Figure 1A

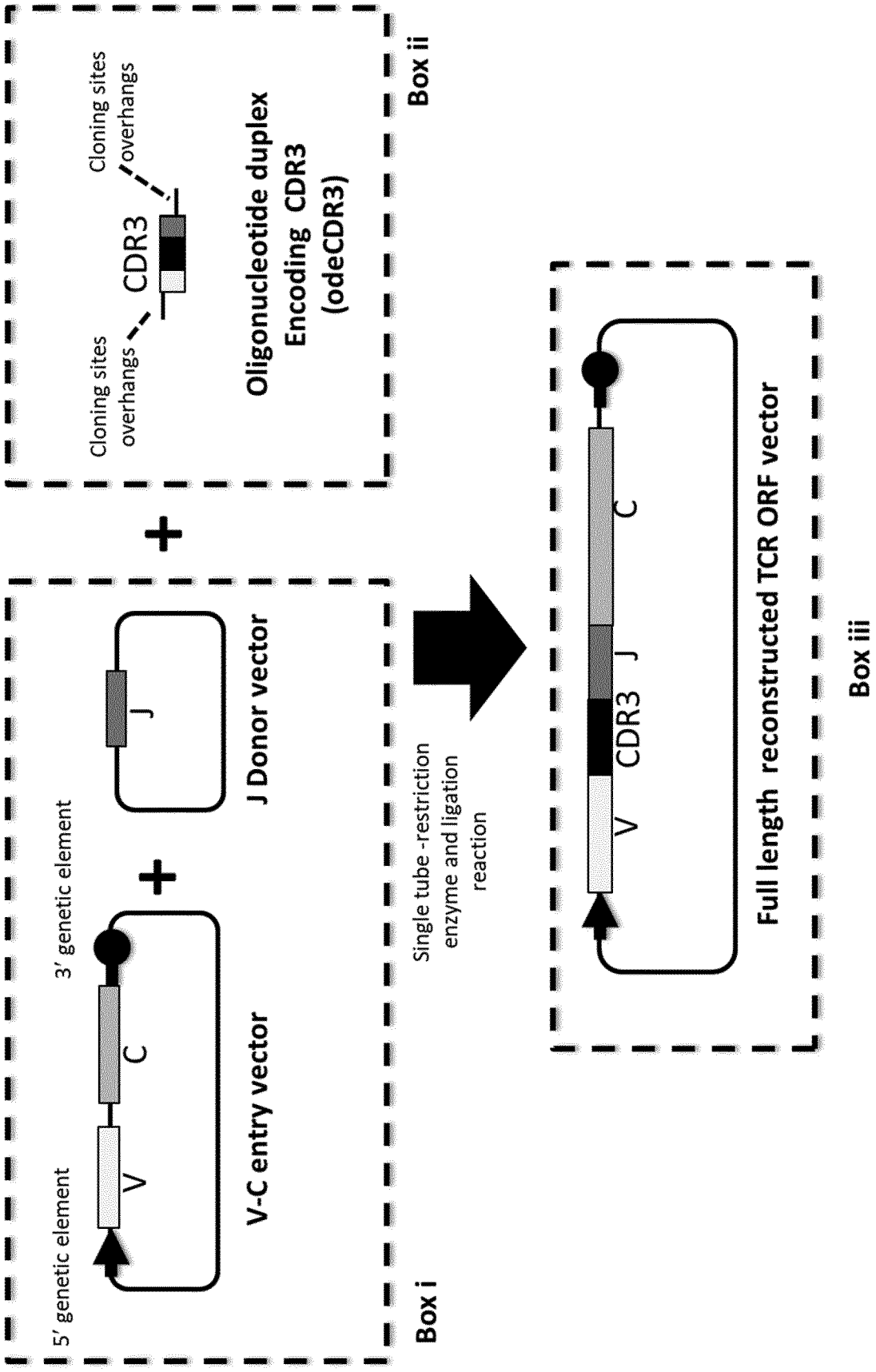

Box i
5′ genetic element
V
C
3′ genetic element
V-C entry vector
J
J Donor vector
Box ii
Cloning sites overhangs
CDR3
Cloning sites overhangs
Oligonucleotide duplex
Encoding CDR3
(odeCDR3)
Single tube -restriction enzyme and ligation reaction
Box iii
V
CDR3
J
C
Full length reconstructed TCR ORF vector

Figure 1B

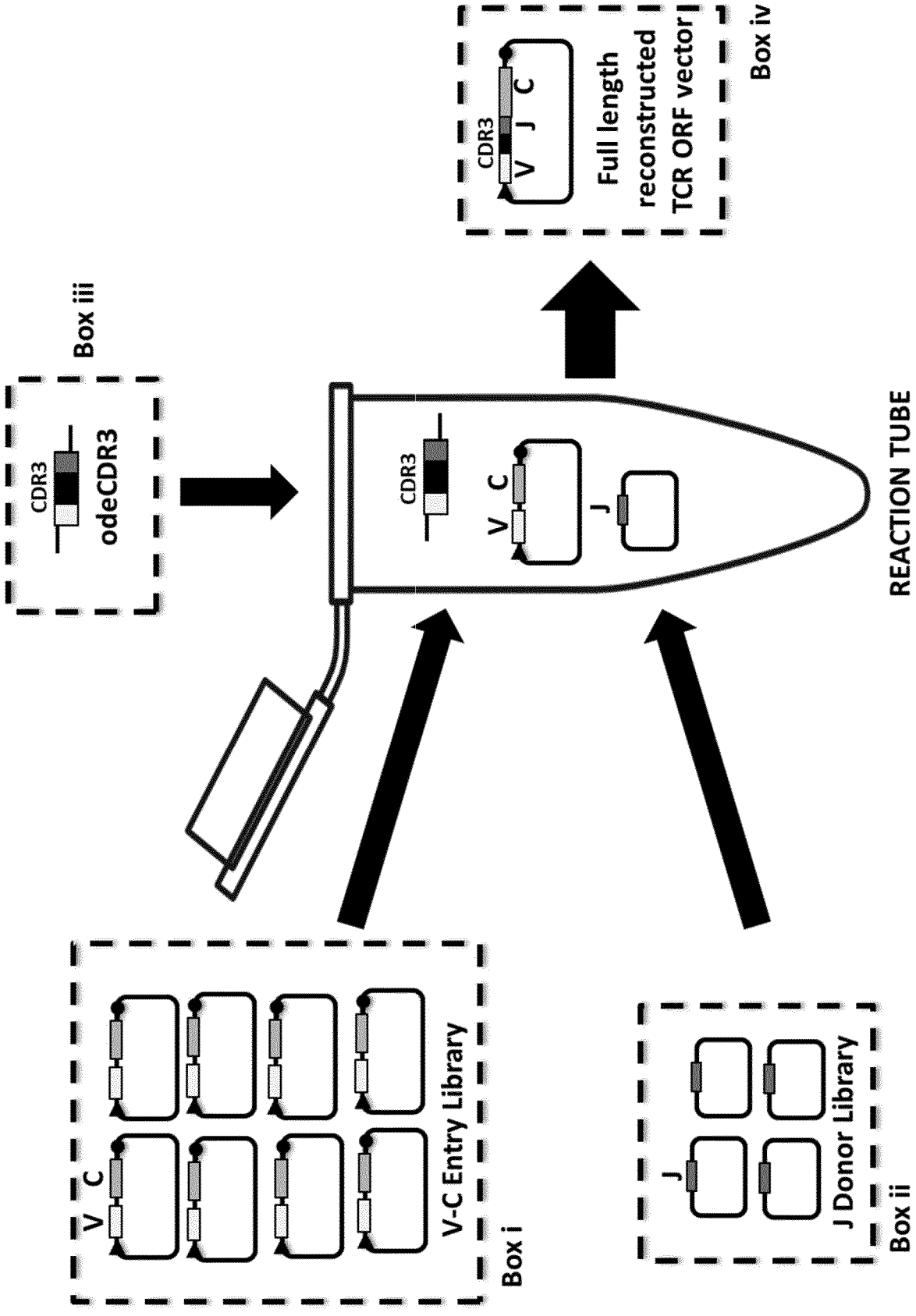

Box iv
Full length reconstructed TCR ORF vector
CDR3
V J C
Box iii
odeCDR3
CDR3
REACTION TUBE
CDR3
V C
J
Box i
V-C Entry Library
V C
Box ii
J Donor Library
J

Figure 2

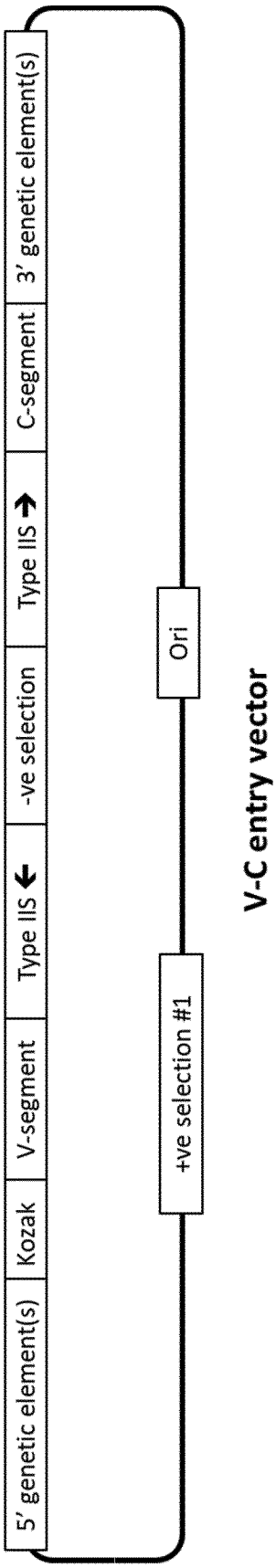

5' genetic element(s)
Kozak
V-segment
Type IIS
-ve selection
Type IIS
C-segment
3' genetic element(s)
+ve selection #1
Ori
V-C entry vector

