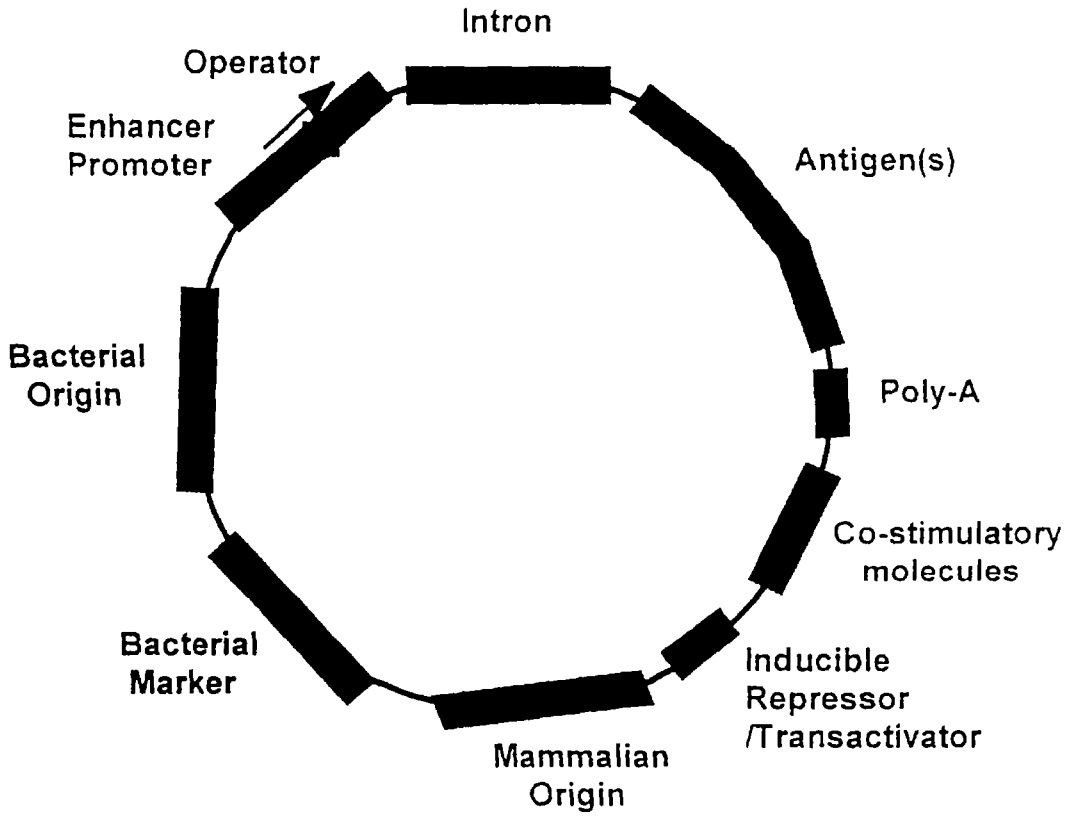
Figure 1: Schematic representation of a multimodule genetic vaccine vector.



G00002

163163

Figure 2

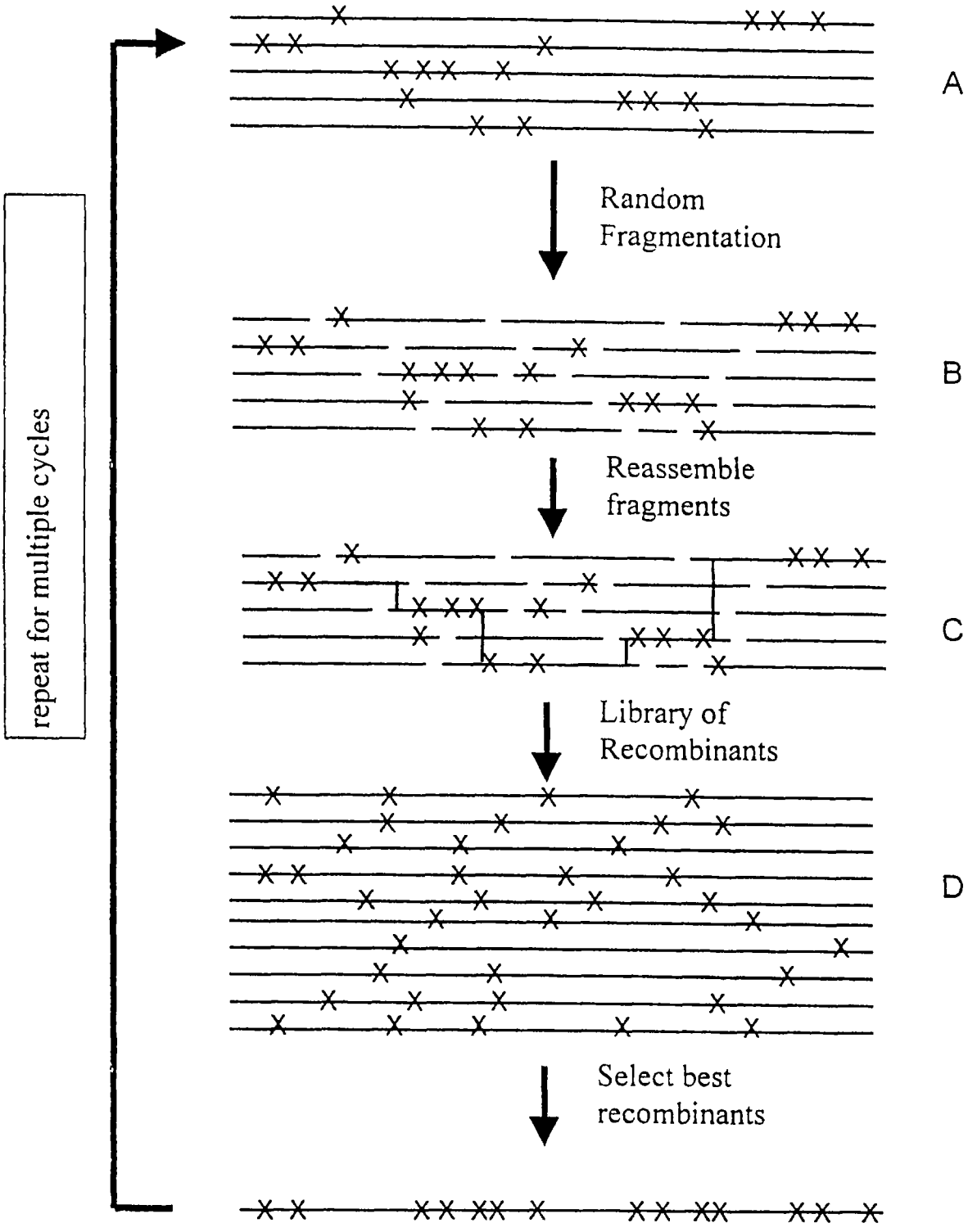

A
Random
Fragmentation
B
Reassemble
fragments
C
Library of
Recombinants
D
Select best
recombinants
repeat for multiple cycles

