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(54) **DIMERIC AND MULTIMERIC ANTIGEN BINDING STRUCTURE**

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(30) **Foreign Application Priority Data**

Sep. 14, 2001 (EP) 01122104.1

Publication Classification

(51) **Int. Cl.**[7] **A61K 39/395**; C07K 16/44

(52) **U.S. Cl.** **424/132.1**; 530/387.3

(57) **ABSTRACT**

The present invention relates to dimeric and multimeric antigen binding structures, expression vectors encoding said structures, and diagnostic, as well as therapeutic, uses of said structures. The antigen binding structures are preferably in the form of a Fv-antibody construct.

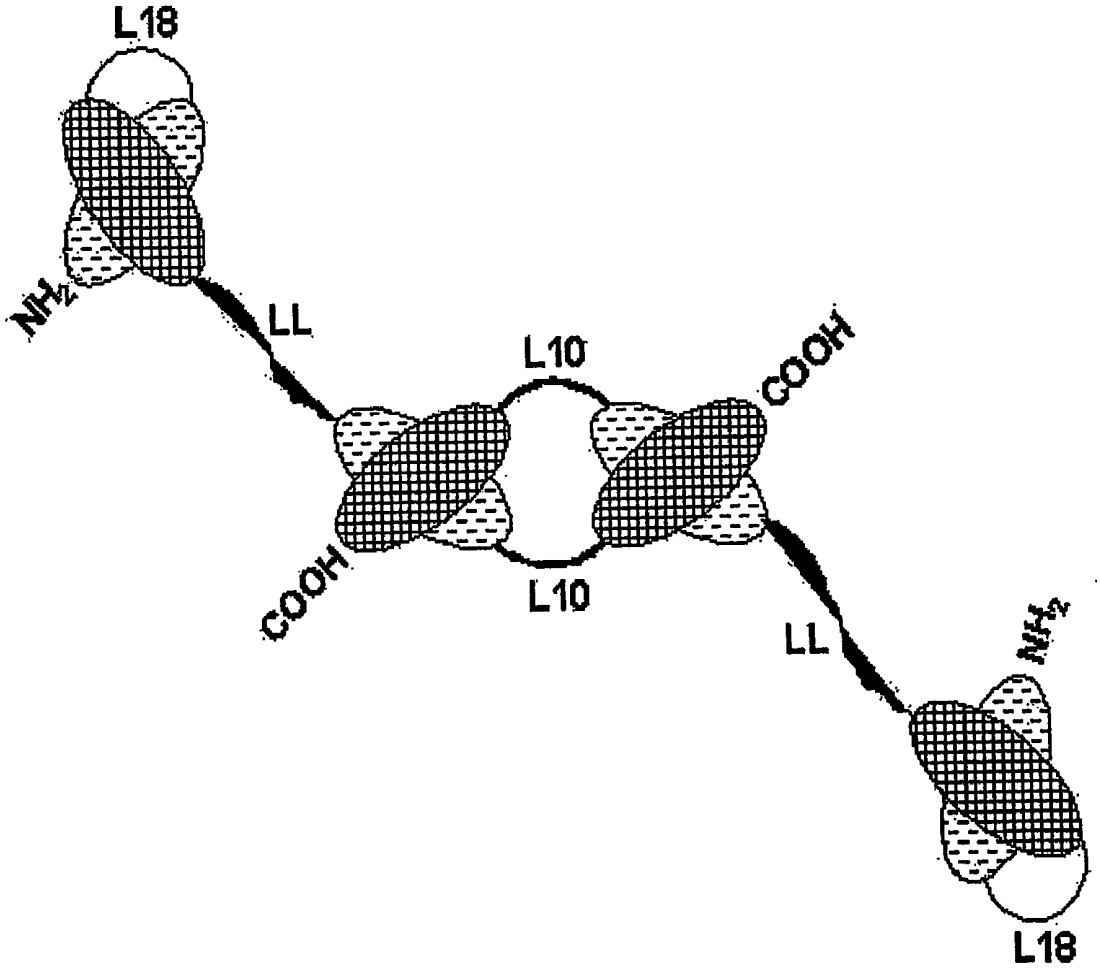


FIGURE 1

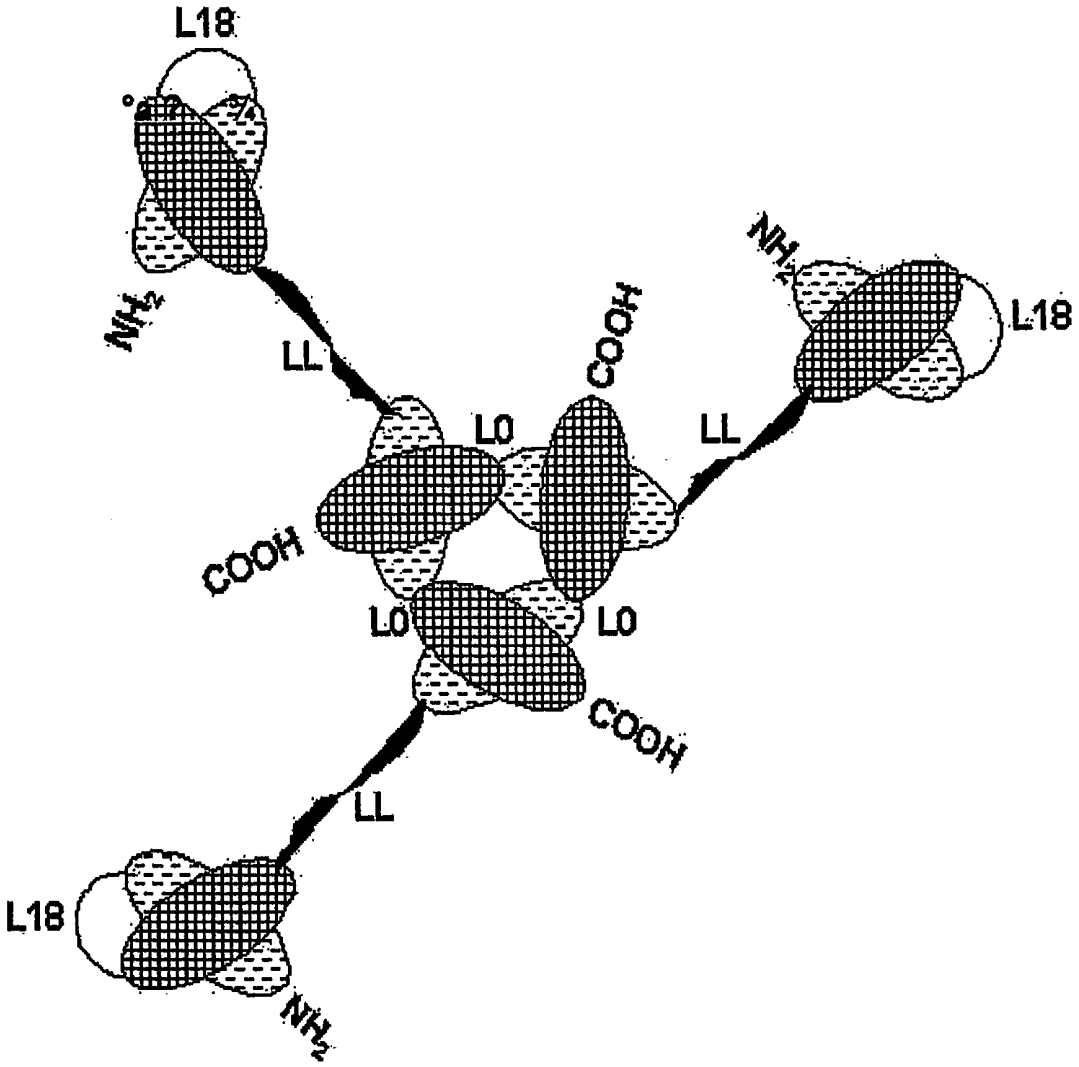
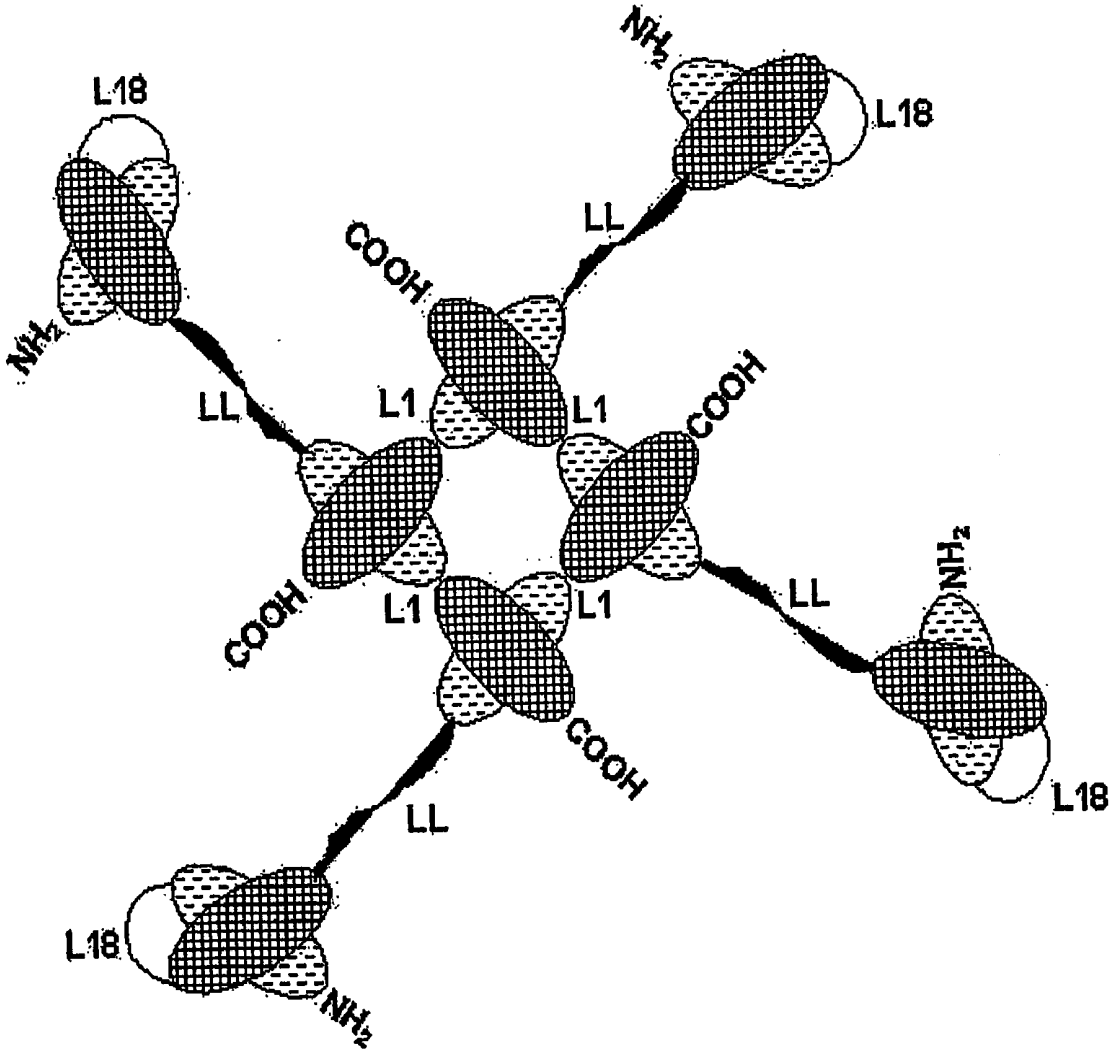


FIGURE 2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NH2
LL
L1
COOH
L1
L1
COOH
L1
L1
COOH
LL
NH2
L18
LL
COOH
L18
NH2

FIGURE 3

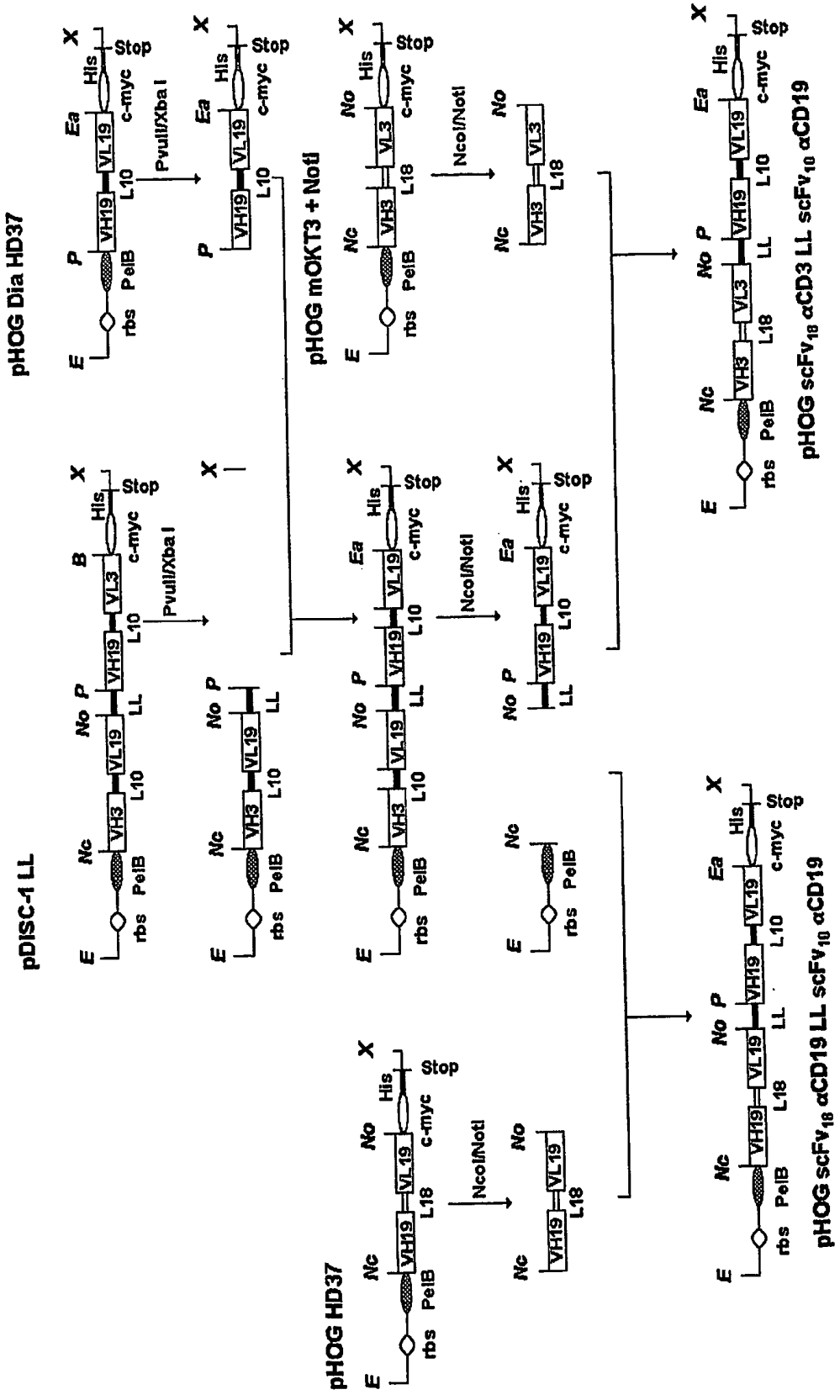

pDISC-1 LL
pHOG Dia HD37
pHOG mOKT3 + NotI
pHOG HD37
pHOG scFv18 αCD3 LL scFv10 αCD19
pHOG scFv18 αCD19 LL scFv10 αCD19
PvuII/XbaI
NcoI/NotI
rbs
PelB
VH3
VH19
VL19
VL3
L10
L18
LL
c-myc
His
Stop

FIGURE 4

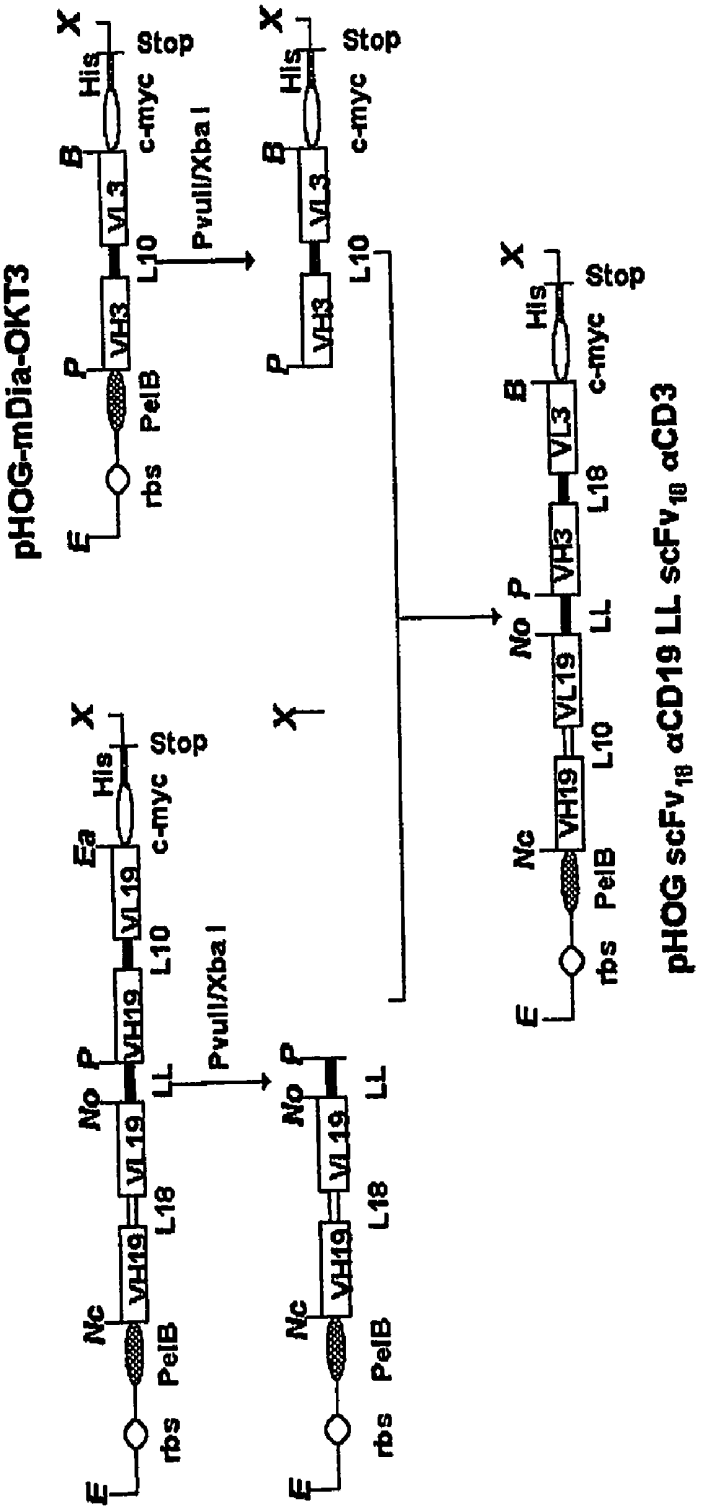

pHOG-mDia-OKT3
pHOG scFv18 αCD19 LL scFv18 αCD3
X
Stop
His
c-myc
B
VL3
L10
VH3
P
PelB
rbs
E
PvuII/Xba I
Ea
VL19
VH19
LL
No
Nc
L18

FIGURE 5

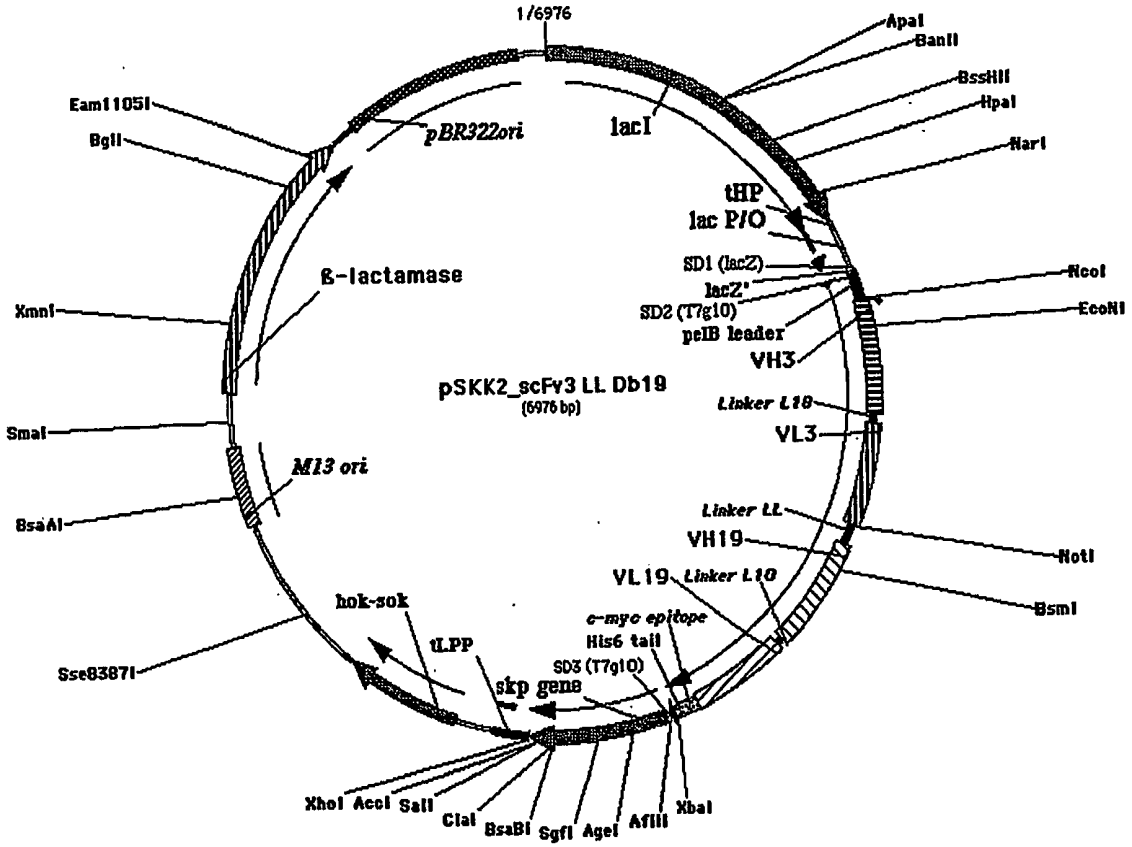
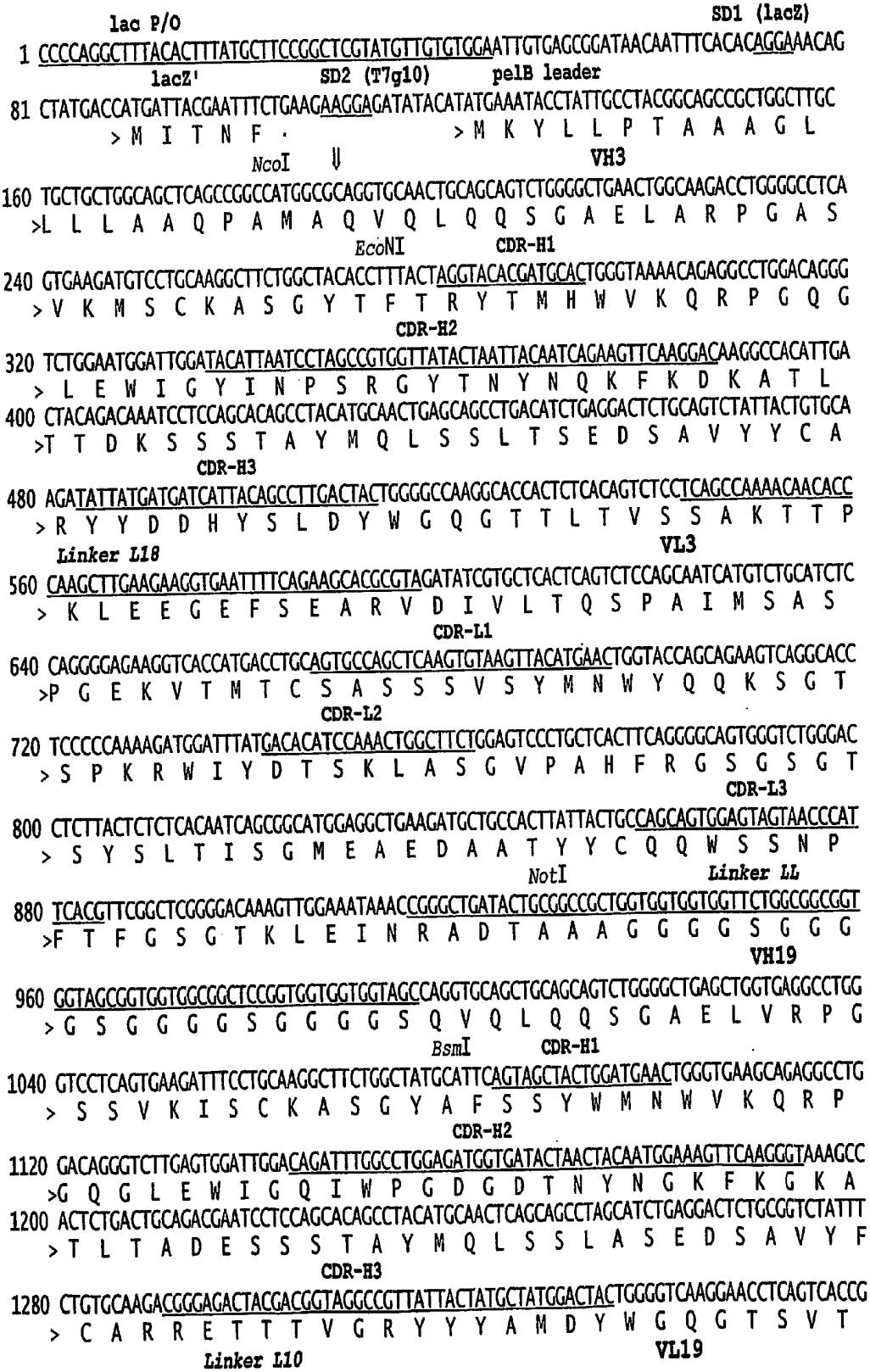

1/6976
ApaI
BanII
BssHII
HpaI
NarI
NcoI
EcoNI
NotI
BsmI
Eam1105I
BglI
pBR322ori
lacI
tHP
lac P/O
SD1 (lacZ)
lacZ'
SD2 (T7g10)
pelB leader
VH3
Linker L18
VL3
Linker LL
VH19
VL19
Linker L18
c-myc epitope
His6 tail
SD3 (T7g10)
skp gene
tLPP
hok-sok
Sse8387I
XmnI
ß-lactamase
SmaI
M13 ori
BsaAI
pSKK2_scFv3 LL Db19
(6976 bp)
XhoI
AccI
SalI
ClaI
BsaBI
SgfI
AgeI
AflII
XbaI

