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Targeted knockdown of canine KIT (stem cell factor receptor) using RNA interference

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1   # Targeted knockdown of canine KIT (stem cell factor receptor) using

2   # RNA interference

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24

1   **Abstract**

2   Canine mast cell tumours often express mutations in KIT exon 11 that result in

3   autophosphorylation and constitutive activation of the c-kit receptor, even in the absence of

4   stem cell factor, its natural ligand. Such activating mutations have been associated with more

5   aggressive neoplastic disease. The aim of the current study was to determine whether small

6   inhibitory RNA (SiRNA) molecules could be used to specifically knock-down canine KIT

7   expression. Canine beta-2 microglobulin (B2M), glyceraldehyde-3-phosphate dehydrogenase

8   (GAPDH) and KIT coding sequences were cloned downstream of the renilla luciferase

9   element in the psiCHECK™-2 bi-cistronic vector, that also expresses firefly luciferase.

10 SiRNA molecules were designed to target gene-specific sequences and were co-transfected

11 with plasmid DNA into Chinese hamster ovary (CHO) cells. Renilla and firefly luciferase

12 activity was subsequently measured using the Dual-GLO$^{®}$ Luciferase Assay (Promega).

13 Using this luciferase reporter system, canine housekeeping gene-specific SiRNA molecules

14 demonstrated knockdown of their targets (72.0% knockdown for B2M and 94.5% knockdown

15 for GAPDH). An SiRNA molecule targeting exon 2 of canine KIT successfully knocked-

16 down renilla luciferase expression of a KIT^{26-407} construct (90.8% knockdown). An SiRNA

17 molecule targeting a common 48 base-pair in-tandem duplication mutation in KIT exon 11

18 selectively knocked down expression of the $KIT^{1569-1966mutant}$ construct (93.1% knockdown)

19 but had no effect on the $KIT^{1569-1918wild-type}$ construct. The results show that RNA interference

20 can be used to inhibit canine KIT mRNA expression and has the potential to selectively target

21 the mutant version of KIT that is expressed by some malignant mast cells.

22

1   **1. Introduction**

2   Mast cell tumours (MCT) are the most common skin malignancy in dogs and are thought to

3   arise from neoplastic transformation of tissue mast cells, although the precise aetiology is

4   uncharacterised (reviewed by Dobson and Scase, 2007, Welle et al., 2008). In order to

5   become malignant, mast cells must be able to resist those mechanisms associated with

6   programmed cell death, to allow survival and proliferation. Developing a greater

7   understanding of how such cells prevent apoptosis might enable us to design more rational

8   treatment strategies for this type of cancer.

9   Mast cell viability in healthy tissues is regulated by stem cell factor (SCF) via its

10   action on the KIT receptor (CD117), which has tyrosine kinase activity (Qiu et al., 1988).

11   SCF binding to KIT is thought to provide survival signals, by inhibiting apoptosis through

12   modulation of apoptosis-regulatory proteins (Kitamura et al., 1978, Kitamura & Go, 1979,

13   Galli & Kitamura, 1987, Tsai et al., 1991, Galli et al., 1994, Serve et al., 1995, Linnekin et

14   al., 1997, Vosseller et al., 1997, Timokhina et al., 1998, Brazis et al., 2000, Taylor &

15   Metcalfe, 2000). In human mast cells, overexpression of Bcl-2 or Bcl-x_L, protects against

16   programmed cell death and is thought to contribute to oncogenesis (Cerveró et al., 1999,

17   Hartmann et al., 2003). Mutations in the *KIT* gene are found in 15-50% of canine MCT,

18   which typically consist of in-tandem duplications, located in or around exon 11 (London et

19   al., 1999, Ma et al., 1999, Reguera et al., 2000, Downing et al., 2002, Reguera et al., 2002,

20   Zemke et al., 2002, Turin et al 2006, Webster et al., 2006 A & B). When expressed, such

21   mutations often result in autophosphorylation of the cytoplasmic kinase domain of the