Figure 3

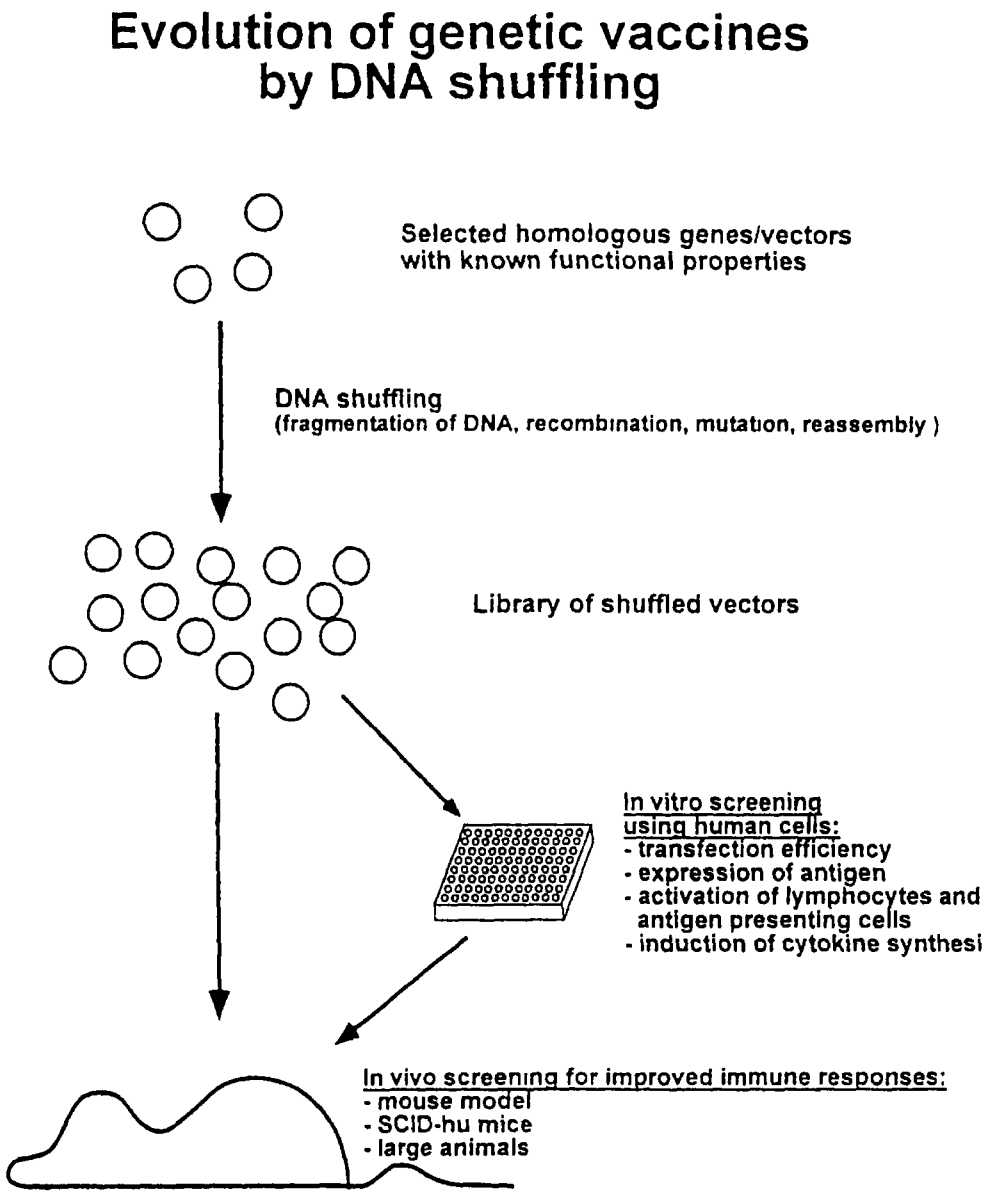

Evolution of genetic vaccines
by DNA shuffling
Selected homologous genes/vectors
with known functional properties
DNA shuffling
(fragmentation of DNA, recombination, mutation, reassembly)
Library of shuffled vectors
In vitro screening
using human cells:
- transfection efficiency
- expression of antigen
- activation of lymphocytes and
antigen presenting cells
- induction of cytokine synthesi
In vivo screening for improved immune responses:
- mouse model
- SCID-hu mice
- large animals

Figure 4. Recursive shuffling and selection of evolved promoter sequences as an example of flow cytometry-based screening methods.

