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(54) **RECOMBINANT PAPILLOMAVIRUS VACCINE AND METHOD FOR PRODUCTION AND TREATMENT**

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(57) **ABSTRACT**

The present invention relates to fusions proteins, comprising a protein or part of a protein that provides T helper epitopes and an antigen from a human-papilloma virus. In particular the invention relates to fusion proteins comprising an E6 or E7 protein from HPV strain 16 or 18 linked to protein D from Haemophilus influenza B. The invention also provides vaccine compositions that are useful in the treatment or prophylaxis of human papilloma induced tumors.

Protein D1/3 E7 his

1 MDPSSHSSNM ANTQMKSDKI IIAHRGASGY LPEHTLESKA LAFAQQADYL

51 EQDLAMTKDG RLVVIHDHFL DGLTDVAKKF PHRHRKDGRY YVIDFTLKEI

101 QSLEMTENFE TMAMHGDTPT LHEYMLDLQP ETTDLYCYEQ LNDSSEEEDE

151 IDGPAGQAEP DRAHYNIVTF CCKCDSTLRL CVQSTHVDIR TLEDLLMGTL

201 GIVCPICSQK PTSGHHHHHH *

Protein D1/3 E7 his

1 MDPSSHSSNM ANTQMKSDKI IIAHRGASGY LPEHTLESKA LAFAQQADYL

51 EQDLAMTKDG RLVVIHDHFL DGLTDVAKKF PHRHRKDGRY YVIDFTLKEI

101 QSLEMTENFE TMAMHGDTPT LHEYMLDLQP ETTDLYCYEQ LNDSSEEEDE

151 IDGPAGQAEP DRAHYNIVTF CCKCDSTLRL CVQSTHVDIR TLEDLLMGTL

201 GIVCPICSQK PTSGHHHHHH *

Figure 1A

Sequence of plasmid expressing fusion protein ProtDthr126-E7-His tail (E7 from HPV16).

1 ATGGATCCAA GCAGCCATTC ATCAAATATG GCGAATACCC AAATGAAATC
51 AGACAAAATC ATTATTGCTC ACCGTGGTGC TAGCGGTTAT TTACCAGAGC
101 ATACGTTAGA ATCTAAAGCA CTTGCGTTTG CACAACAGGC TGATTATTTA
151 GAGCAAGATT TAGCAATGAC TAAGGATGGT CGTTTAGTGG TTATTCACGA
201 TCACTTTTTA GATGGCTTGA CTGATGTTGC GAAAAAATTC CCACATCGTC
251 ATCGTAAAGA TGGCCGTTAC TATGTCATCG ACTTTACCTT AAAAGAAATT
301 CAAAGTTTAG AAATGACAGA AACTTTGAA ACCATGGCCA TGCATGGAGA
351 TACACCTACA TTGCATGAAT ATATGTTAGA TTTGCAACCA GAGACAACTG
401 ATCTCTACTG TTATGAGCAA TTAAATGACA GCTCAGAGGA GGAGGATGAA
451 ATAGATGGTC CAGCTGGACA AGCAGAACCG GACAGAGCCC ATTACAATAT
501 TGTAACCTTT TGTTGCAAGT GTGACTCTAC GCTTCGGTTG TGCGTACAAA
551 GCACACACGT AGACATTCGT ACTTTGGAAG ACCTGTTAAT GGGCACACTA
601 GGAATTGTGT GCCCCATCTG TTCTCAGAAA CCAACTAGTG GCCACCATCA
651 CCATCACCAT TAA

Figure 1B

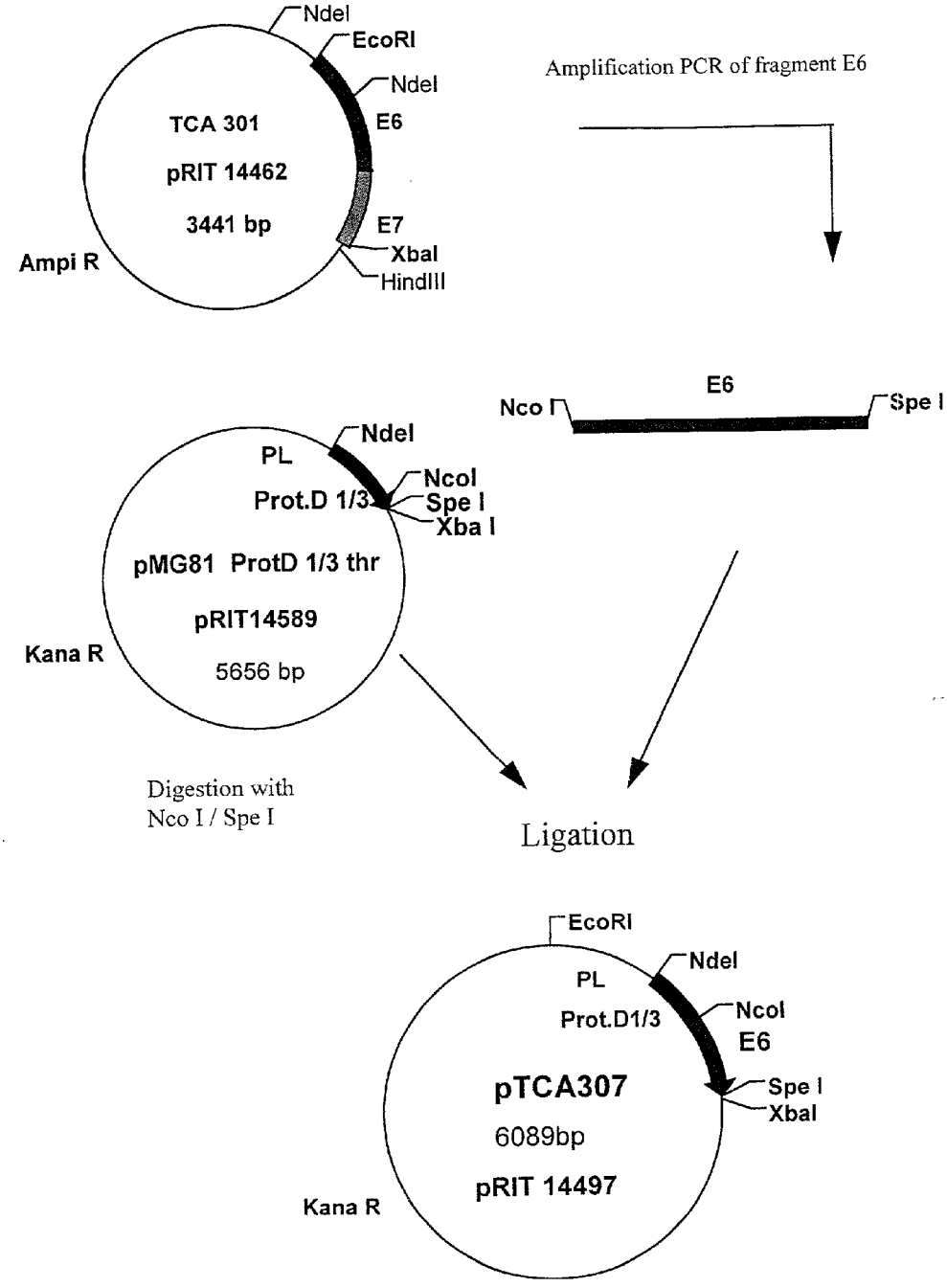

Construction of plasmid pRIT 14497 (TCA 307)
NdeI
EcoRI
NdeI
TCA 301
pRIT 14462
3441 bp
E6
E7
XbaI
HindIII
Ampi R
Amplification PCR of fragment E6
E6
Nco I
Spe I
NdeI
PL
Prot.D 1/3
NcoI
Spe I
Xba I
pMG81 ProtD 1/3 thr
pRIT14589
5656 bp
Kana R
Digestion with
Nco I / Spe I
Ligation
EcoRI
NdeI
PL
Prot.D1/3
NcoI
E6
pTCA307
6089bp
pRIT 14497
Spe I
XbaI
Kana R

Figure 2

SEQUENCE OF PROT.D1/3 E6 His / HPV 16.

Nucleotidic sequence

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATC 50

AGACAAAATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGC 100

ATACGTTAGAATCTAAAGCACTTGCGTTTGCACAACAGGCTGATTATTTA 150

GAGCAAGATTTAGCAATGACTAAGGATGGTCGTTTAGTGGTTATTCACGA 200

TCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAATTCCCACATCGTC 250

ATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAAGAAATT 300

CAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGCCATGTTTCAGGA 350

CCCACAGGAGCGACCCAGAAAGTTACCACAGTTATGCACAGAGCTGCAAA 400

CAACTATACATGATATAATATTAGAATGTGTGTACTGCAAGCAACAGTTA 450

CTGCGACGTGAGGTATATGACTTTGCTTTTCGGGATTTATGCATAGTATA 500

TAGAGATGGGAATCCATATGCTGTATGTGATAAATGTTTAAAGTTTTATT 550

CTAAAATTAGTGAGTATAGACATTATTGTTATAGTTTGTATGGAACAACA 600

TTAGAACAGCAATACAACAAACCGTTGTGTGATTTGTTAATTAGGTGTAT 650

TAACTGTCAAAAGCCACTGTGTCCTGAAGAAAAGCAAAGACATCTGGACA 700

AAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGACCGGTCGATGTATG 750

TCTTGTTGCAGATCATCAAGAACACGTAGAGAAACCCAGCTGACTAGTGG 800

CCACCATCACCATCACCATTAA 822

Figure 3A

Peptidic sequence

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL 50

EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEI 100

QSLEMTENFETMAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQL 150

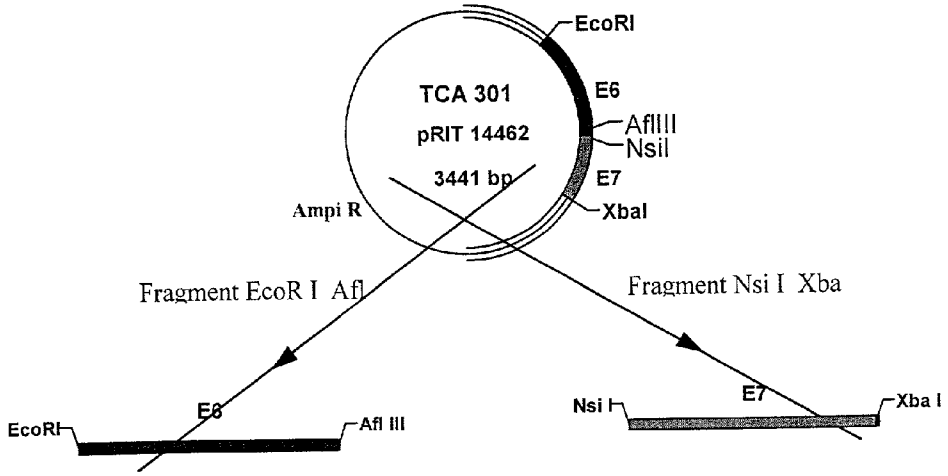
LRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYSKISEYRHYCYSLYGTT 200

LEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDKKQRFHNIRGRWTGRCM 250

SCCRSSRTRRETQLTSGHHHHHH. 274

Figure 3B

Construction of plasmid pRIT 14556 (TCA 309)



Constitution of a fusion protein between E6 and E7: deletion of 5 nucleotides by insertion of adaptor between Afl III and

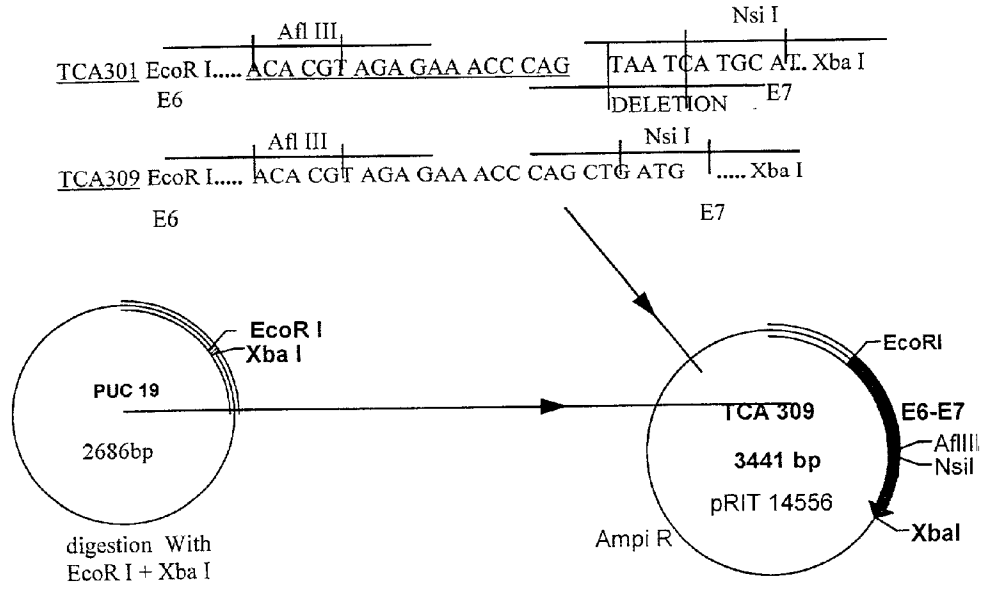


Figure 4

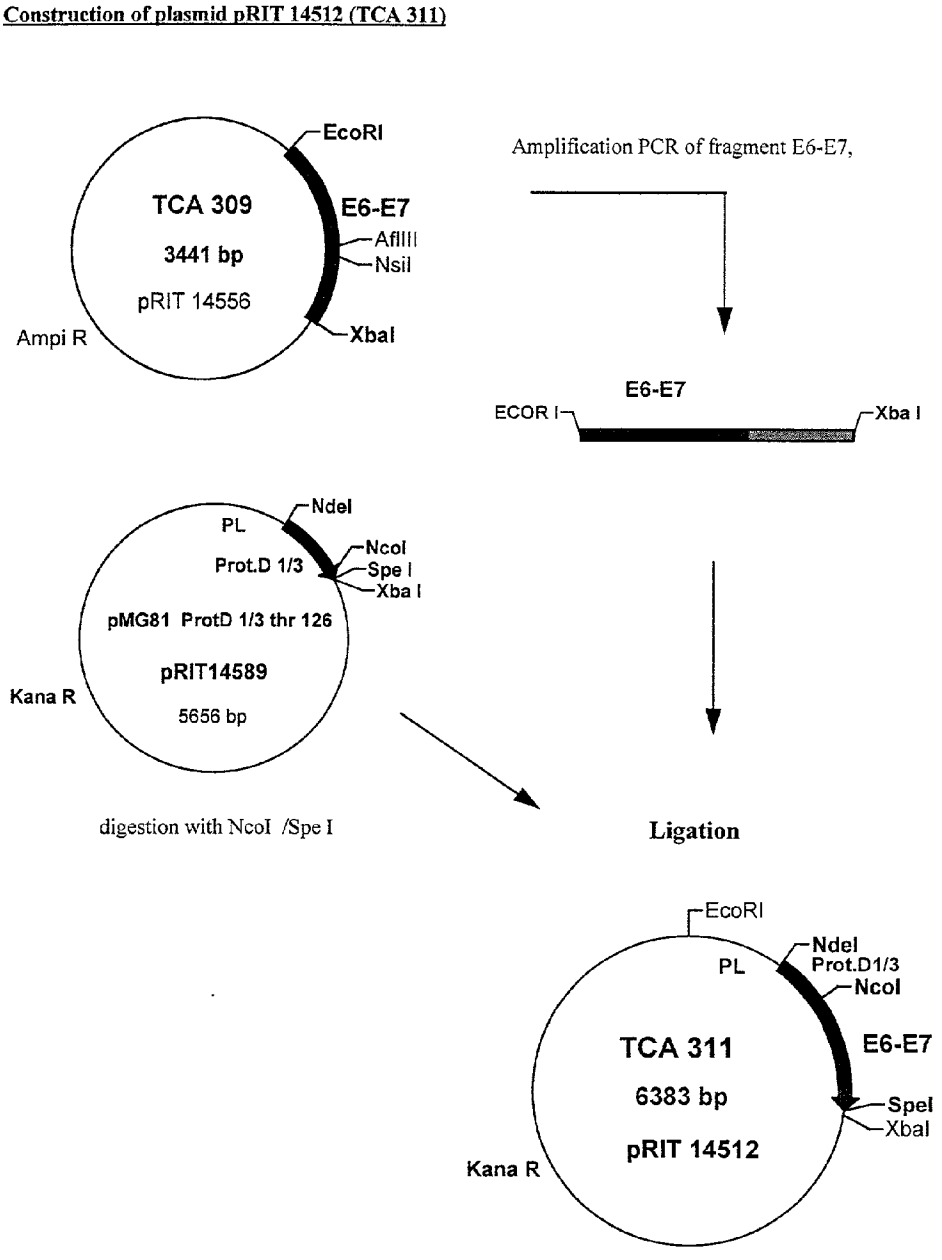

Construction of plasmid pRIT 14512 (TCA 311)
EcoRI
TCA 309
3441 bp
pRIT 14556
E6-E7
AflIII
NsiI
XbaI
Ampi R
Amplification PCR of fragment E6-E7,
E6-E7
ECOR I
Xba I
NdeI
PL
NcoI
Prot.D 1/3
Spe I
Xba I
pMG81 ProtD 1/3 thr 126
pRIT14589
5656 bp
Kana R
digestion with NcoI /Spe I
Ligation
EcoRI
NdeI
PL
Prot.D1/3
NcoI
TCA 311
6383 bp
pRIT 14512
E6-E7
SpeI
XbaI
Kana R

Figure 5

SEQUENCE OF PROT.D1/3 - E6 - E7 - His / HPV 16

Peptidic sequence

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATC 50
AGACAAAATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGC 100
ATACGTTAGAATCTAAAGCACTTGCGTTTGCACAACAGGCTGATTATTTA 150
GAGCAAGATTTAGCAATGACTAAGGATGGTCGTTTAGTGGTTATTCACGA 200
TCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAATTCCCACATCGTC 250
ATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAAGAAATT 300
CAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGCCATGTTTCAGGA 350
CCCACAGGAGCGACCCAGAAAGTTACCACAGTTATGCACAGAGCTGCAAA 400
CAACTATACATGATATAATATTAGAATGTGTGTACTGCAAGCAACAGTTA 450
CTGCGACGTGAGGTATATGACTTTGCTTTTCGGGATTTATGCATAGTATA 500
TAGAGATGGGAATCCATATGCTGTATGTGATAAATGTTTAAAGTTTTATT 550
CTAAAATTAGTGAGTATAGACATTATTGTTATAGTTTGTATGGAACAACA 600
TTAGAACAGCAATACAACAAACCGTTGTGTGATTTGTTAATTAGGTGTAT 650
TAACTGTCAAAAGCCACTGTGTCCTGAAGAAAAGCAAAGACATCTGGACA 700
AAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGACCGGTCGATGTATG 750
TCTTGTTGCAGATCATCAAGAACACGTAGAGAAACCCAGCTGATGCATGG 800
AGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAGACAA 850
CTGATCTCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGAGGAGGAT 900
GAAATAGATGGTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATTACAA 950
TATTGTAACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTGTGCGTAC 1000
AAAGCACACACGTAGACATTCGTACTTTGGAAGACCTGTTAATGGGCACA 1050
CTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCAACTAGTGGCCACCA 1100
TCACCATCACCATTAA 1116

Figure 6A

Peptidic sequence

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL 50
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEI 100
QSLEMTENFETMAMFQDPQERPRKLPQLCTELQTTIHDIILECVYCKQQL 150
LRREVYDFAFRDLCIVYRDGNPYAVCDKCLKFYSKISEYRHYCYSLYGTT 200
LEQQYNKPLCDLLIRCINCQKPLCPEEKQRHLDKKQRFHNIRGRWTGRCM 250
SCCRSSRTRRETQLMHGDTPTLHEYMLDLQPETTDLYCYEQLNDSSEEED 300
EIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIRTLEDLLMGT 350
LGIVCPICSQKPTSGHHHHHH. 372

Figure 6B

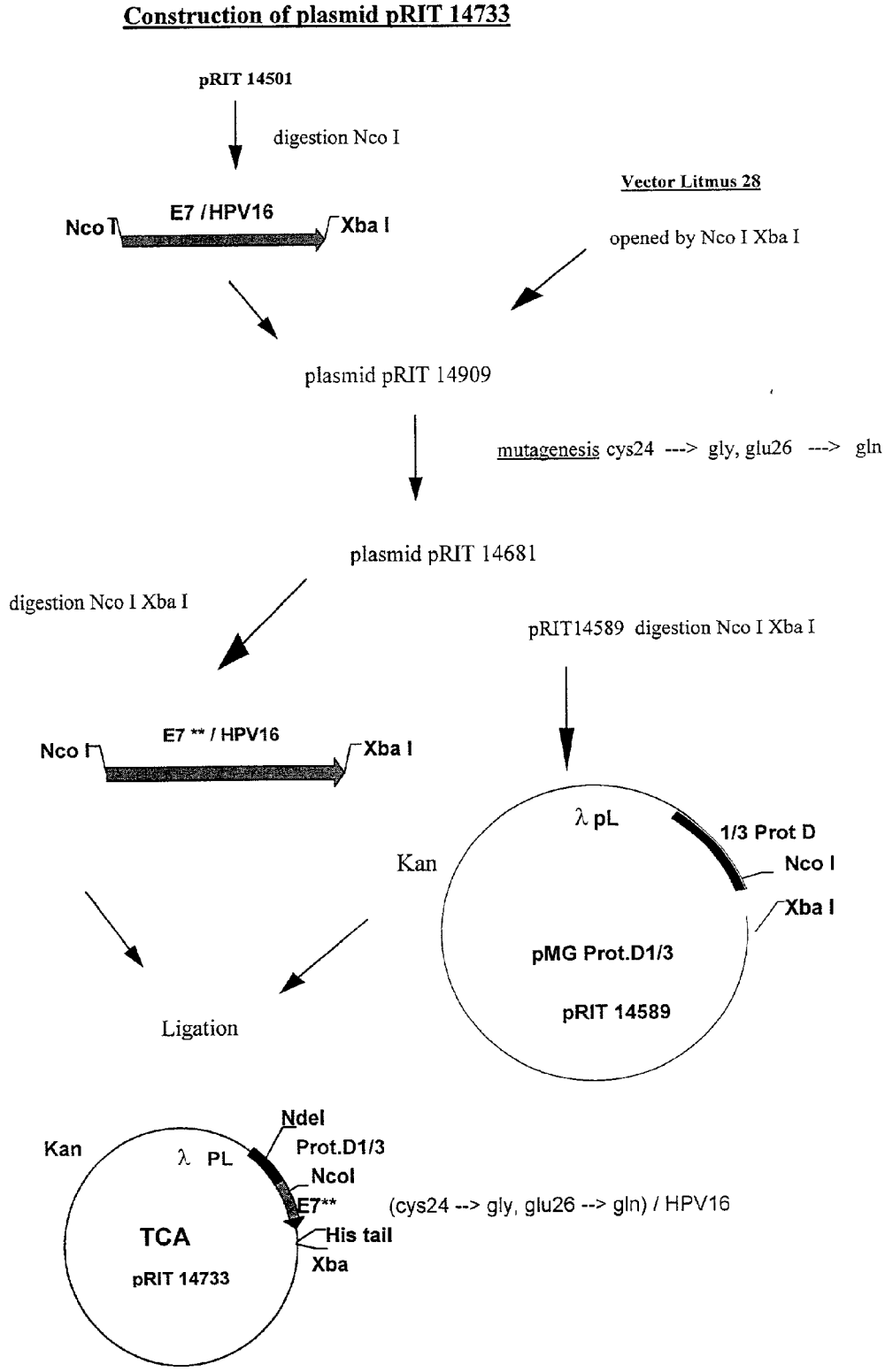

Construction of plasmid pRIT 14733
pRIT 14501
digestion Nco I
Nco I
E7 /HPV16
Xba I
Vector Litmus 28
opened by Nco I Xba I
plasmid pRIT 14909
mutagenesis cys24 ---> gly, glu26 ---> gln
plasmid pRIT 14681
digestion Nco I Xba I
pRIT14589 digestion Nco I Xba I
Nco I
E7 ** / HPV16
Xba I
λ pL
1/3 Prot D
Nco I
Xba I
Kan
pMG Prot.D1/3
pRIT 14589
Ligation
Kan
λ PL
NdeI
Prot.D1/3
NcoI
E7**
(cys24 --> gly, glu26 --> gln) / HPV16
His tail
Xba
TCA
pRIT 14733

Figure 7

SEQUENCE OF PROT.D1/3 - E7 mutated (cys24 → gly, glu26 → gln) HPV16.