22   receptor protein, leading to constitutive activation, even in the absence of SCF (London et

23   al., 1999, Ma et al., 1999, Pryer et al., 2003). The C2 mastocytoma cell line, which is

24   commonly used to study canine malignant mast cell biology *in vitro*, expresses a 48 base-

25   pair in-tandem duplication (London et al., 1999, Ma et al., 1999), although the precise

1　location, nature and size of mutations can vary between different MCTs (Reguera et al.,

2　2000, Downing et al., 2002, Pryer et al., 2003, Riva et al., 2005, Roskoski et al., 2005, Turin

3　et al., 2006, Webster et al., 2006A, Letard et al., 2008).

4　　　KIT activating mutations have been shown to be associated with MCT of a more

5　aggressive phenotype, which is likely due to increased proliferation and resistance to

6　apoptosis (Hirota et al., 1998, Corless et al., 2004, Gleixner et al., 2007, Webster et al.,

7　2007, Letard et al., 2008). This has led to several investigations into the therapeutic

8　potential of receptor tyrosine kinase inhibitors (RTKi), designed to specifically target this

9　molecule. There are currently two drugs (masitinib, Masivet®, AB Science; toceranib,

10　Palladia®, Pfizer) that have recently been licensed for canine MCT. However, these

11　compounds can also impact on other receptor tyrosine kinases (Rubin et al., 2001, Hayes &

12　Thor, 2002, Humbert et al., 2009, London et al., 2009), potentially leading to adverse

13　effects. More specific targeting of the mutant KIT receptor is likely to be more selective in

14　having anti-cancer effects.

15　　　RNA interference can be used to target specific mRNA for degradation (Spee et al.,

16　2006, Tsuchiya et al., 2006, Watanabe et al., 2009. Thus, gene expression can be selectively

17　silenced, preventing production of the encoded protein, which allows experiments to be

18　designed to determine the role of that specific gene product on cellular function. The aim of

19　the current study was to determine whether small inhibitory RNA (SiRNA) molecules could

20　be designed to specifically knock-down canine KIT expression. In particular we aimed to

21　design novel SiRNA molecules that specifically targeted the mutant KIT expressed by C2

22　cells that would spare expression of the wild-type receptor. However, since C2 cells proved

23　to be resistant to chemical transfection, we used a reporter gene assay to assess the ability of

24　canine KIT-specific SiRNA molecules to knock-down their target.

25

1

2 **2. Materials and Methods**

3 *2.1 Plasmid DNA constructs and SiRNA molecules*

4 Partial coding regions for selected canine genes were amplified by PCR from cDNA prepared

5 from the C2 canine mastocytoma cell line (a generous gift from Dr B. Helm, University of

6 Sheffield; originally generated by Prof W. Gold, University of California (Lazarus et al.,

7 1986), which expresses both wild-type KIT as well as a KIT exon 11 mutation (Figure 1).

8 PCR products representing canine glyceraldehyde-3-phosphate dehydrogenase; GAPDH$^{520\text{-}971}$

9 (sense   primer: ACCACCGTCCATGCCATCAC;   antisense   primer:

10 TCCACCACCCGGTTGCTGTA; 452bp amplicon), beta-2 microglobulin; B2M$^{47\text{-}379}$ (sense

11 primer: TCCTCATCCTCCTCGCT; antisense primer: GTCAGTTGTCTCGGTCCCAC;

12 333bp amplicon), KIT$^{26\text{-}407}$ (KIT1: sense primer: ATTTTCTCTGCGTCCTGCTC; antisense

13 primer: ACCAGCGTATCATTGCCTTC; 382bp amplicon), KIT$^{1569\text{-}1920\text{wild-type}}$ and KIT$^{1569\text{-}}$

14 $^{1968\text{mutant}}$ (KIT2: sense primer: CCTGTTCACACCTTTGCTGA; antisense primer

15 TAGGGCTTCTCGTTCGGTTA; 352bp amplicon for wild-type, 400bp amplicon for mutant)

16 were initially cloned into the pSC-A vector (Stratagene, La Jolla, CA). Plasmid DNA from

