

1/35

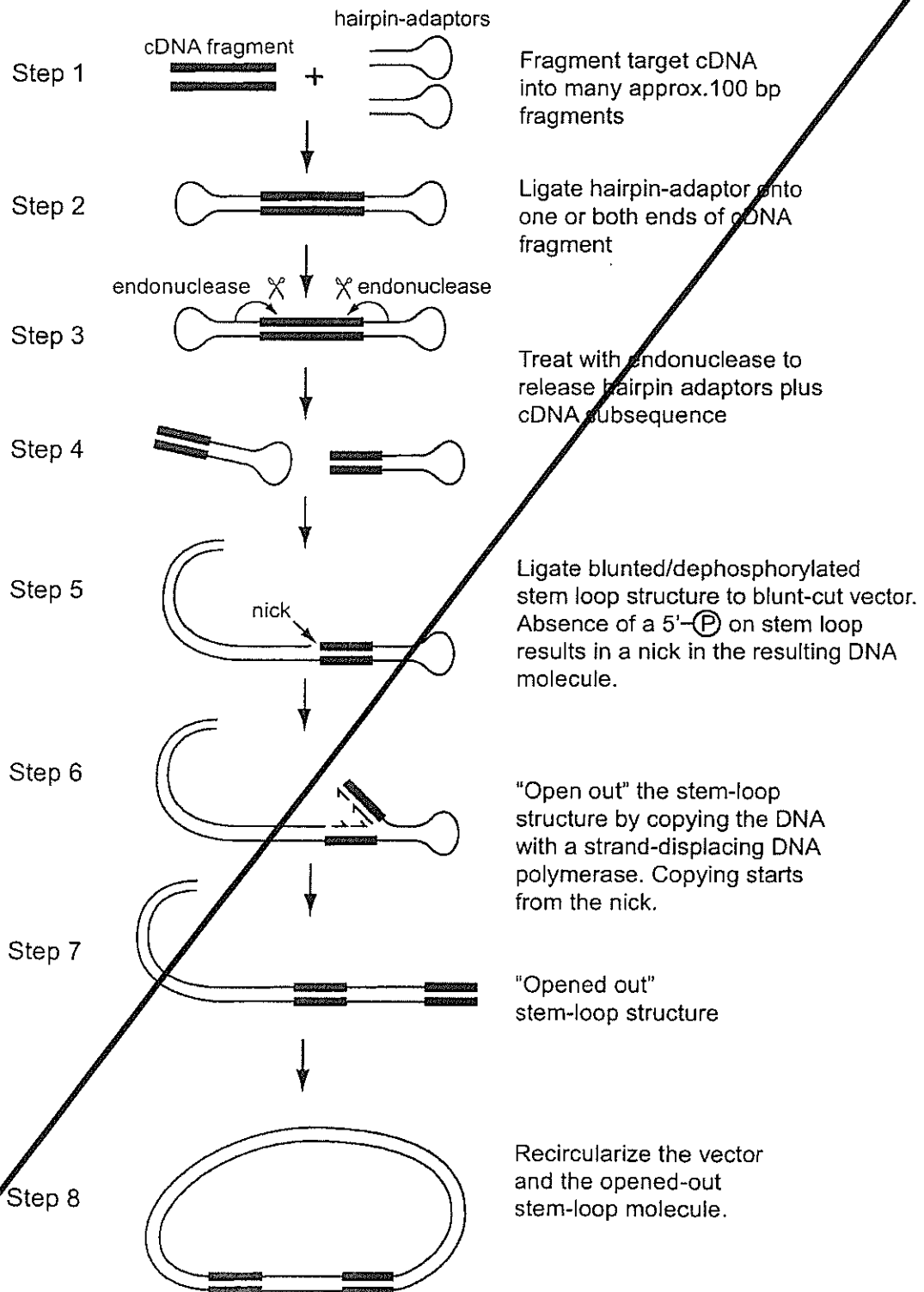


Fig. 1

2/35

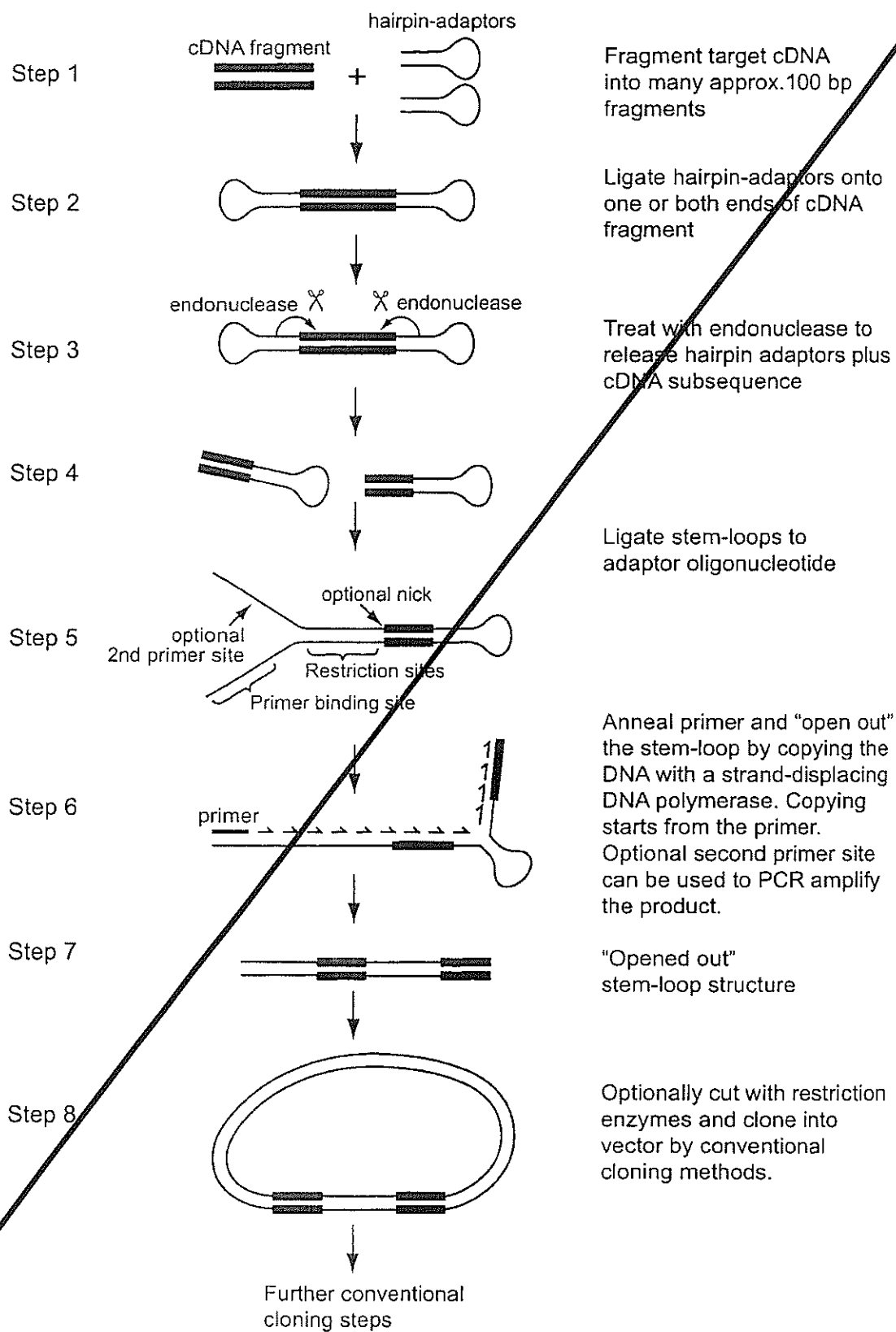


Fig. 2

3/35

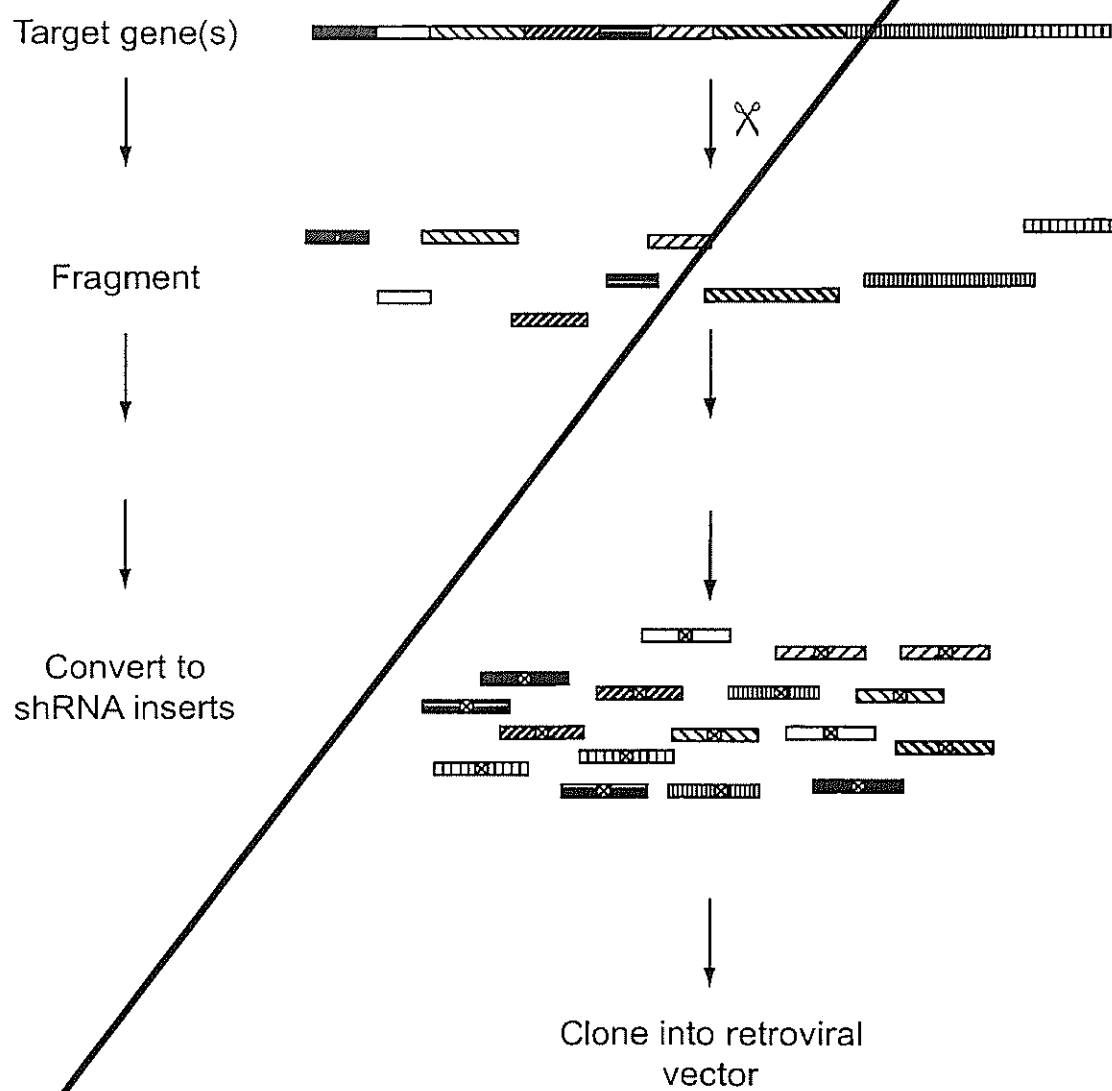
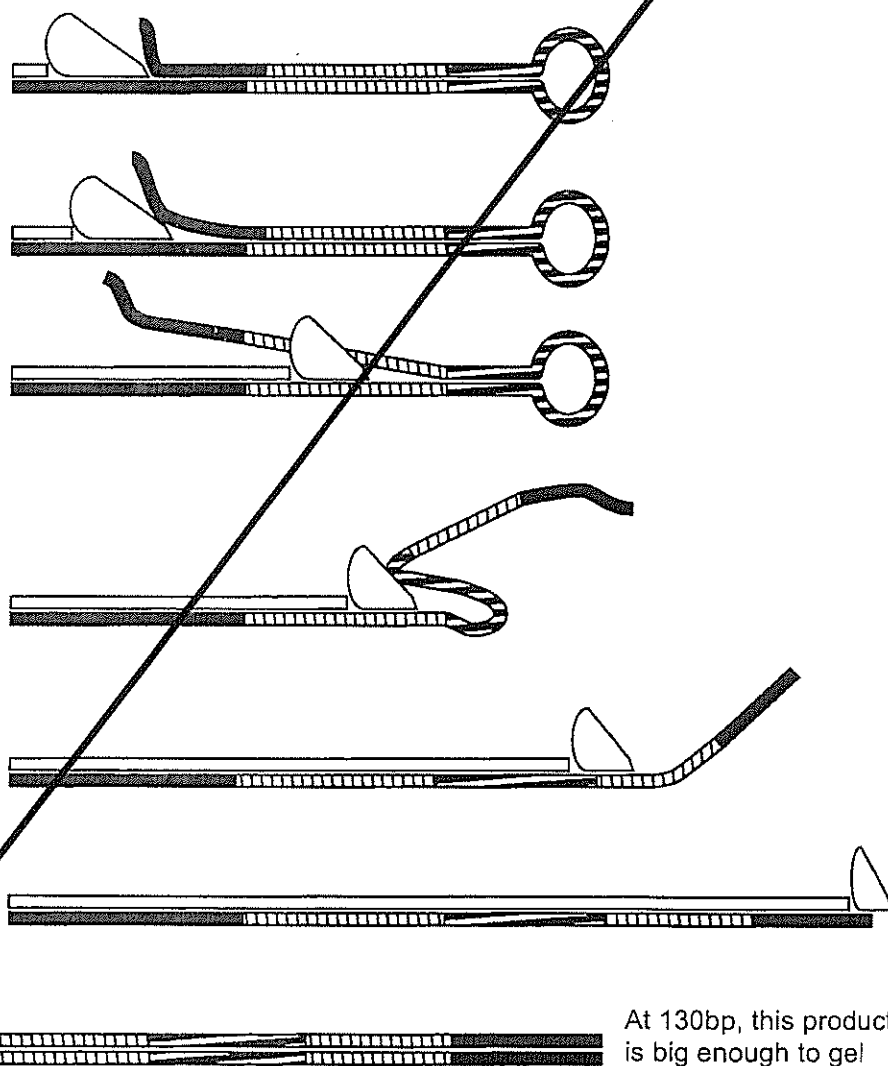


Fig. 3

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Generating shRNA expression cassette from
hairpin tags

- A) Ligate on cloning adaptor loop.
B) Anneal primer and extend (or extend from a nick between
vector and stem-loop). Bst DNA Polymerase can efficiently copy
the whole loop because it can displace the base-paired DNA.
No amplification avoids bias.



At 130bp, this product
is big enough to gel
purify efficiently.

Fig. 4A

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Generating shRNA expression cassette from hairpin tags

- C) Gel purify.
- D) Subclone into a suitable expression cassette by digesting with cloning enzymes. In a preferred method, this isn't required as the opened out loop is already attached to the vector. All that is required is to circularize the vector by ligation.
- E) If necessary, subclone the expression cassette into an appropriate vector, e.g., a retroviral expression vector.
- F) Transcribe the expression cassette in a host cell to produce a candidate shRNA.

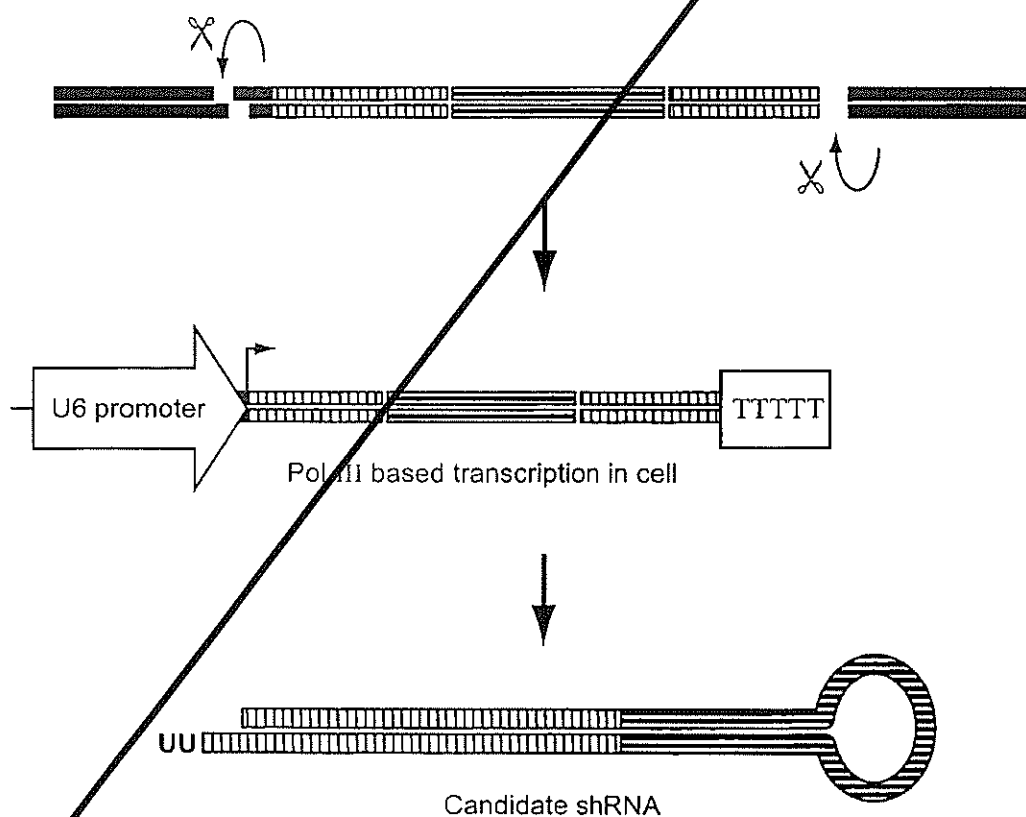


Fig. 4B

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Fig. 5A

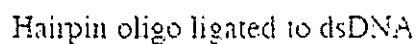
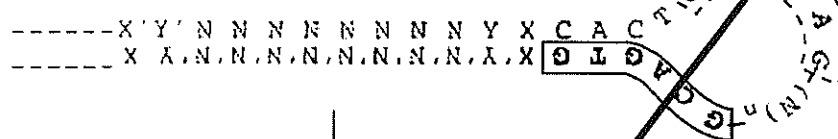
C G T T G G A - - -
 V V C C L - - -

Fig. 5B

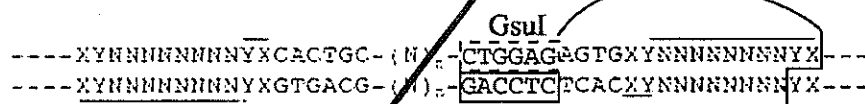
N1N2N3N4N5N6N7N8Y'X'CACTCTCCAG -
X'Y'N'N'N'N'N'N'N'N'N'YXGTG)
ACG - (N)n -/

Fig. 5C

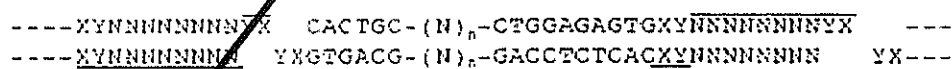
7/35



'Open-out' and
clone into circular
vector



Cut with BtsI, GsuI



Recircularise

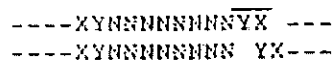


Fig. 5D

8/35

Use of restriction enzymes that cut outside their recognition sequence to seamlessly juxtapose sequence elements

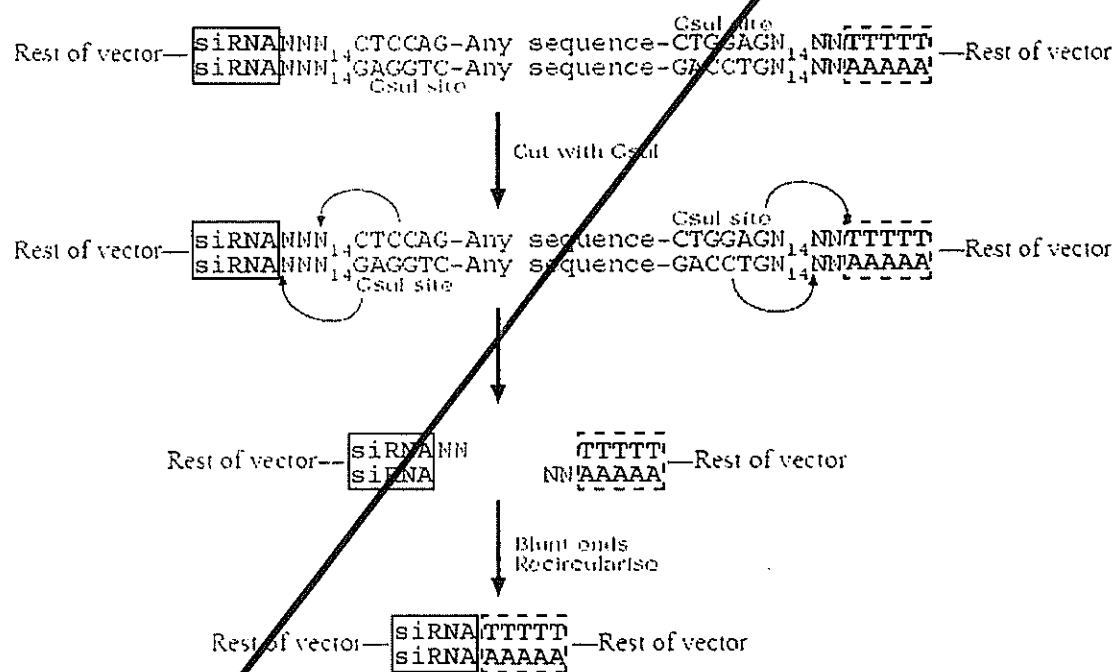


Fig. 6

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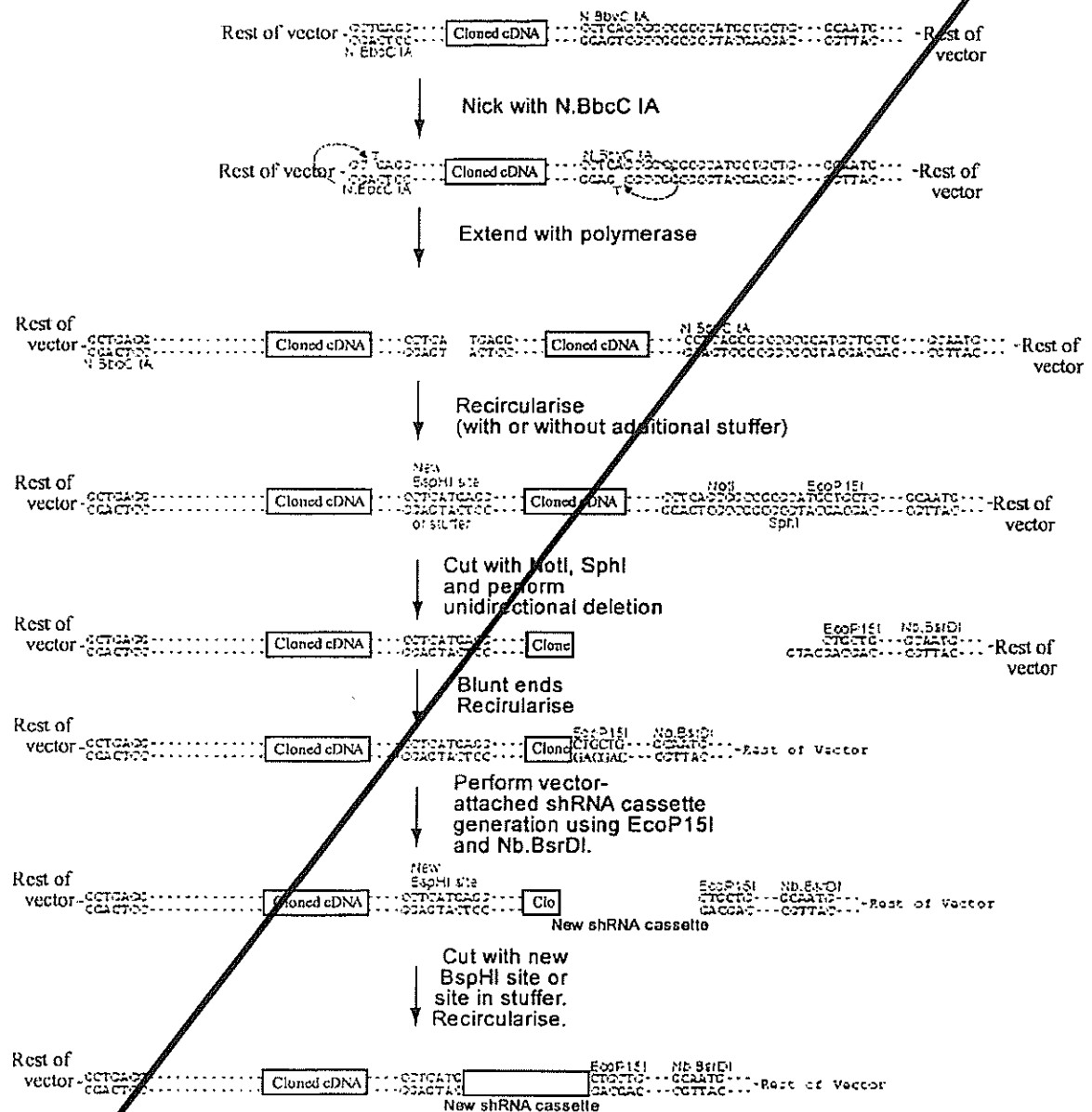