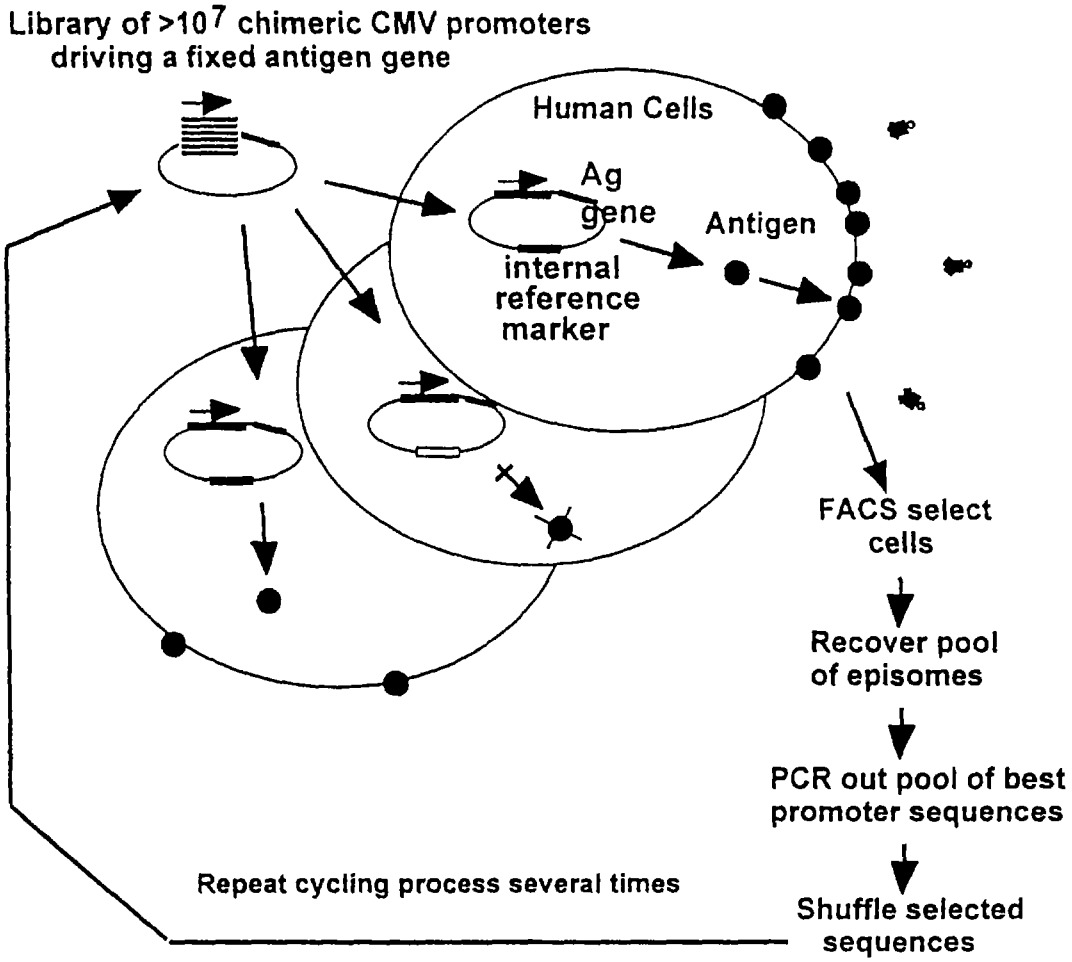


Figure 5

An apparatus for microinjections of skin and muscle

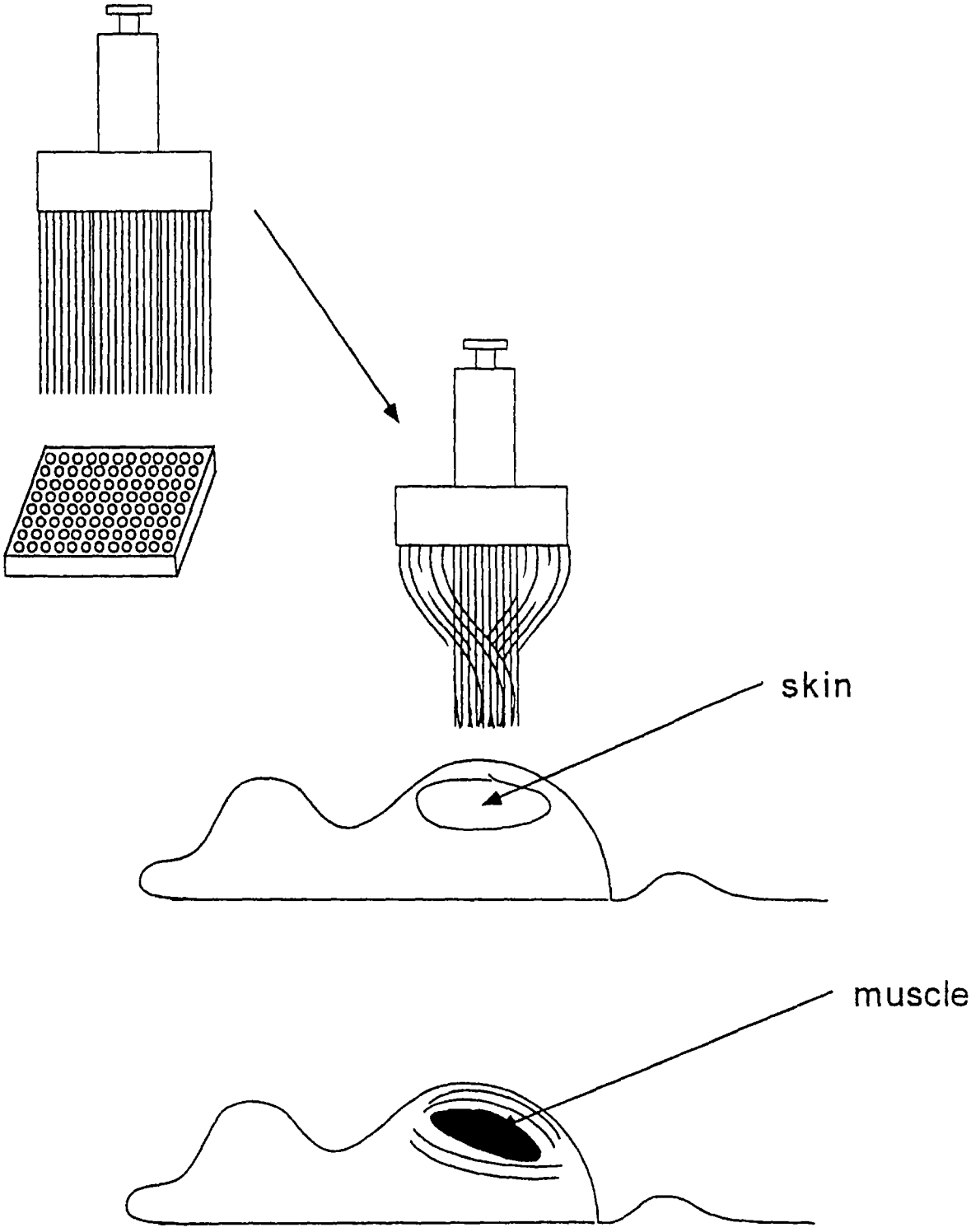


Figure 6

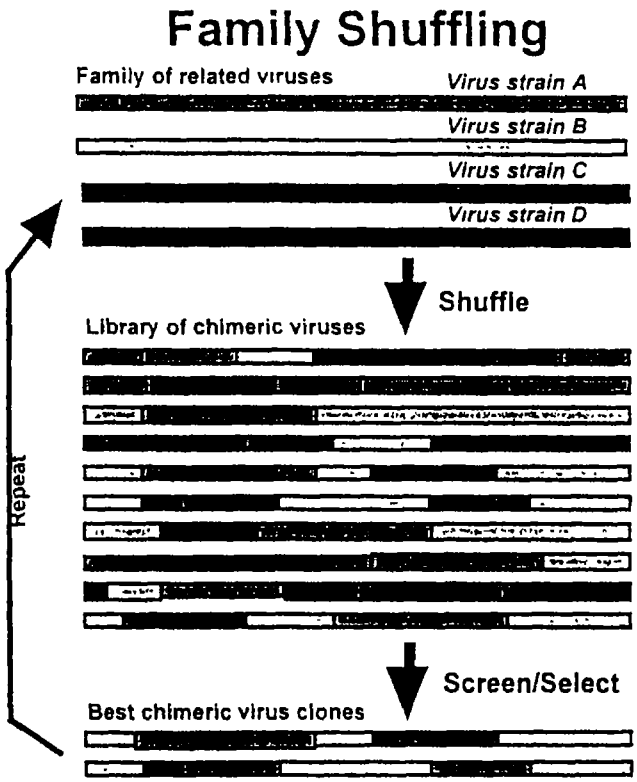

Family Shuffling
Family of related viruses
Virus strain A
Virus strain B
Virus strain C
Virus strain D
Shuffle
Library of chimeric viruses
Repeat
Screen/Select
Best chimeric virus clones