17 recombinant clones was sequenced to confirm the integrity of the inserts, which were then

18 sub-cloned into the psi-CHECK™-2 bi-cistronic vector (Promega, Southampton, UK),

19 downstream of the renilla luciferase element using *Not*I and *Xho*I restriction sites. Plasmid

20 DNA was then extracted from recombinant *E.coli* using the GenElute™ Endotoxin-free

21 Miniprep Kit (Sigma, Poole, UK) in preparation for transfection studies. The SiRNA

22 molecules used in the study are shown in Table 1. In addition, a scrambled SiRNA molecule

23 (AllStars Negative Control SiRNA, Qiagen, Crawley, UK) was used as a negative control.

24

25 *2.2 Cell culture and transfections*

1   Chinese hamster ovary (CHO) cells were maintained in 75 cm^2 flasks of culture medium

2   consisting of Minimal Essential Medium (Sigma. Poole, UK) supplemented with 10 mM

3   Glutamax-1 (Invitrogen) 10% foetal bovine serum (Serotec, Kidlington, UK) and 25 μg/ml

4   gentamicin (Sigma). Cells for transfection studies were plated out in triplicate wells in

5   Corning 96 well clear bottom, white microtitre plates (Sigma) at 1 x10^4 cells in a 100 μl

6   volume of culture medium lacking serum and antibiotics. Cells in each well were transfected

7   with 200 ng plasmid DNA and 5 pmol SiRNA using Lipofectamine 2000™ (Invitrogen)

8   according to the manufacturer's instructions. Plates were incubated for 4 h then the complex-

9   containing medium was replaced with culture medium lacking antibiotics.

10

11   *2.3 Luciferase assay*

12   Twenty four hours after transfection, cells were assayed for both firefly and renilla luciferase

13   activity using the Dual-GLO$^®$ Luciferase Assay System (Promega). Briefly, cells were lysed

14   and the firefly luciferase substrate added (50 μl per well Dual-GLO$^®$ Substrate/Buffer). After

15   15 min, luciferase activity was measured using a luminometer (Spectramax M2, Molecular

16   Devices). Next the renilla luciferase substrate was added (50 μl per well Stop & GLO$^®$

17   Substrate/Buffer) and the luminescence measured after a further 15 min incubation. The

18   renilla / firefly luciferase ratio was calculated from the mean luminescence values of triplicate

19   wells, after blanking against values from untransfected cells. The percentage knockdown

20   using test SiRNA molecules was calculated compared to the control scrambled SiRNA and

21   data are shown as the mean of three experimental replicates.

22

23   **3. Results**

24   *3.1 Knock-down in expression of canine housekeeping genes and KIT$^{wild-type}$*

1   Since there are few studies using SiRNA to specifically knock-down canine gene expression,

2   several SiRNA molecules were initially assessed that were designed to target housekeeping

3   genes. Using a renilla luciferase SiRNA molecule as a positive control, efficient knock-down

4   of canine GAPDH was demonstrated, although SiRNA molecules targeting beta-2

5   microglobulin were less efficient (Table 2). A validated human KIT SiRNA molecule (which

6   has complete identity to the target sequence in canine KIT) was evaluated and showed >90%

7   knock-down efficiency (Table 2).

8

9   *3.2 Knock-down in expression of the C2 KIT mutant*

10   The mutation present in the KIT gene expressed by C2 cells consists of a 48 bp in-tandem

11   duplication in exon 11. Since this generates a unique sequence at the 5' junction of the

12   insertion, an SiRNA molecule was designed that spanned this region (Figure 1, Table 1).

13   When tested against constructs containing either the wild-type sequence or the mutant

14   version, this SiRNA molecule demonstrated efficient knock-down when using the KIT2$^{\text{mutant}}$

15   construct (mean 93.1% knock-down) but had little effect on the KIT2$^{\text{wild-type}}$ construct (mean -

16   2.98% knock-down) (Figure 2).