Figure 3

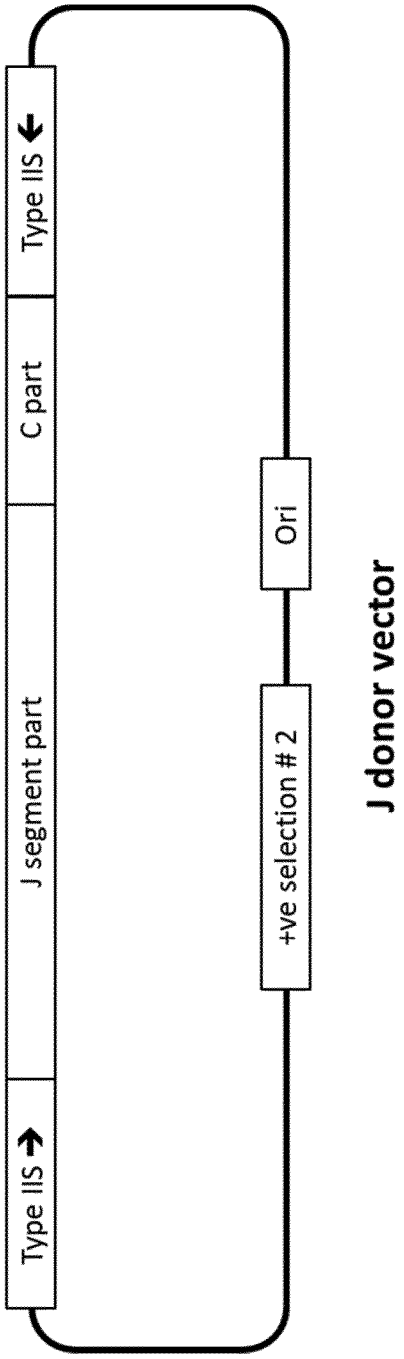

Type IIS
C part
J segment part
Ori
+ve selection # 2
Type IIS
J donor vector

Figure 4

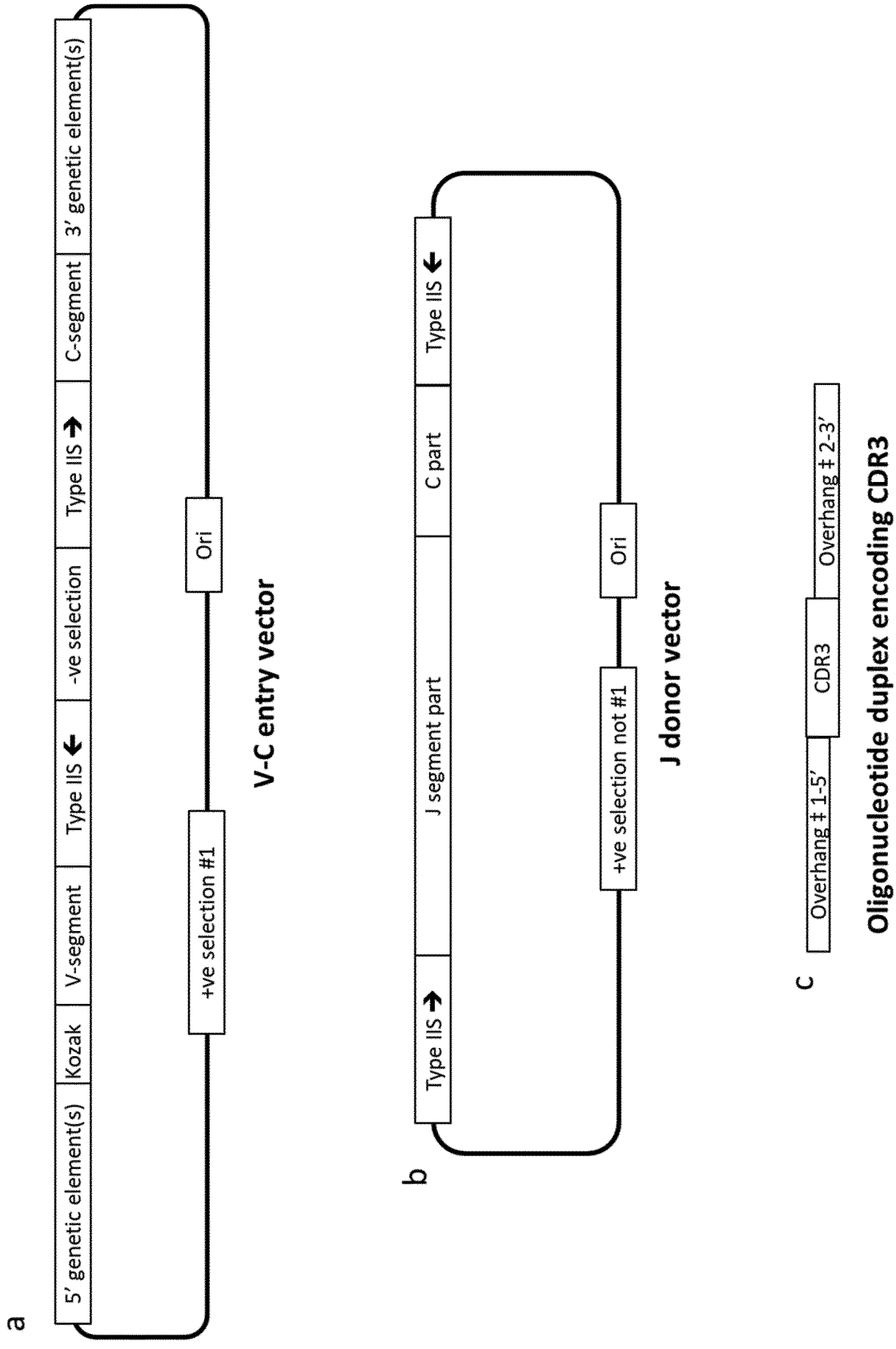

a
5' genetic element(s)
Kozak
V-segment
Type IIS
-ve selection
Type IIS
C-segment
3' genetic element(s)
+ve selection #1
Ori
V-C entry vector
b
Type IIS
J segment part
C part
Type IIS
+ve selection not #1
Ori
J donor vector
c
Overhang ‡ 1-5'
CDR3
Overhang ‡ 2-3'
Oligonucleotide duplex encoding CDR3

Figure 4

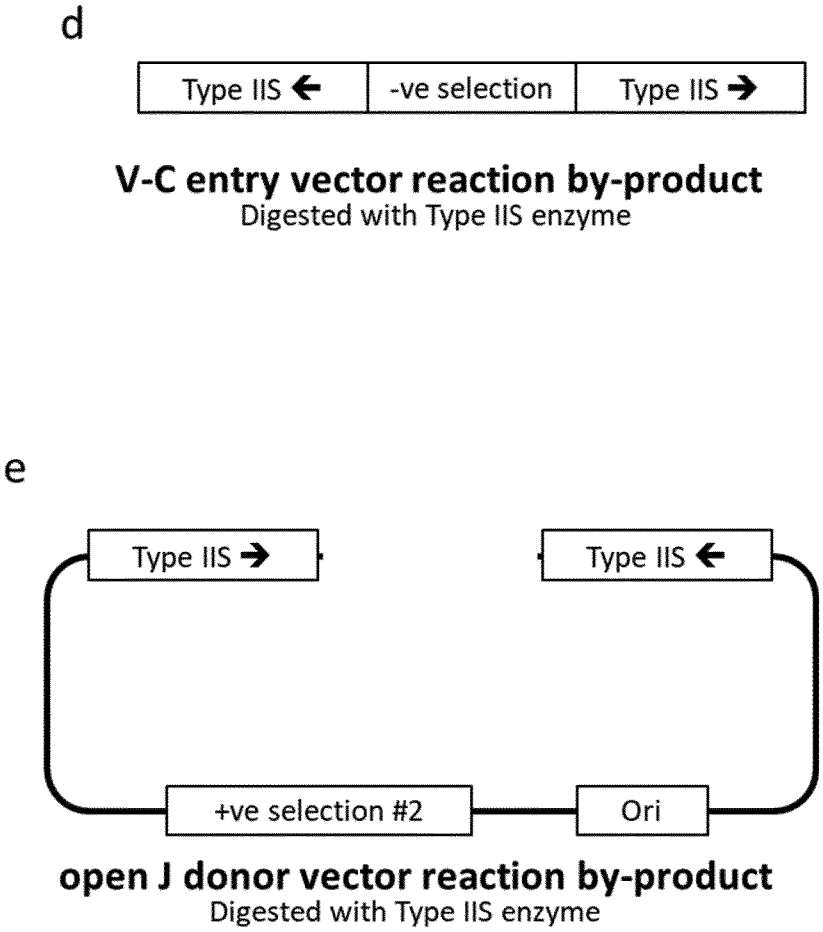

d
Type IIS
-ve selection
Type IIS
V-C entry vector reaction by-product
Digested with Type IIS enzyme
e
Type IIS
Type IIS
+ve selection #2
Ori
open J donor vector reaction by-product
Digested with Type IIS enzyme

Figure 4

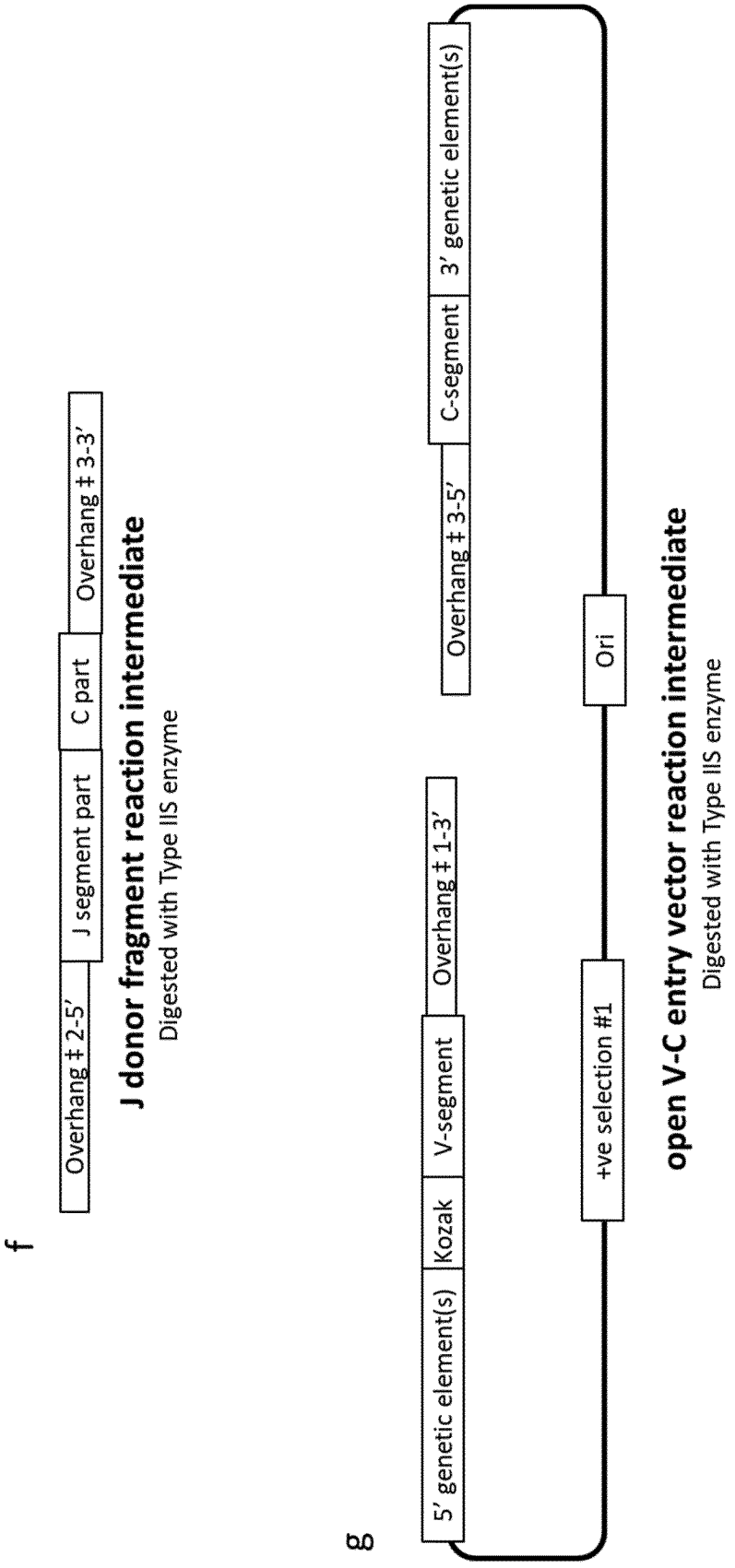

f
Overhang ǂ 2-5'
J segment part
C part
Overhang ǂ 3-3'
J donor fragment reaction intermediate
Digested with Type IIS enzyme
g
5' genetic element(s)
Kozak
V-segment
Overhang ǂ 1-3'
+ve selection #1
Ori
Overhang ǂ 3-5'
C-segment
3' genetic element(s)
open V-C entry vector reaction intermediate
Digested with Type IIS enzyme

Figure 4

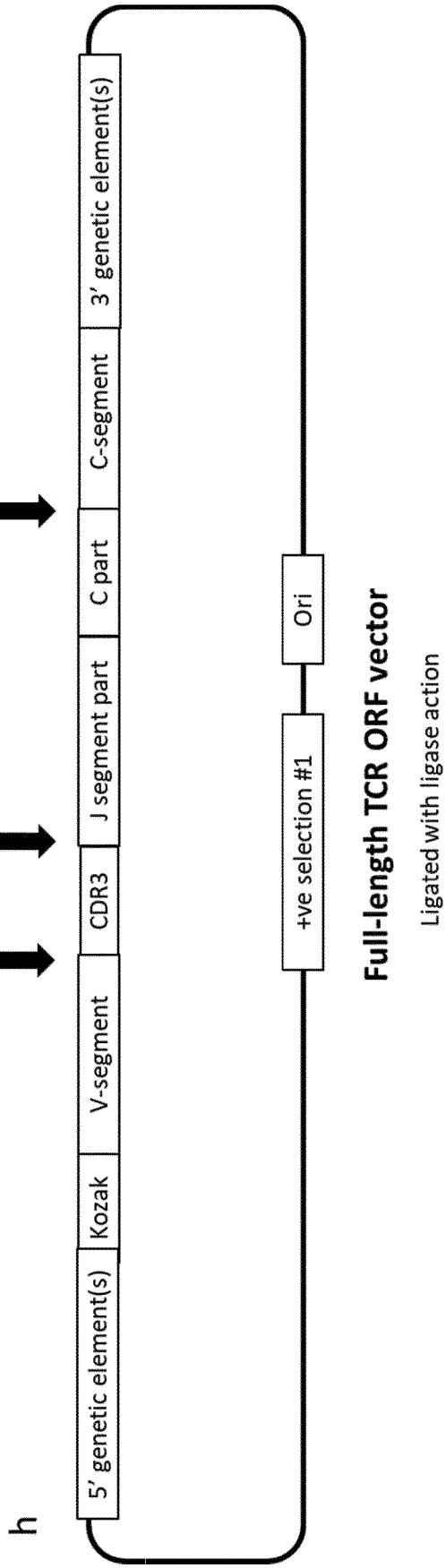

h
5' genetic element(s)
Kozak
V-segment
CDR3
J segment part
C part
C-segment
3' genetic element(s)
‡1
‡2
‡3
+ve selection #1
Ori
Full-length TCR ORF vector
Ligated with ligase action