FIGURE 6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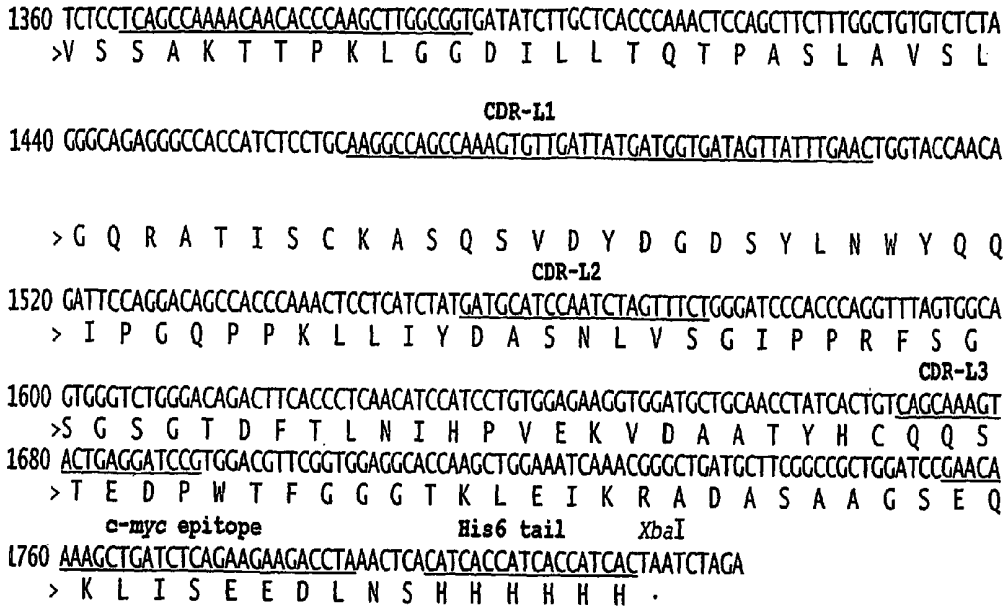
SD1 (lacZ)
1 CCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG
lacZ'
SD2 (T7g10)
pelB leader
81 CTATGACCATGATTACGAATTTCTGAAGAAGGAGATATACATATGAAATACCTATTGCCTACGGCAGCCGCTGGCTTGC
> M I T N F ·
> M K Y L L P T A A A G L
NcoI
VH3
160 TGCTGCTGGCAGCTCAGCCGGCCATGGCGCAGGTGCAACTGCAGCAGTCTGGGGCTGAACTGGCAAGACCTGGGGCCTCA
>L L L A A Q P A M A Q V Q L Q Q S G A E L A R P G A S
EcoNI
CDR-H1
240 GTGAAGATGTCCTGCAAGGCTTCTGGCTACACCTTTACTAGGTACACGATGCACTGGGTAAAACAGAGGCCTGGACAGGG
>V K M S C K A S G Y T F T R Y T M H W V K Q R P G Q G
CDR-H2
320 TCTGGAATGGATTGGATACATTAATCCTAGCCGTGGTTATACTAATTACAATCAGAAGTTCAAGGACAAGGCCACATTGA
> L E W I G Y I N P S R G Y T N Y N Q K F K D K A T L
400 CTACAGACAAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGACATCTGAGGACTCTGCAGTCTATTACTGTGCA
>T T D K S S S T A Y M Q L S S L T S E D S A V Y Y C A
CDR-H3
480 AGATATTATGATGATCATTACAGCCTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCAGCCAAAACAACACC
> R Y Y D D H Y S L D Y W G Q G T T L T V S S A K T T P
Linker L18
VL3
560 CAAGCTTGAAGAAGGTGAATTTTCAGAAGCACGCGTAGATATCGTGCTCACTCAGTCTCCAGCAATCATGTCTGCATCTC
> K L E E G E F S E A R V D I V L T Q S P A I M S A S
CDR-L1
640 CAGGGGAGAAGGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGAACTGGTACCAGCAGAAGTCAGGCACC
>P G E K V T M T C S A S S S V S Y M N W Y Q Q K S G T
CDR-L2
720 TCCCCCAAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCACTTCAGGGGCAGTGGGTCTGGGAC
> S P K R W I Y D T S K L A S G V P A H F R G S G S G T
CDR-L3
800 CTCTTACTCTCTCACAATCAGCGGCATGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGCAGTGGAGTAGTAACCCAT
> S Y S L T I S G M E A E D A A T Y Y C Q Q W S S N P
NotI
Linker LL
880 TCACGTTCGGCTCGGGGACAAAGTTGGAAATAAACCGGGCTGATACTGCGGCCGCTGGTGGTGGTGGTTCTGGCGGCGGT
>F T F G S G T K L E I N R A D T A A A G G G G S G G G
VH19
960 GGTAGCGGTGGTGGCGGCTCCGGTGGTGGTGGTAGCCAGGTGCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAGGCCTGG
> G S G G G G S G G G G S Q V Q L Q Q S G A E L V R P G
BsmI
CDR-H1
1040 GTCCTCAGTGAAGATTTCCTGCAAGGCTTCTGGCTATGCATTCAGTAGCTACTGGATGAACTGGGTGAAGCAGAGGCCTG
> S S V K I S C K A S G Y A F S S Y W M N W V K Q R P
CDR-H2
1120 GACAGGGTCTTGAGTGGATTGGACAGATTTGGCCTGGAGATGGTGATACTAACTACAATGGAAAGTTCAAGGGTAAAGCC
>G Q G L E W I G Q I W P G D G D T N Y N G K F K G K A
1200 ACTCTGACTGCAGACGAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCCTAGCATCTGAGGACTCTGCGGTCTATTT
> T L T A D E S S S T A Y M Q L S S L A S E D S A V Y F
CDR-H3
1280 CTGTGCAAGACGGGAGACTACGACGGTAGGCCGTTATTACTATGCTATGGACTACTGGGGTCAAGGAACCTCAGTCACCG
> C A R R E T T T V G R Y Y Y A M D Y W G Q G T S V T
Linker L10
VL19

FIGURE 6A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>V S S A K T T P K L G G D I L L T Q T P A S L A V S L
CDR-L1
1440 GGGCAGAGGGCCACCATCTCCTGCAAGGCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATTTGAACTGGTACCAACA
>G Q R A T I S C K A S Q S V D Y D G D S Y L N W Y Q Q
CDR-L2
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> I P G Q P P K L L I Y D A S N L V S G I P P R F S G
CDR-L3
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>S G S G T D F T L N I H P V E K V D A A T Y H C Q Q S
1680 ACTGAGGATCCGTGGACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCTTCGGCCGCTGGATCCGAACA
>T E D P W T F G G G T K L E I K R A D A S A A G S E Q
c-myc epitope
His6 tail
XbaI
1760 AAAGCTGATCTCAGAAGAAGACCTAAACTCACATCACCATCACCATCACTAATCTAGA
> K L I S E E D L N S H H H H H H .

FIGURE 6B

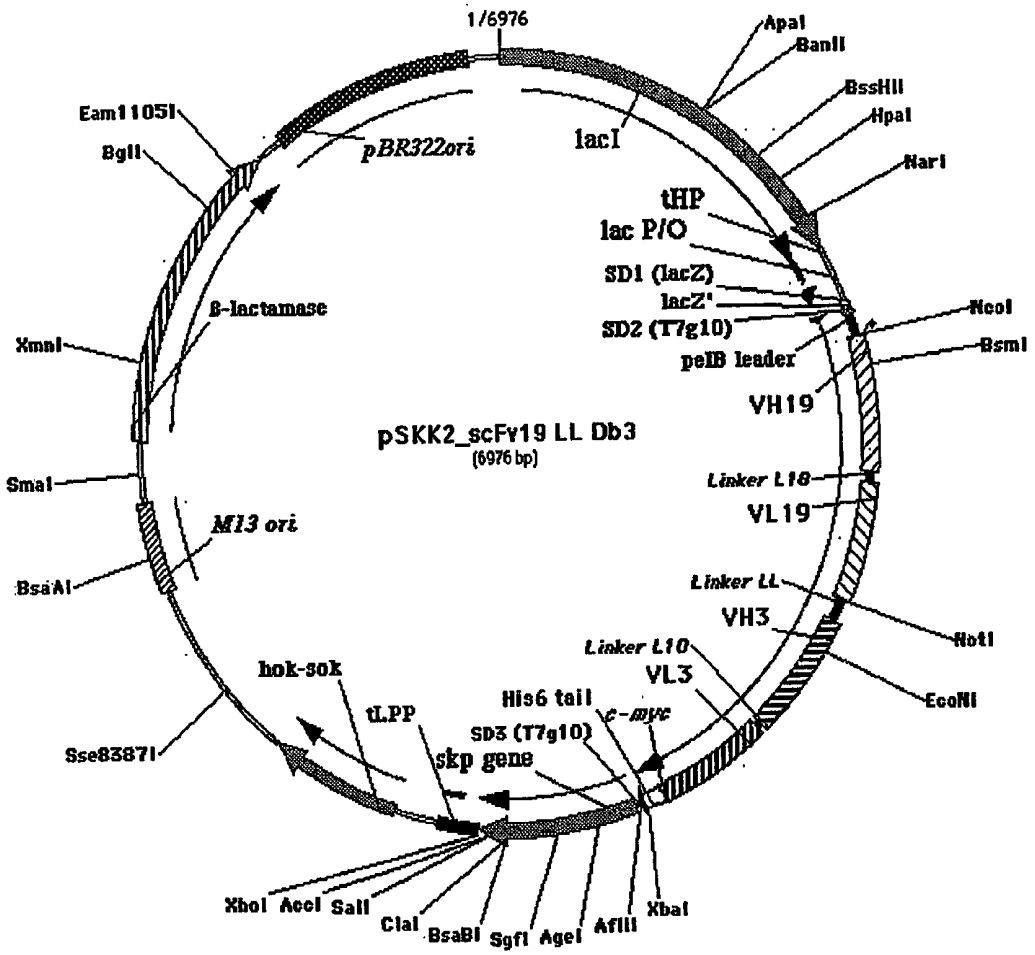

1/6976
ApaI
BanII
BssHII
HpaI
NarI
NcoI
BsmI
Eam1105I
BglI
pBR322ori
lacI
tHP
lac P/O
SD1 (lacZ)
lacZ'
SD2 (T7g10)
pelB leader
VH19
pSKK2_scFv19 LL Db3
(6976 bp)
Linker L18
VL19
Linker LL
VH3
NotI
EcoNI
Linker L10
VL3
His6 tail
c-myc
SD3 (T7g10)
skp gene
tLPP
hok-sok
Sse8387I
BsaAI
M13 ori
SmaI
XmnI
ß-lactamase
XhoI
AccI
SalI
ClaI
BsaBI
SgfI
AgeI
AflII
XbaI

FIGURE 7

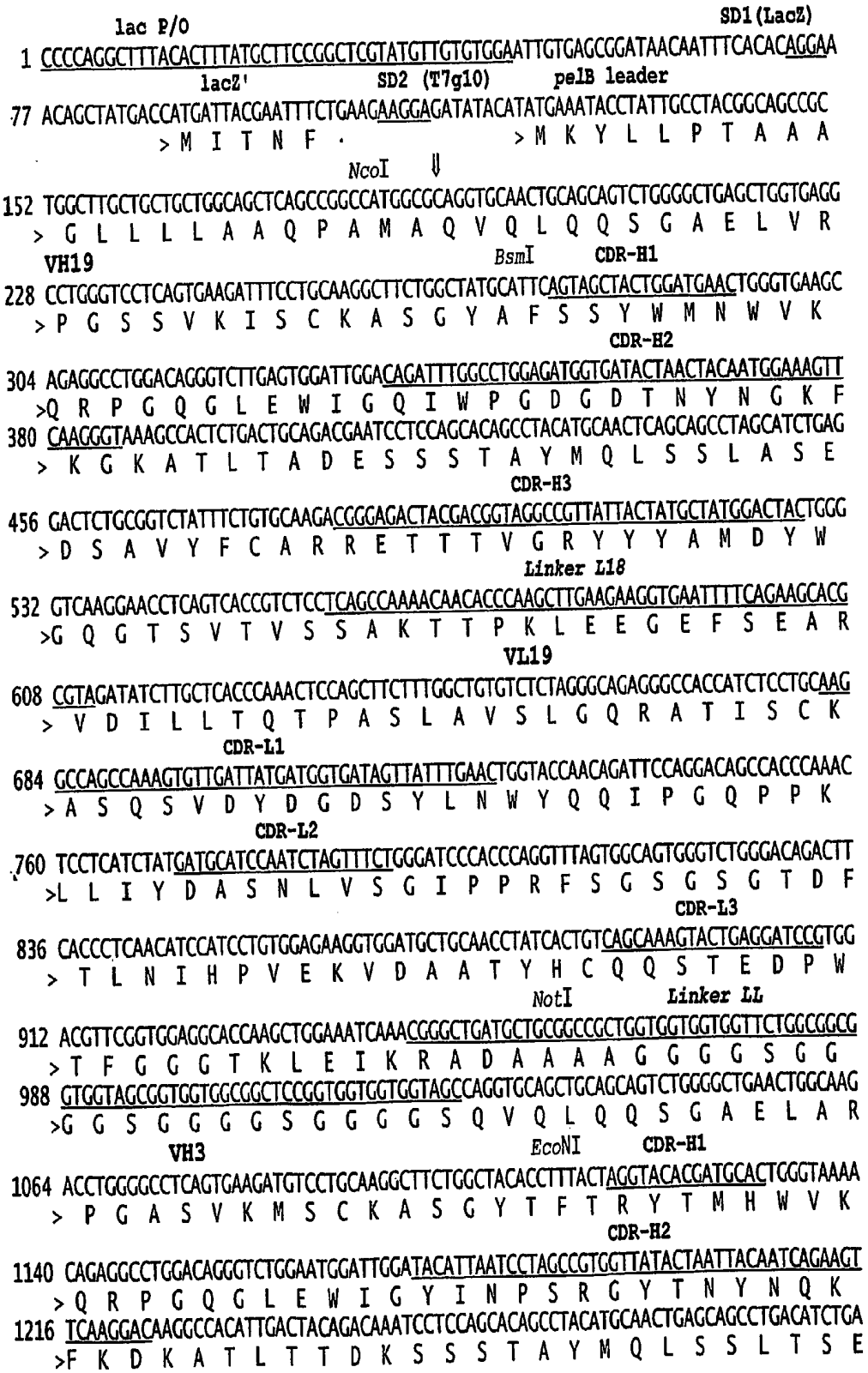
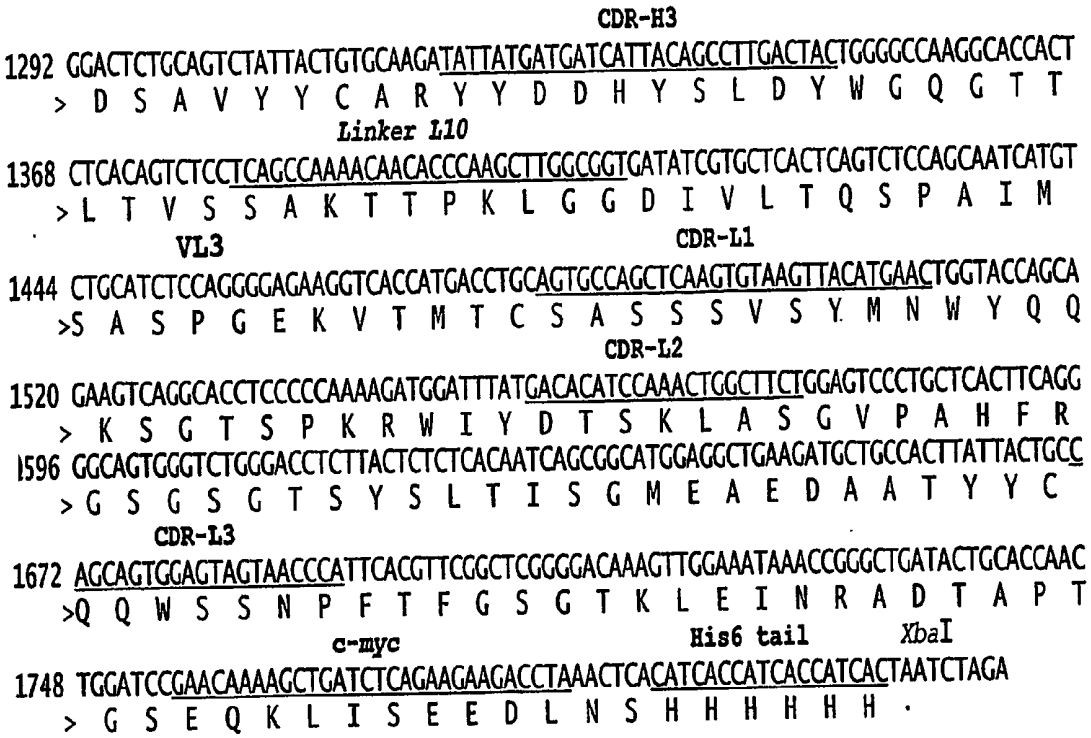

lac P/O
SD1(LacZ)
1 CCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAA
lacZ'
SD2 (T7g10)
pelB leader
77 ACAGCTATGACCATGATTACGAATTTCTGAAGAAGGAGATATACATATGAAATACCTATTGCCTACGGCAGCCGC
>M I T N F ·
>M K Y L L P T A A A
NcoI
152 TGGCTTGCTGCTGCTGGCAGCTCAGCCGGCCATGGCGCAGGTGCAACTGCAGCAGTCTGGGGCTGAGCTGGTGAGG
> G L L L L A A Q P A M A Q V Q L Q Q S G A E L V R
VH19
BsmI
CDR-H1
228 CCTGGGTCCTCAGTGAAGATTTCCTGCAAGGCTTCTGGCTATGCATTCAGTAGCTACTGGATGAACTGGGTGAAGC
> P G S S V K I S C K A S G Y A F S S Y W M N W V K
CDR-H2
304 AGAGGCCTGGACAGGGTCTTGAGTGGATTGGACAGATTTGGCCTGGAGATGGTGATACTAACTACAATGGAAAGTT
>Q R P G Q G L E W I G Q I W P G D G D T N Y N G K F
380 CAAGGGTAAAGCCACTCTGACTGCAGACGAATCCTCCAGCACAGCCTACATGCAACTCAGCAGCCTAGCATCTGAG
> K G K A T L T A D E S S S T A Y M Q L S S L A S E
CDR-H3
456 GACTCTGCGGTCTATTTCTGTGCAAGACGGGAGACTACGACGGTAGGCCGTTATTACTATGCTATGGACTACTGGG
> D S A V Y F C A R R E T T T V G R Y Y Y A M D Y W
Linker L18
532 GTCAAGGAACCTCAGTCACCGTCTCCTCAGCCAAAACAACACCCAAGCTTGAAGAAGGTGAATTTTCAGAAGCACG
>G Q G T S V T V S S A K T T P K L E E G E F S E A R
VL19
608 CGTAGATATCTTGCTCACCCAAACTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACCATCTCCTGCAAG
> V D I L L T Q T P A S L A V S L G Q R A T I S C K
CDR-L1
684 GCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATTTGAACTGGTACCAACAGATTCCAGGACAGCCACCCAAAC
>A S Q S V D Y D G D S Y L N W Y Q Q I P G Q P P K
CDR-L2
760 TCCTCATCTATGATGCATCCAATCTAGTTTCTGGGATCCCACCCAGGTTTAGTGGCAGTGGGTCTGGGACAGACTT
>L L I Y D A S N L V S G I P P R F S G S G S G T D F
CDR-L3
836 CACCCTCAACATCCATCCTGTGGAGAAGGTGGATGCTGCAACCTATCACTGTCAGCAAAGTACTGAGGATCCGTGG
> T L N I H P V E K V D A A T Y H C Q Q S T E D P W
NotI
Linker LL
912 ACGTTCGGTGGAGGCACCAAGCTGGAAATCAAACGGGCTGATGCTGCGGCCGCTGGTGGTGGTGGTTCTGGCGGCG
>T F G G G T K L E I K R A D A A A A G G G G S G G
988 GTGGTAGCGGTGGTGGCGGCTCCGGTGGTGGTGGTAGCCAGGTGCAGCTGCAGCAGTCTGGGGCTGAACTGGCAAG
>G G S G G G G S G G G G S Q V Q L Q Q S G A E L A R
VH3
EcoNI
CDR-H1
1064 ACCTGGGGCCTCAGTGAAGATGTCCTGCAAGGCTTCTGGCTACACCTTTACTAGGTACACGATGCACTGGGTAAAA
> P G A S V K M S C K A S G Y T F T R Y T M H W V K
CDR-H2
1140 CAGAGGCCTGGACAGGGTCTGGAATGGATTGGATACATTAATCCTAGCCGTGGTTATACTAATTACAATCAGAAGT
>Q R P G Q G L E W I G Y I N P S R G Y T N Y N Q K
1216 TCAAGGACAAGGCCACATTGACTACAGACAAATCCTCCAGCACAGCCTACATGCAACTGAGCAGCCTGACATCTGA
>F K D K A T L T T D K S S S T A Y M Q L S S L T S E

FIGURE 7A


CDR-H3
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> D S A V Y Y C A R Y Y D D H Y S L D Y W G Q G T T
Linker L10
1368 CTCACAGTCTCCTCAGCCAAAACAACACCCAAGCTTGGCGGTGATATCGTGCTCACTCAGTCTCCAGCAATCATGT
> L T V S S A K T T P K L G G D I V L T Q S P A I M
VL3
CDR-L1
1444 CTGCATCTCCAGGGGAGAAGGTCACCATGACCTGCAGTGCCAGCTCAAGTGTAAGTTACATGAACTGGTACCAGCA
>S A S P G E K V T M T C S A S S S V S Y M N W Y Q Q
CDR-L2
1520 GAAGTCAGGCACCTCCCCCAAAAGATGGATTTATGACACATCCAAACTGGCTTCTGGAGTCCCTGCTCACTTCAGG
> K S G T S P K R W I Y D T S K L A S G V P A H F R
1596 GGCAGTGGGTCTGGGACCTCTTACTCTCTCACAATCAGCGGCATGGAGGCTGAAGATGCTGCCACTTATTACTGCC
> G S G S G T S Y S L T I S G M E A E D A A T Y Y C
CDR-L3
1672 AGCAGTGGAGTAGTAACCCATTCACGTTCGGCTCGGGGACAAAGTTGGAAATAAACCGGGCTGATACTGCACCAAC
>Q Q W S S N P F T F G S G T K L E I N R A D T A P T
c-myc
His6 tail
XbaI
1748 TGGATCCGAACAAAAGCTGATCTCAGAAGAAGACCTAAACTCACATCACCATCACCATCACTAATCTAGA
> G S E Q K L I S E E D L N S H H H H H H ·

FIGURE 7B

A

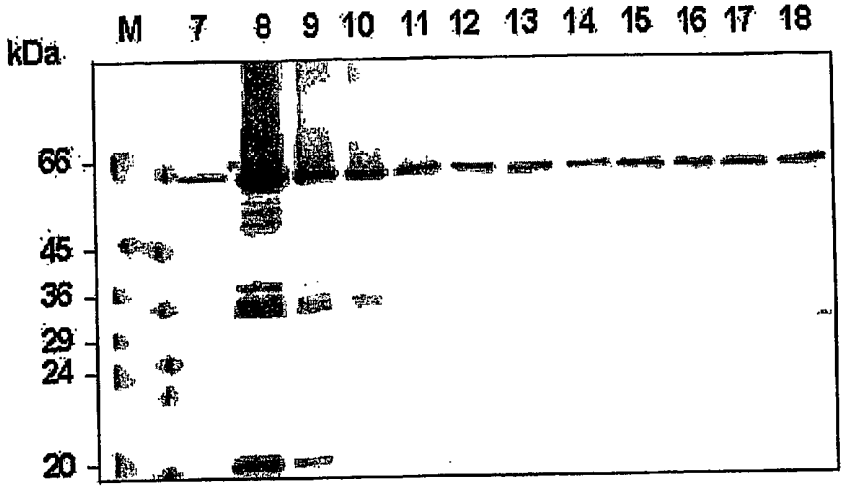

FIGURE 8A

B

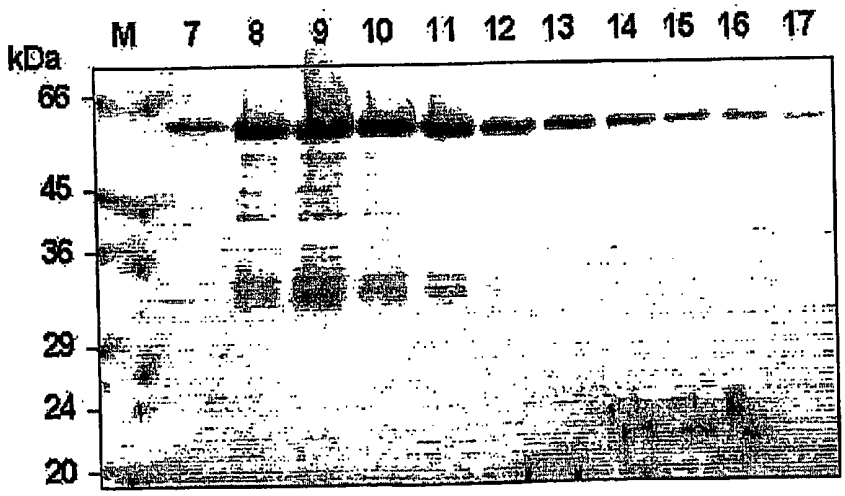

FIGURE 8B

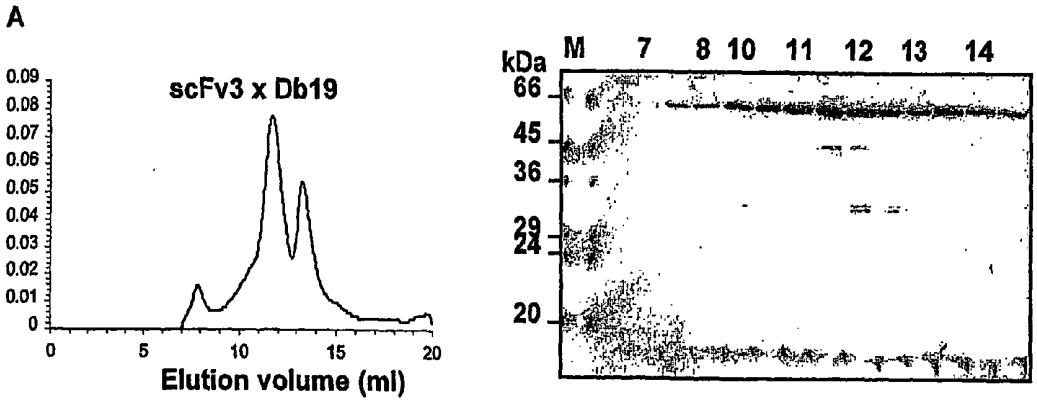

FIGURE 9A

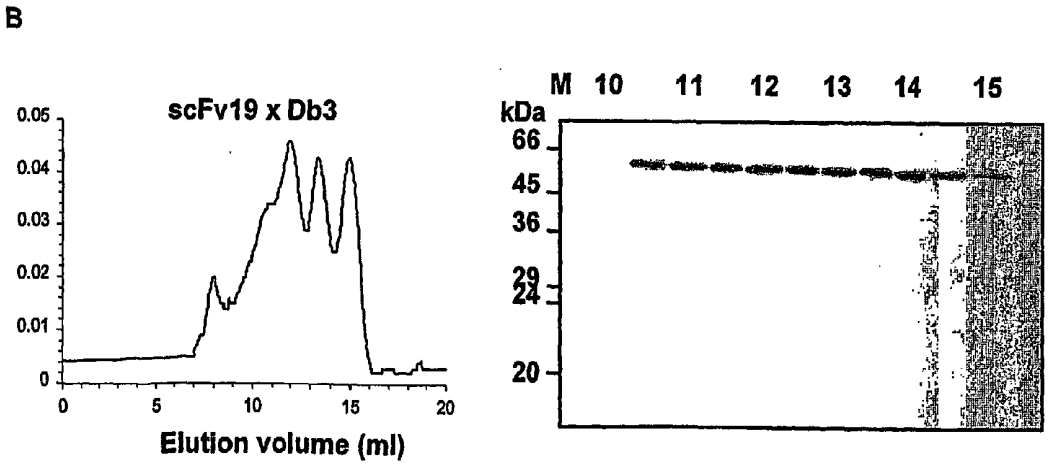

FIGURE 9B

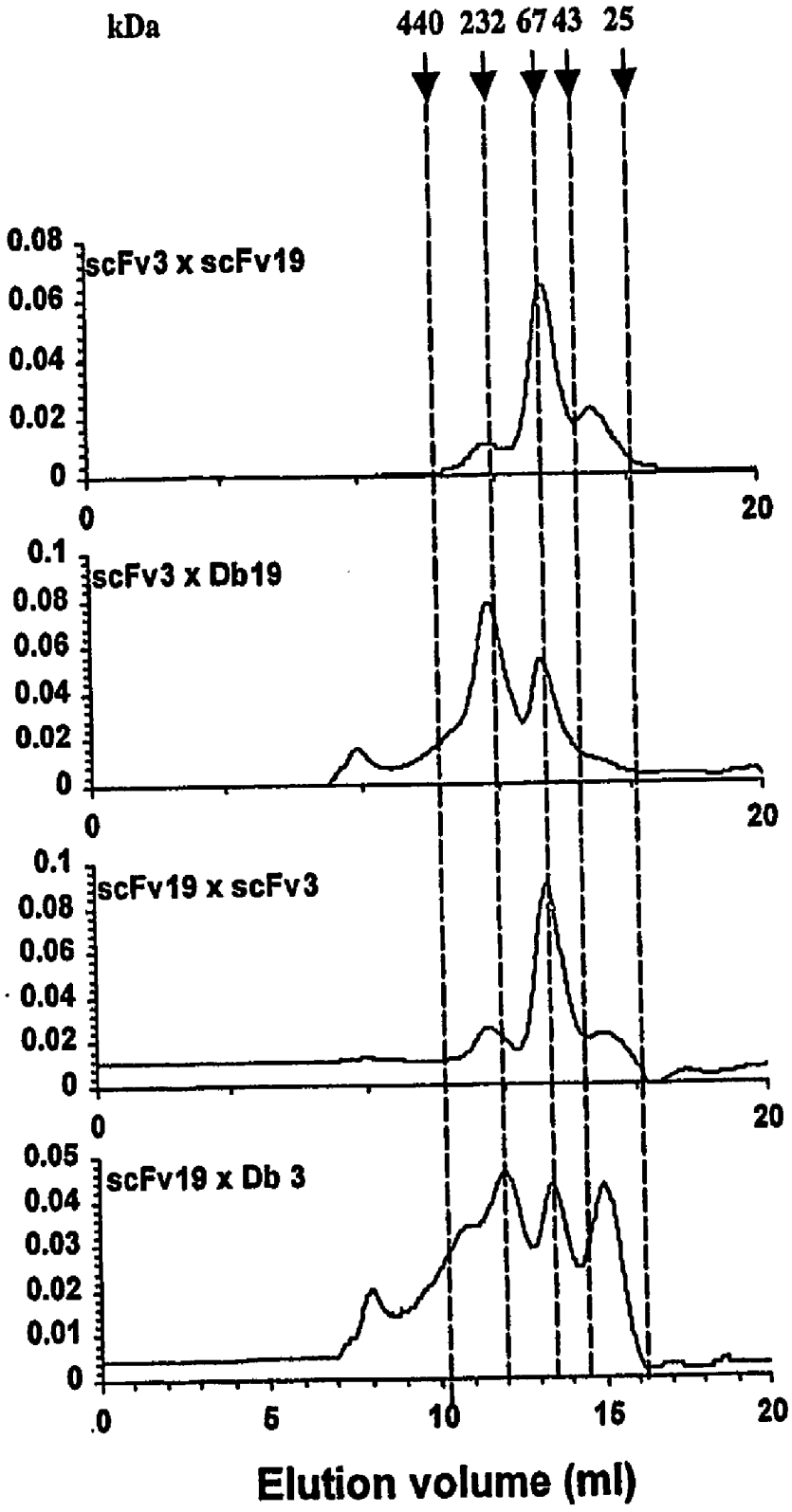
kDa
440 232 67 43 25
scFv3 x scFv19
0.08
0.06
0.04
0.02
0
0
20
scFv3 x Db19
0.1
0.08
0.06
0.04
0.02
0
0
20
scFv19 x scFv3
0.1
0.08
0.06
0.04
0.02
0
0
20
scFv19 x Db 3
0.05
0.04
0.03
0.02
0.01
0
0
5
10
15
20
Elution volume (ml)