17

18   **4. Discussion**

19   Recent advances in treatment of canine MCT have focused on the use of RTKi (e.g

20   masitinib and toceranib). However, these compounds are not specific for KIT and also inhibit

21   other receptor tyrosine kinases, such as platelet-derived growth factor receptor (PDGFR) and

22   vascular growth factor receptor (VEGFR2) (Rubin et al., 2001, Hayes & Thor, 2002, Humbert

23   et al., 2009, London et al., 2009). Although the lack of specificity might enhance the anti-

24   cancer efficacy of these drugs, this could also increase the risk of adverse effects in the

25   patient. In addition, these RTKi will not have specific effects on malignant mast cells, as they

1   will also inhibit wild-type KIT, expressed by tissue mast cells and other cell types including

2   haematopoietic stem cells and melanocytes (Galli et al., 1994, Serve et al., 1995, Linnekin et

3   al., 1997, Vosseller et al., 1997, Timokhina et al., 1998, Brazis et al., 2000) Molecular

4   techniques, such as gene silencing by RNA interference, offer an alternative, more specific

5   method for targeting mutant KIT that might be applicable for canine MCT, as well as for

6   canine gastrointestinal stromal cell tumours that also commonly express KIT mutations in

7   humans (Lasota et al., 2003, Steigen et al., 2007, Du et al., 2008).

8   There are relatively few published papers describing RNA interference in the canine

9   system. In terms of studying the biology of canine malignant cells, siRNA targeting of

10   apoptosis-modulator molecules including Bcl-2 (Watanabe et al., 2009), Bcl-xL (Tsuchiya et

11   al., 2006) and XIAP (Spee et al., 2006) have previously been undertaken in canine cancer cell

12   lines. Knockdown of canine (housekeeping) gene(s), not involved in malignancy, would be

13   useful as negative controls for RNA interference studies, although there were no published

14   reports of their use prior to commencing the current study.

15   To investigate the biological effect of KIT RNA interference, the C2 canine

16   mastocytoma cell line was chosen for *in vitro* studies. The C2 cells used in the current study

17   expressed both wild-type and mutant KIT mRNA (Figure 1), although wild-type KIT seems

18   to be absent in the C2 cells used in some (Ma et al., 1999) but not all previous studies

19   (London et al., 1999). Since no KIT siRNA molecules had been validated for the dog, several

20   human KIT siRNAs, that had been shown to increase apoptosis in human cell lines (Lefevre

21   et al., 2004; Li et al., 2007; Sikarwar & Reddy 2008) were assessed for sequence identity with

22   the canine KIT sequence. One commercially available KIT SiRNA molecule (Silencer[®]

23   SiRNA 35, Ambion), which demonstrated complete sequence identity between human and

24   canine target sequences, was identified. For the other targets (GAPDH, B2M and KIT exon

25   11 mutation), novel SiRNA molecules were designed for evaluation.

1   Chemical transfection of C2 cells proved to be problematic, despite repeated attempts

2   using various transfection reagents and protocols (data not shown). Therefore, to allow

3   investigation of the activity of the siRNA molecules to specifically interfere with their target

4   canine mRNA, an alternative assay was established. This assay was based on use of the

5   psiCHECK-2^{TM} reporter system in the CHO cell line, which had been shown to be readily

6   transfectable with Lipofectamine^{TM} 2000. This reporter assay allows rapid and relatively

7   simple assessment of the activity of novel SiRNA molecules against their target sequences,

8   prior to evaluation of RNA interference in the target cells. One disadvantage, however, is that

9   it does not provide information as to any off-target effects of the SiRNA molecule.

