR-431-5p MIMAT0001418 Mus musculus	OS-6mer	yes
62	>mmu-miR-1941-3p MIMAT0009406 Mus musculus	7mer-A1	no
69	>mmu-miR-10a-3p MIMAT0004659 Mus musculus	OS-6mer	yes
69	>mmu-miR-32-3p MIMAT0017050 Mus musculus	6mer	yes
71	>mmu-miR-183-3p MIMAT0004539 Mus musculus	7mer-A1	no
71	>mmu-miR-3068-3p MIMAT0014843 Mus musculus	OS-6mer	no
73	>mmu-miR-470-5p MIMAT0002111 Mus musculus	OS-6mer	yes

73	>mmu-miR-547-5p	MIMAT0017210	Mus musculus	OS-6mer	yes
74	>mmu-miR-212-5p	MIMAT0017053	Mus musculus	7mer-A1	yes
74	>mmu-miR-3075-3p	MIMAT0014859	Mus musculus	7mer-m8	yes
75	>mmu-miR-362-5p	MIMAT0000706	Mus musculus	OS-6mer	no
75	>mmu-miR-500-5p	MIMAT0017258	Mus musculus	OS-6mer	no
76	>mmu-miR-130a-5p	MIMAT0016983	Mus musculus	7mer-A1	yes
76	>mmu-miR-130b-5p	MIMAT0004583	Mus musculus	7mer-A1	yes
76	>mmu-miR-103-1-5p	MIMAT0017024	Mus musculus	OS-6mer	yes
76	>mmu-miR-103-2-5p	MIMAT0017025	Mus musculus	OS-6mer	yes
76	>mmu-miR-107-5p	MIMAT0017048	Mus musculus	OS-6mer	yes
78	>mmu-miR-22-5p	MIMAT0004629	Mus musculus	OS-6mer	yes
79	>mmu-miR-103-1-5p	MIMAT0017024	Mus musculus	OS-6mer	yes
79	>mmu-miR-103-2-5p	MIMAT0017025	Mus musculus	OS-6mer	yes
79	>mmu-miR-107-5p	MIMAT0017048	Mus musculus	OS-6mer	yes
79	>mmu-miR-320-5p	MIMAT0017057	Mus musculus	OS-6mer	no
81	>mmu-miR-3099-5p	MIMAT0014815	Mus musculus	8mer	yes
81	>mmu-miR-1964-3p	MIMAT0009437	Mus musculus	8mer	yes
83	>mmu-miR-434-5p	MIMAT0001421	Mus musculus	OS-6mer	yes
84	>mmu-miR-34a-3p	MIMAT0017022	Mus musculus	7mer-A1	yes
84	>mmu-miR-369-5p	MIMAT0003185	Mus musculus	OS-6mer	yes
84	>mmu-miR-505-3p	MIMAT0003513	Mus musculus	7mer-A1	yes
85	>mmu-miR-361-5p	MIMAT0000704	Mus musculus	6mer	yes
85	>mmu-miR-3074-1-3p	MIMAT0014857	Mus musculus	OS-6mer	yes
86	>mmu-miR-215-3p	MIMAT0017169	Mus musculus	6mer	yes
88	>mmu-miR-101a-5p	MIMAT0004526	Mus musculus	OS-6mer	yes
94	>mmu-miR-130a-5p	MIMAT0016983	Mus musculus	OS-6mer	yes
95	>mmu-miR-511-5p	MIMAT0004940	Mus musculus	7mer-m8	yes
95	>mmu-miR-3061-3p	MIMAT0014829	Mus musculus	7mer-m8	no
96	>mmu-miR-532-5p	MIMAT0002889	Mus musculus	OS-6mer	yes
97	>mmu-miR-3106-3p	MIMAT0014818	Mus musculus	7mer-A1	yes
97	>mmu-miR-3110-5p	MIMAT0014951	Mus musculus	7mer-m8	yes
98	>mmu-miR-346-5p	MIMAT0000597	Mus musculus	OS-6mer	yes
99	>mmu-miR-676-5p	MIMAT0003781	Mus musculus	6mer	no
102	>mmu-miR-181a-5p	MIMAT0000210	Mus musculus	6mer	yes
102	>mmu-miR-181c-5p	MIMAT0000674	Mus musculus	6mer	yes
102	>mmu-miR-181b-5p	MIMAT0000673	Mus musculus	6mer	yes
102	>mmu-miR-543-3p	MIMAT0003168	Mus musculus	OS-6mer	yes
102	>mmu-miR-181d-5p	MIMAT0004324	Mus musculus	6mer	yes
102	>mmu-miR-574-3p	MIMAT0004894	Mus musculus	6mer	yes
102	>mmu-miR-3572-3p	MIMAT0020636	Mus musculus	6mer	yes
103	>mmu-miR-23b-3p	MIMAT0000125	Mus musculus	OS-6mer	yes