FIGURE 10

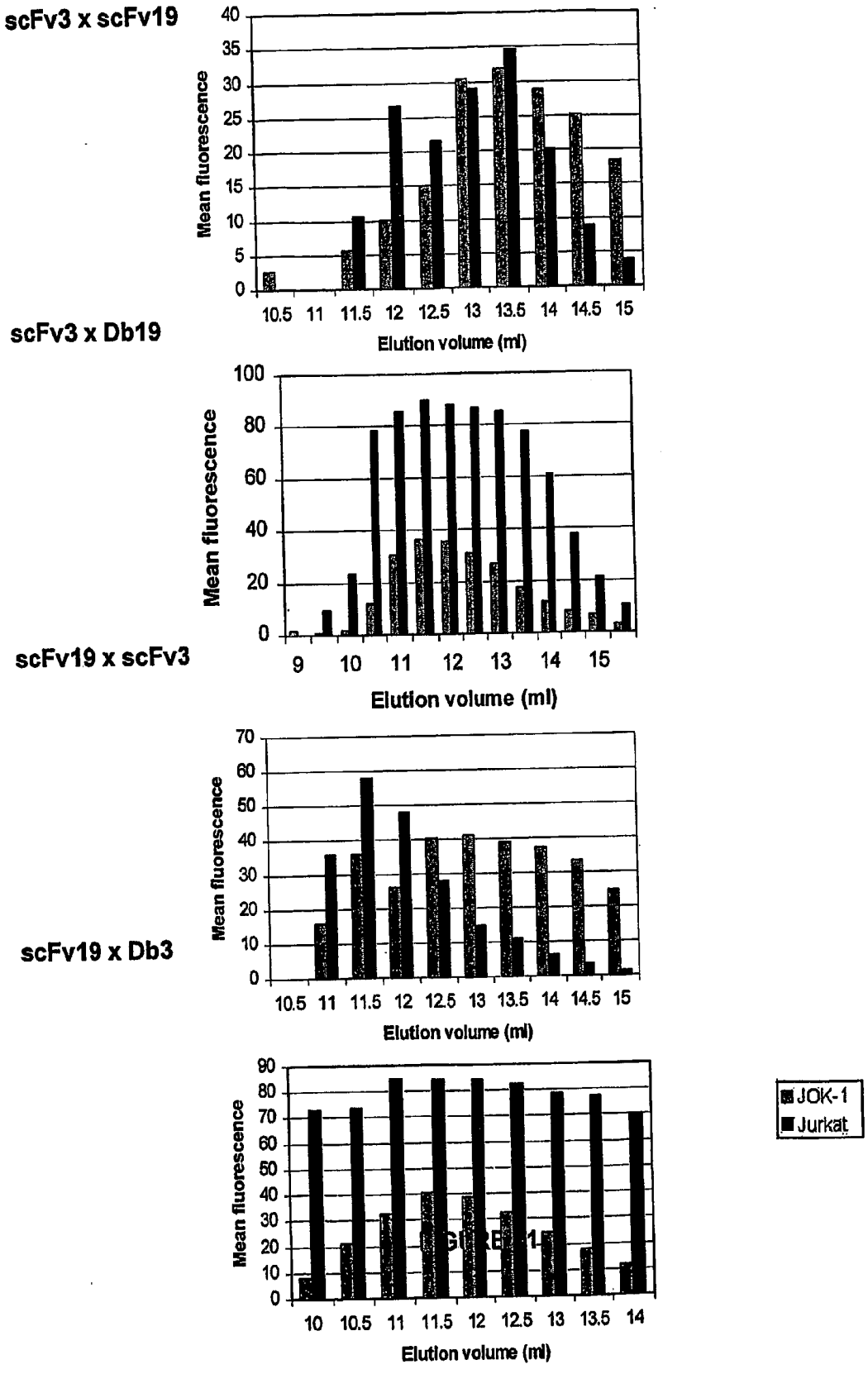
scFv3 x scFv19
Mean fluorescence
40
35
30
25
20
15
10
5
0
10.5 11 11.5 12 12.5 13 13.5 14 14.5 15
Elution volume (ml)
scFv3 x Db19
Mean fluorescence
100
80
60
40
20
0
9 10 11 12 13 14 15
Elution volume (ml)
scFv19 x scFv3
Mean fluorescence
70
60
50
40
30
20
10
0
10.5 11 11.5 12 12.5 13 13.5 14 14.5 15
Elution volume (ml)
scFv19 x Db3
Mean fluorescence
90
80
70
60
50
40
30
20
10
0
10 10.5 11 11.5 12 12.5 13 13.5 14
Elution volume (ml)
JOK-1
Jurkat

FIGURE 11

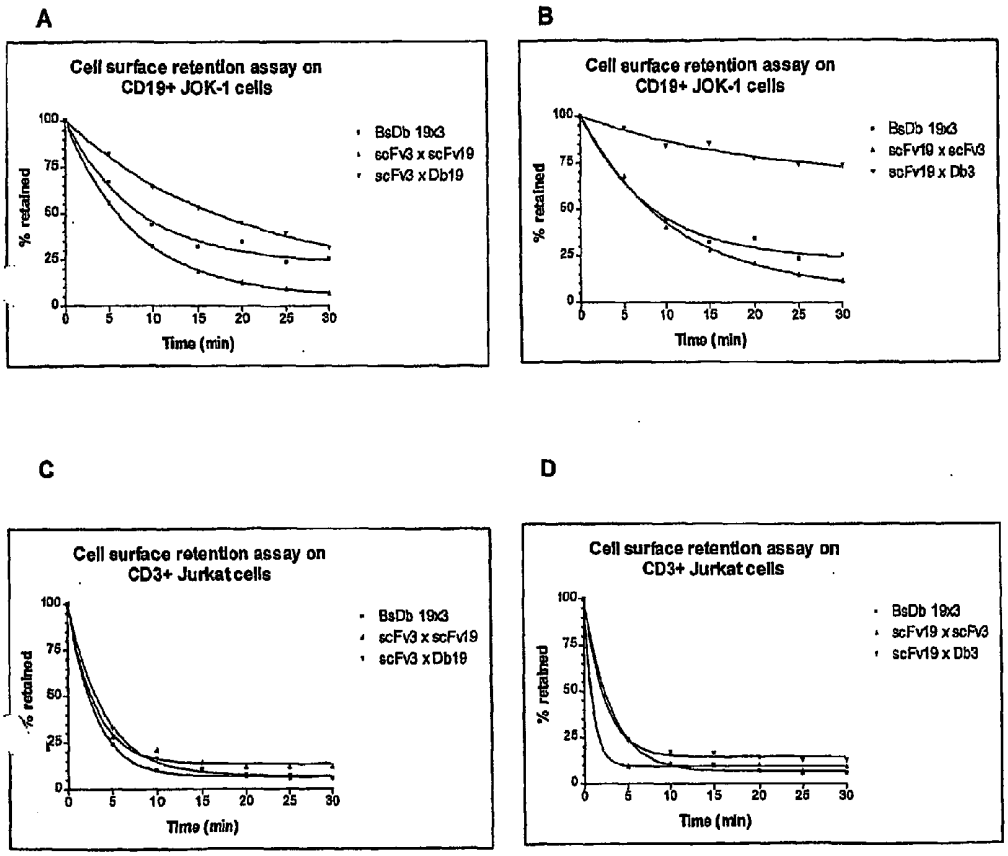
A
Cell surface retention assay on CD19+ JOK-1 cells
% retained
Time (min)
BsDb 19x3
scFv3 x scFv19
scFv3 x Db19
B
Cell surface retention assay on CD19+ JOK-1 cells
% retained
Time (min)
BsDb 19x3
scFv19 x scFv3
scFv19 x Db3
C
Cell surface retention assay on CD3+ Jurkat cells
% retained
Time (min)
BsDb 19x3
scFv3 x scFv19
scFv3 x Db19
D
Cell surface retention assay on CD3+ Jurkat cells
% retained
Time (min)
BsDb 19x3
scFv19 x scFv3
scFv19 x Db3

FIGURE 12

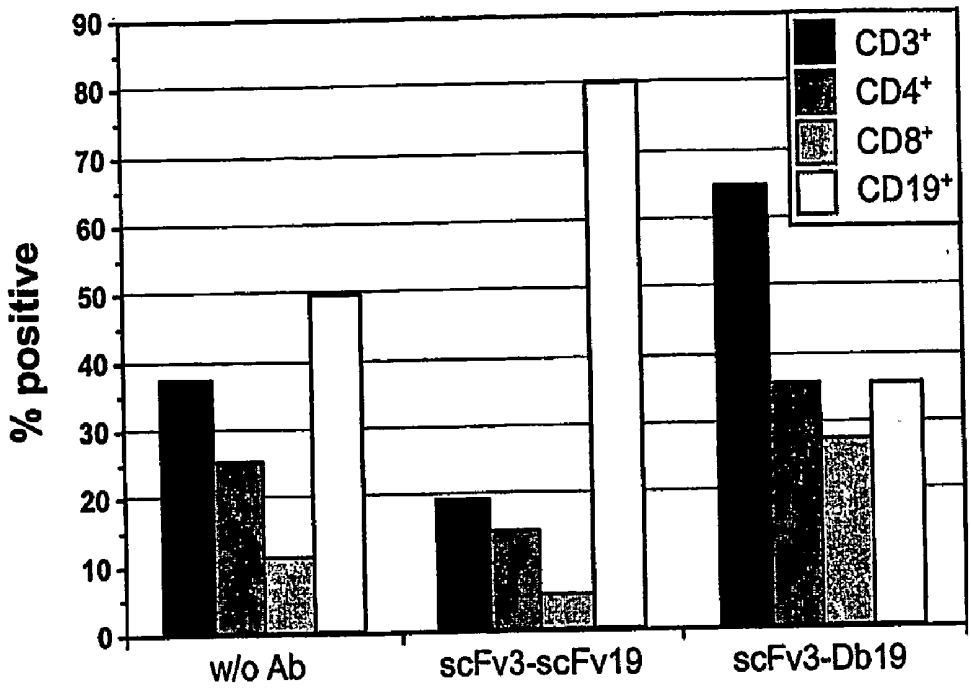
90
80
70
60
50
40
30
20
10
0
% positive
CD3+
CD4+
CD8+
CD19+
w/o Ab
scFv3-scFv19
scFv3-Db19

FIGURE 13

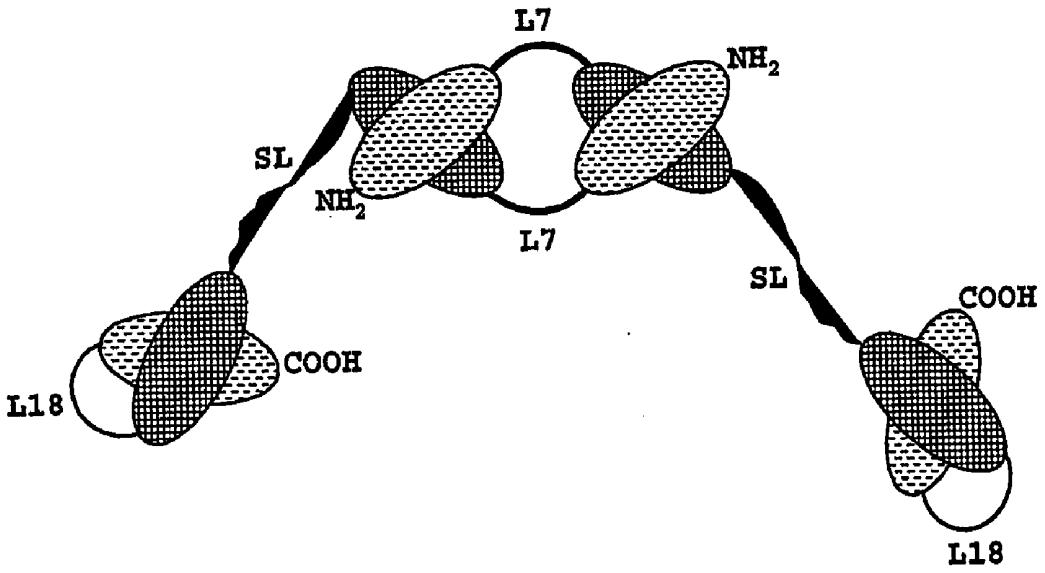

FIGURE 14

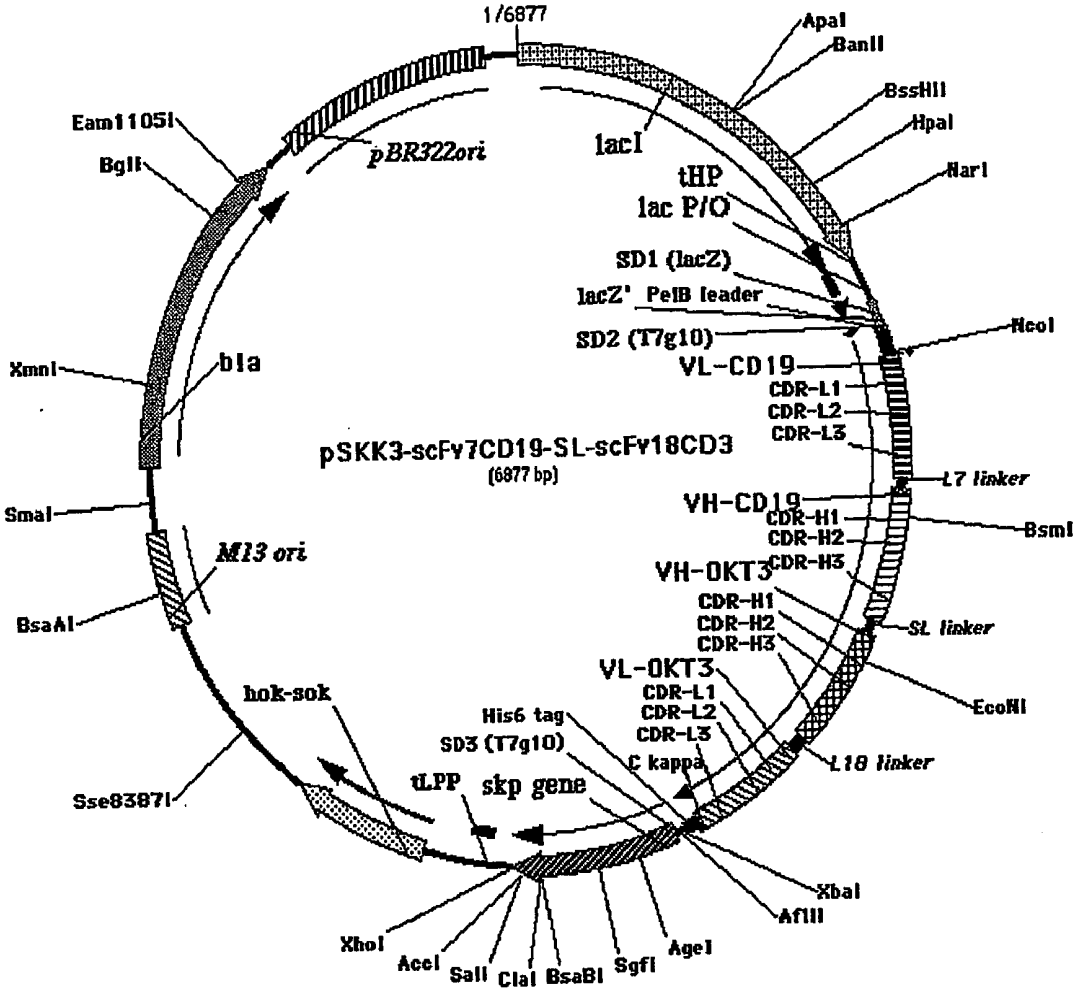
1/6877
ApaI
BanII
BssHII
HpaI
NarI
NcoI
Eam1105I
BglI
pBR322ori
lacI
tHP
lac P/O
SD1 (lacZ)
lacZ' PelB leader
SD2 (T7g10)
VL-CD19
CDR-L1
CDR-L2
CDR-L3
L7 linker
VH-CD19
CDR-H1
CDR-H2
CDR-H3
BsmI
VH-OKT3
CDR-H1
CDR-H2
CDR-H3
SL linker
VL-OKT3
CDR-L1
CDR-L2
CDR-L3
EcoNI
L18 linker
C kappa
His6 tag
SD3 (T7g10)
tLPP
skp gene
XbaI
AflII
AgeI
SgfI
BsaBI
ClaI
SalI
AccI
XhoI
Sse8387I
hok-sok
BsaAI
M13 ori
SmaI
bla
XmnI
pSKK3-scFv7CD19-SL-scFv18CD3
(6877 bp)

Figure 15

FIGURE 16

<110> Affimed Therapeutics AG

<120> Title : Dimeric amd multimeric Antigen Binding Structure

<130>

<140> EP 01122104.1

<141>

<160>2

Figure 16a:

<210> 1
<211> 6844
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the Artificial Sequence:pSKK3 scFv7 anti-CD19-L6-scFv10 anti-CD3
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gagtcaattc agggtggtga atgtgaaacc agtaacgtta tacgatgtcg cagagtatgc 120

cggtgtctct tatcagaccg tttcccgcgt ggtgaaccag gccagccacg tttctgcgaa 180

aacgcgggaa aaagtggaag cggcgatggc ggagctgaat tacattccca accgggtggc 240

acaacaactg gcgggcaaac agtcgttgct gattggcgtt gccacctcca gtctggccct 300

gcacgcgccg tcgcaaattg tcgcggcgat taaatctcgc gccgatcaac tgggtgccag 360

cgtggtggtg tcgatggtag aacgaagcgg cgtcgaagcc tgtaaagcgg cggtgcacaa 420

tcttctcgcg caacgcgtca gtgggctgat cattaactat ccgctggatg accaggatgc 480

cattgctgtg gaagctgcct gcactaatgt tccggcgtta tttcttgatg tctctgacca 540

gacacccatc aacagtatta ttttctccca tgaagacggt acgcgactgg gcgtggagca 600

tctggtcgca ttgggtcacc agcaaatcgc gctgttagcg ggcccattaa gttctgtctc 660

ggcgcgtctg cgtctggctg gctggcataa atatctcact cgcaatcaaa ttcagccgat 720

agcggaacgg gaaggcgact ggagtgccat gtccggtttt caacaaacca tgcaaatgct 780

gaatgagggc atcgttccca ctgcgatgct ggttgccaac gatcagatgg cgctgggcgc 840

aatgcgcgcc attaccgagt ccgggctgcg cgttggtgcg gatatctcgg tagtgggata 900

cgacgatacc gaagacagct catgttatat cccgccgtta accaccatca aacaggattt 960

tcgcctgctg gggcaaacca gcgtggaccg cttgctgcaa ctctctcagg gccaggcggt 1020

gaagggcaat cagctgttgc ccgtctcact ggtgaaaaga aaaaccaccc tggcgcccaa 1080

tacgcaaacc gcctctcccc gcgcgttggc cgattcatta atgcagctgg cacgacaggt 1140

ttcccgactg gaaagcgggc agtgagcggt acccgataaa agcggcttcc tgacaggagg 1200

ccgttttgtt ttgcagccca cctcaacgca attaatgtga gttagctcac tcattaggca 1260

ccccaggctt tacactttat gcttccggct cgtatgttgt gtggaattgt gagcggataa 1320

caatttcaca caggaaacag ctatgaccat gattacgaat ttctgaagaa ggagatatac 1380

atatgaaata cctattgcct acggcagccg ctggcttgct gctgctggca gctcagccgg 1440

ccatggcgga tatcttgctc acccaaactc cagcttcttt ggctgtgtct ctagggcaga 1500

gggccaccat ctcctgcaag gccagccaaa gtgttgatta tgatggtgat agttatttga 1560

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acaatggaaa gttcaagggt aaagccactc tgactgcaga cgaatcctcc agcacagcct 2040

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tgagttcaaa ggccgtgcca gcgaactgca gcgtatggaa accgatctgc aggctaaaat 3180

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caacagccag gatatcgatc tggttgttga tgcaaacgcc gttgcttaca acagcagcga 3420

tgtaaaagac atcactgtcg acgtactgaa acaggttaaa taatgctcga ggaactgctg 3480

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atggcgcaca ttgtgcgaca tttttttgt ctgccgttta ccgctactgc gtcacggatc 3600

cggccgaaca aactccggga ggcagcgtga tgcggcaaca atcacacgga tttcccgtga 3660

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ccgtgttgtg tggcagaaag aagatagccc cgtagtaagt taattttcat taaccaccac 3840

gaggcatccc tatgtctagt ccacatcagg atagcctctt accgcgcttt gcgcaaggag 3900

aagaaggcca tgaaactacc acgaagttcc cttgtctggt gtgtgttgat cgtgtgtctc 3960

acactgttga tattcactta tctgacacga aaatcgctgt gcgagattcg ttacagagac 4020

ggacacaggg aggtggcggc tttcatggct tacgaatccg gtaagtagca acctagaggc 4080

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ggggctgctg gttcgccggt agccccttc tccttgctga tgttgtggga atttcgagca 4200

agacgtttcc cgttgaatat ggctcataac accccttgta ttactgttta tgtaagcaga 4260

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cgcgccctgt agcggcgcat taagcgcggc gggtgtggtg gttacgcgca gcgtgaccgc 4620

tacacttgcc agcgccctag cgcccgctcc tttcgctttc ttcccttcct ttctcgccac 4680

gttcgccggc tttccccgtc aagctctaaa tcggggcatc cctttagggt tccgatttag 4740

tgctttacgg cacctcgacc ccaaaaaact tgattagggt gatggttcac gtagtgggcc 4800

atcgccctga tagacggttt ttcgcccttt gacgttggag tccacgttct ttaatagtgg 4860

actcttgttc caaactggaa caacactcaa ccctatctcg gtctattctt ttgatttata 4920

agggattttg ccgatttcgg cctattggtt aaaaaatgag ctgatttaac aaaaatttaa 4980

cgcgaatttt aacaaaatat taacgtttac aatttcaggt ggcgaattcc ccggggaatt 5040

cacttttcgg ggaaatgtgc gcggaacccc tatttgttta ttttctaaa tacattcaaa 5100

tatgtatccg ctcatgagac aataaccctg ataaatgctt caataatatt gaaaaaggaa 5160

gagtatgagt attcaacatt tccgtgtcgc ccttattccc ttttttgcgg cattttgcct 5220

tcctgttttt gctcacccag aaacgctggt gaaagtaaaa gatgctgaag atcagttggg 5280

tgcacgagtg ggttacatcg aactggatct caacagcggt aagatccttg agagttttcg 5340

ccccgaagaa cgttttccaa tgatgagcac ttttaaagtt ctgctatgtg gcgcggtatt 5400

atcccctatt gacgccgggc aagagcaact cggtcgccgc atacactatt ctcagaatga 5460

cttggttgag tactcaccag tcacagaaaa gcatcttacg gatggcatga cagtaagaga 5520

attatgcagt gctgccataa ccatgagtga taacactgcg gccaacttac ttctgacaac 5580

gatcggagga ccgaaggagc taaccgcttt tttgcacaac atgggggatc atgtaactcg 5640

ccttgatcgt tgggaaccgg agctgaatga agccatacca aacgacgagc gtgacaccac 5700

gatgcctgta gcaatggcaa caacgttgcg caaactatta actggcgaac tacttactct 5760

agcttcccgg caacaattaa tagactggat ggaggcggat aaagttgcag gaccacttct 5820

gcgctcggcc cttccggctg gctggtttat tgctgataaa tctggagccg gtgagcgtgg 5880

gtctcgcggt atcattgcag cactggggcc agatggtaag ccctcccgta tcgtagttat 5940

ctacacgacg gggagtcagg caactatgga tgaacgaaat agacagatcg ctgagatagg 6000

tgcctcactg attaagcatt ggtaactgtc agaccaagtt tactcatata tactttagat 6060

tgatttaaaa cttcattttt aatttaaaag gatctaggtg aagatccttt ttgataatct 6120

catgaccaaa atcccttaac gtgagttttc gttccactga gcgtcagacc ccgtagaaaa 6180

gatcaaagga tcttcttgag atccttttt tctgcgcgta atctgctgct tgcaaacaaa 6240

aaaaccaccg ctaccagcgg tggtttgttt gccggatcaa gagctaccaa ctctttttcc 6300

gaaggtaact ggcttcagca gagcgcagat accaaatact gtccttctag tgtagccgta 6360

gttaggccac cacttcaaga actctgtagc accgcctaca tacctcgctc tgctaatcct 6420

gttaccagtg gctgctgcca gtggcgataa gtcgtgtctt accgggttgg actcaagacg 6480

atagttaccg gataaggcgc agcggtcggg ctgaacgggg ggttcgtgca cacagcccag 6540

cttggagcga acgacctaca ccgaactgag atacctacag cgtgagctat gagaaagcgc 6600

cacgcttccc gaagggagaa aggcggacag gtatccggta agcggcaggg tcggaacagg 6660

agagcgcacg agggagcttc caggggaaa cgcctggtat ctttatagtc ctgtcgggtt 6720

tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcaggggggc ggagcctatg 6780

gaaaaacgcc agcaacgcgg cctttttacg gttcctggcc ttttgctggc cttttgctca 6840

catg 6844

Figure 16b:

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<211>496
<212> PRT
<213>Artificial Squence

<220>
<223>Description of the Artificial Sequence:Db19-L6-scFv3

<400>2

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GIPPRFSGSG SGTDFTLNIH PVEKVDAATY HCQQSTEDPW TFGGGTKLEI KRTVAAPSQV 120