Figure 7

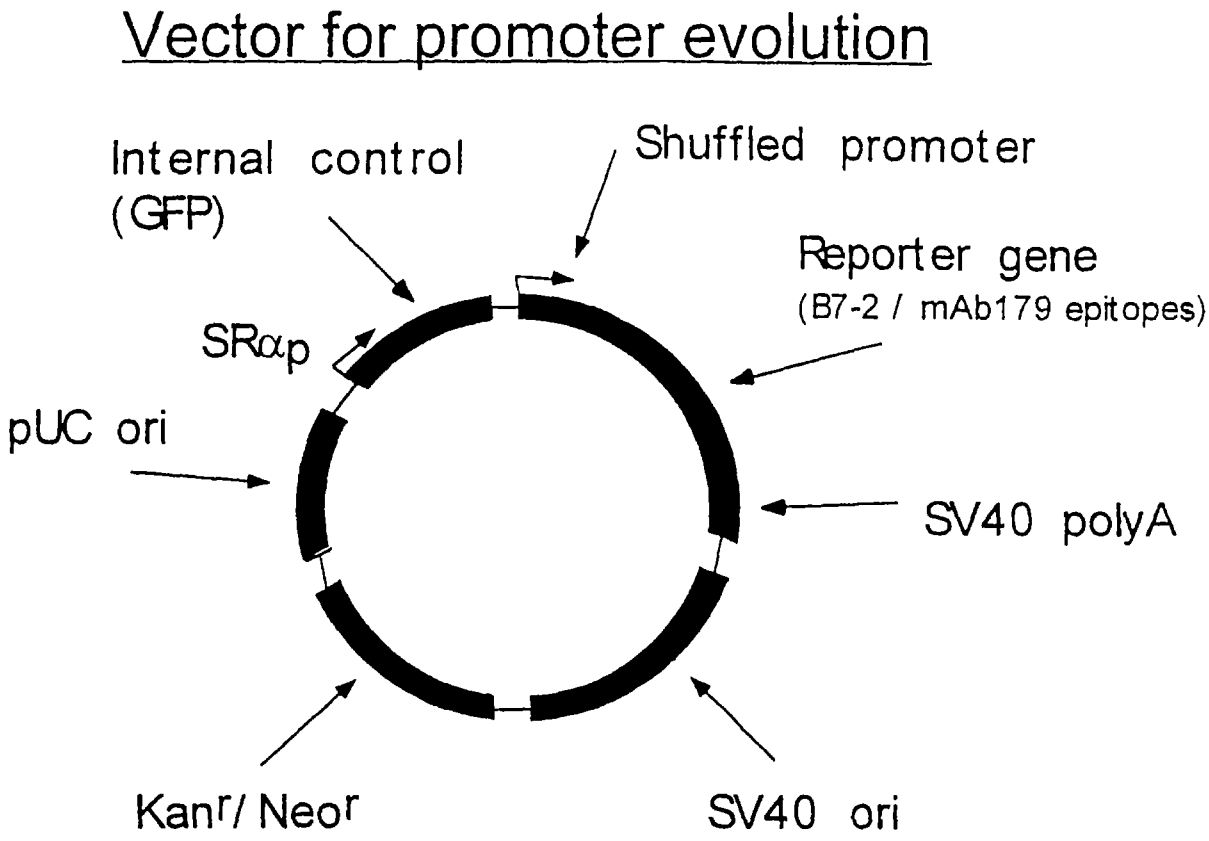

Vector for promoter evolution
Internal control
(GFP)
Shuffled promoter
Reporter gene
(B7-2 / mAb179 epitopes)
SRαp
pUC ori
SV40 polyA
Kanr/ Neor
SV40 ori

Figure 8: Cycling evolution of inducible promoters using DNA shuffling and flow cytometry-based selection.

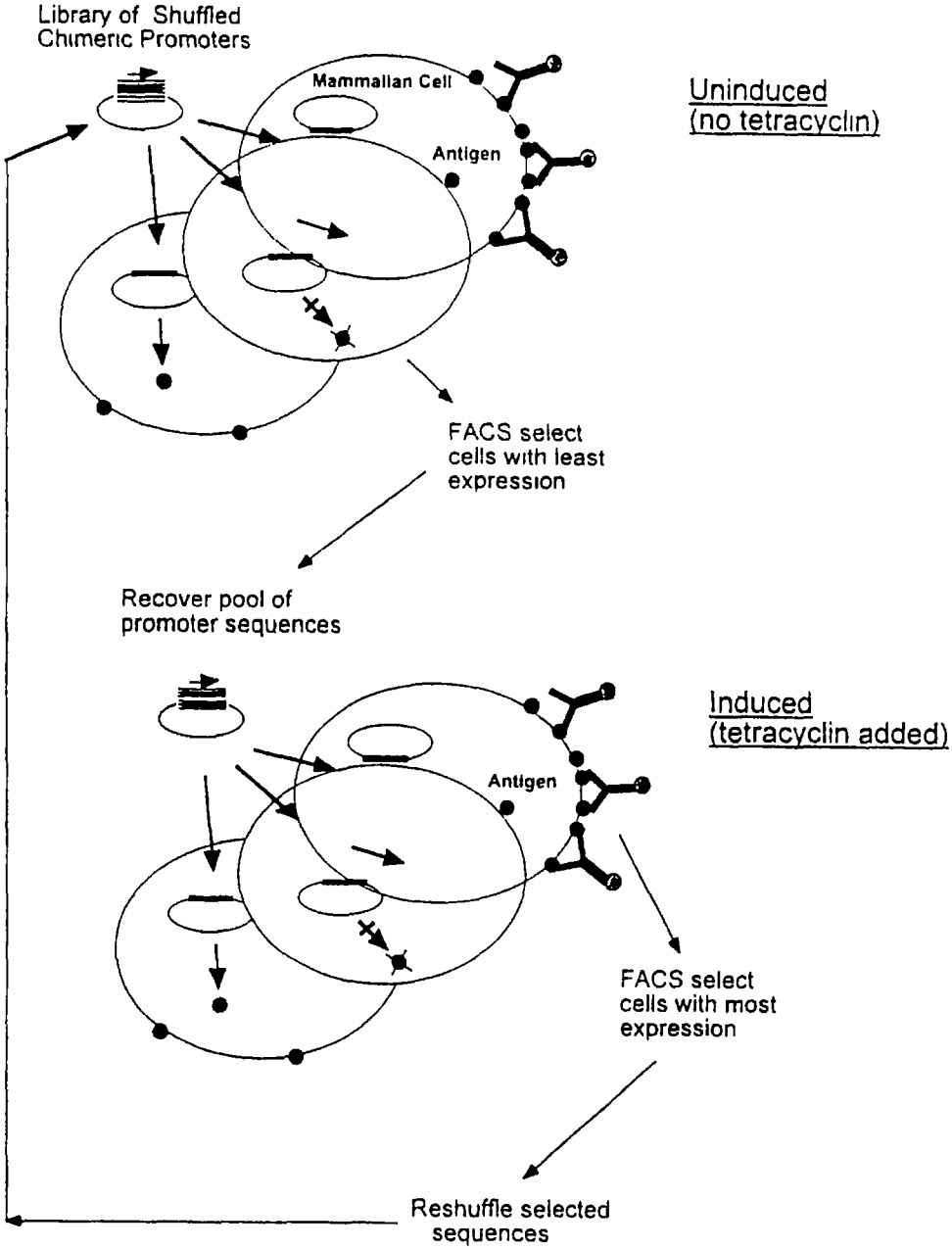
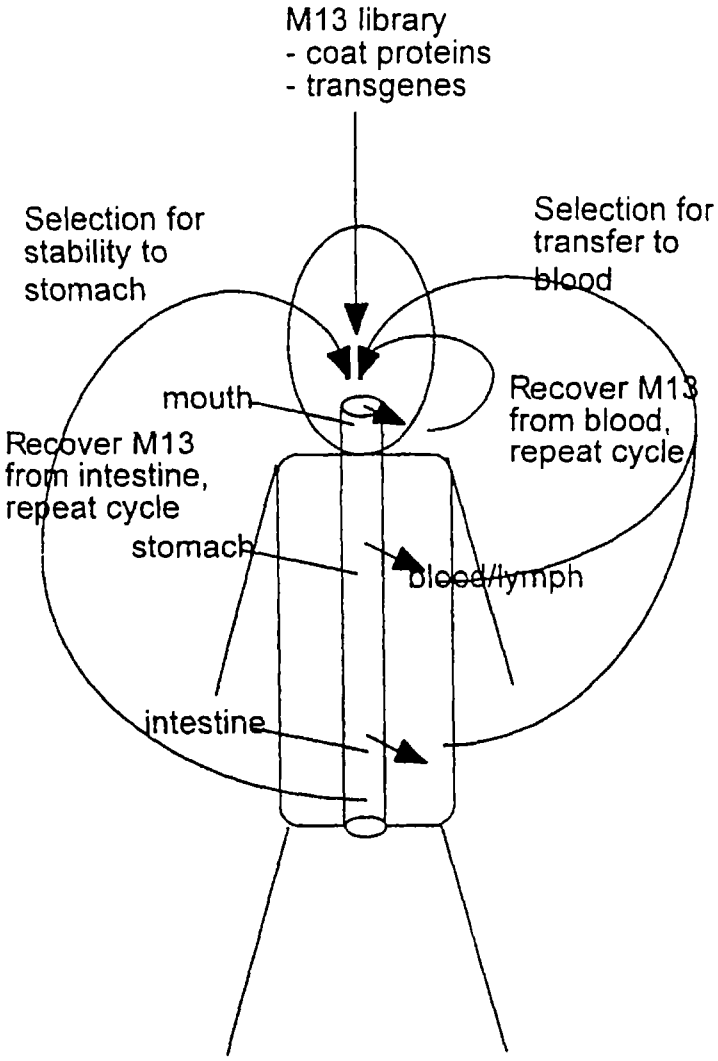