Nucleotidic sequence:

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATC 50
AGACAAAATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGC 100
ATACGTTAGAATCTAAAGCACTTGCGTTTGCACAACAGGCTGATTATTTA 150
GAGCAAGATTTAGCAATGACTAAGGATGGTCGTTTAGTGGTTATTCACGA 200
CACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAATTCCCACATCGTC 250
ATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAAGAAATT 300
CAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGCCATGCATGGAGA 350
TACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAGACAACTG 400
ATCTCTAC**G**GTTAT**C**AGCAATTAAATGACAGCTCAGAGGAGGAGGATGAA 450
ATAGATGGTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATTACAATAT 500
TGTAACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTGTGCGTACAAA 550
GCACACACGTAGACATTCGTACTTTGGAAGACCTGTTAATGGGCACACTA 600
GGAATTGTGTGCCCCATCTGTTCTCAGAAACCAACTAGTGGCCACCATCA 650
CCATCACCATTAA 663

Mutations: T409 → G
G415 → C

Figure 8A

Peptidic sequence:

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEI
QSLEMTENFETMAMHGDTPTLHEYMLDLQPETTDLY**GYQ**QLNDSSEEEDE
IDGPAGQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIRTLEDLLMGTL
GIVCPICSQKPTSGHHHHHH. 221

mutated amino acids: cys24 → gly (=C24→G), glu26 → gln (=E26→Q) of E7 are residues 137 and 139 of the fusion protein.

N term M D P -**ProtD1/3**(aa4 --> 111)-M A-**mutated E7**(aa 114 --> 211)-TSGHHHHHH Cterm.

Figure 8B

Construction of plasmid pRIT 14634 (TCA332)

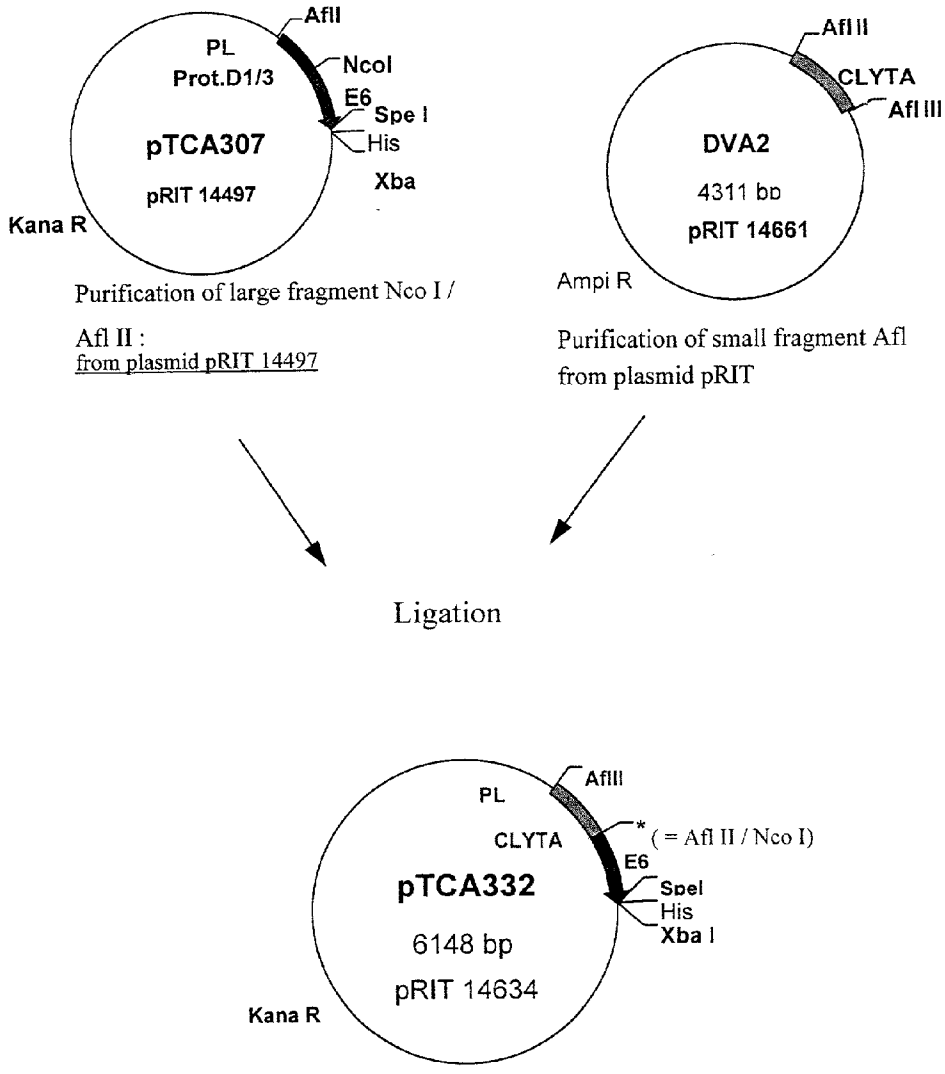


Figure 9

SEQUENCE OF CLYTA - E6 - His
Nucleotidic sequence

ATGAAAGGGGGAATTGTACATTCAGACGGCTCTTATCCAAAAGACAAGTT 50
TGAGAAAATCAATGGCACTTGGTACTACTTTGACAGTTCAGGCTATATGC 100
TTGCAGACCGCTGGAGGAAGCACACAGACGGCAACTGGTACTGGTTCGAC 150
AACTCAGGCGAAATGGCTACAGGCTGGAAGAAAATCGCTGATAAGTGGTA 200
CTATTTCAACGAAGAAGGTGCCATGAAGACAGGCTGGGTCAAGTACAAGG 250
ACACTTGGTACTACTTAGACGCTAAAGAAGGCGCCATGGTATCAAATGCC 300
TTTATCCAGTCAGCGGACGGAACAGGCTGGTACTACCTCAAACCAGACGG 350
AACACTGGCAGACAGGCCAGAATTGGCCAGCATGCTGGACATGGCCATGT 400
TTCAGGACCCACAGGAGCGACCCAGAAAGTTACCACAGTTATGCACAGAG 450
CTGCAAACAACTATACATGATATAATATTAGAATGTGTGTACTGCAAGCA 500
ACAGTTACTGCGACGTGAGGTATATGACTTTGCTTTTCGGGATTTATGCA 550
TAGTATATAGAGATGGGAATCCATATGCTGTATGTGATAAATGTTTAAAG 600
TTTTATTCTAAAATTAGTGAGTATAGACATTATTGTTATAGTTTGTATGG 650
AACAACATTAGAACAGCAATACAACAAACCGTTGTGTGATTTGTTAATTA 700
GGTGTATTAACTGTCAAAAGCCACTGTGTCCTGAAGAAAAGCAAAGACAT 750
CTGGACAAAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGACCGGTCG 800
ATGTATGTCTTGTTGCAGATCATCAAGAACACGTAGAGAAACCCAGCTGA 850
CTAGTGGCCACCATCACCATCACCATTAA 879

Figure 10A

Peptidic sequence

MKGGIVHSDGSYPKDKFEKINGTWYYFDSSGYMLADRWRKHTDGNWYWFD 50
NSGEMATGWKKIADKWYYFNEEGAMKTGWVKYKDTWYYLDAKEGAMVSNA 100
FIQSADGTGWYYLKPDGTLADRPELASMLDMAMFQDPQERPRKLPQLCTE 150
LQTTIHDIILECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLK 200
FYSKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRH 250
LDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQLTSGHHHHHH. 293

Figure 10B

Construction of plasmid pRiT 14626 (TCA330)

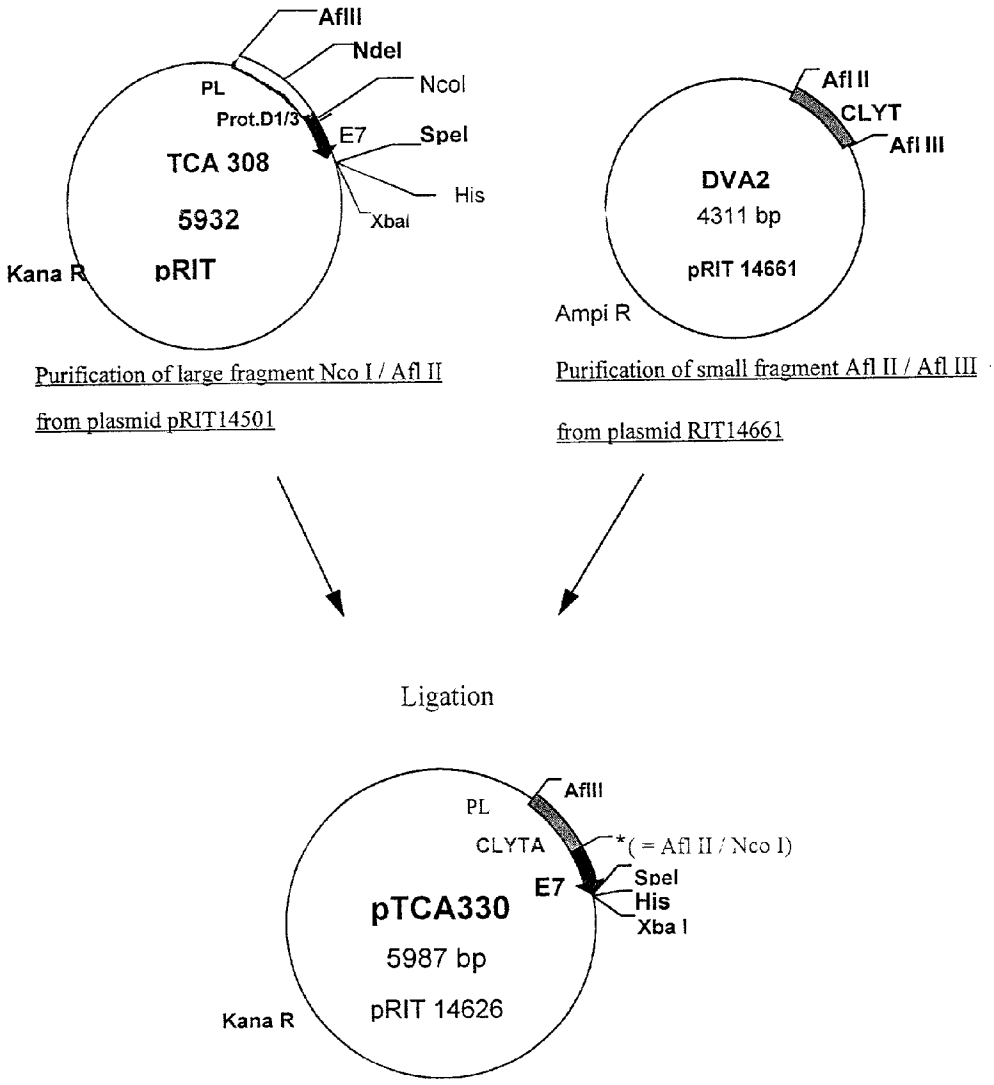


Figure 11

SEQUENCE OF CLYTA - E7 - His.

Nucleotidic sequence

ATGAAAGGGGGAATTGTACATTCAGACGGCTCTTATCCAAAAGACAAGTT 50
TGAGAAAATCAATGGCACTTGGTACTACTTTGACAGTTCAGGCTATATGC 100
TTGCAGACCGCTGGAGGAAGCACACAGACGGCAACTGGTACTGGTTCGAC 150
AACTCAGGCGAAATGGCTACAGGCTGGAAGAAAATCGCTGATAAGTGGTA 200
CTATTTCAACGAAGAAGGTGCCATGAAGACAGGCTGGGTCAAGTACAAGG 250
ACACTTGGTACTACTTAGACGCTAAAGAAGGCGCCATGGTATCAAATGCC 300
TTTATCCAGTCAGCGGACGGAACAGGCTGGTACTACCTCAAACCAGACGG 350
AACACTGGCAGACAGGCCAGAATTGGCCAGCATGCTGGACATGGCCATGC 400
ATGGAGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCAGAG 450
ACAACTGATCTCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGAGGA 500
GGATGAAATAGATGGTCCAGCTGGACAAGCAGAACCGGACAGAGCCCATT 550
ACAATATTGTAACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTGTGC 600
GTACAAAGCACACACGTAGACATTCGTACTTTGGAAGACCTGTTAATGGG 650
CACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCAACTAGTGGCC 700
ACCATCACCATCACCATTAA 720

Figure 12A

Peptidic sequence

MKGGIVHSDGSYPKDKFEKINGTWYYFDSSGYMLADRWRKHTDGNWYWFD 50

NSGEMATGWKKIADKWYYFNEEGAMKTGWVKYKDTWYYLDAKEGAMVSNA 100

FIQSADGTGWYYLKPDGTLADRPELASMLDMAMHGDTPTLHEYMLDLQPE 150

TTDLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRLC 200

VQSTHVDIRTLEDLLMGTLGIVCPICSQKPTSGHHHHHH. 240

Figure 12B

Construction of plasmid pRIT 14634 (TCA331)

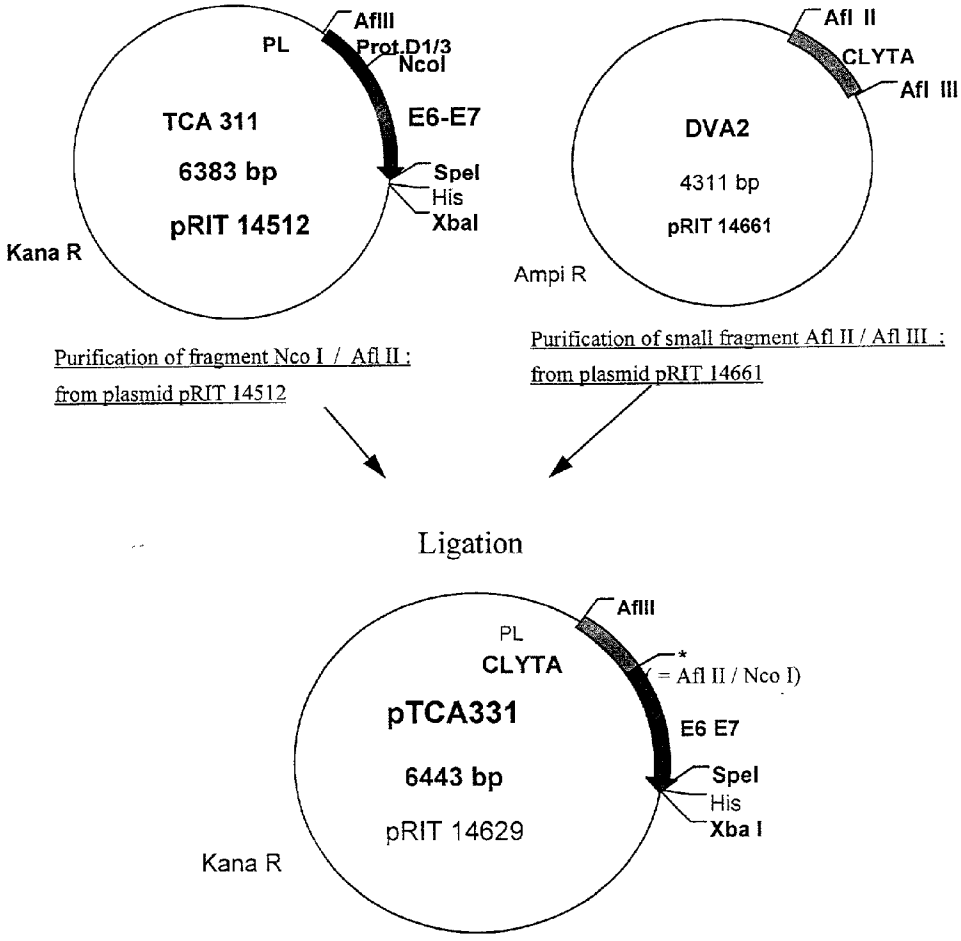


Figure 13

SEQUENCE OF CLYTA - E6E7 - His.

Nucleotidic sequence

ATGAAAGGGGGAATTGTACATTCAGACGGCTCTTATCCAAAAGACAAGTT 50
TGAGAAAATCAATGGCACTTGGTACTACTTTGACAGTTCAGGCTATATGC 100
TTGCAGACCGCTGGAGGAAGCACACAGACGGCAACTGGTACTGGTTCGAC 150
AACTCAGGCGAAATGGCTACAGGCTGGAAGAAAATCGCTGATAAGTGGTA 200
CTATTTCAACGAAGAAGGTGCCATGAAGACAGGCTGGGTCAAGTACAAGG 250
ACACTTGGTACTACTTAGACGCTAAAGAAGGCGCCATGGTATCAAATGCC 300
TTTATCCAGTCAGCGGACGGAACAGGCTGGTACTACCTCAAACCAGACGG 350
AACACTGGCAGACAGGCCAGAATTGGCCAGCATGCTGGACATGGCCATGT 400
TTCAGGACCCACAGGAGCGACCCAGAAAGTTACCACAGTTATGCACAGAG 450
CTGCAAACAACTATACATGATATAATATTAGAATGTGTGTACTGCAAGCA 500
ACAGTTACTGCGACGTGAGGTATATGACTTTGCTTTTCGGGATTTATGCA 550
TAGTATATAGAGATGGGAATCCATATGCTGTATGTGATAAATGTTTAAAG 600
TTTTATTCTAAAATTAGTGAGTATAGACATTATTGTTATAGTTTGTATGG 650
AACAACATTAGAACAGCAATACAACAAACCGTTGTGTGATTTGTTAATTA 700
GGTGTATTAACTGTCAAAAGCCACTGTGTCCTGAAGAAAAGCAAAGACAT 750
CTGGACAAAAAAGCAAAGATTCCATAATATAAGGGGTCGGTGGACCGGTCG 800
ATGTATGTCTTGTTGCAGATCATCAAGAACACGTAGAGAAACCCAGCTGA 850
TGCATGGAGATACACCTACATTGCATGAATATATGTTAGATTTGCAACCA 900
GAGACAACTGATCTCTACTGTTATGAGCAATTAAATGACAGCTCAGAGGA 950
GGAGGATGAAATAGATGGTCCAGCTGGACAAGCAGAACCGGACAGAGCCC 1000
ATTACAATATTGTAACCTTTTGTTGCAAGTGTGACTCTACGCTTCGGTTG 1050
TGCGTACAAAGCACACACGTAGACATTCGTACTTTGGAAGACCTGTTAAT 1100
GGGCACACTAGGAATTGTGTGCCCCATCTGTTCTCAGAAACCAACTAGTG 1150
GCCACCATCACCATCACCATTAA 1173

Figure 14A

Peptidic sequence

MKGGIVHSDGSYPKDKFEKINGTWYYFDSSGYMLADRWRKHTDGNWYWFD 50

NSGEMATGWKKIADKWYYFNEEGAMKTGWVKYKDTWYYLDAKEGAMVSNA 100

FIQSADGTGWYYLKPDGTLADRPELASMLDMAMFQDPQERPRKLPQLCTE 150

LQTTIHDIILECVYCKQQLLRREVYDFAFRDLCIVYRDGNPYAVCDKCLK 200

FYSKISEYRHYCYSLYGTTLEQQYNKPLCDLLIRCINCQKPLCPEEKQRH 250

LDKKQRFHNIRGRWTGRCMSCCRSSRTRRETQLMHGDTPTLHEYMLDLQP 300

ETTDLYCYEQLNDSSEEEDEIDGPAGQAEPDRAHYNIVTFCCKCDSTLRL 350

CVQSTHVDIRTLEDLLMGTLGIVCPICSQKPTSGHHHHHH. 391

Figure 14B

Construction of pLASMID pRIT 14532 (TCA 316)

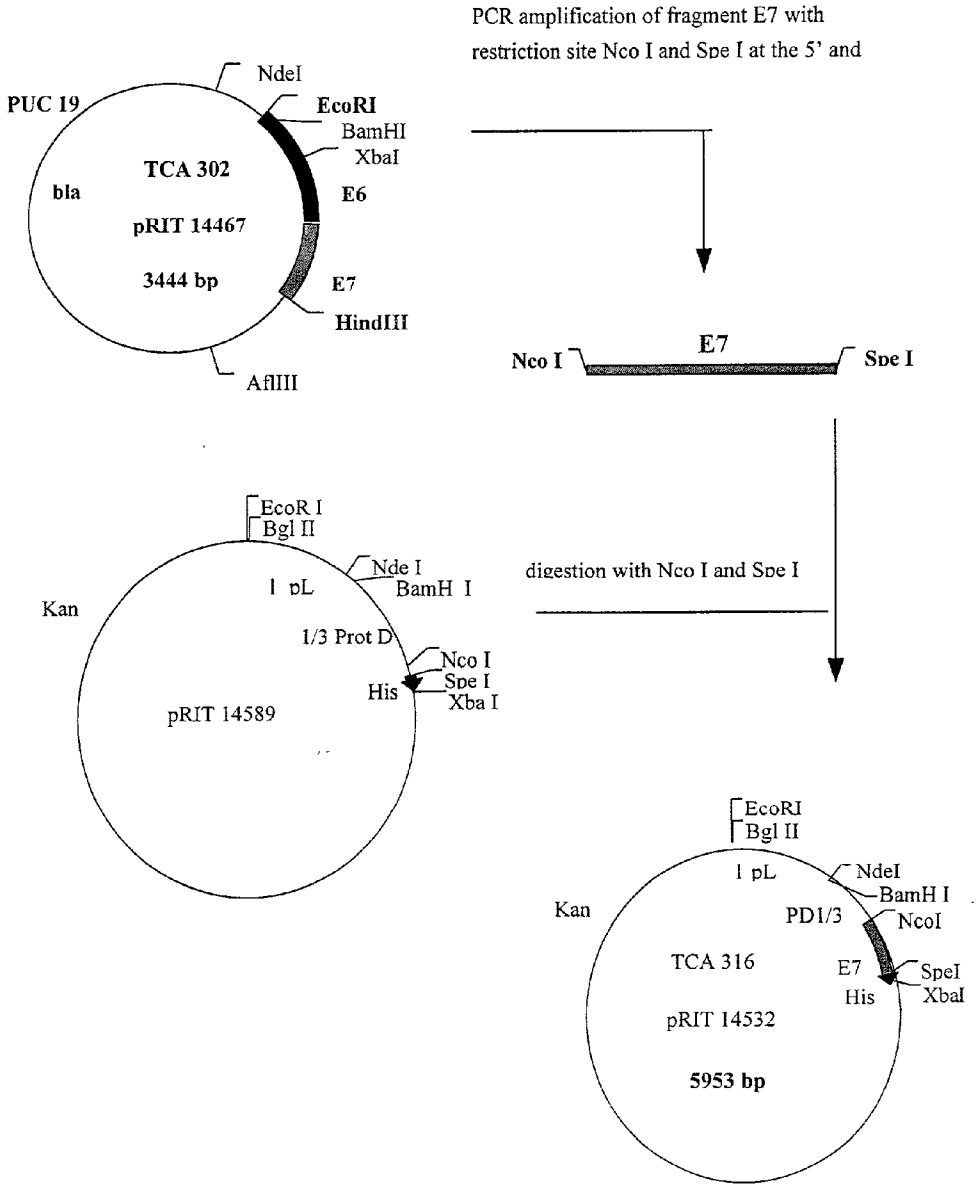


Figure 15

SEQUENCE OF PROT.D1/3 -E7-HIS /HPV18

Nucleotidic Sequence

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATC 50
AGACAAAATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGC 100
ATACGTTAGAATCTAAAGCACTTGCGTTTGCACAACAGGCTGATTATTTA 150
GAGCAAGATTTAGCAATGACTAAGGATGGTCGTTTAGTGGTTATTCACGA 200
TCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAATTCCCACATCGTC 250
ATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAAGAAATT 300
CAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGCCATGCATGGACC 350
TAAGGCAACATTGCAAGACATTGTATTGCATTTAGAGCCCCAAAATGAAA 400
TTCCGGTTGACCTTCTATGTCACGAGCAATTAAGCGACTCAGAGGAAGAA 450
AACGATGAAATAGATGAAGTTAATCATCAACATTTACCAGCCCGACGAGC 500
CGAACCACAACGTCACACAATGTTGTGTATGTGTTGTAAGTGTGAAGCCA 550
GAATTGAGCTAGTAGTAGAAAGCTCAGCAGACGACCTTCGAGCATTCCAG 600
CAGCTGTTTCTGAACACCCTGTCCTTTGTGTGTCCGTGGTGTGCATCCCA 650
GCAGACTAGTGGCCACCATCACCATCACCATTAA 684