Figure 5

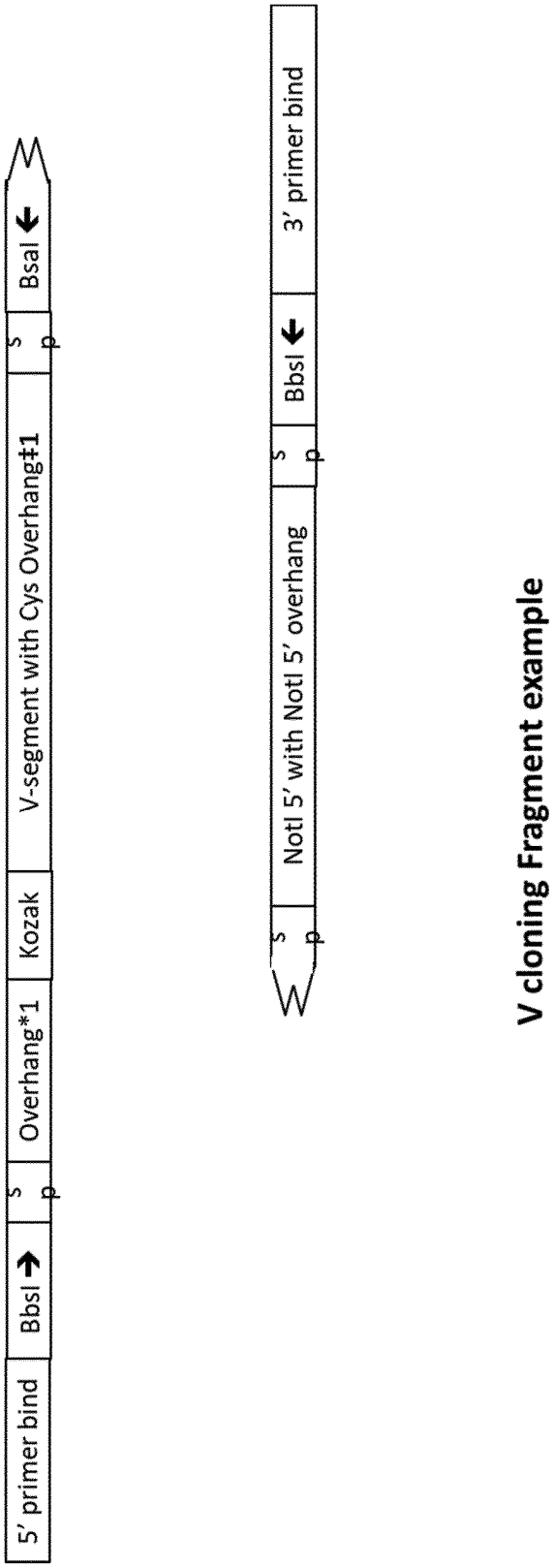
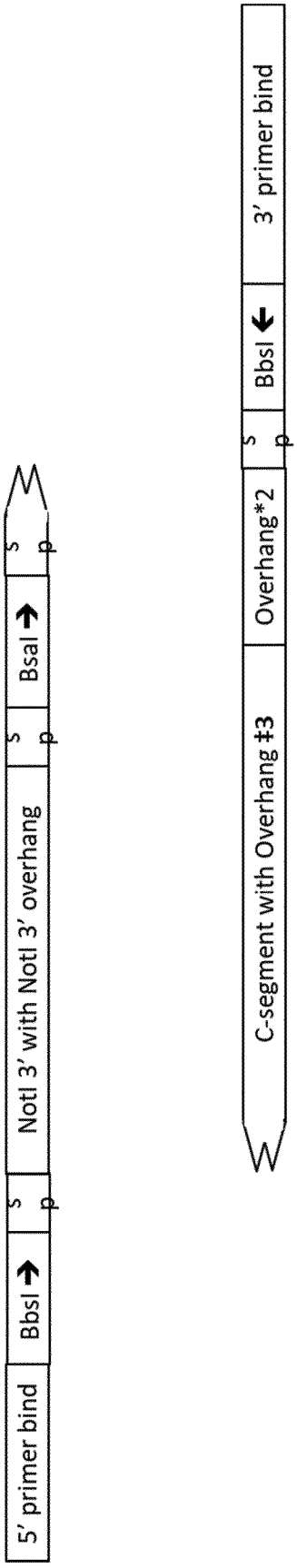
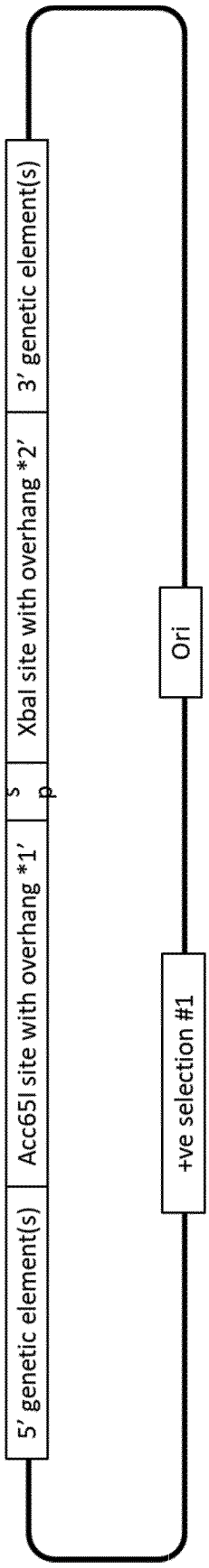

5' primer bind
BbsI
Sp
Overhang*1
Kozak
V-segment with Cys Overhang‡1
Sp
BsaI
Sp
NotI 5' with NotI 5' overhang
Sp
BbsI
Sp
3' primer bind
V cloning Fragment example

Figure 6



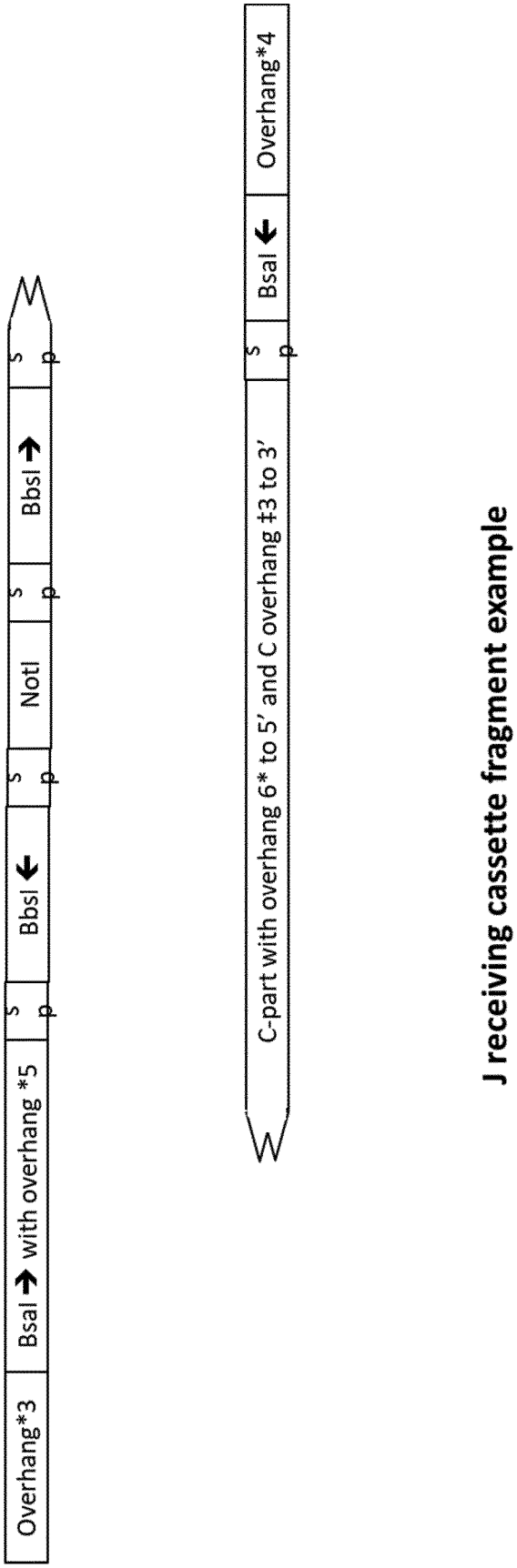
C cloning Fragment example

Figure 7



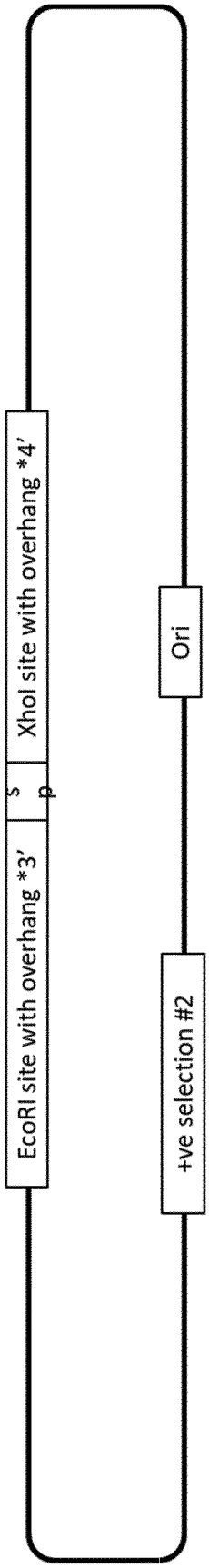
V-C entry vector backbone example

Figure 8



J receiving cassette fragment example

Figure 9



J Donor vector backbone example

Figure 10

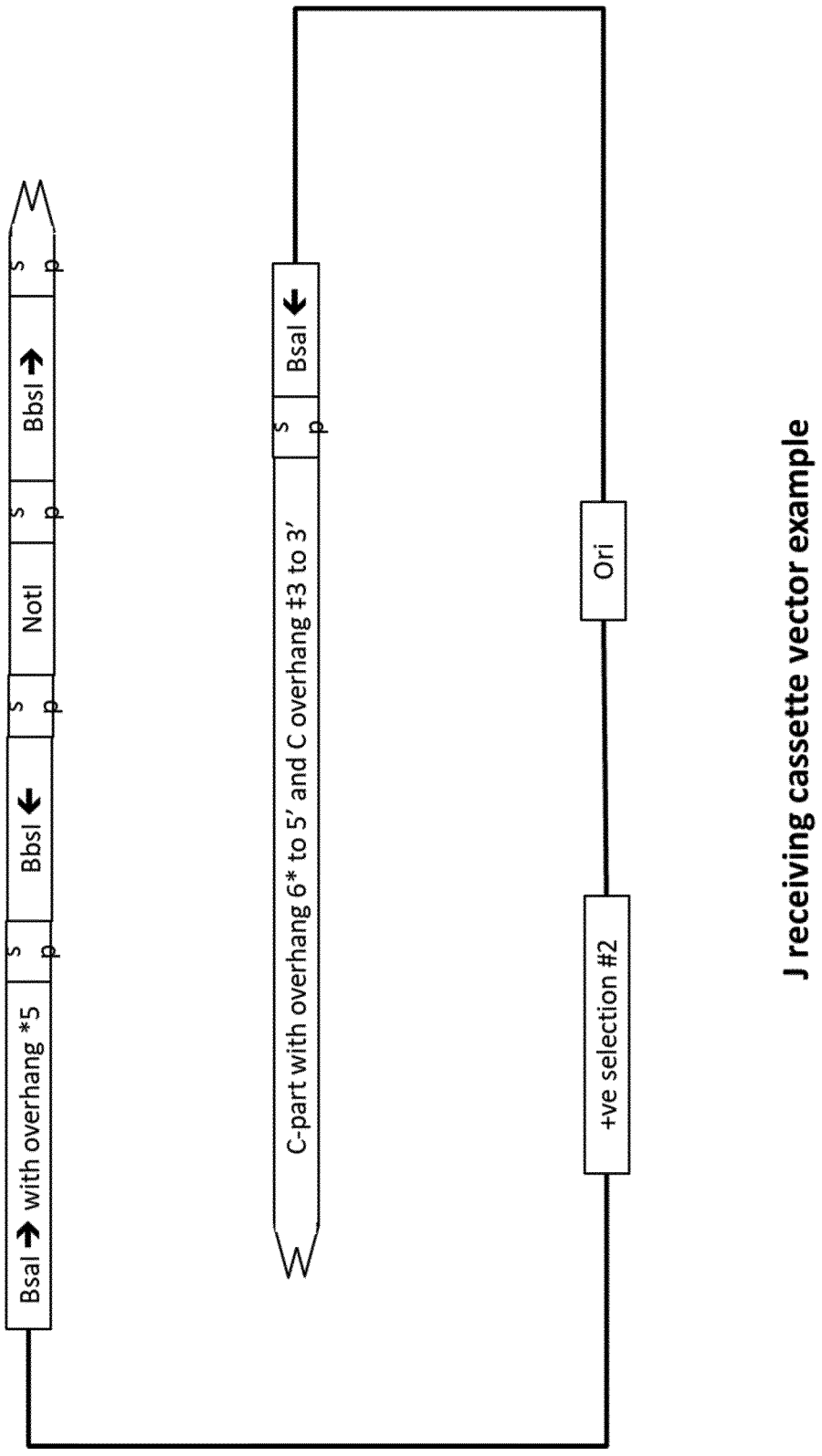
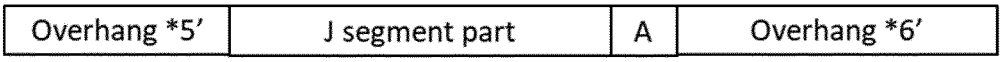