QLQQSGAELV RPGSSVKISC KASGYAFSSY WMNWVKQRPG QGLEWIGQIW PGDGDTNYNG 180

KFKGKATLTA DESSSTAYMQ LSSLASEDSA VYFCARRETT TVGRYYYAMD YWGQGTSVTV 240

SSAKTTPQVQ LQQSGAELAR PGASVKMSCK ASGYTFTRYT MHWVKQRPGQ GLEWIGYINP 300

SRGYTNYNQK FKDKATLTTD KSSSTAYMQL SSLTSEDSAV YYCARYYDDH YSLDYWGQGT 360

TLTVSSAKTT PKLGGDIVLT QSPAIMSASP GEKVTMTCSA SSSVSYMNWY QQKSGTSPKR 420

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NRADTAAAGS HHHHHH 496

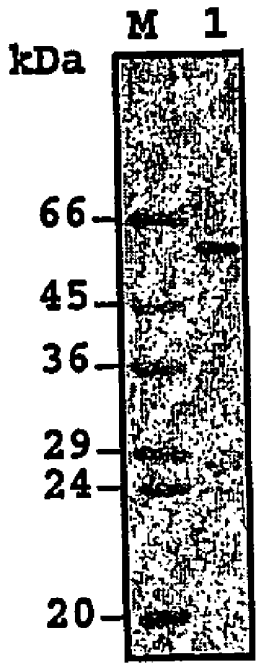


Figure 17

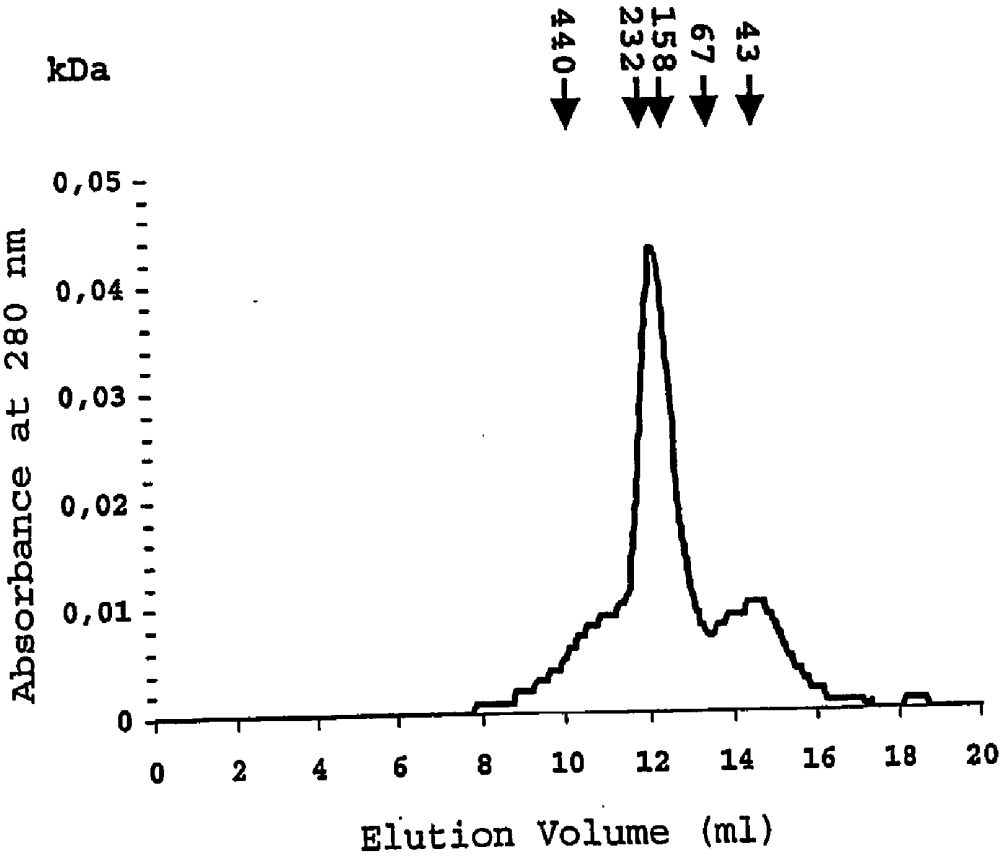
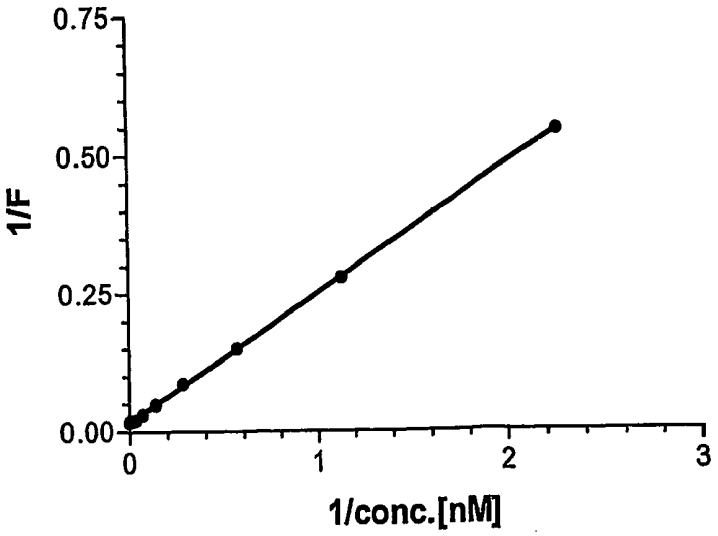
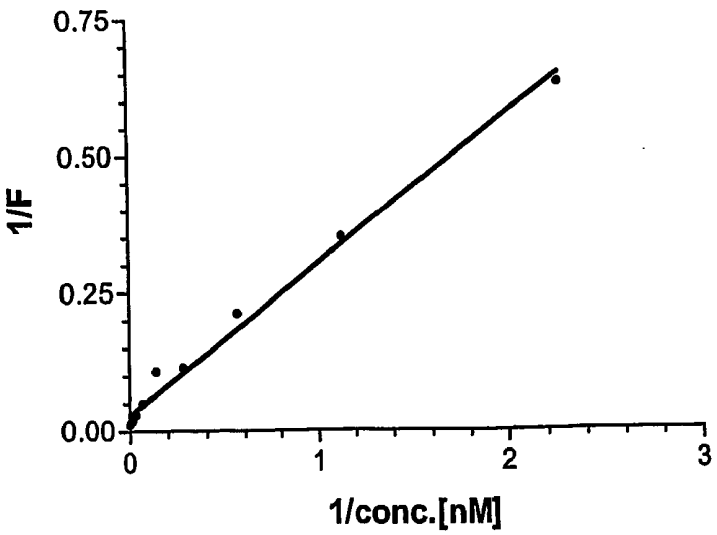

kDa
440
232
158
67
43
0,05
0,04
0,03
0,02
0,01
0
Absorbance at 280 nm
0
2
4
6
8
10
12
14
16
18
20
Elution Volume (ml)

FIGURE 18

A



B



FIGURES 19 A and B

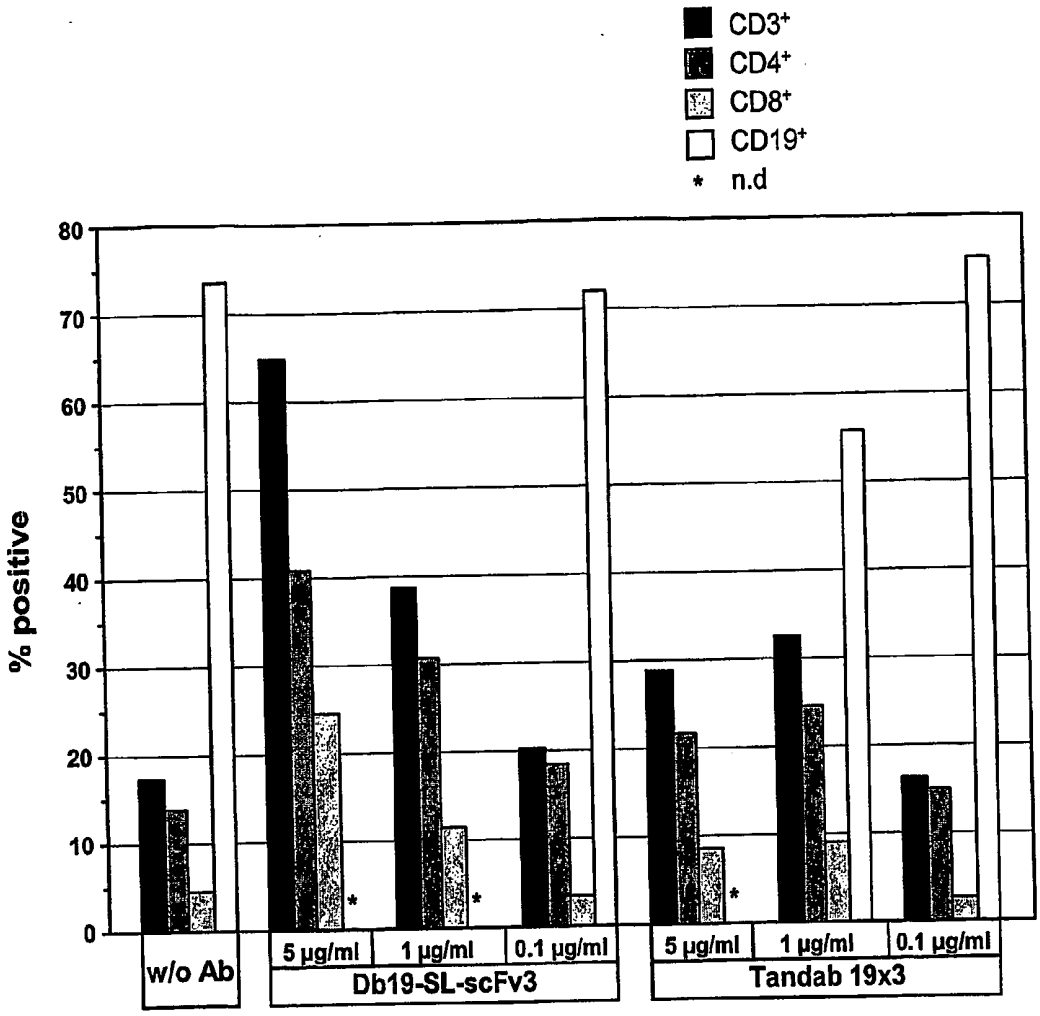

CD3+
CD4+
CD8+
CD19+
* n.d
% positive
80
70
60
50
40
30
20
10
0
w/o Ab
5 µg/ml
1 µg/ml
0.1 µg/ml
Db19-SL-scFv3
5 µg/ml
1 µg/ml
0.1 µg/ml
Tandab 19x3

FIGURE 20

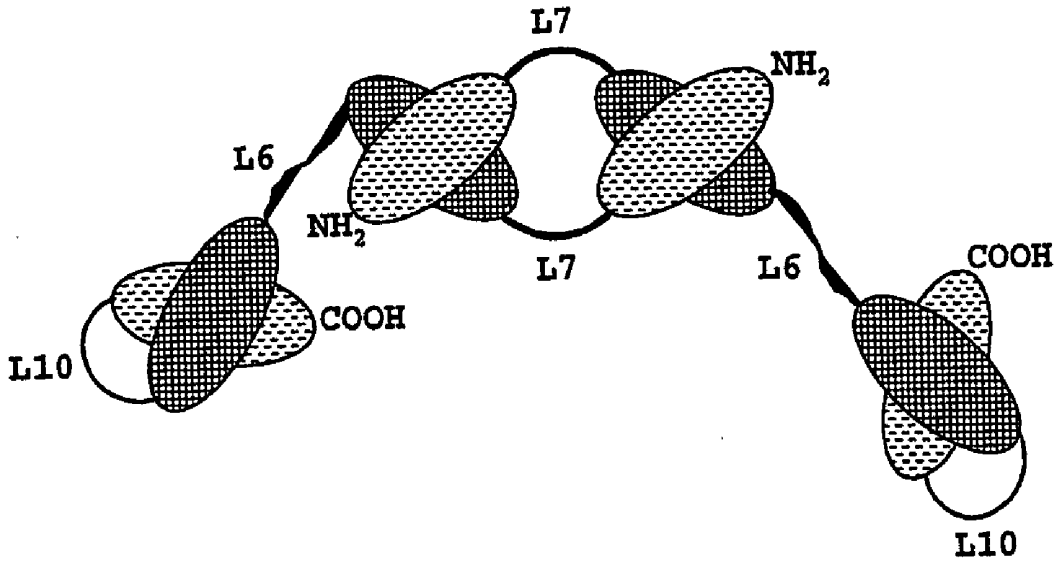
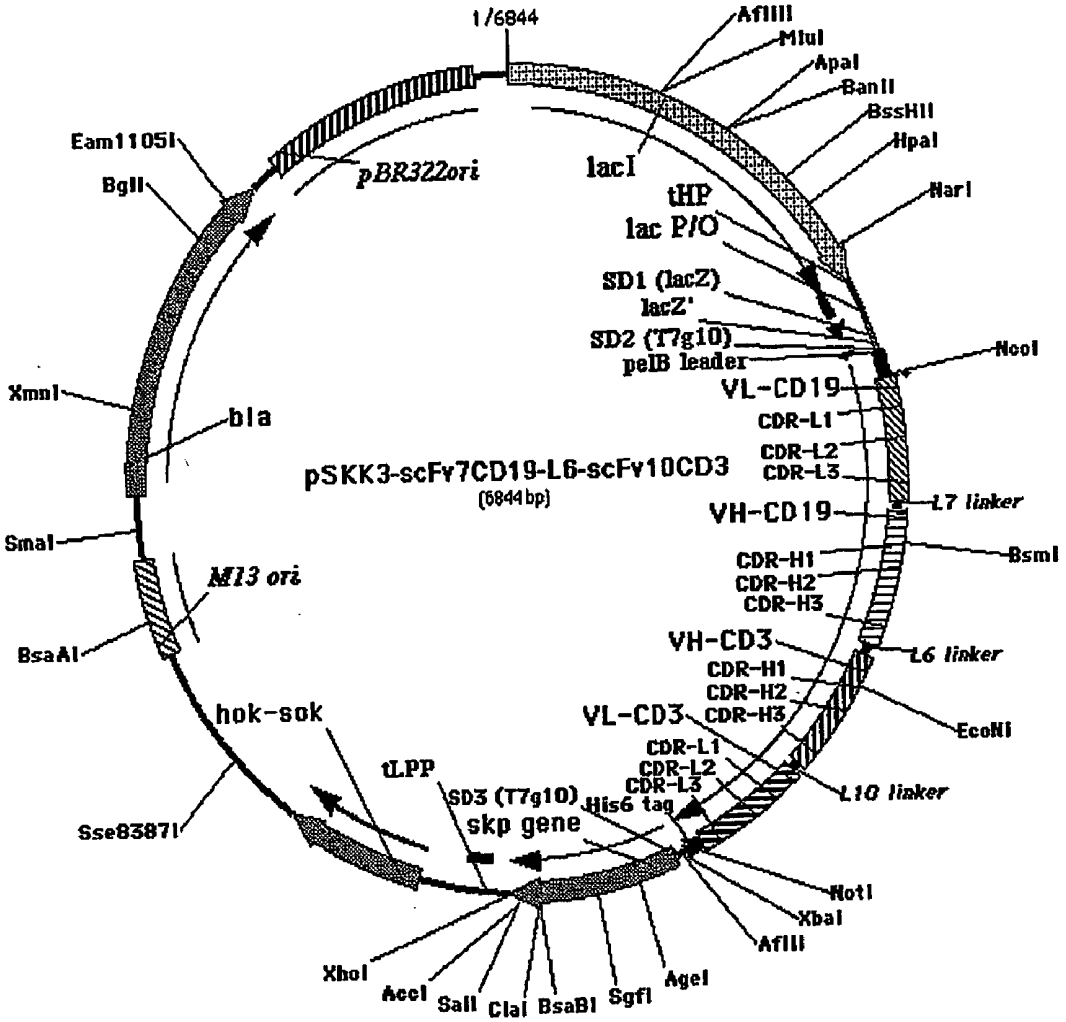


FIGURE 21


1/6844
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MluI
ApaI
BanII
BssHII
HpaI
HarI
NcoI
Eam1105I
BglI
pBR322ori
lacI
tHP
lac P/O
SD1 (lacZ)
lacZ'
SD2 (T7g10)
pelB leader
VL-CD19
CDR-L1
CDR-L2
CDR-L3
L7 linker
VH-CD19
BsmI
CDR-H1
CDR-H2
CDR-H3
VH-CD3
L6 linker
CDR-H1
CDR-H2
CDR-H3
EcoNI
VL-CD3
CDR-L1
CDR-L2
CDR-L3
L10 linker
His6 tag
SD3 (T7g10)
skp gene
tLPP
hok-sok
Sse8387I
BsaAI
SmaI
M13 ori
bla
XmnI
pSKK3-scFv7CD19-L6-scFv10CD3
(6844 bp)
NotI
XbaI
AflII
AgeI
SgfI
BsaBI
ClaI
SalI
AccI
XhoI

FIGURE 22

FIGURE 23

<110> Affimed Therapeutics AG

<120> Dimeric and multimeric Antigen Binding Structure

<130>

<140> EP 01122104.1
<141>

<160>2

Figure23a

<210>1
<211>6844
<212>DNA
<213> Artificial Sequence

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cggtgtctct tatcagaccg tttcccgcgt ggtgaaccag gccagccacg tttctgcgaa 180

aacgcgggaa aaagtggaag cggcgatggc ggagctgaat tacattccca accgggtggc 240

acaacaactg gcgggcaaac agtcgttgct gattggcgtt gccacctcca gtctggccct 300

gcacgcgccg tcgcaaattg tcgcggcgat taaatctcgc gccgatcaac tgggtgccag 360

cgtggtggtg tcgatggtag aacgaagcgg cgtcgaagcc tgtaaagcgg cggtgcacaa 420

tcttctcgcg caacgcgtca gtgggctgat cattaactat ccgctggatg accaggatgc 480

cattgctgtg gaagctgcct gcactaatgt tccggcgtta tttcttgatg tctctgacca 540

gacacccatc aacagtatta ttttctccca tgaagacggt acgcgactgg gcgtggagca 600

tctggtcgca ttgggtcacc agcaaatcgc gctgttagcg ggcccattaa gttctgtctc 660

ggcgcgtctg cgtctggctg gctggcataa atatctcact cgcaatcaaa ttcagccgat 720

agcggaacgg gaaggcgact ggagtgccat gtccggtttt caacaaacca tgcaaatgct 780

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cgacgatacc gaagacagct catgttatat cccgccgtta accaccatca aacaggattt 960

tcgcctgctg gggcaaacca gcgtggaccg cttgctgcaa ctctctcagg gccaggcggt 1020

gaagggcaat cagctgttgc ccgtctcact ggtgaaaaga aaaaccaccc tggcgcccaa 1080

tacgcaaacc gcctctcccc gcgcgttggc cgattcatta atgcagctgg cacgacaggt 1140

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ccgttttgtt ttgcagccca cctcaacgca attaatgtga gttagctcac tcattaggca 1260

ccccaggctt tacactttat gcttccggct cgtatgttgt gtggaattgt gagcggataa 1320

caatttcaca caggaaacag ctatgaccat gattacgaat ttctgaagaa ggagatatac 1380

atatgaaata cctattgcct acggcagccg ctggcttgct gctgctggca gctcagccgg 1440

ccatggcgga tatcttgctc acccaaactc cagcttcttt ggctgtgtct ctagggcaga 1500

gggccaccat ctcctgcaag gccagccaaa gtgttgatta tgatggtgat agttatttga 1560

actggtacca acagattcca ggacagccac ccaaactcct catctatgat gcatccaatc 1620

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gaggcatccc tatgtctagt ccacatcagg atagcctctt accgcgcttt gcgcaaggag 3900

aagaaggcca tgaaactacc acgaagttcc cttgtctggt gtgtgttgat cgtgtgtctc 3960

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catg 6844

FIGURE 23b:

<210>2
<211>496
<212>PRT
<213>Artificial Sequence
<400>2:

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QLQQSGAELV RPGSSVKISC KASGYAFSSY WMNWVKQRPG QGLEWIGQIW PGDGDTNYNG 180

KFKGKATLTA DESSSTAYMQ LSSLASEDSA VYFCARRETT TVGRYYYAMD YWGQGTSVTV 240

SSAKTTPQVQ LQQSGAELAR PGASVKMSCK ASGYTFTRYT MHWVKQRPGQ GLEWIGYINP 300

SRGYTNYNQK FKDKATLTTD KSSSTAYMQL SSLTSEDSAV YYCARYYDDH YSLDYWGQGT 360

TLTVSSAKTT PKLGGDIVLT QSPAIMSASP GEKVTMTCSA SSSVSYMNWY QQKSGTSPKR 420

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NRADTAAAGS HHHHHH 496

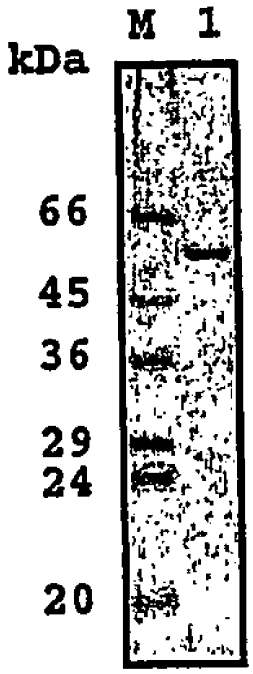
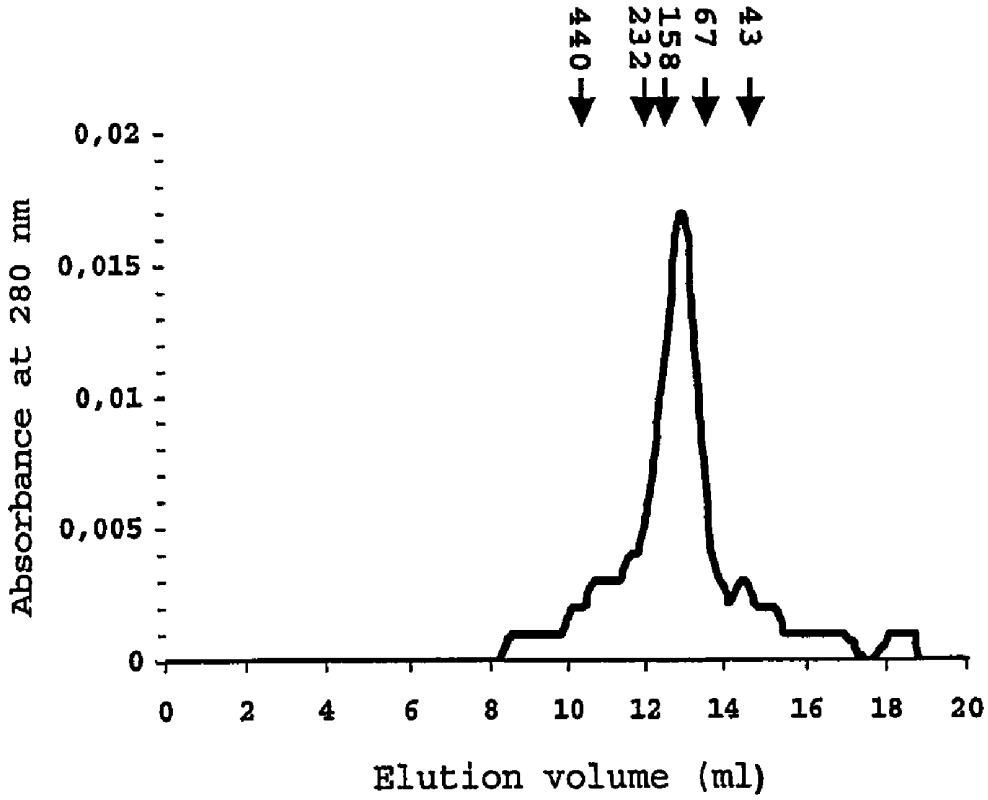


Figure 24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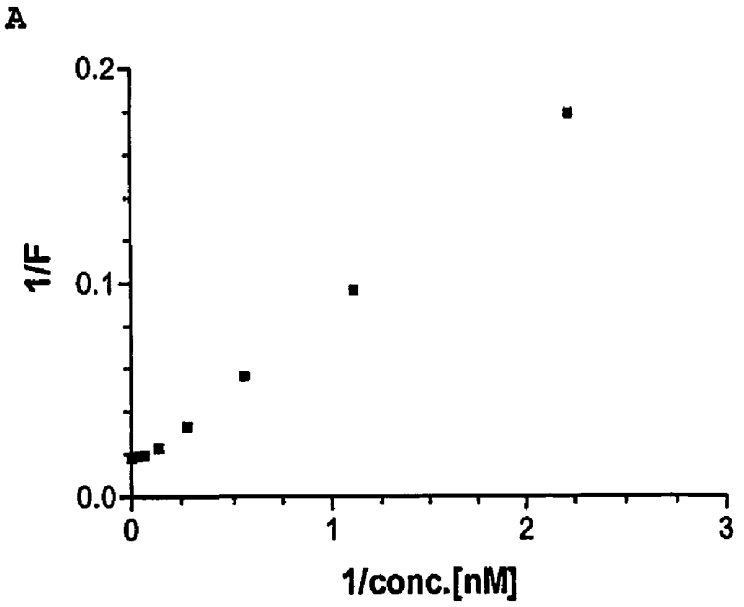
158
67
43
0,02
0,015
0,01
0,005
0
Absorbance at 280 nm
0
2
4
6
8
10
12
14
16
18
20
Elution volume (ml)

FIGURE 25



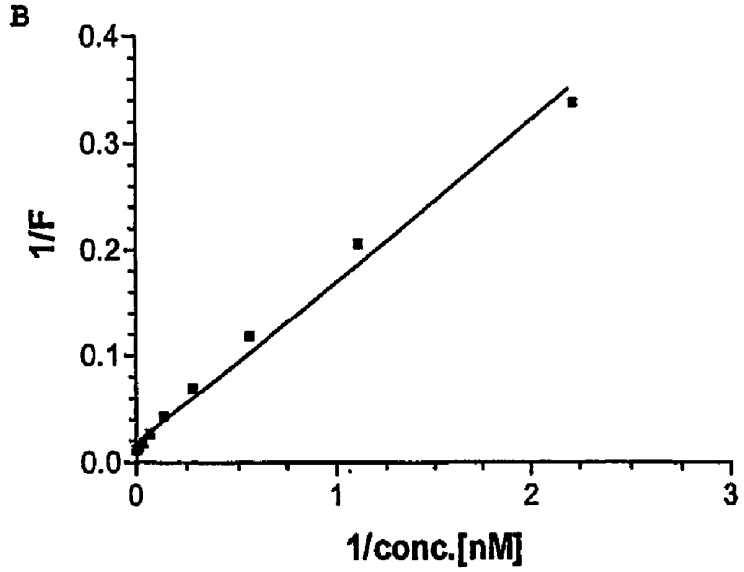


FIGURE 26

DIMERIC AND MULTIMERIC ANTIGEN BINDING STRUCTURE

[0001] This application is a National Stage of International Application PCT/EP02/10307, filed Sep. 13, 2002, published Mar. 27, 2003 under PCT Article 21(2) in English; which claims the priority of EP 01122104.1 filed Sep. 14, 2001.

FIELD OF THE INVENTION

[0002] The present invention relates to dimeric and multimeric antigen binding structures, expression vectors encoding said structures, and diagnostic as well as therapeutic uses of said structures. The antigen binding structures are preferably in the form of a Fv-antibody construct.

[0003] Natural antibodies are themselves dimers, and thus, bivalent. If two hybridoma cells producing different antibodies are artificially fused, some of the antibodies produced by the hybrid hybridoma are composed of two monomers with different specificities. Such bispecific antibodies can also be produced by chemically conjugating two antibodies. Natural antibodies and their bispecific derivatives are relatively large and expensive to produce. The constant domains of mouse antibodies are also a major cause of the human anti-mouse antibody (HAMA) response, which prevents their extensive use as therapeutic agents. They can also give rise to unwanted effects due to their binding of Fc-receptors. For these reasons, molecular immunologists have been concentrating on the production of the much smaller Fab- and Fv-fragments in microorganisms. These smaller fragments are not only much easier to produce, they are also less immunogenic, have no effector functions, and, because of their relatively small size, they are better able to penetrate tissues and tumors. In the case of the Fab-fragments, the constant domains adjacent to the variable domains play a major role in stabilizing the heavy and light chain dimer.

[0004] The Fv-fragment is much less stable, and a peptide linker was therefore introduced between the heavy and light chain variable domains to increase stability. This construct is known as a single chain Fv(scFv)-fragment. A disulfide bond is sometimes introduced between the two domains for extra stability. Thus far, tetravalent scFv-based antibodies have been produced by fusion to extra polymerizing domains such as the streptavidin monomer that forms tetramers, and to amphipathic alpha helices. However, these extra domains can increase the immunogenicity of the tetravalent molecule.

[0005] Bivalent and bispecific antibodies can be constructed using only antibody variable domains. A fairly efficient and relatively simple method is to make the linker sequence between the V_H and V_L domains so short that they cannot fold over and bind one another. Reduction of the linker length to 3-12 residues prevents the monomeric configuration of the scFv molecule and favors intermolecular V_H-V_L pairings with formation of a 60 kDa non-covalent scFv dimer "diabody" (Holliger et al., 1993, *Proc. Natl. Acad. Sci. USA* 90, 6444-6448). The diabody format can also be used for generation of recombinant bispecific antibodies, which are obtained by the noncovalent association of two single-chain fusion products, consisting of the V_H domain from one antibody connected by a short linker to the V_L domain of another antibody. Reducing the linker length still further below three residues can result in the formation of trimers ("triabody", ~90 kDa) or tetramers ("tetrabody", ~120 kDa) (Le Gall et al., 1999, *FEBS Letters* 453, 164-168). However, the small size of bispecific diabodies (50-60 kDa) leads to their rapid clearance from the blood stream through the kidneys, thus requiring the application of relatively high doses for therapy. Moreover, bispecific diabodies have only one binding domain for each specificity. However, bivalent binding is an important means of increasing the functional affinity, and possibly the selectivity, for particular cell types carrying densely clustered antigens.

[0006] Thus, the technical problem underlying the present invention is to provide new dimeric and multimeric antigen binding structures which overcome the disadvantages of the Fv-antibodies of the prior art, and to provide a general way to form a structure with at least four binding domains which is monospecific or multispecific.