Figure 16A

Peptidic Sequence

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKA

40LAFAQQADYLEQDLAMTKDGRLVVIHDHFLDGLTDVAKKF

80PHRHRKDGRYYVIDFTLKEIQSLEMTENFETMAMHGPKAT

120LQDIVLHLEPQNEIPVDLLCHEQLSDSEEENDEIDEVNHQ

160HLPARRAEPQRHTMLCMCCKCEARIELVVESSADDLRAFQ

200QLFLNTLSFVCPWCASQQTSGHHHHHH. 228

Figure 16B

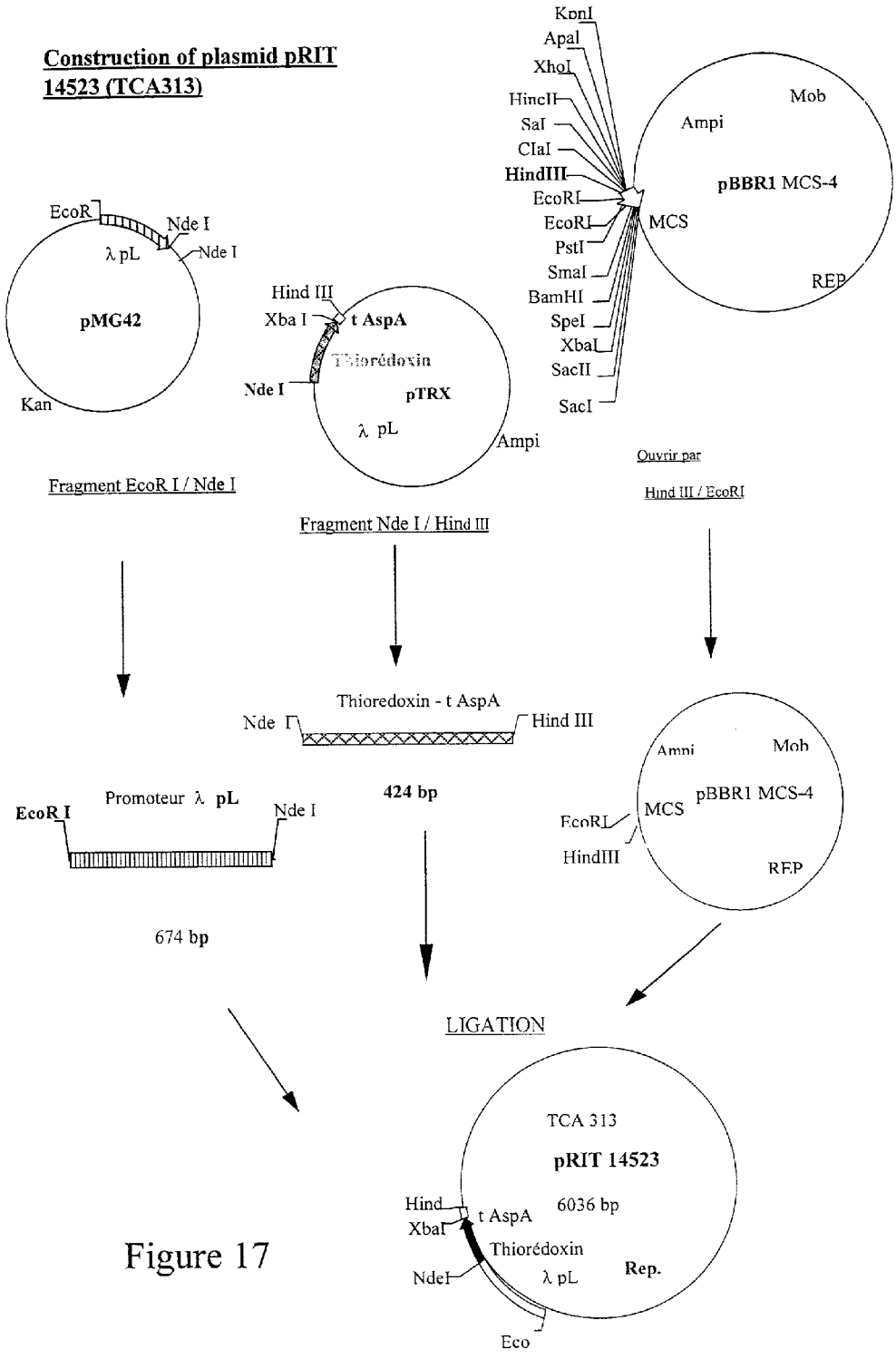

Construction of plasmid pRIT 14523 (TCA313)
KpnI
ApaI
XhoI
HincII
SaI
ClaI
HindIII
EcoRI
EcoRI
PstI
SmaI
BamHI
SpeI
XbaI
SacII
SacI
Mob
Ampi
pBBR1 MCS-4
MCS
REP
EcoR
Nde I
λ pL
Nde I
pMG42
Kan
Hind III
Xba I
t AspA
Thiorédoxin
Nde I
pTRX
λ pL
Ampi
Ouvrir par
Hind III / EcoRI
Fragment EcoR I / Nde I
Fragment Nde I / Hind III
Thioredoxin - t AspA
Nde
Hind III
424 bp
Promoteur λ pL
EcoR I
Nde I
674 bp
Amni
Mob
MCS
pBBR1 MCS-4
EcoRI
HindIII
REP
LIGATION
TCA 313
pRIT 14523
6036 bp
Hind
XbaI
t AspA
Thiorédoxin
NdeI
λ pL
Rep.
Eco

Figure 17

SEQUENCE OF THIOREDOXIN

MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIA 40
PILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLL 80
LFKNGEVAATKVGALSKGQLKEFLDANLA. 110

Figure 18

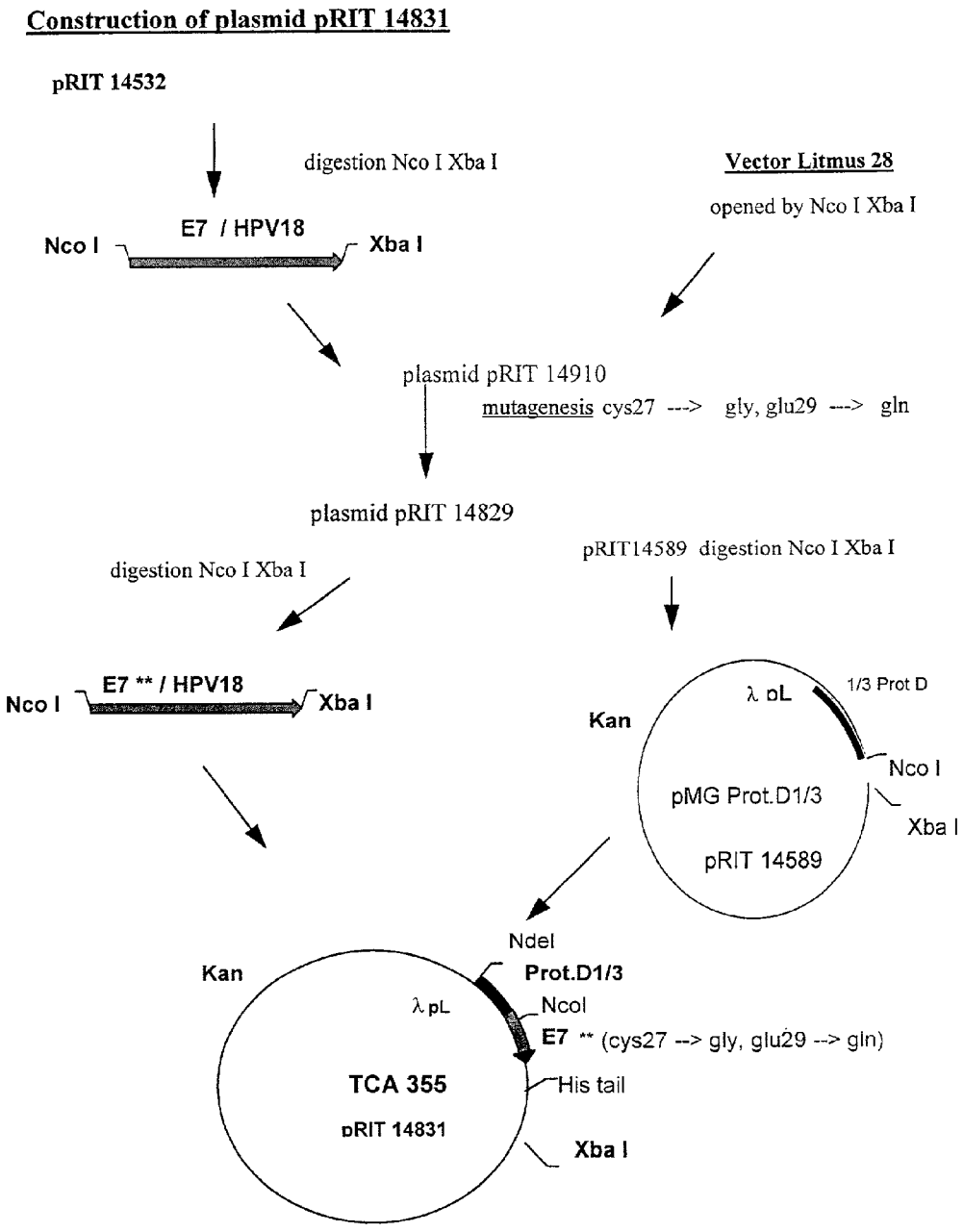

Construction of plasmid pRIT 14831
pRIT 14532
digestion Nco I Xba I
Vector Litmus 28
opened by Nco I Xba I
Nco I
E7 / HPV18
Xba I
plasmid pRIT 14910
mutagenesis cys27 ---> gly, glu29 ---> gln
plasmid pRIT 14829
digestion Nco I Xba I
pRIT14589 digestion Nco I Xba I
Nco I
E7 ** / HPV18
Xba I
Kan
λ pL
1/3 Prot D
Nco I
pMG Prot.D1/3
Xba I
pRIT 14589
NdeI
Kan
Prot.D1/3
λ pL
NcoI
E7 ** (cys27 --> gly, glu29 --> gln)
TCA 355
His tail
pRIT 14831
Xba I

Figure 19

SEQUENCE OF PROT.D1/3 - E7 mutated (cys27 → gly, glu29 → gln) HPV18.

Nucleotidic sequence:

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATC 50
AGACAAAATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGC 100
ATACGTTAGAATCTAAAGCACTTGCGTTTGCACAACAGGCTGATTATTTA 150
GAGCAAGATTTAGCAATGACTAAGGATGGTCGTTTAGTGGTTATTCACGA 200
TCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAATTCCCACATCGTC 250
ATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAAGAAATT 300
CAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGCCATGCATGGACC 350
TAAGGCAACATTGCAAGACATTGTATTGCATTTAGAGCCCCAAAATGAAA 400
TTCCGGTTGACCTTCTAGGTCACCAGCAATTAAGCGACTCAGAGGAAGAA 450
AACGATGAAATAGATGGAGTTAATCATCAACATTTACCAGCCCGACGAGC 500
CGAACCACAACGTCACACAATGTTGTGTATGTGTTGTAAGTGTGAAGCCA 550
GAATTGAGCTAGTAGTAGAAAGCTCAGCAGACGACCTTCGAGCATTCCAG 600
CAGCTGTTTCTGAACACCCTGTCCTTTGTGTGTCCGTGGTGTGCATCCCA 650
GCAGACTAGTGGCCACCATCACCATCACCATTAA 684

Mutations: T418 → G

G424 → C

Figure 20A

SEQUENCE OF PROT.D1/3 - E6 - His / HPV18.

Nucleotidic sequence

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATC 50
AGACAAAATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGC 100
ATACGTTAGAATCTAAAGCACTTGCGTTTGCACAACAGGCTGATTATTTA 150
GAGCAAGATTTAGCAATGACTAAGGATGGTCGTTTAGTGGTTATTCACGA 200
TCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAAATTCCCACATCGTC 250
ATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAAGAAATT 300
CAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGCGCGCTTTGAGGA 350
TCCAACACGGCGACCCTACAAGCTACCTGATCTGTGCACGGAACTGAACA 400
CTTCACTGCAAGACATAGAAATAACCTGTGTATATTGCAAGACAGTATTG 450
GAACTTACAGAGGTATTTGAATTTGCATTTAAAGATTTATTTGTGGTGTA 500
TAGAGACAGTATACCGCATGCTGCATGCCATAAATGTATAGATTTTTATT 550
CTAGAATTAGAGAATTAAGACATTATTCAGACTCTGTGTATGGAGACACA 600
TTGGAAAAACTAACTAACACTGGGTTATACAATTTATTAATAAGGTGCCT 650
GCGGTGCCAGAAACCGTTGAATCCAGCAGAAAAACTTAGACACCTTAATG 700
AAAAACGACGATTTCACAACATAGCTGGGCACTATAGAGGCCAGTGCCAT 750
TCGTGCTGCAACCGAGCACGACAGGAACGACTCCAACGACGCAGAGAAAC 800
ACAAGTAACTAGTGGCCACCATCACCATCACCATTAA 837

Figure 20A

Peptidic sequence:

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL
EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEI
QSLEMTENFETMAMHGPKATLQDIVLHLEPQNEIPVDLLGHQQLSDSEEE
NDEIDGVNHQHLPARRAEPQRHTMLCMCCKCEARIELVVESSADDLRAFQ
QLFLNTLSFVCPWCASQQTSGHHHHHH. 228

mutated amino acids: cys27 → gly (=C27→G), glu29 → gln (=E29→Q) of E7 are residues 140 and 142 of the fusion protein.

N term M D P -**ProtD1/3**(aa4 --> 111)-M A-**mutated E7**(aa 114 --> 218)-TSGHHHHHH Cterm.

Figure 20B

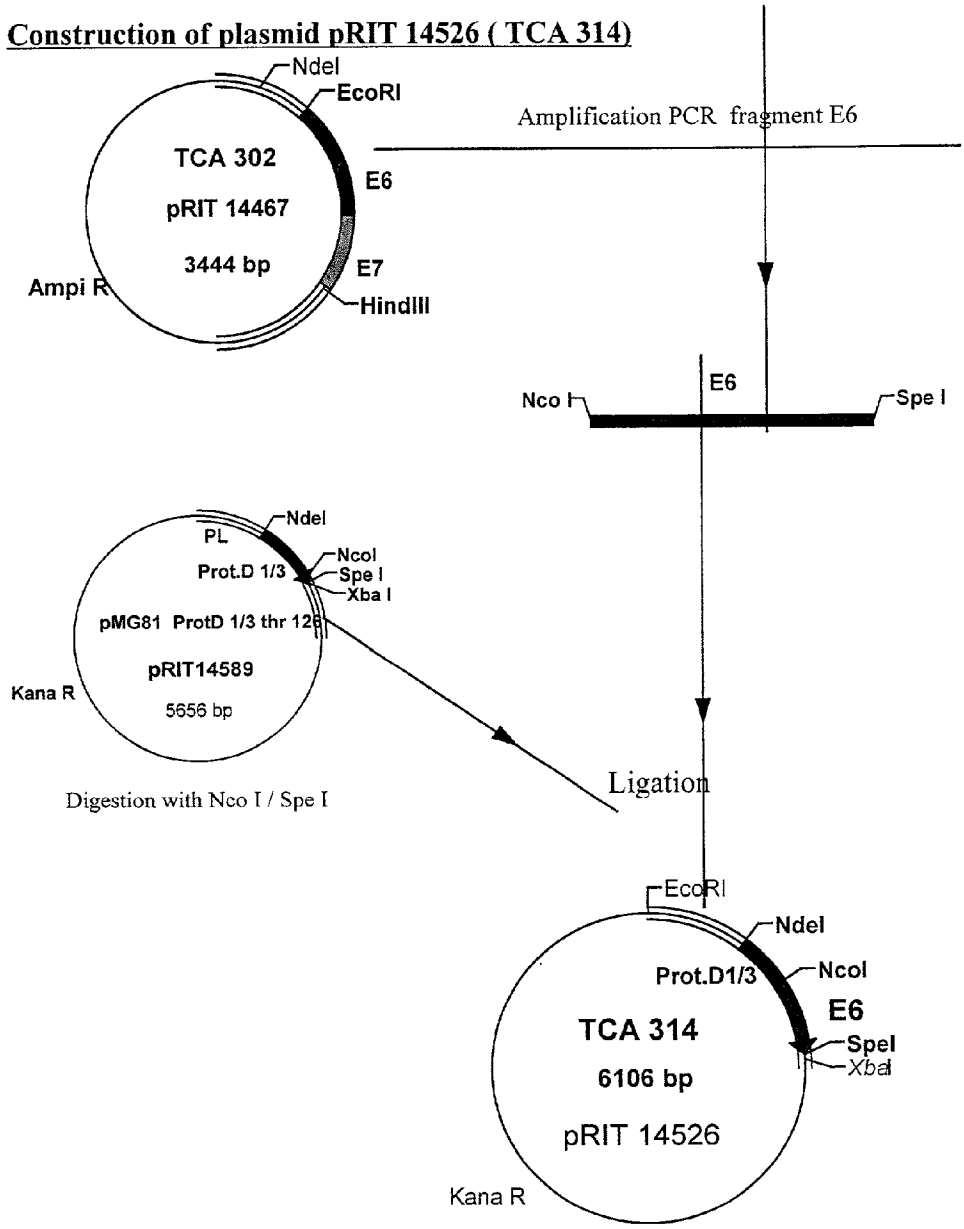

Construction of plasmid pRIT 14526 (TCA 314)
NdeI
EcoRI
TCA 302
pRIT 14467
3444 bp
E6
E7
Ampi R
HindIII
Amplification PCR fragment E6
E6
Nco I
Spe I
NdeI
PL
Prot.D 1/3
NcoI
Spe I
Xba I
pMG81 ProtD 1/3 thr 128
pRIT14589
5656 bp
Kana R
Digestion with Nco I / Spe I
Ligation
EcoRI
NdeI
Prot.D1/3
NcoI
E6
TCA 314
6106 bp
pRIT 14526
SpeI
XbaI
Kana R

Figure 21

Peptidic sequence

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL 50

EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEI 100

QSLEMTENFETMARFEDPTRRPYKLPDLCTELNTSLQDIEITCVYCKTVL 150

ELTEVFEFAFKDLFVVYRDSIPHAACHKCIDFYSRIRELRHYSDSVYGDT 200

LEKLTNTGLYNLLIRCLRCQKPLNPAEKLRHLNEKRRFHNIAGHYRGQCH 250

SCCNRARQERLQRRRETQVTSGHHHHHH. 279

Figure 22B

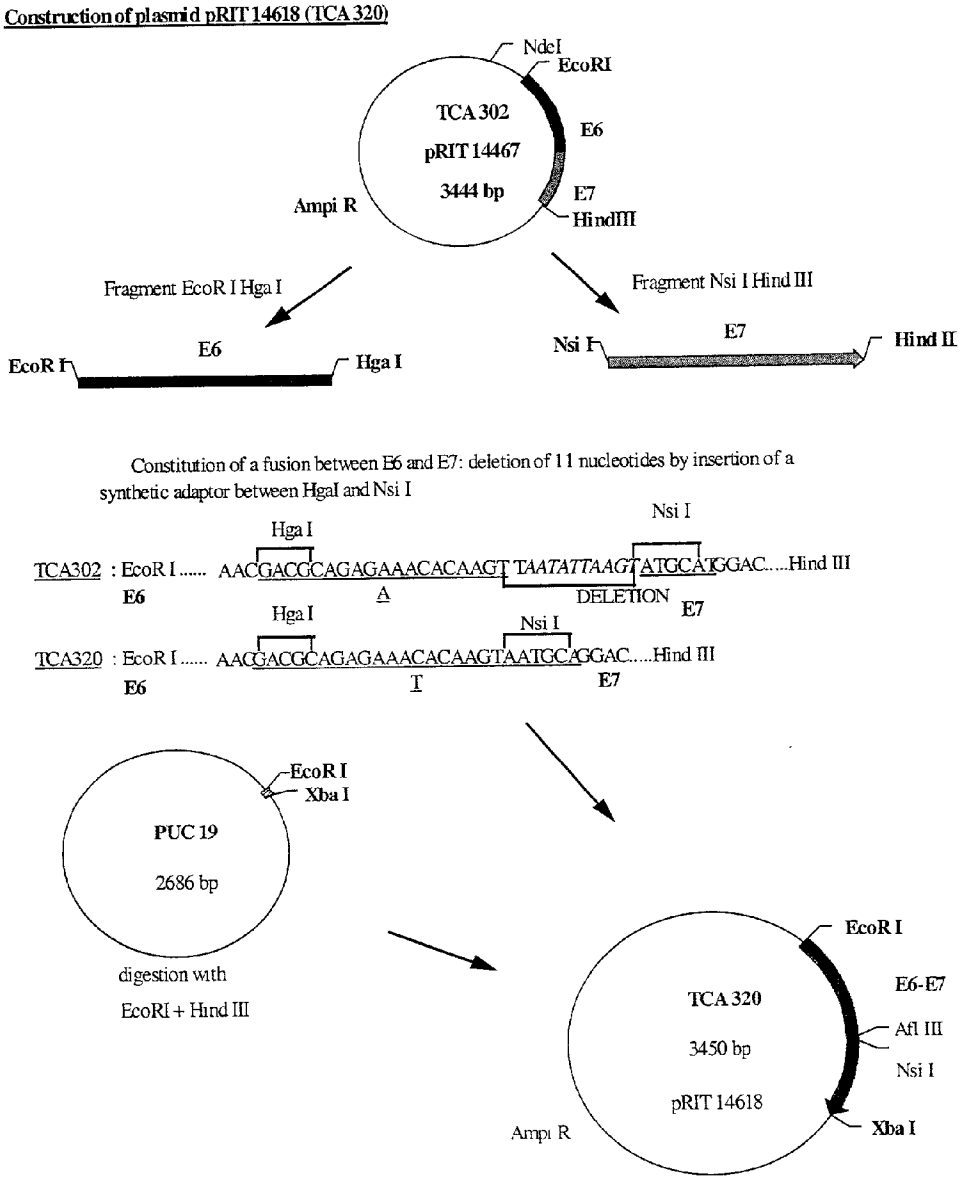

Construction of plasmid pRIT 14618 (TCA 320)
NdeI
EcoRI
TCA 302
pRIT 14467
3444 bp
E6
E7
HindIII
Ampi R
Fragment EcoR I Hga I
E6
EcoR I
Hga I
Fragment Nsi I Hind III
E7
Nsi I
Hind II
Constitution of a fusion between E6 and E7: deletion of 11 nucleotides by insertion of a synthetic adaptor between HgaI and Nsi I
Hga I
Nsi I
TCA302 : EcoR I..... AACGACGCAGAGAAACACAAGT TAATATTAAGTATGCATGGAC.....Hind III
E6
A
DELETION
E7
Hga I
Nsi I
TCA320 : EcoR I..... AACGACGCAGAGAAACACAAGTAATGCAGGAC.....Hind III
E6
T
E7
EcoR I
Xba I
PUC 19
2686 bp
digestion with
EcoRI + Hind III
EcoR I
E6-E7
TCA 320
3450 bp
Afl III
Nsi I
pRIT 14618
Ampi R
Xba I

Figure 23

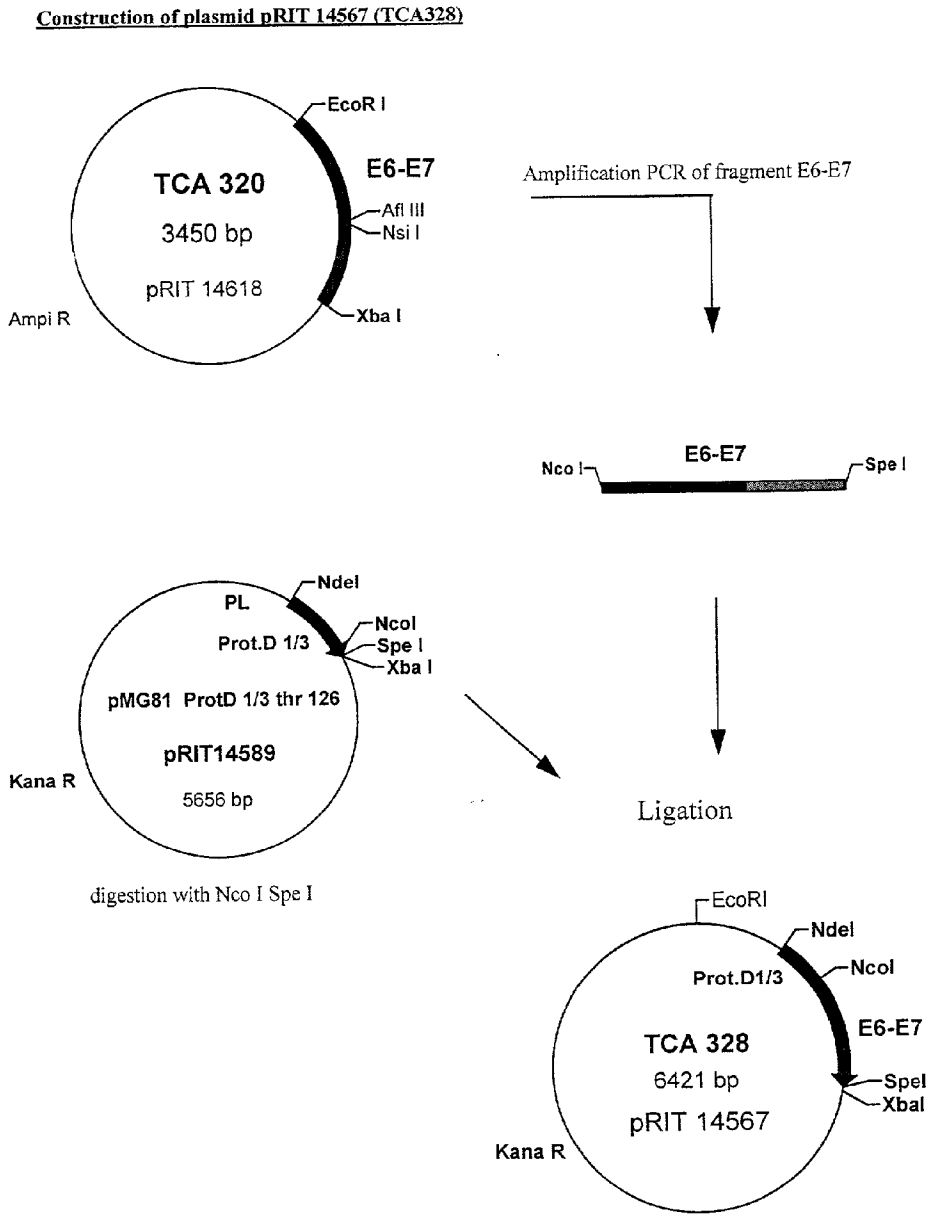

Construction of plasmid pRIT 14567 (TCA328)
EcoR I
E6-E7
TCA 320
3450 bp
pRIT 14618
Afl III
Nsi I
Ampi R
Xba I
Amplification PCR of fragment E6-E7
Nco I
E6-E7
Spe I
Ndel
PL
Prot.D 1/3
NcoI
Spe I
Xba I
pMG81 ProtD 1/3 thr 126
pRIT14589
Kana R
5656 bp
Ligation
digestion with Nco I Spe I
EcoRI
Ndel
Prot.D1/3
NcoI
E6-E7
TCA 328
6421 bp
pRIT 14567
SpeI
XbaI
Kana R

Figure 24

SEQUENCE OF PROT.D1/3 - E6 - E7 - His / HPV18.