Figure 9

M13 Evolved for Oral Delivery



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Figure 10: Promoter sequences of the immediate/early gene of two human CMV strains (AD169 and Towne) and two Monkey strains (rhesus monkey and Vervet monkey) were PCRed; the alignment demonstrates the homologies between the PCR products from Towne strain and the two monkey strains illustrating the feasibility of family shuffling.

```
                                  1                                                60
Towne_promoter_fr_PCR_prod_seq    ATA....TGAGGCTATATCGCCGATAGAGGCGACATCAAGCTGGCACATGGCCAATGCAT
  Rhesus_monkey_PCR_prod_821bp    ACT....TGGCACGGTGCCAA.GTTTGGGGCGGGGTC...TTGGCACCGTGCCAA.....
Vervet_(Simian)_PCR_product_seq   ATTGAATTGGCATGGTGCCAATAATGGCGGC..CATA...TTGGCTATATGCCA......

                                  61                                              120
Towne_promoter_fr_PCR_prod_seq    ATCGATCTATACATTGAATCAATATTGGCAATTAGCCATATTAGTCATTGGTTATATAGC
  Rhesus_monkey_PCR_prod_821bp    ...GTCCGCCATATTGGTTTGGCAT.....ATGTCCAATATTATTGAT...CCATATAGC
Vervet_(Simian)_PCR_product_seq   ..............GGATCAATAT.....ATAGGCAATATC.........CAATATGGC

                                  121                                             180
Towne_promoter_fr_PCR_prod_seq    ATAAATCAATATTGGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACAT
  Rhesus_monkey_PCR_prod_821bp    CAATATCCAATATGGCTAATAGCCA..........GGTTCAATAGAATGGCCAATAAGC
Vervet_(Simian)_PCR_product_seq   CCTATGCCAATATGGCTATTGGCCA..........GGTTCAATACTATGTATTGGCCCT

                                  181                                             240
Towne_promoter_fr_PCR_prod_seq    TTATATTGGCTCATGTCCAATATGACCGCCATGTTGACATTGATTATTGACTAGTT..AT
  Rhesus_monkey_PCR_prod_821bp    CAATAT..GCCATTGGCCAACATGGCAA.TGGGCCAGTATTGATTATAGCCAATAT..AT
Vervet_(Simian)_PCR_product_seq   ATGCCA..TATAGTATTCCATATATGGGTTTTCCTATTGACGTAGATAGCCCCTCCCAAT
```

Figure 10 (continued)

```
                                241                                                300
 Towne_promoter_fr_PCR_prod_seq TAATAGTA......ATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGC
   Rhesus_monkey_PCR_prod_821bp AGGCAATA......ATCCATATTGG...CATATGTCCATATTGCCTATAGCCATATTGGC
Vervet_(Simian)_PCR_product_seq GGGCGGTCCCATATACCATATATGG...GGCTTCCTAATACCGCCCATAGCCACTCCCCC

                                301                                                360
 Towne_promoter_fr_PCR_prod_seq GT...T..ACATAACTTACGGTAAATGGCCCGCCTCGTGACCGCCCAACGACCCCCGCCC
   Rhesus_monkey_PCR_prod_821bp TTATGT..CCATTACCAATACCATATATGGGTCTTCCTATATACGTCATAGGTACCGCCC
Vervet_(Simian)_PCR_product_seq AT...TGACGTCAATGGTCTCTATATATGGTCTTTCCTATTGACGTCATATGGGCGGTCC

                                361                                                420
 Towne_promoter_fr_PCR_prod_seq .ATTGACGT.....................................................CAA
   Rhesus_monkey_PCR_prod_821bp .ATTGACGTAATATGGATACGCCTCCATTGACGTCAATGGGAGGGATTAATATACGTCAC
Vervet_(Simian)_PCR_product_seq TATTGACGTA.TATGGCGCCTCCCCCATTGACGTCAATTACGGTAAATGGCCCGCCTGGC

                                421                                                480
 Towne_promoter_fr_PCR_prod_seq TAATGACGTATGTTCCCAT..............AGTAACGCCAATAGGG..ACTTTCCA
   Rhesus_monkey_PCR_prod_821bp TAATACCGCCCATTGACGTGTATAGGACCGTCCCATTGACGTCAATAGGCCCACCTCCCA
Vervet_(Simian)_PCR_product_seq T..CAATGCCCATTGACGT.....................CAATAGGACCACCCACCA

                                481                                                540
 Towne_promoter_fr_PCR_prod_seq TTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTT.........GGCAGTAC
   Rhesus_monkey_PCR_prod_821bp TTGACGTCAATGGG.........GTGGCCCATTGCCCATTC.................
Vervet_(Simian)_PCR_product_seq TTGACGTCAATGGG.........ATGGCTCATTGCCCATTCATATCCGTTC........

                                541                                                600
 Towne_promoter_fr_PCR_prod_seq ATCAAGTGTATCATATGCCAAGTCCGGCCCCCTATTGACGTCAATGACGGTAAATGGCCC
   Rhesus_monkey_PCR_prod_821bp .....................CCACGCCCCCTATTGACGTCAATGACGGTAAATGGCC.
Vervet_(Simian)_PCR_product_seq .....................TCACGCCCCCTATTGACGTCAATGACGGTAAATGGCC.
```