103	>mmu-miR-106b-3p	MIMAT0004582	Mus musculus	6mer	yes
103	>mmu-miR-23a-3p	MIMAT0000532	Mus musculus	OS-6mer	yes
103	>mmu-miR-1b-5p	MIMAT0005835	Mus musculus	7mer-m8	yes
104	>mmu-miR-329-3p	MIMAT0000567	Mus musculus	6mer	no
104	>mmu-miR-362-3p	MIMAT0004684	Mus musculus	6mer	no
104	>mmu-miR-466b-3p	MIMAT0004876	Mus musculus	OS-6mer	yes
104	>mmu-miR-466c-3p	MIMAT0004878	Mus musculus	OS-6mer	yes
104	>mmu-miR-466m-3p	MIMAT0014883	Mus musculus	6mer	yes
104	>mmu-miR-466o-3p	MIMAT0014886	Mus musculus	6mer	yes
106	>mmu-miR-124-3p	MIMAT0000134	Mus musculus	7mer-m8	yes
106	>mmu-miR-3095-3p	MIMAT0014912	Mus musculus	6mer	yes
107	>mmu-miR-208a-3p	MIMAT0000520	Mus musculus	OS-6mer	yes
107	>mmu-miR-743a-3p	MIMAT0004238	Mus musculus	OS-6mer	no
107	>mmu-miR-743b-3p	MIMAT0004840	Mus musculus	OS-6mer	no
107	>mmu-miR-208b-3p	MIMAT0004939	Mus musculus	OS-6mer	yes
107	>mmu-miR-6540-5p	MIMAT0025585	Mus musculus	OS-6mer	yes
108	>mmu-miR-7a-5p	MIMAT0000677	Mus musculus	OS-6mer	no
109	>mmu-miR-151-5p	MIMAT0004536	Mus musculus	7mer-A1	yes
109	>mmu-miR-802-3p	MIMAT0017240	Mus musculus	7mer-A1	yes
112	>mmu-miR-325-3p	MIMAT0004640	Mus musculus	OS-6mer	yes
118	>mmu-miR-503-3p	MIMAT0004790	Mus musculus	OS-6mer	yes
119	>mmu-miR-223-5p	MIMAT0017056	Mus musculus	OS-6mer	no
119	>mmu-miR-1198-5p	MIMAT0005859	Mus musculus	OS-6mer	yes

1c) 150 nucleotide segment of fly *Ey* 3'UTR

TATCTAAAGGAGGCCATAATAAATCGAATTTACATATCTCTTGAAAAATA
 ATGGAGGTTGTAGAAAATAACATTTGTATGTATAAATTATATAGTTCCGC
 CCATTAAATCCAATCTATAGTGTAGAATAATTGGTGTAATTAATGATA

Start Position	miRNA	Site Type	Predicted by
16	dme-miR-1000	7mer-A1+G:U	PITA
26	dme-miR-1014	7mer-A1	TS
32	dme-miR-190	7mer-m8	TS
34	dme-miR-977	7mer-m8	TS

Summary of ImiRP predictions for wild type fly *Ey* sequence:

position	mirna	type	gu
1	>dme-miR-983-3p MIMAT0020880 <i>Drosophila melanogaster</i>	8mer	yes
2	>dme-miR-303-5p MIMAT0000388 <i>Drosophila melanogaster</i>	7mer-A1	yes
4	>dme-miR-9a-5p MIMAT0000114 <i>Drosophila melanogaster</i>	6mer	yes
4	>dme-miR-79-5p MIMAT0020814 <i>Drosophila melanogaster</i>	6mer	yes
4	>dme-miR-9c-5p MIMAT0000392 <i>Drosophila melanogaster</i>	6mer	yes
4	>dme-miR-9b-5p MIMAT0000395 <i>Drosophila melanogaster</i>	6mer	yes
6	>dme-miR-316-5p MIMAT0000408 <i>Drosophila melanogaster</i>	OS-6mer	yes
9	>dme-miR-282-5p MIMAT0000346 <i>Drosophila melanogaster</i>	OS-6mer	yes
16	>dme-miR-1000-5p MIMAT0005520 <i>Drosophila melanogaster</i>	7mer-A1	yes
21	>dme-bantam-5p MIMAT0020823 <i>Drosophila melanogaster</i>	7mer-A1	yes
22	>dme-miR-315-5p MIMAT0000407 <i>Drosophila melanogaster</i>	7mer-m8	yes
24	>dme-miR-314-3p MIMAT0000406 <i>Drosophila melanogaster</i>	6mer	no
25	>dme-miR-10-3p MIMAT0012531 <i>Drosophila melanogaster</i>	8mer	no
25	>dme-miR-1006-3p MIMAT0005019 <i>Drosophila melanogaster</i>	8mer	no
26	>dme-miR-958-3p MIMAT0005471 <i>Drosophila melanogaster</i>	OS-6mer	yes
26	>dme-miR-1014-3p MIMAT0005027 <i>Drosophila melanogaster</i>	7mer-A1	no
27	>dme-miR-998-5p MIMAT0020898 <i>Drosophila melanogaster</i>	OS-6mer	yes
28	>dme-miR-4969-5p MIMAT0020195 <i>Drosophila melanogaster</i>	OS-6mer	no
29	>dme-miR-971-5p MIMAT0020868 <i>Drosophila melanogaster</i>	OS-6mer	no
31	>dme-miR-9388-5p MIMAT0035238 <i>Drosophila melanogaster</i>	6mer	no
32	>dme-miR-190-5p MIMAT0005467 <i>Drosophila melanogaster</i>	7mer-m8	no
34	>dme-miR-975-3p MIMAT0020872 <i>Drosophila melanogaster</i>	OS-6mer	yes
34	>dme-miR-977-3p MIMAT0005493 <i>Drosophila melanogaster</i>	7mer-m8	no
35	>dme-let-7-5p MIMAT0000396 <i>Drosophila melanogaster</i>	6mer	yes
35	>dme-miR-317-5p MIMAT0020843 <i>Drosophila melanogaster</i>	6mer	yes
35	>dme-miR-967-5p MIMAT0005482 <i>Drosophila melanogaster</i>	6mer	no
35	>dme-miR-984-5p MIMAT0005500 <i>Drosophila melanogaster</i>	6mer	yes

37	>dme-miR-281-1-5p	MIMAT0000344	Drosophila melanogaster	6mer	no
40	>dme-miR-314-3p	MIMAT0000406	Drosophila melanogaster	OS-6mer	yes
40	>dme-miR-315-3p	MIMAT0020841	Drosophila melanogaster	8mer	yes
43	>dme-miR-980-5p	MIMAT0020877	Drosophila melanogaster	8mer	yes

Supplementary Figure 4. Testing the ImiRP Target Site Predictor

Several 3'UTR segments from three different species were provided as input to ImiRP. ImiRP target site predictions were compared to predictions made by PITA and TargetScan for each sequence. MiRBase was used to validate the ImiRP target site predictions. Three sample test results are provided. a) 150 nucleotide segment of the human *PAX6* 3'UTR: ImiRP successfully identified target sites for miR-190a, miR-140-5p, miR-96, miR-182, and miR-1271. Target sites for miR-190b and miR-507 were not detected because these miRNAs are not high confidence miRNAs. miR-190b has less than five reads mapping to its 3' arm, and miR-507 has no reads mapping to the 5' arm. b) 150 nucleotide segment of the mouse *Pax6* 3'UTR: ImiRP successfully identified predicted target sites for miR-7a, miR-9, miR-376c, miR-450b-5p, miR-129-5p, and miR-329. miR-7b was not detected because this miRNA is not present in the miRBase version 21 high confidence dataset. c) 150 nucleotide segment of the fly *Ey* 3'UTR: ImiRP successfully identified predicted target sites for miR-1000, miR-1014, miR-190, and miR-977.