Nucleotidic sequence

ATGGATCCAAGCAGCCATTCATCAAATATGGCGAATACCCAAATGAAATC 50
AGACAAAATCATTATTGCTCACCGTGGTGCTAGCGGTTATTTACCAGAGC 100
ATACGTTAGAATCTAAAGCACTTGCGTTTGCACAACAGGCTGATTATTTA 150
GAGCAAGATTTAGCAATGACTAAGGATGGTCGTTTAGTGGTTATTCACGA 200
TCACTTTTTAGATGGCTTGACTGATGTTGCGAAAAAATTCCCACATCGTC 250
ATCGTAAAGATGGCCGTTACTATGTCATCGACTTTACCTTAAAAGAAATT 300
CAAAGTTTAGAAATGACAGAAAACTTTGAAACCATGGCGCGCTTTGAGGA 350
TCCAACACGGCGACCCTACAAGCTACCTGATCTGTGCACGGAACTGAACA 400
CTTCACTGCAAGACATAGAAATAACCTGTGTATATTGCAAGACAGTATTG 450
GAACTTACAGAGGTATTTGAATTTGCATTTAAAGATTTATTTGTGGTGTA 500
TAGAGACAGTATACCGCATGCTGCATGCCATAAATGTATAGATTTTTATT 550
CTAGAATTAGAGAATTAAGACATTATTCAGACTCTGTGTATGGAGACACA 600
TTGGAAAAACTAACTAACACTGGGTTATACAATTTATTAATAAGGTGCCT 650
GCGGTGCCAGAAACCGTTGAATCCAGCAGAAAAACTTAGACACCTTAATG 700
AAAAACGACGATTTCACAACATAGCTGGGCACTATAGAGGCCAGTGCCAT 750
TCGTGCTGCAACCGAGCACGACAGGAACGACTCCAACGACGCAGAGAAAC 800
ACAAGTAATGCATGGACCTAAGGCAACATTGCAAGACATTGTATTGCATT 850
TAGAGCCCCAAAATGAAATTCCGGTTGACCTTCTATGTCACGAGCAATTA 900
AGCGACTCAGAGGAAGAAAACGATGAAATAGATGGAGTTAATCATCAACA 950
TTTACCAGCCCGACGAGCCGAACCACAACGTCACACAATGTTGTGTATGT 1000
GTTGTAAGTGTGAAGCCAGAATTGAGCTAGTAGTAGAAAGCTCAGCAGAC 1050
GACCTTCGAGCATTCCAGCAGCTGTTTCTGAACACCCTGTCCTTTGTGTG 1100
TCCGTGGTGTGCATCCCAGCAGACTAGTGGCCACCATCACCATCACCATT 1150
AA 1152

Figure 25A

Peptidic sequence

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFAQQADYL 50

EQDLAMTKDGRLVVIHDHFLDGLTDVAKKFPHRHRKDGRYYVIDFTLKEI 100

QSLEMTENFETMARFEDPTRRPYKLPDLCTELNTSLQDIEITCVYCKTVL 150

ELTEVFEFAFKDLFVVYRDSIPHAACHKCIDFYSRIRELRHYSDSVYGDT 200

LEKLTNTGLYNLLIRCLRCQKPLNPAEKLRHLNEKRRFHNIAGHYRGQCH 250

SCCNRARQERLQRRRETQVMHGPKATLQDIVLHLEPQNEIPVDLLCHEQL 300

SDSEEENDEIDGVNHQHLPARRAEPQRHTMLCMCCKCEARIELVVESSAD 350

DLRAFQQLFLNTLSFVCPWCASQQTSGHHHHHH. 384

Figure 25B

Therapeutic effect of vaccination with ProtD1/3 E7 of HPV16 formulations, on TC1 tumor growth

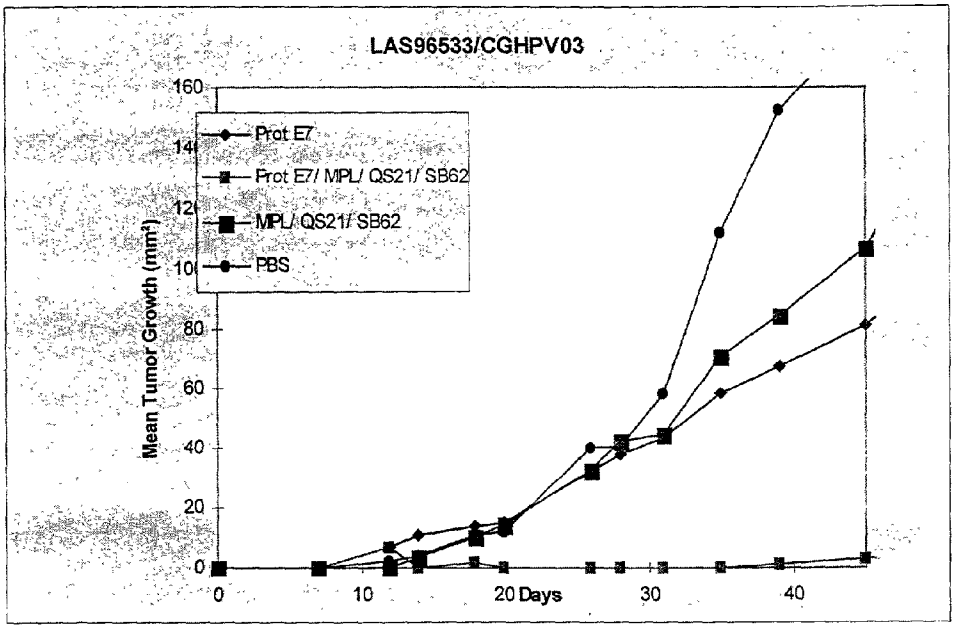


Figure 26

Lymphoproliferation on spleen cells
(stimulation index)
72 Hrs *in vitro* restimulation with ProtD1/3E7 (0.1; 1 µg/ml)
(exp 96533)

Group 1: ProtD 1/3 E7
Group 2: ProtD 1/3 E7 + SB 62 Qs21 & 3 D MPL
Group 3: SB 62 Qs21 & 3 D MPL
Group 4: PBS

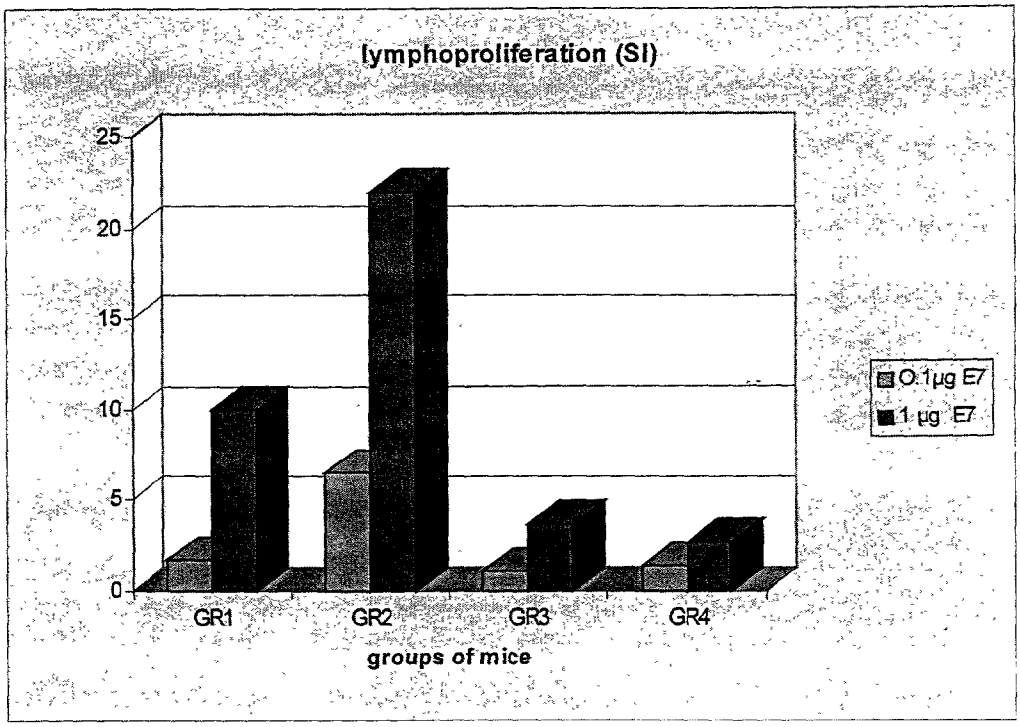


Figure 27

Lymphoproliferation on lymph node cells
(stimulation index)
72 Hrs *in vitro* restimulation with ProtD1/3E7 (1 µg/ml)
(exp 96533)

Group 1: ProtD 1/3 E7
Group 2: ProtD 1/3 E7 + SB 62 Qs21 & 3 D MPL
Group 3: SB 62 Qs21 & 3 D MPL
Group 4: PBS

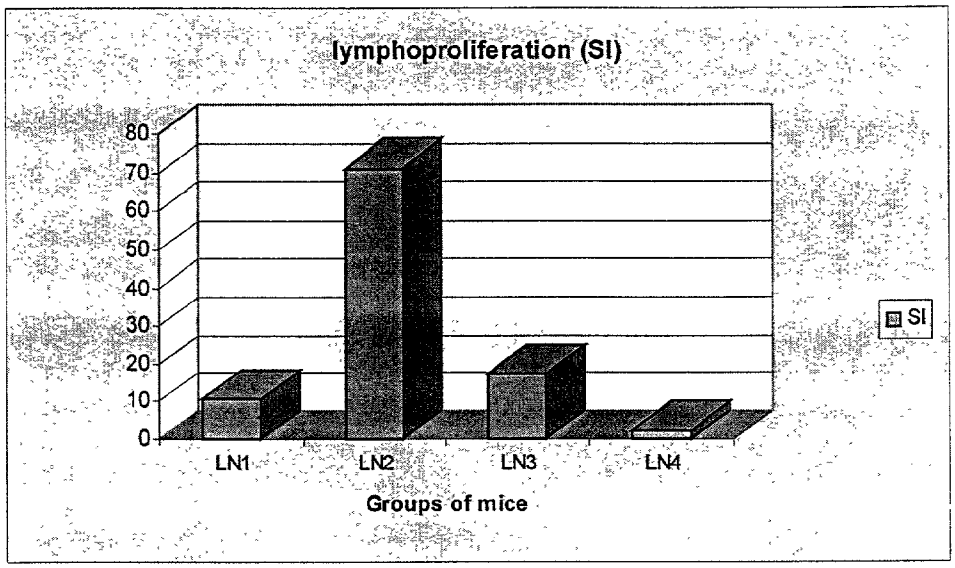


Figure 28

Subclass-specific antibody response (exp 96533)

group 1: ProtD1/3 E7 HPV16
group 2: ProtD1/3 E7 HPV16+ SB 62 Qs21 & 3 D MPL
group 3: SB 62 Qs21 & 3 D MPL
group 4: PBS

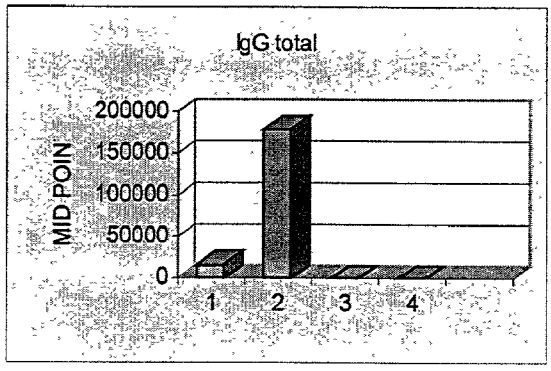


Figure 29A

Subclass-specific antibody response (exp 96533)

group 1: ProtD1/3 E7 HPV16
group 2: ProtD1/3 E7 HPV16+ SB 62 Qs21 & 3 D MPL
group 3: SB 62 Qs21 & 3 D MPL
group 4: PBS

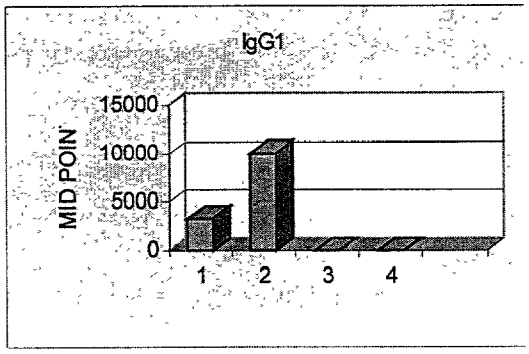


Figure 29B

Subclass-specific antibody response (exp 96533)

group 1: ProtD1/3 E7 HPV16
group 2: ProtD1/3 E7 HPV16+ SB 62 Qs21 & 3 D MPL
group 3: SB 62 Qs21 & 3 D MPL
group 4: PBS

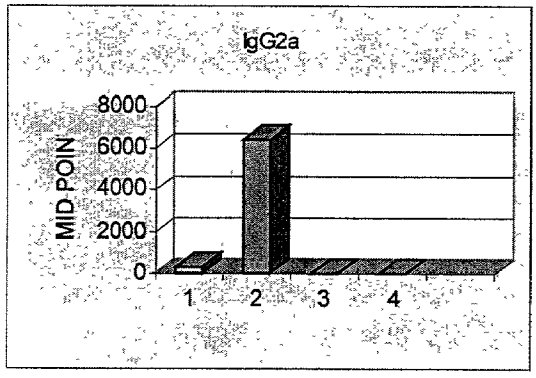


Figure 29C

Subclass-specific antibody response (exp 96533)

group 1: ProtD1/3 E7 HPV16

group 2: ProtD1/3 E7 HPV16+ SB 62 Qs21 & 3 D MPL

group 3: SB 62 Qs21 & 3 D MPL

group 4: PBS

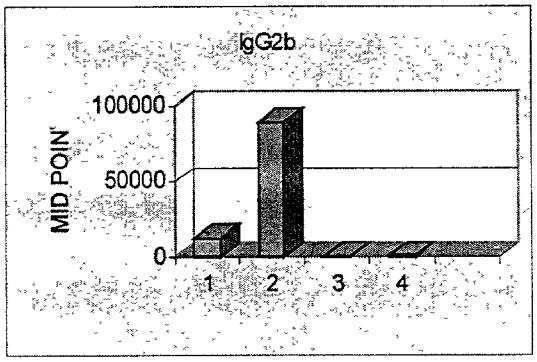


Figure 29D

Protective effect of vaccination with ProtD1/3 E7 HPV16 formulations against a TC1 tumor challenge (2 10e5 cells) (exp 96532)

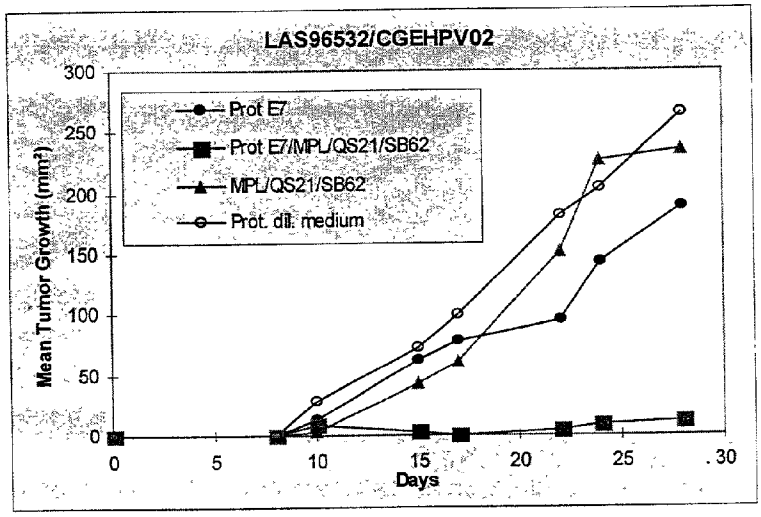


Figure 30

Lymphoproliferation on spleen cells (Stimulation index) (Exp. 96532)

72 Hrs *in vitro* re-stimulation with

A) ProtD1/3 E7 (1; 0.1 µg/ml)

B) ProtD1/3 E7 (0.1; 0.01 µg/ml) coated on latex µbeads

Group 1: ProtD1/3 E7 HPV16

Group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL

Group 3: SB 62 Qs21 & 3 D MPL

Group 4: PBS

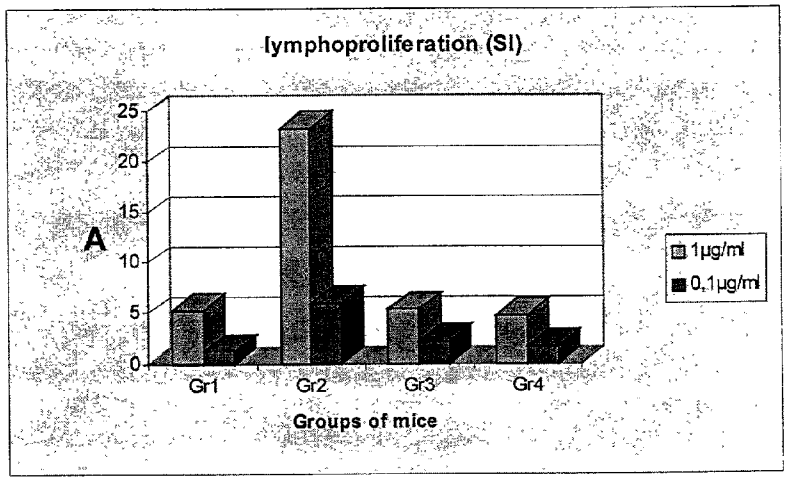


Figure 31A

Lymphoproliferation on spleen cells (Stimulation index) (Exp. 96532)

72 Hrs *in vitro* re-stimulation with

A) ProtD1/3 E7 (1; 0.1 μg/ml)

B) ProtD1/3 E7 (0.1; 0.01 μg/ml) coated on latex μbeads

Group 1: ProtD1/3 E7 HPV16

Group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL

Group 3: SB 62 Qs21 & 3 D MPL

Group 4: PBS

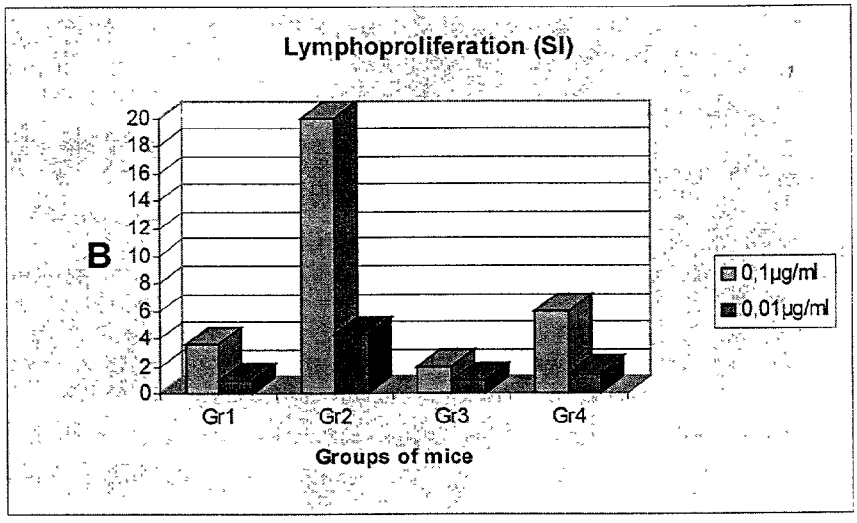
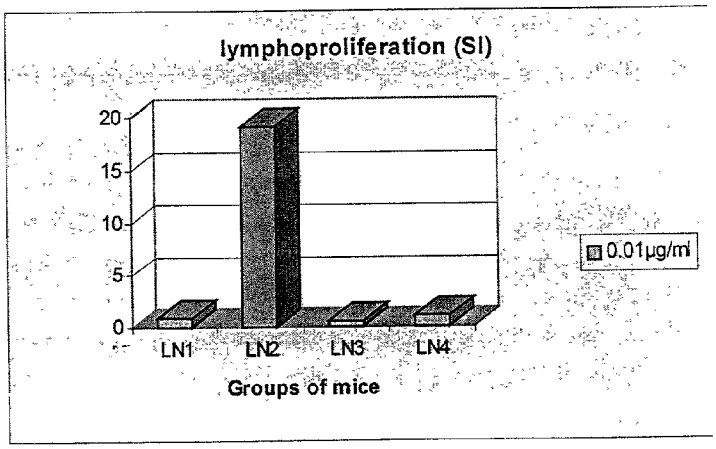


Figure 31B

Lymphoproliferation on lymph node cells
(Stimulation index) (Exp. 96532)
72 Hrs *in vitro* re-stimulation with

A) ProtD1/3 E7 (0.01 µg/ml)
B) ProtD1/3 E7 (0.01 µg/ml) coated on latex µbeads

Group 1: ProtD1/3 E7 HPV16
Group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL
Group 3: SB 62 Qs21 & 3 D MPL
Group 4: PBS



A

Figure 32 A

Lymphoproliferation on lymph node cells
(Stimulation index) (Exp. 96532)
72 Hrs *in vitro* re-stimulation with

A) ProtD1/3 E7 (0.01 µg/ml)
B) ProtD1/3 E7 (0.01 µg/ml) coated on latex µbeads

Group 1: ProtD1/3 E7 HPV16
Group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL
Group 3: SB 62 Qs21 & 3 D MPL
Group 4: PBS

B

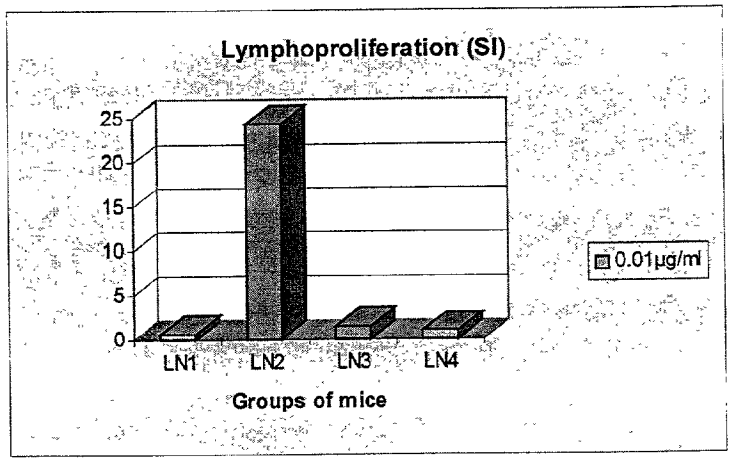


Figure 32B

Subclass-specific antibody response (exp 96532)

group 1: ProtD/3 E7 HPV16
group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL
group 3: SB 62 Qs21 & 3 D MPL
group 4: medium