Figure 10 (continued)

```
                                  601                                                660
Towne_promoter_fr_PCR_prod_seq    GCCTGGCATTATGCCCAGTACATGACCTTACGGGACTTTCCTACTTGGCAGTACATC..T
  Rhesus_monkey_PCR_prod_821bp    ..........................................CACTTGGCAGTACATCAAT
Vervet_(Simian)_PCR_product_seq   ..........................................CACTTGGCAGTACATCAAT

                                  661                                                720
Towne_promoter_fr_PCR_prod_seq    ACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACACCAA........
  Rhesus_monkey_PCR_prod_821bp    ACCTATTAATAGTAACT..TGGCAAGTAAATGGGTACTTGGCAGTACACCAAGG.TACAT
Vervet_(Simian)_PCR_product_seq   ATCTATTAATAGTAACT..TGGCAAGTACATTACTATTGGCAAGTACGCCAAGGGTACAT

                                  721                                                780
Towne_promoter_fr_PCR_prod_seq    ..............TGGGCGTGGATAGCGGT..TTGACTCACGGGGATTTCCAAGTCTC
  Rhesus_monkey_PCR_prod_821bp    TGGCAG.TACTCCCATTGACGTCAATGGCGGTAAATGGCCCGCAATGGCTGCCAAGTACA
Vervet_(Simian)_PCR_product_seq   TGGCAGGTACTCCCATTGACGTCAATGGCGGTAAATGGCCCGGCATGGCTGCCAAGTACA

                                  781                                                840
Towne_promoter_fr_PCR_prod_seq    ...CACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCA
  Rhesus_monkey_PCR_prod_821bp    ...TGCCC.ATTGACGTCAATGGGG.....................................
Vervet_(Simian)_PCR_product_seq   ACATCCCC.ATTGACGTCAATGGGAA.....................................

                                  841                                                900
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  Rhesus_monkey_PCR_prod_821bp    ..............CGGTCCTATGACGTCAATGGGCG.....................
Vervet_(Simian)_PCR_product_seq   ..............GGGGCAATGACGCAAATGGGCGTTCCATTGACGTAAATGGCG

                                  901                                                960
Towne_promoter_fr_PCR_prod_seq    GTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCG
  Rhesus_monkey_PCR_prod_821bp    GTAGGCGTGC.CTATGGGCGGTCTATATAAGCAATGCACGTTTAGGGAACCGCCATTCTG
```

Figure 10 (continued)

```
Vervet_(Simian)_PCR_product_seq   GTAGGCGTGCCTAATGGGAGGTCTATATAAGCAATGCTCGTTTAGGGAACCGCCATTCTG

                                  961                                                    1020
 Towne_promoter_fr_PCR_prod_seq   CCTGGAGACGCCATCCACGCTGTTTTGACCTCCAT.AGAAGACACCGGG.ACCGATCCAG
   Rhesus_monkey_PCR_prod_821bp   CCTGGGGACGTCG.........GAGGAGCACCAT.AGAAGGTACCGGGGACCGATCCAG
Vervet_(Simian)_PCR_product_seq   CCTGGGGACGTCG.........GAGGAGCTCCATTGGAAGAGACCGGG.ACCGATCCAG

                                  1021                                  1057
 Towne_promoter_fr_PCR_prod_seq   CCTCCGCGGCCGGGAACGGTGCATTGGAACGCGGATT
   Rhesus_monkey_PCR_prod_821bp   CCTCCATAGCCGGGAAGGGTGCATTGGAACGCGGATA
Vervet_(Simian)_PCR_product_seq   CCTCCATAGCCGGGGACGGTGCATTGGAATGCGGATA
```


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Figure 11: Alignment of Intron A sequences from CMV strains Towne and AD169 illustrating the feasibility of family shuffling.

```
                          1                                                  60
  AD_169_Intron_A_seq     GTAAGTACCGCCTATAGAGTCTATAGGCCCACCCCCTTGGCTTCTTATGCATGCTATACT
Towne_Intron_A_fr_map     GTAAGTACCGCCTATAGACTCTATAGGCACACCCCTTTGGCT.CTTATGCATGCTATACT

                          61                                                 120
  AD_169_Intron_A_seq     GTTTTTGGCTTGGGGTCTATACACCCCCGCTTCCTCATGTTATAGGTGATGGTATAGCTT
Towne_Intron_A_fr_map     GTTTTTGGCTTGGGGCCTATACACCCCCGCT.CCTTATGCTATAGGTGATGGTATAGCTT

                          121                                                180
  AD_169_Intron_A_seq     AGCCTATAGGTGTGGGTTATTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTT
Towne_Intron_A_fr_map     AGCCTATAGGTGTGGGTTATTGACCATTATTGACCACTCCCCTATTGGTGACGATACTTT

                          181                                                240
  AD_169_Intron_A_seq     CCATTACTAATCCATAACATGGCTCTTTGCCACAACTCTCTTTATTGGCTATATGCCAAT
Towne_Intron_A_fr_map     CCATTACTAATCCATAACATGGCTCTTTGCCACAACTATCTCTATTGGCTATATGCCAAT

                          241                                                300
  AD_169_Intron_A_seq     ACACTGTCCTTCAGAGACTGACACGGACTCTGTATTTTTACAGGATGGGGTCTCATTTAT
Towne_Intron_A_fr_map     ACTCTGTCCTTCAGAGACTGACACGGACTCTGTATTTTTACAGGATGGGGTCCCATTTAT

                          301                                                360
  AD_169_Intron_A_seq     TATTTACAAATTCACATATACAACACCACCGTCCCCAGTGCCCGCAGTTTTTATTAAACA
Towne_Intron_A_fr_map     TATTTACAAATTCACATATACAACAACGCCGTCCCCCGTGCCCGCAGTTTTTATTAAACA

                          361                                                420
  AD_169_Intron_A_seq     TAACGTGGGATCTCCACGCGAATCTCGGGTACGTGTTCCGGACATGGGCTCTTCTCCGGT
Towne_Intron_A_fr_map     TAGCGTGGGATCTCCACGCGAATCTCGGGTACGTGTTCCGGACATGGGCTCTTCTCCGGT
```