Fig. 7

10/35

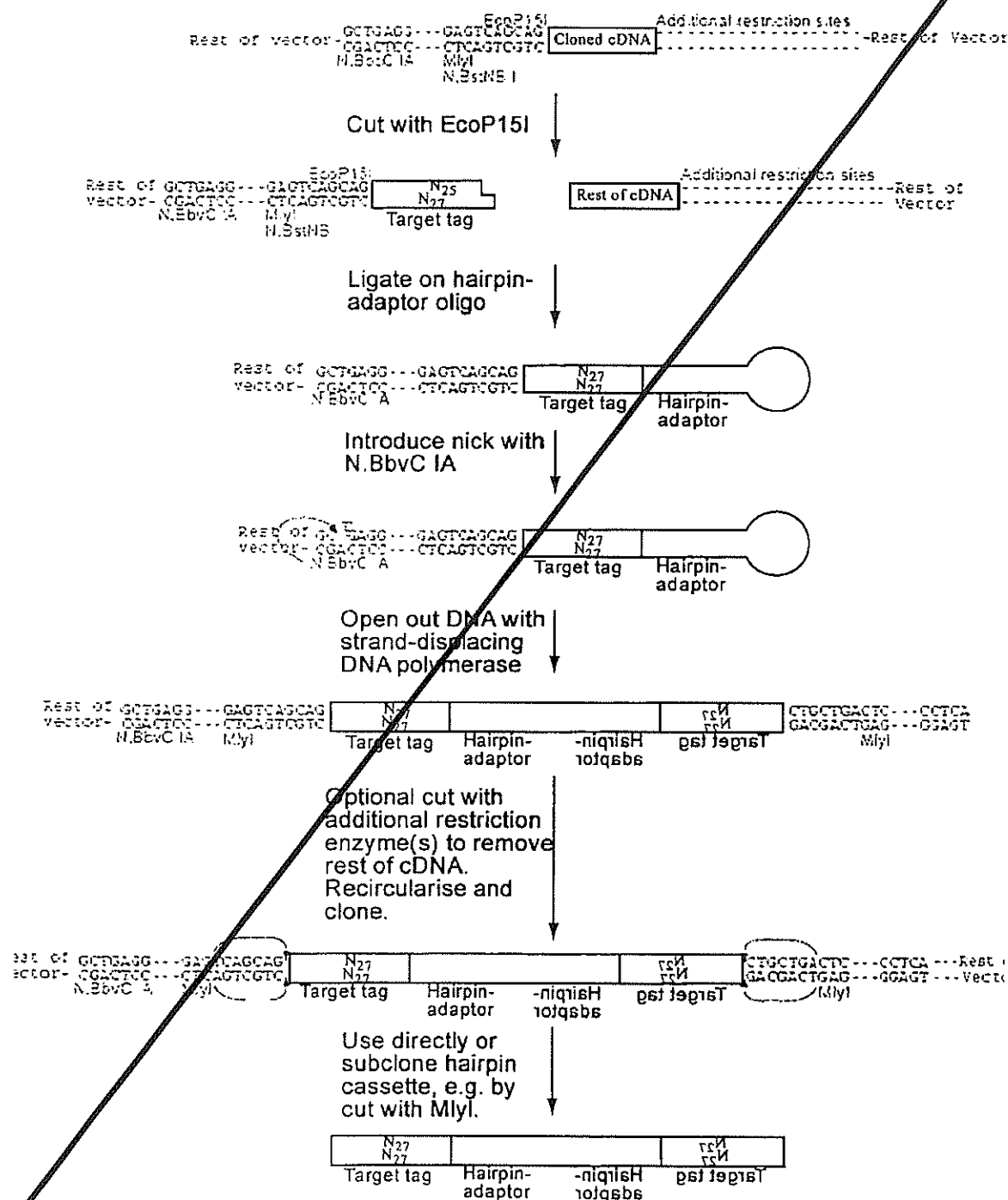


Fig. 8

11/35

Fig. 9

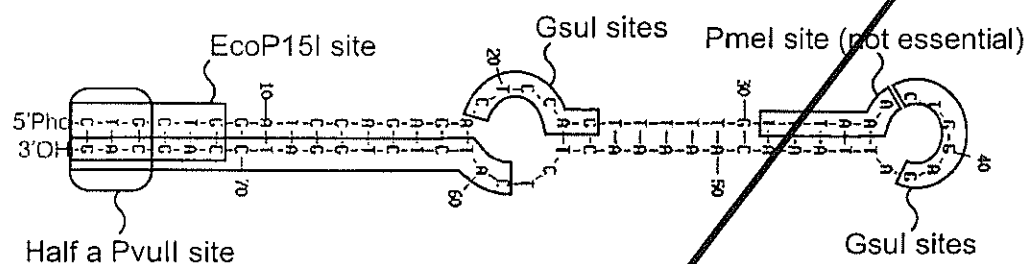


Fig. 10

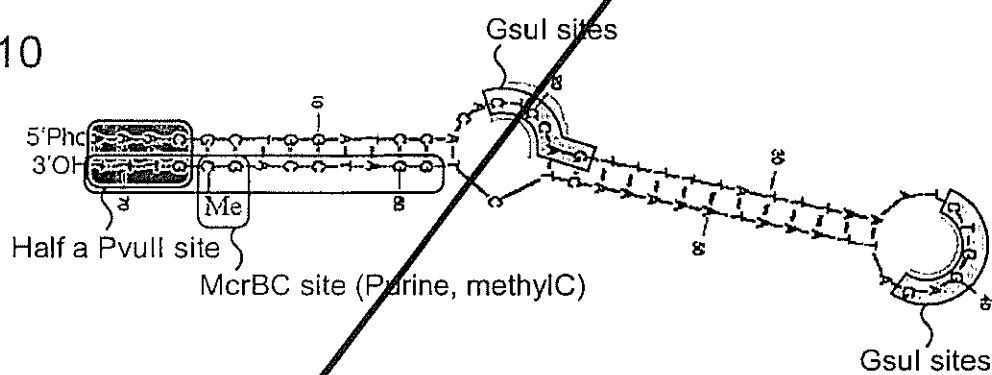
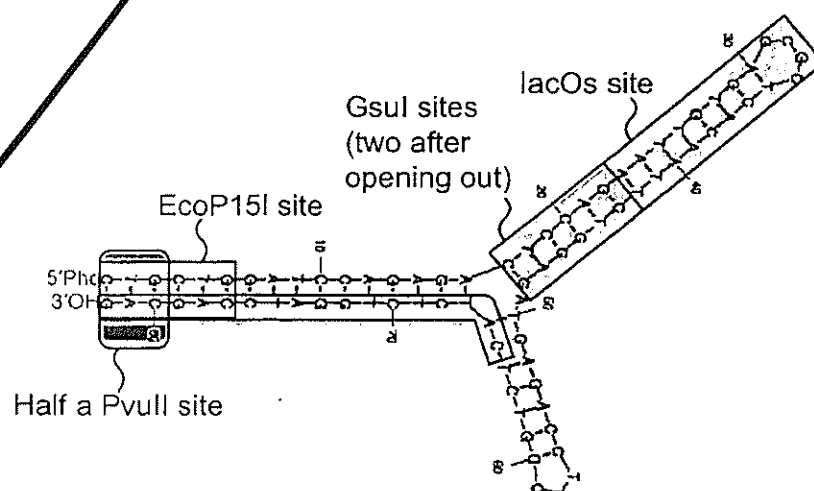


Fig. 11



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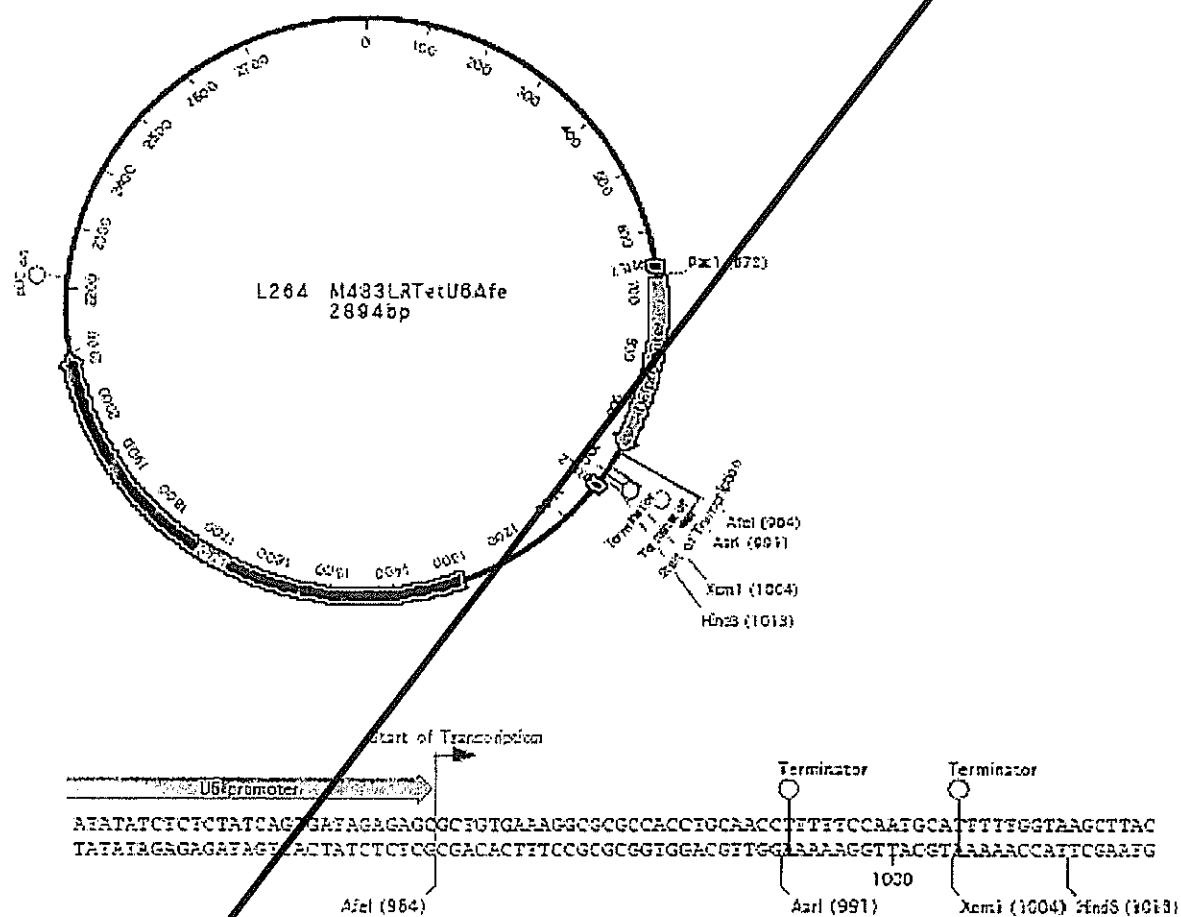


Fig. 12

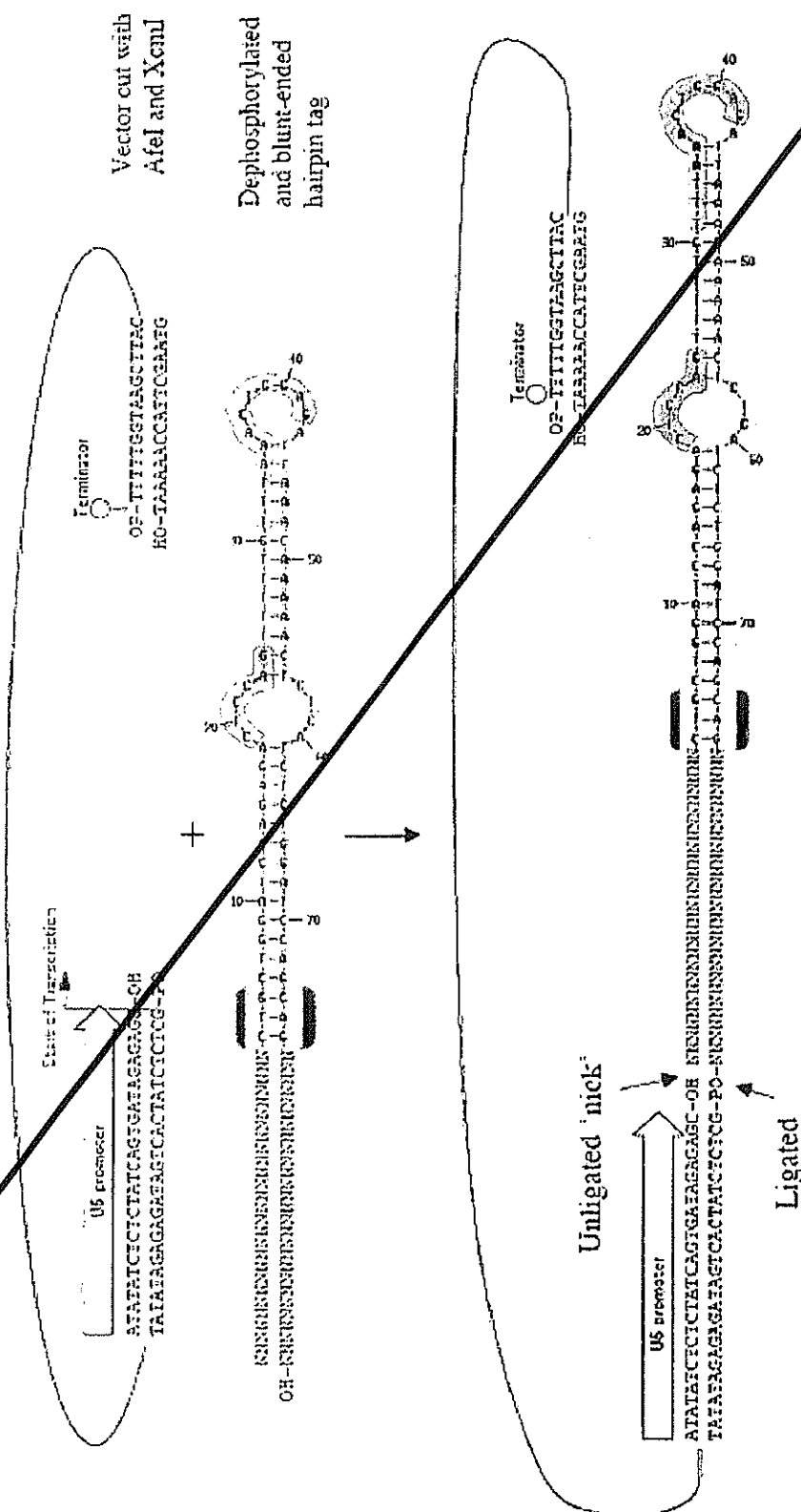


Fig. 13

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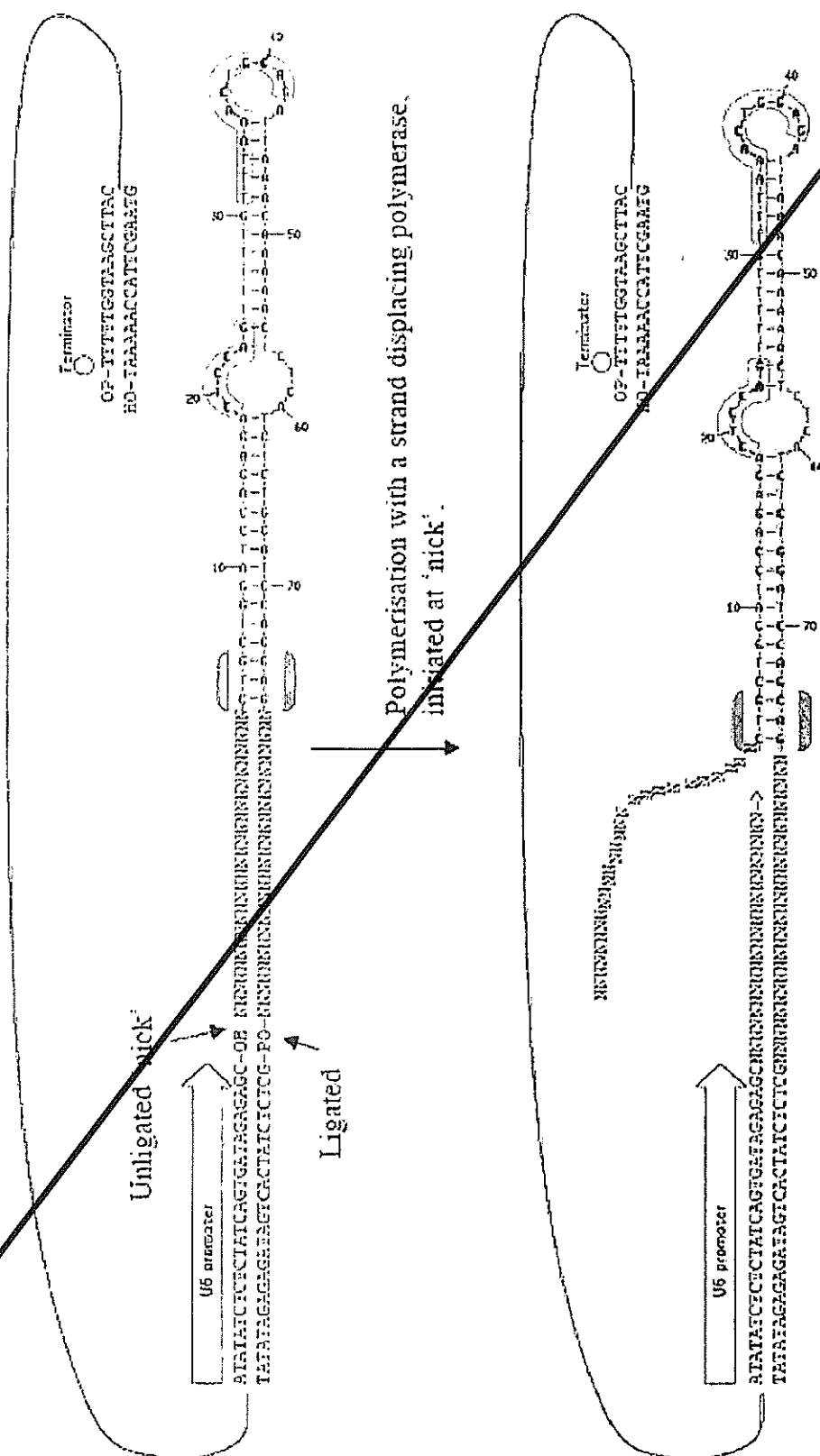


Fig. 14

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Fig. 17

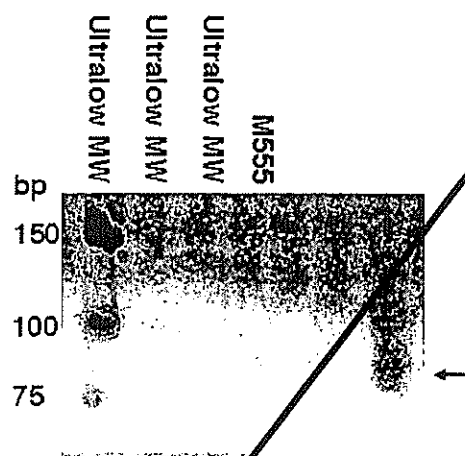
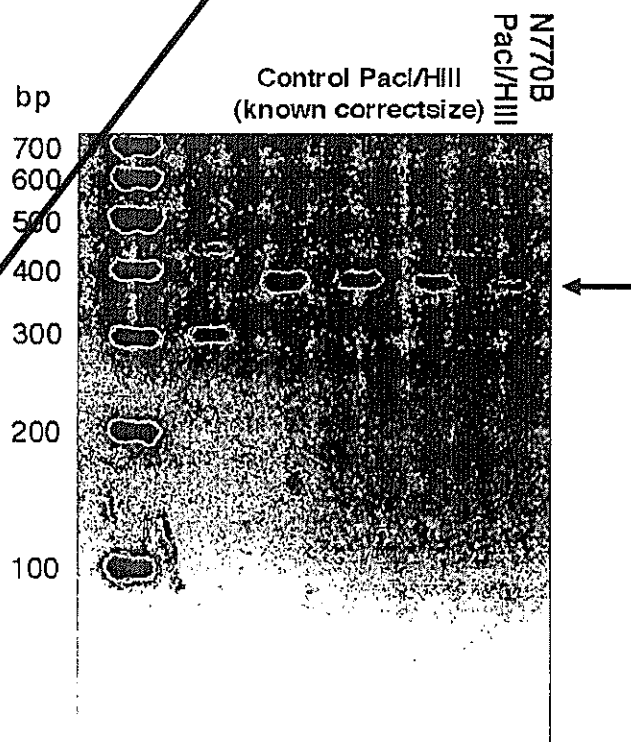


Fig. 18



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N783
PacI/HII
100bp
ladder

bp
3000
1000
500
400
300
200
100

Fig. 19

TCAGTGATGTAGAGAGAGGAG	CTGCTGGATCCAGAGATG	CTTCCTCTCTTCTTACATCACTGA	GG	TTTTT Matches	KDR NM002253.1	3251-3274
TTCACCTCACTTCCATATCGTCAGT	CTGCTGGATCCAGAGATG	ACTGACGATTATGGAAGTGAAGTGA	AG	TTTTT Matches	KDR NM002253.1	1473-1449
ATTATGTCATATGTTCAAGATTACAG	CTGCTGGATCCAGAGATG	CTGTAATCTTTGAACATAGACATAAAT		TTTTT Matches	KDR NM002253.1	643-669
TTATGATGCCAGCAATGGGAATT	CTGCTGGATCCAGAGATG	AATCCCATTTGCTGGCATCATAA	GG	TTTTT Matches	KDR NM002253.1	2766-2789
TCAGTGATGTAGAGAGAGAGGAG	CTGCTGGATCCAGAGATG	CTTCCTCTCTTCTTACATCACTGA	GG	TTTTT Matches	KDR NM002253.1	3251-3274
CCGCTCGGCCACCCAGCTTTCTTGT	CTGCTGGATCCAGAGAT	ACAAGAAAGCTGGGTGGCGAGGCGG	C	TTTTT Matches	vector L304	
TCCACGACAGAGCCACAGGGCGACGG	CTGCTGGATCCAGAGAT	CCGTGCGCCCTGNTGGCTCTGCGTGA	GA	TTTTT Matches	KDR NM002253.1	350-326
TCCCTCAGTGATGTAGAGAGAGGAG	CTGCTGGATCCAGAGAT	CTTCCTCTCTTCTTACATCACTGAGGA		TTTTT Matches	KDR NM002253.1	3247-3274

Fig. 20

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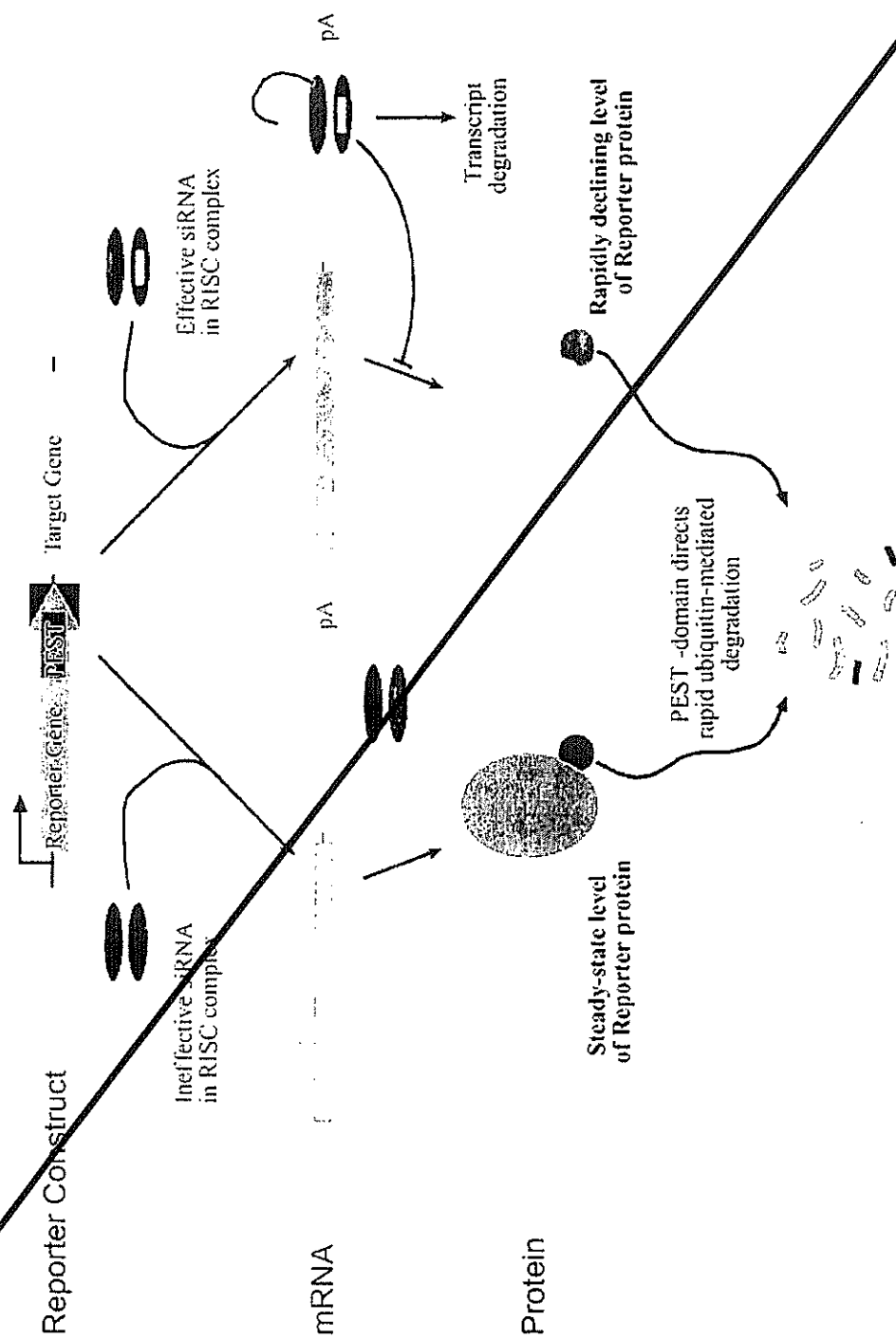