J receiving cassette vector example
Bsal with overhang *5
s
p
BbsI
BbsI
s
p
NotI
s
p
BbsI
s
p
C-part with overhang 6* to 5' and C overhang ‡3 to 3'
s
p
Bsal
Ori
+ve selection #2

Figure 11



J segment part example

Figure 12

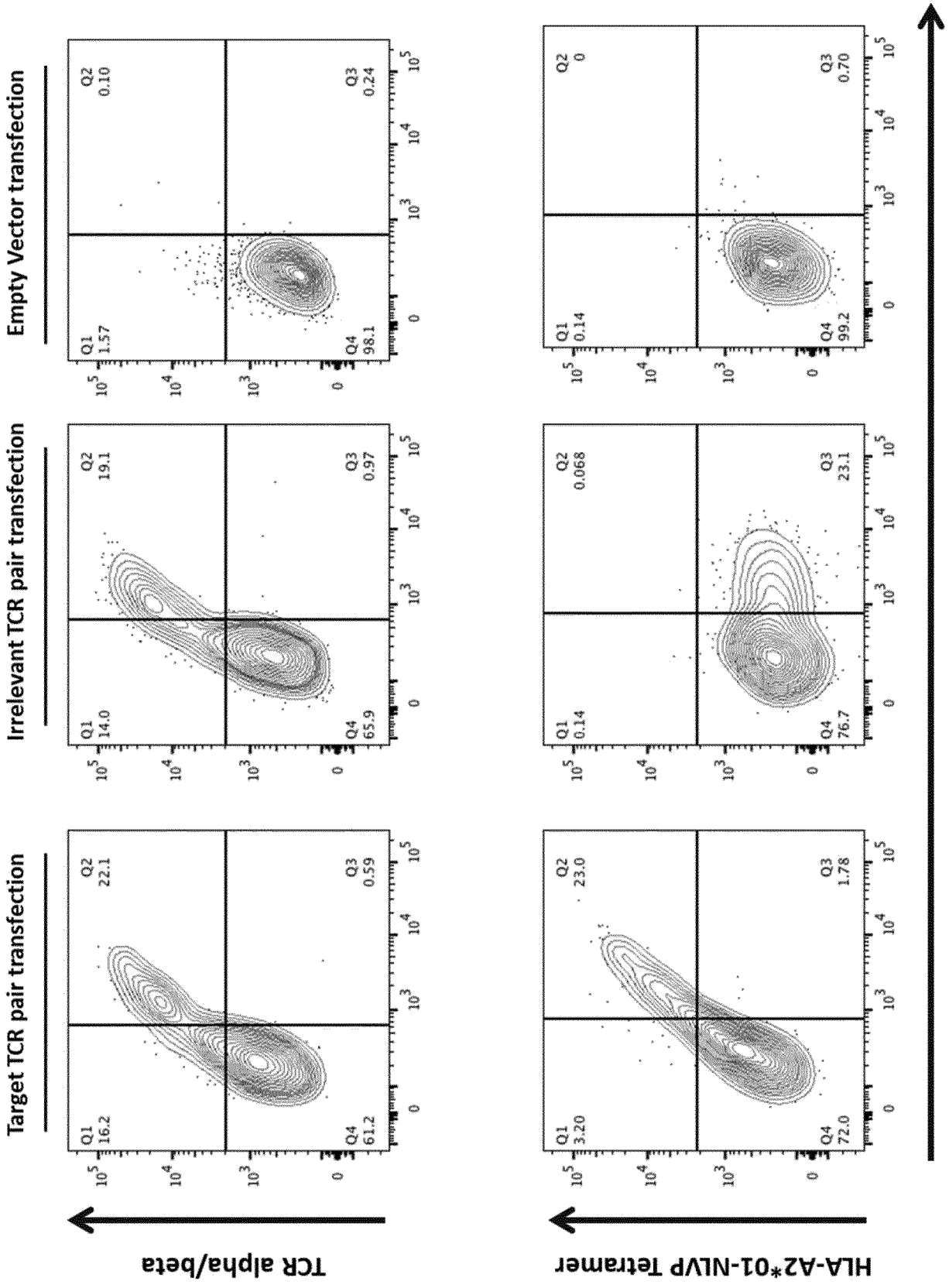

Empty Vector transfection
Irrelevant TCR pair transfection
Target TCR pair transfection
TCR alpha/beta
HLA-A2*01-NLVP Tetramer
CD8
Q1 1.57
Q2 0.10
Q3 0.24
Q4 98.1
Q1 14.0
Q2 19.1
Q3 0.97
Q4 65.9
Q1 16.2
Q2 22.1
Q3 0.59
Q4 61.2
Q1 0.14
Q2 0
Q3 0.70
Q4 99.2
Q1 0.14
Q2 0.068
Q3 23.1
Q4 76.7
Q1 3.20
Q2 23.0
Q3 1.78
Q4 72.0

Figure 13

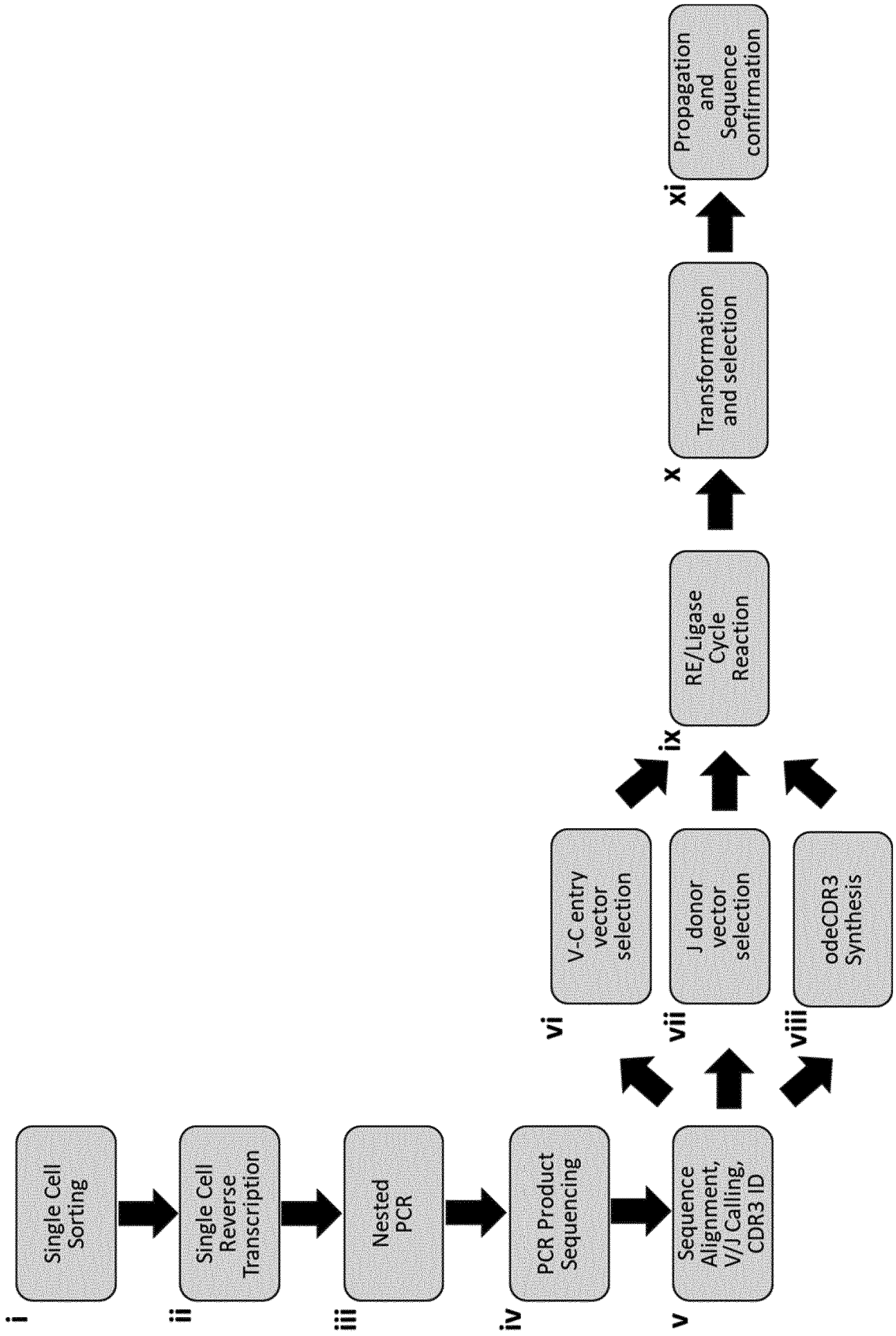

Single Cell Sorting
i
Single Cell Reverse Transcription
ii
Nested PCR
iii
PCR Product Sequencing
iv
Sequence Alignment, V/J Calling, CDR3 ID
v
V-C entry vector selection
vi
J donor vector selection
vii
odeCDR3 Synthesis
viii
RE/Ligase Cycle Reaction
ix
Transformation and selection
x
Propagation and Sequence confirmation
xi

Figure 14

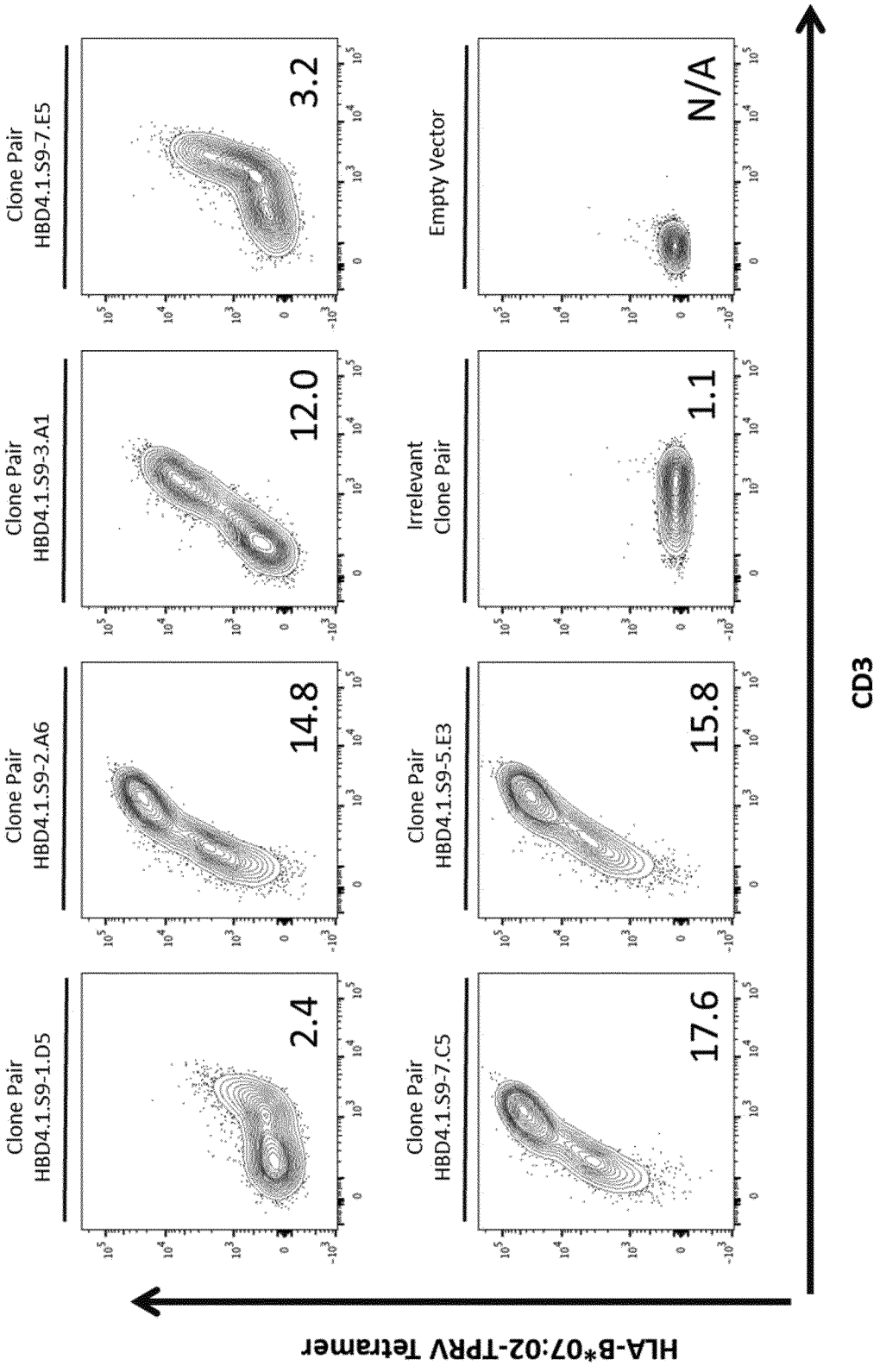

Clone Pair HBD4.1.S9-1.D5
2.4
Clone Pair HBD4.1.S9-2.A6
14.8
Clone Pair HBD4.1.S9-3.A1
12.0
Clone Pair HBD4.1.S9-7.E5
3.2
Clone Pair HBD4.1.S9-7.C5
17.6
Clone Pair HBD4.1.S9-5.E3
15.8
Irrelevant Clone Pair
1.1
Empty Vector
N/A
HLA-B*07:02-TPRV Tetramer
CD3