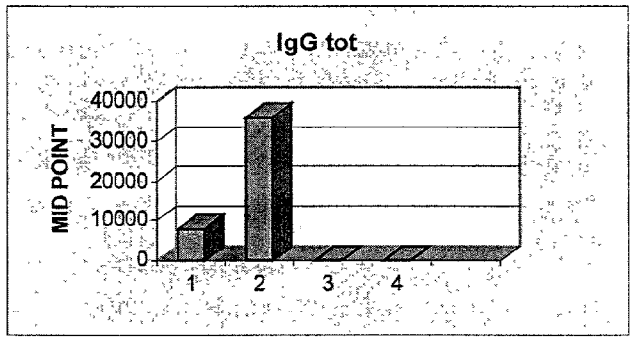


Figure 33A

Subclass-specific antibody response (exp 96532)

group 1: ProtD/3 E7 HPV16
group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL
group 3: SB 62 Qs21 & 3 D MPL
group 4: medium

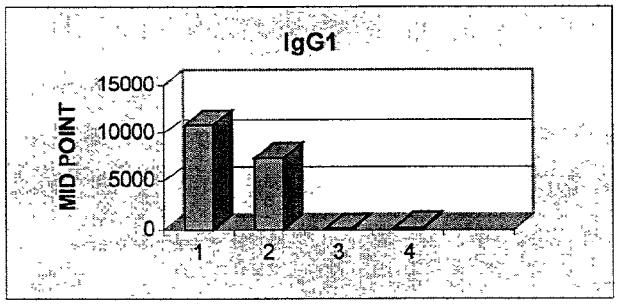


Figure 33B

Subclass-specific antibody response (exp 96532)

group 1: ProtD/3 E7 HPV16

group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL

group 3: SB 62 Qs21 & 3 D MPL

group 4: medium

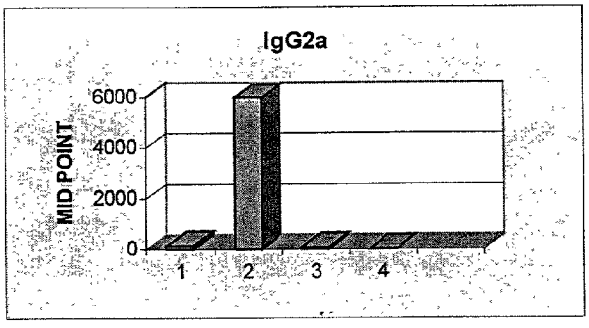


Figure 33C

Subclass-specific antibody response (exp 96532)

group 1: ProtD/3 E7 HPV16

group 2: ProtD1/3 E7 HPV16 + SB 62 Qs21 & 3 D MPL

group 3: SB 62 Qs21 & 3 D MPL

group 4: medium

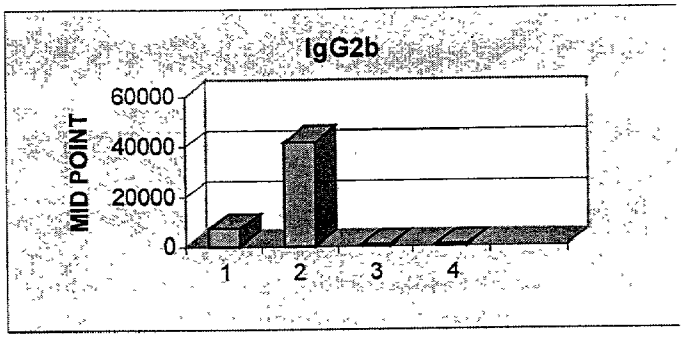


Figure 33D

Lymphoproliferation on spleen cells (stimulation index)

72HRs in vitro re-stimulation with PD1/3 18E7 (10, 1, 0.1, 0.01 µg/ml)

(Exp 98038)

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQS21 + 3D-MPL

Group 3: ProtD 1/3 18 E7 + QS21 + 3D-MPL + SB62 O/W

Group 4: ProtD 1/3 18 E7 + DQS21 alum

spleen Gr	1	2	3	4
18E7 10µg	6	27	23	20
18E7 1µg	5	23	25	23
18E7 0.1µg	5	21	21	23
18 E70.01µg	4	14	15	18
baseline/cpm	1168	1359	1025	1268

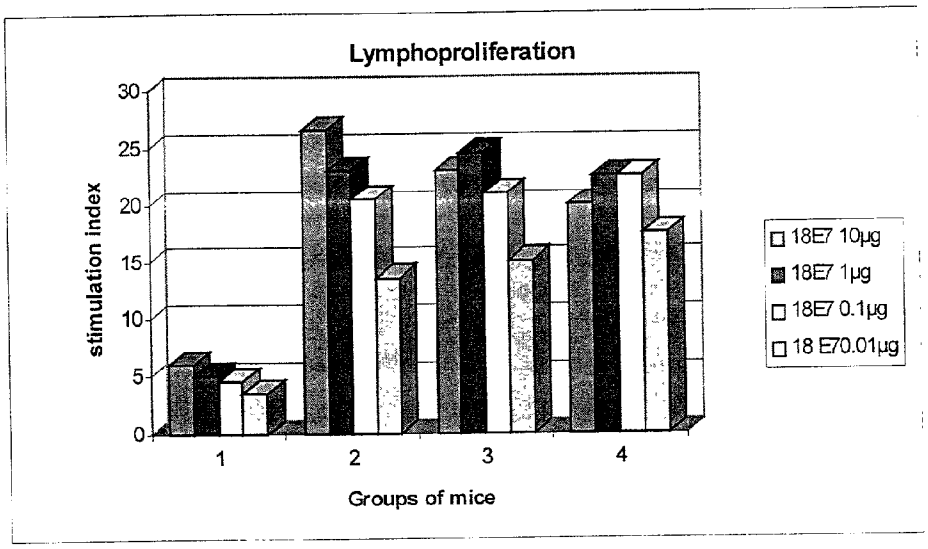


Figure 34

Lymphoproliferation on popliteal Lymph nodes

72HRs in vitro re-stimulation with PD1/3 18E7 (10, 1, 0.1, 0.01 μg/ml)

(Exp 98038)

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQS21 + 3D-MPL

Group 3: ProtD 1/3 18 E7 + QS21 + 3D-MPL + SB62 O/W

Group 4: ProtD 1/3 18 E7 + DQS21 alum

LN Group	1	2	3	4
18 E7 10	33	117	108	203
18E7 1	8	110	108	208
18 E7 0.1	4	95	95	196
18 E70.01	2	75	81	141
baseline	325	161	131	607

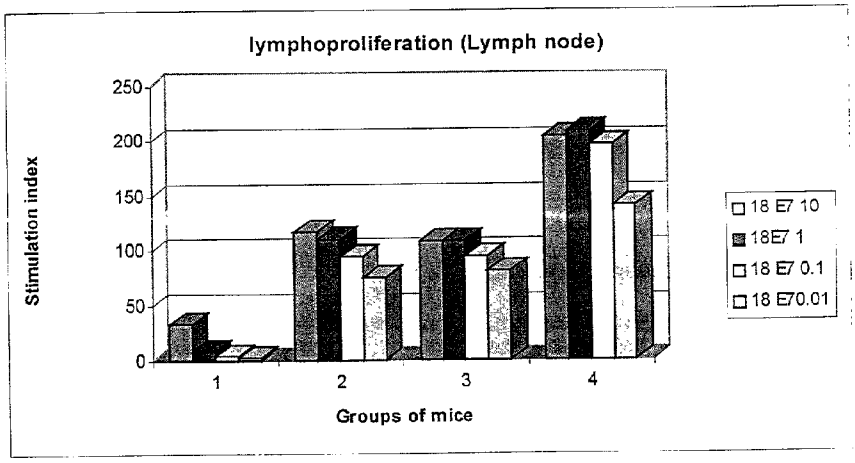


Figure 35

Cytokine production in the culture supernatant of spleen cells after 96 Hrs in vitro re-stimulation (ProtD1/3 18E7 1 , 3µg/ml)

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQ 3D-MPL

Group 3: ProtD 1/3 18 E7 + QS21, 3D-MPL, SB62 O/W

Group 4: ProtD 1/3 18 E7 + DQ, 3D-MPL alum

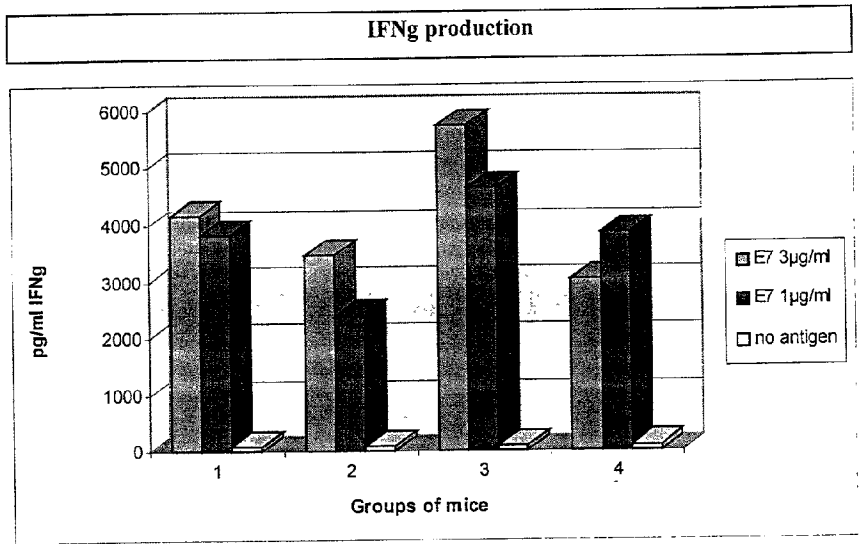


Figure 36A

Cytokine production in the culture supernatant of spleen cells after 96 Hrs in vitro re-stimulation (ProtD1/3 18E7 1 , 3µg/ml)

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQ 3D-MPL

Group 3: ProtD 1/3 18 E7 + QS21, 3D-MPL, SB62 O/W

Group 4: ProtD 1/3 18 E7 + DQ, 3D-MPL alum

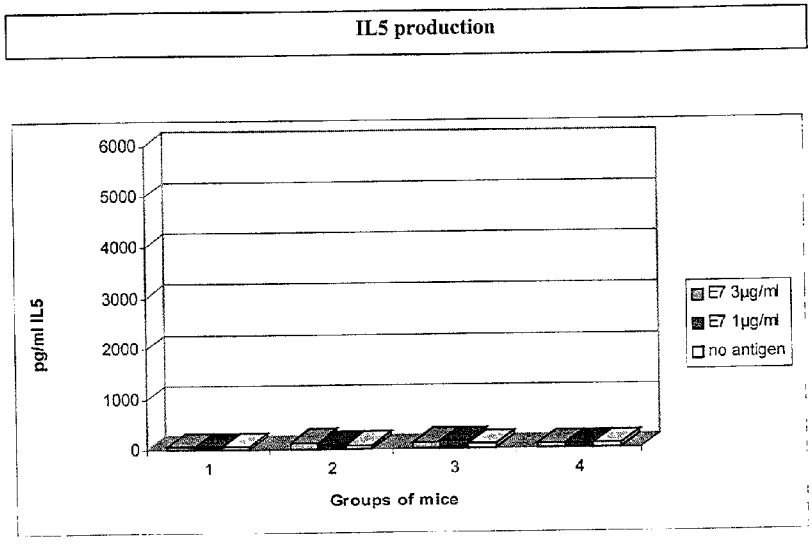


Figure 36B

Cytokine production in the culture supernatant of Lymph ode cells after 96 Hrs in vitro re-stimulation with ProtD1/3 18E7

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQS21 3DMPL +

Group 3: ProtD 1/3 18 E7 + SB62 QS21/3DMPL

Group 4: ProtD 1/3 18 E7 + DQ Alum

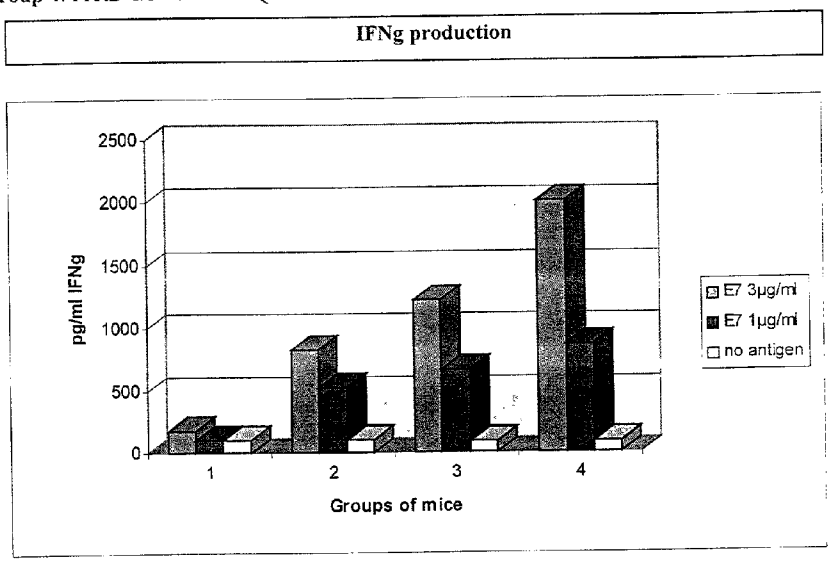


Figure 37A

Cytokine production in the culture supernatant of Lymph ode cells after 96 Hrs in vitro re-stimulation with ProtD1/3 18E7

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQS21 3DMPL +

Group 3: ProtD 1/3 18 E7 + SB62 QS21/3DMPL

Group 4: ProtD 1/3 18 E7 + DQ Alum

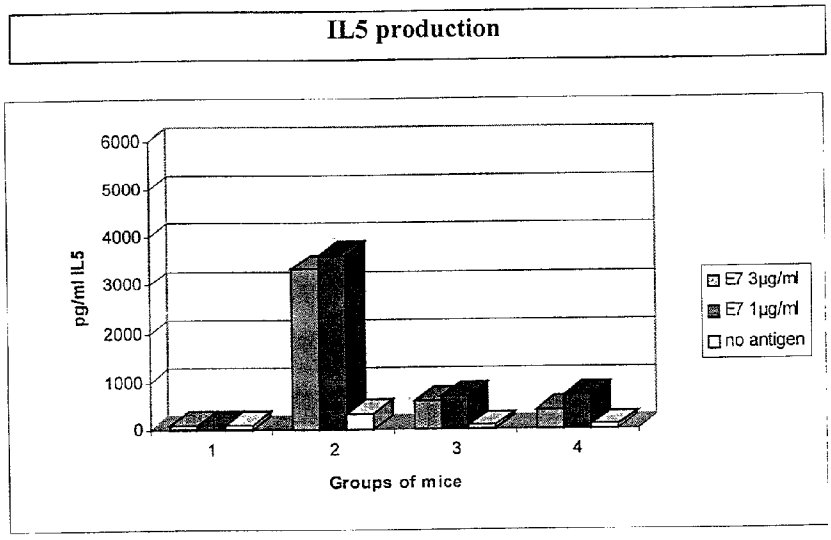


Figure 37B

Antibody response and Isotypic (exp 98038)

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQS21 3DMPL

Group 3: ProtD 1/3 18 E7 + SB62 QS21/3DMPL

Group 4: ProtD 1/3 18 E7 + MPL DQ alum

Groups	mid. Dil IgGt tot	IgG1 %	IgG2a %	IgG2b %
1	1500	46	32	22
2	84172	28	48	23
3	80545	43	44	13
4	213685	82	8	10

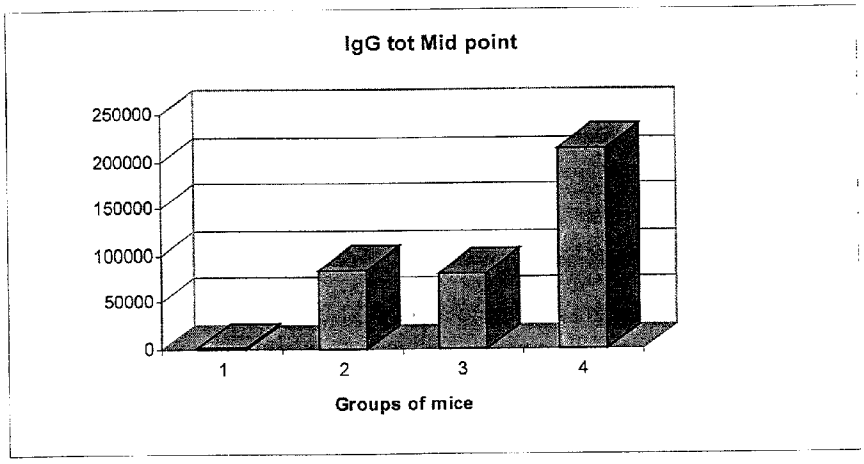


Figure 38A

Antibody response and Isotypic (exp 98038)

Group 1: ProtD 1/3 18 E7

Group 2: ProtD 1/3 18 E7 + DQS21 3DMPL

Group 3: ProtD 1/3 18 E7 + SB62 QS21/3DMPL

Group 4: ProtD 1/3 18 E7 + MPL DQ alum

Groups	mid. Dil IgGt tot	IgG1 %	IgG2a %	IgG2b %
1	1500	46	32	22
2	84172	28	48	23
3	80545	43	44	13
4	213685	82	8	10

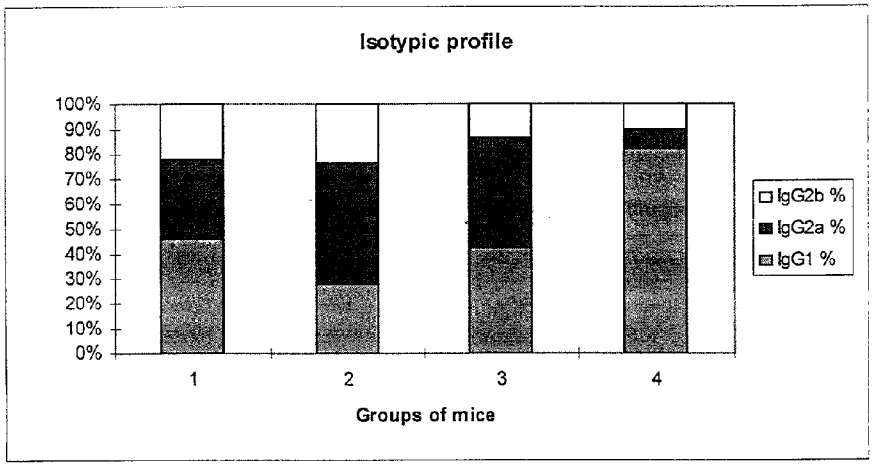


Figure 38B

RECOMBINANT PAPILLOMAVIRUS VACCINE AND METHOD FOR PRODUCTION AND TREATMENT

[0001] The present invention relates to fusions proteins, comprising a protein or part of a protein that provides T helper epitopes and an antigen from a human-papilloma virus that find utility in the treatment or prophylaxis of human papilloma induced tumours. In particular the invention relates to fusion proteins comprising an E6 or E7 protein from HPV strain 16 or 18 linked to protein D from Heamophilius influenza B.

[0002] Papillomaviruses are small naked DNA tumour viruses (7.9 kilobases, double strand), which are highly species-specific. Over 70 individual human papillomavirus (HPV) genotypes have been described. Papillomaviruses are classified on the basis of species of origin (human, bovine etc.) and of the degree of genetic relatedness with other papillomaviruses from the same species. HPVs are generally specific for the skin or mucosal surfaces and have been broadly classified into "low" and "high" risk on the basis of rare and common, respectively, detection in abnormal or tumour tissue. Low risk HPVs usually cause benign lesions (warts or papillomas) that persist for several months or years. High risk HPVs are associated with cancer. The strongest positive association between an HPV virus and human cancer is that which exist between HPV 16 and 18 and cervical carcinoma. More than ten other HPV types have also been found in cervical carcinomas including HPV 31 and HPV 33 although at less frequency.

[0003] Genital HPV infection in young sexually active women is common and most individuals either clear the infection, or if lesions develop, these regress. Only a subset of infected individuals has lesions which progress to high grade intraephithelial neoplasia and only a fraction of these progress further to invasive carcinoma.

[0004] The molecular events leading to HPV infection have not been clearly established. The lack of an adequate in vitro system to propagate human papillomaviruses has hampered the progress to a best information about the viral cycle.

[0005] Today, the different types of HPVs have been isolated and characterised with the help of cloning systems in bacteria and more recently by PCR amplification. The molecular organisation of the HPV genomes has been defined on a comparative basis with that of the well characterised bovine papillomavirus type 1 (BPV 1).

[0006] Although minor variations do occur, all HPVs genomes described have at least seven early genes, E1 to E7 and two late genes L1 and L2. In addition, an upstream regulatory region harbors the regulatory sequences which appears to control most transcriptional events of the HPV genome.

[0007] E1 and E2 genes are involved in viral replication and transcriptional control, respectively and tend to be disrupted by viral integration. E6 and E7 are involved in viral transformation. E5 has also been implicated in this process.

[0008] In the HPVs involved in cervical carcinoma such as HPV 16 and 18, the oncogenic process starts after integration of viral DNA. The integration results in the inactivation of genes coding for the capsid proteins L1 and L2 and loss of E2 repressor function leads to deregulation of the E6/E7 open reading frame installing continuously overexpression of the two early proteins E6 and E7 that will lead to gradually loss of the normal cellular differentiation and the development of the carcinoma. E6 and E7 overcome normal cell cycle by inactivating major tumor suppressor proteins, p53 and pRB, the retinoblastoma gene product, respectively.

[0009] Carcinoma of the cervix is common in women and develops through a pre-cancerous intermediate stage to the invasive carcinoma which frequently leads to death. The intermediate stages of the disease is known as cervical intraepithelial neoplasia and is graded I to III in terms of increasing severity (CIN I-III).

[0010] Clinically, HPV infection of the female anogenital tract manifests as cervical flat condylomas, the hallmark of which is the koilocytosis affecting predominantly the superficial and intermediate cells of the cervical squamous epithelium.

[0011] Koilocytes which are the consequence of a cytopathic effect of the virus, appear as multinucleated cells with a perinuclear clear haloe. The epithelium is thickened with abnormal keratinisation responsible for the warty appearance of the lesion.

[0012] Such flat condylomas when positive for the HPV 16 or 18 serotypes, are high-risk factors for the evolution toward cervical intraepithelial neoplasia (CIN) and carcinoma in situ (CIS) which are themselves regarded as precursor lesions of invasive cervix carcinoma.

[0013] The natural history of oncogenic HPV infection presents 3 consecutive phases, namely:

[0014] (1) a latent infection phase,

[0015] (2) a phase of intranuclear viral replication with product of complete virions, which corresponds to the occurrence of koilocytes. At this stage, the HPV is producing its full range of proteins including E2, E5, E6, E7, L1 and L2.

[0016] (3) a phase of viral integration into the cellular genome, which triggers the onset of malignant transformation, and corresponds to CIN II and CIN III/CIS with progressive disappearance of koilocytes. At this stage, the expression of E2 is down-regulated, the expression of E6 and E7 is enhanced. Between CIN II/III and CIN III/Cervix carcinoma the viral DNA changes from being episomal in the basal cells to integration of E6 and E7 genes only (tumoral cells). 85% of all cervix carcinomas are squamos cell carcinomas most predominantly related to the HPV16 serotype. 10% and 5% are adenocarcinomas and adenosquamos cell carcinomas respectively, and both types are predominantly related to HPV 18 serotype. Nevertheless other oncogenic HPV's exist.

[0017] International Patent Application No. WO 96/19496 discloses variants of human papilloma virus E6 and E7 proteins, particularly fusion proteins of E6/E7 with a deletion in both the E6 and E7 proteins. These deletion fusion proteins are said to be immunogenic.

[0018] The present invention provides compositions comprising either an E6 or E7 or an E6/E7 fusion protein linked to an immunological fusion partner having T cell epitopes.

[0019] In a preferred form of the invention, the immunological fusion partner is derived from protein D of Heamophilus influenza B. Preferably the protein D derivative comprises approximately the first ⅓ of the protein, in particular approximately the first N-terminal 100-110 amino acids. The protein D may be lipidated (Lipo Protein D). Other immunological fusion partners include the non-structural protein from influenzae virus, NS1 (hemagglutinin). Typically the N terminal 81 amino acids are utilised, although different fragments may be used provided they include T-helper epitopes.