Fig. 21

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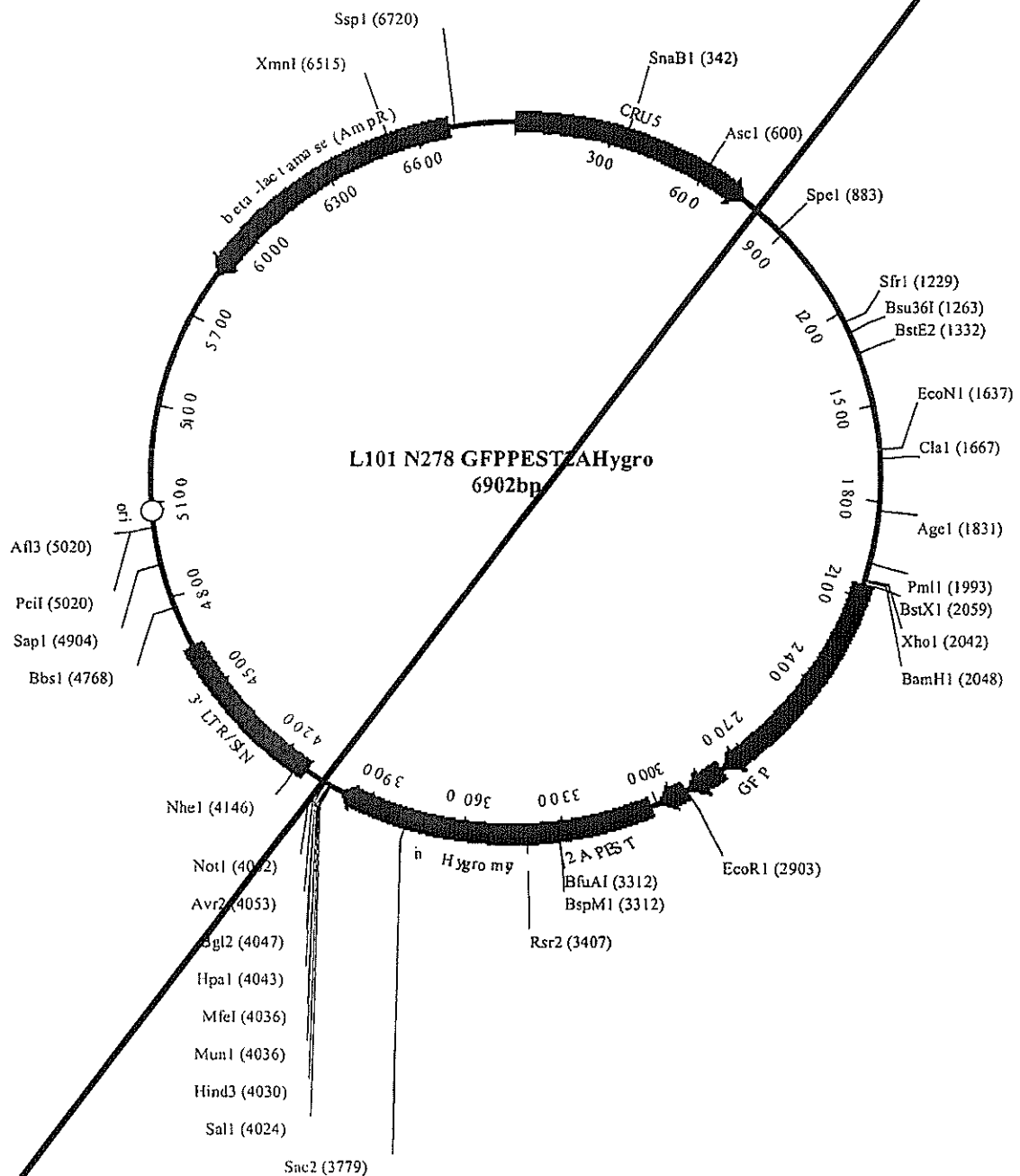
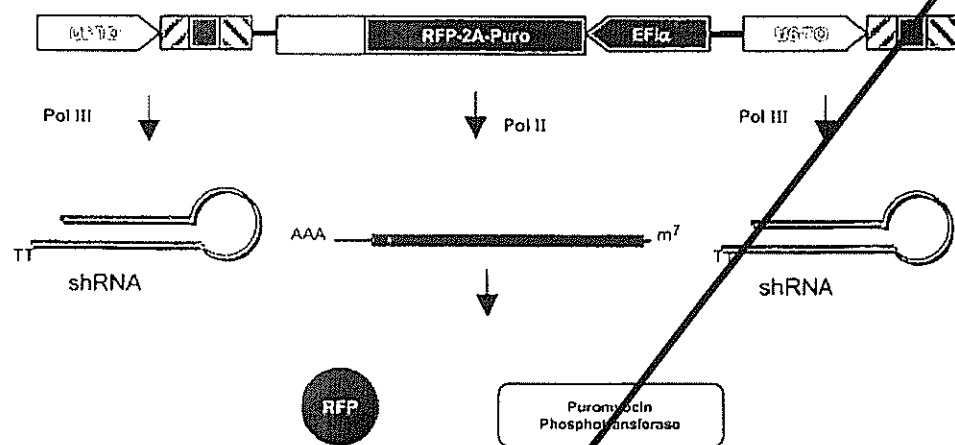


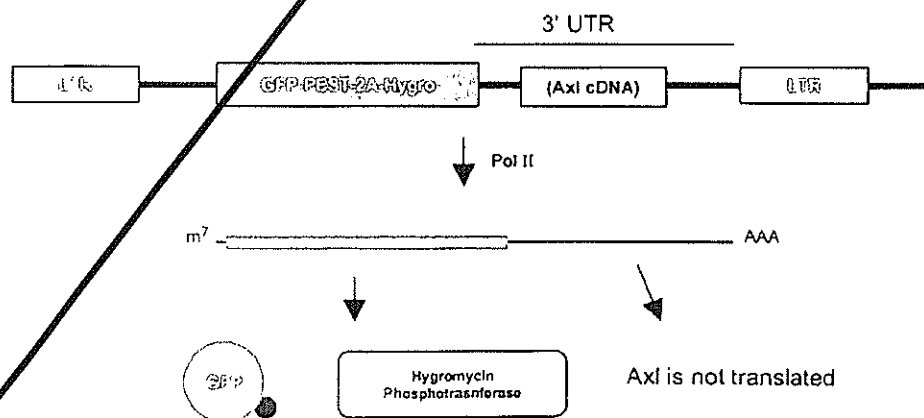
Fig. 22

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shRNA targeting Axl or Luciferase (control) in an RRI vector

Fig. 23A



GFP-PEST reporter with or without Axl cDNA

Fig. 23B

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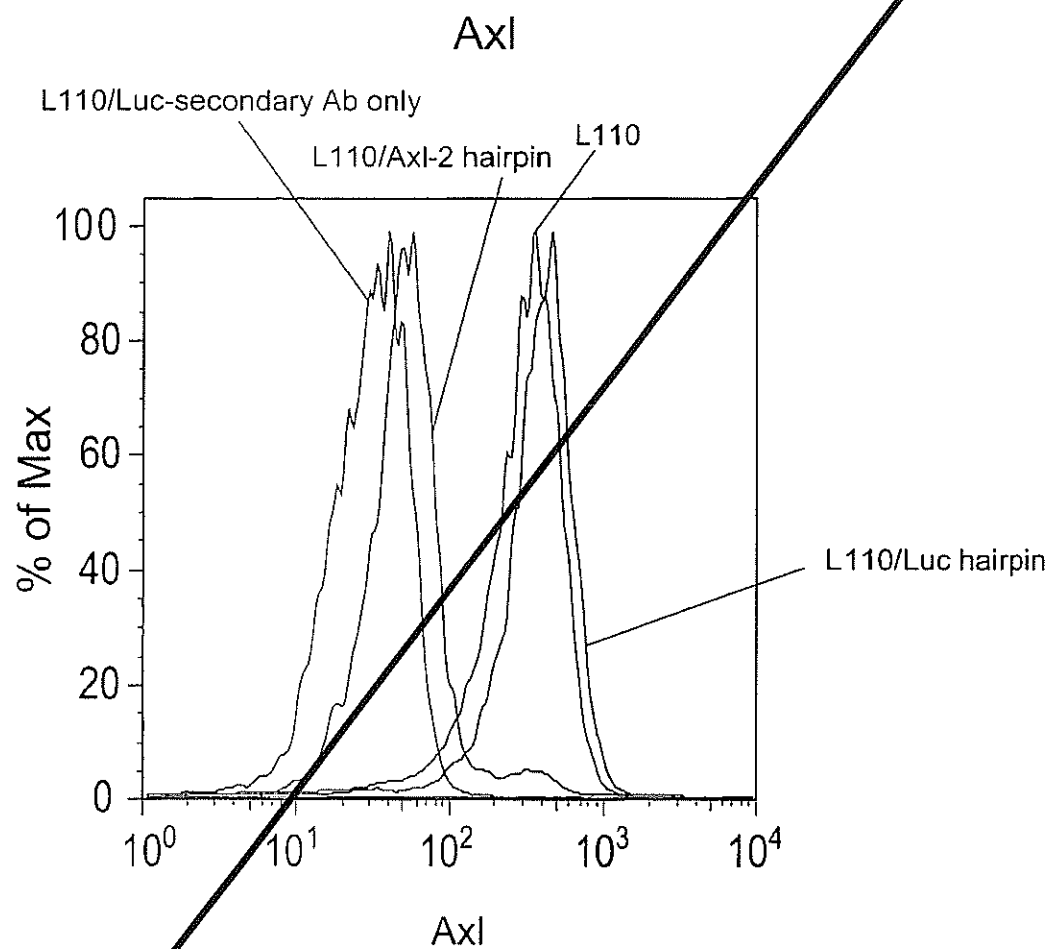


Fig. 24

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Fig. 25A

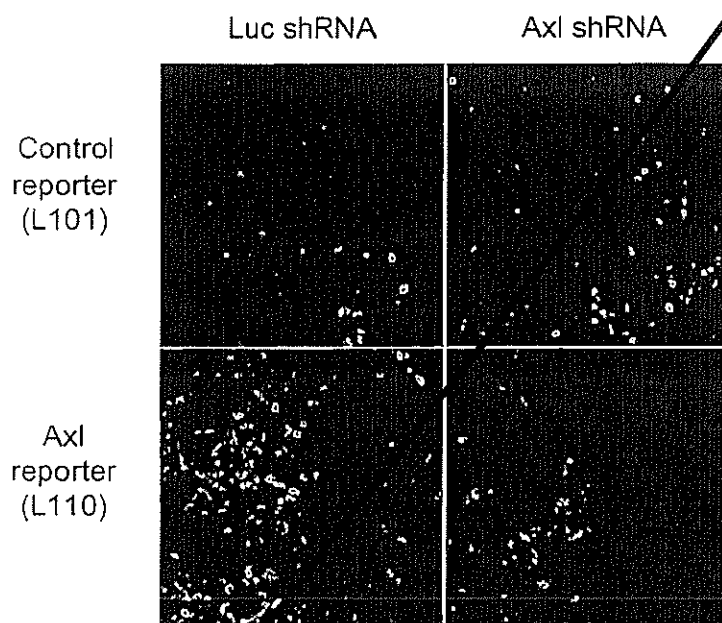
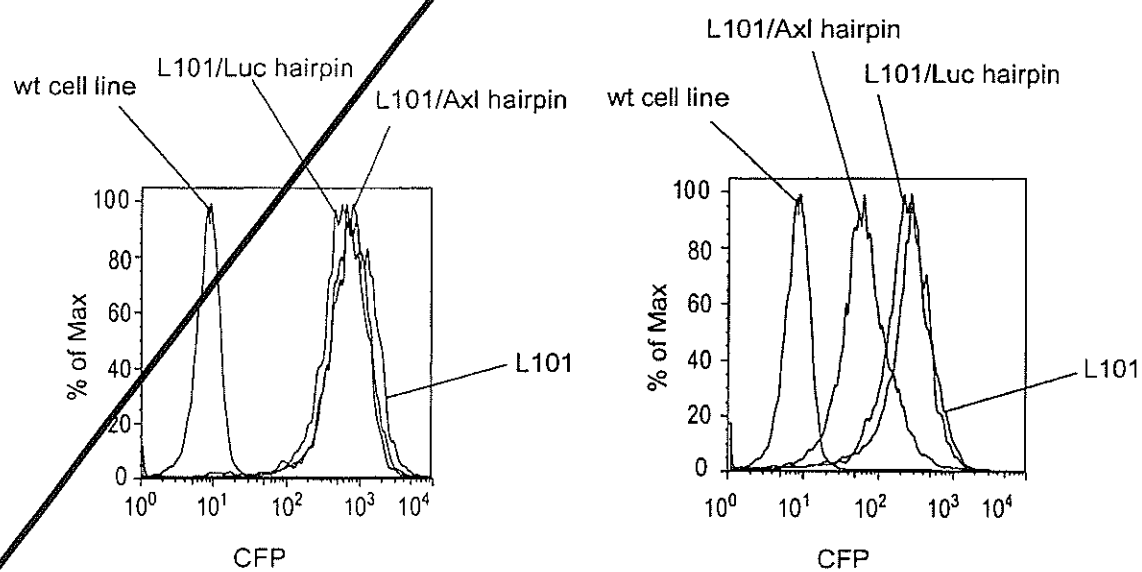


Fig. 25B

23/35

Fig. 26A

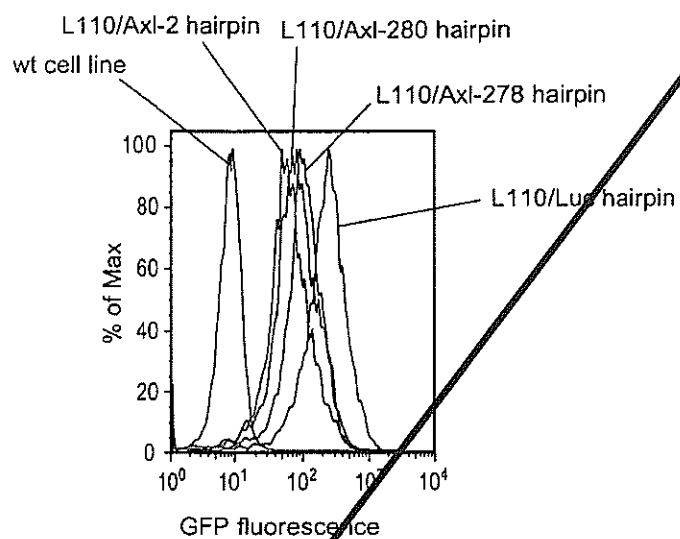


Fig. 26B

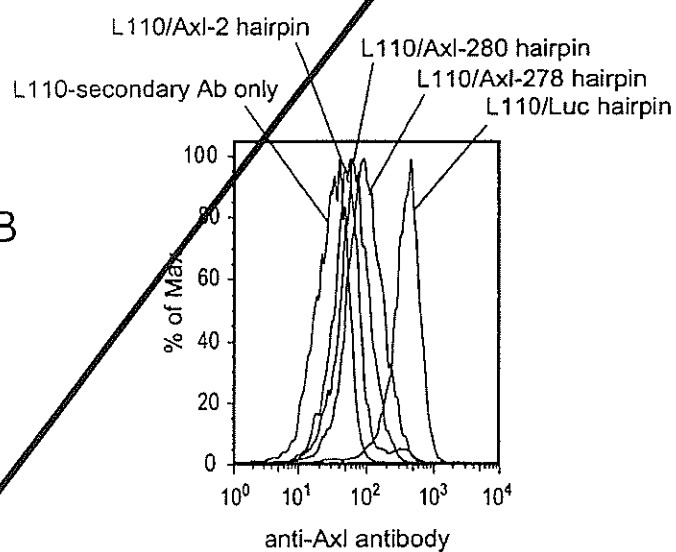
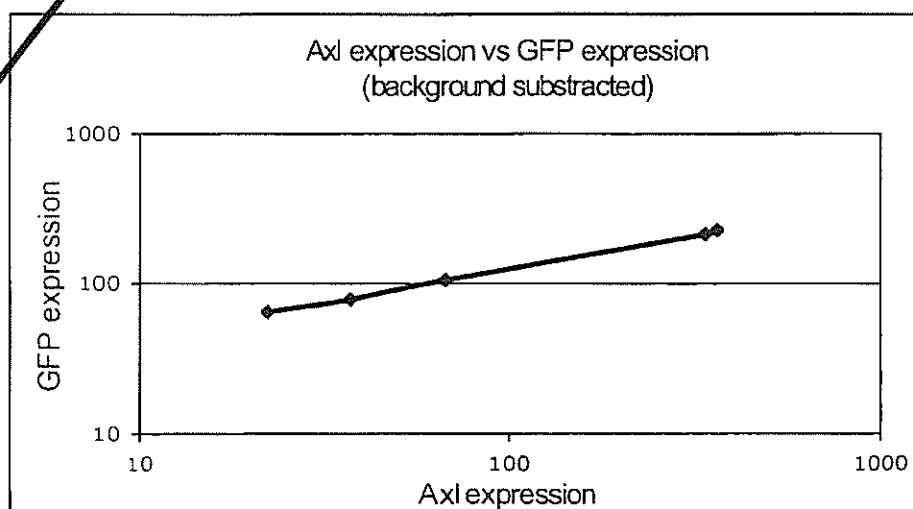


Fig. 26C



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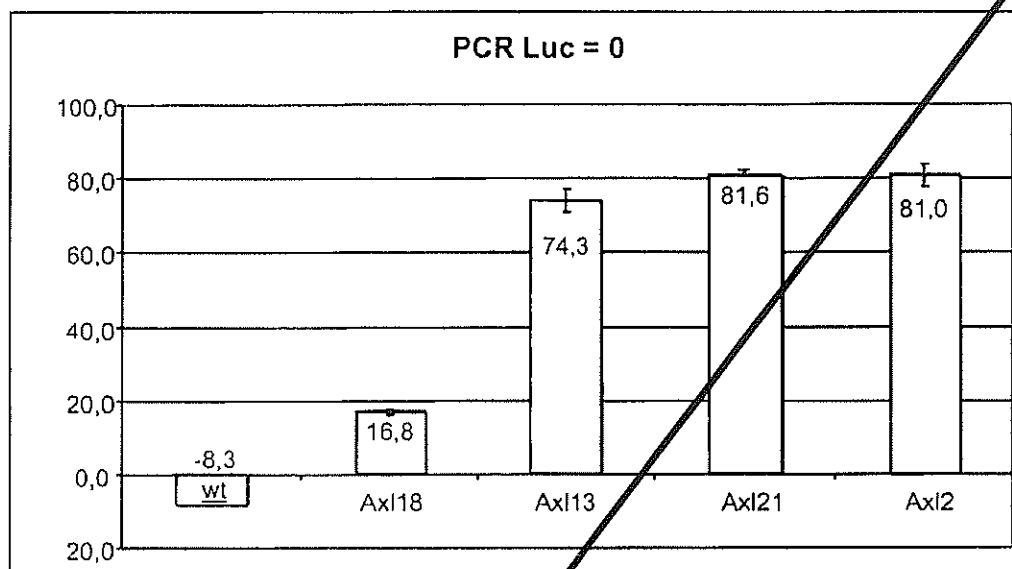