Figure 15

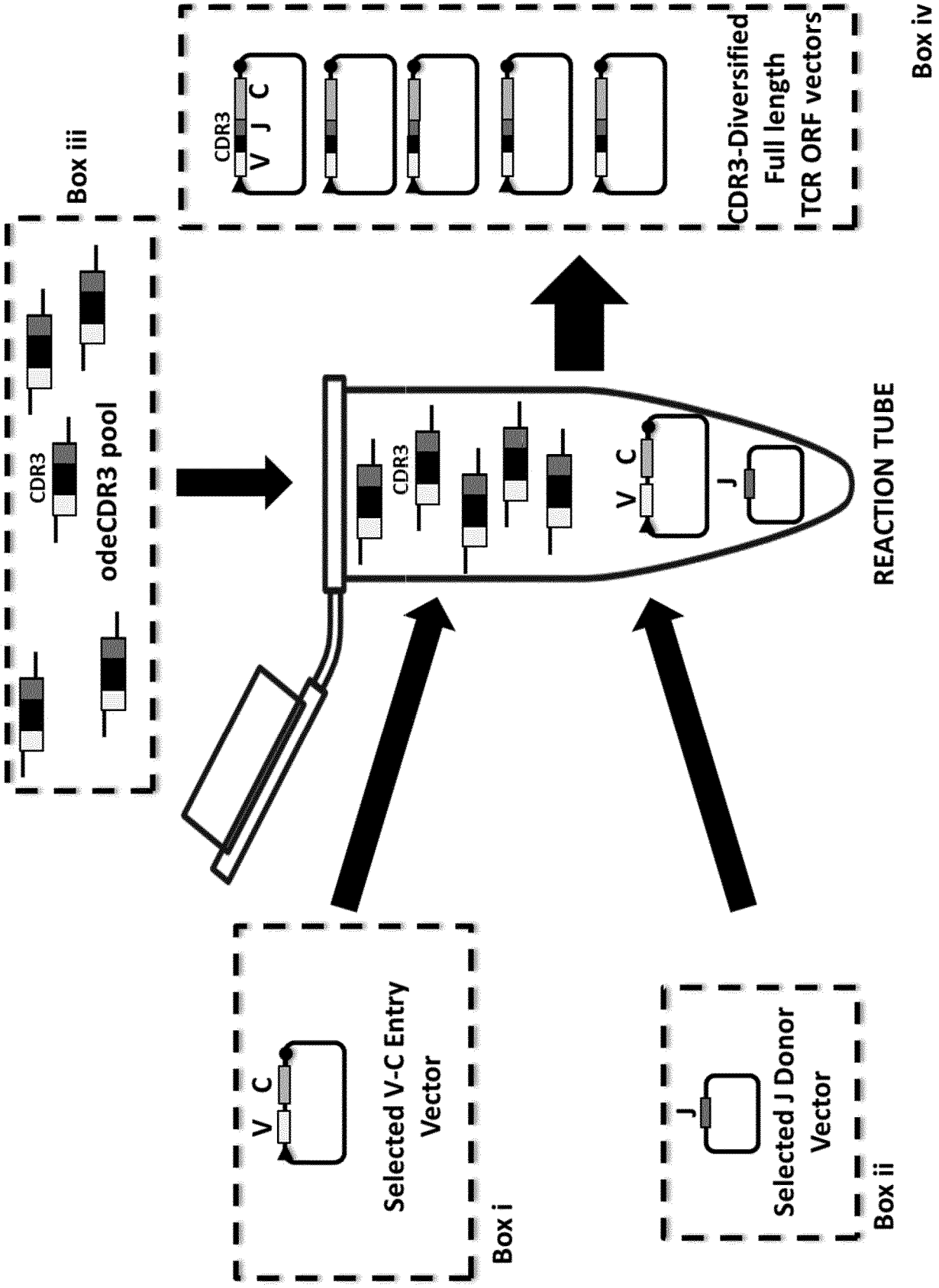

Box iii
odeCDR3 pool
CDR3
REACTION TUBE
CDR3
V C
J
CDR3
V J C
CDR3-Diversified
Full length
TCR ORF vectors
Box iv
Selected V-C Entry
Vector
V C
Box i
Selected J Donor
Vector
J
Box ii

Figure 16A

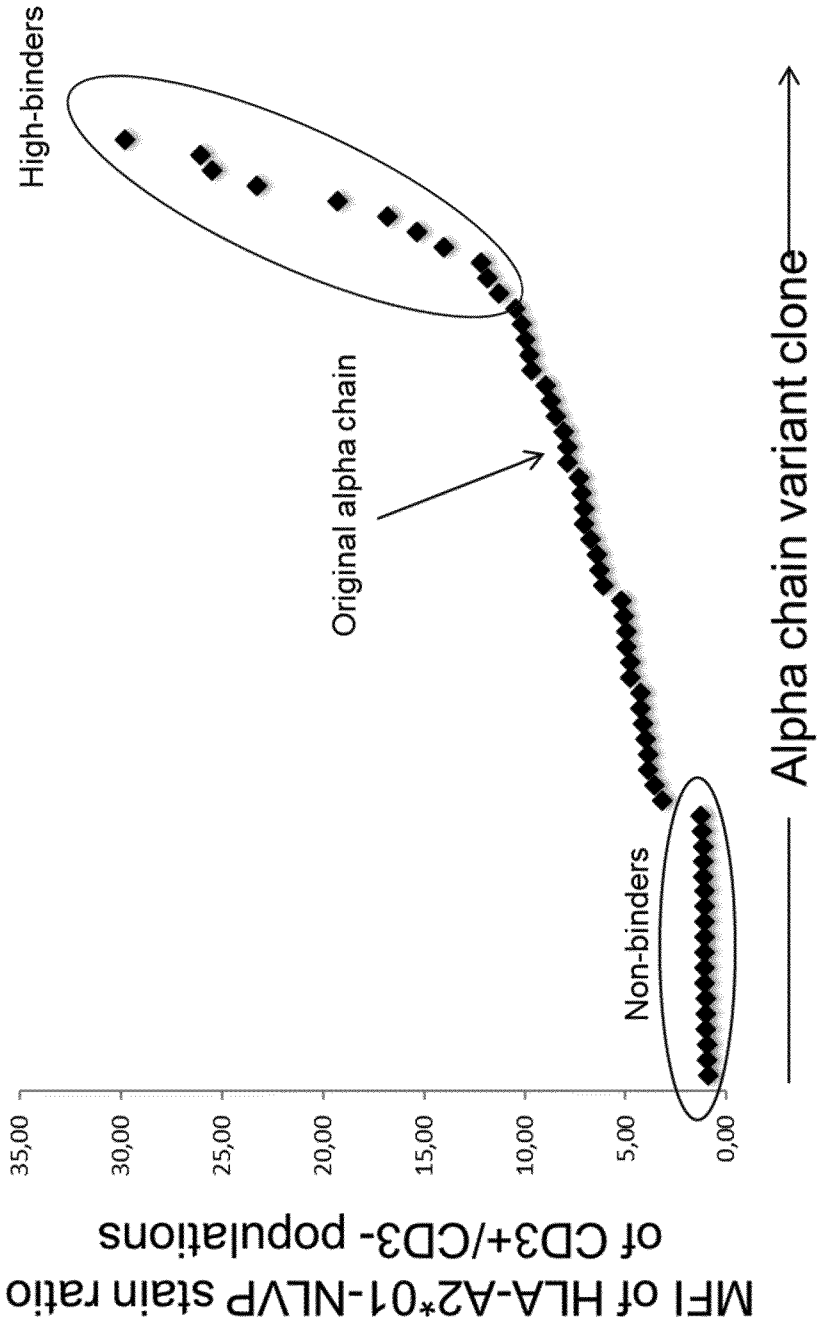

MFI of HLA-A2*01-NLVP stain ratio of CD3+/CD3- populations
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
Non-binders
Original alpha chain
High-binders
Alpha chain variant clone

Figure 16B

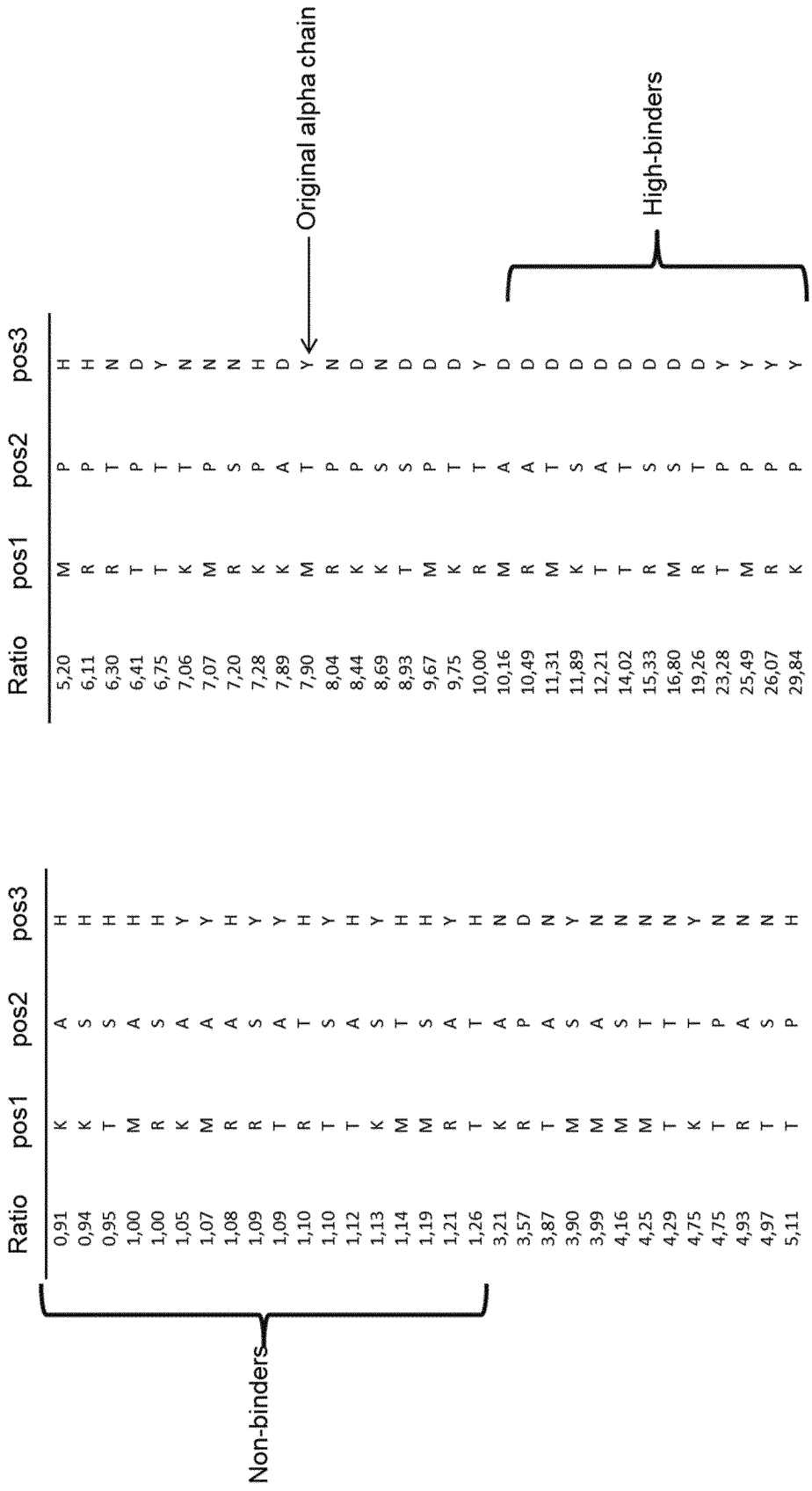

Non-binders
Original alpha chain
High-binders
Ratio pos1 pos2 pos3
0,91 K A H
0,94 K S H
0,95 T S H
1,00 M A H
1,00 R S H
1,05 K A Y
1,07 M A Y
1,08 R A H
1,09 R S Y
1,09 T A Y
1,10 R T H
1,10 T S Y
1,12 T A H
1,13 K S Y
1,14 M T H
1,19 M S H
1,21 R A Y
1,26 T T H
3,21 K A N
3,57 R P D
3,87 T A N
3,90 M S Y
3,99 M A N
4,16 M S N
4,25 M T N
4,29 T T N
4,75 K T Y
4,75 T P N
4,93 R A N
4,97 T S N
5,11 T P H
Ratio pos1 pos2 pos3
5,20 M P H
6,11 R P H
6,30 R T N
6,41 T P D
6,75 T T Y
7,06 K T N
7,07 M P N
7,20 R S N
7,28 K P H
7,89 K A D
7,90 M T Y
8,04 R P N
8,44 K P D
8,69 K S N
8,93 T S D
9,67 M P D
9,75 K T D
10,00 R T Y
10,16 M A D
10,49 R A D
11,31 M T D
11,89 K S D
12,21 T A D
14,02 T T D
15,33 R S D
16,80 M S D
19,26 R T D
23,28 T P Y
25,49 M P Y
26,07 R P Y
29,84 K P Y