[0007] The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

[0008] The present invention is further described with regard to the figures.

BRIEF DESCRIPTION OF THE DRAWINGS

[0009] **FIGS. 1, 2,** and **3** depict schemes of the multimeric Fv molecules depending on the particular antibody domains and the length of the peptide linker LL between the variable domains that comprise the multimerization motif.

[0010] Abbreviations L0: The V_H and V_L domains are directly connected without intervening linker peptide; L1: linker sequence coding for Ser residue; L10: linker sequence coding for SerAlaLysThrThrProLysLeuGlyGly polypeptide (SEQ ID NO:1) connecting V_H and V_L domains; LL: linker sequence coding for $(Gly_4Ser)_4$ polypeptide (SEQ ID NO:2) connecting hybrid scFv fragments; L18: linker sequence coding for SerAlaLysThrThrProLysLeuGlu-GluGlyGluPheSerGluAlaArgVal polypeptide (SEQ ID NO:3) connecting V_H and V_L domains.

[0011] **FIGS. 4 and 5** depict schemes of construction of the plasmids pHOG $scFv_{18}\alpha CD3$-LL-$scFv_{10}\alpha CD19$ and pHOG $scFv_{18}\alpha CD19$-LL-$scFv_{10}\alpha CD3$.

[0012] Abbreviations c-myc: sequence coding for an epitope recognized by mAb 9E10; His: sequence coding for six C-terminal histidine residues; PelB: signal peptide sequence of the bacterial pectate lyase (PelB leader); rbs: ribosome binding site; Stop: stop codon (TAA); V_H and V_L: variable region of the heavy and light chain; L10: linker sequence coding for SerAlaLysThrThrProLysLeuGlyGly polypeptide connecting V_H and V_L domains; LL: linker sequence coding for $(Gly_4Ser)_4$ polypeptide connecting hybrid scFv-fragments; L18: linker sequence coding for SerAlaLysThrThrProLysLeuGlu-GluGlyGluPheSerGluAlaArgVal polypeptide connecting V_H and V_L domains; B: BamHI, Ea: EagI, E: EcoRI, Nc: NcoI; N. NotI, P: PvuII, X: XbaI.

[0013] **FIG. 6** shows nucleotide (SEQ ID NO:4) and deduced amino acid sequences (SEQ ID NO:5) of the plasmid pSKK2 scFv3–LL-Db19.

[0014] Abbreviations His6 tail: sequence coding for six C-terminal histidine residues; β-lactamase: gene encoding β-lactamase that confers resistance to ampicillin resistance; bp: base pairs; c-myc epitope: sequence coding for an

epitope recognized by mAb 9E10; Lac P/0: wt lac operon promoter/operator; Pel B leader: signal peptide sequence of the bacterial pectate lyase; V_H and H_L: variable region of the heavy and light chain; L10: linker sequence coding for SerAlaLysThrThrProLysLeuGlyGly polypeptide connecting V_H and V_L domains; LL: linker sequence coding for $(Gly_4Ser)_4$ polypeptide connecting hybrid scFv-fragments; LI8: linker sequence coding for SerAlaLysThrThrProLysLeuGluGluGlyGluPheSerGluAlaArgVal polypeptide connecting V_H and V_L domains; rbs: ribosome binding site; V_H and V_L: variable region of the heavy and light chain; hok-sok: plasmid stabilizing DNA locus; lacI: gene coding for lac-repressor; lac P/0: wt lac operon promoter/operator; lacZ': gene coding for α-peptide of β-galactosidase; skp gene: gene encoding bacterial periplasmic factor Skp/OmpH; tLPP: nucleotide sequence of the lipoprotein terminator; M13 ori: origin of the DNA replication; pBR322ori: origin of the DNA replication; tHP: strong terminator of transcription; SD1: ribosome binding site (Shine Dalgarno) derived from *E. coli* lacZ gene (lacZ); SD2 and SD3: Shine Dalgarno sequence for the strongly expressed T7 gene 10 protein (T7g10).

[**0015**] **FIG. 7** shows nucleotide (SEQ ID NO:6; **FIG. 7***a*) and deduced amino acid (SEQ ID NO:7; **FIG. 7***b*) sequences of the plasmid pSKK2 scFv19-LL-Db3.

[**0016**] Abbreviations His6 tail: sequence coding for six C-terminal histidine residues; β-lactamase: gene encoding β-lactamase that confers resistance to ampicillin resistance; bp: base pairs; c-myc epitope: sequence coding for an epitope recognized by mAb 9E10; Lac P/0: wt lac operon promoter/operator; PelB leader: signal peptide sequence of the bacterial pectate lyase; V_H and V_L: variable region of the heavy and light chain; L10: linker sequence coding for SerAlaLysThrThrProLysLeuGlyGly polypeptide connecting V_H and V_L domains; LL: linker sequence coding for (Gly4Ser)4 polypeptide connecting hybrid scFv-fragments; Ll8: linker sequence encoding SerAlaLysThrThrProLysLeuGluGluGlyGluPheSerGluAlaArgVal polypeptide connecting V_H and V_L domains; rbs: ribosome binding site; hok-sok: plasmid stabilizing DNA locus; lacI: gene coding for lac-repressor; lac P/0: wt lac operon promoter/operator; lacZ': gene coding for α-peptide of β-galactosidase; skp gene: gene encoding bacterial periplasmic factor Skp/OmpH; tLPP: nucleotide sequence of the lipoprotein terminator; M13 ori: origin of the DNA replication, pBR322ori: origin of the DNA replication; tHP: strong terminator of transcription; SD1: ribosome binding site (Shine Dalgarno) derived from *E. coli* lacZ gene (lacZ); SD2 and SD3: Shine Dalgarno sequence for the strongly expressed T7 gene 10 protein (T7g10).

[**0017**] **FIG. 8** shows analyses of protein contents of peaks after IMAC.

[**0018**] Electrophoresis was carried out under reducing conditions; Western blot with anti-c-myc monoclonal antibody, in the case of scFv3-Db19 (**FIG. 8A**) and scFv 19×Db3 (**FIG. 8B**) molecules.

[**0019**] **FIG. 9** shows size-exclusion FPLC chromatography elution profiles.

[**0020**] A calibrated Superdex 200 HR10/30 column was used and the analysis of protein contents of peaks by Western blot was carried out with anti-c-myc monoclonal antibody, in the case of scFv3-Db19 (**FIG. 9A**) and scFv19-Db3 (**FIG. 9B**) molecules.

[**0021**] **FIG. 10** shows size-exclusion FPLC chromatography elution profiles. A calibrated Superdex 200 HR10/30 column for the scFv3-Db19, scFv19-Db3, scFv19-scFv3 and scFv3-scFv19 molecules was used.

[**0022**] **FIG. 11** shows flow cytometry results on $CD3^+$ Jurkat and $CD19^+$JOK-1 cells.

[**0023**] **FIG. 12** shows an analysis of cell surface retention on $CD19^+$JOK-1 (A,B) and $CD3^+$Jurkat cells (C,D) for the scFv3-scFv19 and scFv3-Db19 molecules (A,C) and for scFv19-scFv3 and scFv 19×Db3 molecules (B,D).

[**0024**] **FIG. 13** shows depletion of primary malignant $CD19^+$ CLL-cells by recruitment of autologous T-lymphocytes through CD19×CD3 bispecific molecules.

[**0025**] Freshly isolated peripheral blood mononuclear cells (PBMC) from patient with chronic lymphocytic leukemia (CLL) were seeded in individual wells of a 12-well plate in 2 ml RPMI-Medium/10% FCS at a density of 2×10^6 cells/ml. The recombinant antibodies scFv3-scFv19 and scFv3-Db19 were added at concentration of 5 μg/ml. After 5 day incubation, the cells were harvested, counted, and stained with anti-CD3 MAb OKT3, anti-CD4 MAb Edu-2, anti-CD8 MAb UCH-T4, and anti-CD19 MAb HD37 for flow cytometric analysis. 10^4 living cells were analyzed using a Beckman-Coulter flow cytometer and the relative amounts of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells were plotted.

[**0026**] **FIG. 14** is a schematic representation of the multimeric Fv-antibody construct formed by dimerizing via N-terminal "diabody" motif.

[**0027**] Abbreviations L7: 7 amino acid linker peptide Arg-Thr-Val-Ala-Ala-Pro-Ser (SEQ 10 NO:8) connecting the V_L and V_H domains in the dimerizing "diabody" motif; SL: 8 amino acid linker peptide Ala-Ala-Ala-Gly-Gly-Pro-Gly-Ser (SEQ ID NO:9) between the dimerizing motif and scFvs; L18: 18 amino acid linker peptide Ser-Ala-Lys-Thr-Thr-Pro-Lys-Leu-Glu-Glu-Gly-Glu-Phe-Ser-Glu-Ala-Arg-Val connecting the V_H and V_L domains in scFvs.

[**0028**] **FIG. 15** is a diagram of the expression plasmid pSKK3-$scFv_{L7}$anti-CD19-SL-$scFv_{L18}$anti-CD3.

[**0029**] Abbreviations bla: gene of beta-lactamase responsible for ampicillin resistance; bp: base pairs; CDR-H1, CDR-H2 and CDR-H3: sequence encoding the complementarity determining regions (CDR) 1-3 of the heavy chain; CDR-L1, CDR-L2 and CDR-L3: sequence encoding the complementarity determining regions (CDR) 1-3 of the light chain; His6 tag: sequence encoding six C-terminal histidine residues; hok-sok: plasmid stabilizing DNA locus; L7 linker: sequence which encodes the 7 amino acid peptide Arg-Thr-Val-Ala-Ala-Pro-Ser connecting the anti-CD19 V_L and V_H domains; L18 linker: sequence which encodes the 18 amino acid peptide Ser-Ala-Lys-Thr-Thr-Pro-Lys-Leu-Glu-Glu-Gly-Glu-Phe-Ser-Glu-Ala-Arg-Val connecting the anti-CD3 V_H and V_L domains; lacI: gene encoding lac-repressor; lac P/O: wild-type lac-operon promoter/operator; M13ori: intergenic region of bacteriophage M13; pBR322ori: origin of the DNA replication; PelB leader: signal peptide sequence of the bacterial pectate lyase; SD1: ribosome binding site derived from *E. coli* lacZ gene (lacZ); SD2 and SD3: ribosome binding site derived from the strongly expressed gene 10 of bacteriophage T7 (T7g10); skp gene: gene

encoding bacterial periplasmic factor Skp/OmpH; SL linker: sequence which encodes the 9 amino acid peptide Ser-Ala-Ala-Ala-Gly-Gly-Pro-Gly-Ser (SEQ 10 No:10) connecting the anti-CD19 and anti-CD3 V_H domains; tHP: strong transcriptional terminator; tLPP: lipoprotein terminator of transcription; V_H and V_L: sequence coding for the variable region of the immunoglobulin heavy and light chain, respectively. Unique restriction sites are indicated.

[0030] **FIG. 16** shows nucleotide (**FIG. 16***a*) and deduced amino acid (**FIG. 16***b*) sequences of the plasmid pSKK3-$scFv_{L7}$anti-CD19-SL-$scFv_{L18}$anti-CD3.

[0031] **FIG. 17** shows an analysis of purified Db19-SL-scFv3 molecule by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

[0032] Lane 1: M_r markers (kDa, M_r in thousands) Lane 2: Db19-SL-scFv3. The gel was stained with Coomassie Blue.

[0033] **FIG. 18** shows an analysis of purified Db19-SL-scFv3 molecule by size exclusion chromatography on a calibrated Superdex 200 column.

[0034] The elution positions of molecular mass standards are indicated.

[0035] **FIG. 19** shows a Lineweaver-Burk analysis of fluorescence dependence on antibody concentration as determined by flow cytometry.

[0036] Binding of Db19-SL-scFv3 to $CD3^+$ Jurkat (A) and $CD19^+$ JOK-1 cells (B) was measured.

[0037] **FIG. 20** shows depletion of primary malignant $CD19^+$ CLL-cells by recruitment of autologous T-lymphocytes through Db19-SL-scFv3 molecule.

[0038] Freshly isolated peripheral blood mononuclear cells (PBMC) from a patient with chronic lymphocytic leukemia (CLL) were seeded in individual wells of a 12-well plate in 2 ml RPMI-Medium/10% FCS at a density of 2×10^6 cells/ml. The recombinant scFv-antibody Db19-SL-scFv3 or CD19×CD3 tandem diabody (Tandab; Kipriyanov et al. 1999, J. Mol. Biol. 293, 41-56; Cochlovius et al. 2000, Cancer Res. 60, 4336-4341) was added at concentrations of 5 μg/ml, 1 μg/ml, and 0.1 μg/ml. After 5 day incubation, the cells were harvested, counted, and stained with anti-CD3 MAb OKT3, anti-CD4 MAb Edu-2, anti-CD8 MAb UCH-T4, and anti-CD19 MAb HD37 for flow cytometric analysis. 10^4 living cells were analyzed using a Beckman-Coulter flow cytometer and the relative amounts of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells were plotted. n.d.: not determined due to CD19 coating and/or modulation.

[0039] **FIG. 21** is a schematic representation of the multimeric $scFv_7$-L_6-$scFv_{10}$ Fv-antibody construct formed by dimerizing via N-terminal "diabody" motif.

[0040] Abbreviations L7: 7 amino acid linker peptide Arg-Thr-Val-Ala-Ala-Pro-Ser connecting the V_L and V_H domains in the dimerizing "diabody" motif; L6: 6 amino acid linker peptide Ser-Ala-Lys-Thr-Thr-Pro (SEQ ID NO:13) between the dimerizing motif and scFvs; L10: 10 amino acid linker peptide Ser-Ala-Lys-Thr-Thr-Pro-Lys-Leu-Gly-Gly connecting the V_H and V_L domains in the scFvs.

[0041] **FIG. 22** is a diagram of the expression plasmid pSKK3-$scFv_{L7}$anti-CD19-L6-$scFv_{L10}$anti-CD3.

[0042] Abbreviations bla: gene of beta-lactamase responsible for ampicillin resistance; bp: base pairs; CDR-H1, CDR-H2 and CDR-H3: sequence encoding the complementarity determining regions (CDR) 1-3 of the heavy chain; CDR-L1, CDR-L2 and CDR-L3: sequence encoding the complementarity determining regions (CDR) 1-3 of the light chain; His6 tag: sequence encoding six C-terminal histidine residues; hok-sok: plasmid stabilizing DNA locus; L6 linker: sequence which encodes the 6 amino acid peptide Ser-Ala-Lys-Thr-Thr-Pro connecting the anti-CD19 and anti-CD3 V_H domains; L7 linker: sequence which encodes the 7 amino acid peptide Arg-Thr-Val-Ala-Ala-Pro-Ser connecting the anti-CD19 V_L and V_H domains; L10 linker: sequence which encodes the 10 amino acid peptide Ser-Ala-Lys-Thr-Thr-Pro-Lys-Leu-Gly-Gly connecting the anti-CD3 V_H and V_L domains; lacI: gene encoding lac-repressor; lac P/O: wild-type lac-operon promoter/operator; M13ori: intergenic region of bacteriophage M13; pBR322ori: origin of the DNA replication; PelB leader: signal peptide sequence of the bacterial pectate lyase; SD1: ribosome binding site derived from *E. coli* lacZ gene (lacZ); SD2 and SD3: ribosome binding site derived from the strongly expressed gene 10 of bacteriophage T7 (T7g10); skp gene: gene encoding bacterial periplasmic factor Skp/OmpH; tHP: strong transcriptional terminator; tLPP: lipoprotein terminator of transcription; V_H and V_L: sequence coding for the variable region of the immunoglobulin heavy and light chain, respectively. Unique restriction sites are indicated.

[0043] **FIG. 23** shows nucleotide (SEQ ID NO:11; **FIG. 23***a*) and deduced amino acid (SEQ ID NO:1; **FIG. 23***b*) sequences of the plasmid pSKK3-$scFv_{L7}$anti-CD19-L6-$scFv_{L10}$anti-CD3

[0044] **FIG. 24** shows an analysis of purified Db19-L6-scFv3 molecule by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

[0045] Lane 1: M_r markers (kDa, M_r in thousands) Lane 2: Db19-L6-scFv3. The gel was stained with Coomassie Blue.

[0046] **FIG. 25** shows an analysis of purified Db19-L6-scFv3 molecule by size exclusion chromatography on a calibrated Superdex 200 column.

[0047] The elution positions of molecular mass standards are indicated.

[0048] **FIG. 26** shows a Lineweaver-Burk analysis of fluorescence dependence on concentration of Db19-L6-scFv3 as determined by flow cytometry.

[0049] Binding of Db19-L6-scFv3 to $CD3^+$ Jurkat (A) and $CD19^+$JOK-1 cells (B) was measured.

DETAILED DESCRIPTION OF THE INVENTION

[0050] The present invention is based on the observation that scFv-dimers, -trimers and -tetramers that are placed in the N-terminal or C-terminal part of the molecule can be used as multimerization motifs for construction of multimeric Fv-molecules. Thus, the present invention provides a general way to form a multimeric Fv molecule with at least four binding domains which is monospecific or multispe-

cific. Each monomer of the Fv molecule of the present invention is characterized by a V_H/V_L antigen-binding unit and two antibody variable domains that form V_H/V_L antigen-binding units after binding non-covalently to the variable domains of other monomers (multimerization motif). Dimers, trimers or tetramers are formed depending on the variable domains and the length of the peptide linkers between the variable domains that comprise the multimerization motif (see **FIGS. 1, 2, 3, 14, 21**).

[**0051**] The dimeric or multimeric antigen binding structures of the present invention, preferably in form of multimeric Fv-antibodies, are expected to be very stable and have a higher binding capacity. They should also be particularly useful for therapeutic purposes, since the dimeric diabodies used so far are small and remove fairly quickly from the blood stream through the kidneys. Moreover, the single chain format of the multimeric Fv-antibodies of the present invention allows them to be made in eukaryotic organisms and not only in bacteria.

[**0052**] Accordingly, the present invention relates to a dimeric or multimeric structure comprising a single chain molecule that comprises four antibody variable domains, wherein

[**0053**] (a) either the first two or the last two of the four variable domains bind intramolecularly to one another within the same chain by forming an antigen binding scFv in the orientation V_H/V_L or V_L/V_H

[**0054**] (b) the other two domains bind intermolecularly with the corresponding V_H or V_L domains of another chain to form antigen binding V_H/V_L pairs.

[**0055**] In a particularly preferred embodiment the present invention relates to a multimeric Fv-antibody, characterized by the following features:

[**0056**] (a) the monomers of said Fv-antibody comprise at least four variable domains of which two neighboring domains of one monomer form an antigen-binding V_H-V_L or V_L-V_H scFv unit; these two variable domains are linked by a peptide linker of at least 5 amino acid residues, preferably of at least 6, 7, 8, 9, 10, 11, or 12 amino acids, which does not prevent the intramolecular formation of a scFv,

[**0057**] (b) at least two variable domains of the monomer are non-covalently bound to two variable domains of another monomer resulting in the formation of at least two additional antigen binding sites to form the multimerization motif; these two variable domains of each monomer are linked by a peptide linker of a maximum of 12, preferably a maximum of 10 amino acid residues.

[**0058**] A further preferred feature is that the antigen-binding V_H-V_L or V_L-V_H scFv unit formed by the two neighbouring domains of one monomer is linked to the other variable domains of the multimerization motif by a peptide linker of at least 5 amino acid residues, preferably of at least 6, 7, 8, 9, 10, 11, or 12 amino acid residues.

[**0059**] The term "Fv-antibody" relates to an antibody containing variable domains but not constant domains.

[**0060**] The term "peptide linker" relates to any peptide capable of connecting two variable domains with its length depending on the kinds of variable domains to be connected. The peptide linker might contain any amino acid residue with the amino acid residues glycine, serine and proline being preferred for the peptide linker linking the second and third variable domain.

[**0061**] The term "intramolecularly" means interaction between V_H and V_L domains which belong to the same polypeptide chain (monomer) with the formation of a functional antigen binding site.

[**0062**] The term "intermolecularly" means interaction of the V_H and V_L domains which belong to different monomers.

[**0063**] The dimeric or multimeric antigen binding construct, e.g., the multimeric Fv-antibody of the present invention, can be prepared according to standard methods. Preferably, said Fv-antibody is prepared by ligating DNA sequences encoding the peptide linkers with the DNA sequences encoding the variable domains, such that the peptide linkers connect the variable domains, resulting in the formation of a DNA sequence encoding a monomer of the multimeric Fv-antibody and expressing DNA sequences encoding the various monomers in a suitable expression system as described in the Examples below.

[**0064**] The antigen binding structures, in particular the Fv-antibodies, of the present invention can be further modified using conventional techniques known in the art, for example, by using amino acid deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s), and/or any other modification(s) known in the art either alone or in combination. Methods for introducing such modifications in the DNA sequence underlying the amino acid sequence of a variable domain or peptide linker are well known to the person skilled in the art; see, e.g., Sambrook, Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory (1989) N.Y.

[**0065**] The antigen binding structures of the present invention can comprise at least one further protein domain, said protein domain being linked by covalent or non-covalent bonds. The linkage can be based on genetic fusion according to the methods known in the art and described above, or can be performed by, e.g., chemical cross-linking as described in, e.g., WO 94/04686. The additional domain present in the fusion protein comprising the structure employed in accordance with the invention may be linked preferably by a flexible linker, and advantageously by a peptide linker, wherein said peptide linker comprises plural, hydrophilic, peptide-bonded amino acids of a length sufficient to span the distance between the C-terminal end of said further protein domain and the N-terminal end of the Fv-antibody, or vice versa. The above described fusion protein may further comprise a cleavable linker or cleavage site for proteinases. The fusion protein may also comprise a tag, like a histidine-tag, e.g., $(His)_6$.

[**0066**] In a preferred embodiment of the present invention, the monomers of the antigen binding structure comprise four variable domains, and either the first and second, or the third and fourth, variable domains of the monomers are linked by a peptide linker of 12, 11, 10, or less amino acid residues, preferably less than five amino acid residues. In an even more preferred embodiment, either the first and second or the third and fourth domain are linked directly without intervening amino acid residues.

[0067] In another preferred embodiment of the present invention, the second and third variable domains of the monomers are linked by a peptide linker of at least 5 amino acid residues, preferably of at least 6, 7, 8, 9, 10, 11, or 12. Preferably, the maximum number of amino acid residues is 30.

[0068] In another preferred embodiment of the present invention, any variable domain is shortened by at least one amino acid residue at its N- and/or C-terminus. In some circumstances, this truncated form gives a better stability of the molecule, as described in German patent application 100 63 048.0.

[0069] In a particularly preferred embodiment of the present invention, the order of domains of a monomer is V_H-V_L-V_H-V_L,V_L-V_H-V_H-V_L, V_H-V_L-V_L-V_H or V_L-V_H-V_L-V_H.

[0070] In some cases, it might be desirable to strengthen the association of two variable domains. Accordingly, in a further preferred embodiment of the multimeric Fv-antibody of the present invention, the binding of at least one pair of variable domains is strengthened by at least one intermolecular disulfide bridge. This can be achieved by modifying the DNA sequences encoding the variable domains accordingly, i.e., by introducing cysteine codons. The two most promising sites for introducing disulfide bridges appeared to be V_H44-V_L100 connecting framework 2 of the heavy chain with framework 4 of the light chain and V_H105-V_L43 that links framework 4 of the heavy chain with framework 2 of the light chain.

[0071] In a further preferred embodiment of the present invention, the multimeric Fv-antibody is a tetravalent dimer, hexavalent trimer, or octavalent tetramer. The formation of such forms is preferably determined by particular V_H and V_L domains comprising the multimerization motif and by the length of the linker.

[0072] In another preferred embodiment of the present invention, the multimeric Fv-antibody is a bispecific, trispecific, tetraspecific, . . . etc. antibody.

[0073] Multimerization of the monomeric subunits can be facilitated by the presence of a dimerization motif at the C-terminus of the fourth variable domain, which is, preferably, a (poly)peptide directly linked via a peptide bond. Examples of such dimerization motifs are known to the person skilled in the art and include streptavidin and amphipathic alpha helixes. Accordingly, in a further preferred embodiment, a dimerization motive is fused to the last domain of at least two monomers of the multimeric Fv-antibody of the present invention.

[0074] For particular therapeutic applications, at least one monomer of the multimeric antibody of the invention can be linked non-covalently or covalently to a biologically active substance (e.g., cytokines or growth hormones), a chemical agent (e.g. doxorubicin, cyclosporin), a peptide (e.g., α-Amanitin), a protein (e.g., granzyme A and B).

[0075] In an even more preferred embodiment, the multimeric Fv-antibody of the present invention is (I) a monospecific antibody capable of specifically binding the CD19 antigen of B-lymphocytes or the carcinoma embryonic antigen (CEA); or (II) a bispecific antibody capable of specifically binding (a) CD19 and the CD3 complex of the T-cell receptor, (b) CD19 and the CD5 complex of the T-cell receptor, (c) CD19 and the CD28 antigen on T-lymphocytes, (d) CD19 and CD16 on natural killer cells, macrophages and activated monocytes, (e) CEA and CD3, (f) CEA and CD28, or (g) CEA and CD16. The nucleotide sequences of the variable domains have already been obtained and described in the case of the antibody anti-CD19 (Kipriyanov et al., 1996, J. Immunol. Methods 200, 51-62), anti-CD3 (Kipriyanov et al., 1997, Protein Engineer. 10, 445-453), anti-CD28 (Takemura et al.; 2000, FEBS Lett. 476, 266-271), anti-CD16 (German Patent Application DE 199 37 264 A1), anti-CEA (Griffiths et al., 1993, EMBO J. 12, 725-734), and anti-CD5 (Better et al., 1993, Proc. Natl. Acad. Sci. U.S.A. 90, 457-461).

[0076] Surprisingly, a tetravalent structure as defined in claim 1 with the specificities anti-CD3 and anti-CD19 showed a much higher efficacy in vitro than a corresponding bivalent (scFv)x2 structure and a tetravalent structure in which all of the four domains formed pairs with corresponding domains of another chain.

[0077] Another object of the present invention is a process for the preparation of a multimeric Fv-antibody according to the present invention, wherein (a) DNA sequences encoding the peptid linkers are ligated with the DNA sequences encoding the variable domains, such that the peptide linkers connect the variable domains, resulting in the formation of a DNA sequence encoding a monomer of the multimeric Fv-antibody, and (b) the DNA sequences encoding the various monomers are expressed in a suitable expression system. The various steps of this process can be carried according to standard methods, e.g., methods described in Sambrook et al., or described in the Examples below.

[0078] The present invention also relates to DNA sequences encoding the multimeric Fv-antibody of the present invention and vectors, preferably expression vectors containing said DNA sequences.

[0079] A variety of expression vector/host systems may be utilized to contain and express sequences encoding the multimeric Fv-antibody. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems. The invention is not limited by the host cell employed.

[0080] The "control elements" or "regulatory sequences" are those non-translated regions of the vector-enhancers, promoters, 5' and 3' untranslated regions-which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the Bluescript.R™ phagemid (Stratagene, LaJolla, Calif.) or pSport1.™ plasmid (Gibco BRL) and the like may be used. The baculovirus polyhedrin promoter may be used in insect

cells. Promoters or enhancers derived from the genomes of plant cells (e.g., heat shock, RUBISCO, and storage protein genes) or from plant viruses (e.g., viral promoters or leader sequences) may be cloned into the vector. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are preferable. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding the multimeric Fv-antibody, vectors based on SV40 or EBV may be used with an appropriate selectable marker.

[0081] In bacterial systems, a number of expression vectors may be selected depending upon the use intended for the multimeric Fv-antibody. Vectors suitable for use in the present invention include, but are not limited to, the pSKK expression vector for expression in bacteria.

[0082] In the yeast, *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Grant et al. (1987) Methods Enzymol. 153:516-544.

[0083] In cases where plant expression vectors are used, the expression of sequences encoding the multimeric Fv-antibody may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) EMBO J. 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, G. et al. (1984) EMBO J. 3:1671-1680; Broglie, R. et al. (1984) Science 224:838-843; and Winter, J. et al. (1991) Results Probl. Cell Differ. 17:85-105). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs, S. or Murry, L. E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, N.Y.; pp. 191-196.

[0084] An insect system may also be used to express the multimeric Fv-antibody. For example, in one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia larvae*. The sequences encoding the multimeric Fv-antibody may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the polyhedrin promoter. Successful insertion of the multimeric Fv-antibody will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses may then be used to infect, for example, *S. frugiperda* cells or *Trichoplusia larvae* in which APOP may be expressed (Engelhard, E. K. et al. (1994) Proc. Nat. Acad. Sci. 91:3224-3227).