Fig. 27A

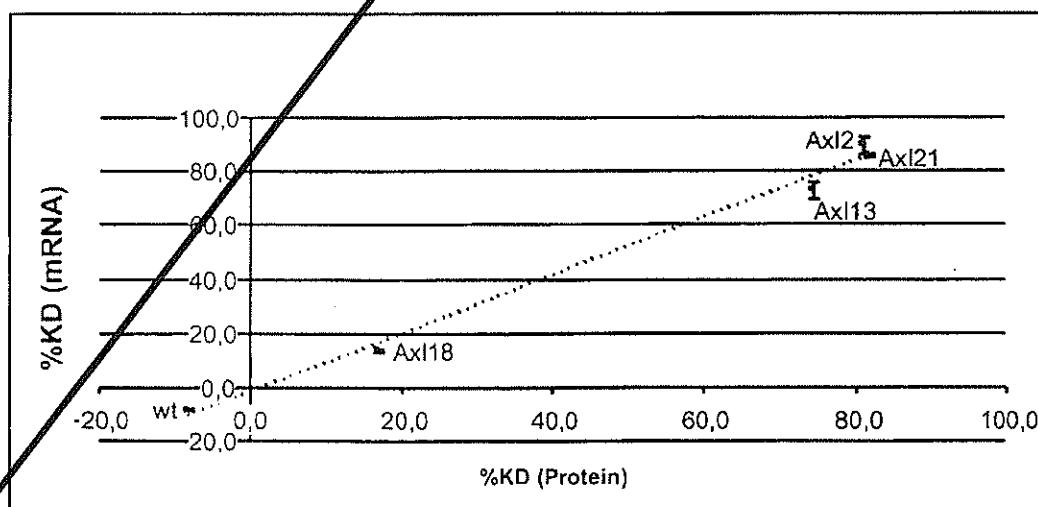


Fig. 27B

25/35

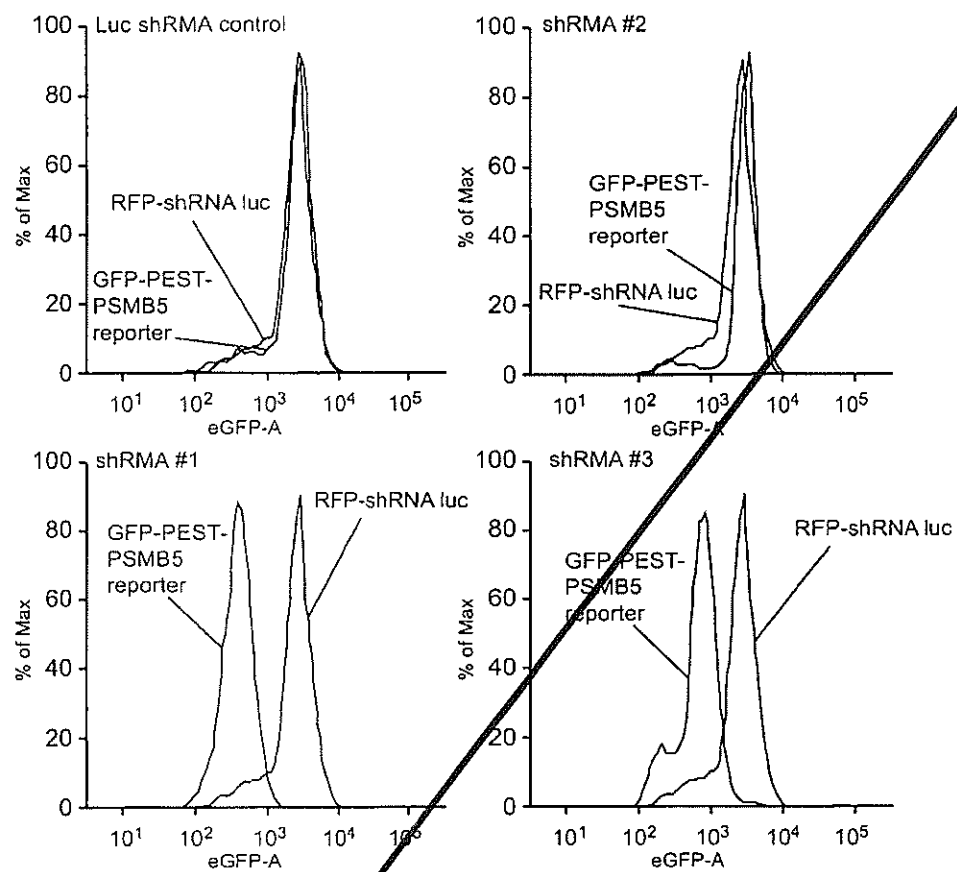


Fig. 28A

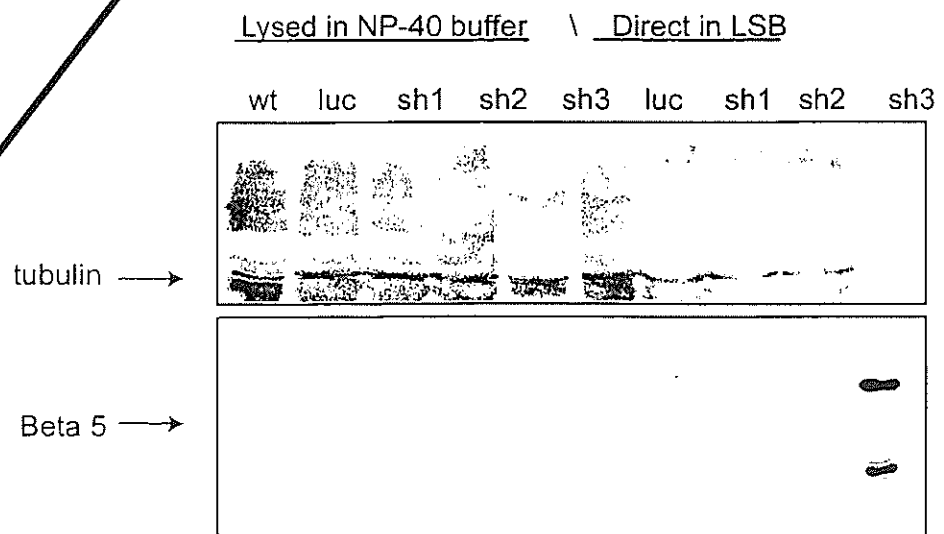


Fig. 28B

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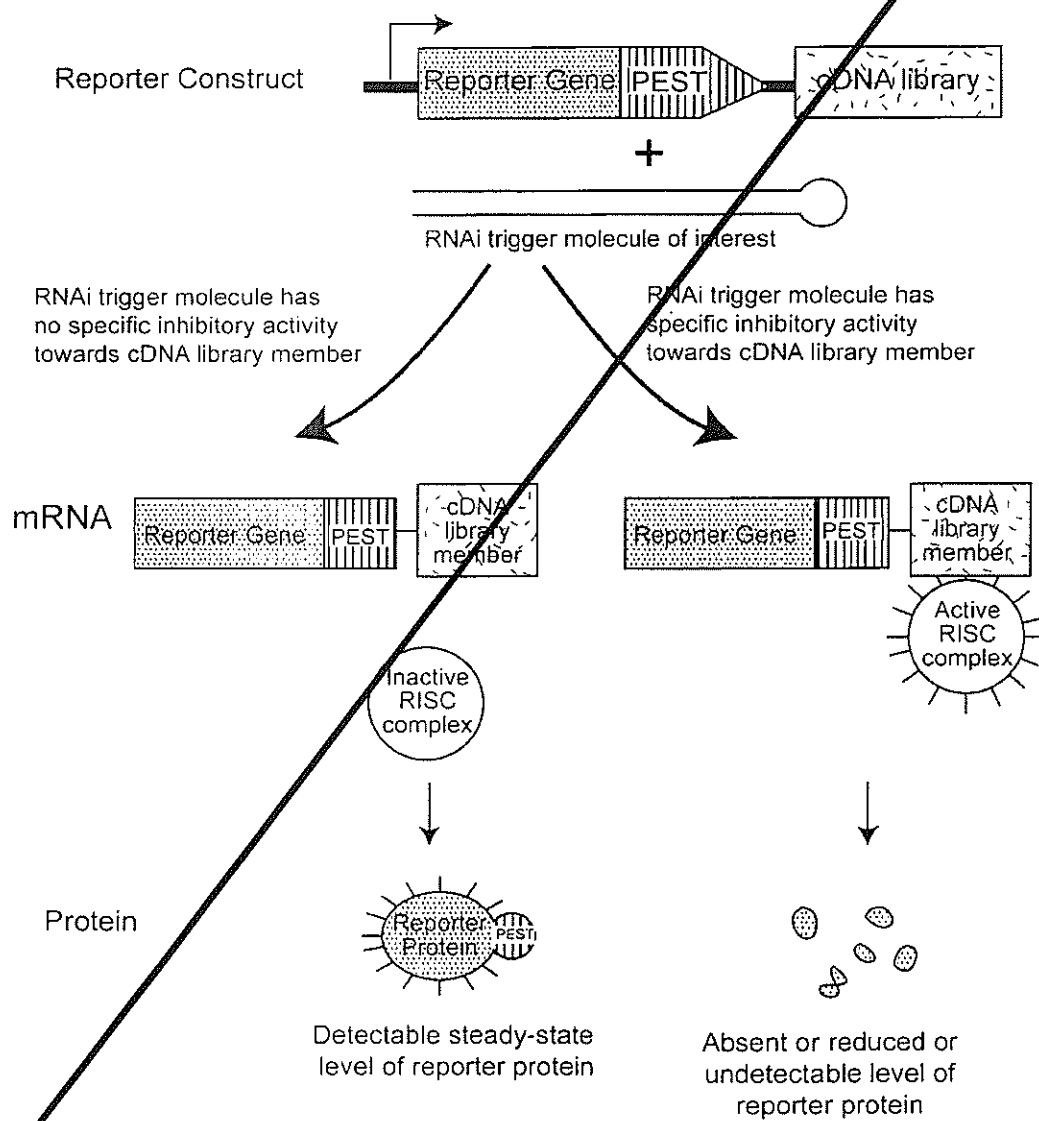


Fig. 29

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Fig. 30

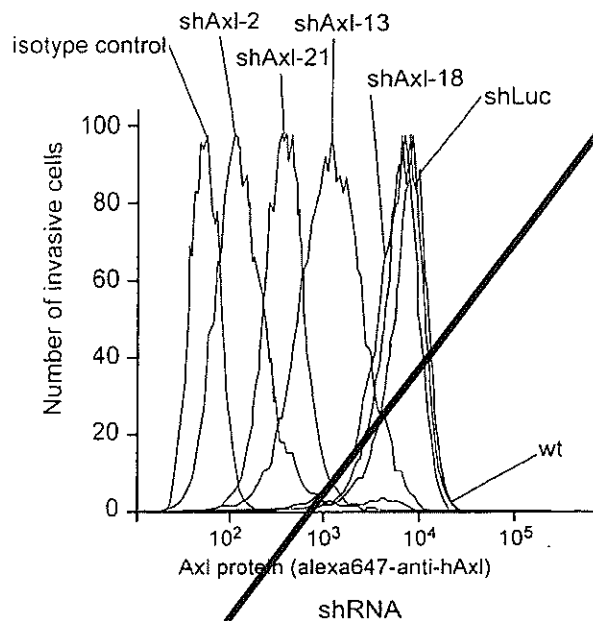


Fig. 31

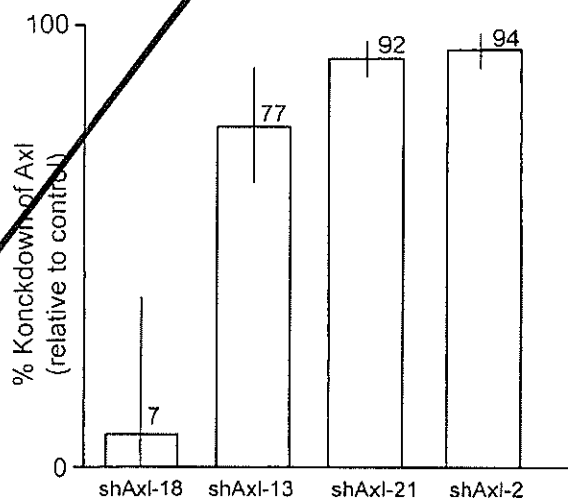
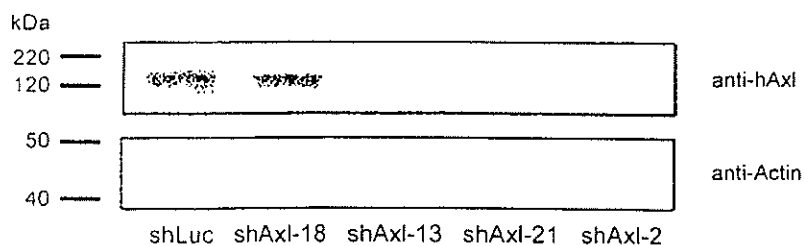
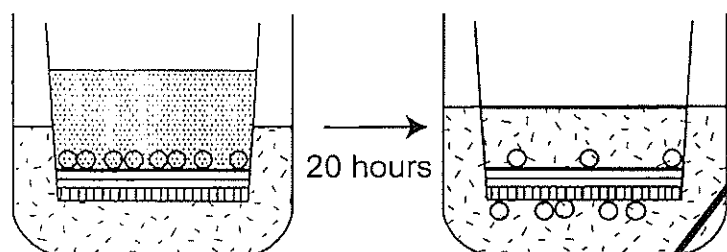


Fig. 32



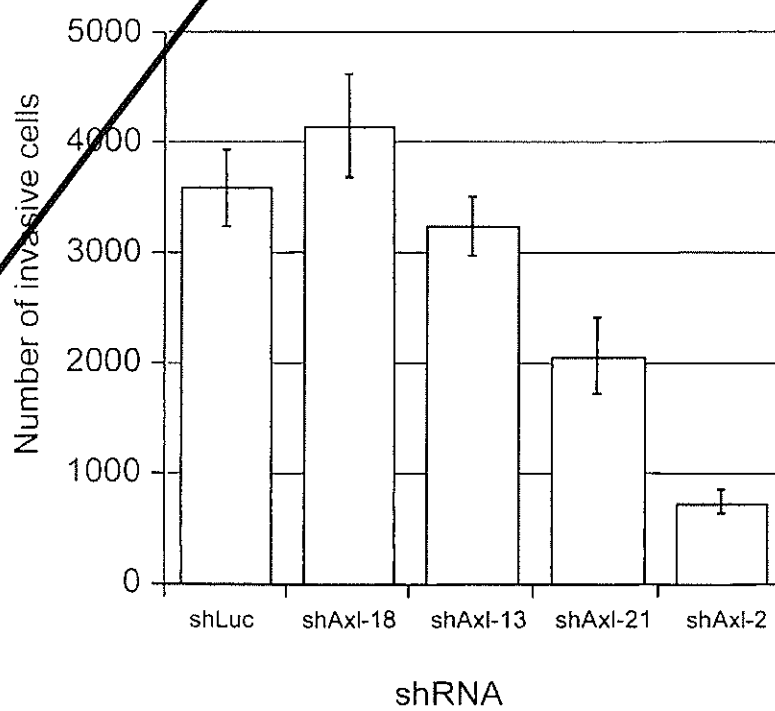
28/35

Fig. 33



- Medium with FBS
- ▨ Growth Factor Reduced Matrigel™ Matrix
- ▧ Membrane with 8 mm pores
- ▩ Serum-free medium
- Cells

Fig. 34



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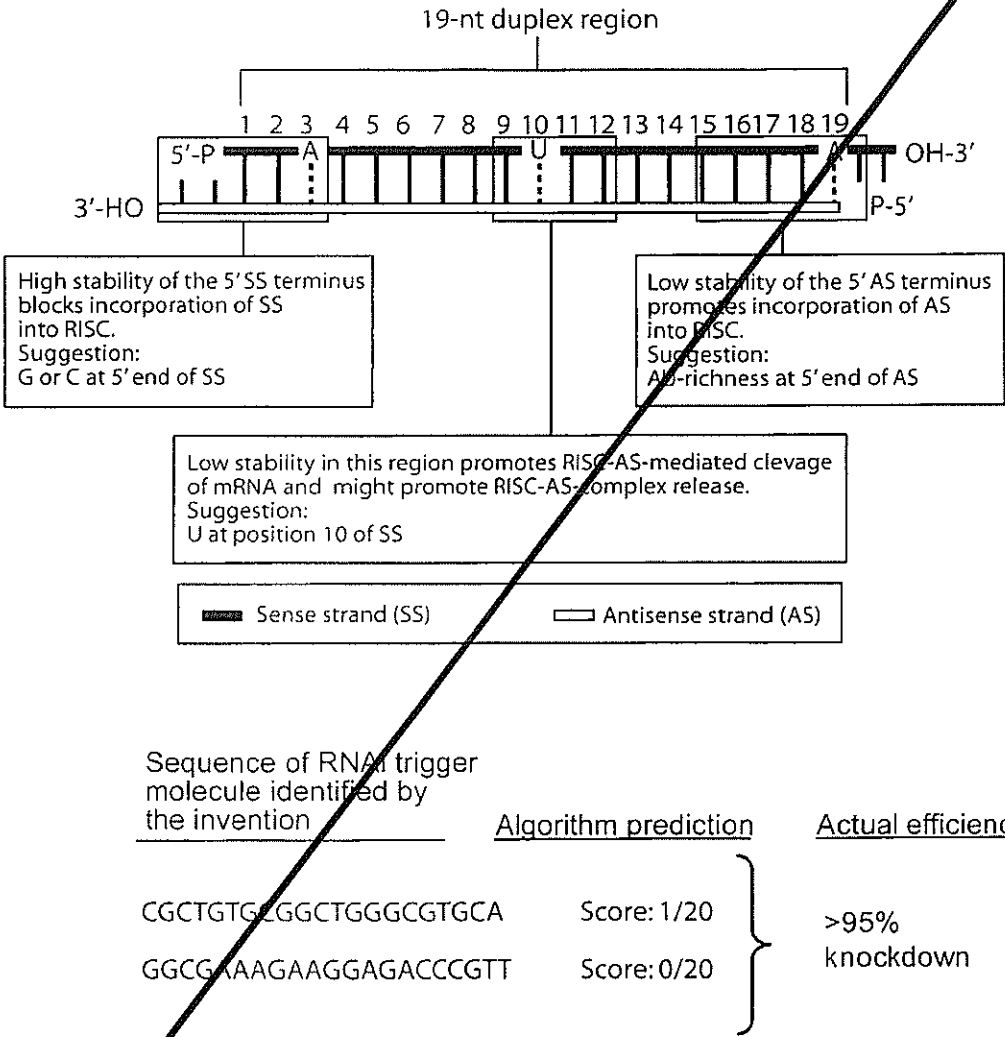


Fig. 35

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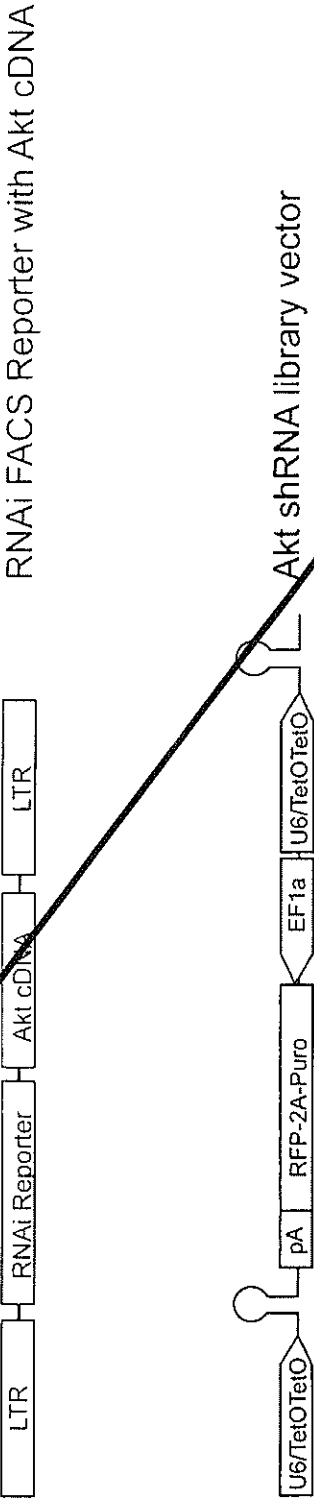


Fig. 36

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Proteasomal subunit beta 5			
shRNA Hairpin Sequence	Corresponding mRNA target sequence	Relative Activity	
gacctctatgtgttaccagtcatttaataatgaCTGGTctcattaatg actggttaacacatagaggtc (SEQ ID NO: 1) Orientation: sense-LOOP-antisense	GACCTCTATGTGTACCAGTCATTAAATGA (SEQ ID NO: 16)	Strong	
gaatctatgagcttcgaaataaaggaaacgcCTGGTCgcgttcctt atttcgaaagctcatagattc (SEQ ID NO: 2) Orientation: sense-LOOP-antisense	GAATCTATGAGCTTCGAAATAAGGAACGC (SEQ ID NO: 17)	Strong	
gatctacatgagaagtatatagtggtctctacCTGGTCgtagagcca ctatacttctcatgtagatc (SEQ ID NO: 3) Orientation: sense-LOOP-antisense	GATCTACATGAGAAGTATAGTGGCTCTAC (SEQ ID NO: 18)	Intermediate	

Fig. 37

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Axl Receptor Tyrosine Kinase			
Name	shRNA Hairpin Sequence	Corresponding mRNA target sequence	Relative Activity
shAxl21	atgcacgccccagccgacacagcgTTGGATCCCTGGTCGGATCCAA cgctgtgcggctggcgatgcac (SEQ ID NO: 4) Orientation: antisense-LOOP-sense	CGCTGTGCGGCTGGGCGTGTCAT (SEQ ID NO: 19)	Strong
shAxl13	acgggtctctccttcttttcgcccgtTTGGATCCCTGGTCGGATCCAA ggcgaaagaaggagaccggt (SEQ ID NO: 5) Orientation: antisense-LOOP-sense	CGGCGAAAGAAGGAGACCCGT (SEQ ID NO: 20)	Intermediate
shAxl18	gcttcaggcgatttccccggcgTTGGATCCCTGGTCGGATCCAA cgccgggggaaatcgccctgaagc (SEQ ID NO: 6) Orientation: antisense-LOOP-sense	CGCCGGGGGAATCGCCCTGAAGC (SEQ ID NO: 21)	Weak

Fig. 37 cont'd

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Akt serine/threonine-specific protein kinase		
shRNA Hairpin Sequence	Corresponding mRNA target sequence	
atcctcatggaggagatccgcttcccgctgctggatccagagatgcgggaagc ggatcctccatgaggat (SEQ ID NO: 7) Orientation: sense-LOOP-antisense	ATCCTCATGGAGGAGATCCGCTTCCCCGC (SEQ ID NO: 22)	
cccagccccaccagatccactgcacggctgctggatccagagatgccgtgcag tggactggtggggctggg (SEQ ID NO: 8) Orientation: antisense-LOOP-sense	GCCGTGCAGTGGACTGCTGGGGGCTGGG (SEQ ID NO: 23)	
gtcacgcggtgcttggccttggtgctggatccagagatgccaaagcccaagca ccgcgtgac (SEQ ID NO: 9) Orientation: antisense-LOOP-sense	GCCAAGCCCCAAGCACCGCGTGAC (SEQ ID NO: 24)	
cttcattgtagtagcggctgctggatccagagatccgctactactacgccatga ag (SEQ ID NO: 10) Orientation: antisense-LOOP-sense	CCGCTACTACGCCCATGAAG (SEQ ID NO: 25)	
gtggccacacactcaccgagaaaccgctgctggatccagagatggttctcgg gagtgtgtggggccac (SEQ ID NO: 11) Orientation: sense-LOOP-antisense	GTGGCCACACACTCACCCGAGAACCGC (SEQ ID NO: 26)	
ccttttgcggcacacctgagtacctggctgctggatccagagatgccaggtag tcagggtgtgccgcaaaagg (SEQ ID NO: 12) Orientation: sense-LOOP-antisense	CCTTTTGGGGCACACCTGAGTACCTGGC (SEQ ID NO: 27)	

Fig. 37 cont'd

Akt serine/threonine-specific protein kinase		
shRNA Hairpin Sequence	Corresponding mRNA target sequence	
ccatgaacgagtttgagtacacctgaagcTGCTGGATCCAGAGATctttcaggtac tcaactcgttcacatgg (SEQ ID NO: 13) Orientation: sense-LOOP-antisense	CCATGAACGAGTTTGAGTACCTGAAGC (SEQ ID NO: 28)	
aagatcctcaagaaggaagtcacgtgctgctggATCCAGAGATgcccacgat gacttccttttgaggatcctt (SEQ ID NO: 14) Orientation: sense-LOOP-antisense	AAGATCCTCAAGAAGGAAGTCATCGTGGC (SEQ ID NO: 29)	
cctgcactcggagaagaacgtggtgtCTGCTGGATCCAGAGATacaccacgtt cttctccgagtgacagg (SEQ ID NO: 15) Orientation: sense-LOOP-antisense	CCTGCACCTCGGAGAAGAACGTGGTGT (SEQ ID NO: 30)	

Fig. 37 cont'd

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Subcutaneous growth of MB-MDA-231/CSI-Axl shRNA epi-allelic
breast carcinoma cell series in NOD/SCID mice