10   Co-transfection of CHO cells with recombinant plasmid DNA containing canine target

11   sequences and either scrambled or targeted siRNA molecules demonstrated that GAPDH and

12   KIT^{exon2} siRNAs induced a knockdown efficiency of greater than 80%, although the canine

13   B2M SiRNA molecules were somewhat less effective. The increase in firefly luciferase

14   activity seen in CHO cells co-transfected with plasmid DNA with scrambled siRNA,

15   compared to those cells transfected with plasmid DNA alone, might be explained by the

16   greater amount of transfection reagent used, leading to increased transfection efficiency. A

17   further increase in firefly luminescence was seen in CHO cells co-transfected with targeted

18   siRNA compared to scrambled siRNA. This pattern of reactivity might be explained if firefly

19   and renilla luciferase mRNA compete for translation. Thus, following successful targeting of

20   the renilla-fusion mRNA for destruction, this could lead to enhanced firefly luciferase protein

21   expression.

22   The KIT^{exon2} SiRNA molecule is not specific to the mutant KIT sequence and would

23   be expected to knock-down both wild-type and mutant KIT mRNAs. Therefore, a novel

24   canine KIT SiRNA molecule was designed, that targeted the 5' junction of the insertion

25   mutation to determine whether it might be possible to specifically knock-down the mutant

1 version, while sparing expression of wild-type KIT. Both firefly and renilla luciferase activity

2 were consistently lower when using the psiCHECK-2^{TM} construct containing canine mutant

3 KIT, compared to the native vector or vector containing wild-type KIT sequences. Endotoxin

4 contamination of the plasmid DNA was considered, but a new batch of plasmid DNA

5 demonstrated the same response. The explanation for this reduced efficiency of luciferase

6 activity with this particular construct remains unclear. Despite this, the KIT^{exon11mut} SiRNA

7 molecule demonstrated specificity of activity against the mutant KIT construct, without

8 affecting expression from the construct containing the equivalent wild-type KIT sequence.

9   In the current study, we have validated several canine gene-specific SiRNA

10 molecules. These can now be assessed in canine cell lines and it is anticipated that alternative

11 delivery systems (e.g. electroporation) will allow evaluation of these molecules in the C2 cell

12 line. We have shown as "proof-of-concept" that SiRNA molecules can be designed to

13 specifically target KIT mutations, although the heterogeneity of mutations seen in canine

14 MCT means that it is unlikely that a single SiRNA molecule could be designed that would be

15 effective in all cases.

16

1   **Acknowledgements**

4

5   **References**

6

7

1   **Fig. 1.** The C2 cell line expresses both wild-type KIT and a KIT exon 11 mutant. (a) cDNA

2   was prepared from C2 cells and used as the template for PCR using primers spanning KIT

3   exon 11. PCR products were separated by 2% agarose gel electrophoresis. Anticipated

4   amplicon size = 352bp for wild-type KIT and 400bp for the mutant version, which contains a

5   48bp in-tandem duplication. MWt = 200bp molecular weight marker. (b) Sequence of KIT

6   mutation in C2 cells. Partial genomic DNA sequence is shown with exon 11 highlighted. The

7   48 bp mutation is shown in bold and the region targeted by the SiRNA molecule is

8   underlined.

9

10 **Fig. 2**. Knockdown efficiency of an SiRNA molecule that targets the C2 KIT mutation.

11 Recombinant psiCHECK-2 plasmid DNA was generated containing either the 5' region of

12 canine KIT (KIT1) or the region containing exon 11 (KIT2) of either the wild-type or mutant

13 version of the gene. Triplicate wells of CHO cells were transfected with recombinant

14 psiCHECK-2 plasmid DNA and selected wells were co-transfected with scrambled siRNA

15 (negative control), renilla SiRNA (positive control) or siRNA targeted to KIT (either exon 2

16 or the in-tandem duplication mutation within exon 11). Both firefly and renilla luminescence

17 were measured after 24 h incubation. The renilla/firefly luciferase ratio are shown which

18 represents the knock-down capability of the SiRNA molecule on the target mRNA. A

19 representative experiment is shown of three replicates.