[0020] In another embodiment the immunological fusion partner is the protein known as LYTA. Preferably the C terminal portion of the molecule is used. Lyta is derived from *Streptococcus pneumoniae* which synthesize an N-acetyl-L-alanine amidase, amidase LYTA, (coded by the lytA gen {Gene, 43 (1986) page 265-272} an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E.coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at its amino terminus has been described {Biotechnology: 10, (1992) page 795-798}. As used herein a preferred embodiment utilises the repeat portion of the Lyta molecule found in the C terminal end starting at residue 178. A particularly preferred form incorporates residues 188-305.

[0021] Accordingly, the present invention in preferred embodiment provides fusion proteins comprising Protein D-E6 from HPV 16, Protein D-E7 from HPV 16 Protein D-E7 from HPV 18, Protein D-E6 from HPV 18, and Protein D E6 E7 from both HPV 16 and 18. The protein D part preferably comprises the first ⅓ of protein D. It will be appreciated that other E6 and E7 proteins may be utilised from other HPV subtypes.

[0022] The proteins of the present invention preferably are expressed in *E. coli*. In a preferred embodiment the proteins are expressed with a Histidine tail comprising between 5 to 9 and preferably six Histidine residues. These are advantageous in aiding purification.

[0023] The protein E7 may in a preferred embodiment carry a mutation to reduce the binding for the rb site (retinoblastoma gene product) and hence eliminate any potential transforming capacity. Preferred mutations for HPV 16 E7 involve replacing Cys_{24} with Glycine, or Glutamic $acid_{26}$ with Glutamine. In a preferred embodiment the E7 protein contains both these mutations.

[0024] Preferred mutations for the HPV 18 E_7 involve replacing Cys_{27} with Glycine and/or Glutamic $acid_{26}$ with Glutamine. Again preferably both mutations are present.

[0025] Single or double mutations may also be introduced p53 region of E_6 to eliminate any potential transforming ability.

[0026] In a further embodiment of the invention there is provided and E6 E7 fusion protein from HPV linked to an immunological fusion partner. A preferred Immunological fusion partner is Protein D, more preferable the first ⅓ of protein D.

[0027] The present invention also provides a DNA encoding the proteins of the present invention. Such sequences can be inserted into a suitable expression vector and expressed in a suitable host.

[0028] A DNA sequence encoding the proteins of the present invention can be synthesized using standard DNA synthesis techniques, such as by enzymatic ligation as described by D. M. Roberts et al. in Biochemistry 1985, 24, 5090-5098, by chemical synthesis, by in vitro enzymatic polymerization, or by PCR technology utilising for example a heat stable polymerase, or by a combination of these techniques.

[0029] Enzymatic polymerisation of DNA may be carried out in vitro using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37° C., generally in a volume of 50 μl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M $MgCl_2$, 0.01M dithiothreitol, 1 mM spermidine, 1 mM ATP and 0.1 mg/ml bovine serum albumin, at a temperature of 4° C. to ambient, generally in a volume of 50 ml or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments—A Laboratory Manual' (ed. H. G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M. J. Gait, H. W. D. Matthes, M. Singh, B. S. Sproat, and R. C. Titmas, Nucleic Acids Research, 1982, 10, 6243; B. S. Sproat, and W. Bannwarth, Tetrahedron Letters, 1983, 24, 5771; M. D. Matteucci and M. H. Caruthers, Tetrahedron Letters, 1980, 21, 719; M. D. Matteucci and M. H. Caruthers, Journal of the American Chemical Society, 1981, 103, 3185; S. P. Adams et al., Journal of the American Chemical Society, 1983, 105, 661; N. D. Sinha, J. Biernat, J. McMannus, and H. Koester, Nucleic Acids Research, 1984, 12, 4539; and H. W. D. Matthes et al., EMBO Journal, 1984, 3, 801.

[0030] The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis et al., Molecular Cloning—A Laboratory Manual; Cold Spring Harbor, 1982-1989.

[0031] In particular, the process may comprise the steps of:

[0032] i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the protein or an immunogenic derivative thereof;

[0033] ii) transforming a host cell with said vector;

[0034] iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and

[0035] iv) recovering said protein.

[0036] The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell. This can be achieved for example by transformation, transfection or

infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S. M. Kingsman and A. J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

[0037] Preferably recombinant antigen of the invention are expressed in *E. coli*. The expression strategy include fusion of E7, E6 or E6/E7 fusion to the 1/3-N-terminal portion of protein D from Haemophilus influenzae B, an immunological fusion partner providing T cell helper epitopes. An affinity polyhistidine tail is engineered at the carboxy terminus of the fusion protein allowing for simplified purification. Such recombinant antigen is overexpressed in *E. coli* as insoluble protein.

[0038] Preferably the proteins of the invention are coexpressed with thioredoxin in trans (TIT). Coexpression of thioredoxin in trans versus in cis is preferred to keep antigen free of thioredoxin without the need for protease. Thioredoxin coexpression eases the solubilisation of the proteins of the invention. Thioredoxin coexpression has also a significant impact on protein purification yield, on purified-protein solubility and quality.

[0039] The expression vectors are novel and also form part of the invention.

[0040] The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions.

[0041] Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

[0042] The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic but preferably is *E. coli*. Suitable vectors include plasmids, bacteriophages, cosmids and recombinant viruses.

[0043] The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis et al. cited above.

[0044] The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis et al. cited above, or "DNA Cloning" Vol. II, D. M. Glover ed., IRL Press Ltd, 1985.

[0045] The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E. coli* may be treated with a solution of $CaCl_2$ (Cohen et al., Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl, $MnCl_2$, potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid, RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

[0046] Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis et al. and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50° C.

[0047] The product is recovered by conventional methods according to the host cell. Thus, where the host cell is bacterial, such as *E. coli* it may be lysed physically, chemically or enzymatically and the protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

[0048] When the proteins of the present invention are expressed with a hisitidine tail (His tag). The proteins can easily be purified by affinity chromatography using an ion metal affinity chromatography column (IMAC) column.

[0049] A second chromatographic step, such as Q-sepharose may be utilised either before or after the IMAC column to yield highly purified protein. If the immunological fusion partner is C-LYTA, then it is possible to exploit the affinity of CLYTA for choline and/or DEAE to purify this product. Products containing both C-LYTA and his tags can be easily and efficiently purified in a two step process involving differential affinity chromatography. One step involves the affinity of the His tag to IMAC columns, the other involves the affinity of the C-terminal domain of LYTA for choline or DEAE.

[0050] Proteins comprising both a C-LYTA and Hisitidine tag are new and accordingly form one aspect of the invention. These may be purified to high levels (greater than 80% preferably greater than 90%) by a simple two step differential affinity procedure.

[0051] The proteins of the present invention are provided preferably at least 80% pure more preferably 90% pure as visualized by SDS PAGE. The protein present a major single band when analysed by SDS PAGE under reducing conditions, and western blot analysis show less than 5% host cell protein contamination.

[0052] The present invention also provides pharmaceutical composition comprising a protein of the present invention in a pharmaceutically acceptable excipient. A preferred vaccine composition comprises at least Protein D-E6 from HPV 16 or derivative thereof together with Protein D-E7 from HPV 16. Alternatively the E6 and E7 may be presented in a single molecule, preferably a Protein D E6/E7 fusion. Such vaccine may optionally contain either or both E6 and E7 proteins from HPV 18, preferably in the form of a Protein D-E6 or Protein D-E7 fusion protein or Protein D E6/E7 fusion protein. The vaccines of the present invention may contain other HPV antigens from HPV 16 or 18. In particular, the vaccine may contain L1 or L2 antigen monomers. Alternatively such L1 or L2 antigens may be presented together as a virus like particle or the L1 alone protein may be presented as virus like particle or caposmer structure.

Such antigens, virus like particles and capsomer are per se known. See for example WO94/00152, WO94/20137, WO94/05792, & WO93/02184. Additional early proteins may be included such as E2 or preferably E5 for example The vaccine of the present invention may additionally comprise antigens from other HPV strains, preferably from strains HPV 6 11, HPV 31 or 33.

[0053] Vaccine preparation is generally described in Vaccine Design—The subunit and adjuvant approach (Ed. Powell and Newman) Pharmaceutical Biotechnology Vol. 6 Plenum Press 1995. Encapsulation within liposomes is described by Fullerton, U.S. Pat. No. 4,235,877.

[0054] The proteins of the present invention are preferably adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

[0055] In the formulation of the inventions it is preferred that the adjuvant composition induces a preferential TH 1 response. Suitable adjuvant systems include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt.

[0056] An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739.

[0057] A particularly potent adjuvant formulation involving QS21, 3D-MPL & tocopherol in an oil in water emulsion is described in WO 95/17210 and is a preferred formulation.

[0058] Accordingly in one embodiment of the present invention there is provided a vaccine comprising a protein D (or derivative thereof)—E6 or protein D (or derivative thereof)—E7 adjuvanted with a monophosphoryl lipid A or derivative thereof.

[0059] Preferably the vaccine additionally comprises a saponin, more preferably QS21.

[0060] Preferably the formulation additional comprises an oil in water emulsion and tocopherol. The present invention also provides a method for producing a vaccine formulation comprising mixing a protein of the present invention together with a pharmaceutically acceptable excipient, such as 3D-MPL.

[0061] The invention will be further described by reference to the following examples:

EXAMPLE I

Construction of an *E. coli* Strain Expressing Fusion Protein-D1/3-E7-His (HPV16)

[0062] 1)—Construction of Expression Plasmid

[0063] a)—Plasmid pMG MCS prot D1/3 (=pRIT14589) is a derivative of pMG81 (described in UK patent application n° 951 3261.9 published as WO97/01640) in which the codons 4-81 of NS 1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thrl27 of mature protein D of *Haemophilus Influenzae* strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E7-His.

[0064] b)—HPV genomic E6 and E7 sequences type HPV 16 (See Dorf et al., Virology 1985, 145, p. 181-185) were amplified from HPV 16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses—D 69120—Heidelberg) and were subcloned into pUC19 to give TCA 301 (=pRIT14462).

[0065] Construction of Plasmid TCA 308 (=pRIT14501): a Plasmid Expressing the Fusion Protein-D1/3-E7-His

[0066] The nucleotides sequences corresponding to amino acids 1→98 of E7 protein are amplified from pRIT14462. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E7 sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA308 (=pRIT14501). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The sequence for the fusion protein-D1/3-E7-His (HPV 16) is described in **FIG. 1**.

[0067] 2)—Transformation of AR58 Strain

[0068] Plasmid pRIT14501 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

[0069] 3)—Growth and Induction of Bacterial Strain—Expression of Prot -D1/3-E7-His

[0070] Cells of AR58 transformed with plasmid pRIT14501 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein D1/3-E7-His. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20° C.

EXAMPLE II

Characterisation of fusion Protein D1/3-E7-His (HPV 16)

[0071] Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4° C.

[0072] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting. A major band of about 33 kDa, localised in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phos-

phatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 5% of total protein as shown on a Coomassie-stained SDS-polyacrylamide gel.

EXAMPLE III

Protein -D1/3-E7-His (HPV 16) Purification

[0073] One liter culture of bacteria expressing protein-D1/3-E7-His, is centrifuged at 11,300 g for 30 min at 4° C. and cell pellet is kept at −80° C. until further treatment. After resuspension in 75 ml PBS buffer, *E. coli* cells are broken in a French pressure cell press (SLM Aminco®) at 20,000 psi. Lysed cells are pelleted by centrifugation at 17,000 g for 30 minutes. Pellet, containing the protein-D1/3-E7-His, is washed once in 30 ml of 2M NaCI, 50 mM Phosphate pH 7.5, then twice in 30 ml 50 mM Phosphate pH 7.5. Proteins are solubilised after 2 hours incubation of the pellet in 30 ml of 8 M urea, 50 mM phosphate pH 7.5 at RT. Cells debris are eliminated by 15 min centrifugation at 17,000 g, 4° C. Protein purification is carried out at RT°, 15 ml of solubilised protein are applied onto a 5 ml Ni2+NTA (Qiagen) resin (Pharmacia column XK 16/20) preequilibrated in 8M urea, 50 mM phosphate pH 7.5 at a flow rate of 0.2 ml/min. The column is washed in the same buffer until the absorbance at 280 nm reaches the base line. The protein is eluted with a 0-600 mM Imidazole gradient in 8M urea, 50 mM phosphate pH 7.5. The flow rate of these two last steps is brought to 1 ml/min. Eluted fractions are analysed by SDS polyacrylamide gel electrophoresis and by Western blotting. ProtD1/3-E7-His, visualised by Coomassie blue staining, by a polyclonal anti protein D or by a monoclonal anti E7 antibody, appears as a major single band at about 32 kDalton and is estimated as a 95% pure protein. No *E. coli* contaminants, traced with a polyclonal anti *E. coli* proteins antibody, are observed.

[0074] In order to eliminate urea, 9 ml of purified antigen, at 1.33 mg/ml (Bradford), is dialysed against 3 liters of PBS buffer overnight at RT° followed by a 4 hours dialysis against a fresh PBS buffer. 80% of urea free protein is recovered as soluble protein. To eliminate contaminating endotoxins, 6 ml of dialysed protein are incubated with 1 ml of Affiprep polymixin gel (Biorad), for 3 hours at 4° C. under gentle stirring. A second incubation with 500 μl of Affiprep polymixin resin is performed to minimise the endotoxin level to 8.8 EU/μg protein. After sterile filtration on a 0.22 μm filter device (Millex 0.22 GV, Millipore), prot-D1/3-E7-His at 0.665 mg/ml is assayed for stability. SDS PAGE analysis showed no evolution of the protein after 7 days incubation at −20° C., 4° C., RT° or **37°** C.

EXAMPLE IV

Construction of an *E.coli* strain expressing fusion Protein-D1/3-E6-his/HPV16

[0075] 1. Construction of Expression Plasmid

[0076] a) Plasmid pMG MCS prot D1/3 (=pRIT14589) is a derivative of pMG81 (described in WO97/01640 in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6-his.

[0077] b) HPV genomic E6 and E7 sequences type HPV 16 (Seedorf et al., Virology 1985, 145, p.181-185) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses—

[0078] c) D 69120—Heidelberg) and were subcloned into pUC19 to give TCA 301 (=pRIT14462).

[0079] Construction of Plasmid TCA 307 (=pRIT14497): a Plasmid Expressing the Fusion Protein-D1/3-E6-His/HPV16

[0080] The nucleotides sequences corresponding to amino acid.

[0081] 1→151 of E6 protein were amplified from pRIT 14462. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6 sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA307 (=pRIT14497) (see **FIG. 2**). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The coding sequence for the fusion protein-D1/3-E6-His is described in **FIG. 3**.

[0082] 2. Transformation of AR58 Strain

[0083] Plasmid pRIT14497 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

[0084] 3. Growth and Induction of Bacterial Strain—Expression of Prot-D1/3-E6-His

[0085] Cells of AR58 transformed with plasmid pRIT14497 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6-his. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20C.

[0086] 4. Characterization of Fusion Protein D1/3-E6-his (HPV 16) Preparation of Extracts

[0087] Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4° C.

[0088] Analysis on Coomassie-Stained SDS-Polyacrylamide Gels and Western Blots

[0089] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

[0090] A major band of about 32 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which

detects accessible histidine tail. The level of expression represents about 5% of total protein.

[0091] 5. Coexpression with Thioredoxin

[0092] In an analagons fashion to the expression of prot D 1/3 E7 His from HPV 18 (example XIII) an *E.coli* strain AR58 was transformed with a plasmid encoding thioredoxin and protein D 1/3 E7 His (HPV 16).

EXAMPLE V

Purification of Prot D 1/3 E6 His (HPV 16)

[0093] HPV-16 ProtD1/3 E6 recombinant antigen was expressed in *E. coli* (AR58). Expression strategy included fusion of E6 to the 1/3-N-terminal portion of protein D from Haemophilus influenzae, an immunological fusion partner providing T cell helper epitopes. An affinity polyhistidine tail was engineered at the carboxy terminus of the fusion protein. The recombinant antigen was overexpressed in *E. coli* as insoluble proteins.

[0094] Solubilisation of the antigen required denaturing agents. In absence of denaturing agent, ProtD1/3-E6-His precipitated at neutral pH. To circumvent the solubility problems, co-expression of these proteins with Thioredoxin in Trans (TIT), a folding partner was carried out.

[0095] Bacterial expressions are conducted in LB media in presence of 0.05 mg/ml of kanamycin at 30° C. plus 0.2 mg/ml of Ampicillin when Thioredoxin is coexpressed. Recombinant protein expression is thermally induced by transferring the cells to 42° C., when cell optical density (OD 600 nm) of 0.4 is reached. Protein expression is maintained for 4 hours. Purification was carried out according to the following protocol.

Cell Culture Pellet	60 OD_{600} 1 mM pefabloc, 2 M NaCl, PBS pH 7.4 (Buffer A)
French Press Disruptor	Three passes 20,000 psi
Centrifugation	17,000 g 30 min, 4° C.
Pellet Washes	2 M NaCl, PBS pH 7.4 (Buffer B) x1 PBS pH 7.4 (Buffer C) x2
Centrifugation	17,000 g 30 min, 4° C.
Pellet Solubilisation	6 M Guanidine Chloride, 20 mM PO4, pH 7.0 (Buffer D) Overnight at 4° C.
Centrifugation	17,000 g 30 min, 4° C.
Supernatant on IMAC	Equilibration: 6 M Guanidine Chloride, 20 mM PO4, pH 7.0 (Buffer D) Elution: Imidazole steps (0.025 M, 0.1 M, 0.5 M) in 8 M Urea, 20 mM PO4, pH 7.0
Affiprep Polymyxin	8 M Urea, 20 mM PO4, pH 7.0 (Buffer E) 2 h RT°
Dialysis	4 M Urea, 0.5 M Arginine, 150 mM NaCl, 10 mM PO4, pH 6.8 (Buffer I) 2 M Urea, 0.5 M Arginine, 150 mM NaCl, 10 mM PO4 pH 6.8, (Buffer J) 0 M Urea, 0.5 M Arginine, 150 mM NaCl, 10 mM PO4 pH 6.8 (Buffer K)

[0096] Cells are efficiently broken by high-pressure homogenisation using a French pressure cell device. Antigen is extracted with high concentration of protein denaturant. This first step breaks open the bacterial cell wall and antigen is extracted from the bacterial insoluble fraction. The following purification was carried out on 4 liter culture.

[0097] Buffers

[0098] A. PBS/2M NaCl/1 mM Pefabloc

[0099] B. PBS/2MNaCl

[0100] C. PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM NaH2PO4, 1.47 mM KH2PO4 pH 7.4.

[0101] D. 6 M Guanidium Chloride, 20 mM PO4 (NaH2PO4 (2H2O)/K2HPO4 (3H2O)) pH 7.0

[0102] Starting material is 10 flasks of 400 ml culture each.

[0103] Cell paste is suspended to 60 OD_{600} in Buffer A (240 ml of Buffer A in this case), prior cell lysis by three passes through a French press disrupter (20,000 psi). Lysed cells are pelleted 30 min at 15,000 g at 4° C. Bacterial cell pellet containing the recombinant protein is washed once in 240 ml Buffer B, then twice in 240 ml Buffer C.

[0104] Prot D E6-His (TIT) is solubilised by 240 ml Buffer D overnight at 4° C. on a rotating wheel. Cell debris are pelleted 30 min at 15,000 g at 4° C. Supernatant (230 ml) is stored at −20° C. The material is then subjected to IMAC chromatography.

[0105] The chelating ligand NTA (nitrilo-tri-acetic-acid) is attached to an Agarose support (Qiagen). NTA ligand is charged with nickel metal ion with which it interacts through 4 of the 6 coordination sites of the nickel. The two remaining coordination sites of nickel interact strongly with histidine residues of the 6× His-tagged protein. Elution is achieved by competition with Imidazole which bind to the Ni-NTA and displace the tagged antigen.

[0106] Ni-NTA Agarose Qiagen (catalogue number: 30 250) was used.

[0107] Solutions

D:	6 M Guanidium Chloride, 20 mM PO4 (NaH_2PO4 ($2H_2O$)/K_2HPO4 ($3H_2O$)), pH 7.0
E:	8 M Urea, 20 mM PO4 (NaH_2PO4 ($2H_2O$)/K_2HPO4 ($3H_2O$)), pH 7.0
F:	E + 0.025 M Imidazole
G:	E + 0.1 M Imidazole
H:	E + 0.5 M Imidazole

[0108] 0.5 M NaOH

[0109] Deionized water

[0110] 0.02% NaN_3

[0111] Purification

[0112] a) The resin (15 ml resin/230 ml sample) is packed and equilibrated in 10 column volumes (C.V.) of Buffer D at 15 cm h^{-1}.

[0113] b) Supernatant from solubilised fraction is injected onto the column at 15 cm h^{-1}.

[0114] c) Column is washed at 15 cm h^{-1} with buffer D until OD 280 nm returns to the baseline.

[0115] d) Column is washed with 2 CV of Buffer E at 15 cm h^{-1}. The wash fraction is recovered.

[0116] e) Column is first eluted with 5 CV of Buffer F. Elimination of 25 kD major contaminant.

[0117] f) Column is then eluted with 2 CV of Buffer G.

[0118] g) Column is finally eluted with 3 CV of Buffer H. Elution of the antigen.

[0119] Antigen positive fractions are pooled (30 ml).

[0120] Endotoxin is removed by affiprep chromatography.

[0121] Affi-Prep® Polymyxin support consists of USP Grade Polymyxin B coupled to the Affi-Prep® Matrix. Due to its high affinity to the lipid A moiety of endotoxins, polymixin B binds endotoxin molecules with high capacity and selectivity.

[0122] Solutions

[0123] E: 8M Urea, 20 mM PO4 (NaH_2PO4 ($2H_2O$)/ K_2HPO4 ($3H_2O$)), pH 7.0 (apyrogenic).

[0124] 0.5 M Na OH

[0125] Deionized apyrogenic water

[0126] Procedure

[0127] 1) Affi-Prep® Polymyxin resin is washed in 10 volumes of 0.1 M NaOH, followed by 10 volumes of pyrogen free water.

[0128] 2) Resin is equilibrated in 10 volumes of Buffer E.

[0129] 3) 15 ml (half-pool) of IMAC-eluted sample is incubated with 3 ml of Affi-Prep® Polymyxin resin in a batch mode.

[0130] 4) Incubation is pursued 4 hours at Room Temperature or O/N at 4° C. on a rotating wheel.

[0131] 5) Sample is centrifuged 10 min at 2000 g (Beckman GS-6R).

[0132] 6) Supernatant containing the antigen is collected and submitted to endotoxins and protein assays.

[0133] 7) Resin is discarded.

[0134] Small molecules diffuse through a semi-permeable membrane while large molecules are retained. The process of dialysis is driven by the difference in concentration of the solutes on the two sides of the membrane. New buffer solution is introduced until buffer composition on each side equalises.

[0135] Buffers

I: 4 M Urea, 0.5 M Arginine, 0.15 M NaCl, 10 mM PO4 (NaH_2PO4 ($2H_2O$)/K_2HPO4 ($3H_2O$)) pH 6.8
J: 2 M Urea, 0.5 M Arginine, 0.15 M NaCl, 10 mM PO4 (NaH_2PO4 ($2H_2O$)/K_2HPO4 ($3H_2O$)) pH 6.8
K: 0 M Urea, 0.5 M Arginine, 0.15 M NaCl, 10 mM PO4 (NaH_2PO4 ($2H_2O$)/K_2HPO4 ($3H_2O$)) pH 6.8

[0136] 1) The Sample (15 ml) is introduced into a dialysis tubing (20.4 mm diameter and 6 cm height).

[0137] 2) Dialysis tubing is placed in a 2 liters cylinder containing Buffer I under stirring at 4° C. for 2 hours.

[0138] 3) Dialysis tubing is placed in a 2 liters cylinder (under stirring) containing Buffer J; at 4° C. for 2 hours.

[0139] 4) Dialysis tubing is placed in a 2 liters cylinder containing Buffer K (under stirring) at 4° C. O/N. Buffer is changed and dialysis is pursued 2 more hours at 4° C.

[0140] Millipore Sterile Millex-GV 0.22μ, 13 mm. Catalogue number: SLGV0130S.

[0141] All steps are performed at room temperature (RT≅22° C.), the antigen appears stable.

[0142] Antigen solution is filtered through a 0.2 μm filter to prevent any bacterial growth. Antigen is kept at −20° C. in Nunc vials.

[0143] Characterisation:

[0144] Protein D1/3 E6 His is characterised as follows:

[0145] ProteinD1/3-E6-His is a 273 amino acids long peptide with 112 amino acids coming from Protein D part. ProteinD1/3-E6-His has a theoretical Molecular Weight of 32 kD and migrates on SDS-PAGE as a 33 kD protein. ProteinD1/3-E6-His theoretical isoelectric point is 8.17.