[0085] In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, sequences encoding the multimeric Fv-antibody may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the multimeric Fv-antibody in infected host cells (Logan, J. and Shenk, T. (1984) Proc. Natl. Acad. Sci. 81:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

[0086] Human artificial chromosomes (HACs) may also be employed to deliver larger fragments of DNA than can be contained and expressed in a plasmid. HACs of 6 to 10M are constructed and delivered via conventional delivery methods (liposomes, polycationic amino polymers, or vesicles) for therapeutic purposes.

[0087] Specific initiation signals may also be used to achieve more efficient translation of sequences encoding the multimeric Fv-antibody. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the multimeric Fv-antibody, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in the case where only the coding sequence is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) Results Probl. Cell Differ. 20:125-162).

[0088] In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells which have specific cellular machinery and characteristic mechanisms for post-translational activities (e.g., CHO, HeLa, MDCK, HEK293, and W138), are available from the American Type Culture Collection (ATCC; Bethesda, Md.), and may be chosen to ensure the correct modification and processing of the foreign protein.

[0089] For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the multimeric Fv-antibody may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

[0090] Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) Cell 11:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1980) Cell 22:817-23) genes which can be employed in tk.sup.- or

aprt.sup.- cells, respectively. Also, antimetabolite, antibiotic, or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) Proc. Natl. Acad. Sci. 77:3567-70); npt, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. et al (1981) J. Mol. Biol. 150:1-14) and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, supra). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S. C. and R. C. Mulligan (1988) Proc. Natl. Acad. Sci. 85:8047-51). Recently, the use of visible markers has gained popularity with such markers as anthocyanins, β-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C. A. et al. (1995) Methods Mol. Biol. 55:121-131).

[**0091**] A particular expression vector is pSKK2-scFv$_{L18}$anti-CD3-LL-scFv$_{L10}$anti-CD19(pSKK2-scFv3LL Db19) (deposited with the DSMZ according to the Budapest Treaty under DSM 14470 at Aug. 22, 2001 or pSKK2-scFv$_{L18}$anti-CD19-LL-scFv$_{L10}$anti-CD3(pSKK2-scFv19LL Db3) (deposited with the DSMZ according to the Budapest Treaty under DSM 14471 at Aug. 22, 2001.

[**0092**] The present invention also relates to a pharmaceutical composition containing a multimeric Fv-antibody of the present invention, a DNA sequence, or an expression vector, preferably combined with suitable pharmaceutical carriers. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emuslions, various types of wetting agents, sterile solutions etc. Such carriers can be formulated by conventional methods and can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperetoneal, subcutaneous, intramuscular, topical, or intradermal administration. The route of administration, of course, depends on the nature of the disease, e.g., tumor, and the kind of compound contained in the pharmaceutical composition. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depend on many factors, including the patient's size, body surface area, age, sex, the particular compound to be administered, time and route of administration, the kind of the disorder, general health, and other drugs being administered concurrently.

[**0093**] Preferred medical uses of the compounds of the present invention are: (a) the treatment of a viral, bacterial, tumoral, or prion related diseases, (b) the agglutination of red blood cells, (c) linking cytotoxic cells, e.g., T or Natural killer cells of the immune system to tumor cells, or (d) linking activating cytokines, preferably IL-1, IL-2, IFNγ, TNFα, or GM-CSF, cytotoxic substances (e.g., doxorubicin, cyclosporin, α-Amanitin), or a protease, preferably Granzyme B, to a target cell.

[**0094**] A further object of the present invention is the use of a multimeric Fv-antibody of the present invention for diagnosis. For use in the diagnostic research, kits are also provided by the present invention, said kits comprising a multimeric antibody of the present invention. The Fv-antibody can be detectably labeled. In a preferred embodiment, said kit allows diagnosis by ELISA, and contains the Fv-antibody bound to a solid support, for example, a polystyrene microtiter dish or nitrocellulose paper, using techniques known in the art. Alternatively, said kit is based on a RIA, and contains said Fv-antibody marked with a radioactive isotope. In a preferred embodiment of the kit of the invention, the antibody is labelled with enzymes, fluorescent compounds, luminescent compounds, ferromagnetic probes, or radioactive compounds.

[**0095**] The following Examples illustrate the invention.

EXAMPLES

Example 1

Construction of the plasmids pSKK2 scFv$_{L18}$anti-CD3-LL-scFv$_{L10}$anti-CD19 (scFv3–Db19) and pSKK2 scFv$_{L18}$anti-CD19-LL-scFv$_{L10}$anti-CD3 (scFv19–Db3) for Expression of Multimeric Fv Molecules in Bacteria

[**0096**] For generation of multimeric Fv constructs, the plasmids pHOG_HD37, pHOG_Dia_HD37, pHOG_mOKT3+NotI and pHOG_Dia_mOKT3 encoding the antibody fragments were derived either from hybridoma HD37 specific to human CD19 (Kipriyanov et al., 1996, J. Immunol. Meth. 196, 51-62; Le Gall et al., 1999, FEBS Lett., 453, 164-168) or from hybridoma OKT3 specific to human CD3 (Kipriyanov et al., 1997, Protein Eng. 10, 445-453) were used.

[**0097**] The anti-CD19 ScFV$_{L10}$ gene followed by a segment coding for a c-myc epitope and a hexahistidinyl tail was cut with PvuII/XbaI from the plasmid pHOG Dia HD37, and recloned into the PvuII/XbaI linearized vector pDISC-1 LL (Kipriyanov et al., 1999, J. Mol. Biol. 293, 41-56) (**FIG. 4**). This hybrid plasmid was linearized by NcoI/NotI and the gene coding for the scFV$_{L18}$ (cut by NcoI/NotI from the plasmid pHOG mOKT3+NotI) was ligated into this plasmid. The plasmid obtained is the pHOG scFv$_{L18}$αCD3-LL-scFv$_{L10}$αCD19 (scFv3–Db19) (**FIG. 4**).

[**0098**] The linearized hybrid plasmid NcoI/NotI was also used for the ligation of the gene coding for the scFv$_{L18}$αCD19 from the plasmid pHOG HD37, and the plasmid obtained is the pHOG scFv$_{L18α}$CD19-LL-scFv$_{L10}$αCD19 (scFv19×Db19) (**FIG. 4**). This plasmid was linearized by PvuII/XbaI and the scFV$_{L10}$ gene followed by a segment coding for a c-myc epitope and a hexahistidinyl tail was cut with PvuII/XbaI from the plasmid pHOG mDia OKT3. The plasmid obtained is the PHOG scFv$_{L18}$αCD19-LL-scFv$_{L10}$αCD3 (scFv19–Db3) (**FIG. 5**).

[**0099**] To increase the yield of functional antibody fragments in the bacterial periplasm, an optimized expression vector pSKK2 was generated. This vector was constructed on the base of plasmid pHKK (Horn, 1996, Appl. Microbiol. Biotechnol., 46, 524-532) containing hok/sok plasmid-free cell suicide system (Thisted et al., 1994, EMBO J., 13, 1950-1956). First, the gene coding for hybrid scFv V$_H$3-V$_L$19 was amplified by PCR from the plasmid pHOG3-19 (Kipriyanov et al., 1998, Int. J. Cancer 77, 763-772) using the primers 5-NDE, 5'-GATATACATATGAAATACCTAT-

TGCCTACGGC (SEQ ID NO:14), and 3-AFL, 5'-CGAATTCTTAAGTTAGCACAGGCCTCTAGAGACACACAGATCTTTAG (SEQ ID NO:15). The resulting 921 bp PCR fragment was digested with NdeI and AflII, and cloned into the NdeI/AflII linearized plasmid PHKK, generating the vector pHKK3-19. To delete an extra XbaI site, a fragment of pHKK plasmid containing 3'-terminal part of the lacI gene (encodes the lac repressor), the strong transcriptional terminator t_{HP} and wild-type lac promoter/operator was amplified by PCR using primers 5-NAR, 5'-CACCCTGGCGCCCAATACGCAAACCGCC (SEQ ID NO:16), and 3-NDE, 5'-GGTATTTCATATGTATATCTCCTTCTTCAGAAATTCGTAATCATGG (SEQ ID NO:17). The resulting 329 bp DNA fragment was digested with NarI and NdeI, and cloned into NarI/NdeI linearized plasmid pHKK3-19 generating the vector pHKKΔXba. To introduce a gene encoding the Skp/OmpH periplasmic factor for higher recombinant antibody production (Bothmann and Plückthun, 1998, Nat. Biotechnol., 16, 376-380), the skp gene was amplified by PCR with primers skp-3, 5'-CGAATTCTTAAGAAGGAGATATACATATGAAAAAGTGGTTATTAGCTGCAGG (SEQ ID NO:18) and skp-4, 5'-CGAATTCTCGAGCATTATTTAACCTGTTTCAGTACGTCGG (SEQ ID NO:19) using as a template the plasmid pGAH317 (Holck and Kleppe, 1988, Gene, 67, 117-124). The resulting 528 bp PCR fragment was digested with AflII and XhoI and cloned into the AflII/XhoI digested plasmid pHKKΔXba resulting in the expression plasmid pSKK2.

[**0100**] The plasmids pHOG $scFv_{L18}\alpha CD3$-LL-$scFv_{L10}\alpha CD19$ (scFv3-Db19) and pHOG $scFv_{L18}\alpha CD19$-LL-$scFv_{L10}\alpha CD3$ (scFv19-Db3) were cut by NcoI/XbaI, and ligated in the NcoI/XbaI linearized plasmid pSKK2. The resulting plasmids are pSKK2 $scFv_{L18}\alpha CD3$-LL-$scFv_{L10}\alpha CD19$ and pSKK2 $scFv_{L18}\alpha CD19$-LL-$scFv_{L10}\alpha CD3$. The complete nucleotide and amino acid sequences are given in **FIGS. 6 and 7**, respectively.

Example 2

Expression and Purification of the Multimeric Fv Molecules in Bacteria

[**0101**] The *E. coli* K12 strain RV308 (Maurer et al., 1980, J. Mol. Biol. 139, 147-161), transformed with the expression plasmids pSKK2 $scFv_{L18}\alpha CD3$-LL-$scFv_{L10}\alpha CD19$ and pSKK2 $scFv_{L18}\alpha CD19$-LL-$scFv_{L10}\alpha CD3$, was grown overnight in 2xYT medium with 50 μg/ml ampicillin and 100 mM glucose ($2xYT_{GA}$) at 28° C. Dilutions (1:50) of the overnight cultures in $2xYT_{GA}$ were grown as flask cultures at 28° C. with shaking at 200 rpm. When cultures reached OD_{600}=0.8, bacteria were pelleted by centrifugation at 5,000×g for 10 min at 20° C., and resuspended in the same volume of fresh YTBS medium (2xYT containing 1 M sorbitol and 2.5 mM glycine betaine; Blacwell & Horgan, 1991, FEBS Letters. 295, 10-12) containing 50 μg/ml ampicillin. IPTG was added to a final concentration of 0.2 mM, and growth was continued at 20° C. for 18-20 h. Cells were harvested by centrifugation at 9,000×g for 20 min and 4° C. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 50 mM Tris-HCl, 20% sucrose, and 1 mM EDTA, pH 8.0. After a 1 h incubation on ice with occasional stirring, the spheroplasts were centrifuged at 30,000×g for 30 min at 4° C., leaving the soluble periplasmic extract as the supernatant and spheroplasts plus the insoluble periplasmic material as the pellet. The periplasmic fractions were dialyzed against start buffer (50 mM Tris-HCl, 1 M NaCl, 50 mM Imidazole, pH 7.0) at 4° C. The dialyzed solution containing recombinant product was centrifuged at 30,000×g for 30 min at 4° C. The recombinant product was concentrated by ammonium sulfate precipitation (final concentration 70% of saturation). The protein precipitate was collected by centrifugation (10,000×g, 4° C., 40 min), and dissolved in 10% of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0. Immobilized metal affinity chromatography (IMAC) was performed at 4° C. using a 5 ml column of Chelating Sepharose (Pharmacia) charged with Cu^{2+} and equilibrated with 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (start buffer). The sample was loaded by passing the sample over the column. It was then washed with twenty column volumes of start buffer followed by start buffer containing 50 mM imidazole until the absorbency (280 nm) of the effluent was minimal (about thirty column volumes). Absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 250 mM imidazole, pH 7.0. The elution fractions containing the multimeric Fv-molecules were identified by Western-blot analysis using anti-c-myc Mab 9E10, performed as previously described (Kipriyanov et al., 1994, Mol. Immunol. 31, 1047-1058) and as illustrated in **FIG. 8A** for scFv3-Db19 and **FIG. 8B** for scFv19-Db3.

[**0102**] The positive fractions were collected and concentrated on an Ultrafree-15 centrifugal filter device (Millipore Corporation, Eschborn, Germany) until 0.5 ml was collected.

[**0103**] Further purification of the multimeric Fv-molecules was done by size-exclusion FPLC on a Superdex 200 HR10/30 column (Pharmacia) in PBSI (15 mM sodium phosphate, 0,15 M NaCl, 50 mM Imidazole, pH 7.0). Sample volumes for preparative chromatography were 500 μl, and the flow rate was 0.5 ml/min, respectively. The column was calibrated with High and Low Molecular Weight Gel Filtration Calibration Kits (Pharmacia). The elution fractions containing the multimeric Fv-molecules were identified by Western-blot analysis using anti-c-myc Mab 9E10 performed as previously described (Kipriyanov et al., 1994, Mol. Immunol. 31, 1047-1058), and the results are presented in **FIGS. 9A and 9B** for scFv3-Db19 and scFv19-Db3 molecules, respectively. The fractions were collected and stored individually on ice.

[**0104**] The generated Fv molecules were compared with two scFv-scFv tandems, scFv3-scFv19 and scFv19-scFv3 (**FIG. 10**), produced and purified under the same conditions. **FIG. 10** clearly demonstrates that higher molecular forms were obtained for the scFv3×Db 19 and scFv 19×Db3 in comparison with scFv3×scFv 19 and scFv 19×scFv3. The main peak for scFv3-scFv19 and scFv19-scFv3 molecules correspond to a molecular weight of about 67 kDa, and to about 232 kDa for the scFv3-Db19 and scFv19-Db3. The presence of the dimerization motif on the C-terminus of the molecule has a positive effect for the multimerisation of the molecules.

Example 3

Characterization of the Multimeric Fv Molecules by Flow Cytometry

[0105] The human $CD3^+/CD19^-$ acute T cell leukemia line Jurkat and the $CD19^+/CD3^-$B cell line JOK-1 were used for flow cytometry. In brief, 5×10^5 cells in 50 μl RPMI 1640 medium (GIBCO BRL, Eggenstein, Germany) supplemented with 10% FCS and 0.1% sodium azide (referred to as complete medium) were incubated with 100 μl of a multimeric Fv molecule preparation for 45 min on ice. After washing with complete medium, the cells were incubated with 100 μl of 10 μg/ml anti c-myc MAb 9E10 (IC Chemikalien, Ismaning, Germany) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 100 μl of FITC-labeled goat anti-mouse IgG (GIBCO BRL) under the same conditions as before. The cells were then washed again and resuspended in 100 μl of 1 μg/ml solution of propidium iodide (Sigma, Deisenhofen, Germany) in complete medium to exclude dead cells. The relative fluorescence of stained cells was measured using a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) or Epics XL flow cytometer systems (Beckman Coulter, Miami, Fla.).

[0106] Flow cytometry experiments demonstrated specific interactions with both human $CD3^+$Jurkat and the $CD19^+$ JOK-1 cells for all the multimeric Fv-molecules (**FIG. 11**).

[0107] For the CD19 and CD3 binding affinities, we decided to use the fractions corresponding to the monomers for scFv3×scFv 19 and scFv19–scFv3, and to the multimers for scFv3×Db 19 and scFv 19×Db3.

Example 4

In Vitro Cell Surface Retention Assay of the Multimeric Fv Molecules

[0108] Cell surface retention assays were performed at 37° C. essentially as described (Adams et al., 1998, Cancer Res. 58, 485-490), except that the detection of the retained antibody fragments was performed using anti c-myc MAb 9E10 followed by FITC-labeled anti-mouse IgG. Kinetic dissociation constant (k_{off}) and the half-life ($t_{1/2}$) for dissociation of the multimeric Fv-molecules were deduced from a one-phase exponential decay fit of experimental data using "GraphPad" Prism (GraphPad Software, San Diego, Calif.). For control, the bispecific diabody CD19×CD3 (BsDb 19×3) described previously (Kipriyanov et al., 1998, Int. J. Cancer 77, 763-777; Cochlovius et al., 2000, J. Immunol. 165, 888-895) was used. The results of the experiments are shown in **FIG. 12** and summarized in Table 1.

[0109] The scFv3–scFv19 had a relatively short retention half-life ($t_{1/2}$) on $CD19^+$JOK-1 cells, almost two time less than with the $t_{1/2}$ of the BsDb 19×3 (Table 1). In contrast, the scFv3–Db19 was retained longer on the surface of JOK-1 cells. For the scFv19–scFv3, the $t_{1/2}$ is in the same range as the $t_{1/2}$ of the BsDb 19×3. The retention of the scFv3–Db19 is significantly higher, with $t_{1/2}$=65.71 min, in comparison with the others molecules (Table 1). The half-lives of all the multispecific Fv-molecules on the surface of $CD3^+$Jurkat cells were relatively short. The length of the linker appeared to have some influence on antigen binding, since the scFv3–Db19 and scFv19–Db3 showed a significantly slower k_{off} for CD19-positive cells than for the BsDb 19×3, scFv3×scFv 19 and scFv19–scFv3.

TABLE 1

Binding kinetics of recombinant bispecific molecules

Antibody	k_{off} ($s^{-1}/10^{-3}$)	$t_{1/2}$ (min)	Antibody	k_{off} ($s^{-1}/10^{-3}$)	$t_{1/2}$ (min)
A. JOK-1 cells ($CD3^-/CD19^+$)			B. JOK-1 cells ($CD3^-/CD19^+$)		
BsDb 19x3	0.9945	11.62	BsDb 19x3	0.1512	10.68
scFv3-scFv19	1.814	6.368	scFv19-scFv3	0.05547	8.622
scFv3-Db19	0.6563	17.6	scFv19-Db3	0.01171	65.71
C. Jurkat cells ($CD3^+/CD19^-$)			D. Jurkat cells ($CD3^+/CD19^-$)		
BsDb 19x3	4.268	2.707	BsDb 19x3	4.268	2.707
scFv3-scFv19	2.912	3.967	scFv19-scFv3	6.91	1.672
scFv3-Db19	3.161	3.655	scFv19-Db3	3.4	3.394

Example 5

In Vitro Analysis of Anti-Tumor Activity of Recombinant Multivalent Molecules

[0110] Freshly isolated peripheral blood mononuclear cells (PBMC) from a patient with chronic lymphocytic leukemia (CLL) were seeded in individual wells of a 12-well plate in 2 ml RPMI-Medium/10% FCS (Invitrogen, Breda, The Netherlands) at a density of 2×10^6 cells/ml. The recombinant antibodies scFv3–scFv19 and scFv3–Db19 were added at concentration of 5 μg/ml. After a 5 day incubation, the cells were harvested, counted, and stained with anti-CD3 MAb OKT3 (DKFZ, Heidelberg, Germany), anti-CD4 MAb Edu-2 (Chemicon, Hofheim, Germany), anti-CD8 MAb UCH-T4 (Chemicon, Hofheim, Germany), and anti-CD19 MAb HD37 (DKFZ, Heidelberg, Germany) for flow cytometric analysis. 10^4 living cells were analyzed using a Beckman-Coulter flow cytometer and the relative and absolute amounts of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells were plotted.

[0111] The results shown in **FIG. 13** demonstrated that tetravalent scFv3–Db19 molecules caused vigorous proliferation of autologous T cells and killing of $C19^+$ tumor cells. In contrast, bivalent scFv3–scFv19 molecules had nearly no effect.

Example 6

Construction of the Plasmid pSKK3-scFv$_{L7}$Anti-CD19-SL-scFv$_{L18}$Anti-CD3 for the Expression of Multimeric Fv-Antibody (Db19-SL-scFv3) in Bacteria

[0112] For generation of multimeric Fv constructs, the plasmids pHOG_HD37, pHOG_Dia_HD37, pHOG_mOKT3+NotI, and pHOG_Dia_mOKT3 encoding the antibody fragments derived either from hybridoma HD37 specific to human CD19 (Kipriyanov et al., 1996, J. Immunol. Meth. 196, 51-62; Le Gall et al., 1999, FEBS Lett., 453, 164-168) or from hybridoma OKT3 specific to human CD3 (Kipriyanov et al., 1997, Protein Eng. 10, 445-453) were used.

[0113] To generate a gene encoding the anti-CD19 scFv$_{L7}$ with the V_L-V_H orientation, the V_L-HD37 gene was ampli-

fied by PCR using as a template the plasmid DNA pHOG_HD37 (Kipriyanov et al., 1996, J. Immunol. Meth. 196, 51-62) and primers VL_Nco, 5'-CAGCCGGCCATGGCGGATATCTTGCTCACCCAAACTCCAGC (SEQ ID NO:20) and 3_Ck, 5'-AGACGGTGCAGCAACAGTACGTTTGATTTCCAGC (SEQ ID NO:21). The resulting 371 bp PCR fragments code for the anti-CD19 VL domain followed by 7 amino acid Arg-Thr-Val-Ala-Ala-Pro-Ser linker. In turn, the V_H-HD37 gene was amplified by PCR using as a template the plasmid DNA pHOG_HD37 (Kipriyanov et al., 1996, J. Immunol. Meth. 196, 51-62) and primers 5_Ck, 5'-CGTACTGTTGCTGCACCGTCTCAGGTGCAACTGCAGCAGTC (SEQ ID NO:22) and VH_Not, 5'-GAAGATGGATCCAGCGGCCGCTGAGGAGACGGTGACTGAGGTTCC (SEQ ID NO:23). The resulting 416 bp PCR fragment codes for the anti-CD19 VH domain preceded by 7 amino acid Arg-Thr-Val-Ala-Ala-Pro-Ser linker. The whole gene for anti-CD19 $scFv_{L7}$ was assembled by PCR from 371 bp and 416 bp DNA fragments using primers VL_Nco and VH_Not. The resulting 764 bp PCR fragment was digested with NcoI and NotI and cloned into NcoI/NotI-linearized plasmid pDISC2/SL (Kipriyanov et al., 1999, J. Mol. Biol. 293, 41-56), thus generating the plasmid pDISC-$scFv_{L7}$anti-CD19-SL-$scFV_{L10}$anti-CD3.

[0114] To increase the yield of functional scFv-antibodies in the bacterial periplasm, an optimized expression vector pSKK3 was generated (**FIG. 15**). This vector was constructed on the basis of plasmid pHKK (Horn et al., 1996, Appl. Microbiol. Biotechnol. 46, 524-532) containing hok/sok plasmid-free cell suicide system (Thisted et al., 1994, EMBO J. 13, 1960-1968). First, the gene coding for hybrid scFv V_H3-V_L19 was amplified by PCR from the plasmid pHOG3-19 (Kipriyanov et al., 1998, Int. J. Cancer 77, 763-772) using the primers 5-NDE, 5'-GATATACATATGAAATACCTATTGCCTACGGC, (SEQ ID NO:24) and 3-AFL, 5'-CGAATTCTTAAGTTAGCACAGGCCTCTAGAGACACACAGATCTTTAG (SEQ ID NO:25). The resulting 921 bp PCR fragment was digested with NdeI and AflII and cloned into the NdeI/AflII linearized plasmid pHKK, generating the vector pHKK3-19. To delete an extra XbaI site, a fragment of the PHKK plasmid containing the 3'-terminal part of the lacI gene (encoding the lac repressor), the strong transcriptional terminator tHP, and wild-type lac promoter/operator was amplified by PCR using primers 5-NAR, 5'-CACCCTGGCGCCCAATACGCAAACCGCC, (SEQ ID NO:16) and 3-NDE, 5'-GGTATTTCATATGTATATCTCCTTCTTCAGAAATTCGTAATCATGG (SEQ ID NO:17). The resulting 329 bp DNA fragment was digested with NarI and NdeI and cloned into NarI/NdeI-linearized plasmid pHKK3-19, generating the vector pHKKΔXba. To introduce a gene encoding the Skp/OmpH periplasmic factor for higher recombinant antibody production (Bothmann and Plückthun, 1998, Nat. Biotechnol. 16, 376-380), the skp gene was amplified by PCR with primers skp-3, 5'-CGAATTCTTAAGAAGGAGATATACATATGAAAAAGTGGTTATTAGCTGCAGG (SEQ ID NO:18), and skp-4, 5'-CGAATTCTCGAGCATTATTTAACCTGTTTCAGTACGTCGG (SEQ ID NO:19), using as a template the plasmid pGAH317 (Holck and Kleppe, 1988, Gene 67, 117-124). The resulting 528 bp PCR fragment was digested with AflII and XhoI and cloned into the AflII/XhoI digested plasmid pHKKΔXba resulting in the expression plasmid pSKK2.

[0115] For removing the sequence encoding the potentially immunogenic c-myc epitope, the NcoI/XbaI-linearized plasmid pSKK2 was used for cloning the NcoI/XbaI-digested 902 bp PCR fragment encoding the scFv phOx31E (Marks et al., 1997, BioTechnology 10, 779-783), which was amplified with primers DP1 and His-Xba, 5'-CAGGCCTCTAGATTAGTGATGGTGATGGTGATGGG (SEQ ID NO:26). The resulting plasmid pSKK3 was digested with NcoI and NotI and used as a vector for cloning the gene coding for anti-CD3 $scFv_{18}$, which was isolated as a 751 bp DNA fragment after digestion of plasmid pHOG21_dmOKT3+NotI (Kipriyanov et al., 1997, Protein Eng. 10, 445-453) with NcoI and NotI. The resulting plasmid pSKK3_$scFv_{L18}$anti-CD3 was used as a template for PCR amplification of the gene encoding the anti-CD3 $scFv_{18}$ with primers Bi3h, 5'-CCGGCCATGGCGCAGGTGCAGCTGCAGCAGTCTGG (SEQ ID NO:27), and P-skp 5'-GCTGCCCATGTTGACGATTGC (SEQ ID NO:28). The generated 919 bp PCR fragment was digested with PvuII and XbaI and cloned into PvuII/XbaI-cut plasmid pDISC-$scFv_{L7}$anti-CD19-SL-$scFv_{L10}$anti-CD3. The resulting plasmid pDISC-$scFv_{L7}$anti-CD19-SL-$scFv_{L18}$anti-CD3 was digested with NcoI and XbaI, and the 1536 bp DNA fragment was isolated and cloned into NcoI/XbaI-linearized vector pSKK3.

[0116] The generated plasmid pSKK3-$scFv_{L7}$anti-CD19-SL-$scFv_{L18}$anti-CD3 (**FIG. 15**) contains several features that improve plasmid performance and lead to increased accumulation of functional bivalent product in the *E. coli* periplasm under conditions of both shake-flask cultivation and high cell density fermentation. These are the hok/sok post-segregation killing system, which prevents plasmid loss, strong tandem ribosome-binding sites, and a gene encoding the periplasmic factor Skp/OmpH that increases the functional yield of antibody fragments in bacteria. The expression cassette is under the transcriptional control of the wt lac promoter/operator system and includes a short sequence coding for the N-terminal peptide of β-galactosidase (lacZ') with a first rbs derived from the *E. coli* lacZ gene, followed by genes encoding the scFv-antibody and Skp/OmpH periplasmic factor under the translational control of strong rbs from gene 10 of phage T7 (T7g10). In addition, the gene of scFv-antibody is followed by a nucleotide sequence encoding six histidine residues for both immunodetection and purification of recombinant product by immobilized metal-affinity chromatography (IMAC).