24	>mmu-miR-1264-3p	OS-6mer	yes			
24				>mmu-miR-883a-3p	6mer	yes
24				>mmu-miR-883b-3p	6mer	yes
24				>mmu-miR-872-3p	OS-6mer	yes
26				>mmu-miR-674-3p	6mer	yes
27	>mmu-miR-196a-1-3p	7mer-A1	yes	>mmu-miR-196a-1-3p	7mer-A1	yes
27	>mmu-miR-21a-3p	7mer-A1	yes	>mmu-miR-21a-3p	7mer-A1	yes
28	>mmu-miR-194-5p	OS-6mer	yes	>mmu-miR-194-5p	OS-6mer	yes
30	>mmu-miR-758-5p	OS-6mer	yes	>mmu-miR-758-5p	OS-6mer	yes
32	>mmu-miR-544-5p	OS-6mer	yes	>mmu-miR-544-5p	OS-6mer	yes
38	>mmu-miR-205-5p	OS-6mer	no	>mmu-miR-205-5p	OS-6mer	no
38	>mmu-miR-335-3p	OS-6mer	yes	>mmu-miR-335-3p	OS-6mer	yes
39	>mmu-miR-205-3p	6mer	yes	>mmu-miR-205-3p	6mer	yes
40	>mmu-miR-3099-5p	OS-6mer	yes			
40	>mmu-miR-1964-3p	OS-6mer	yes			
41	>mmu-miR-100-3p	OS-6mer	yes			
42	>mmu-miR-151-3p	6mer	yes			
42	>mmu-miR-345-3p	OS-6mer	yes			
43	>mmu-miR-30a-5p	6mer	yes			
43	>mmu-miR-30b-5p	6mer	yes			
43	>mmu-miR-30e-5p	6mer	yes			
43	>mmu-miR-30c-5p	6mer	yes			
43	>mmu-miR-30d-5p	6mer	yes			
43	>mmu-miR-384-5p	6mer	yes			
43	>mmu-miR-463-3p	OS-6mer	yes			
43				>mmu-miR-742-5p	6mer	yes
43				>mmu-miR-1839-3p	OS-6mer	yes
44	>mmu-miR-340-5p	6mer	yes			
44	>mmu-miR-376b-3p	OS-6mer	yes			
44	>mmu-miR-376c-3p	7mer-m8	yes			
45	>mmu-miR-322-3p	OS-6mer	yes			
45	>mmu-miR-410-3p	6mer	yes			
45	>mmu-miR-344d-3p	6mer	yes			
45				>mmu-miR-448-5p	OS-6mer	yes
45				>mmu-miR-667-3p	OS-6mer	yes
45				>mmu-miR-3093-5p	6mer	yes
46				>mmu-miR-329-3p	OS-6mer	no
46				>mmu-miR-362-3p	OS-6mer	no
46	>mmu-miR-466b-3p	6mer	yes			
46	>mmu-miR-466c-3p	6mer	yes			
46	>mmu-miR-466e-3p	OS-6mer	yes			
46	>mmu-miR-669d-3p	OS-6mer	yes			
47	>mmu-miR-448-3p	8mer	yes			

48	>mmu-let-7g-3p	OS-6mer	yes			
48	>mmu-let-7i-3p	7mer-A1	yes			
48	>mmu-let-7a-2-3p	7mer-A1	yes			
48	>mmu-let-7c-1-3p	7mer-A1	yes			
49	>mmu-miR-92a-3p	OS-6mer	no			
49	>mmu-miR-25-3p	OS-6mer	no			
49	>mmu-miR-32-5p	OS-6mer	no			
49	>mmu-miR-363-3p	OS-6mer	no			
49	>mmu-miR-92b-3p	OS-6mer	no			
49				>mmu-miR-500-3p	6mer	yes
50	>mmu-miR-450a-5p	7mer-A1	yes			
50	>mmu-miR-452-5p	OS-6mer	no			
50	>mmu-miR-450b-5p	7mer-A1	no			
51	>mmu-miR-129-5p	8mer	no			
51	>mmu-miR-19b-1-5p	OS-6mer	no			
51	>mmu-miR-669b-5p	OS-6mer	yes			
51				>mmu-miR-652-3p	OS-6mer	yes
55	>mmu-miR-130a-5p	7mer-m8	yes			
56				>mmu-miR-130a-5p	6mer	yes
56	>mmu-miR-204-5p	OS-6mer	no	>mmu-miR-204-5p	OS-6mer	no
56	>mmu-miR-130b-5p	6mer	yes	>mmu-miR-130b-5p	6mer	yes
56	>mmu-miR-211-5p	OS-6mer	no	>mmu-miR-211-5p	OS-6mer	no
...						
98	>mmu-miR-346-5p	OS-6mer	yes	>mmu-miR-346-5p	OS-6mer	yes
99	>mmu-miR-676-5p	6mer	no	>mmu-miR-676-5p	6mer	no
102	>mmu-miR-181a-5p	6mer	yes			
102	>mmu-miR-181c-5p	6mer	yes			
102	>mmu-miR-181b-5p	6mer	yes			
102	>mmu-miR-543-3p	OS-6mer	yes			
102	>mmu-miR-181d-5p	6mer	yes			
102	>mmu-miR-574-3p	6mer	yes			
102	>mmu-miR-3572-3p	6mer	yes			
102				>mmu-miR-668-3p	OS-6mer	yes
103				>mmu-miR-136-5p	OS-6mer	yes
103	>mmu-miR-23b-3p	OS-6mer	yes			
103	>mmu-miR-106b-3p	6mer	yes			
103	>mmu-miR-23a-3p	OS-6mer	yes			
103	>mmu-miR-1b-5p	7mer-m8	yes			
104	>mmu-miR-329-3p	6mer	no			
104	>mmu-miR-362-3p	6mer	no			
104	>mmu-miR-466b-3p	OS-6mer	yes			
104	>mmu-miR-466c-3p	OS-6mer	yes			
104	>mmu-miR-466m-3p	6mer	yes			