[0146] The viral Protein E6 is a basic protein containing 14 cystein residues, eight of them (Cys 30,33,63,66 and Cys 103,106,136,139) are involved in two C-terminal zinc binding motifs.

[0147] Protein D 1/3-E6-His is expressed as insoluble protein, in *E. coli*-AR 58 strain, with Thioredoxin in Trans, a folding partner. Cell culture is produced in 400 ml flask.

[0148] 5.4 mg of 95% pure protein is obtained per liter of culture.

EXAMPLE VI

Construction of an *E. coli* Strain Expressing Fusion Protein-D1/3-E6E7-his/HPV16

[0149] 1. Construction of Expression Plasmid

[0150] a) Plasmid pMG MCS prot D1/3 (=pRIT14589) is a derivative of pMG81 (described Supra) in which the codons 4-81 of NS 1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thr 127 of mature protein D of *Haemophilus Influenzae* strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6E7-his.

[0151] b) HPV genomic E6 and E7 sequences type HPV16 (Seedorf et al., Virology 1985, 145, p.181-185) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses—D 69120—Heidelberg) and were subcloned into pUC19 to give TCA 301 (=pRIT14462).

[0152] c) The coding sequences for E6 and E7 in TCA301 (=pRIT 14462) were modified with a synthetic oligonucleotides adaptor (inserted between Afl III and Nsi I sites) introducing a deletion of 5 nucleotides between E6 and E7 genes to remove the stop codon of E6 and create fused E6 and E7 coding sequences in the plasmid TCA309(=pRIT 14556) see **FIG. 4**.

[0153] Construction of Plasmid TCA 311(=pRIT14512): a Plasmid Expressing the Fusion Protein-D1/3-E6E7-His/HPV16

[0154] The nucleotides sequences corresponding to amino acids 1→249 of fused E6E7 protein were amplified from pRIT14556. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6E7 fused sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA311 (=pRIT14512) (see **FIG. 5**). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The coding sequence for the fusion protein-D1/3-His is described **FIG. 6**.

[0155] 2. Transformation of AR58 Strain

[0156] Plasmid pRIT14512 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

[0157] 3. Growth and Induction of Bacterial Strain—Expression of Prot-D1/3-E6E7-His

[0158] Cells of AR58 transformed with plasmid pRIT14512 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6E7-his. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20C.

[0159] 4. Characterization of Fusion Protein D1/3-E6E7-his

[0160] Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4° C.

[0161] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

[0162] A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1% of total protein.

EXAMPLE: VIb

[0163] In an analagous fashion the fusion protein of Lipo D 1/3 and E6-E7 from HPV 16 was expressed in *E. coli* in the presence of thioredoxin. The N-terminal of the pre-protein (388 aa) contains MDP residues followed by 16 amino acids of signal peptide of lipoprotein D (from Haemophilus Influenzae) which is cleaved in vivo to give the mature protein (370 aa). Lipoprotein portion (aa 1 to 127) is followed by the proteins E6 and E7 in fusion. The C terminal of the protein is elongated by TSGHHHHHH.

[0164] The protein was purified by the following protocol:

EXAMPLE VII

Lipoprotein D1/3-E6-E7-HIs (TIT) Purification

[0165] A) Solubilisation.

[0166] Cell paste is suspended to 60 OD_{600} in 2 M NaCl, 20 mM Phosphate (NaH_2PO4/K_2HPO4) pH 7.5 in presence of 1 mM Pefabloc as protease inhibitor prior cell lysis by three passes through a French press disruptor (20,000 psi). Lysed cells are pelleted 30 min at 15,000 g at 4° C. In order to reduce endotoxin level, bacterial cell pellet containing the recombinant protein is washed twice in 4 M urea, 2 M NaCl, 20 mM Phosphate pH 7.5, once in 2% Empigen BB, 20 mM Phosphate pH 7.5 and finally twice in 20 mM Phosphate buffer pH 7.0 to eliminate trace of detergent (each wash is performed in the same volume used for cell suspension). LipoProt.D1/3-E6-E7-His (TIT) is solubilised (in the same volume used for cell suspension) by 8 M urea in 0.2 M βMercaptoEthanol (=βMeOH), 20 mM PO4 pH 12 overnight at 4° C. followed by a two hours incubation at RT° versus the same buffer. Cell debris are pelleted 30 min at 15,000 g at 4° C. Supernatant is kept at −20° C.

[0167] B) Purification

[0168] 1) Anion Exchange Chromatography on Q-Sepharose Fast Flow.

[0169] 225 ml of frozen sample is thawed at room temperature in a cold water bath and is applied onto a Q-Sepharose fast flow column (Pharmacia, XK 26/20) pre-equilibrated in 8 M urea, 0.2 M βMEOH, 20 mM PO4 pH 12 (30 ml resin/225 ml supernatant) at 45 cm/h. Column is washed by 8 M urea, 0.2 M MEOH, 20 mM PO4 pH 12, until OD 280 nm reaches the baseline, followed by a second wash in 8 M urea, 20 mM Phosphate pH 12 (in 2 column volumes) Elution is performed by NaCl steps (0.1 M, 0.25 M, 0.5 M NaCl, each step in about 2 column volumes) in 8 M urea, 20 mM Phosphate pH 12, at 45 cm/h. 0.5 M NaCl-eluted fractions are pooled.

[0170] 2) Ion Metal Affinity Chromatography (IMAC).

[0171] 0.5 M NaCl-eluted fractions from Q Sepharose step are pooled and dialyzed versus 0.2 M NaCl, 8 M urea, 20 mM Phosphate pH 10 before loading onto a Ni2+-NTA (Qiagen) column (XK 26/20, Pharmacia) preequilibrated in 8 M urea, 20 mM PO4 pH 12 (30 ml resin/61 ml sample) at 5.6 cm/h. Column is washed in 8 M urea, 20 mM PO4 pH 12 until the base line is reached then by 8 M urea, 20 mM PO4 pH 10. Antigen is eluted by Imidazole steps (0.025 M, 0.05 M, 0.1 M, 0.15 M, 0.2 M, 0.5 M Imidazole, each step in two column volumes) in 8 M urea, 20 mM PO4 pH 10, at 45 cm/h. 0.05 M Imidazole-eluted fractions are pooled.

[0172] C) Concentration.

[0173] Imac sample is concentrated about 5 times (to 0.407 mg/ml) on a 5 kDa Filtron Omega membrane in a stirred cell from AMICON at RT°.

[0174] D) Dialysis

[0175] Concentrated sample is dialyzed at RT versus decreasing-urea-concentration steps (4 M, 2 M urea) in 0.5 M Arginine, 150 mM NaCl, 10 mM PO4 pH 6.8. Last dialysis against 0.5 M Arginine, 150 mM NaCl, 10 mM PO4 pH 6.8 is achieved at 4° C.

[0176] Results:

[0177] IMAC step is able to eliminate a 32 kD contaminant at 0.025 M Imidazole which eluted also some antigen. 0.05 M Imidazole-eluted Antigen is estimated pure at 90% by Coomassie blue staining of SDS-PAGE. After these two purification steps, sample is free of *E. coli* contaminants. Western blotting analysis using specific antigen-N and/or C terminus antibodies shows a heterogeneous pattern of bands with higher and lower MW than the full length protein. This pattern suggests the presence of aggregates and incompletely processed protein and/or degraded one, copurified with the full length protein.

EXAMPLE VIII

Construction of *E.coli* Strain B1002 Expressing Fusion ProtD1/3-E7

[0178] Mutated (cys24→gly,glu26→gln) type HPV16

[0179] 1)-Construction of Expression Plasmid

[0180] Starting Material:

[0181] a)—Plasmid pRIT 14501 (=TCA 308) which codes for fusion ProtD1/3-E7-His

[0182] b)—Plasmid LITMUS 28 (New England Biolabs cat n° 306-28), a cloning vector pUC-derived

[0183] c)—Plasmid pMG MCS ProtD1/3 (pRIT 14589), a derivative of pMG81 (described Supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His) Construction of plasmid pRIT 14733(=TCA347): a plasmid expressing the fusion Protein-D1/3-E7 mutated (cys24→gly, glu26→gln) with His tail

[0184] The NcoI—XbaI fragment from pRIT 14501 (=TCA 308), bearing the coding sequence of E7 gene from HPV16, elongated with an His tail, was subcloned in an intermediate vector Litmus 28 useful for mutagenesis to give pRIT 14909 (=TCA337) Double mutations cys24-->gly (Edmonds and Vousden, J. Virology 63: 2650 (1989) and glu26-->gln (Phelps et al, J. Virology 66: 2418-27 (1992) were chosen to impair the binding to the antioncogene product of Retinoblastome gene (pRB). The introduction of mutations in E7 gene was realized with the kit "Quick Change Site directed Mutagenesis (Stratagene cat n° 200518) to give plasmid pRIT 14681 (=TCA343) After verification of presence of mutations and integrity of the complete E7 gene by sequencing, the mutated E7 gene was introduced into vector pRIT 14589 (=pMG MCS ProtD1/3) to give plasmid pRIT 14733 (=TCA347) (**FIG. 7**).

[0185] The sequence for the fusion protein-D1/3-E 7 mutated (cys24→gly, glu26→gln)-His is described in the **FIG. 8**.

[0186] 2)—Construction of Strain B1002 Expressing ProtD1/3-E7 Mutated (cys 24-->gly, glu26-->gln)-His/HPV16

[0187] Plasmid pRIT 14733 was introduced into *E.coli* AR58 (Mott et al.,1985, Proc. Natl. Acad. Sci., 82:88) a defective X lysogen containing a thermosensitive repressor of the λ pL promoter, to give strain B1002, by selection for transformants resistant to kanamycine

[0188] 3)—Growth and Induction of Bacterial Strain B1002—Expression of ProtD1/3-E7 Mutated (cys 24→gly, glu26→gln)-His/HPV16

[0189] Cells of AR58 transformed with plasmid pRIT 14733 (B1002 strain) were grown at 30° C. in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of ProtD1/3-E7 mutated-His/HPV16. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20° C.

[0190] 4)-Characterization of Fusion ProtD1/3-E7 mut (cys24→gly, glu26→gln)-His type HPV16.

[0191] Frozen cells were thawed and resuspended in 10 ml of PBS buffer.Cells were broken in a French Pressure cell press SLM Aminco at 20 000 psi (three passages). The extract was centrifuged at 16000 g for 30 minutes at 4° C.

[0192] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

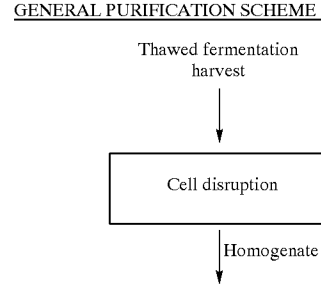
[0193] A major band of about 33 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal 22 J 70 anti-protein D, by monoclonal anti E7/HPV16 from Zymed and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3 to5% of total protein.

[0194] Cells of B1002 were separated from the culture broth by centrifugation. The concentrated cells of B1002 were stored at −65° C.

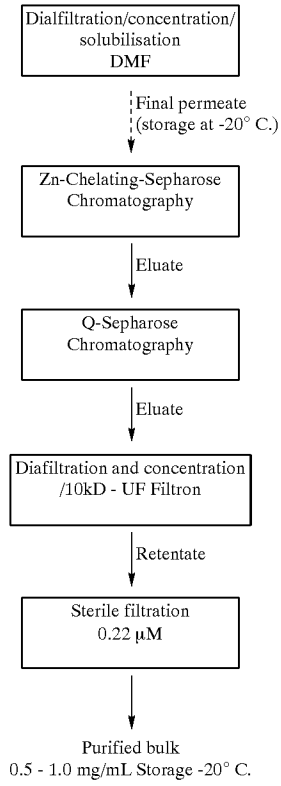
EXAMPLE IX

Purification PROTD1/3 E7 (Dmutant) HPV 16

[0195]



-continued



[0196] a) Preparation of Cell Suspension

[0197] The frozen concentrated cells of B1002 were thawed and resuspended in a cell disruption buffer at +4° C. (see table 1) to a final optical density OD_{650} of 60 (corresponding to a cell concentration of approximately 25 g DCW L^{-1})

[0198] b) Cell Disruption

[0199] The cells were disrupted by two passes at 1000 bar through a high-pressure homogeniser (Rannie). The broken cell suspension was collected in a flask maintained at 4° C. CELL DISRUPTION BUFFER: Na_2 HPO_4 (0.02N), NaCl (2M) pH adjusted to 7.5 with HCl 3N (Merck)

[0200] Purification

[0201] 2a) Dynamic Membrane Filtration (DMF®-PALL FILTRON)

[0202] 2 Litres of broken cell suspension (OD 60) is loaded on the DMF®, a dynamic filtration system from PALL, mounted with a 0.2 μm cut-off membrane.

[0203] concentration from 2 Litres to 1L to give sample PCC1

[0204] washing at constant volume with 3 volumes (3L) of empigen-EDTA buffer (concentration EDTA 1.86 g, Empigen (30%) 3.33 mL, PO_4^{3-}0.5M 40.00 mL 40.00 mL) gave sample PD!

[0205] concentration from 1L to 300 mL gave sample PCC2

[0206] washing at constant volume with 10 volumes (3L) of empigen buffer (concentration L^{-1}: Empigen 30%, 3.33 mL, PO_4^{3-}0.5M 40 mL) pH 7.5 gave sample PD2

[0207] solubilisation of the protein in by addition of the same volume (300 mL) of Guanidine hydrochloride 8M buffer (concentration L^{-1} Gu.HCl 764 g;

[0208] Empigen 30% 3.33 mL, PO_4^{3-}0.5M 40 mL) pH 7.5

[0209] recovery of the protein: Collection of the permeate—sample P3 during Concentration to initial volume (300 mL) and

[0210] Diafiltration with 3 volume of Guanidine hydrochloride 4M buffer (concentration L^{-1}: Gu.HCl 328.12 g, Empigen (30%) 3.33 mL Po43-0.5M 40.00 mL) pH 7.5.

[0211] All these steps are made in a cold room (2-8° C.), pH adjusted with 0.5M PO_4^{3-}.

[0212] The P3 fraction is store at −20° C. waiting for the next purification step.

[0213] 2b) Zn-Chelating Sepharose Chromatography

[0214] The P3 fraction is thawed and injected in a packed and equilibrated Zn-chelating sepharose FF.

[0215] After that, the column is:

[0216] Washed with around 3 volumes of Guanidine hydrochloride 4M buffer (see above)—sample Zn-FT

[0217] Washed with around 5 volumes of Urea 4M buffer (concentration L^{-1}: Urea 240.24 g Empigen 3.33 mL PO_4^{3-}0.5M 40.00 mL)—sample Zn-W

[0218] Eluted with around 3 volumes of Urea 4M-Imidazole 20 mM buffer (concentration L^{-1} Urea 240.24 g, Empigen (30%) 3.33 mL Imidazole (1.36 g) PO_4^{3-}0.5M 40.00 mL pH 7.5) buffer as above, but concentration L^{-1} of Imidazole 34.04 g—sample Zn-20

[0219] Eluted with Urea 4M-Imidazole 500 mM to the end of the UV peak—sample Zn-500

[0220] The column is the washed with EDTA 50 mM and NaOH 0.5M. Zn chelating sepharose eluate (Zn-500) is stored between 2-8° c. before the next purification step.

[0221] The Zn-chelating sepharose chromatography operations are carried out at room temperature.

[0222] 2c) Q-sepharose Chromatography

[0223] The Zn-500 fraction is injected in a packed and equilibrated Q-sepharose FF.

[0224] After that, the column is:

[0225] Washed with around 7 volumes of Urea 4M buffer (see above) - sample QS-FT

[0226] Washed with around 10 volumes of Urea 4M buffer without empigen (concentration L^{-1} Urea 240.24 g PO_4^{3-}0.5M 40.00 mL)—sample QS-W1

[0227] Washed with around 10 volumes of Urea 6M buffer without empigen (Urea 360.36 g/L)—sample QS-W2

[0228] Eluted with around 5 volumes of Urea 6M-NaCl 200 mM buffer (conctration L^{-1}: Urea 360.36 g NaCl 11.69 g, 40.00 MnL PO_4^{3-}.

[0229] Eluted with around 3 volumes of Urea 6M-NaCl 500 mM buffer (as above, but NaCl 29.22 g/L). The exact end of the fraction is determined by the end of the UV peak.—sample QS-500

[0230] Eluted with 4 volumes of Urea 4M-NaCl 1M buffer (conctration L^{-1} Urea 360.36, NaCl 58.44 g 40.00 mL PO_4^{3-}(0.5)—sample QS-1M

[0231] The column is then washed with NaOH 0.5M

[0232] QS-sepharose eluate (QS-500) is stored between 2-8°c before the next purification step.

[0233] The Q-sepharose chromatography operations are carried out at room temperature.

[0234] 2d) Ultrafiltration

[0235] The QS-500 fraction is then treated on a 10 kD utrafiltration unit (Ultrasette—Pall Filtron)

[0236] The product is first concentrated to around 1 mg/mL of protein and then diafiltrated against 10 volumes of phosphate buffer.

[0237] The permeate (fraction UF-P) is discarded and the retentate (fraction UF-R) is stored at 2-8° c. waiting for final filtration.

[0238] Ultrafiltration operations are carried out at 2-8° C.

[0239] 2e) Final Filtration

[0240] The final bulk (UF-R fraction) is filtered through a 0.22 cm sterile filter (Millipak-Millipore) under laminar flow and in an aseptic class 100 room.

[0241] The final concentration is between 0.5 and 1.0 μg/mL.

[0242] The sterile bulk is stored at −20° C.

EXAMPLE X

Construction of an *E. coli* Strain Expressing Fusion clyta-E6-his (HPV 16)

[0243] 1. Construction of Expression Plasmid

[0244] a)—Plasmid pRIT14497 (=TCA307), that codes for fusion ProtD1/3-E6-His/HPV16

[0245] b)—Plasmid pRIT14661 (=DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of *Streptococcus Pneumoniae.* Lyta is derived from *Streptococcus pneumoniae* which synthesize an N-acetyl-L-alanine amidase, amidase LYTA, (coded by the lytA gene {Gene, 43 (1986) pag 265-272} an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE.

[0246] 1.b Construction of Plasmid pRIT14634 (=TCA332): a Plasmid Expressing the Fusion clyta-E6-His/HPV16

[0247] a) The first step was the purification of the large NcoI-AflII restriction fragment from plasmid pRIT14497 and the purification of the small AflII-AflIII restriction fragment from pRIT14661

[0248] b) The second step was linking of clyta sequences to the E7-His sequences (NcoI and AfilIII are compatible restriction sites) that gave rise to the plasmid pRIT 14634 (=TCA332), coding for the fusion protein clyta-E6-His under the control of the pL promoter. (see **FIG. 9**) The coding sequence for the fusion protein clyta-E6-His is decribed in **FIG. 10**.

[0249] Transformation of AR58 Strain

[0250] Plasmid pRIT14634 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

[0251] Growth and Induction of Bacterial Strain—Expression of clyta-E6-His

[0252] Cells of AR58 transformed with plasmid pRIT14634 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein clyta-E6-his. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20° C.

[0253] 4. Characterization of Fusion clyta-E6-his

[0254] Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4° C. After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

[0255] A major band of about 33 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3% of total protein.

EXAMPLE XI

Construction of an *E. coli* strain expressing fusion clyta-E7-his (HPV 16)

[0256] 1. Construction of Expression Plasmid

[0257] 1.a Starting Materials

[0258] a)—Plasmid pRIT14501 (=TCA308), that codes for fusion ProtD1/3-E7-His/HPV16

[0259] b)-Plasmid pRIT14661 (=DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of *Streptococcus Pneumoniae.*

[0260] 1.b Construction of Plasmid pRIT14626 (=TCA330): a plasmid expressing the fusion clyta-E7-His/HPV16

[0261] a) The first step was the purification of the large NcoI-AflIII restriction fragment from plasmid pRIT14501 and the purification of the small AflII-AflIII restriction fragment from pRIT14661

[0262] b) The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites)that gave rise to the plasmid pRIT 14626 (=TCA330), coding for the fusion protein clyta-E7-His under the control of the pL promoter.

[0263] **(FIG. 11)**

[0264] The coding sequence for the fusion protein clyta-E7-His is decribed in **FIG. 12**.

[0265] 2. Transformation of AR58 Strain

[0266] Plasmid pRIT14626 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective ? lysogen containing a thermosensitive repressor of the λ pL promoter.

[0267] 3. Growth and Induction of Bacterial Strain—Expression of clyta-E7-His

[0268] Cells of AR58 transformed with plasmid pRIT14626 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein clyta-E7-his. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20° C.

[0269] 4. Characterization of Fusion clyta-E7-his

[0270] Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4° C. After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

[0271] A major band of about 35 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 5% of total protein.

EXAMPLE XII

Construction of an *E. coli* Strain Expressing Fusion clyta-E6E7-his (HPV 16)

[0272] 1. Construction of Expression Plasmid

[0273] 1.a Starting Materials

[0274] a)—Plasmid pRIT14512 (=TCA311), that codes for fusion ProtD1/3-E6E7-His/HPV16

[0275] b)—Plasmid pRIT14661 (=DVA2), an intermediate vector containing the coding sequence for the 117 C-terminal codons of LytA of Streptococcus Pneumoniae.

[0276] 1.b Construction of Plasmid pRIT14629 (=TCA331): a Plasmid Expressing the Fusion clyta-E6E7-His/HPV16

[0277] a) The first step was the purification of the large NcoI-AflII restriction fragment from plasmid pRIT14512 and the purification of the small AflII-AflIII restriction fragment from pRIT14661

[0278] b) The second step was linking of clyta sequences to the E7-His sequences (NcoI and AflIII are compatible restriction sites)that gave rise to the plasmid pRIT 14629 (=TCA33 1), coding for the fusion protein clyta-E6E7-His under the control of the pL promoter. (see **FIG. 13**)

[0279] The coding sequence for the fusion protein clyta-E6E7-His is decribed in **FIG. 14**.

[0280] 2. Transformation of AR58 Strain

[0281] Plasmid pRIT14629 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

[0282] 3. Growth and Induction of Bacterial Strain—Expression of clyta-E6E7-His

[0283] Cells of AR58 transformed with plasmid pRIT14629 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein clyta-E6E7-his. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20° C.

[0284] 4. Characterization of Fusion clyta-E6E7-his

[0285] Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract was centrifuged at 16.000 g for 30 minutes at 4° C.

[0286] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

[0287] A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-clyta antibodies and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1% of total protein.

EXAMPLE XIII

Prot D1/3 E7 his (HPV 18) (*E.Coli* B1011) Protein D1/3 E7 his HPV expressed with Thioredoxin in Trans (*E.Coli* B1012)

[0288] 1)—Construction of Expression Plasmids

[0289] 1).a. Construction of Plasmid TCA316(=pRIT 14532) a Plasmid Expressing the Fusion Protein-D1/3-E7-His IHPV18

[0290] Starting Materials

[0291] a)—Plasmid pMG MCS prot D1/3 (=pRIT14589) is a derivative of pMG81 (described in UK patent application n° 951 3261.9 published as WO97/01640 in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thr 127 of mature protein D of *Haemophilus Influenzae* strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His) (see **FIG. 15**). This plasmid is used to express the fusion protein D1/3-E7-his.

[0292] b)—HPV genomic E6 and E7 sequences of prototype HPV18 (Cole et al, J. Mol. Biol. (1987) 193,599-608) were amplified from HPV16 full length genome cloned in pBR322 (obtained from Deutsche Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses—D 69120—Heidelberg) and were subcloned into pUC19 to give TCA 302 (=pRIT14467).

[0293] Construction of Plasmid TCA 316(=pRIT14532)

[0294] The nucleotides sequences corresponding to amino acids 1→105 of E7 protein were amplified from pRIT14467. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E7 sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA316 (=pRIT14532). The insert was sequenced and a modification versus E7/HPV18 prototype sequence was identified in E7 gene (nucleotide 128 G→A) generating a substitution of a glycine by a glutamic acid (aa 43 in E7, position 156 in fusion protein). The sequence for the fusion protein-D1/3-E7-His/HPV18 is described in **FIG. 16**.

[0295] 1).b. Construction of Plasmid TCA313 (=pRIT14523): a Plasmid Expressing Thioredoxin

[0296] Starting Materials

[0297] a)—Plasmid pBBR1MCS4(Antoine R. and C. Locht, Mol. Microbiol. 1992, 6, 1785-1799 ; M. E. Kovach et al. Biotechniques 16, (5), 800-802)which is compatible with plasmids containing ColE1 or P15a origins of replication.