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Figure 11 (continued)

```
                    421                                                 480
AD_169_Intron_A_seq AGCGGCGGAGCTTCTACATCCGAGCCCTGCTCCCATGCCTCCAGCGACTCATGGTCGCTC
Towne_Intron_A_fr_map AGCGGCGGAGCTTCCACATCCGAGCCCTGGTCCCATGCCTCCAGCGGCTCATGGTCGCTC

                    481                                                 540
AD_169_Intron_A_seq GGCAGCTCCTTGCTCCTAACAGTGGAGGCCAGACTTAGGCACAGCACGATGCCCACCACC
Towne_Intron_A_fr_map GGCAGCTCCTTGCTCCTAACAGTGGAGGCCAGACTTAGGCACAGCACAATGCCCACCACC

                    541                                                 600
AD_169_Intron_A_seq ACCAGTGTGCCGCACAAGGCCGTGGCGGTAGGGTATGTGTCTGAAAATGAGCTCGGGGAG
Towne_Intron_A_fr_map ACCAGTGTGCCGCACAAGGCCGTGGCGGTAGGGTATGTGTCTGAAAATGAGCTCGGAGAT

                    601                                                 660
AD_169_Intron_A_seq CGGGCTTGCACCGCTGACGCATTTGGAAGACTTAAGGCAGCGGCAGAAGAAGATGCAGGC
Towne_Intron_A_fr_map TGGGCTCGCACCG.TGACGCAGATGGAAGACTTAAGGCAGCGGCAGAAGAAGATGCAGGC

                    661                                                 720
AD_169_Intron_A_seq AGCTGAGTTGTTGTGTTCTGATAAGAGTCAGAGGTAACTCCCGTTGCGGTGCTGTTAACG
Towne_Intron_A_fr_map AGCTGAGTTGTTGTATTCTGATAAGAGTCAGAGGTAACTCCCGTTGCGGTGCTGTTAACG

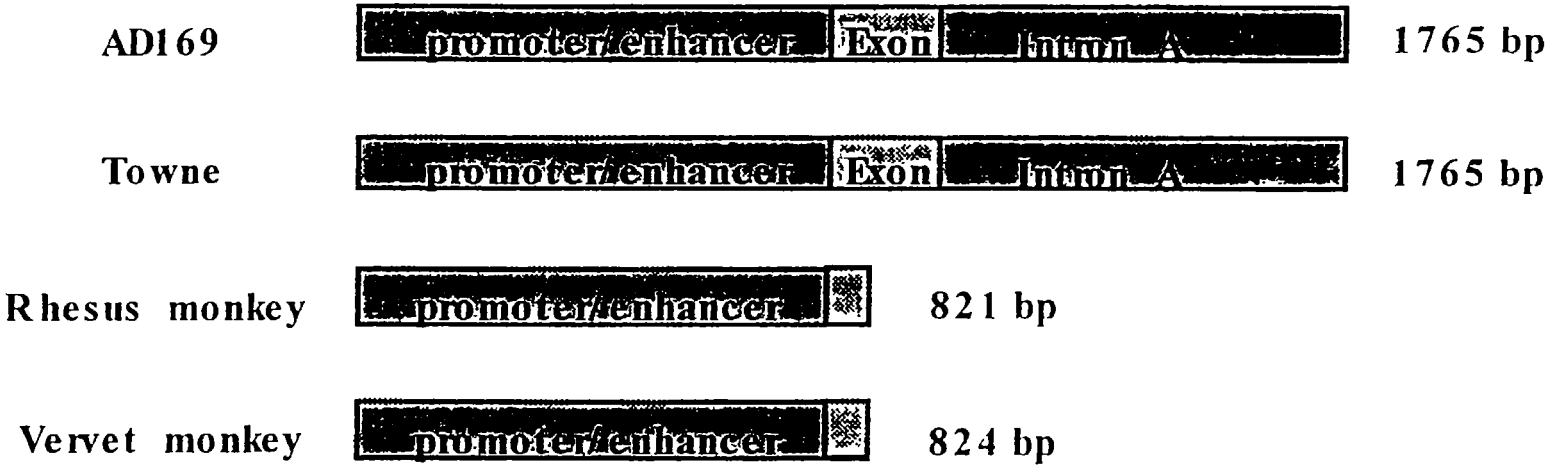
                    721                                                 780
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Towne_Intron_A_fr_map GTGGAGGGCAGTGTAGTCTGAGCAGTACTCGTTGCTGCCGCGCGCGCCACCAGACATAAT

                    781                                         827
AD_169_Intron_A_seq AGCTGACAGACTAACAGACTGTTCCTTTCCATGGGTCTTTTCTGCAG
Towne_Intron_A_fr_map AGCTGACAGACTAACAGACTGTTCCTTTCCATGGGTCTTTTCTGCAG
```


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Figure 12

Generation of a Library of Shuffled CMV Promoters



- AD169 vs. Towne 95%
- Rhesus vs. vervet 78%
- Human vs. primate 70%

- Library size 5×10^5

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PCT/US99/03022

Figure 13

Enrichment of Promoter Library by Cell Sorting

	Library		FACS → Sorted	
High activity (~ wild-type)	55/162	34%	348/705	49%
Intermediate	36/162	22%	183/705	26%
Low	71/162	44%	168/705	24%
Non-transfected	30/192	16%	43/748	6%