20

21

1

Target gene	Plasmid DNA construct	SiRNA molecule	SiRNA target sequence	SiRNA source
Renilla luciferase	psiCHECK-2	Renilla	tggcctttcactactcctacg	Custom synthesized (Ambion)
GAPDH	psiCHECK-2/GAPDH$^{520\text{-}971}$	GAPDH#1^{exon8a}	ccactttgtcaagctcatt	Custom synthesized (Ambion)
GAPDH	psiCHECK-2/GAPDH$^{520\text{-}971}$	GAPDH#2^{exon8b}	cgacatcaagaaggtagtg	Custom synthesized (Ambion)
B2M	psiCHECK-2/B2M$^{47\text{-}379}$	B2M#1^{exon2a}	ctcccaatgagcaggatga	Custom synthesized (Sigma)
B2M	psiCHECK-2/B2M$^{47\text{-}379}$	B2M#2^{exon2b}	ccgtgtaaagcatgttact	Custom synthesized (Sigma)
KIT	psiCHECK-2/KIT$^{26\text{-}407}$	KIT^{exon2}	ggctcttctcaaccatctg	Silencer® SiRNA 35 (Ambion)
KIT	psiCHECK-2/KIT$^{1569\text{-}1966mutant}$	KIT^{exon11mut}	agaacatacccaacacagc	Custom synthesized (Ambion)

2

3   **Table 1.** Plasmid DNA constructs and SiRNA molecules used in knock-down studies.
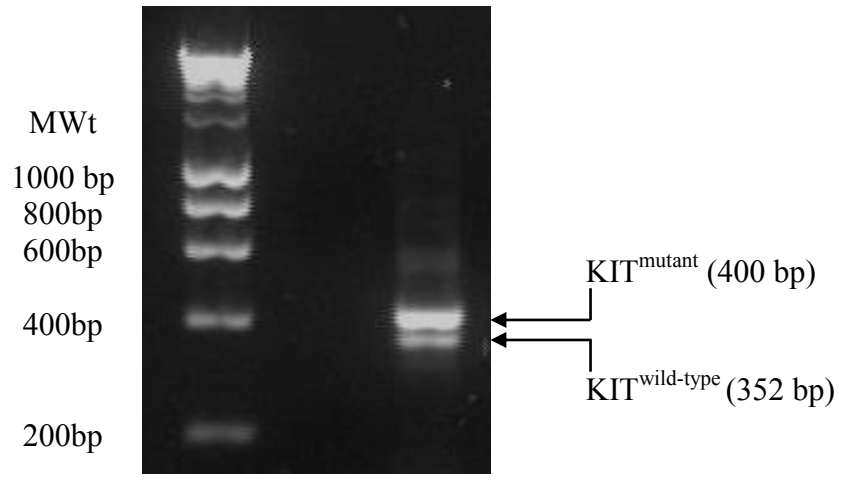
4

| Transfection conditions | Exp #1 | | | Exp #2 | | | Exp #3 | | | Mean |
	Firefly (LU)	Renilla (LU)	KD (%)	Firefly (LU)	Renilla (LU)	KD (%)	Firefly (LU)	Renilla (LU)	KD (%)	KD (%)
psiCHECK™-2	2981	1493		1023	550		407	372		
psiCHECK™-2 + Scrambled siRNA	7528	3307		2662	1474		1536	1316		
psiCHECK™-2 + Renilla siRNA	10474	240	94.8	2794	91	94.1	3014	19	99.3	96.1
psiCHECK™-2/B2M	2505	1586		2137	1305		1873	998		
psiCHECK™-2/B2M + Scrambled siRNA	9952	5278		7329	3733		7001	3509		
psiCHECK™-2/B2M + B2M#1 siRNA	6175	1858	43.3	5657	1296	55.0	5593	1206	57.0	51.8
psiCHECK™-2/B2M + B2M#2 siRNA	8532	1300	71.3	7452	1126	70.3	7478	958	74.4	72.0
psiCHECK™-2/GAPDH	369	1045		535	896		474	703		
psiCHECK™-2/GAPDH + Scrambled siRNA	1236	2620		843	1034		1102	1133		
psiCHECK™-2/GAPDH + GAPDH#1 siRNA	1503	101	96.8	1354	139	91.6	1774	90	95.07	94.5
psiCHECK™-2/GAPDH + GAPDH#2 siRNA	2162	767	83.3	2052	489	80.6	2225	466	79.63	81.2
psiCHECK™-2/KIT1	524	1105		506	751		619	682		
psiCHECK™-2/KIT1 + Scrambled siRNA	1512	2580		1130	1199		1390	1325		
psiCHECK™-2/KIT1 + KITexon2 siRNA	1933	241	92.7	1526	202	87.5	2368	174	92.28	90.8