Example 7

Expression in Bacteria and Purification of the Multimeric Fv-Antibodies

[0117] The *E. coli* K12 strain RV308 (Δlac$_\chi$74 galISII::OP308strA) (Maurer et al., 1980, J. Mol. Biol. 139, 147-161) (ATCC 31608) was used for functional expression of scFv-antibodies. The bacteria transformed with the expression plasmid pSKK3-$scFv_7$anti-CD19-SL-$scFv_{18}$anti-CD3 were grown overnight in 2xYT medium with 100 μg/ml ampicillin and 100 mM glucose ($2xYT_{GA}$) at 28° C. The overnight culture was diluted in fresh $2xYT_{GA}$ medium to an optical density at 600 nm (OD_{600}) of 0.1, and continued to grow as flask cultures at 28° C. with vigorous shaking (180-220 rpm) until OD_{600} reached 0.8. Bacteria were harvested by centrifugation at 5,000 g for 15 min at 20°

C., and resuspended in the same volume of fresh YTBS medium (2xYT containing 1 M sorbitol, 2.5 mM glycine betaine and 100 μg/ml ampicillin). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2 mM, and growth was continued at 21° C. for 18-20 h. Cells were harvested by centrifugation at 9,000 g for 20 min at 4° C. To isolate soluble periplasmic proteins, the pelleted bacteria were resuspended in 5% of the initial volume of ice-cold 200 mM Tris-HCl, 20% sucrose, 1 mM EDTA, pH 8.0. After 1 h incubation on ice with occasional stirring, the spheroplasts were centrifuged at 30,000 g for 30 min at 4° C. leaving the soluble periplasmic extract as the supernatant and spheroplasts plus the insoluble periplasmic material as the pellet. The periplasmic extract was thoroughly dialyzed against 50 mM Tris-HCl, 1 M NaCl, pH 7.0, and used as a starting material for isolating scFv-antibodies. The recombinant product was concentrated by ammonium sulfate precipitation (final concentration 70% of saturation). The protein precipitate was collected by centrifugation (10,000 g, 4° C., 40 min) and dissolved in 2.5% of the initial volume of 50 mM Tris-HCl, 1 M NaCl, pH 7.0, followed by thorough dialysis against the same buffer. Immobilized metal affinity chromatography (IMAC) was performed at 4° C. using a 5 ml column of Chelating Sepharose (Amersham Pharmacia, Freiburg, Germany) charged with Cu^{2+} and equilibrated with 50 mM Tris-HCl, 1 M NaCl, pH 7.0 (start buffer). The sample was loaded by passing the sample over the column by gravity flow. The column was then washed with twenty column volumes of start buffer followed by start buffer containing 50 mM imidazole until the absorbance (280 nm) of the effluent was minimal (about thirty column volumes). Absorbed material was eluted with 50 mM Tris-HCl, 1 M NaCl, 300 mM imidazole, pH 7.0, as 1 ml fractions. The eluted fractions containing recombinant protein were identified by Western-blot analysis using Anti-penta-His mAb (QIAGEN, Hilden, Germany) and goat anti-mouse IgG HRP-conjugated antibodies (Dianova, Hamburg, Germany) as previously described (Kipriyanov et al., 1994, Mol. Immunol. 31, 1047-1058). The positive fractions were pooled and subjected to buffer exchange for 50 mM imidazole-HCl, 50 mM NaCl (pH 6.0) using pre-packed PD-10 columns (Pharmacia Biotech, Freiburg, Germany). The turbidity of protein solution was removed by centrifugation (30,000 g, 1 h, 4° C.).

[0118] The final purification was achieved by ion-exchange chromatography on a Mono S HR 5/5 column (Amersham Biosciences, Freiburg, Germany) in 50 mM imidazole-HCl, 50 mM NaCl, pH 6.0, with a linear 0.05-1 M NaCl gradient. The fractions containing multimeric Fv-antibodies were concentrated with simultaneous buffer exchange for PBS containing 50 mM imidazole, pH 7.0 (PBSI buffer), using an Ultrafree-15 centrifugal filter device (Millipore, Eschborn, Germany). Protein concentrations were determined by the Bradford dye-binding assay (Bradford, 1976, Anal. Biochem., 72, 248-254) using the Bio-Rad (Munich, Germany) protein assay kit. SDS-PAGE analysis demonstrated that Db19-SL-scFv3 migrated as single band with a molecular mass (M_r) around 56 kDa (**FIG. 17**). Size-exclusion chromatography on a calibrated Superdex 200 HR 10/30 column (Amersham Biosciences, Freiburg, Germany) demonstrated that Db19-SL-scFv3 was mainly in a dimeric form with M_r around 150 kDa(**FIG. 18**).

Example 8

Cell Binding Measurements

[0119] The human $CD3^+$ T-cell leukemia cell line Jurkat and human $CD19^+$ B-cell cell line JOK-1 were used for flow cytometry experiments. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin G sodium and 100 μg/ml streptomycin sulfate (all from Invitrogen, Groningen, The Netherlands) at 37° C. in a humidified atmosphere with 5% CO_2. 1×10^6 cells were incubated with 0.1 ml phosphate buffered saline (PBS, Invitrogen, Groningen, The Netherlands) supplemented with 2% heat-inactivated fetal calf serum (FCS, Invitrogen, Groningen, The Netherlands) and 0.1% sodium azide (Roth, Karlsruhe, Germany) (referred to as FACS buffer) containing diluted Db19-SL-scFv3 for 45 min on ice. After washing with FACS buffer, the cells were incubated with 0.1 ml of 0.01 mg/ml anti-$(His)_6$ mouse mAb 13/45/31-2 (Dianova, Hamburg, Germany) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 0.1 ml of 0.015 mg/ml FITC-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) under the same conditions as before. The cells were then washed again and resuspended in 0.5 ml of FACS buffer containing 2 μg/ml propidium iodide (Sigma-Aldrich, Taufkirchen, Germany) to exclude dead cells. The fluorescence of 1×10^4 stained cells was measured using a Beckman-Coulter Epics XL flow cytometer (Beckman-Coulter, Krefeld, Germany). Mean fluorescence (F) was calculated using System-II and Expo32 software (Beckman-Coulter, Krefeld, Germany) and the background fluorescence was subtracted. Equilibrium dissociation constants (K_d) were determined by fitting the experimental values to the Lineweaver-Burk equation: $1/F = 1/F_{max} + (K_d/F_{max})\ (1/[Ab])$ using the software program PRISM (GraphPad Software, San Diego, Calif.).

[0120] The flow cytometry experiments demonstrated a specific interaction of Db19-SL-scFv3 molecule to Jurkat cells expressing CD3 on their surface and to JOK-1 cells expressing CD19 on their surface (**FIG. 19**, A and B). The measured affinity constants proved to be fairly comparable for both CD3 and CD19-binding parts of the molecule (Table 2).

TABLE 2

Affinity of Db19-SL-scFv3 multimeric antibody binding to $CD3^+$ Jurkat cells and $CD19^+$ JOK-1 cells

Cell line	K_d (nM)
Jurkat ($CD3^+$)	14.67
JOK-1 ($CD19^+$)	10.02

[0121] The dissociation constants (K_d) were deduced from Lineweaver-Burk plots shown in **FIG. 19**.

Example 9

In Vitro Analysis of Anti-Tumor Activity of Recombinant Multivalent Molecules

[0122] Freshly isolated peripheral blood mononuclear cells (PBMC) from a patient with chronic lymphocytic leukemia (CLL) were seeded in individual wells of a 12-well

plate in 2 ml RPMI-Medium/10% FCS (Invitrogen, Breda, The Netherlands) at a density of 2×10^6 cells/ml. The recombinant antibodies Db19-SL-scFv3 were added at concentration of 5, 1, 0.1 μg/ml. After 6 days incubation, the cells were harvested, counted, and stained with anti-CD3 MAb OKT3 (DKFZ, Heidelberg, Germany), anti-CD4 MAb Edu-2 (Chemicon, Hofheim, Germany), anti-CD8 MAb UCH-T4 (Chemicon, Hofheim, Germany), and anti-CD19 MAb HD37 (DKFZ, Heidelberg, Germany) for flow cytometric analysis. 10^4 living cells were analyzed using a Beckman-Coulter flow cytometer, and the relative amounts of $CD3^+$, $CD4^+$, $CD8^+$ and $CD19^+$ cells were plotted.

[0123] The results, shown in **FIG. 20**, demonstrated that tetravalent Db19-SL-scFv3 molecule caused vigorous proliferation of autologous T cells and killing of $C19^+$ tumor cells. The observed T cell proliferation and killing CLL cells was even higher than those observed for previously described CD19×CD3 tandem diabody (Tandab; Kipriyanov et al. 1999, J. Mol. Biol. 293, 41-56; Cochlovius et al. 2000, Cancer Res. 60, 4336-4341).

Example 10

Construction of the Plasmid pSKK3-scFv$_{L7}$Anti-CD19-L6-scFv$_{L10}$anti-CD3 for the Expression of Multimeric Fv-Antibody (Db19-L6-scFv3) in Bacteria

[0124] For constructing the gene encoding the anti-CD3 scFV$_{L10}$, the plasmid pHOG21-dmOKT3 containing the gene for anti-human CD3 scFv$_{18}$ (Kipriyanov et al., 1997, Protein Engineering 10, 445-453) was used. To facilitate the cloning procedures, a NotI restriction site was introduced into the plasmid pHOG21-dmOKT3 by PCR amplification of scFv$_{18}$ gene using primers Bi3sk, 5'-CAGCCGGCCATGGCGCAGGTGCAACTGCAGCAG (SEQ ID NO:29) and Bi9sk, 5'-GAAGATGGATCCAGCGGCCGCAGTATCAGCCCGGTT (SEQ ID NO:30). The resulting 776 bp PCR fragment was digested with NcoI and NotI, and cloned into the NcoI/NotI-linearized vector pHOG21-CD19 (Kipriyanov et al., 1996, J. Immunol. Methods 196, 51-62), thus generating the plasmid pHOG21-dmOKT3+Not. The gene coding for OKT3 V_H domain with a Cys-Ser substitution at position 100A according to Kabat numbering scheme (Kipriyanov et al., 1997, Protein Engineering 10, 445-453) was amplified by PCR with primers DP1, 5'-TCACACAGAATTCTTAGATCTATTAAAGAGGAGAAATTAACC(SEQ ID NO:31) and DP2, 5'-AGCACACGATATCACCGCCAAGCTTGGGTGTTGTTTTGGC (SEQ ID NO:32), to generate the gene for anti-CD3 V_H followed by linker of 10 amino acids Ser-Ala-Lys-Thr-Thr-Pro-Lys-Leu-Gly-Gly. The resulting 507 bp PCR fragment was digested with NcoI and EcoRV, and cloned into NcoI/EcoRV-linearized plasmid pHOG21-dmOKT3+Not, thus generating the plasmid pHOG21-scFv10/anti-CD3.

[0125] To generate a gene encoding the anti-CD19 scFV$_{L7}$ with the V_L-V_H orientation followed by 6 amino acid linker peptide Ser-Ala-Lys-Thr-Thr-Pro, the V_L-HD37 gene was amplified by PCR using as a template the plasmid DNA pHOG_HD37 (Kipriyanov et al., 1996, J. Immunol. Meth. 196, 51-62) and primers VL_Nco, 5'-CAGCCGGCCATGGCGGATATCTTGCTCACCCAAACTCCAGC and 3_Ck, 5'-AGACGGTGCAGCAACAGTACGTTTGATTTCCAGC. The resulting 371 bp PCR fragment codes for the anti-CD19 VL domain followed by 7 amino acid Arg-Thr-Val-Ala-Ala-Pro-Ser linker. In turn, the V_H-HD37 gene was amplified by PCR using as a template the plasmid DNA pHOG_HD37 (Kipriyanov et al., 1996, J. Immunol. Meth. 196, 51-62) and primers 5_Ck, 5'-CGTACTGTTGCTGCACCGTCTCAGGTGCAACTGCAGCAGTC and VH-L6_Pvu, 5'-CTGCTGCAGCTGCACCTGGGGTGTTGTTTTGGCTGAGGAG (SEQ ID NO:33). The resulting 428 bp PCR fragment codes for the anti-CD19 VH domain preceded by 7 amino acid Arg-Thr-Val-Ala-Ala-Pro-Ser linker and followed by 6 amino acid linker peptide Ser-Ala-Lys-Thr-Thr-Pro. The whole gene for anti-CD19 scFv$_{L7}$-L_6 was assembled by PCR from 371 bp and 428 bp DNA fragments using primers VL_Nco and VH-L6_Pvu. The resulting 790 bp PCR fragment was digested with NcoI and PvuII and cloned into NcoI/PvuII-linearized plasmid pHOG21-scFv10/anti-CD3, thus generating the plasmid pDISC-scFv$_{L7}$anti-CD19-L6-scFv$_{L10}$anti-CD3.

[0126] To increase the yield of functional scFv-antibodies in the bacterial periplasm, the plasmid pDISC-scFv$_{L7}$anti-CD19-L6-scFv$_{L10}$anti-CD3 was digested with NcoI and XbaI, and the 1503 bp DNA fragment was isolated and cloned into NcoI/XbaI-linearized vector pSKK3 (see Example 6). The generated plasmid pSKK3-scFv$_{L7}$anti-CD19-L6-scFv$_{L10}$anti-CD3 (**FIG. 22**) is suitable for expression of functional bivalent product in the *E. coli* periplasm under conditions of both shake-flask cultivation and high cell density fermentation.

Example 11

Characterization of Db19-L6-scFv3 Antibody

[0127] The recombinant scFv-antibody Db19-L6-scFv3 was expressed in *E. coli* RV308 cells transformed with the plasmid pSKK3-scFv$_{L7}$anti-CD19-L6-scFv$_{L10}$anti-CD3 and purified from soluble periplasmic fraction essentially as described in Example 7. SDS-PAGE analysis demonstrated that Db19-L6-scFv3 migrated as single band with a molecular mass (M_r) around 56 kDa (**FIG. 24**). Size-exclusion chromatography on a calibrated Superdex 200 HR 10/30 column (Amersham Biosciences, Freiburg, Germany) demonstrated that Db19-L6-scFv3 was mainly in a dimeric form with M_r around 150 kDa (**FIG. 25**).

[0128] The human $CD3^+$ T-cell leukemia cell line Jurkat and human $CD19^+$ B-cell cell line JOK-1 were used for flow cytometry experiments. The cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/mL penicillin G sodium, and 100 μg/ml streptomycin sulfate (all from Invitrogen, Groningen, The Netherlands) at 37° C. in a humidified atmosphere with 5% CO_2. 1×10^6 cells were incubated with 0.1 ml phosphate buffered saline (PBS, Invitrogen, Groningen, The Netherlands) supplemented with 2% heat-inactivated fetal calf serum (FCS, Invitrogen, Groningen, The Netherlands) and 0.1% sodium azide (Roth, Karlsruhe, Germany) (referred to as FACS buffer) containing diluted Db19-SL-scFv3 for 45 min on ice. After washing with FACS buffer, the cells were incubated with 0.1 ml of 0.01 mg/ml anti-$(His)_6$ mouse mAb 13/45/31-2 (Dianova, Hamburg, Germany) in the same buffer for 45 min on ice. After a second washing cycle, the cells were incubated with 0.1 ml of 0.015 mg/ml FITC-conjugated goat anti-mouse

IgG (Dianova, Hamburg, Germany) under the same conditions as before. The cells were then washed again and resuspended in 0.5 ml of FACS buffer containing 2 μg/ml propidium iodide (Sigma-Aldrich, Taufkirchen, Germany) to exclude dead cells. The fluorescence of 1×10^4 stained cells was measured using a Beckman-Coulter Epics XL flow cytometer (Beckman-Coulter, Krefeld, Germany). Mean fluorescence (F) was calculated using System-II and Expo32 software (Beckman-Coulter, Krefeld, Germany), and the background fluorescence was subtracted. Equilibrium dissociation constants (K_d) were determined by fitting the experimental values to the Lineweaver-Burk equation: $1/F=1/F_{max}+(K_d/F_{max})$ $(1/[Ab])$ using the software program PRISM (GraphPad Software, San Diego, Calif.).

[0129] The flow cytometry experiments demonstrated a specific interaction of Db19-L6-scFv3 molecule to Jurkat cells expressing CD3 on their surface and to JOK-1 cells expressing CD19 on their surface (FIGS. **26**,A and B). The measured affinity constants proved to be fairly comparable for both CD3 and CD19-binding parts of the molecule (Table 3).

TABLE 3

Affinity of Db19-L6-scFv3 multimeric antibody binding to $CD3^+$ Jurkat cells and $CD19^+$ JOK-1 cells

Cell line	K_d (nM)
Jurkat ($CD3^+$)	4.42
JOK-1 ($CD19^+$)	8.49

[0130] The dissociation constants (K_d) were deduced from Lineweaver-Burk plots shown in **FIG. 26**.

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ccatggcgca ggtgcaactg cagcagtctg gggctgagct ggtgaggcct gggtcctcag 240

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Gly Leu Tyr Ala Leu Ala Ser Glu Arg Val Ala Leu Leu Tyr Ser Met
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Arg Leu Glu Thr His Arg Thr His Arg Ala Ser Pro Leu Tyr Ser Ser
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Glu Arg Ser Glu Arg Ser Glu Arg Thr His Arg Ala Leu Ala Thr Tyr
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Glu Thr His Arg Ser Glu Arg Gly Leu Ala Ser Pro Ser Glu Arg Ala
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Arg Ala Leu Ala Leu Tyr Ser Thr His Arg Thr His Arg Pro Arg Leu
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Tyr Ser Leu Glu Gly Leu Gly Leu Gly Leu Tyr Gly Leu Pro His Glu
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gacagatttg gcctggagat ggtgatacta actacaatgg aaagttcaag ggtaaagcca   1200

ctctgactgc agacgaatcc tccagcacag cctacatgca actcagcagc ctagcatctg   1260

aggactctgc ggtctatttc tgtgcaagac gggagactac gacggtaggc cgttattact   1320

atgctatgga ctactggggt caaggaacct cagtcaccgt ctcctcagcc aaaacaacac   1380

ccaagcttgg cggtgatatc ttgctcaccc aaactccagc ttctttggct gtgtctctag   1440

ggcagagggc caccatctcc tgcaaggcca gccaaagtgt tgattatgat ggtgatagtt   1500
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atttgaactg gtaccaacag attccaggac agccacccaa actcctcatc tatgatgcat 1560

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ccctcaacat ccatcctgtg gagaaggtgg atgctgcaac ctatcactgt cagcaaagta 1680

ctgacgatcc gtggacgttc ggtggaggca ccaagctgga aatcaaacgg gctgatgctt 1740

cggccgctgg atccgaacaa aagctgatct cagaagaaga cctaaactca catcaccatc 1800

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<210> SEQ ID NO 7
<211> LENGTH: 1602
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Plasmid

<400> SEQUENCE: 7

Met Glu Thr Leu Tyr Ser Thr Tyr Arg Leu Glu Leu Glu Pro Arg Thr
1 5 10 15

His Arg Ala Leu Ala Ala Leu Ala Ala Leu Ala Gly Leu Tyr Leu Glu
20 25 30

Leu Glu Leu Glu Leu Glu Ala Leu Ala Ala Leu Ala Gly Leu Asn Pro
35 40 45

Arg Ala Leu Ala Met Glu Thr Ala Leu Ala Gly Leu Asn Val Ala Leu
50 55 60

Gly Leu Asn Leu Glu Gly Leu Asn Gly Leu Asn Ser Glu Arg Gly Leu
65 70 75 80

Tyr Ala Leu Ala Gly Leu Leu Glu Val Ala Leu Ala Arg Gly Pro Arg
85 90 95

Gly Leu Tyr Ser Glu Arg Ser Glu Arg Val Ala Leu Leu Tyr Ser Ile
100 105 110

Leu Glu Ser Glu Arg Cys Tyr Ser Leu Tyr Ser Ala Leu Ala Ser Glu
115 120 125

Arg Gly Leu Tyr Thr Tyr Arg Ala Leu Ala Pro His Glu Ser Glu Arg
130 135 140

Ser Glu Arg Thr Tyr Arg Thr Arg Pro Met Glu Thr Ala Ser Asn Thr
145 150 155 160

Arg Pro Val Ala Leu Leu Tyr Ser Gly Leu Asn Ala Arg Gly Pro Arg
165 170 175

Gly Leu Tyr Gly Leu Asn Gly Leu Tyr Leu Glu Gly Leu Thr Arg Pro
180 185 190

Ile Leu Glu Gly Leu Tyr Gly Leu Asn Ile Leu Glu Thr Arg Pro Pro
195 200 205

Arg Gly Leu Tyr Ala Ser Pro Gly Leu Tyr Ala Ser Pro Thr His Arg
210 215 220

Ala Ser Asn Thr Tyr Arg Ala Ser Asn Gly Leu Tyr Leu Tyr Ser Pro
225 230 235 240

His Glu Leu Tyr Ser Gly Leu Tyr Leu Tyr Ser Ala Leu Ala Thr His
245 250 255

Arg Leu Glu Thr His Arg Ala Leu Ala Ala Ser Pro Gly Leu Ser Glu
260 265 270

Arg Ser Glu Arg Ser Glu Arg Thr His Arg Ala Leu Ala Thr Tyr Arg
275 280 285

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Met Glu Thr Gly Leu Asn Leu Glu Ser Glu Arg Ser Glu Arg Leu Glu
    290                 295                 300

Ala Leu Ala Ser Glu Arg Gly Leu Ala Ser Pro Ser Glu Arg Ala Leu
305                 310                 315                 320

Ala Val Ala Leu Thr Tyr Arg Pro His Glu Cys Tyr Ser Ala Leu Ala
                325                 330                 335

Ala Arg Gly Ala Arg Gly Gly Leu Thr His Arg Thr His Arg Thr His
            340                 345                 350

Arg Val Ala Leu Gly Leu Tyr Ala Arg Gly Thr Tyr Arg Thr Tyr Arg
        355                 360                 365

Thr Tyr Arg Ala Leu Ala Met Glu Thr Ala Ser Pro Thr Tyr Arg Thr
    370                 375                 380

Arg Pro Gly Leu Tyr Gly Leu Asn Gly Leu Tyr Thr His Arg Ser Glu
385                 390                 395                 400

Arg Val Ala Leu Thr His Arg Val Ala Leu Ser Glu Arg Ser Glu Arg
                405                 410                 415

Ala Leu Ala Leu Tyr Ser Thr His Arg Thr His Arg Pro Arg Leu Tyr
            420                 425                 430

Ser Leu Glu Gly Leu Gly Leu Gly Leu Tyr Gly Leu Pro His Glu Ser
        435                 440                 445

Glu Arg Gly Leu Ala Leu Ala Ala Arg Gly Val Ala Leu Ala Ser Pro
    450                 455                 460

Ile Leu Glu Leu Glu Leu Glu Thr His Arg Gly Leu Asn Thr His Arg
465                 470                 475                 480

Pro Arg Ala Leu Ala Ser Glu Arg Leu Glu Ala Leu Ala Val Ala Leu
                485                 490                 495

Ser Glu Arg Leu Glu Gly Leu Tyr Gly Leu Asn Ala Arg Gly Ala Leu
            500                 505                 510

Ala Thr His Arg Ile Leu Glu Ser Glu Arg Cys Tyr Ser Leu Tyr Ser
        515                 520                 525

Ala Leu Ala Ser Glu Arg Gly Leu Asn Ser Glu Arg Val Ala Leu Ala
    530                 535                 540

Ser Pro Thr Tyr Arg Ala Ser Pro Gly Leu Tyr Ala Ser Pro Ser Glu
545                 550                 555                 560

Arg Thr Tyr Arg Leu Glu Ala Ser Asn Thr Arg Pro Thr Tyr Arg Gly
                565                 570                 575

Leu Asn Gly Leu Asn Ile Leu Glu Pro Arg Gly Leu Tyr Gly Leu Asn
            580                 585                 590

Pro Arg Pro Arg Leu Tyr Ser Leu Glu Leu Glu Ile Leu Glu Thr Tyr
        595                 600                 605

Arg Ala Ser Pro Ala Leu Ala Ser Glu Arg Ala Ser Asn Leu Glu Val
    610                 615                 620

Ala Leu Ser Glu Arg Gly Leu Tyr Ile Leu Glu Pro Arg Pro Arg Ala
625                 630                 635                 640

Arg Gly Pro His Glu Ser Glu Arg Gly Leu Tyr Ser Glu Arg Gly Leu
                645                 650                 655

Tyr Ser Glu Arg Gly Leu Tyr Thr His Arg Ala Ser Pro Pro His Glu
            660                 665                 670

Thr His Arg Leu Glu Ala Ser Asn Ile Leu Glu His Ile Ser Pro Arg
        675                 680                 685

Val Ala Leu Gly Leu Leu Tyr Ser Val Ala Leu Ala Ser Pro Ala Leu
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    690                 695                 700

Ala Ala Leu Ala Thr His Arg Thr Tyr Arg His Ile Ser Cys Tyr Ser
705                 710                 715                 720

Gly Leu Asn Gly Leu Asn Ser Glu Arg Thr His Arg Gly Leu Ala Ser
                725                 730                 735

Pro Pro Arg Thr Arg Pro Thr His Arg Pro His Glu Gly Leu Tyr Gly
            740                 745                 750

Leu Tyr Gly Leu Tyr Thr His Arg Leu Tyr Ser Leu Glu Gly Leu Ile
        755                 760                 765

Leu Glu Leu Tyr Ser Ala Arg Gly Ala Leu Ala Ala Ser Pro Ala Leu
    770                 775                 780

Ala Ala Leu Ala Ala Leu Ala Ala Leu Ala Gly Leu Tyr Gly Leu Tyr
785                 790                 795                 800

Gly Leu Tyr Gly Leu Tyr Ser Glu Arg Gly Leu Tyr Gly Leu Tyr Gly
                805                 810                 815

Leu Tyr Gly Leu Tyr Ser Glu Arg Gly Leu Tyr Gly Leu Tyr Gly Leu
            820                 825                 830

Tyr Gly Leu Tyr Ser Glu Arg Gly Leu Tyr Gly Leu Tyr Gly Leu Tyr
        835                 840                 845

Gly Leu Tyr Ser Glu Arg Gly Leu Asn Val Ala Leu Gly Leu Asn Leu
    850                 855                 860

Glu Gly Leu Asn Gly Leu Asn Ser Glu Arg Gly Leu Tyr Ala Leu Ala
865                 870                 875                 880

Gly Leu Leu Glu Ala Leu Ala Ala Arg Gly Pro Arg Gly Leu Tyr Ala
                885                 890                 895

Leu Ala Ser Glu Arg Val Ala Leu Leu Tyr Ser Met Glu Thr Ser Glu
            900                 905                 910

Arg Cys Tyr Ser Leu Tyr Ser Ala Leu Ala Ser Glu Arg Gly Leu Tyr
        915                 920                 925

Thr Tyr Arg Thr His Arg Pro His Glu Thr His Arg Ala Arg Gly Thr
    930                 935                 940

Tyr Arg Thr His Arg Met Glu Thr His Ile Ser Thr Arg Pro Val Ala
945                 950                 955                 960

Leu Leu Tyr Ser Gly Leu Asn Ala Arg Gly Pro Arg Gly Leu Tyr Gly
                965                 970                 975

Leu Asn Gly Leu Tyr Leu Glu Gly Leu Thr Arg Pro Ile Leu Glu Gly
            980                 985                 990

Leu Tyr Thr Tyr Arg Ile Leu Glu  Ala Ser Asn Pro Arg  Ser Glu Arg
        995                 1000                1005

Ala Arg  Gly Gly Leu Tyr Thr  Tyr Arg Thr His Arg  Ala Ser Asn
    1010                1015                1020

Thr Tyr  Arg Ala Ser Asn Gly  Leu Asn Leu Tyr Ser  Pro His Glu
    1025                1030                1035

Leu Tyr  Ser Ala Ser Pro Leu  Tyr Ser Ala Leu Ala  Thr His Arg
    1040                1045                1050

Leu Glu  Thr His Arg Thr His  Arg Ala Ser Pro Leu  Tyr Ser Ser
    1055                1060                1065

Glu Arg  Ser Glu Arg Ser Glu  Arg Thr His Arg Ala  Leu Ala Thr
    1070                1075                1080

Tyr Arg  Met Glu Thr Gly Leu  Asn Leu Glu Ser Glu  Arg Ser Glu
    1085                1090                1095
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Arg Leu  Glu Thr His Arg Ser  Glu Arg Gly Leu Ala  Ser Pro Ser
    1100                 1105                 1110

Glu Arg  Ala Leu Ala Val Ala  Leu Thr Tyr Arg Thr  Tyr Arg Cys
    1115                 1120                 1125

Tyr Ser  Ala Leu Ala Ala Arg  Gly Thr Tyr Arg Thr  Tyr Arg Ala
    1130                 1135                 1140

Ser Pro  Ala Ser Pro His Ile  Ser Thr Tyr Arg Ser  Glu Arg Leu
    1145                 1150                 1155

Glu Ala  Ser Pro Thr Tyr Arg  Thr Arg Pro Gly Leu  Tyr Gly Leu
    1160                 1165                 1170

Asn Gly  Leu Tyr Thr His Arg  Thr His Arg Leu Glu  Thr His Arg
    1175                 1180                 1185

Val Ala  Leu Ser Glu Arg Ser  Glu Arg Ala Leu Ala  Leu Tyr Ser
    1190                 1195                 1200

Thr His  Arg Thr His Arg Pro  Arg Leu Tyr Ser Leu  Glu Gly Leu
    1205                 1210                 1215

Tyr Gly  Leu Tyr Ala Ser Pro  Ile Leu Glu Val Ala  Leu Leu Glu
    1220                 1225                 1230

Thr His  Arg Gly Leu Asn Ser  Glu Arg Pro Arg Ala  Leu Ala Ile
    1235                 1240                 1245

Leu Glu  Met Glu Thr Ser Glu  Arg Ala Leu Ala Ser  Glu Arg Pro
    1250                 1255                 1260

Arg Gly  Leu Tyr Gly Leu Leu  Tyr Ser Val Ala Leu  Thr His Arg
    1265                 1270                 1275

Met Glu  Thr Thr His Arg Cys  Tyr Ser Ser Glu Arg  Ala Leu Ala
    1280                 1285                 1290