104	>mmu-miR-466o-3p	6mer	yes			
106	>mmu-miR-124-3p	7mer-m8	yes			
106	>mmu-miR-3095-3p	6mer	yes			
106				>mmu-miR-615-5p	OS-6mer	yes
107				>mmu-miR-540-5p	OS-6mer	yes
107	>mmu-miR-208a-3p	OS-6mer	yes			
107	>mmu-miR-743a-3p	OS-6mer	no			
107	>mmu-miR-743b-3p	OS-6mer	no			
107	>mmu-miR-208b-3p	OS-6mer	yes			
107	>mmu-miR-6540-5p	OS-6mer	yes			
108	>mmu-miR-7a-5p	OS-6mer	no			
108				>mmu-miR-1843b-5p	OS-6mer	yes
109	>mmu-miR-151-5p	7mer-A1	yes			
109	>mmu-miR-802-3p	7mer-A1	yes			
112	>mmu-miR-325-3p	OS-6mer	yes	>mmu-miR-325-3p	OS-6mer	yes
118	>mmu-miR-503-3p	OS-6mer	yes	>mmu-miR-503-3p	OS-6mer	yes
119	>mmu-miR-223-5p	OS-6mer	no	>mmu-miR-223-5p	OS-6mer	no
119	>mmu-miR-1198-5p	OS-6mer	yes	>mmu-miR-1198-5p	OS-6mer	yes
120	>mmu-miR-592-5p	6mer	yes	>mmu-miR-592-5p	6mer	yes
120	>mmu-miR-669a-5p	OS-6mer	yes	>mmu-miR-669a-5p	OS-6mer	yes
120	>mmu-miR-669d-5p	OS-6mer	yes	>mmu-miR-669d-5p	OS-6mer	yes
120	>mmu-miR-669f-5p	OS-6mer	yes	>mmu-miR-669f-5p	OS-6mer	yes
120	>mmu-miR-669l-5p	OS-6mer	yes	>mmu-miR-669l-5p	OS-6mer	yes
120	>mmu-miR-669p-5p	OS-6mer	yes	>mmu-miR-669p-5p	OS-6mer	yes

Supplementary Figure 5. Testing the final mutant sequence validity

A 150 nucleotide segment of the mouse *Pax6* 3'UTR was used as input into ImiRP. Six miRNA target sites predicted by PITA and TargetScan (Figure S4b) were selected for mutation. Mutation parameters were set such that three nucleotide changes would be introduced per specified target site, using all four nucleotide bases for mutation. 8mer, 7mer-m8, 7mer-A1 target sites with and without a single G:U pair and 6mer target site without a G:U pair were specified as invalid. As such, the final mutant sequence should not contain any new-created sites of these types. a) Alignment of the 150 nucleotide wild type input sequence with the mutated sequence output by ImiRP. b) Comparison of miRNA target site predictions output by ImiRP for the wild type and mutant mouse *Pax6* 3'UTR segment. All predicted miRNA target sites selected for mutation have been disrupted. Only 6mer+G:U and OS-6mer target sites were newly created upon mutation of the specified target sites.

Supplemental Document:

Instruction on How to Use ImiRP

Creating a New Project

1. Type imirp.org into your web browser. Click on the App tool at the top of the screen, and then select “Create New Project” from the Projects drop-down menu.
2. To create a new project, first provide a project name and a description of the project (optional)

Create Project

Project Description Sequence Input Target Sites 0

Project Name
Pax6 Project

Description
Mutate 6 predicted miRNA target sites in the mouse Pax6 3'UTR: miR-7, miR-9, miR-376c, miR-450b-5p, miR-129-5p, miR-329

✓ Create Project

In this example, we have named the project “Pax6 Project”, and will mutate six predicted target sites in a segment of the mouse *Pax6* 3'UTR.

3. Next, click on the “Sequence Input” tab of the “Create Project” menu, input the desired 3'UTR sequence and select the species of interest. A FASTA format DNA or RNA sequence can be cut and pasted into the “Sequence” text box. The input sequence is then displayed above the create project tabs. To indicate the species, begin typing the scientific name or three letter organism code into the “Species” text box. All species having miRNA sequences available in miRBase version 21 are available for selection.

```
1  A A A A A C T A T C T G T T G G T T T T C C A A A G G T T G T T A A C A G A T G A A G T T T A T G T 50
51  G C A A A A A A G G G T A A G A T A T G A A T T C A A G G A G A A G T T G A T A G C T A A A A G G T 100
101 A G A G T G T G T C T T C G A T A T A A T A C A A T T T G T T T T A T G T C A A A A T G T A A G T A 150
```

Create Project

Project Description Sequence Input Target Sites 0

Species
Macaca mulatta
Mus musculus

Sequence
AAAAACTATCTGTTGGTTTTCCAAAGGTTGTTAACAGATGAAGTTTATGTGCAAAAAGGGTAAGATGAATTCAAG
GAGAAAGTTGATAGCTAAAAGGTAGAGTGTCTTCGATATAATACAATTTGTTTTATGTCAAATGTAAGTA

✓ Create Project

A 150 nucleotide segment of the mouse Pax6 3'UTR has been copied into the sequence text box and is displayed above the project tabs for viewing. We have begun to enter "mmu", the three letter organism code for mouse, into the species text box. Mus musculus is provided as one of the species options for selection, and can be clicked on to define mouse as the desired species.