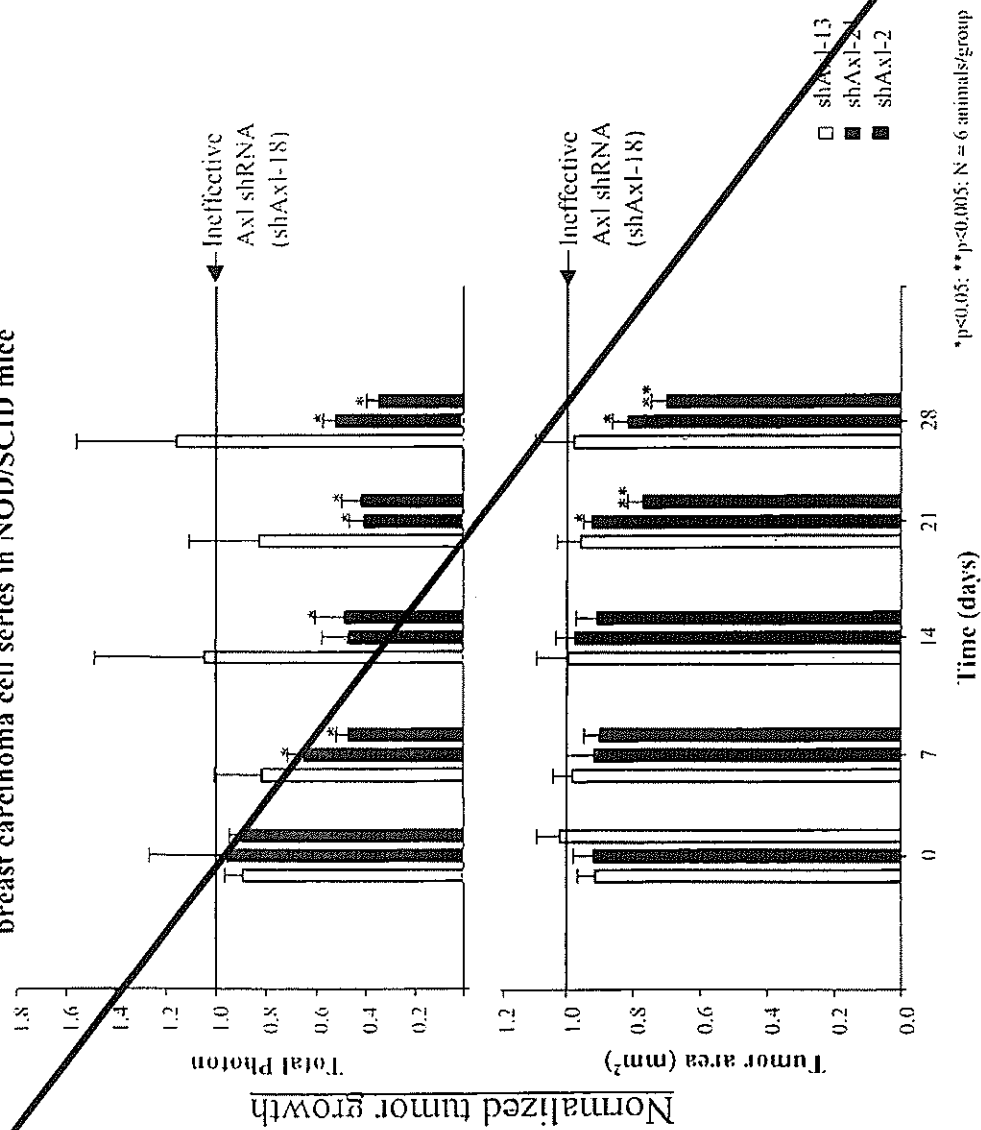


Fig. 38

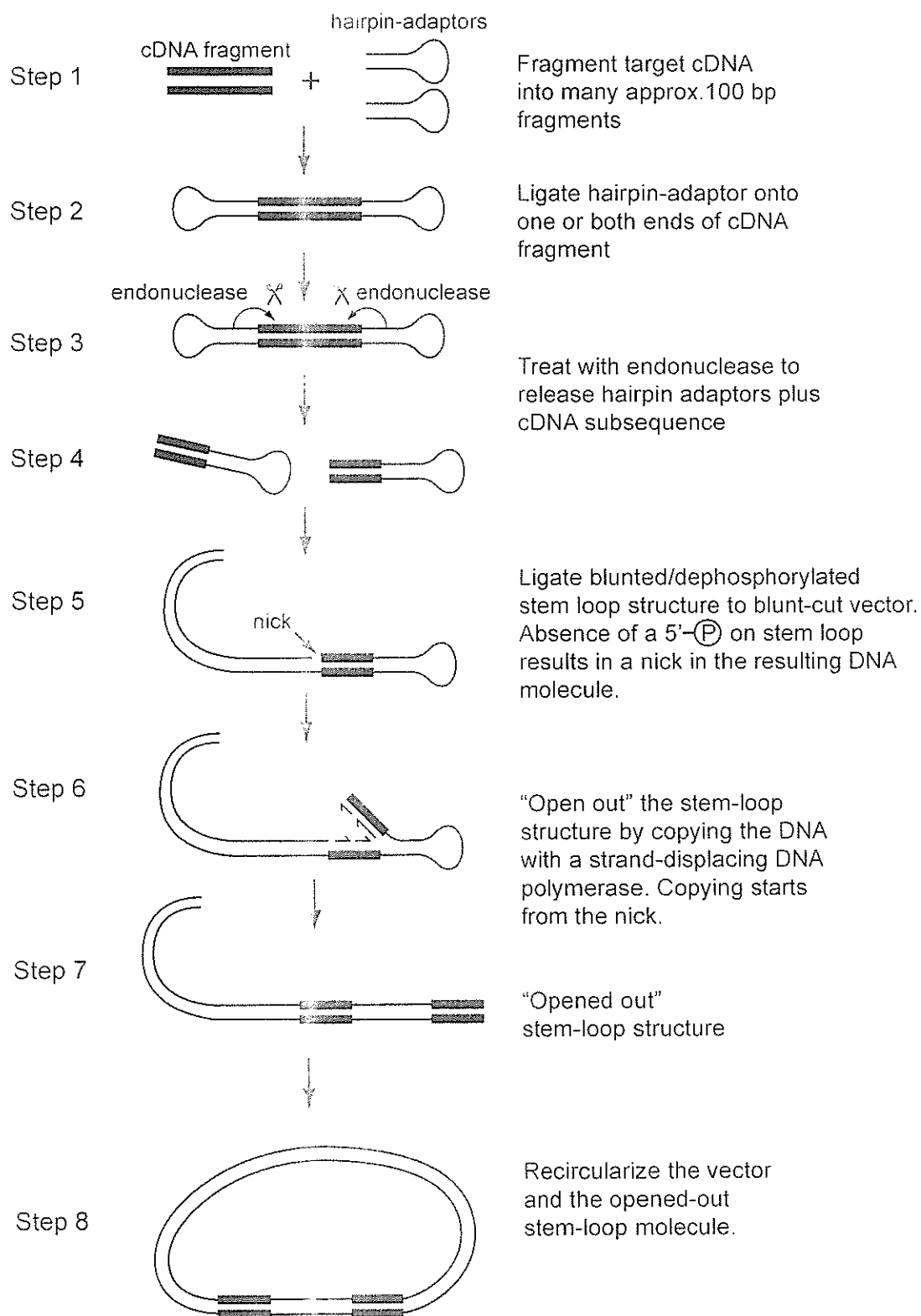


Fig. 1

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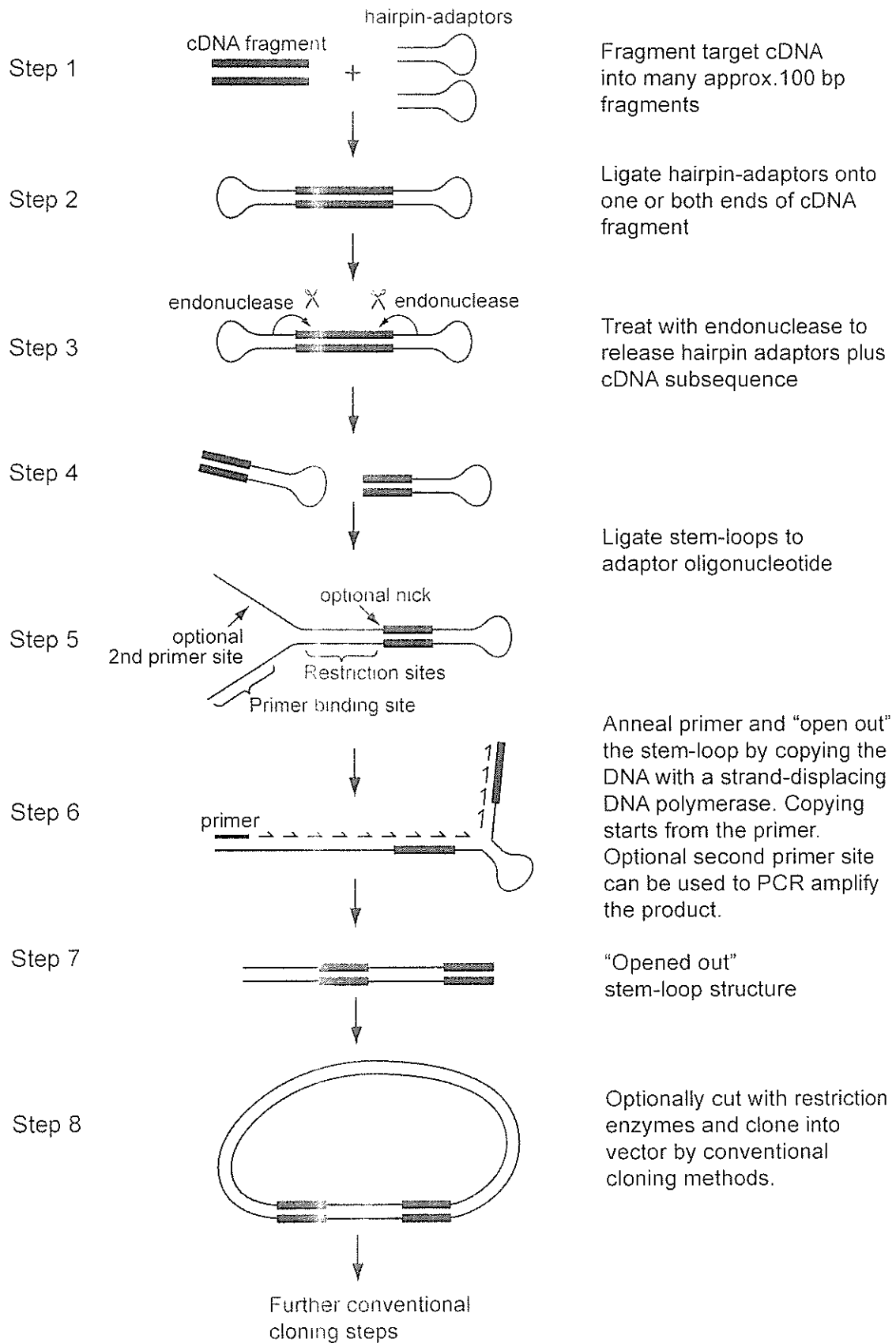


Fig. 2

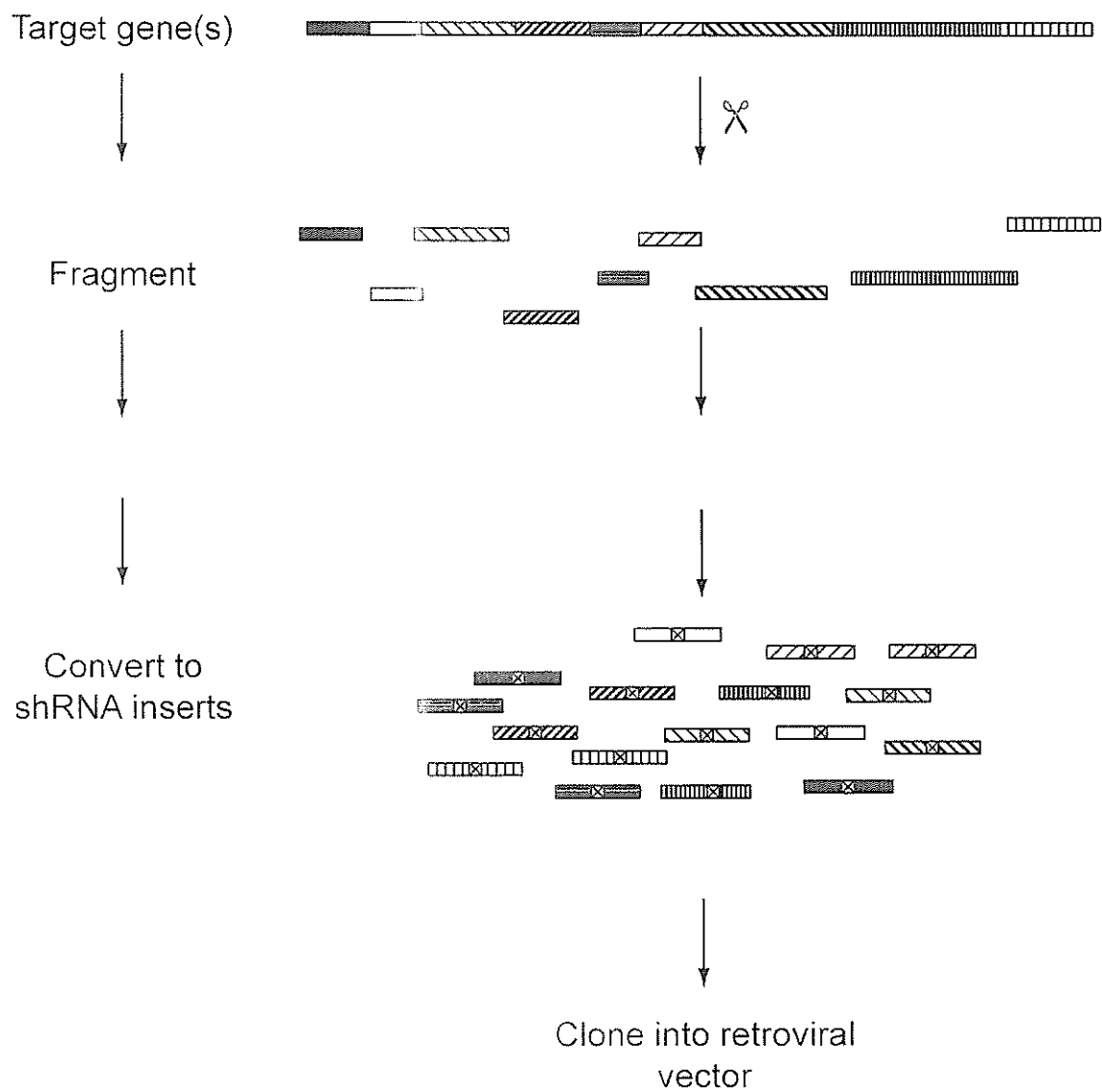
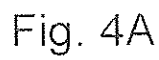


Fig. 3

A) Ligate on cloning adaptor loop
B) Anneal primer and extend (or extend from a nick between vector and stem-loop) Bst DNA Polymerase can efficiently copy the whole loop because it can displace the base-paired DNA. No amplification avoids bias.



Generating shRNA expression cassette from
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- C) Gel purify.
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- E) If necessary, subclone the expression cassette into an appropriate vector, e.g., a retroviral expression vector.
- F) Transcribe the expression cassette in a host cell to produce a candidate shRNA.

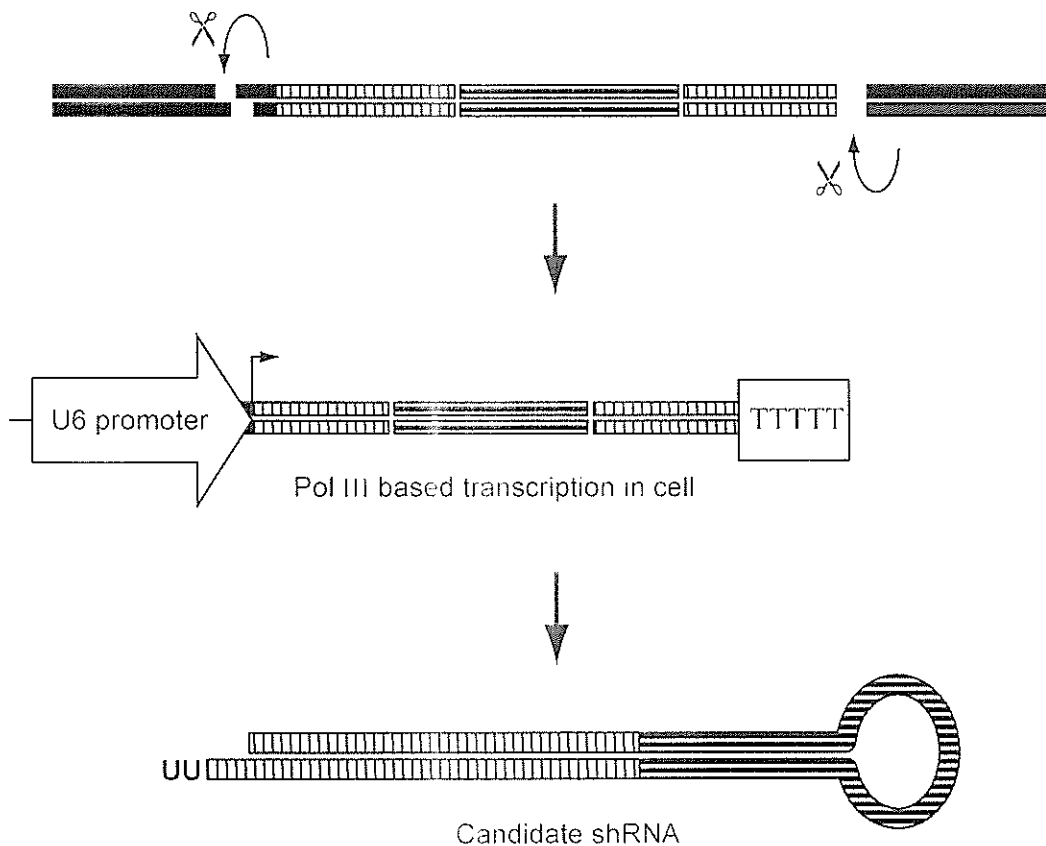


Fig. 4B

Fig. 5A

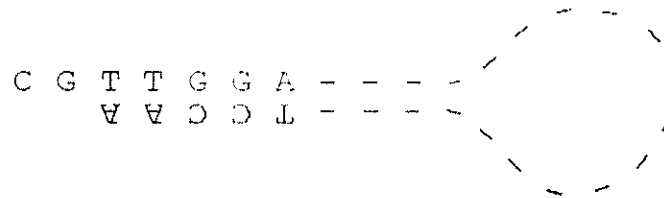


Fig. 5B

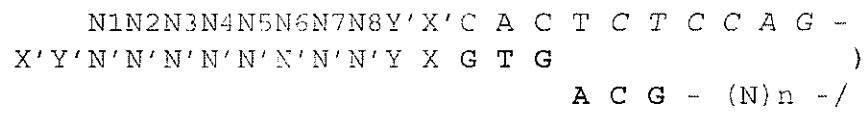
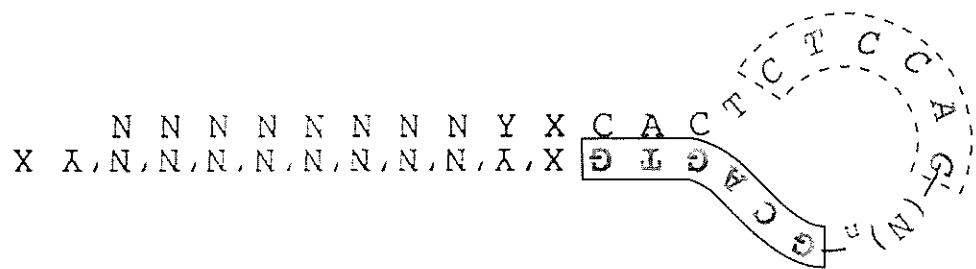


Fig. 5C



Use of restriction enzymes that cut outside their recognition sequence to seamlessly juxtapose sequence elements

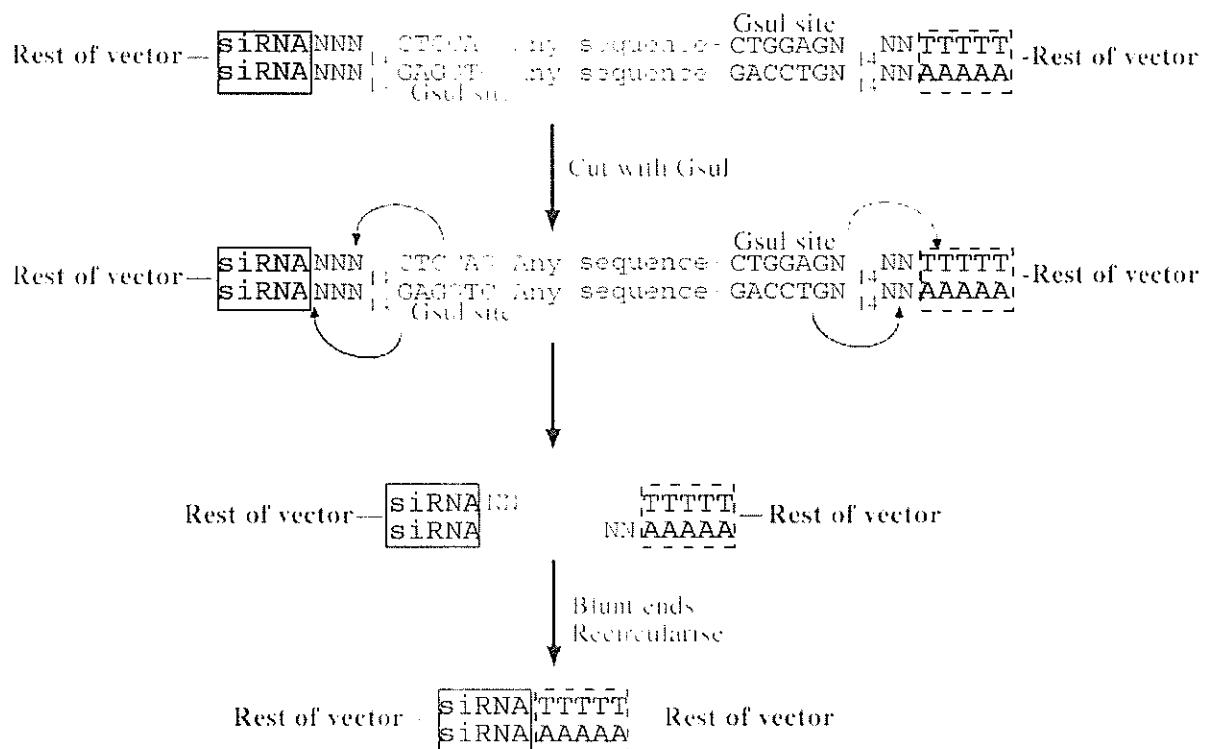


Fig. 6

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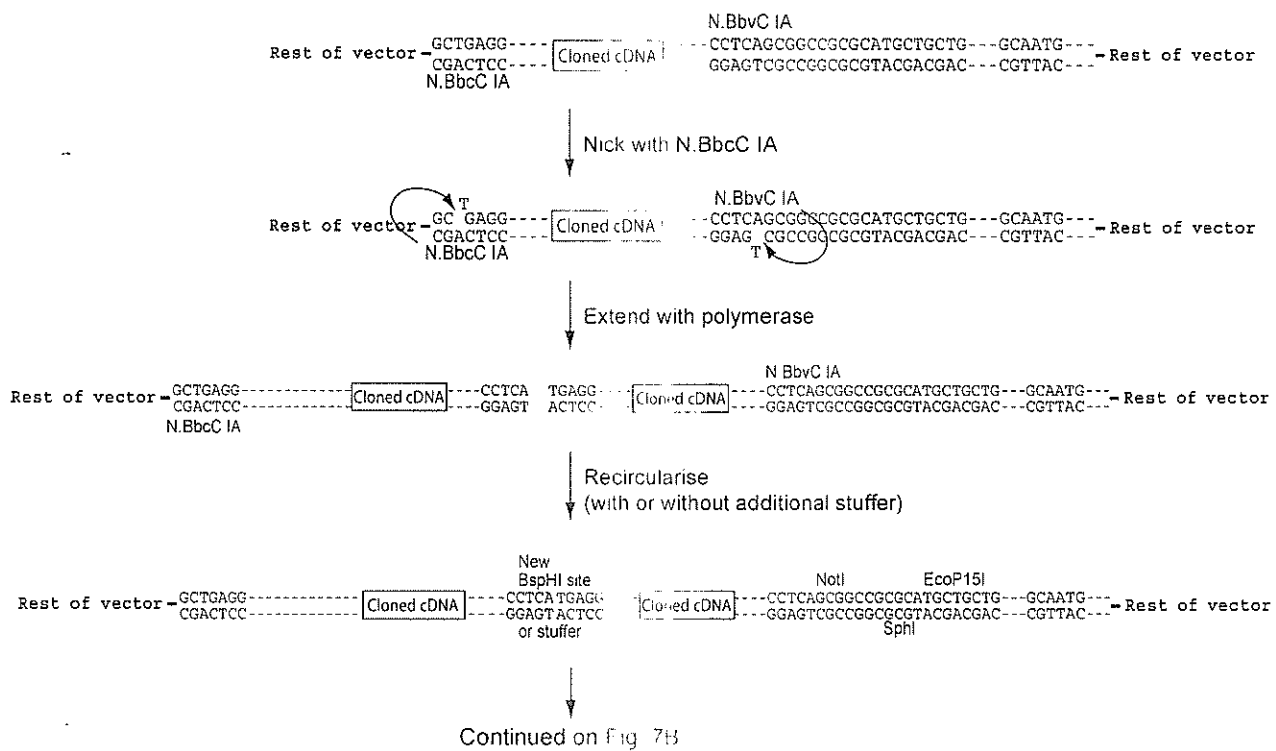