Figure 17

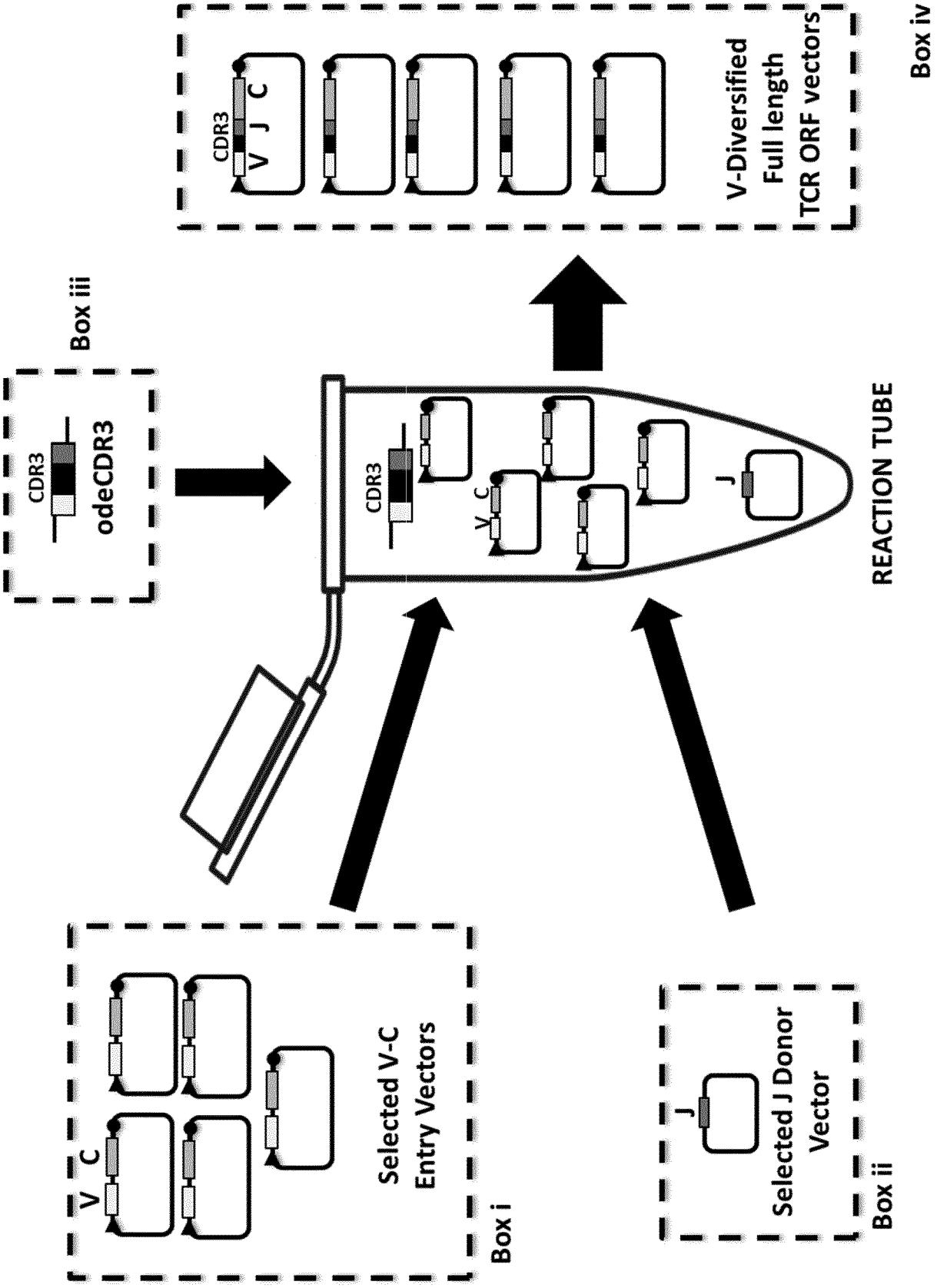

Box iv
V-Diversified
Full length
TCR ORF vectors
CDR3
V J C
Box iii
odeCDR3
CDR3
REACTION TUBE
CDR3
V C
J
Box i
Selected V-C
Entry Vectors
V C
Box ii
Selected J Donor
Vector
J

Figure 18

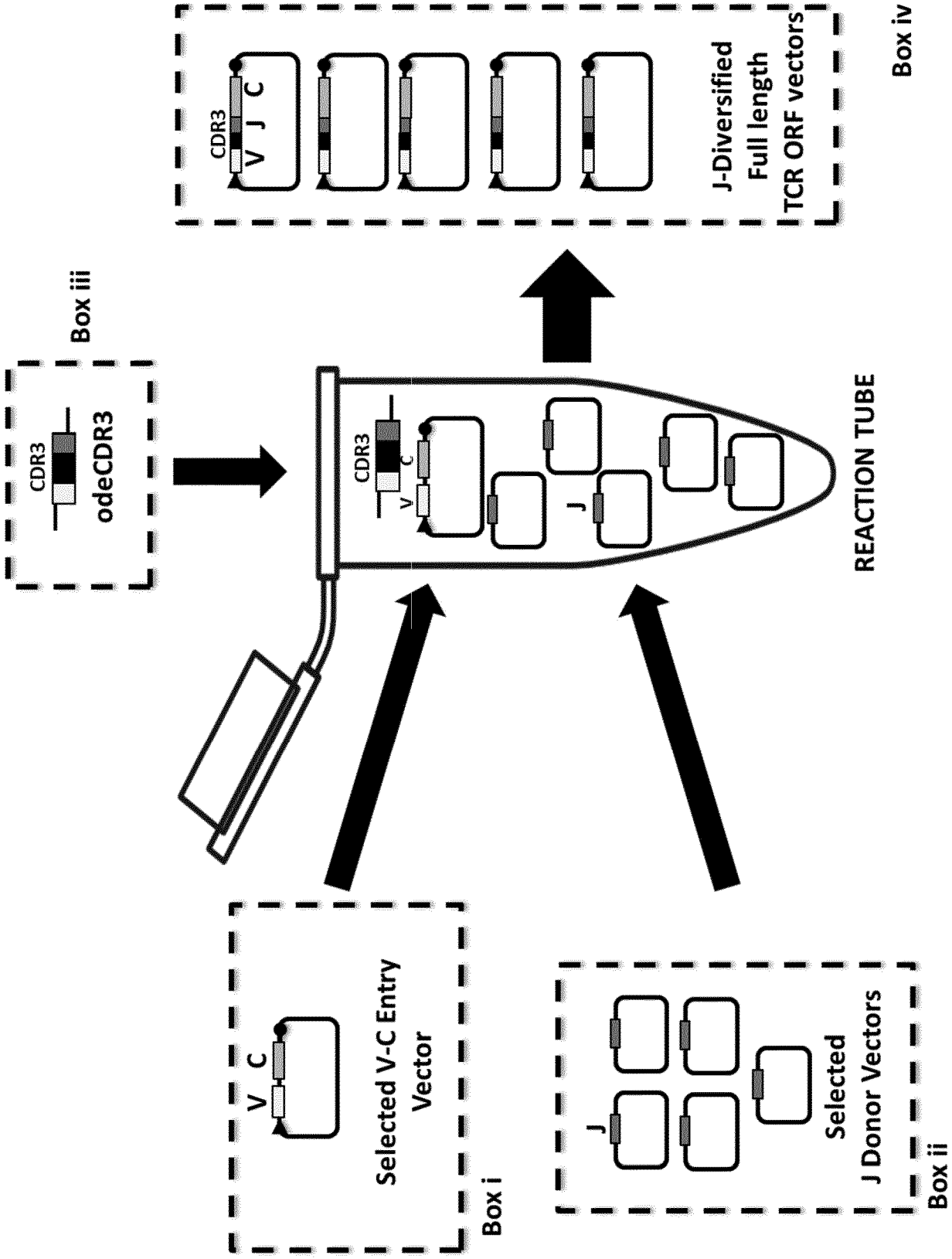

Box iv
J-Diversified
Full length
TCR ORF vectors
CDR3
V J C
Box iii
odeCDR3
CDR3
REACTION TUBE
CDR3
V
C
J
Box i
Selected V-C Entry
Vector
V C
Box ii
Selected
J Donor Vectors
J

Figure 19

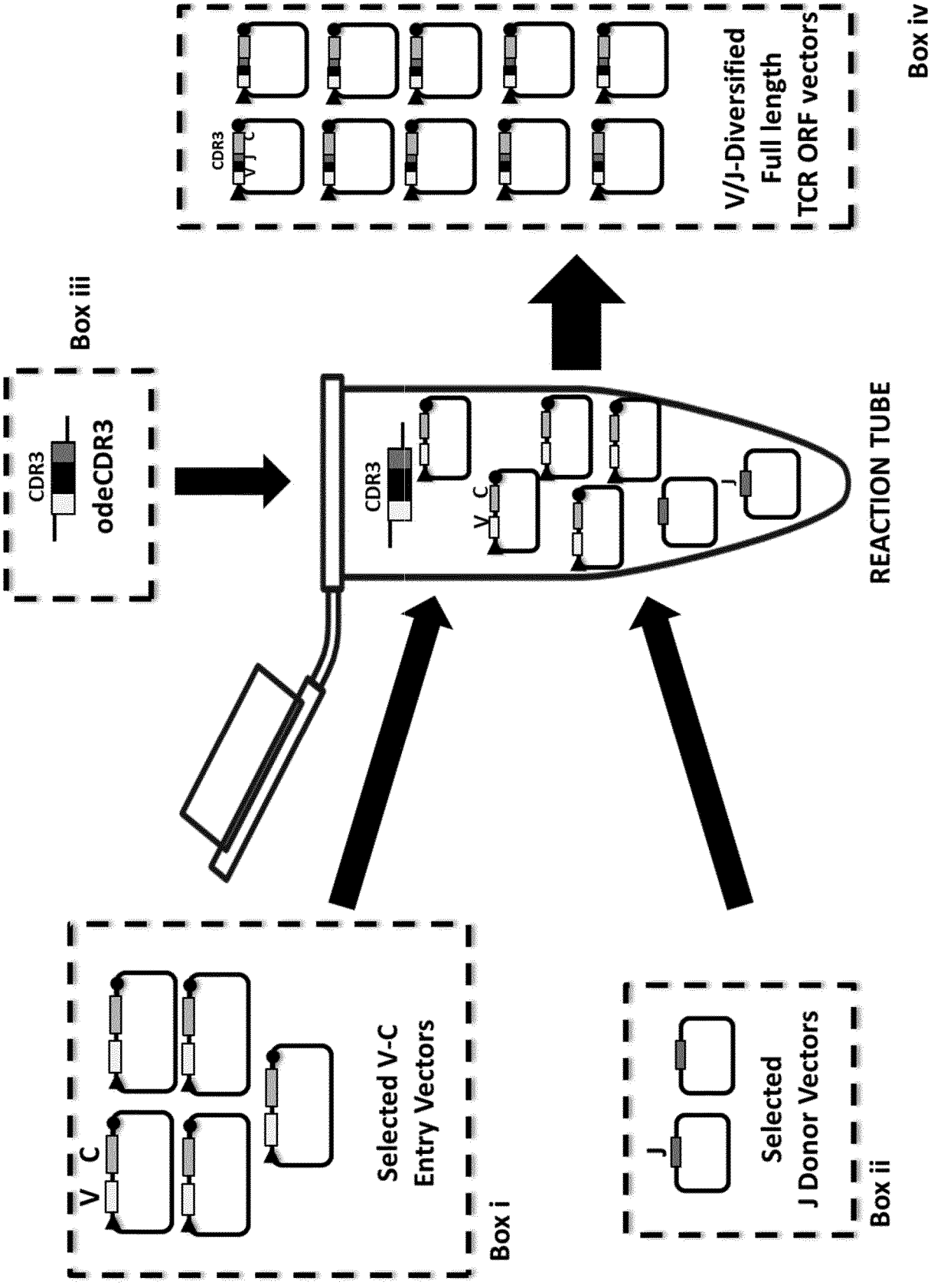

Box iii
CDR3
odeCDR3
CDR3
V C
J
REACTION TUBE
Box iv
CDR3
V J C
V/J-Diversified
Full length
TCR ORF vectors
V C
Selected V-C
Entry Vectors
Box i
J
Selected
J Donor Vectors
Box ii