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Figure 14

Functional Diversity and Enrichment of High-activity Shuffled CMV Promoters

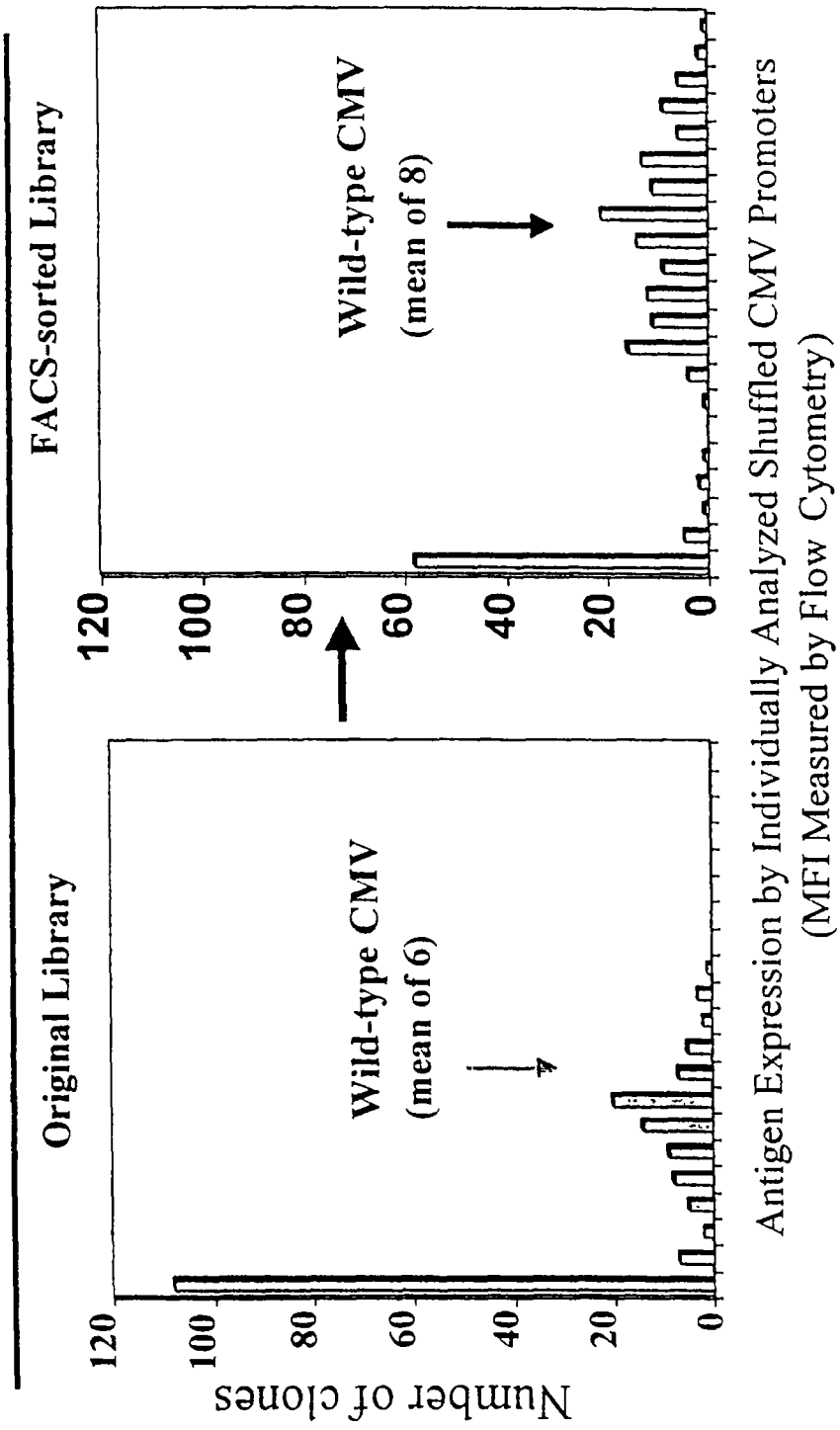


Figure 15

Transgene Expression *in vivo* by a Shuffled CMV Promoter

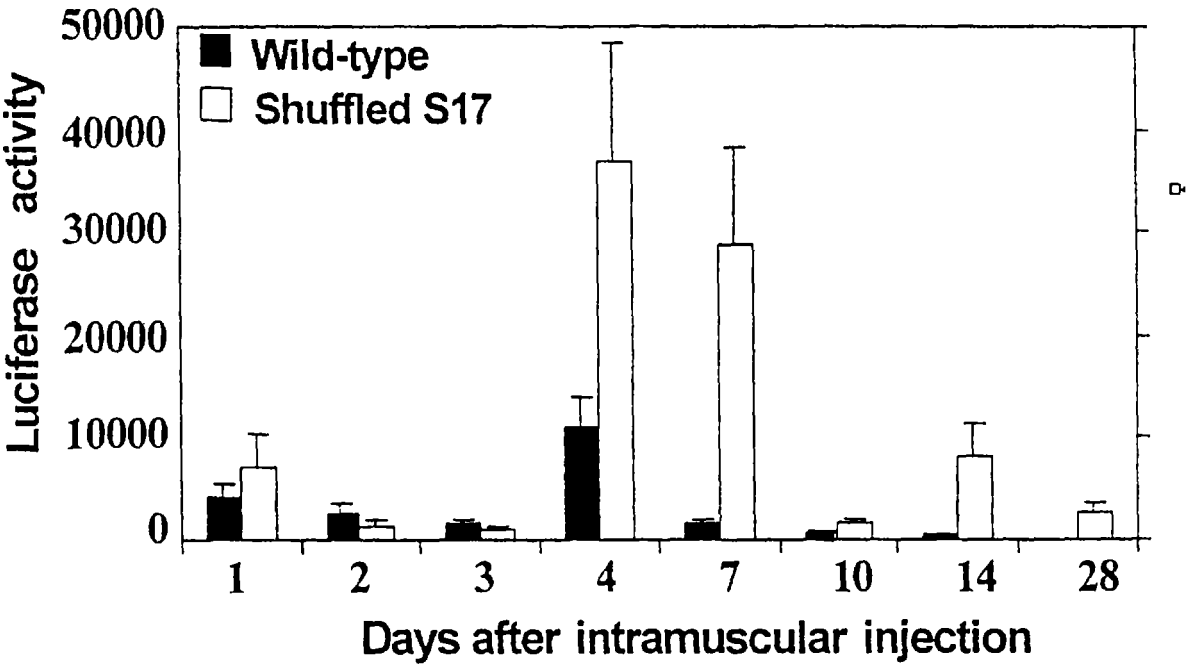
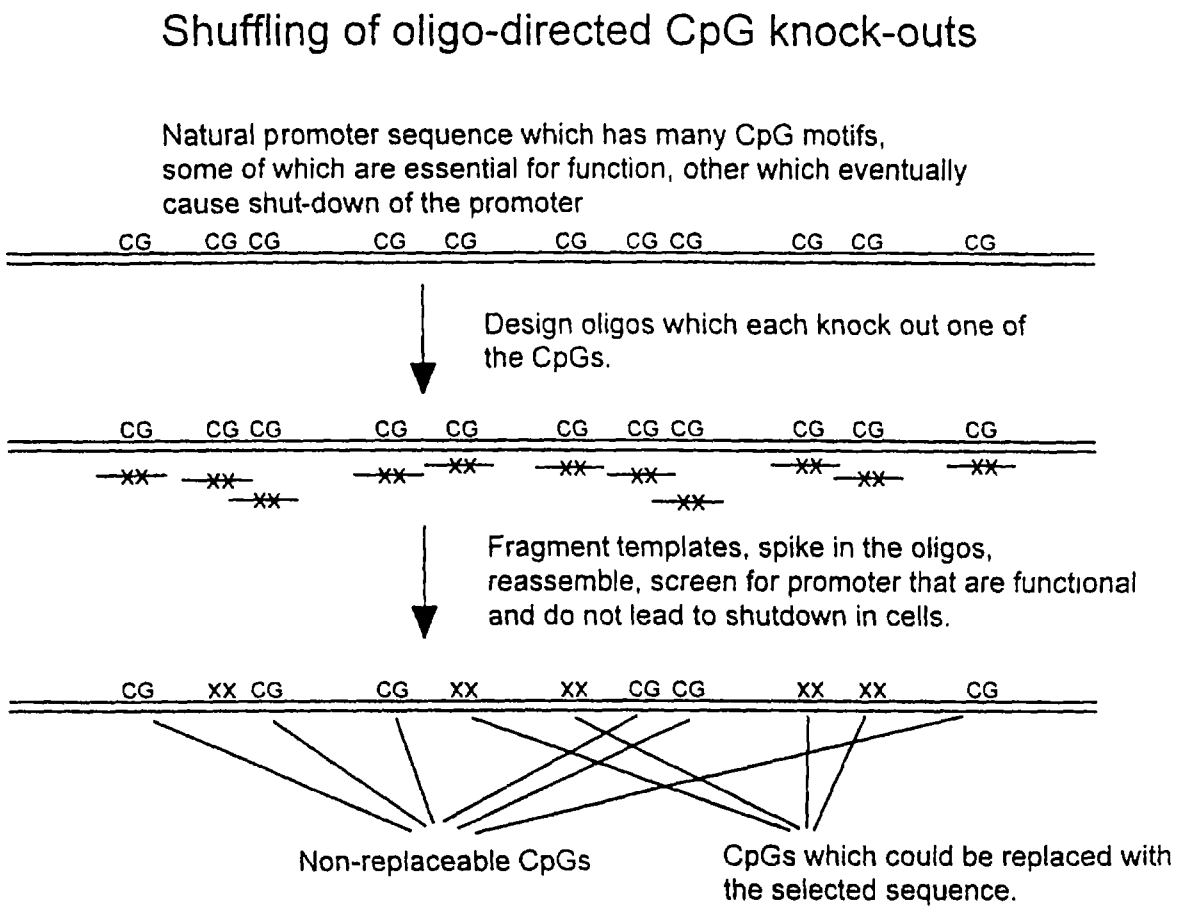


Figure 16


Shuffling of oligo-directed CpG knock-outs
Natural promoter sequence which has many CpG motifs, some of which are essential for function, other which eventually cause shut-down of the promoter
CG CG CG CG CG CG CG CG CG CG
Design oligos which each knock out one of the CpGs.
Fragment templates, spike in the oligos, reassemble, screen for promoter that are functional and do not lead to shutdown in cells.
CG XX CG CG XX XX CG CG XX XX CG
Non-replaceable CpGs
CpGs which could be replaced with the selected sequence.