Table 2. Knockdown efficiency of canine housekeeping gene and KIT SiRNA molecules. Triplicate wells of CHO cells were transfected with native or recombinant psiCHECK-2 plasmid DNA and co-transfected with either scrambled siRNA, siRNA targeted to renilla luciferase, or SiRNA targeting the inserted canine sequence. Both firefly and renilla luciferase activity were measured after 24 h incubation. Mean luminescence data are shown, following subtraction of the luminescence values of untransfected CHO cells. Knockdown efficiency of targeted siRNA was calculated compared to scrambled siRNA. GAPDH = glyceraldehyde-3-phosphate dehydrogenase, B2M = β2microglobulin, Exp = experimental replicate, LU = luminescence units, KD (%) = percentage knockdown efficiency.
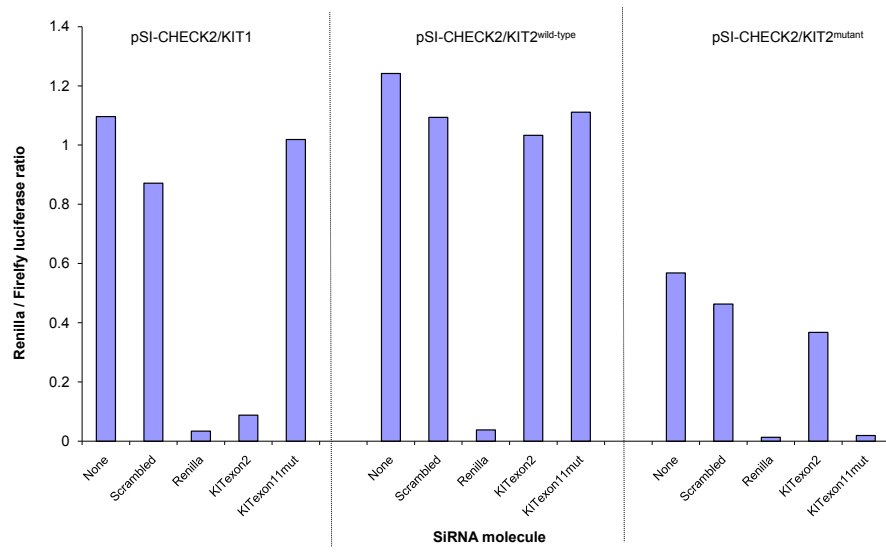
1 FIGURE 1

2 a)

3

4

5 1000 bp

6 800bp

7 600bp

MWt

KIT^{mutant} (400 bp)

8 400bp

9 KIT^{wild-type} (352 bp)

10 200bp

11

12

13 b)

14

15 TCTCTACCCTAAGTGCTATAATGATCGAAATGTTATTCATTAAAAGATG

16 ATCTGTCTCTCTTTTCTCCCCCCACCAGAAACCCATGTATGAAGTACAG

17 TGGAAGGTTGTTGAGGAGATCAATGGAAACAATTATGTTTACATAGACC

18 CAACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAA**CATACCC**

19 **AACACAGCTTCCTTACGATCACAAATGGGAGTTTCCCAGAA**ACAGGCTG

20 AGCTTTGGTCAGTATGAAATAGGGGCTTTCCATGTAACCTTTTTGTGTA

21 CGTGTAACAATGACTTTAGGGAACCCCATTAGCTTCCTTTGTTCTGTTC

22 CAACTGAGACAATAAGTATTTTCTGTGAAGTTTCATCATTTTTGATA

23

1   FIGURE 2

2

3
4