- Specify the regions of the input sequence, termed mutation sites, into which nucleotide substitutions will be introduced. Identify the input sequence nucleotide position of the first predicted target site to mutate that is complementary to position 7 of the targeting miRNA. Type this number into the "mutation site" text box and click "Add Site". The region of the predicted target site complementary to miRNA positions 2-7 will be bolded in the displayed input sequence. Also, the input sequence position of the start and end of the defined mutation site will be represented in (x, y) format below the mutation sites text box, where x is the first and y is the last nucleotide of the mutation site. This process is repeated for each target site to be mutated.

AAAACTATCTGTTGGTTTTCCAAAGGTTGTTAACAGATGAAGTTTATGTT
 GCAAAAAGGGTAAGATATGAATCAAGGAGAAGTTGATAGCTAAAAGGT
 AGAGTGTGTCTTCGATATAATAACAATTTGTTTTATGTCAAAATGTAAGTA

Start Position	miRNA	Site Type
16	mmu-miR-7a/b	8mer+G:U
21	mmu-miR-9	6mer
44	mmu-miR-376c	7mer-m8+G:U
50	mmu-miR-450b-5p	7mer-A1
51	mmu-miR-129-5p	8mer
104	mmu-miR-329	6mer

In this example, six miRNA target sites are to be mutated. The start position indicates the first nucleotide position of each miRNA target site, with the first nucleotide of the 3'UTR being position 1. +G:U indicates that the predicted target site contains a single G:U wobble pair. For each predicted target site, the site position complementary to miRNA position 7 is typed into the mutation site text box.

```

1  A A A A A C T A T C T G T T G G | T T T T | C C | A A A G | G T T G T T A A C A G A T G A A G T | T T A T G | T | 50
51 | G | C | A A A A | A A | A G G G T A A G A T A T G A A T T C A A G G A G A A G T T G A T A G C T A A A A G G T 100
101 A G A G T G T G T C T T C G A T A T A A T A C A A T T T G T T T T A T G T C A A A A T G T A A G T A 150

```

Create Project

Project Description Sequence Input Target Sites **5**

[Help](#)

Mutation sites

104| Add Site

(17,22)	X
(21,26)	X
(45,50)	X
(50,55)	X
(52,57)	X

[✔ Create Project](#)

In the provided example, the mutation site for miR-7 was defined by entering 17 into the mutation site text box, and the mutation site for miR-329 is being defined by typing 104. The mutation site corresponding to each predicted target site is indicated in bold in the sequence display, and positions defining the start and end of the mutation sites are listed below the input text box. For example, the mutation site for miR-7 is listed as (17,22), and is defined in bold in the display sequence as |**TTTTCC**|. To remove a mutation site from the list, click the X.

- Once all the project information has been defined, click the “Create Project” button at the bottom left of the page.

Defining Mutation Parameters

- Once a project has been created, the user is directed to the project page. Here, a mutation request can be submitted.

Pax6 Project

Overview | Project Parameters | Mutation Requests **0**

Mutate

Results

Mutation Sites

- (17, 22)
- (21, 26)
- (45, 50)
- (50, 55)
- (52, 57)
- (104, 109)

Species

Mus musculus

Sequence

```
1  A A A A A C T A T C T G T T G G | T T T T | C C | A A A G | G T T G T T A A C A G A T G A A G T | T T A T G | T | 50
51  | G | C A A A | A A | A G G G T A A G A T A T G A A T T C A A G G A G A A G T T G A T A G C T A A A A G G T | 100
101 A G A | G T G T G T | C T T C G A T A T A A T A C A A T T T G T T T T A T G T C A A A A T G T A A G T A | 150
```

Clicking on “Overview” on the project page will provide a summary of the information that was input when the project was created. It will also provide information about any mutation requests that have been submitted. Since we have not yet submitted a mutation request the number “0” appears on the mutation request tab and no information is available for viewing.

Pax6 Project

Overview | Mutate | Results

Help ?

No results!

Pax6 Project has no results. To get results, submit a new project mutation request.