Fig 7A

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Continued from Fig 7A

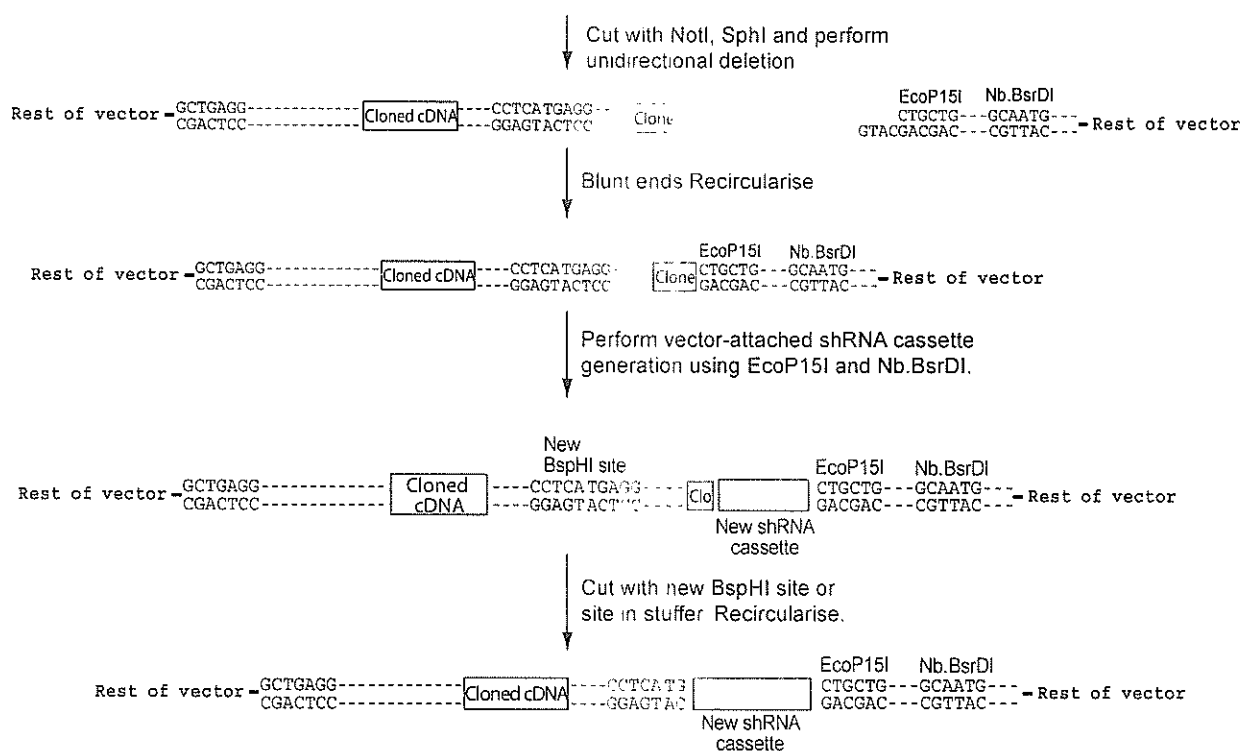


Fig 7B

11/36

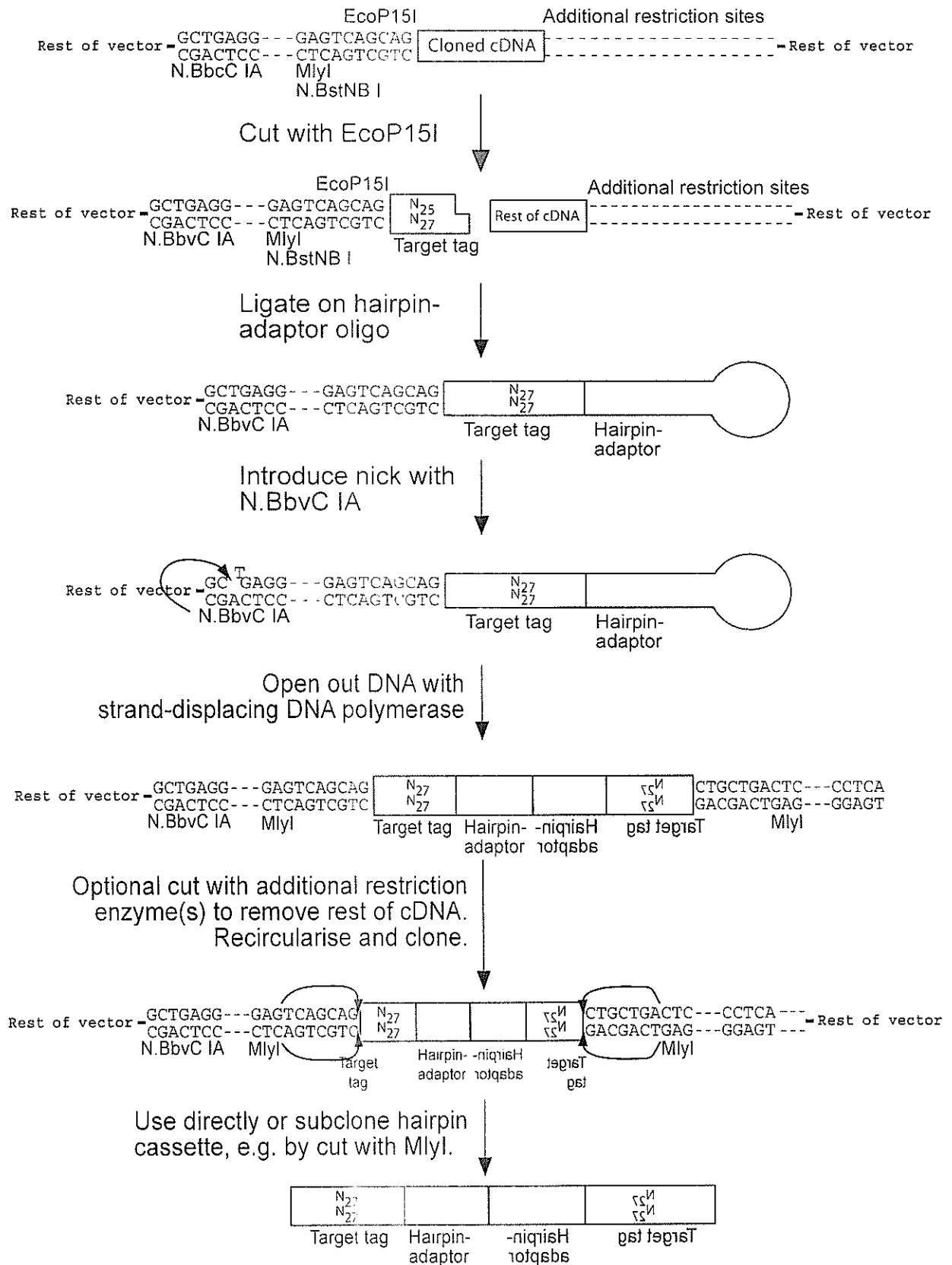


Fig. 8

Fig. 9

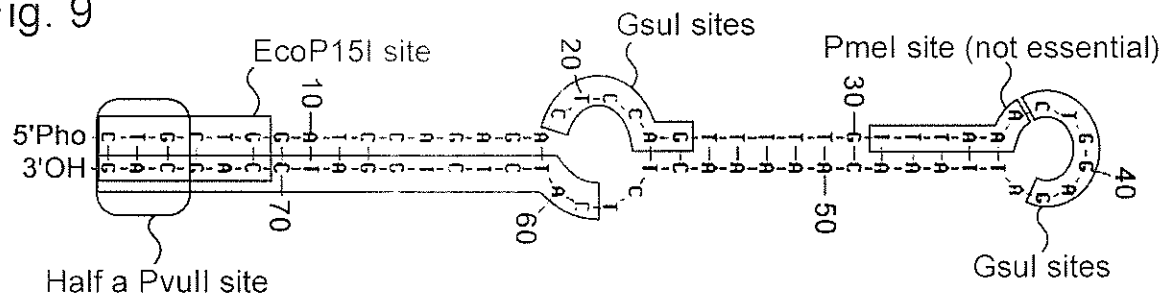


Fig. 10

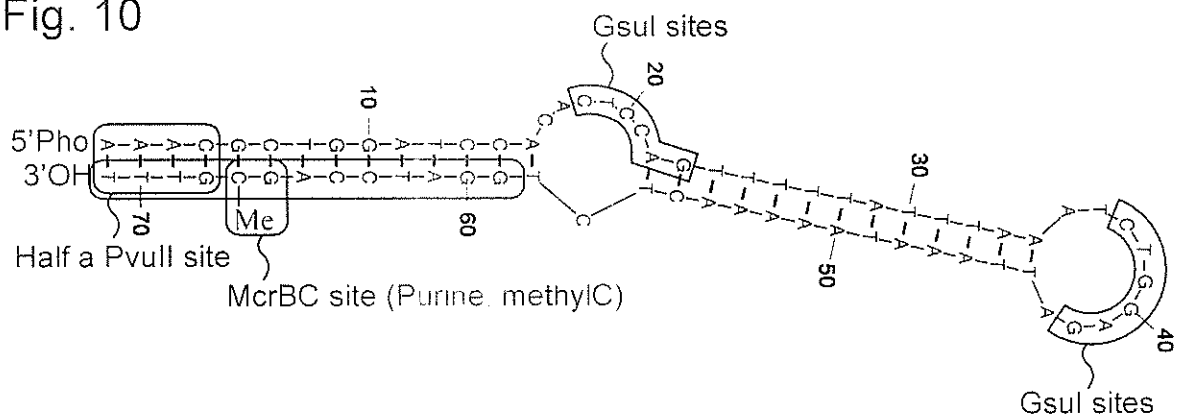
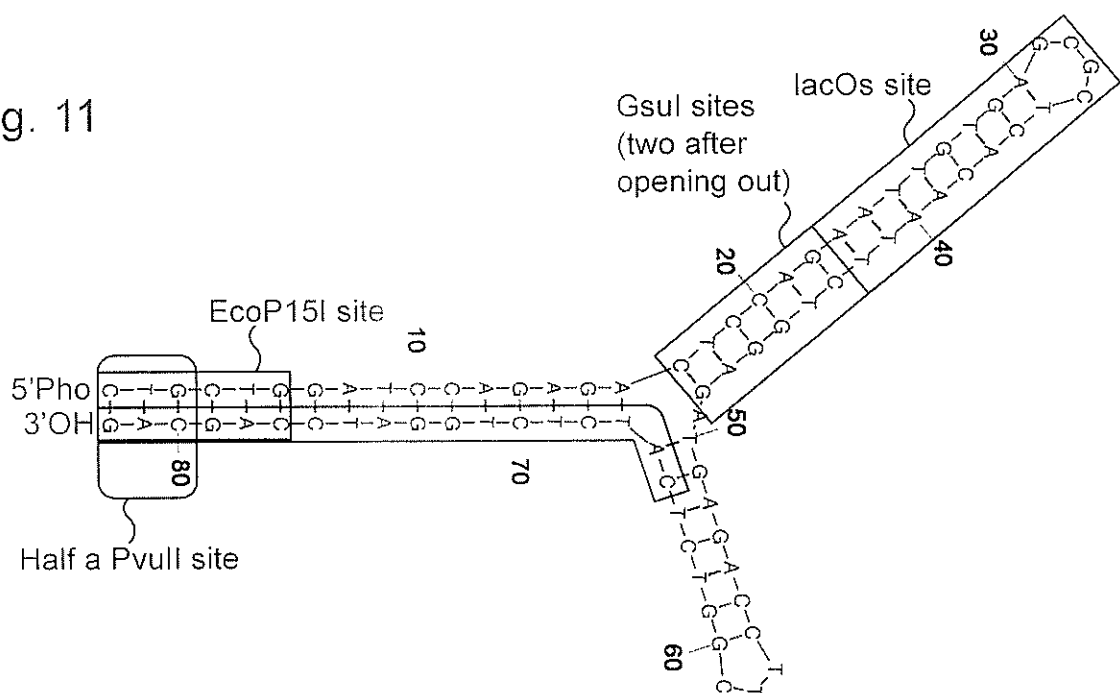


Fig. 11



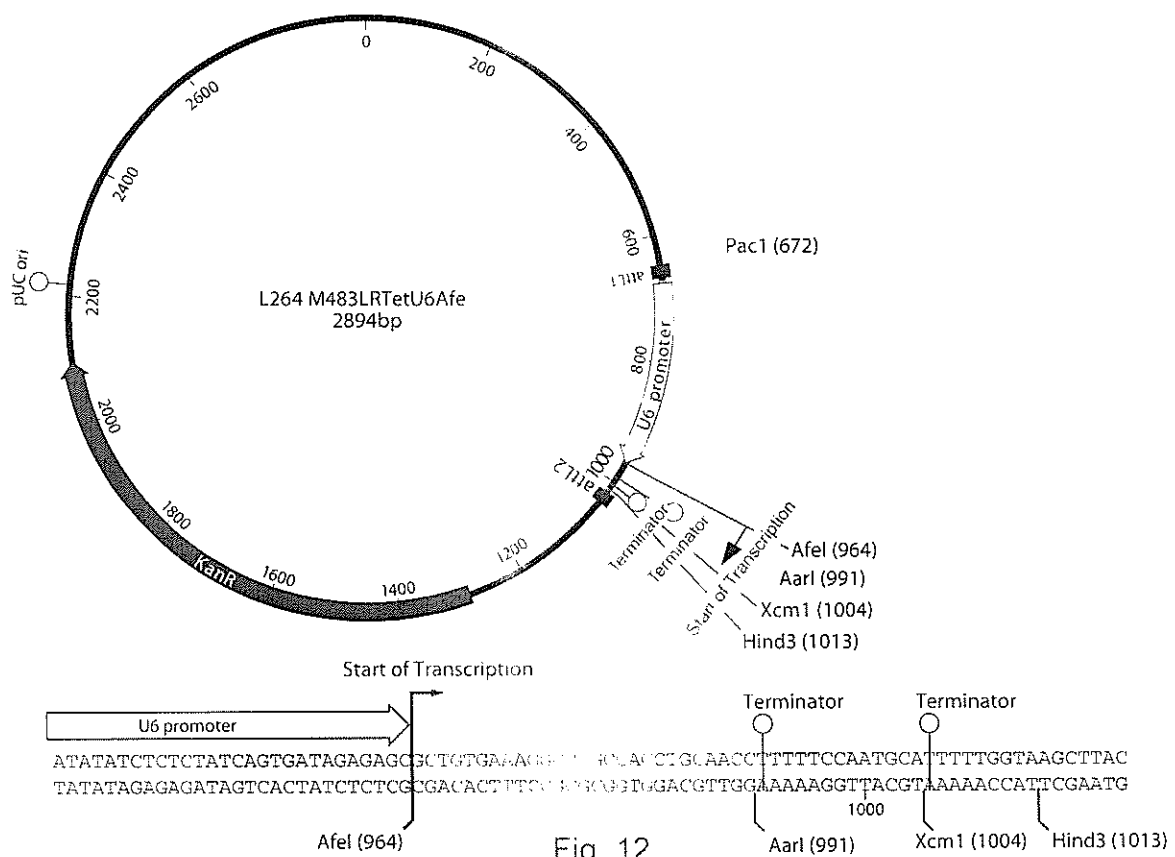
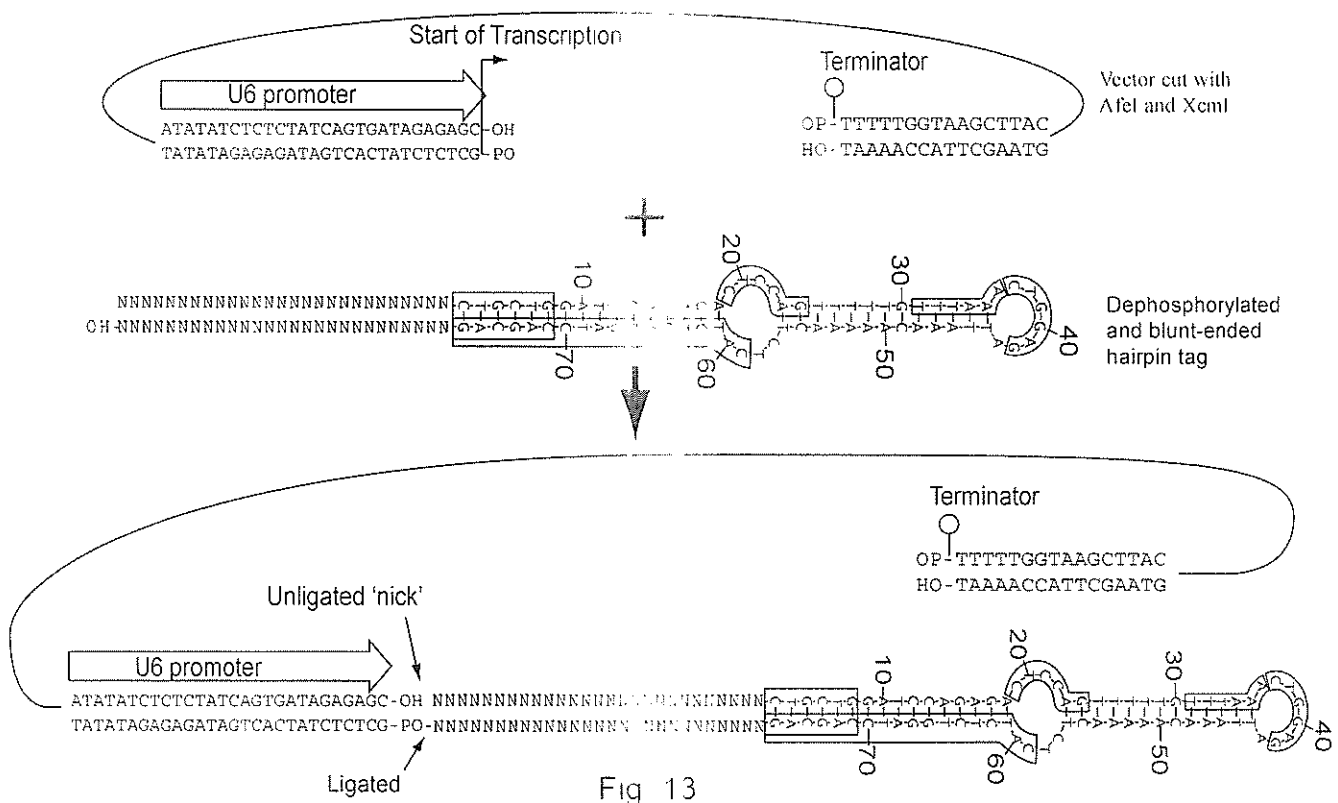


Fig. 12



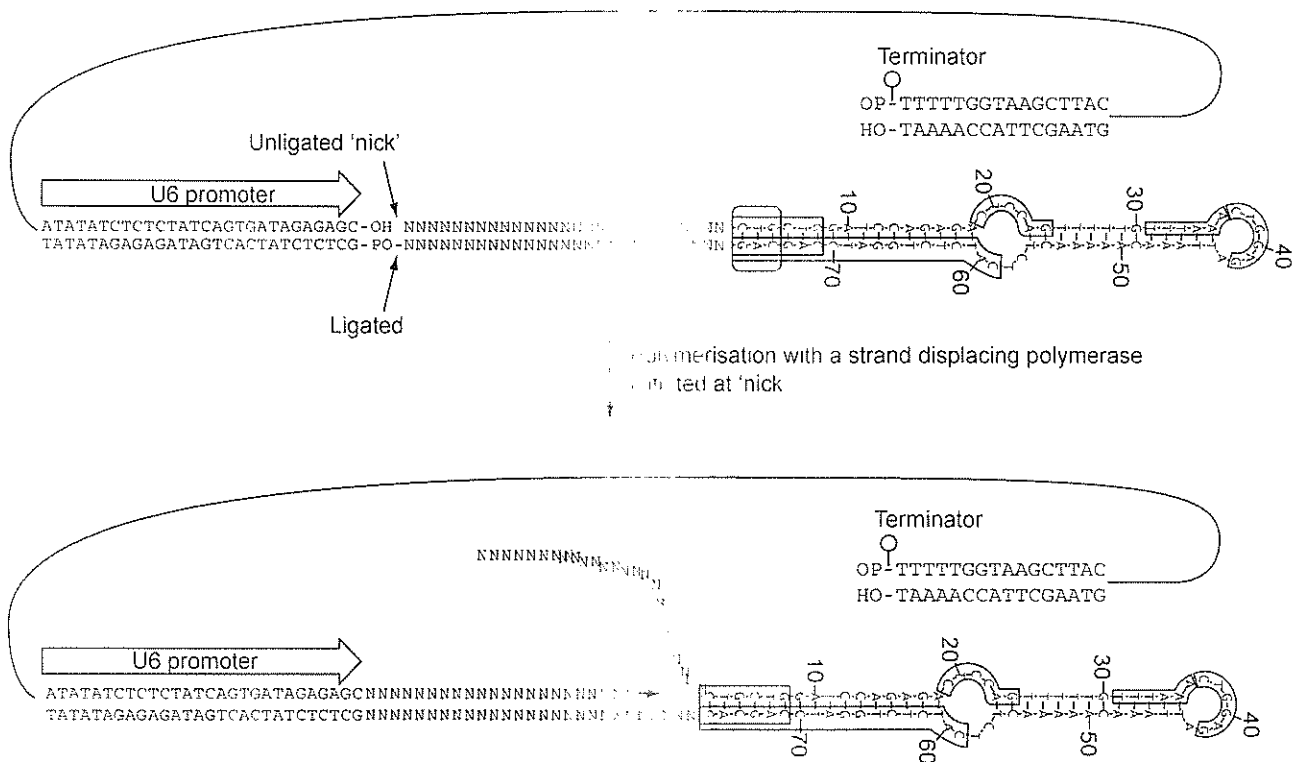


Fig 14

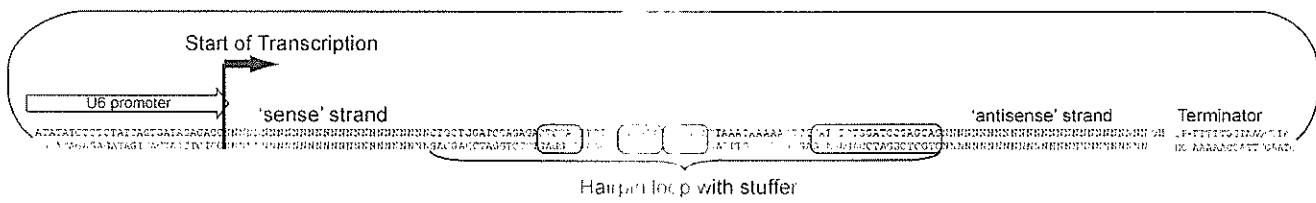


Fig. 15

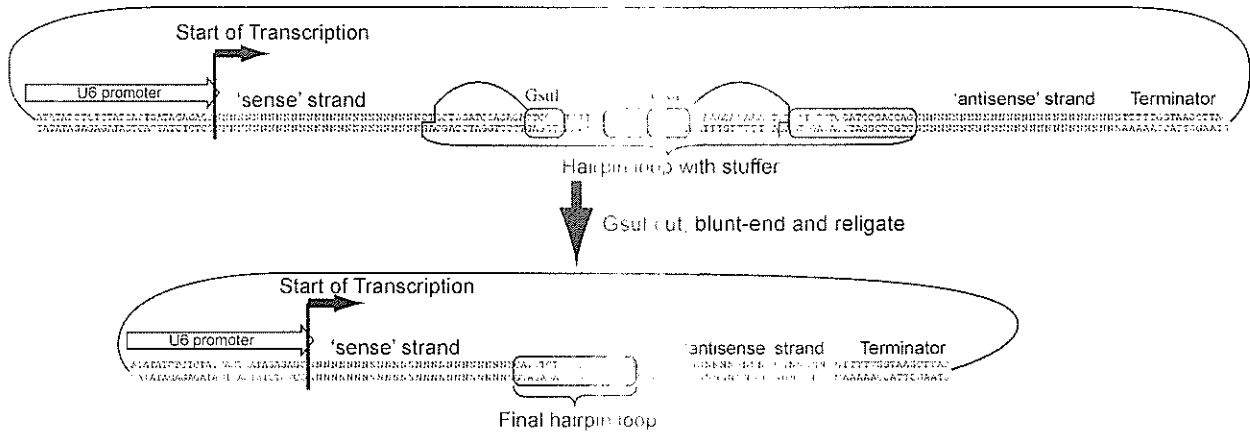


Fig. 16

17/36

Fig. 17

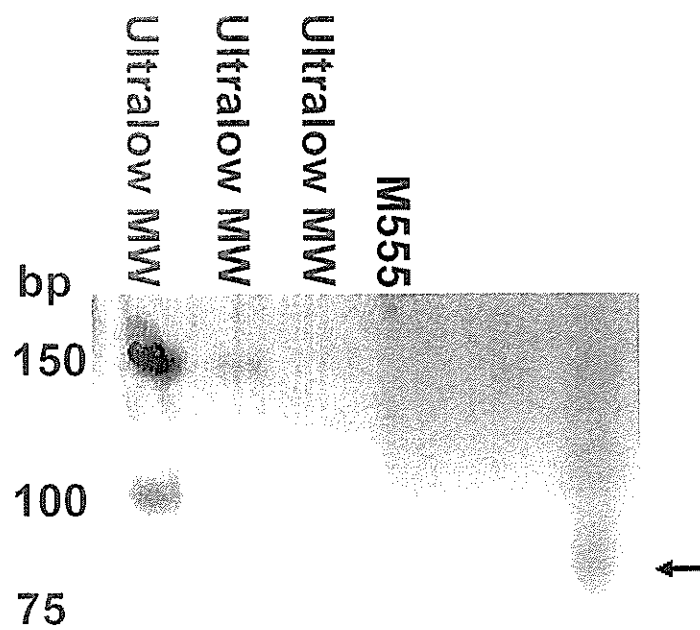
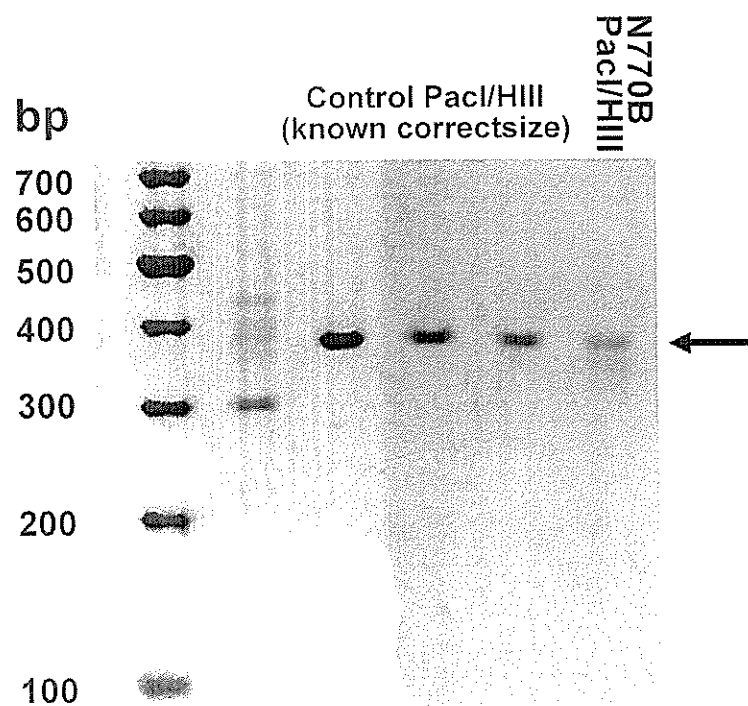


Fig. 18



N783
PacI/HIII
100bp
ladder

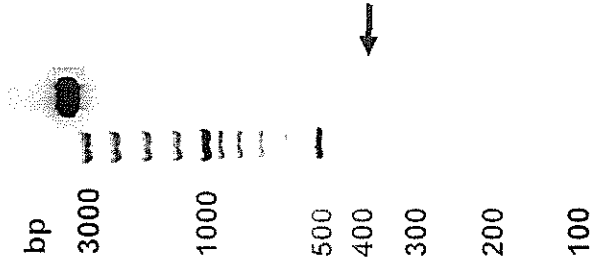


Fig. 19

TCAGTGATGTAGAAGAAGAGGAAG	CTGCTGGATCCAGAGATG	CTTCCTCTCTTCTTCTACATCACTGA	GG	TTTTT	Matches	KDR	NM002253.1	3251-3274
TTCACTCACTTCCATAATCGTCAGT	CTGCTGGATCCAGAGATG	ACTGACGATTATGGAAGTCACTGAA	AG	TTTTT	Matches	KDR	NM002253.1	1473-1449
ATTATATGTCATGTTCAGATTACAG A	CTGCTGGATCCAGAGATG	CTGTAATCTTGAACATAGACATAAAT		TTTTT	Matches	KDR	NM002253.1	643-669
TTATGATGCCAGCAAAATGGGAATT	CTGCTGGATCCAGAGATG	AATTCCTCATTTGCTGGCATCATAA	GG	TTTTT	Matches	KDR	NM002253.1	2766-2789
TCAGTGATGTAGAAGAAGAGGAAG	CTGCTGGATCCAGAGATG	CTTCCTCTCTTCTTCTACATCACTGA	GG	TTTTT	Matches	KDR	NM002253.1	3251-3274
CCGCCTCGGCCACCCAGCTTTCTTGT	CTGCTGGATCCAGAGAT	ACAAGAAGCTGGTGGCCGAGGGCG	C	TTTTT	Matches	vector	L304	
TCCACGCAGAGCCACAGGGCGACGG	CTGCTGGATCCAGAGAT	CCGTGCGCCCTGNTGGCTCTGCGTGGA	GA	TTTTT	Matches	KDR	NM002253.1	350-326
TCCCTCAGTGATGTAGAAGAAGAGGAAG	CTGCTGGATCCAGAGAT	CTTCCTCTCTTCTTCTACATCACTGAGGGA		TTTTT	Matches	KDR	NM002253.1	3247-3274