Ser Glu  Arg Ser Glu Arg Ser  Glu Arg Val Ala Leu  Ser Glu Arg
    1295                 1300                 1305

Thr Tyr  Arg Met Glu Thr Ala  Ser Asn Thr Arg Pro  Thr Tyr Arg
    1310                 1315                 1320

Gly Leu  Asn Gly Leu Asn Leu  Tyr Ser Ser Glu Arg  Gly Leu Tyr
    1325                 1330                 1335

Thr His  Arg Ser Glu Arg Pro  Arg Leu Tyr Ser Ala  Arg Gly Thr
    1340                 1345                 1350

Arg Pro  Ile Leu Glu Thr Tyr  Arg Ala Ser Pro Thr  His Arg Ser
    1355                 1360                 1365

Glu Arg  Leu Tyr Ser Leu Glu  Ala Leu Ala Ser Glu  Arg Gly Leu
    1370                 1375                 1380

Tyr Val  Ala Leu Pro Arg Ala  Leu Ala His Ile Ser  Pro His Glu
    1385                 1390                 1395

Ala Arg  Gly Gly Leu Tyr Ser  Glu Arg Gly Leu Tyr  Ser Glu Arg
    1400                 1405                 1410

Gly Leu  Tyr Thr His Arg Ser  Glu Arg Thr Tyr Arg  Ser Glu Arg
    1415                 1420                 1425

Leu Glu  Thr His Arg Ile Leu  Glu Ser Glu Arg Gly  Leu Tyr Met
    1430                 1435                 1440

Glu Thr  Gly Leu Ala Leu Ala  Gly Leu Ala Ser Pro  Ala Leu Ala
    1445                 1450                 1455

Ala Leu  Ala Thr His Arg Thr  Tyr Arg Thr Tyr Arg  Cys Tyr Ser
    1460                 1465                 1470
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Gly Leu  Asn Gly Leu Asn Thr  Arg Pro Ser Glu Arg  Ser Glu Arg
    1475                 1480                 1485

Ala Ser  Asn Pro Arg Pro His  Glu Thr His Arg Pro  His Glu Gly
    1490                 1495                 1500

Leu Tyr  Ser Glu Arg Gly Leu  Tyr Thr His Arg Leu  Tyr Ser Leu
    1505                 1510                 1515

Glu Gly  Leu Ile Leu Glu Ala  Ser Asn Ala Arg Gly  Ala Leu Ala
    1520                 1525                 1530

Ala Ser  Pro Thr His Arg Ala  Leu Ala Pro Arg Thr  His Arg Gly
    1535                 1540                 1545

Leu Tyr  Ser Glu Arg Gly Leu  Gly Leu Asn Leu Tyr  Ser Leu Glu
    1550                 1555                 1560

Ile Leu  Glu Ser Glu Arg Gly  Leu Gly Leu Ala Ser  Pro Leu Glu
    1565                 1570                 1575

Ala Ser  Asn Ser Glu Arg His  Ile Ser His Ile Ser  His Ile Ser
    1580                 1585                 1590

His Ile  Ser His Ile Ser His  Ile Ser
    1595                 1600


<210> SEQ ID NO 8
<211> LENGTH: 20
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: linker sequence

<400> SEQUENCE: 8

Ala Arg Gly Thr His Arg Val Ala Leu Ala Leu Ala Ala Leu Ala Pro
1               5                   10                  15

Arg Ser Glu Arg
            20


<210> SEQ ID NO 9
<211> LENGTH: 23
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Amino acid linker peptide

<400> SEQUENCE: 9

Ala Leu Ala Ala Leu Ala Ala Leu Ala Gly Leu Tyr Gly Leu Tyr Pro
1               5                   10                  15

Arg Gly Leu Tyr Ser Glu Arg
            20


<210> SEQ ID NO 10
<211> LENGTH: 26
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: linker sequence

<400> SEQUENCE: 10

Ser Glu Arg Ala Leu Ala Ala Leu Ala Ala Leu Ala Gly Leu Tyr Gly
1               5                   10                  15

Leu Tyr Pro Arg Gly Leu Tyr Ser Glu Arg
            20                  25
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<210> SEQ ID NO 11
<211> LENGTH: 6844
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: plasmid

<400> SEQUENCE: 11

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gagtcaattc agggtggtga atgtgaaacc agtaacgtta tacgatgtcg cagagtatgc 120

cggtgtctct tatcagaccg tttcccgcgt ggtgaaccag gccagccacg tttctgcgaa 180

aacgcgggaa aaagtggaag cggcgatggc ggagctgaat tacattccca accgggtggc 240

acaacaactg gcgggcaaac agtcgttgct gattggcgtt gccacctcca gtctggccct 300

gcacgcgccg tcgcaaattg tcgcggcgat taaatctcgc gccgatcaac tgggtgccag 360

cgtggtggtg tcgatggtag aacgaagcgg cgtcgaagcc tgtaaagcgg cggtgcacaa 420

tcttctcgcg caacgcgtca gtgggctgat cattaactat ccgctggatg accaggatgc 480

cattgctgtg gaagctgcct gcactaatgt tccggcgtta tttcttgatg tctctgacca 540

gacacccatc aacagtatta ttttctccca tgaagacggt acgcgactgg gcgtggagca 600

tctggtcgca ttgggtcacc agcaaatcgc gctgttagcg ggcccattaa gttctgtctc 660

ggcgcgtctg cgtctggctg gctggcataa atatctcact cgcaatcaaa ttcagccgat 720

agcggaacgg gaaggcgact ggagtgccat gtccggtttt caacaaacca tgcaaatgct 780

gaatgagggc atcgttccca ctgcgatgct ggttgccaac gatcagatgg cgctgggcgc 840

aatgcgcgcc attaccgagt ccgggctgcg cgttggtgcg gatatctcgg tagtgggata 900

cgacgatacc gaagacagct catgttatat cccgccgtta accaccatca aacaggattt 960

tcgcctgctg gggcaaacca gcgtggaccg cttgctgcaa ctctctcagg gccaggcggt 1020

gaagggcaat cagctgttgc ccgtctcact ggtgaaaaga aaaaccaccc tggcgcccaa 1080

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aggtctcggt ttagcactgg caacttctgc tcaggcggct gacaaaattg caatcgtcaa   3060

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tgcctcactg attaagcatt ggtaactgtc agaccaagtt tactcatata tactttagat 6060

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gaaggtaact ggcttcagca gagcgcagat accaaatact gtccttctag tgtagccgta 6360

gttaggccac cacttcaaga actctgtagc accgcctaca tacctcgctc tgctaatcct 6420

gttaccagtg gctgctgcca gtggcgataa gtcgtgtctt accgggttgg actcaagacg 6480

atagttaccg gataaggcgc agcggtcggg ctgaacgggg ggttcgtgca cacagcccag 6540

cttggagcga acgacctaca ccgaactgag atacctacag cgtgagctat gagaaagcgc 6600

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cacgcttccc gaagggagaa aggcggacag gtatccggta agcggcaggg tcggaacagg 6660

agagcgcacg agggagcttc cagggggaaa cgcctggtat ctttatagtc ctgtcgggtt 6720

tcgccacctc tgacttgagc gtcgattttt gtgatgctcg tcaggggggc ggagcctatg 6780

gaaaaacgcc agcaacgcgg cctttttacg gttcctggcc ttttgctggc cttttgctca 6840

catg 6844

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<212> TYPE: PRT
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<223> OTHER INFORMATION: plasmid

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1 5 10 15

Thr His Arg Pro Arg Ala Leu Ala Ser Glu Arg Leu Glu Ala Leu Ala
20 25 30

Val Ala Leu Ser Glu Arg Leu Glu Gly Leu Tyr Gly Leu Asn Ala Arg
35 40 45

Gly Ala Leu Ala Thr His Arg Ile Leu Glu Ser Glu Arg Cys Tyr Ser
50 55 60

Leu Tyr Ser Ala Leu Ala Ser Glu Arg Gly Leu Asn Ser Glu Arg Val
65 70 75 80

Ala Leu Ala Ser Pro Thr Tyr Arg Ala Ser Pro Gly Leu Tyr Ala Ser
85 90 95

Pro Ser Glu Arg Thr Tyr Arg Leu Glu Ala Ser Asn Thr Arg Pro Thr
100 105 110

Tyr Arg Gly Leu Asn Gly Leu Asn Ile Leu Glu Pro Arg Gly Leu Tyr
115 120 125

Gly Leu Asn Pro Arg Pro Arg Leu Tyr Ser Leu Glu Leu Glu Ile Leu
130 135 140

Glu Thr Tyr Arg Ala Ser Pro Ala Leu Ala Ser Glu Arg Ala Ser Asn
145 150 155 160

Leu Glu Val Ala Leu Ser Glu Arg Gly Leu Tyr Ile Leu Glu Pro Arg
165 170 175

Pro Arg Ala Arg Gly Pro His Glu Ser Glu Arg Gly Leu Tyr Ser Glu
180 185 190

Arg Gly Leu Tyr Ser Glu Arg Gly Leu Tyr Thr His Arg Ala Ser Pro
195 200 205

Pro His Glu Thr His Arg Leu Glu Ala Ser Asn Ile Leu Glu His Ile
210 215 220

Ser Pro Arg Val Ala Leu Gly Leu Leu Tyr Ser Val Ala Leu Ala Ser
225 230 235 240

Pro Ala Leu Ala Ala Leu Ala Thr His Arg Thr Tyr Arg His Ile Ser
245 250 255

Cys Tyr Ser Gly Leu Asn Gly Leu Asn Ser Glu Arg Thr His Arg Gly
260 265 270

Leu Ala Ser Pro Pro Arg Thr Arg Pro Thr His Arg Pro His Glu Gly
275 280 285

Leu Tyr Gly Leu Tyr Gly Leu Tyr Thr His Arg Leu Tyr Ser Leu Glu

-continued

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    290                 295                 300

Gly Leu Ile Leu Glu Leu Tyr Ser Ala Arg Gly Thr His Arg Val Ala
305                 310                 315                 320

Leu Ala Leu Ala Ala Leu Ala Pro Arg Ser Glu Arg Gly Leu Asn Val
                325                 330                 335

Ala Leu Gly Leu Asn Leu Glu Gly Leu Asn Gly Leu Asn Ser Glu Arg
            340                 345                 350

Gly Leu Tyr Ala Leu Ala Gly Leu Leu Glu Val Ala Leu Ala Arg Gly
        355                 360                 365

Pro Arg Gly Leu Tyr Ser Glu Arg Ser Glu Arg Val Ala Leu Leu Tyr
    370                 375                 380

Ser Ile Leu Glu Ser Glu Arg Cys Tyr Ser Leu Tyr Ser Ala Leu Ala
385                 390                 395                 400

Ser Glu Arg Gly Leu Tyr Thr Tyr Arg Ala Leu Ala Pro His Glu Ser
                405                 410                 415

Glu Arg Ser Glu Arg Thr Tyr Arg Thr Arg Pro Met Glu Thr Ala Ser
            420                 425                 430

Asn Thr Arg Pro Val Ala Leu Leu Tyr Ser Gly Leu Asn Ala Arg Gly
        435                 440                 445

Pro Arg Gly Leu Tyr Gly Leu Asn Gly Leu Tyr Leu Glu Gly Leu Thr
    450                 455                 460

Arg Pro Ile Leu Glu Gly Leu Tyr Gly Leu Asn Ile Leu Glu Thr Arg
465                 470                 475                 480

Pro Pro Arg Gly Leu Tyr Ala Ser Pro Gly Leu Tyr Ala Ser Pro Thr
                485                 490                 495

His Arg Ala Ser Asn Thr Tyr Arg Ala Ser Asn Gly Leu Tyr Leu Tyr
            500                 505                 510

Ser Pro His Glu Leu Tyr Ser Gly Leu Tyr Leu Tyr Ser Ala Leu Ala
        515                 520                 525

Thr His Arg Leu Glu Thr His Arg Ala Leu Ala Ala Ser Pro Gly Leu
    530                 535                 540

Ser Glu Arg Ser Glu Arg Ser Glu Arg Thr His Arg Ala Leu Ala Thr
545                 550                 555                 560

Tyr Arg Met Glu Thr Gly Leu Asn Leu Glu Ser Glu Arg Ser Glu Arg
                565                 570                 575

Leu Glu Ala Leu Ala Ser Glu Arg Gly Leu Ala Ser Pro Ser Glu Arg
            580                 585                 590

Ala Leu Ala Val Ala Leu Thr Tyr Arg Pro His Glu Cys Tyr Ser Ala
        595                 600                 605

Leu Ala Ala Arg Gly Ala Arg Gly Gly Leu Thr His Arg Thr His Arg
    610                 615                 620

Thr His Arg Val Ala Leu Gly Leu Tyr Ala Arg Gly Thr Tyr Arg Thr
625                 630                 635                 640

Tyr Arg Thr Tyr Arg Ala Leu Ala Met Glu Thr Ala Ser Pro Thr Tyr
                645                 650                 655

Arg Thr Arg Pro Gly Leu Tyr Gly Leu Asn Gly Leu Tyr Thr His Arg
            660                 665                 670

Ser Glu Arg Val Ala Leu Thr His Arg Val Ala Leu Ser Glu Arg Ser
        675                 680                 685

Glu Arg Ala Leu Ala Leu Tyr Ser Thr His Arg Thr His Arg Pro Arg
    690                 695                 700
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Gly Leu Asn Val Ala Leu Gly Leu Asn Leu Glu Gly Leu Asn Gly Leu
705                 710                 715                 720

Asn Ser Glu Arg Gly Leu Tyr Ala Leu Ala Gly Leu Leu Glu Ala Leu
                725                 730                 735

Ala Ala Arg Gly Pro Arg Gly Leu Tyr Ala Leu Ala Ser Glu Arg Val
            740                 745                 750

Ala Leu Leu Tyr Ser Met Glu Thr Ser Glu Arg Cys Tyr Ser Leu Tyr
        755                 760                 765

Ser Ala Leu Ala Ser Glu Arg Gly Leu Tyr Thr Tyr Arg Thr His Arg
    770                 775                 780

Pro His Glu Thr His Arg Ala Arg Gly Thr Tyr Arg Thr His Arg Met
785                 790                 795                 800

Glu Thr His Ile Ser Thr Arg Pro Val Ala Leu Leu Tyr Ser Gly Leu
                805                 810                 815

Asn Ala Arg Gly Pro Arg Gly Leu Tyr Gly Leu Asn Gly Leu Tyr Leu
            820                 825                 830

Glu Gly Leu Thr Arg Pro Ile Leu Glu Gly Leu Tyr Thr Tyr Arg Ile
        835                 840                 845

Leu Glu Ala Ser Asn Pro Arg Ser Glu Arg Ala Arg Gly Gly Leu Tyr
    850                 855                 860

Thr Tyr Arg Thr His Arg Ala Ser Asn Thr Tyr Arg Ala Ser Asn Gly
865                 870                 875                 880

Leu Asn Leu Tyr Ser Pro His Glu Leu Tyr Ser Ala Ser Pro Leu Tyr
                885                 890                 895

Ser Ala Leu Ala Thr His Arg Leu Glu Thr His Arg Thr His Arg Ala
            900                 905                 910

Ser Pro Leu Tyr Ser Ser Glu Arg Ser Glu Arg Ser Glu Arg Thr His
        915                 920                 925

Arg Ala Leu Ala Thr Tyr Arg Met Glu Thr Gly Leu Asn Leu Glu Ser
    930                 935                 940

Glu Arg Ser Glu Arg Leu Glu Thr His Arg Ser Glu Arg Gly Leu Ala
945                 950                 955                 960

Ser Pro Ser Glu Arg Ala Leu Ala Val Ala Leu Thr Tyr Arg Thr Tyr
                965                 970                 975

Arg Cys Tyr Ser Ala Leu Ala Ala Arg Gly Thr Tyr Arg Thr Tyr Arg
            980                 985                 990

Ala Ser Pro Ala Ser Pro His Ile  Ser Thr Tyr Arg Ser  Glu Arg Leu
        995                 1000                1005

Glu Ala  Ser Pro Thr Tyr Arg  Thr Arg Pro Gly Leu  Tyr Gly Leu
    1010                1015                1020

Asn Gly  Leu Tyr Thr His Arg  Thr His Arg Leu Glu  Thr His Arg
    1025                1030                1035

Val Ala  Leu Ser Glu Arg Ser  Glu Arg Ala Leu Ala  Leu Tyr Ser
    1040                1045                1050

Thr His  Arg Thr His Arg Pro  Arg Leu Tyr Ser Leu  Glu Gly Leu
    1055                1060                1065

Tyr Gly  Leu Tyr Ala Ser Pro  Ile Leu Glu Val Ala  Leu Leu Glu
    1070                1075                1080

Thr His  Arg Gly Leu Asn Ser  Glu Arg Pro Arg Ala  Leu Ala Ile
    1085                1090                1095
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Leu Glu  Met Glu Thr Ser Glu  Arg Ala Leu Ala Ser  Glu Arg Pro
    1100                 1105                 1110

Arg Gly  Leu Tyr Gly Leu Leu  Tyr Ser Val Ala Leu  Thr His Arg
    1115                 1120                 1125

Met Glu  Thr Thr His Arg Cys  Tyr Ser Ser Glu Arg  Ala Leu Ala
    1130                 1135                 1140

Ser Glu  Arg Ser Glu Arg Ser  Glu Arg Val Ala Leu  Ser Glu Arg
    1145                 1150                 1155

Thr Tyr  Arg Met Glu Thr Ala  Ser Asn Thr Arg Pro  Thr Tyr Arg
    1160                 1165                 1170

Gly Leu  Asn Gly Leu Asn Leu  Tyr Ser Ser Glu Arg  Gly Leu Tyr
    1175                 1180                 1185

Thr His  Arg Ser Glu Arg Pro  Arg Leu Tyr Ser Ala  Arg Gly Thr
    1190                 1195                 1200

Arg Pro  Ile Leu Glu Thr Tyr  Arg Ala Ser Pro Thr  His Arg Ser
    1205                 1210                 1215

Glu Arg  Leu Tyr Ser Leu Glu  Ala Leu Ala Ser Glu  Arg Gly Leu
    1220                 1225                 1230

Tyr Val  Ala Leu Pro Arg Ala  Leu Ala His Ile Ser  Pro His Glu
    1235                 1240                 1245

Ala Arg  Gly Gly Leu Tyr Ser  Glu Arg Gly Leu Tyr  Ser Glu Arg
    1250                 1255                 1260

Gly Leu  Tyr Thr His Arg Ser  Glu Arg Thr Tyr Arg  Ser Glu Arg
    1265                 1270                 1275

Leu Glu  Thr His Arg Ile Leu  Glu Ser Glu Arg Gly  Leu Tyr Met
    1280                 1285                 1290

Glu Thr  Gly Leu Ala Leu Ala  Gly Leu Ala Ser Pro  Ala Leu Ala
    1295                 1300                 1305

Ala Leu  Ala Thr His Arg Thr  Tyr Arg Thr Tyr Arg  Cys Tyr Ser
    1310                 1315                 1320

Gly Leu  Asn Gly Leu Asn Thr  Arg Pro Ser Glu Arg  Ser Glu Arg
    1325                 1330                 1335

Ala Ser  Asn Pro Arg Pro His  Glu Thr His Arg Pro  His Glu Gly
    1340                 1345                 1350

Leu Tyr  Ser Glu Arg Gly Leu  Tyr Thr His Arg Leu  Tyr Ser Leu
    1355                 1360                 1365

Glu Gly  Leu Ile Leu Glu Ala  Ser Asn Ala Arg Gly  Ala Leu Ala
    1370                 1375                 1380

Ala Ser  Pro Thr His Arg Ala  Leu Ala Ala Leu Ala  Ala Leu Ala
    1385                 1390                 1395

Gly Leu  Tyr Ser Glu Arg His  Ile Ser His Ile Ser  His Ile Ser
    1400                 1405                 1410

His Ile  Ser His Ile Ser His  Ile Ser
    1415                 1420


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<213> ORGANISM: Artificial
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<223> OTHER INFORMATION: linker sequence

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Ser Glu Arg Ala Leu Ala Leu Tyr Ser Thr His Arg Thr His Arg Pro
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Arg

<210> SEQ ID NO 14
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: primer

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gatatacata tgaaatacct attgcctacg gc 32

<210> SEQ ID NO 15
<211> LENGTH: 47
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 15

cgaattctta agttagcaca ggcctctaga gacacacaga tctttag 47

<210> SEQ ID NO 16
<211> LENGTH: 28
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<400> SEQUENCE: 16

caccctggcg cccaatacgc aaaccgcc 28

<210> SEQ ID NO 17
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<212> TYPE: DNA
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ggtatttcat atgtatatct ccttcttcag aaattcgtaa tcatgg 46

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<211> LENGTH: 52
<212> TYPE: DNA
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<210> SEQ ID NO 19
<211> LENGTH: 40
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<210> SEQ ID NO 20
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<212> TYPE: DNA
<213> ORGANISM: Artificial
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<400> SEQUENCE: 20

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<211> LENGTH: 34
<212> TYPE: DNA
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<400> SEQUENCE: 21

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<212> TYPE: DNA
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<400> SEQUENCE: 22

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<400> SEQUENCE: 23

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<210> SEQ ID NO 24
<211> LENGTH: 32
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<400> SEQUENCE: 24

gatatacata tgaaatacct attgcctacg gc 32

<210> SEQ ID NO 25
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<210> SEQ ID NO 29
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agcacacgat atcaccgcca agcttgggtg ttgttttggc 40

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1. A multimeric structure comprising two or more identical protein monomers, characterized by the following features:

(a) the monomers of said structure comprise at least four variable domains of which the first or last two variable domains of which the first or last two variable domains form an antigen-binding VH-VL or VL-VH scFv unit wherein two variable domains are linked by a peptide linker of at least 5 amino acid residues which does not prevent the intramolecular formation of a scFv;

(b) the other two neighboring variable domains of the monomer are non-covalently bound to the complementary domains of another monomer resulting in the formation of at least two additional antigen binding sites to form the multimerisation motif.

2. The multimeric structure of claim 1, in form of a multimeric Fv-antibody, having the following features:

(a) the monomers of said Fv-antibody comprise at least four variable domains of which the first or last two variable domains are linked by a peptide linker of 5 to 30 acid residues, which does not prevent the intramolecular formation of a scFv.

(b) the other two neighboring variable domains of the monomer are non-covalently bound to two complementary variable domains of another monomer resulting in the formation of at least two additional antigen binding sites to form a multimerization motif, wherein said two variable domains are linked by a peptide linker of a maximum of 12 amino aid residues.

3. The multimeric Fv-antibody of claim 2, wherein a further feature is that the antigen-binding V_H-V_L or V_L-V_H scFv unit formed by the two neighboring domains of one monomer is linked to the other variable domains of the multimerization motif by a peptide linker of 5 to 30 amino acid residues.

4. The mutlimeric structure of claim 1, wherein said monomers comprise four variable domains and wherein the third and fourth variable domains of said one end of the monomers are linked by a peptide linker, said peptide linker having 12 or less amino acid residues.

5. The mutlimeric structure of claim 1, wherein said monomers comprise four variable domains and wherein the first and second variable domains of said one end of the monomers are linked by a peptide linker, said peptide linker having 12 or less amino residues.

6. The multimeric structure of claim 2, wherein the second and third variable domain of the monomers are linked by a peptide linker consisting of 5 to 30 amino acid residues.

7. The multimeric structure of claim 1, wherein any variable domain of the monomers is shortened by at least one amino acid residue at their N- and/or C-terminus.

8. The multimeric structure of claim 1, wherein the order of domains of a monomer is V_H-V_L-V_H-V_L, V_L-V_H-V_H-V_L, V_H-V_L-V_L-V_H or V_L-V_H-V_L-V_H.

9. The multimeric structure of claim 1, wherein the non-covalent binding of at least one pair of variable domains is strengthened by at least one disulfide bridge.

10. The multimeric structure of claim 1, which is a tetravalent dimer, hexavalent trimer or octavalent tetramer.

11. The multimeric structure of claim 1, which is a bisepcific, of trispecific or tetraspecific antibody.

12. The multimeric structure of claim 1, wherein at least one monomer is linked to a biologically active substance, a chemical agent, a peptide, a protein or a drug.

13. The multimeric structure of claim 1, which is a monospecific antibody capable of specifically binding the CD19 antigen of B-lymphocytes or the CEA antigen.

14. The multimeric structure of claim 1, which is a bispecific antibody capable of specifically bi9dning:

(a) CD19 and the CD3 complex of the T-cell receptor;

(b) CD19 and the CD5 complex of the T-cell receptor;

(c) CD19 and the CD28 antigen on T-lymphocytes;

(d) CD19 and the CD16 on natural killer cells, macrophages and activated monocytes;

(e) CEA and CD3;

(f) CEA and CE28; or

(g) CEA and CDE16.

15. A process for the preparation of a multimeric structure of claim 1, wherein (a) DNA sequences encoding the peptide linkers are ligated with the DNA sequences encoding the variable domains such that the peptide linkers connect the variable domains resulting in the formation of a DNA sequence encoding a monomer of the multivalent multimeric structure and (b) the DNA sequences encoding the various monomers are expressed in a suitable expression system.

16. A DNA sequence encoding a multimeric structure of claim 1.

17. An expression vector containing the DNA sequence of claim 16.

18. The expression vector of claim 17, which is pSKK2-scFv$_{L18}$anti-CD3-LL-scFv$_{L10}$anti-CD19 (pSKK2-scFv3LL Db19) (DSM 14470) or psKK2-scFv$_{L18}$antiCD19-LL-scFv$_{L10}$anti-CD3(pSKK2-scFv19LL Db3) (DSM 14471).

19. A host cell containing the expression vector of claim 17.

20. A pharmaceutical composition comprising a dimeric or multimeric structure of claim 1.

21. Use of a dimeric or multimeric structure of claim 1 for diagnosis.

22. Use of a dimeric or multimeric structure of claim 1 for the preparation of a pharmaceutical composition for (a) the treatment of a viral, bacterial, tumoral or prion related disease, (b) the agglutination of red blood cells, (c) linking cytotoxic cells of the immune system to tumor cells, or (d) linking activating cytokines, cytotoxic substances or a protease to a target cell.

23. A diagnostic kit comprising a multimeric structure of claim 1.

24. A pharmaceutical composition comprising a DNA sequence of claim 17.

25. A pharmaceutical composition comprising an expression vector of claim 18.

* * * * *

专利名称(译)	二聚体和多聚体抗原结合结构		
公开(公告)号	US20050079170A1	公开(公告)日	2005-04-14
申请号	US10/489626	申请日	2002-09-13
[标]申请(专利权)人(译)	勒加尔FABRICE KIPRIYANOV SERGEY REUSCH UWE 莫尔登豪尔GERHARD LITTLE梅尔文		
申请(专利权)人(译)	勒加尔FABRICE KIPRIYANOV SERGEY REUSCH UWE 莫尔登豪尔GERHARD LITTLE梅尔文		
当前申请(专利权)人(译)	勒加尔FABRICE KIPRIYANOV SERGEY REUSCH UWE 莫尔登豪尔GERHARD LITTLE梅尔文		
[标]发明人	LE GALL FABRICE KIPRIYANOV SERGEY REUSCH UWE MOLDENHAUER GERHARD LITTLE MELVYN		
发明人	LE GALL, FABRICE KIPRIYANOV, SERGEY REUSCH, UWE MOLDENHAUER, GERHARD LITTLE, MELVYN		
IPC分类号	G01N33/531 A61P31/00 C07K16/00 C07K16/28 C12N1/15 C12N1/19 C12N1/21 C12N5/10 C12N15/09 C12N15/13 A61K39/395 C07K16/44		
CPC分类号	A61K2039/505 C07K16/00 C07K16/28 C07K2317/34 C07K2317/622 C07K2319/00 C07K2317/31 A61P31/00 A61P35/02		
优先权	2001122104 2001-09-14 EP		
外部链接	Espacenet USPTO		

摘要(译)

本发明涉及二聚体和多聚体抗原结合结构，编码所述结构的表达载体，以及所述结构的诊断和治疗用途。抗原结合结构优选为Fv-抗体构建体的形式。

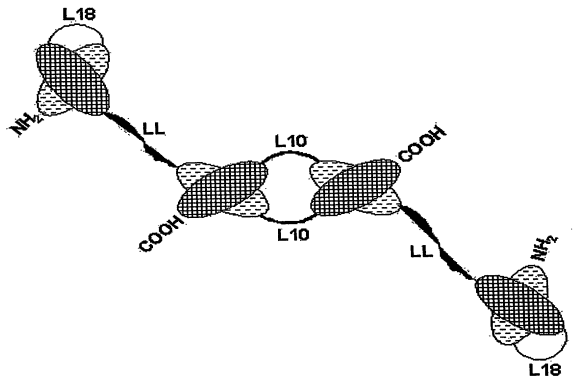

FIGURE 1