Create Mutation Request

Region 17 to 26 **0**

[T T T T [C C] A A A G]

No valid mutations were produced for this region. Try submitting a new mutation request with less stringent parameters.

Region 45 to 57 **0**

[T T A T G T] T [T G [C A A A] A A]

No valid mutations were produced for this region. Try submitting a new mutation request with less stringent parameters.

Region 104 to 109 **0**

[G T G T G T]

No valid mutations were produced for this region. Try submitting a new mutation request with less stringent parameters.

Assembled Mutant Sequence

```
1  A A A A A C T A T C T G T T G G | T T T T | C C | A A A G | G T T G T T A A C A G A T G A A G T | T T A T G | T | 50
51  | G | C A A A | A A | A G G G T A A G A T A T G A A T T C A A G G A G A A G T T G A T A G C T A A A A G G T | 100
101 A G A | G T G T G T | C T T C G A T A T A A T A C A A T T T G T T T T A T G T C A A A A T G T A A G T A | 150
```

Download Sequence Analysis

Clicking on “Results” on the project page shows that no mutant sequences have been generated because a mutation request has not been submitted. Mutation sites are grouped

based on inter-site spacing, with sites spaced less than 7 nucleotides apart being grouped into a single “Region”. The nucleotide position of each region is defined based on the start and end positions of the first and last sites in the region, and the mutation sites are displayed in square brackets. For example, the mutation sites corresponding to the predicted miR-7 and miR-9 target sites are present in the first region, which begins at input sequence position 17 and ends at position 26. The mutation sites are displayed as [TTTT[CC]AAAG], with [TTTTCC] defining the mutation site for miR-7 and [CCAAAG] for miR-9.

2. Clicking the “Mutate” option on the project page enables the user to submit a mutation request.
3. Clicking on the “Mutation Strategy” tab allows the user to define the nucleotides that can be used for introducing substitutions into the mutation sites. Using the drop down menu, the user can also specify the number of nucleotide changes required per mutation site.

Pax6 Project

The screenshot shows the 'Pax6 Project' interface. At the top, there are tabs for 'Overview', 'Mutate', and 'Results'. The 'Mutate' tab is selected. Below the tabs, there is a DNA sequence with mutation sites highlighted in brackets. The sequence is: 1 A A A A A C T A T C T G T T G G [T T T T [C C] A A A G] S T T G T T A A C A G A T G A A G T T T A T G T | 50
51 | S C A A A A A A A G G G T A A G A T A T G A A T T C A A G G A G A A G T T G A T A G C T A A A A G G T 100
101 A G A G T G T G T | C T T C G A T A T A A T A C A A T T T G T T T T A T G T C A A A A T G T A A G T A 150

Below the sequence, there are tabs for 'Project Parameters', 'Mutation Strategy', and 'Define Invalid Sites'. The 'Mutation Strategy' tab is active. Under 'Allowable nucleotides for mutation', there are checkboxes for A, T, C, and G, all of which are checked. Under 'Mutation strategy', there is a dropdown menu set to '3 changes per site'. At the bottom, there is a 'Submit Mutation Request' button.

In this example, we have selected all four nucleotides for mutation and 3 changes per site. This will ensure that three adjacent nucleotide substitutions will be introduced per mutation site using any of the four available nucleotides.

4. Next, clicking on the “Define Invalid Sites” tab enables the user to specify the miRNA target site types that they want to avoid creating upon mutation of the defined mutation sites. By clicking on the target site classes listed in the “Invalid site types” and “Valid site types” lists, and using the arrow buttons, users can move target site types between the two lists.

Pax6 Project

Overview

Mutate

Results

```
1  A A A A A C T A T C T G T T G G | T T T T | C C | A A A G | G T T G T T A A C A G A T G A A G T | T T A T G | T
51 | G | C | A A A | A A | A G G G T A A G A T A T G A A T T C A A G G A G A A G T T G A T A G C T A A A A G G T
101 A G A | G T G T G T | C T T C G A T A T A A T A C A A T T T G T T T T A T G T C A A A A T G T A A G T A
```

Project Parameters

Mutation Strategy

Define Invalid Sites

Help

Invalid site types

7mer-A1
8mer
7mer-m8



Valid site types

OS-6mer + GU
7mer-m8 + GU
OS-6mer
7mer-A1 + GU
6mer
8mer + GU
6mer + GU

Site type key:

6mer: miRNA target site complementary to miRNA positions 2-7

OS-6mer: offset-6mer, miRNA target site complementary to miRNA positions 3-8

7mer-A1: miRNA target site complementary to miRNA positions 2-7 having an A across from miRNA position 1

7mer-m8: miRNA target site complementary to miRNA position 2-8

8mer: miRNA target site complementary to miRNA positions 2-8 and having an A across from miRNA position 1

+GU: site contains a single G:U wobble pair

Submit Mutation Request

In this example, 7mer-A1, 8mer and 7mer-m8 target site types have been defined as invalid. This means that upon mutation of our six miRNA target sites, no new target sites of these types will be created. However, any target site types listed as “Valid site types” may be created upon mutation.