Fig. 20

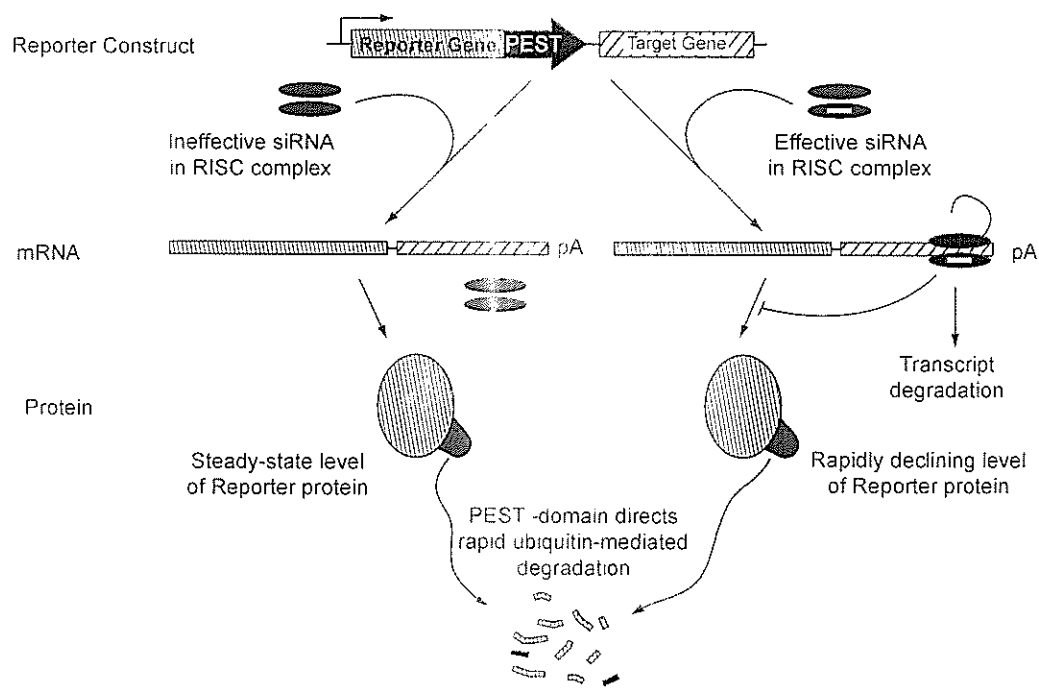


Fig. 21

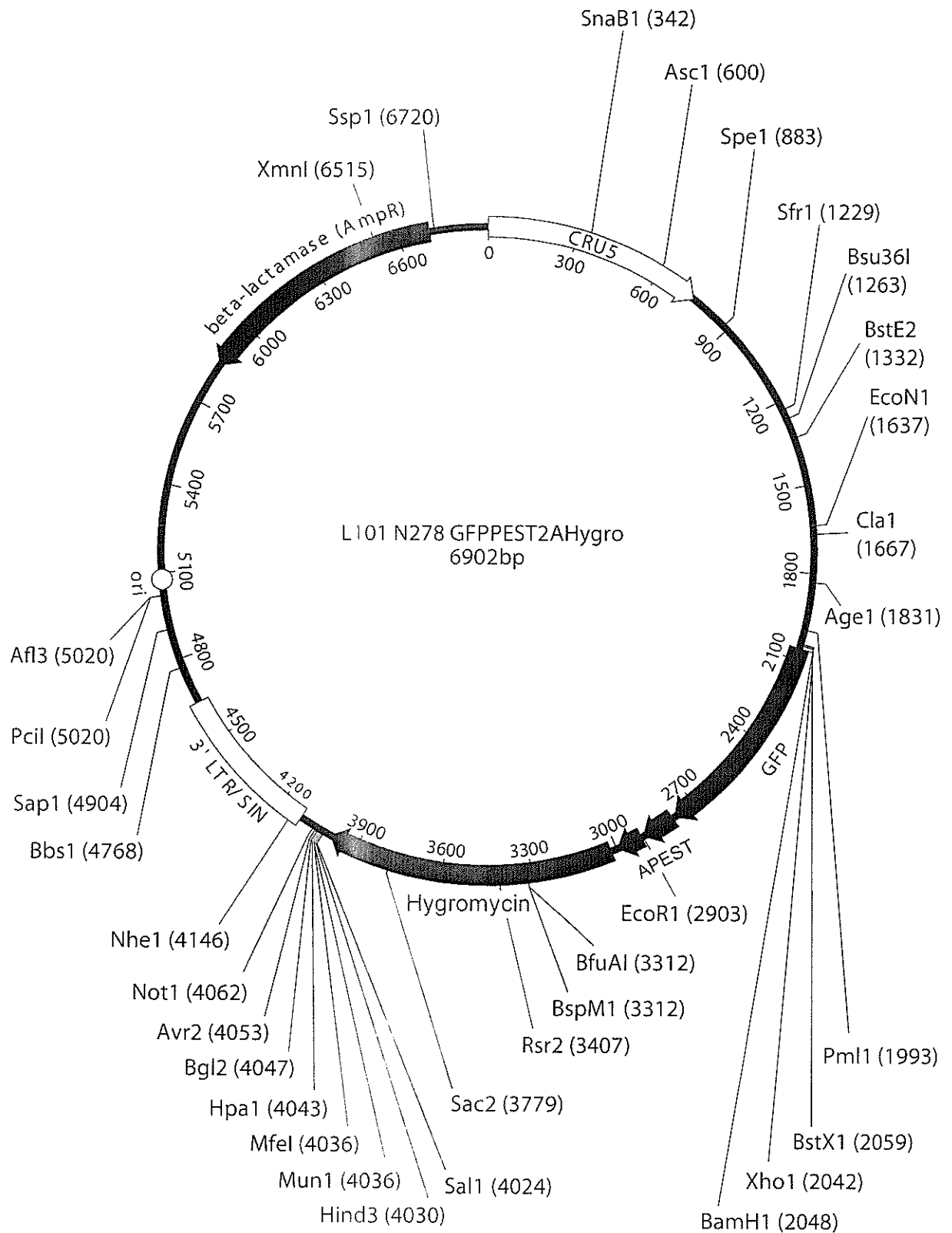
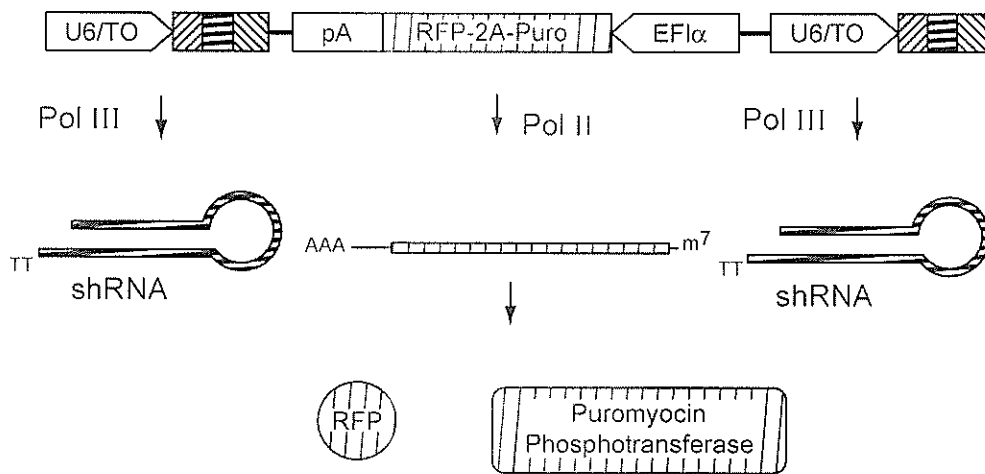
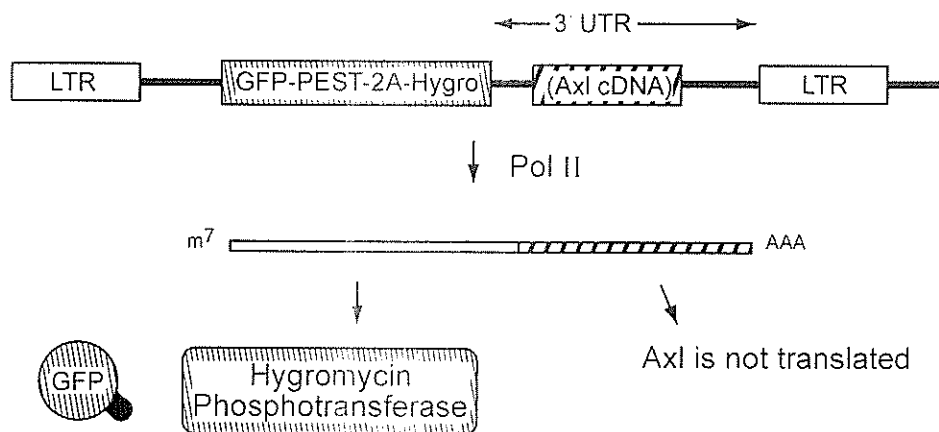


Fig. 22



shRNA targeting Axl or Luciferase (control) in an RRI vector

Fig. 23A



GFP-PEST reporter with or without Axl cDNA

Fig. 23B

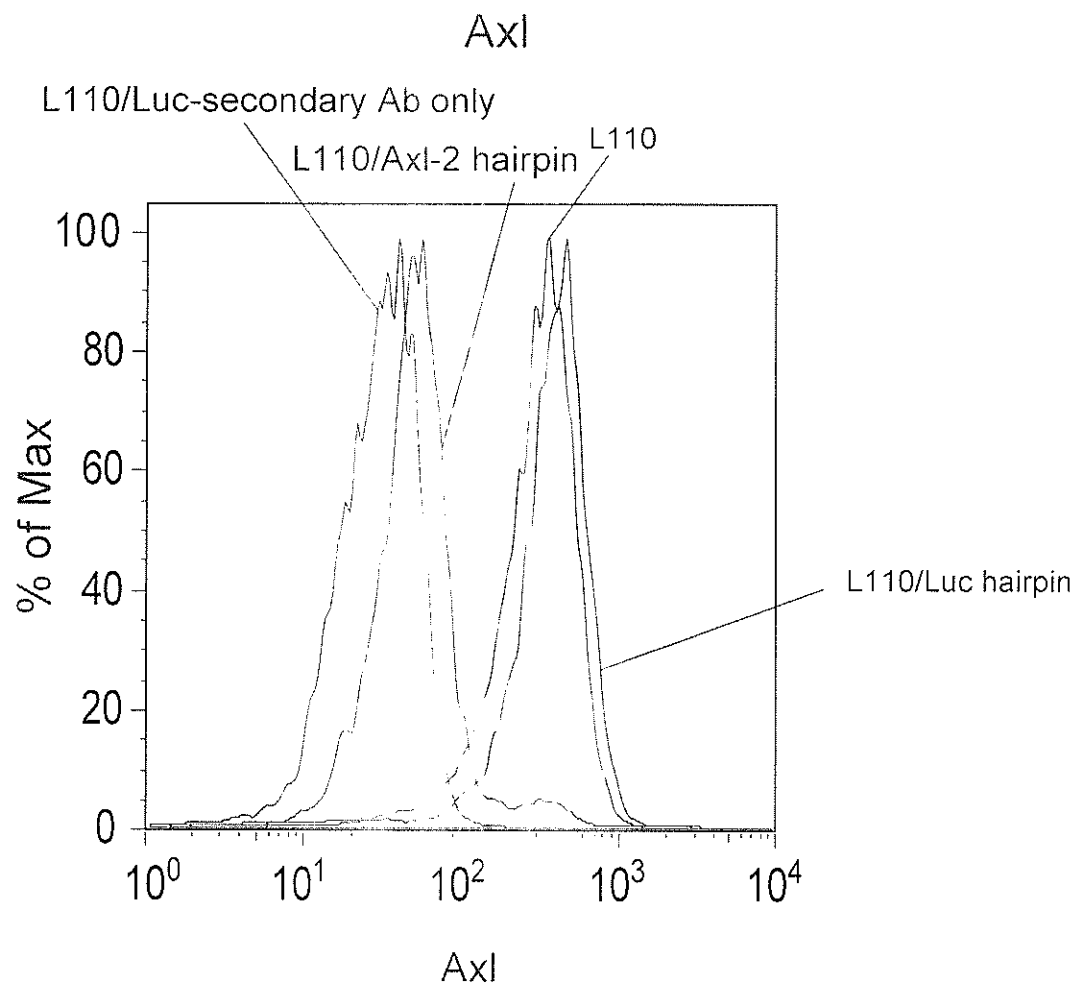


Fig. 24

Fig. 25A

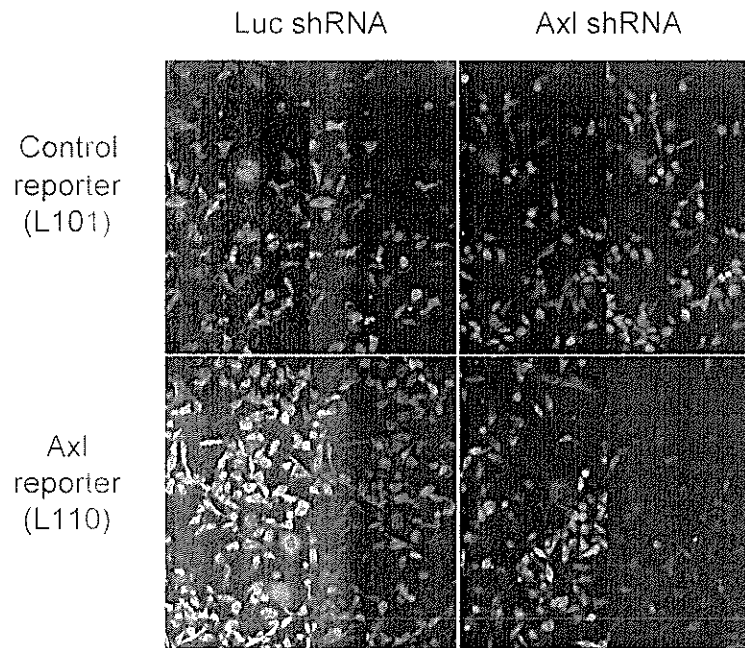
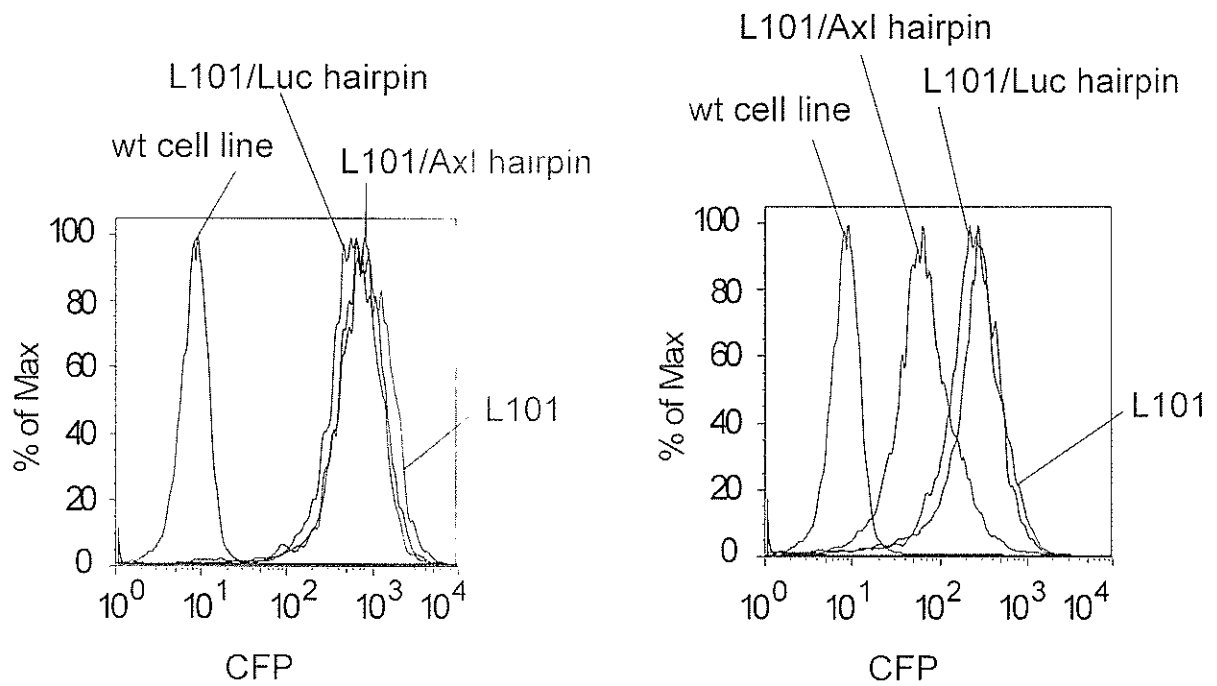


Fig. 25B

Fig. 26A

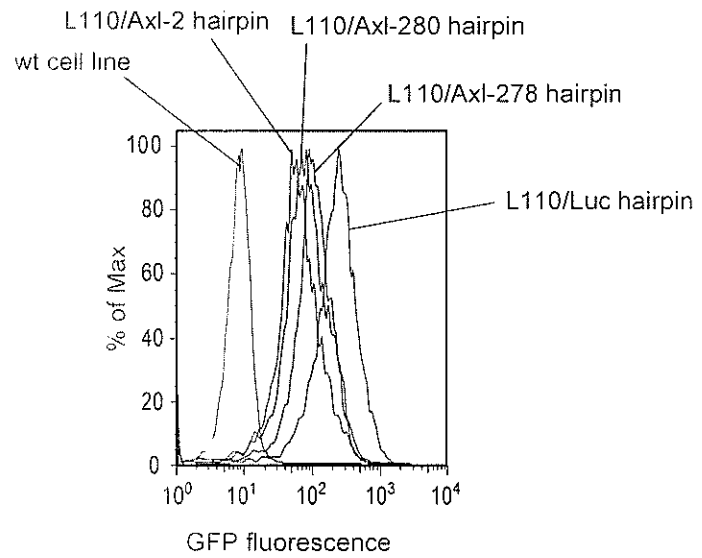


Fig. 26B

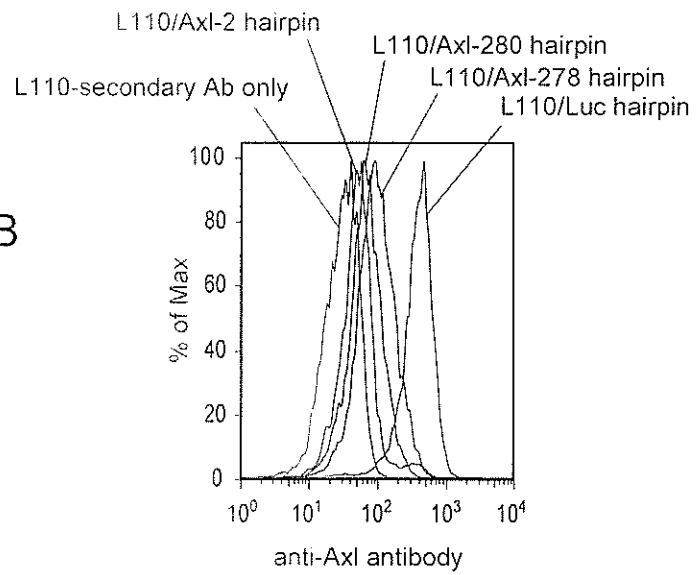
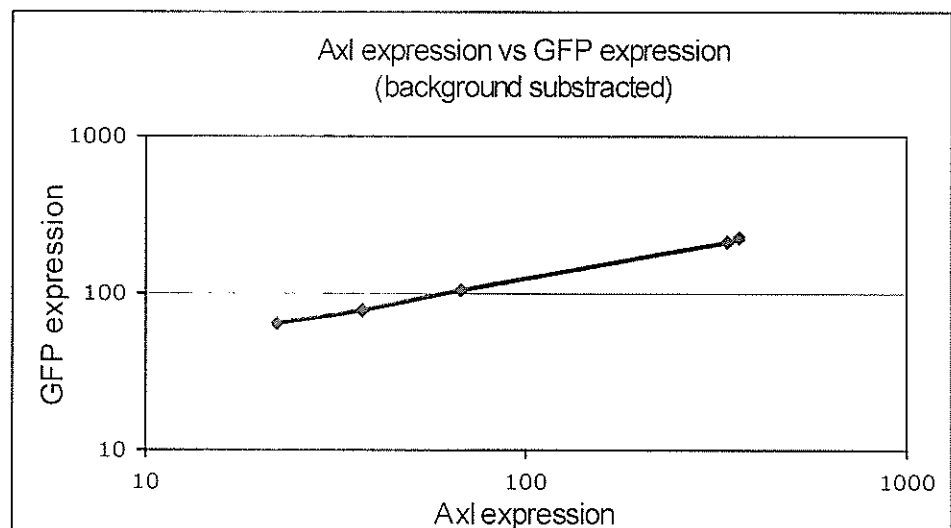