Figure 20

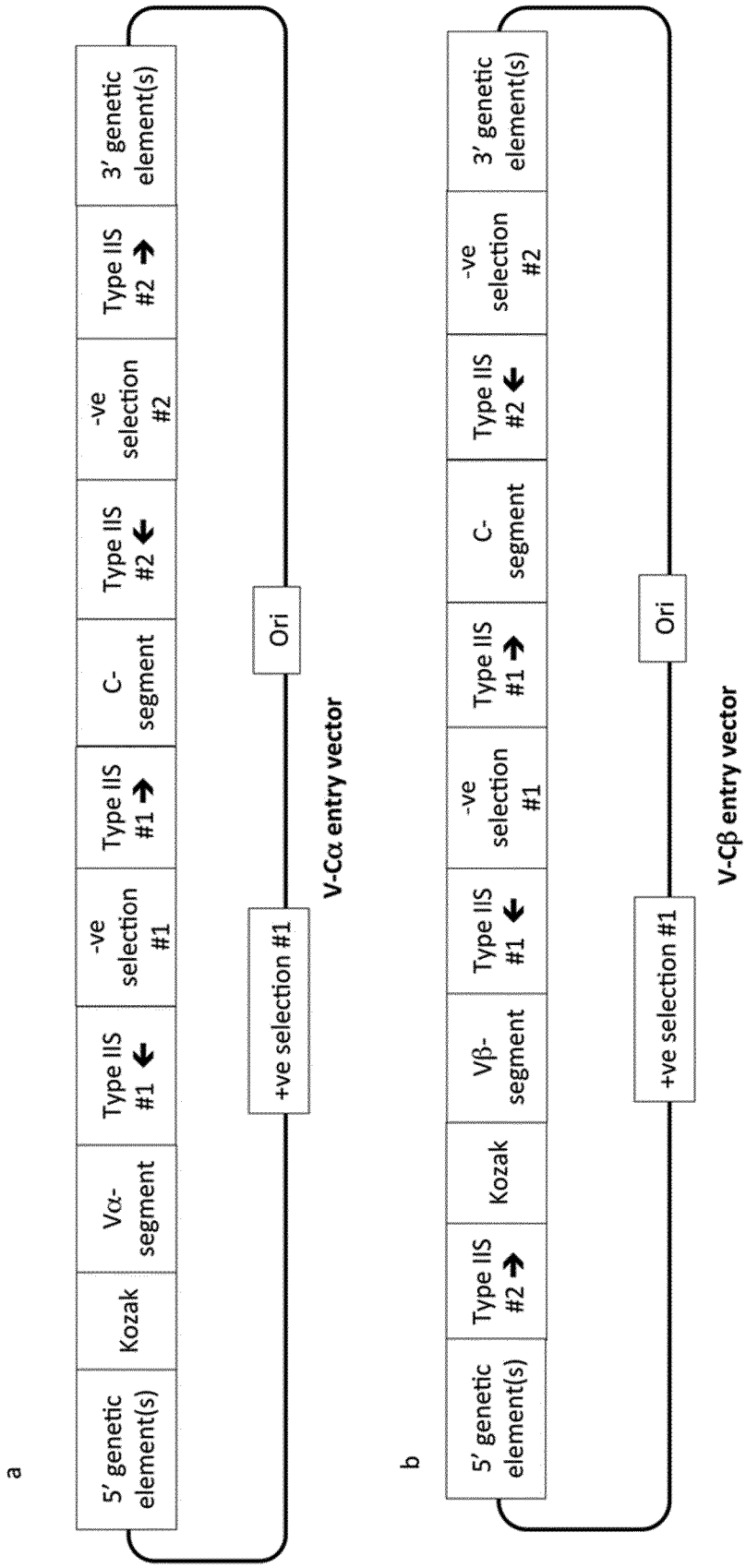
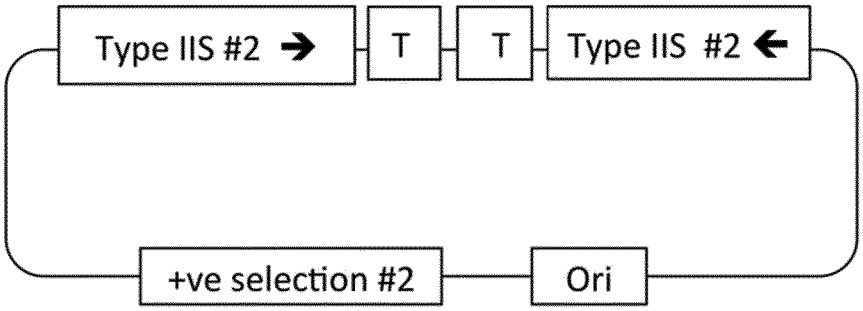

a
5' genetic element(s)
Kozak
Vα-segment
Type IIS #1
-ve selection #1
Type IIS #1
C-segment
Type IIS #2
-ve selection #2
Type IIS #2
3' genetic element(s)
Ori
+ve selection #1
V-Cα entry vector
b
5' genetic element(s)
Type IIS #2
Kozak
Vβ-segment
Type IIS #1
-ve selection #1
Type IIS #1
C-segment
Type IIS #2
-ve selection #2
3' genetic element(s)
Ori
+ve selection #1
V-Cβ entry vector

Figure 21



Bidirectional terminator (BiT) donor vector

Figure 22

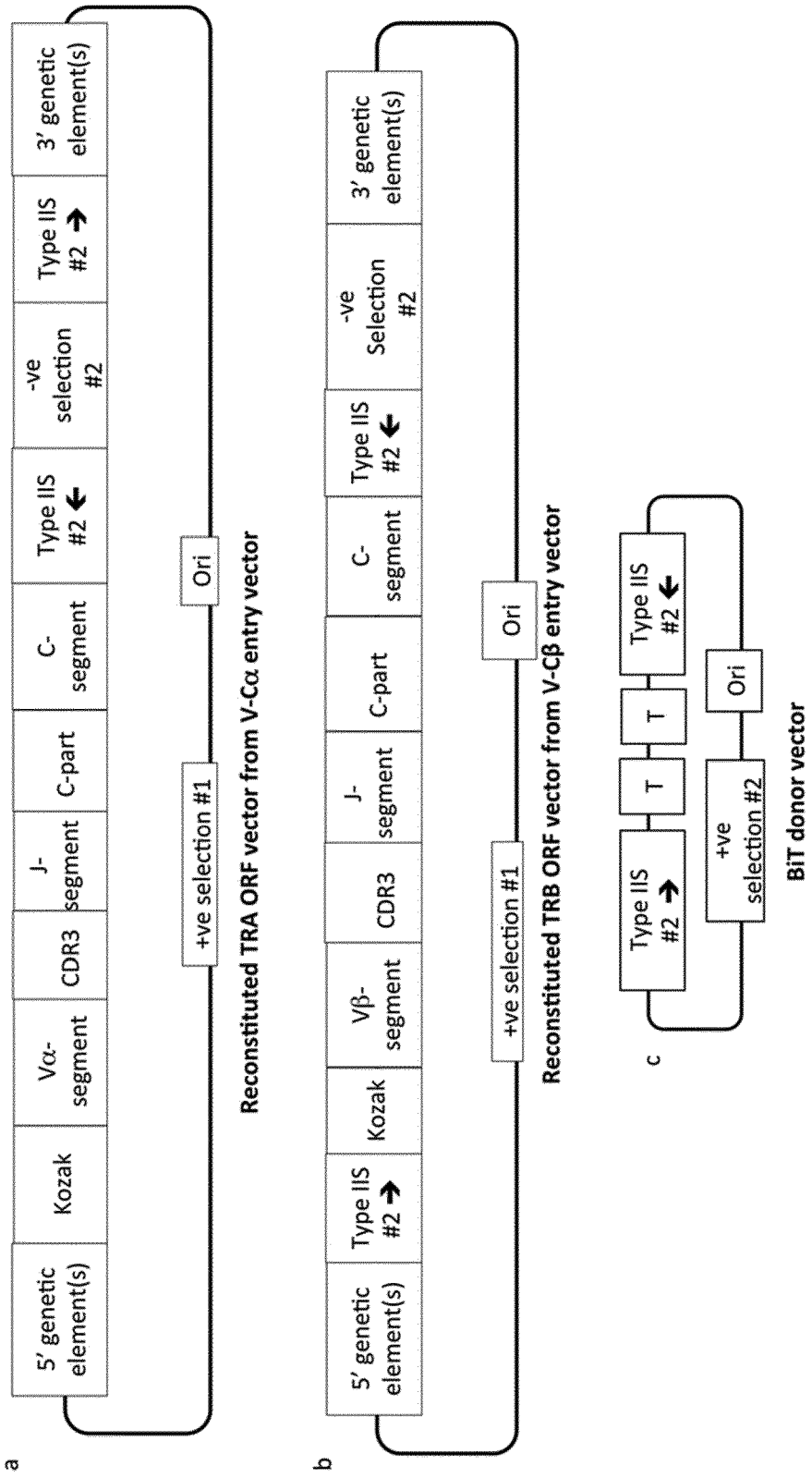

a
5' genetic element(s)
Kozak
Vα-segment
CDR3
J-segment
C-part
C-segment
Type IIS #2
-ve selection #2
Type IIS #2
3' genetic element(s)
+ve selection #1
Ori
Reconstituted TRA ORF vector from V-Cα entry vector
b
5' genetic element(s)
Type IIS #2
Kozak
Vβ-segment
CDR3
J-segment
C-part
C-segment
Type IIS #2
-ve Selection #2
3' genetic element(s)
+ve selection #1
Ori
Reconstituted TRB ORF vector from V-Cβ entry vector
c
Type IIS #2
T
T
Type IIS #2
+ve selection #2
Ori
BiT donor vector

Figure 22

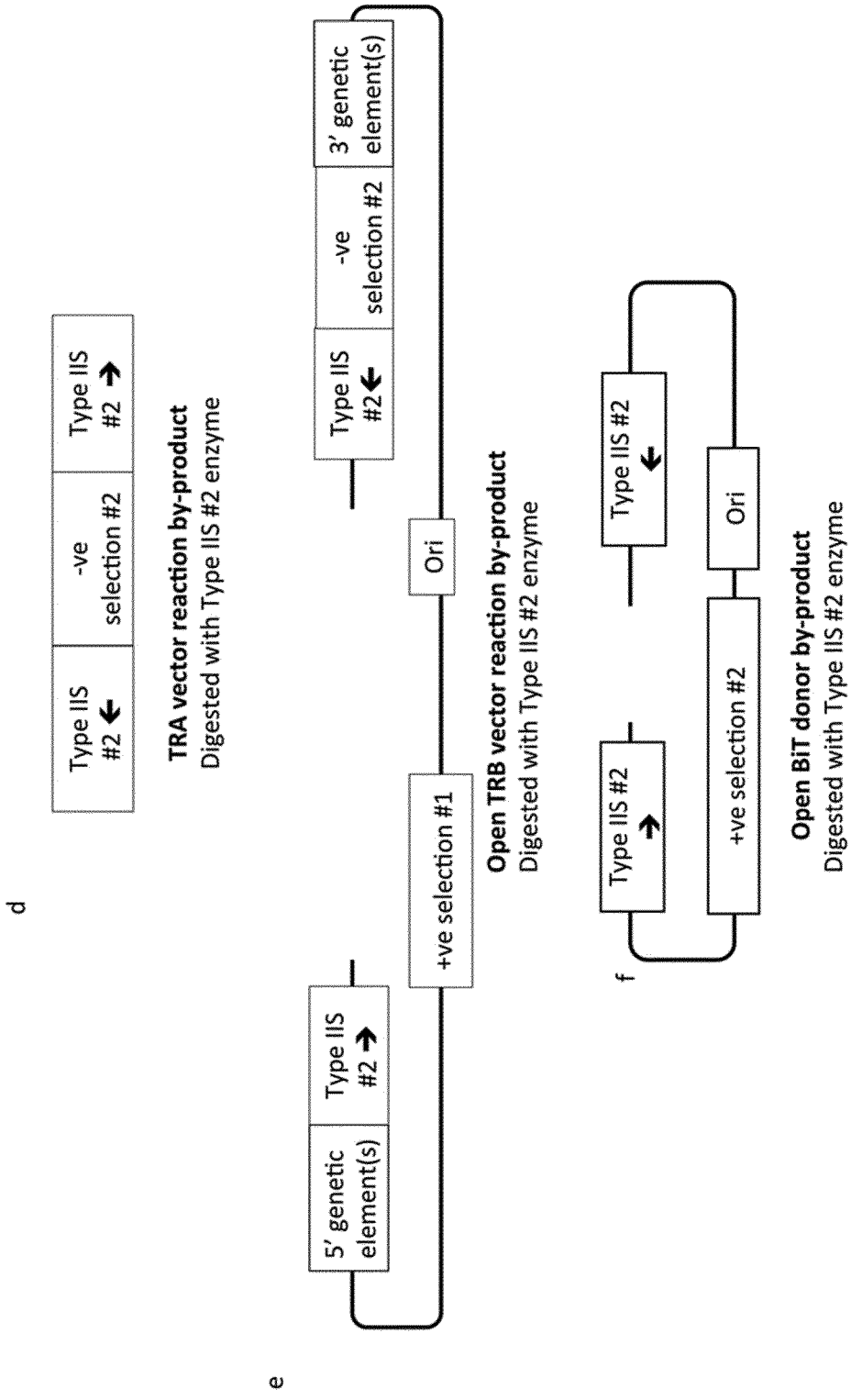

d
Type IIS #2
-ve selection #2
Type IIS #2
TRA vector reaction by-product
Digested with Type IIS #2 enzyme
e
5' genetic element(s)
Type IIS #2
+ve selection #1
Ori
Type IIS #2
-ve selection #2
3' genetic element(s)
Open TRB vector reaction by-product
Digested with Type IIS #2 enzyme
f
Type IIS #2
+ve selection #2
Ori
Type IIS #2
Open BiT donor by-product
Digested with Type IIS #2 enzyme