5. Once mutation parameters have been defined, clicking the “Submit Mutation Request” button will begin the generation of mutant sequences that satisfy the input criteria. The user will be directed to the results page within the project page.

Constructing a Final Mutant Sequence

1. A maximum of five mutations that satisfy the input mutation criteria will be displayed per region. For each region display box, the original input sequence is displayed above, and the mutated sequences are listed below.

Pax6 Project

Overview

Mutate

Results

Help

Region 17 to 26

5

- [T T T T [C C] A A A G]
- [T T T C [A G] C A A G]
- [T T T C [A A] C A A G]
- [T G A A [C C] C C C G]
- [T A C C [C C] A T C C]
- [T T T C [G T] G A A G]

Region 45 to 57

5

- [T T A T G T] T [T G [C A A A] A A]
- [A G G T G T] T [T G [C C T G] A A]
- [T A C C G T] T [T G [A G C A] A A]
- [T G C A G T] T [T G [C C T G] A A]
- [T C G G G T] T [T G [G T C A] A A]
- [A A C T G T] T [T G [C T G G] A A]

Region 104 to 109

5

- [G T G T G T]
- [G T A C T T]
- [G C T G G T]
- [T C T T G T]
- [G T G C C G]
- [G T G A A G]

Assembled Mutant Sequence

```
1  A A A A A C T A T C T G T T G G | T T T T | C C | A A A G | G T T G T T A A C A G A T G A A G T | T T A T G | T | 50
51 | G | C A A A | A A | A G G G T A A G A T A T G A A T T C A A G G A G A A G T T G A T A G C T A A A A G G T 100
101 A G A | G T G T G T | C T T C G A T A T A A T A C A A T T T G T T T T A T G T C A A A A T G T A A G T A 150
```

Download Sequence Analysis

- To select the desired mutant sequence for each region from the list, click on the select bubble to the left of the mutant sequence. One mutant sequence can be selected per region. As mutant sequences are selected for each region the region display box will change green and the “Assembled Mutant Sequence” displayed below will be updated to include the specified nucleotide substitutions.

Pax6 Project

Overview

Help ?

Mutate

Results

▼ Region 17 to 26 5

- [T T T T [C C] A A A G]
- [T T T C [A G] C A A G]
- [T T T C [A A] C A A G]
- [T G A A [C C] C C C G]
- [T A C C [C C] A T C C]
- [T T T C [G T] G A A G]

▼ Region 45 to 57 5

- [T T A T G T] T [T G [C A A A] A A]
- [A G G T G T] T [T G [C C T G] A A]
- [T A C C G T] T [T G [A G C A] A A]
- [T G C A G T] T [T G [C C T G] A A]
- [T C G G G T] T [T G [G T C A] A A]
- [A A C T G T] T [T G [C T G G] A A]

▼ Region 104 to 109 5

- [G T G T G T]
- [G T A C T T]
- [G C T G G T]
- [T C T T G T]
- [G T G C C G]
- [G T G A A G]

Assembled Mutant Sequence

```
1  AAAAACTATCTGTTGG|TTTC|AG|CAAG|CTTGTAAACAGATGAAAGT|TACCGT| 50
51 |G|AGCA|AA|AGGGTAAGATATGAATTCAAGGAGAAAGTTGATAGCTAAAAGGT 100
101 AGA|GTGAAG|CTTCGATATAATAACAATTTGTTTTATGTCAAAAATGTAAGTA 150
```

[Download Sequence Analysis](#)

In this example, we have selected the first mutant sequence from Region 17-26 (which includes mutation sites for miR-7 and miR-9), the second mutant sequence for Region 45-57 (which includes mutation sites for miR-376c, miR-450b-5p and miR-129-5p) and the fifth mutant sequence for Region 104-109 (which includes the mutation site for miR-329). The nucleotide substitutions corresponding to these selections have now been displayed in the assembled mutant sequence.

- Once one mutant sequence has been selected per region, clicking the “Download Sequence Analysis” button will enable the user to download a ZIP folder, entitled mutant_analysis.zip, containing a single TXT file and three CSV files. The TXT file contains the project information, input sequence and final mutant sequence. The CSV files can be opened using Excel and contain information about the predicted miRNA target sites in the input sequence, predicted target sites in the mutant sequence, and all predicted target sites that have been newly created in the mutant sequence.

If the mutation criteria are too stringent and successful mutations are not generated, returning to the mutate page within the project page will enable the user to specify new mutation