Fig. 26C



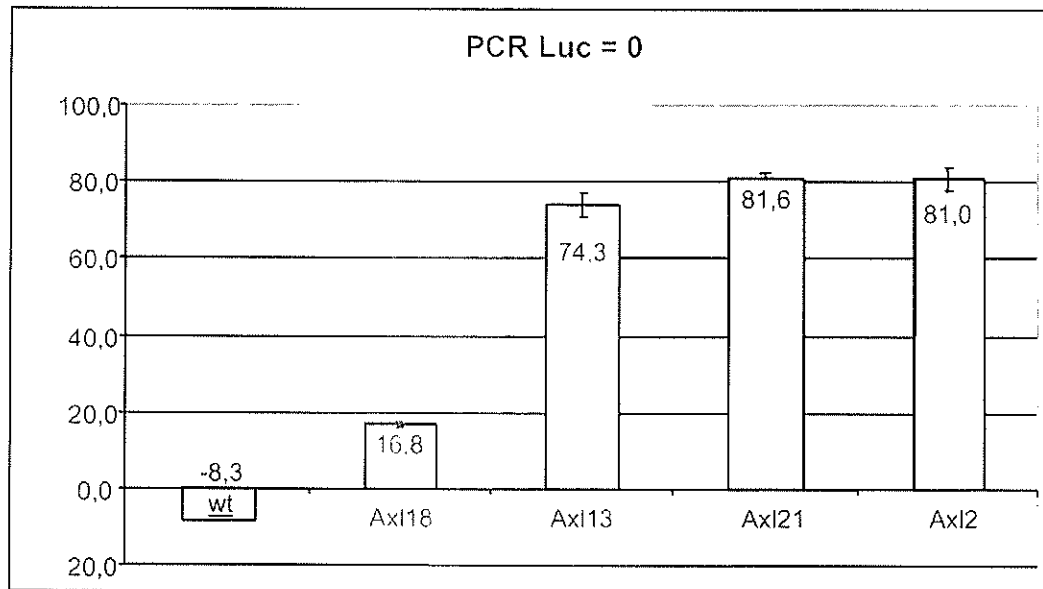


Fig. 27A

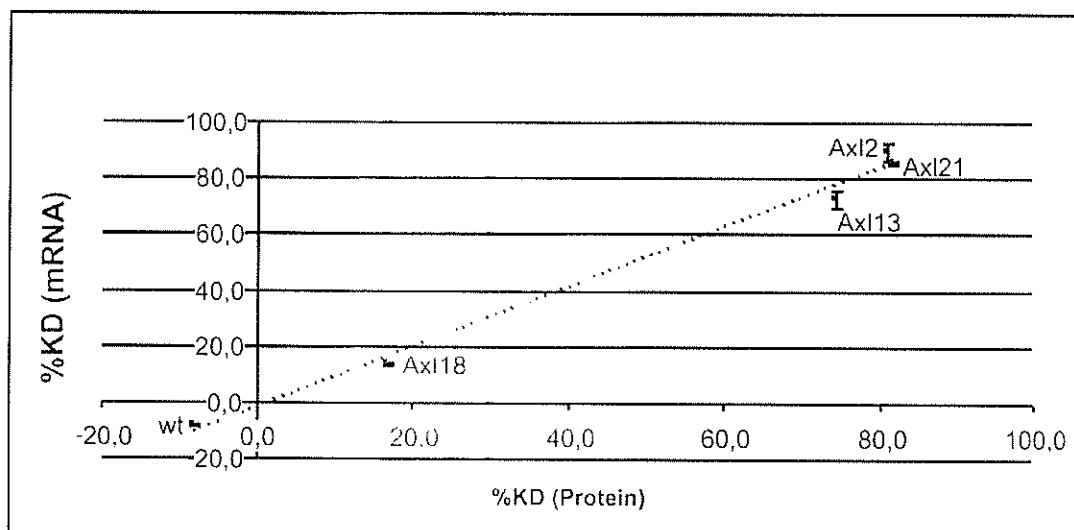


Fig. 27B

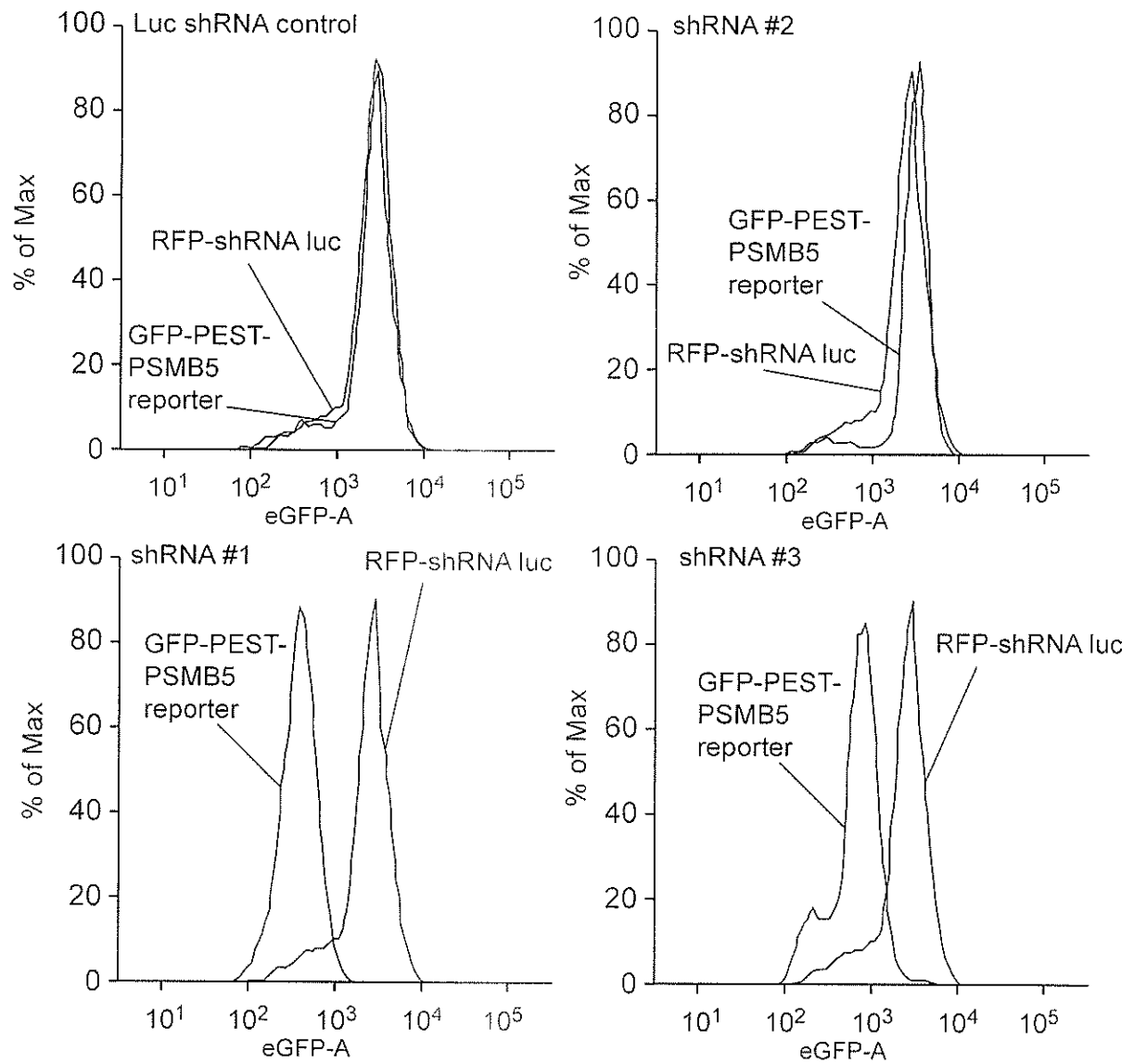
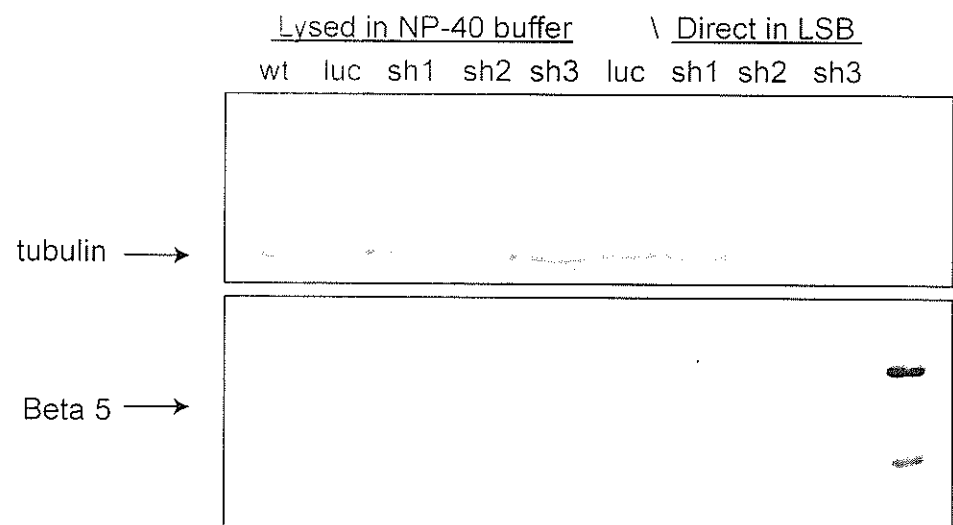


Fig. 28A

Fig. 28B



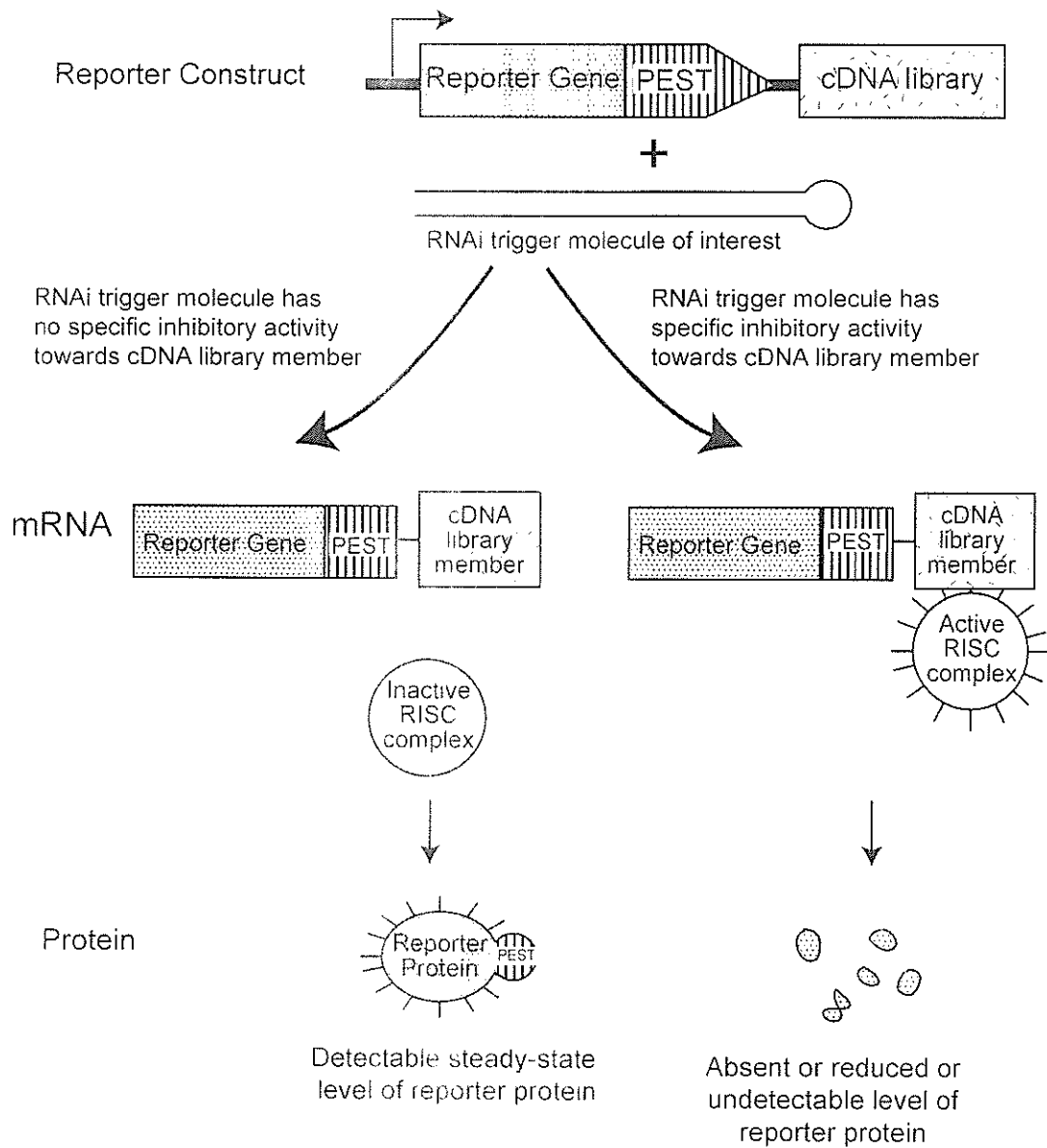


Fig. 29

Fig. 30

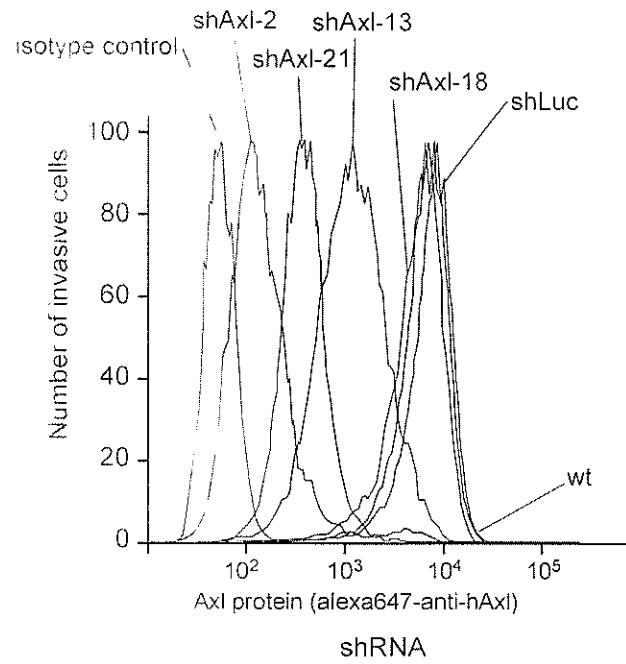


Fig. 31

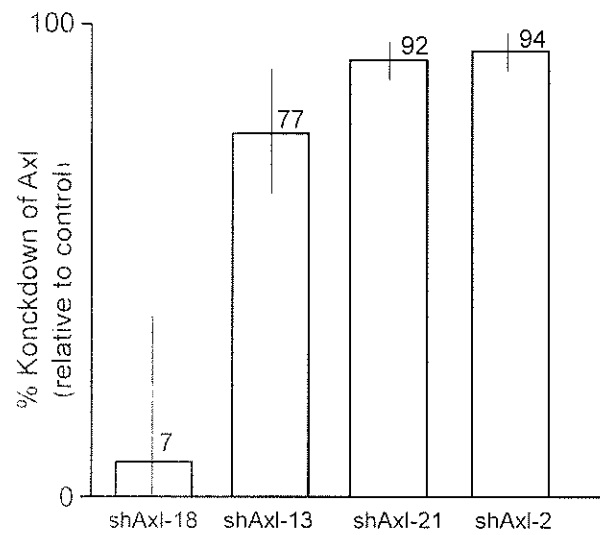


Fig. 32

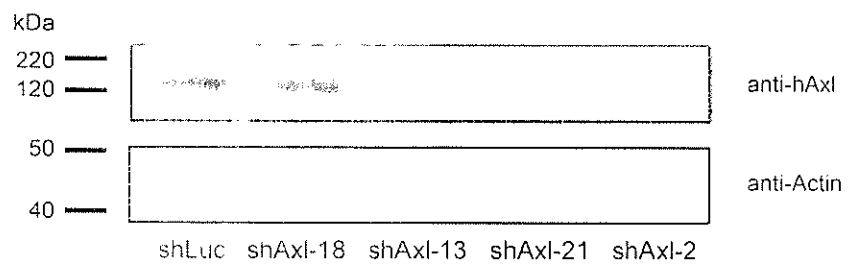
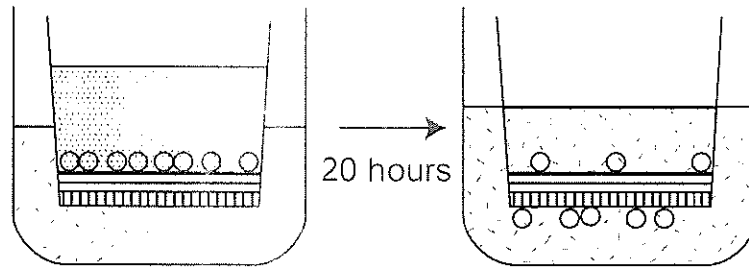
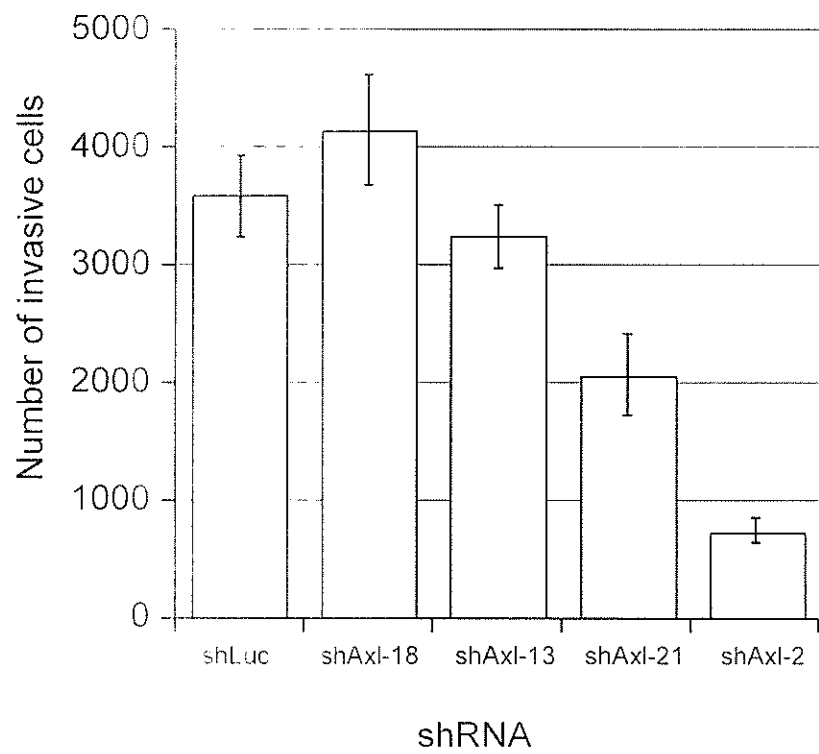


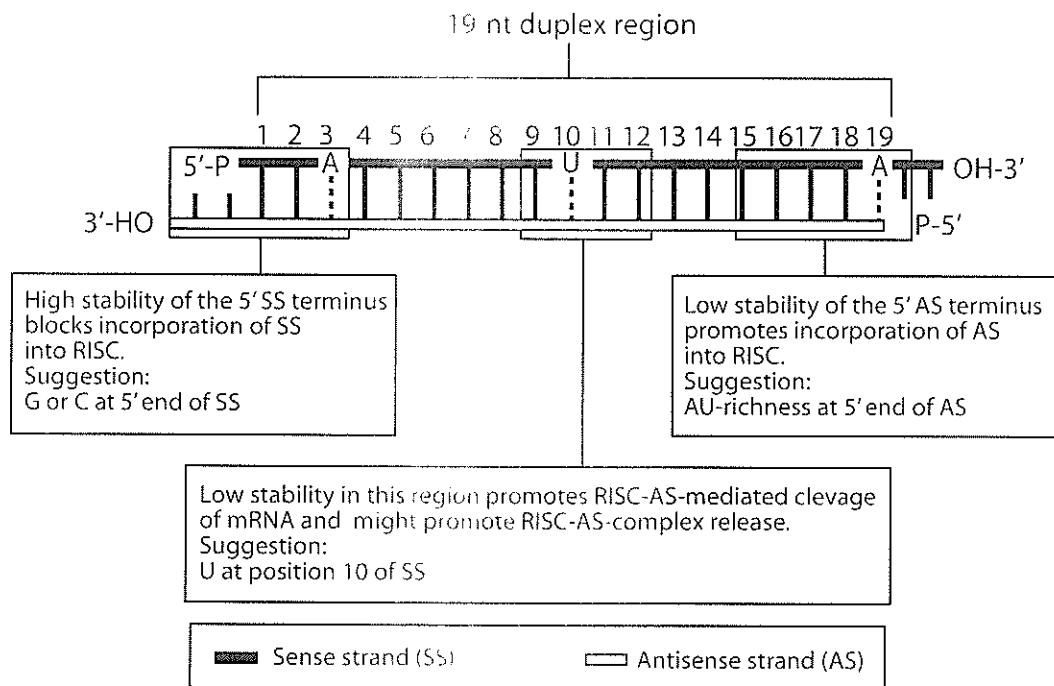
Fig. 33



- Medium with FBS
- ▨ Growth Factor Reduced Matrigel™ Matrix
- ▧ Membrane with 8 mm pores
- Serum-free medium
- Cells

Fig. 34





Sequence of RNAi trigger molecule identified by the invention

Algorithm prediction

Actual efficiency

CGCTGTGCGGCTGGGCGTGCA

Score: 1/20

GGCGAAAGAAGGAGACCCGTT

Score: 0/20

>95%
knockdown

Fig. 35

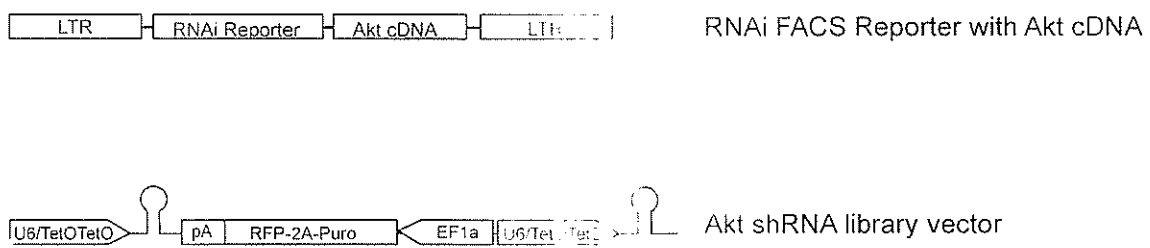


Fig. 36

Proteasomal subunit beta 5		
shRNA Hairpin Sequence	Corresponding mRNA target sequence	Relative Activity
gacctctatgtgttaccagtcattaatgaCTGGTCtcattaatg actggtaacacatagaggtc (SEQ ID NO: 1) Orientation: sense-LOOP-antisense	CACTGTTATGTGTTACCAGTCATTAATGA (SEQ ID NO: 16)	Strong
gaatctatgagcttcgaaaataaggaacgcCTGGTCgcggttcctt attcgaagctcatagattc (SEQ ID NO: 2) Orientation: sense-LOOP-antisense	CACTGTTATGAGCTTCGAAATAAGGAACGC (SEQ ID NO: 17)	Strong
gatctacatgagaagtatagtggctctacCTGGTCgtagagcca ctatacttctcatgtagatc (SEQ ID NO: 3) Orientation: sense-LOOP-antisense	CACTGTTATGAGAAGTATAGTGGCTCTAC (SEQ ID NO: 18)	Intermediate

Fig. 37

Axl Receptor Tyrosine Kinase			
Name	shRNA Hairpin Sequence	Corresponding mRNA target sequence	Relative Activity
shAxl21	atgcacgcccagccgcacagcgTTGGATCCCTGGTGGGATCCAA cgctgtgcggctgggcgtgcat (SEQ ID NO: 4) Orientation: antisense-LOOP-sense	CGCTGTGCGGCTGGGCGTGCA (SEQ ID NO: 19)	Strong
shAxl13	acgggtctccttctttcgccgTTGGATCCCTGGTGGGATCCAAc ggcgaaagaaggagaccg (SEQ ID NO: 18) Orientation: antisense-LOOP-sense	CGGCGAAAGAAGGAGACCCGT (SEQ ID NO: 20)	Intermediate
shAxl18	gcttcaggcgatttccccggcgTTGGATCCCTGGTGGGATCCAA cgccggggaaatgcctgaagc (SEQ ID NO: 17) Orientation: antisense-LOOP-sense	CGCCGGGGAAATCGCCTGAAGC (SEQ ID NO: 21)	Weak

Fig. 37 cont'd

Akt serine/threonine-specific protein kinase	
shRNA Hairpin Sequence	Corresponding mRNA target sequence
atctcatggaggagatccgcttcccgctGCTGGATCCAGAGATccctggagc ggatctcctccatgaggat (SEQ ID NO: 7) Orientation: sense-LOOP-antisense	ATCCTCATGGAGGAGATCCGCTTCCCGC (SEQ ID NO: 22)
cccagccccaccagtcactgcacggcTGCTGGATCCAGAGATccctggagc tggactggtgggggctggg (SEQ ID NO: 8) Orientation: antisense-LOOP-sense	GGCTGCAGTGGACTGGTGGGGGCTGGG (SEQ ID NO: 23)
gtcacgcggtgcttgggcttggcTGCTGGATCCAGAGATgcccaccccaagca ccgctgac (SEQ ID NO: 9) Orientation: antisense-LOOP-sense	GCCAAGCCCAAGCACCGCGTGAC (SEQ ID NO: 24)
cttcattggcgtagtagcggcTGCTGGATCCAGAGATccgctactaagccatga ag (SEQ ID NO: 10) Orientation: antisense-LOOP-sense	CCGCTACTACGCCATGAAG (SEQ ID NO: 25)
gtggcccacacactcaccgagaaccgcTGCTGGATCCAGAGATgcttctgggt gagtgtgtggggccac (SEQ ID NO: 11) Orientation: sense-LOOP-antisense	GTGGCCACACACTCACCAGAAACCGC (SEQ ID NO: 26)
ccttttgccgcacacctgagtacctggcTGCTGGATCCAGAGATgcttctgggt tcagggtgtccgcaaaagg (SEQ ID NO: 12) Orientation: sense-LOOP-antisense	CCTTTTCGGGCACACCTGAGTACCTGGC (SEQ ID NO: 27)

Fig 37 cont'd

Akt serine/threonine-specific protein kinase	
shRNA Hairpin Sequence	Corresponding mRNA target sequence
ccatgaacgagtttgagtacctgaagcTGCTGGATCCAGAGT (SEQ ID NO: 13) tcaaaactcgttcacgg (SEQ ID NO: 14) Orientation: sense-LOOP-antisense	CCATGAACGAGTTTGAGTACCTGAAGC (SEQ ID NO: 28)
aagatcctcaagaaggaagtcacgtggtCTGCTGGATCCAGAGT (SEQ ID NO: 15) gacttccttcttgaggatctt (SEQ ID NO: 16) Orientation: sense-LOOP-antisense	AAGATCCTCAAGAAGGAAGTCATCGTGGC (SEQ ID NO: 29)
cctgcactcggagaagaacgtggtgtCTGCTGGATCCAGAGT (SEQ ID NO: 17) cttctccgagtgacgg (SEQ ID NO: 18) Orientation: sense-LOOP-antisense	CCTGCACTCGGAGAAGAACGTGGTGT (SEQ ID NO: 30)

Fig 37 cont'd

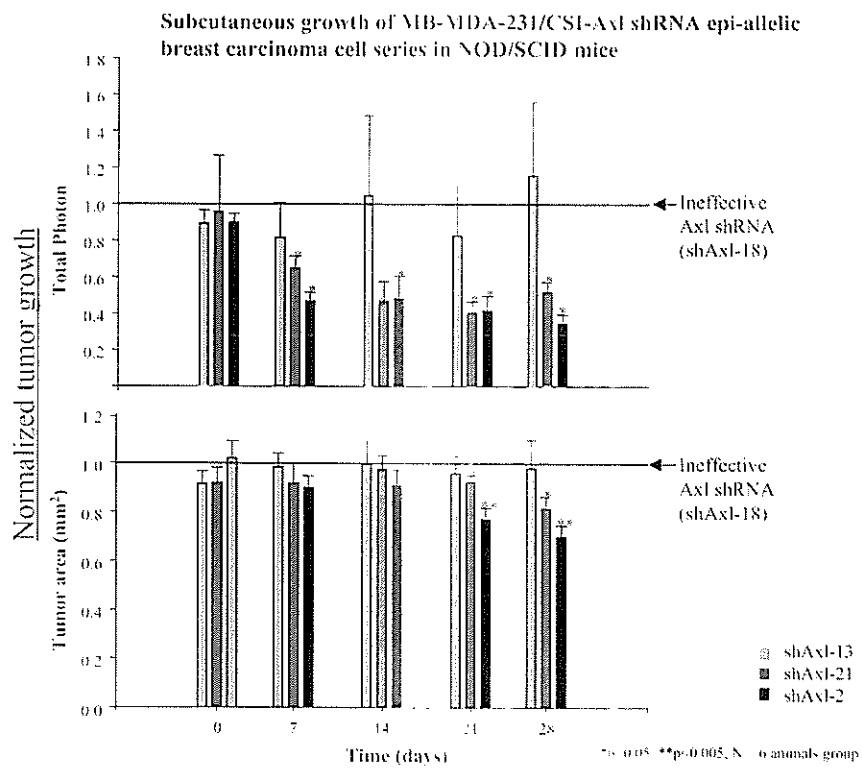


Fig 38