Figure 22

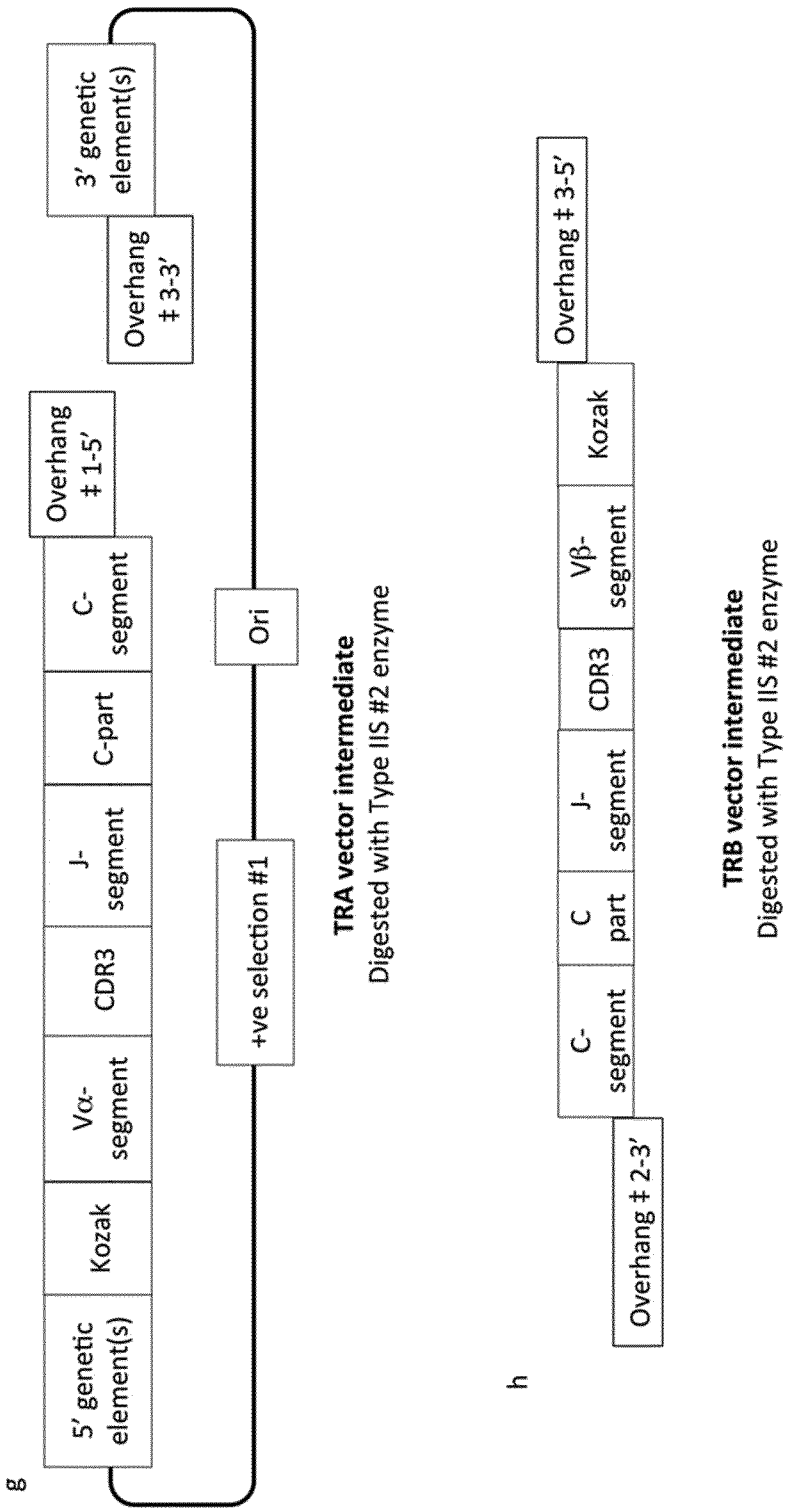
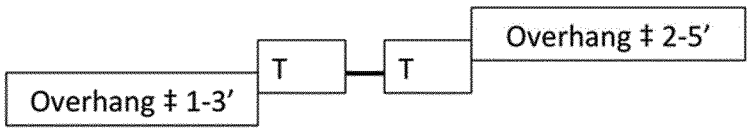

g
5' genetic element(s)
Kozak
Vα-segment
CDR3
J-segment
C-part
C-segment
Overhang ‡ 1-5'
Overhang ‡ 3-3'
3' genetic element(s)
Ori
+ve selection #1
TRA vector intermediate
Digested with Type IIS #2 enzyme
h
Overhang ‡ 2-3'
C-segment
C part
J-segment
CDR3
Vβ-segment
Kozak
Overhang ‡ 3-5'
TRB vector intermediate
Digested with Type IIS #2 enzyme

Figure 22

i



BiT donor intermediate
Digested with Type IIS #2 enzyme

Figure 22

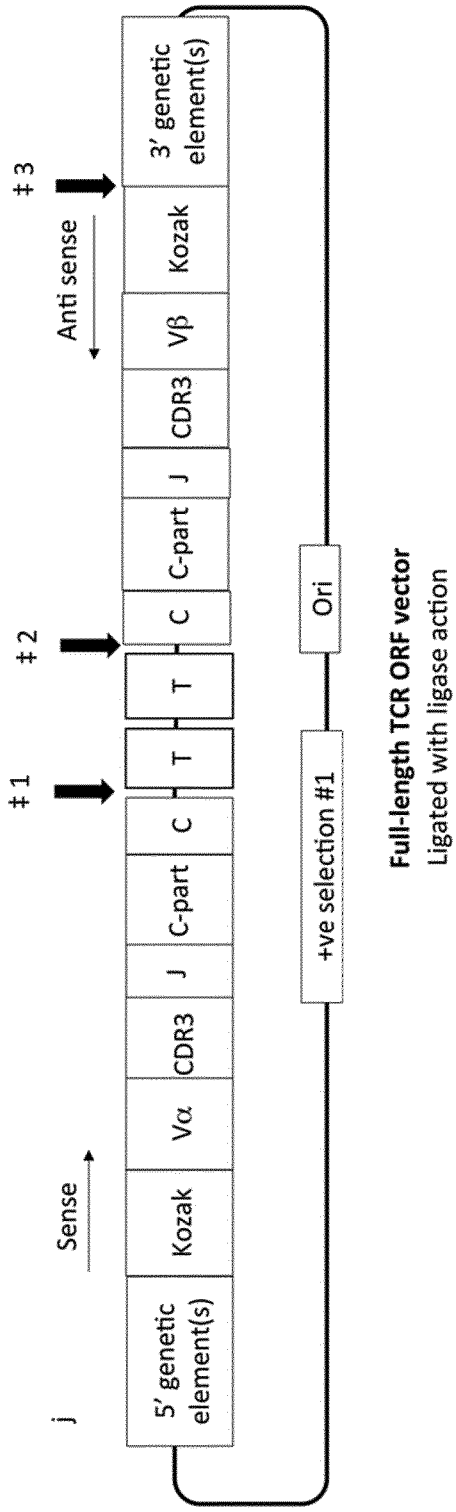
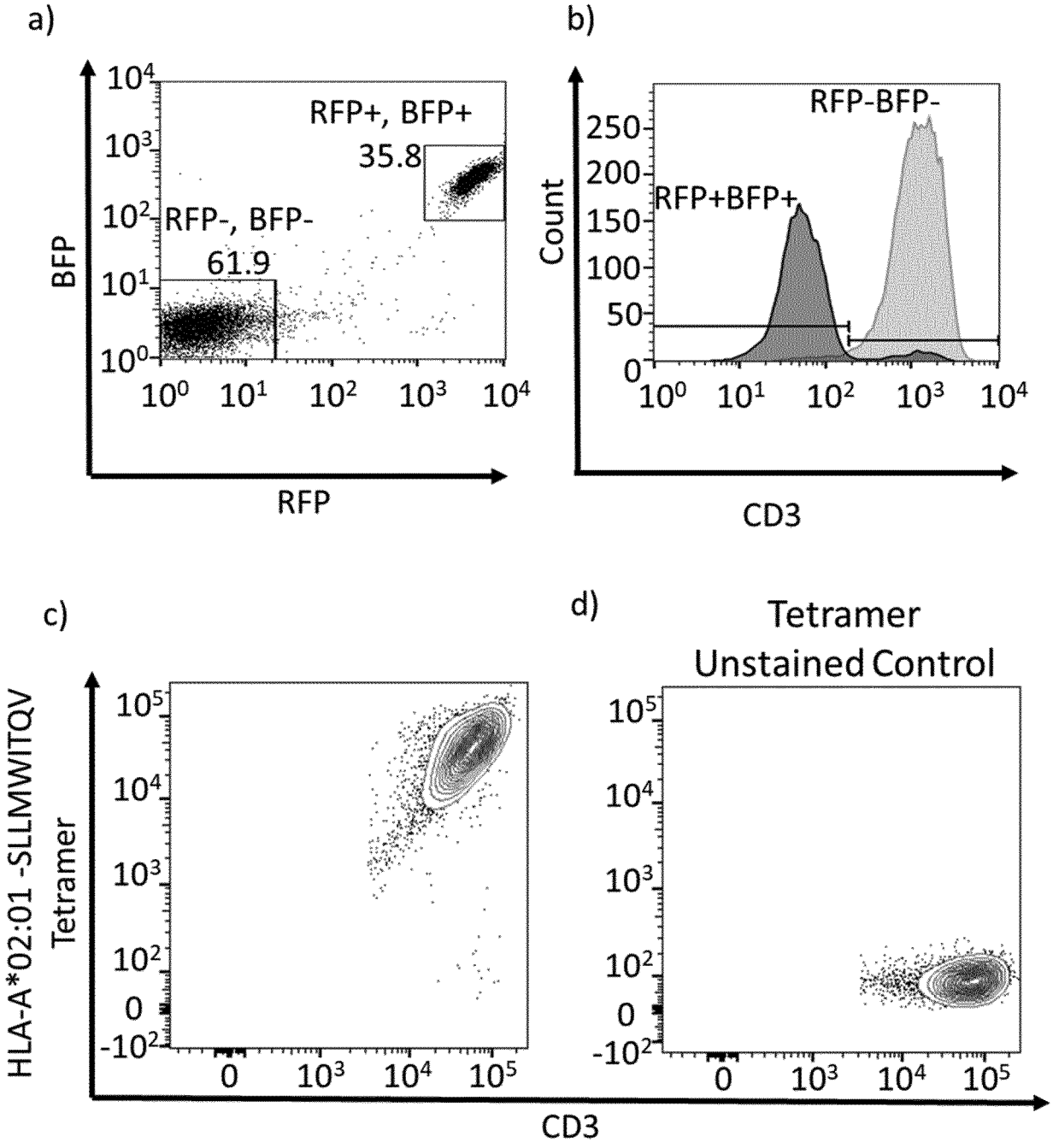

5' genetic element(s)
Kozak
Sense
Vα
CDR3
J
C-part
C
‡ 1
T
‡ 2
T
C
C-part
J
CDR3
Vβ
Anti sense
‡ 3
Kozak
3' genetic element(s)
+ve selection #1
Ori
Full-length TCR ORF vector
Ligated with ligase action

Figure 23


a)
RFP+, BFP+
35.8
RFP-, BFP-
61.9
BFP
RFP
b)
RFP-BFP-
RFP+BFP+
Count
CD3
c)
HLA-A*02:01 -SLLMWITQV Tetramer
CD3
d)
Tetramer
Unstained Control