[0298] b)—Plasmid pMG42 (described in WO93/04175) containing the sequence of promoter pL of Lambda phage

[0299] c)—Plasmid pTRX (Invitrogen, kit Thiofusion K350-01) bearing the coding sequence for thioredoxin followed by AspA transcription terminator.

[0300] Construction of Plasmid TCA313(=pRIT14523)

[0301] The fragment EcoRI-NdeI fragment from pMG42, bearing pL promoter and the NdeI-HindIII fragment from pTRX, bearing the coding sequence for thioredoxin followed by AspA terminator, were purified and ligated into the EcoRI and HindIII sites of plasmid vector pBBR 1MCS4 to give plasmid TCA313(=pRIT14523) (see **FIG. 17**).

[0302] The sequence for thioredoxin is described in **FIG. 18**.

[0303] 2)—Transformation of AR58 Strain

[0304] 2).a. To Obtain Strain B1011 Expressing ProtD1/3-E7-His/HPV18

[0305] Plasmid pRIT14532 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter, by selection for transformants resistant to kanamycine.

[0306] 2).b. Construction of Strain B1012 Expressing ProtD1/3-E7-His/HPV18 and Thioredoxin

[0307] Plasmid pRIT14532 and pRIT14523 were introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter, by double selection for transformants resistant to kanamycin and ampicillin.

[0308] 3)—Growth and Induction of Bacterial Strains B1011 and B1012—Expression of Prot-D1/3-E7-His/HPV18 Without and With Thioredoxin in Trans

[0309] Cells of AR58 transformed with plasmids pRIT14532 (B1011 strain) and Cells of AR58 transformed with plasmids pRIT14532 and pRIT14523 (B1012 strain) were grown at 30° c. in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin for B1011 strain and supplemented 50 μgr/ml of Kanamycin and 100 μgr/ml of Ampicillin for B1012 strain. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein D1/3-E7-his/HPV18 and thioredoxin. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20° C.

[0310] Characterization of Fusion Protein D1/3-E7-his/HPV18

[0311] Preparation of Extracts

[0312] Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4° C.

[0313] Analysis on Coomassie-Stained SDS-Polyacrylamide Gels and Western Blots

[0314] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

[0315] The fusion protD1/3-E7-His (about 31 kDa) was visualised by Coomassie stained gels in the pellet fraction for strain B1011 and partially localized (30%) in the supernatant fraction for strain 1012 and was identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1-3% of total protein as shown on a Coomassie-stained SDS-polyacrylamide gel.

[0316] For the extract of strain B1012 the thioredoxin (about 12 KDa) was visualised by coomassie stained gel in the supernatant and identified in western blots by monoclonal anti thioredoxin (Invitrogen R920-25)

[0317] Purification of Prot D1/3 E7-his/HPV18

[0318] Recombinant HPV 18-ProtD1/3-E7-His is expressed in *E. coli* (as described above) AR58 strain. All steps are performed at room temperature (RT≅22° C.). Proteins are followed by monitoring OD 280 nm. Between steps, antigens positive fractions are kept at −20° C.

[0319] Purified antigen is stable one week at −20° C. and 4° C. (no degradation) but appears more susceptible to oxidation after incubation at 37° C.

[0320] d)—Solubility

[0321] Protein solubility is pH dependent (see below) with decrease of solubility for pH<7.4:

PBS pH 7.4	686 μg/ml	100%
PBS pH 7.2	560 μg/ml	81%
PBS pH 7.0	498 μg/ml	72%
PBS pH 6.8	327 μg/ml	48%

[0322] e)—The HPV 18 Prot D1/3 E7 proteiin is composed of 227 amino acids. Its theoretical molecular weight is 25.9 kDa, and a theoretical isoelectric point of 5.83. It migrates at about 31.5 kDa in reducing SDS PAGE.

EXAMPLE XIV

Purification of HPV 18 Protein D1/3 E7

[0323] a)—Solubilisation

[0324] Cell paste is suspended to 60 OD_{600} in 2 M NaCl, 20 mM Phosphate. (NaH_2PO4/K_2HPO4) pH 7.6 prior cell lysis by two passes through a Rannie disruptor. Lysed cells are pelleted 30 min at 9,000 rpm in a JA 10 rotor at 4° C. In order to reduce endotoxin level, bacterial cell pellet containing the recombinant protein is washed once in 5 mM EDTA, 2 M NaCl, PBS pH 7.4; once in 4 M urea, 20 mM Phosphate pH 7.4 and finally once in PBS pH 7.4 to eliminate trace of EDTA (each wash is performed in twice volume used for cell suspension). HPV18Prot.D1/3-E7-His (TIT for Thioredoxin In Trans) is solubilised (in the same volume used for cell suspension) by 6 M Guanidine-Chloride, 50 mM PO4 pH 7.6 overnight at 4° C. Cell debris are pelleted 30 min at 9,000 rpm in a JA 10 rotor at 4° C. Supernatant is supplemented with 0.5% Empigen BB and incubated 30 min at RT.

[0325] b)—Purification

[0326] 1).a. Immobilized Metal Affinity Chromatography

[0327] 125 .ml of sample are loaded onto a Zn^{2+}-Chelating Sepharose FF column (XK 26/20, Pharmacia; 50 ml gel/125 ml solubilisation) preequilibrated in 0.5% Empigen BB, 6 M Guanidine-Chloride, 50 mM PO4 pH 7.6 at 4 ml/min. Column is washed by Guanidine Chloride 6M, PO4 50 mM pH 7.6 until the base line is reached then by 6 M urea, 0.5 M NaCl, 50 mM PO4 pH 7.6. Antigen is eluted by 0.25 M-Imidazole in 6 M urea, 0.5 M NaCl, 50 mM PO4 pH 7.6, at 2 ml/min (**FIG. 1B**). IMAC-eluted sample is dialyzed at 4° C. versus PBS pH 7.4

[0328] 1).b. Affi-Prep Polymixin (Bio-Rad)

[0329] To reduce endotoxin level, 28 mg (37 ml) of antigen are incubated in batch mode with 2 ml of Affiprep Polymyxin resin prequilibrated in PBS pH 7.4, over night at room temperature. Protein recovery is estimate at 60% and endotoxin content is reduced 6.5 times.

[0330] 1).c. Analysis

[0331] Purified antigen analyzed on reducing-SDS-PAGE presents a major 30 kDa band with a second one at 55 kDa, after Coomassie Blue or Silver Staining. In a non reducing SDS-PAGE, HPV-18-ProtD1/3-E7-His appears mainly like a smear with Molecular Weight≧175 kDa. However this oxidation can be reversed by addition of 5 mM of β-Mercapto-Ethanol. This pattern is confirmed by anti ProtD or by anti His Western Blotting analysis.

[0332] c)—Stability

[0333] Purified antigen is stable one week at −20° C. and 4° C. (no degradation) but appears more susceptible to oxidation after incubation at 37° C.

[0334] d)—Solubility

[0335] Protein solubility is pH dependent (see below) with decrease of solubility for pH <7.4:

PBS pH 7.4	686 μg/ml	100%
PBS pH 7.2	560 μg/ml	81%
PBS pH 7.0	498 μg/ml	72%
PBS pH 6.8	327 μg/ml	48%

[0336] HPV18-ProtD1/3-E7-His protein is composed of 227 amino acids. Its theoretical molecular weight is 25.9 kDa. It migrates at about 31.5 kDa in reducing SDS-PAGE. Theoretical isoelectric point is 5.83.

EXAMPLE XV

Construction of *E.coli* strain B1098 expressing fusion ProtD1/3-E7

[0337] Mutated (cys27→gly,glu29→gln) type HPV18

[0338] 1)-Construction of Expression Plasmid

[0339] Starting Material:

[0340] a)—Plasmid pRIT 14532 (=TCA 316) which codes for fusion ProtD 1/3-E7-His

[0341] b)—Plasmid LITMUS 28 (New England Biolabs cat n° 306-28), a cloning vector pUC-derived

[0342] c)—Plasmid pMG MCS ProtD1/3 (pRIT 14589), a derivative of pMG81 (described supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thr 127 of mature protein D of *Haemophilus Influenzae* strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p. 119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His)

[0343] Construction of Plasmid pRIT 14831(=TCA355): a Plasmid Expressing the Fusion Protein-D1/3-E7 Mutated (cys27→gly, glu29→gln) With His Tail

[0344] The NcoI-XbaI fragment from pRIT 14532 (—TCA 316), bearing the coding sequence of E7 gene from HPV18, elongated with an His tail, was subcloned in an intermediate vector Litmus 28 useful for mutagenesis to give pRIT 14910 (=TCA348) By analogy with E7/HPV 16 mutagenesis, double mutations cys27-->gly and glu29-->gln were chosen to impair the binding to the antioncogene product of Retinoblastome gene (pRB).

[0345] The introduction of mutations in E7 gene was realized with the kit "Quick Change Site directed Mutagenesis (Stratagene cat n° 200518). As the sequencing of pRIT14532 had pointed out the presence of a glutamic acid in position 43 of E7 instead of a glycine in the prototype sequence of HPV 18, a second cycle of mutagenesis was realized to introduce a glycine in position 43. We obtained plasmid pRIT 14829 (=TCA353). After verification of presence of mutations and integrity of the complete E7 gene by sequencing, the mutated E7 gene was introduced into vector pRIT 14589 (=pMG MCS ProtD1/3) to give plasmid pRIT 14831 (=TCA355) (see **FIG. 17**).

[0346] The sequence for the fusion protein-D1/3-E 7 mutated (cys27→gly, glu29→gln)-His is described in the **FIG. 18**.

[0347] 2) Construction of Strain B1098 Expressing ProtD1/3-E7 Mutated (cys 27-->gly, glu29-->gln)-His/HPV18

[0348] Plasmid pRIT 14831 was introduced into *E.coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter, to give strain B1098, by selection for transformants resistant to kanamycin.

[0349] 3)—Growth and Induction of Bacterial Strain B1098—Expression of ProtD1/3-E7 Mutated (cys 27→gly, glu29→gln)-His/HPV18

[0350] Cells of AR58 transformed with plasmid pRIT 14831 (B1098 strain) were grown at 30° C. in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of ProtD1/3-E7 mutated -His/HPV18. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20° C.

[0351] 4)—Characterization of Fusion ProtD1/3-E7 mut (cys24→gly, glu26→gln)-His Type HPV16

[0352] Frozen cells were thawed and resuspended in 10 ml of PBS buffer. Cells were broken in a French Pressure cell press SLM Aminco at 20 000 psi (three passages). The extract was centrifuged at 16000 g for 30 minutes at 4° C.

[0353] Analysis on Coomassie Stained SDS-Polyacrylamide Gels and Western Blots

[0354] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting. A major band of about 31 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal 22 J 70 anti-protein D and by monoclonal Penta-His (Qiagen cat. n° 34660) which detects accessible histidine tail. The level of expression represents about 3 to 5% of total protein.

EXAMPLE XVI

Construction of an *E. coli* Strain Expressing Fusion Protein-D 1/3-E6-his/HPV18

[0355] 1. Construction of Expression Plasmid

[0356] a) Plasmid pMG MCS prot D1/3 (=pRIT14589) is a derivative of pMG81 (described supra) in which the codons 4-81 of NS1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D 1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6-his.

[0357] HPV genomic E6 and E7 sequences type HPV18 (Cole et al., J. Mol. Biol. 1987, 193, p.599-608.) were amplified from HPV18 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses—D 69120—Heidelberg) and were subcloned into pUC19 to give TCA 302 (=pRIT14467).

[0358] Construction of Plasmid TCA 314(=pRIT14526): a Plasmid Expressing the Fusion Protein-D1/3-E6-His/HPV18

[0359] The nucleotides sequences corresponding to amino acids

[0360] 1→158 of E6 protein were amplified from pRIT14467. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6 sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA314 (=pRIT14526) (see **FIG. 21**). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The coding sequence for the fusion protein-D1/3-E6-His is described in **FIG. 22**.

[0361] Transformation of AR58 Strain

[0362] Plasmid pRIT14526 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

[0363] 3. Growth and Induction of Bacterial Strain—Expression of Prot-D1/3-E6-His

[0364] Cells of AR58 transformed with plasmid pRIT14526 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6-his. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20C.

[0365] 4. Characterization of Fusion Protein D1/3-E6-his

[0366] Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The

extract is centrifuged at 16.000 g for 30 minutes at 4° C. After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting.

[0367] A major band of about 32 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 3-5% of total protein.

EXAMPLE XVII

Construction of an *E. coli* strain expressing fusion Protein-D1/3-E6E7-his/HPV18

[0368] 1. Construction of Expression Plasmid

[0369] a) Plasmid pMG MCS prot D1/3 (=pRIT14589) is a derivative of pMG81 (described supra) in which the codons 4-81 of NS 1 coding region from Influenza were replaced by the codons corresponding to residues Ser 20→Thr 127 of mature protein D of Haemophilus Influenzae strain 772, biotype 2 (H. Janson et al., 1991, Infection and Immunity, Jan. p.119-125). The sequence of Prot-D1/3 is followed by a multiple cloning site (11 residues) and a coding region for a C-terminal histidine tail (6 His). This plasmid is used to express the fusion protein D1/3-E6E7-his.

[0370] b) HPV genomic E6 and E7 sequences type HPV18 (Cole et al., J. Mol. Biol. 1987, 193, 599-608) were amplified from HPV18 full length genome cloned in pBR322 (obtained from Deutsches Krebsforschungszentrum (DKFZ), Referenzzentrum für human pathogen Papillomaviruses—D 69120—Heidelberg) and were subcloned into pUC19 to give TCA 302 (=pRIT14467).

[0371] c) The coding sequences for E6 and E7 in TCA302 (=pRIT 14467) were modified with a synthetic oligonucleotides adaptor (inserted between Hga I and Nsi I sites) introducing a deletion of 11 nucleotides between E6 and E7 genes, removing the stop codon of E6 and creating fused E6 and E7 coding sequences in the plasmid TCA320(=pRIT 14618) see **FIG. 23**.

[0372] Construction of Plasmid TCA 328(=pRIT14567): a Plasmid Expressing the Fusion Protein-D1/3-E6E7-His/HPV18

[0373] The nucleotides sequences corresponding to amino acids

[0374] 1→263 of fused E6E7 protein were amplified from pRIT14618. During the polymerase chain reaction, NcoI and SpeI restriction sites were generated at the 5' and 3' ends of the E6E7 fused sequences allowing insertion into the same sites of plasmid pMGMCS Prot D1/3 to give plasmid TCA328 (=pRIT14567) (see **FIG. 24**). The insert was sequenced to verify that no modification had been generated during the polymerase chain reaction. The coding sequence for the fusion protein-D1/3-E6E7-His is described in **FIG. 25**.

[0375] 2. Transformation of AR58 Strain

[0376] Plasmid pRIT14567 was introduced into *E. coli* AR58 (Mott et al., 1985, Proc. Natl. Acad. Sci., 82:88) a defective λ lysogen containing a thermosensitive repressor of the λ pL promoter.

[0377] 3. Growth and Induction of Bacterial Strain—Expression of Prot-D1/3-E6E7-His

[0378] Cells of AR58 transformed with plasmid pRIT14512 were grown in 100 ml of LB medium supplemented with 50 μgr/ml of Kanamycin at 30° C. During the logarithmic phase of growth bacteria were shifted to 39° C. to inactivate the λ repressor and turn on the synthesis of protein D1/3-E6E7-his. The incubation at 39° C. was continued for 4 hours. Bacteria were pelleted and stored at −20C.

[0379] 4. Characterization of Fusion Protein D1/3-E6E7-his

[0380] Frozen cells are thawed and resuspended in 10 ml of PBS buffer. Cells are broken in a French pressure cell press SLM Aminco at 20.000 psi (three passages). The extract is centrifuged at 16.000 g for 30 minutes at 4° C.

[0381] After centrifugation of extracts described above, aliquots of supernatant and pellet were analysed by SDS-polyacrylamide gel electrophoresis and Western blotting. A major band of about 48 kDa, localized in the pellet fraction, was visualised by Coomassie stained gels and identified in Western blots by rabbit polyclonal anti-protein-D and by Ni-NTA conjugate coupled to calf intestinal alkaline phosphatase (Qiagen cat. n° 34510) which detects accessible histidine tail. The level of expression represents about 1% of total protein.

EXAMPLE XVIII

Vaccine Formulations

[0382] Vaccines are formulated with a Protein from the above examples expressed in *E. coli* from the strain AR58, and as adjuvant, the formulation comprising a mixture of 3 de -O-acylated monophosphoryl lipid A (3D-MPL) and aluminium hydroxide or 3D-MPL and/or QS21 optionally in an oil/water emulsion, and optionally formulated with cholesterol.

[0383] 3D-MPL: is a chemically detoxified form of the lipopolysaccharide (LPS) of the Gram-negative bacteria Salmonella minnesota.

[0384] Experiments performed at Smith Kline Beecham Biologicals have shown that 3D-MPL combined with various vehicles strongly enhances both the humoral and a THI type of cellular immunity.

[0385] QS21: is one saponin purified from a crude extract of the bark of the Quillaja Saponaria Molina tree, which has a strong adjuvant activity: it activates both antigen-specific lymphoproliferation and CTLs to several antigens.

[0386] Vaccine containing an antigen of the invention containing 3D-MPL and alum may be prepared in analogous manner to that described in WO93/19780 or 92/16231.

[0387] Experiments performed at Smith Kline Beecham Biologicals have demonstrated a clear synergistic effect of

combinations of 3D-MPL and QS21 in the induction of both humoral and TH1 type cellular immune responses. Vaccines containing an antigen such antigens are described in U.S. Pat. No. 5,750,110.

[0388] The oil/water emulsion is composed of 2 oils (a tocopherol and squalene), and of PBS containing Tween 80 as emulsifier. The emulsion comprised 5% squalene 5% tocopherol 0.4% Tween 80 and had an average particle size of 180 nm and is known as SB62 (see WO 95/17210).

[0389] Experiments performed at Smith Kline Beecham Biologicals have proven that the adjunction of this O/W emulsion to MPL/QS21 further increases their immunostimulant properties.

[0390] Preparation of Emulsion SB62 (2 Fold Concentrate)

[0391] Tween 80 is dissolved in phosphate buffered saline (PBS) to give a 2% solution in the PBS. To provide 100 ml two fold concentrate emulsion 5 g of DL alpha tocopherol and 5 ml of squalene are vortexed to mix thoroughly. 90 ml of PBS/Tween solution is added and mixed thoroughly. The resulting emulsion is then passed through a syringe and finally microfluidised by using an M110S microfluidics machine. The resulting oil droplets have a size of approximately 180 nm.

[0392] Preparation of Prot.D1/3 E7 QS21/3D MPL oil in Water Formulation

[0393] ProtD1/3-E7 (5 μg) was diluted in 10 fold concentrated PBS pH 6.8 and H_2O before consecutive addition of SB62, 3 D MPL (5 μg), QS21 (5 μg) and 50 μg/ml thiomersal as preservative at 5 min interval. The emulsion volume is equal to 50% of the total volume (50 μl for a dose of 100 μl). All incubations were carried out at room temperature with agitation. The adjuvants controls without antigen were prepared by replacing the protein by PBS.

[0394] Tumour Regression Experiments (HPV 16) with PROT D E7

[0395] Vaccine Antigen: Fusion Protein ProtD E7

[0396] Protein D is a lipoprotein exposed on the surface of the Gram-negative bacteria Haemophilus influenzae.

[0397] The inclusion of the 109 first residues of the protein D as fusion partner is incorporated to provide the vaccine antigen with bystander help properties. The antigen was formulated with QS21 3D-MPL and SB62 as described supra.

EXAMPLE XIX

In vivo Tumour Regression Experiments Tumour cell line TC1:

[0398] Primary lung epithelial cells from C57BL.6 mice were immortalised by HPV 16 E6 and E7 and then transformed with an activated ras oncogene, producing a tumourigenic cell line expressing E6 and E7 (Lin KY et al. 1996). The E7 expression has been verified by FACS analysis of fixed and permeabilised TC1 cells using the mouse anti-HPV 16 E7 Mab (Triton Corp. Alameda, Calif.)

[0399] Tumour Growth:

[0400] TC 1 cells growing in vitro culture were trypsinised, washed two times in serum-free medium and were injected S.C. in the right flank of the mice. To assess treatment of established tumours, TC1 cells were injected at a dose of 3×10e4 cells/mouse. One and two weeks after the tumour cell injection, mice were vaccinated with 5 μg in 100 μl of protD 1/3 E7 His intra foot pad (50 μl/foot pad) in PBS or in the 3D-MPL, QS21 and SB62 or with PBS or with the adjuvant alone. Five C57BL/6 mice (Iffa Credo) were used in each group. Mice were monitored twice a week for tumour growth. The mean tumour mass/group is shown in **FIG. 26**, the mice vaccinated with protD 1/3 E7 His in PBS or with PBS or the adjuvant alone developed progressively growing tumours (0-1 tumour-free animal/group). On the contrary, four out of five mice vaccinated with protD1/3 E7 His in adjuvant did not develop a tumour, one animal developed a very small and stable tumour at day 40. This results indicate that the protein protD1/3 E7 His from HPV 16 formulated in adjuvant is able to induce the regression of small established tumours expressing this antigen.

[0401] Immunological Read Out

[0402] Proliferation Assay:

[0403] For in vitro assay, Lymphocytes were prepared by crushing the spleen or the popliteal lymph nodes from the vaccinated mice at day 69.

[0404] An aliquot of 2×10e5 cells was plated in triplicate in 96 well plates with decreasing concentrations (10, 1, 0.1 μg/ml) of protD 1/3 E7 His coated or not onto latex microbeads (Sigma) to restimulate the cells in vitro (72 Hrs). T cell proliferation was measured by 3H thymidine incorporation.

[0405] **FIGS. 27 and 28** compares the ability of protD E7 to stimulate the proliferation of splenocytes and lymph node cells primed in vivo either by PBS, 3D-MPL, QS21 SB62, ProtD1/3 E7 His and ProtD1/3 E7 His+the adjuvant of 3D-MPL, QS21, SB62 and shows that high proliferative responses in spleen were detected only in mice immunised with protD1/3 E7 His in adjuvant compared to the other groups.

[0406] Antibody Response

[0407] Individual serum were taken at the same time as the organs were taken and submitted to indirect ELISAs.

[0408] 5 μg/ml of purified E7 protein was used as coated antigen. After saturation in PBS+1% newborn calf serum 1 Hr at 37° C., the sera were serially diluted (starting at 1/100) in the saturation buffer and incubated O/N at 4° C. or 90 min at 37° C. After washing in PBS Tween 20 0.1%, biotinylated goat Anti mouse Ig (1/1000) or goat anti mouse Ig subclass (total IgG, IgGl, IgG2a, IgG2b) antisera (1/5000) were used as second antibodies, after an incubation of 90 min at 37° C., streptavidin coupled to peroxydase was added and TMB (tetra-methyl-benzidine/peroxide) was used as substrate, after 10 min. the reaction was stopped with H2SO4 0.5 M and the O.D.450 was determined.

[0409] The subclass-specific anti E7 titers elicited by the vaccinations in the different groups of mice are shown in **FIG. 29** as a comparison of the relative mean midpoint dilution of the serum.

[0410] These results show that a weak antibody response is triggered with 2 injections of ProtD 1/3 E7 HPV16 alone.

[0411] Much more anti-E7 antibodies are generated when ProtD1/3 E7 was injected in the presence of the adjuvant SB62, QS21+3D-MPL.

[0412] No IgA nor IgM were detected in any of the serum samples even in the serum of the mice that received ProtD 1/3 E7 in the adjuvant SB62, QS21+3D-MPL (data not shown) On the contrary, the total IgG level was slightly increased by the vaccination of the mice with ProtD 1/3 E7 alone and was greatly increased by the addition of the adjuvant SB62, QS21+3D-MPL to the protein. The analysis of the concentrations of the different IgG subclass show that a mixed antibody response has been induced as the concentration of all types of IgG subclass analyzed (IgG1, IgG2a, and IgG2b) were increased in the serum of the mice that received the adjuvanted antigen, compared to the concentration observed in the serum of mice that received the antigen or the adjuvant alone. The predominant isotype found was IgG2b which represented more than 80% of the total of IgG), this isotype is generally said to be associated with the induction of a TH1 type immune response.

EXAMPLE XX

In vivo Tumour Protection Experiments

[0413] Mice were immunised 2 times at 14 days interval with either PBS, adjuvant of example 1, 5 μg of protD1/3 E7 His or 5 μg of protD1/3 E7 His in the adjuvant of example 1 intra foot pad in a volume of 100 μl (50 μl/foot pad).

[0414] Tumour Growth:

[0415] Four weeks after the latest vaccination mice were challenged with 2×10e5 TC1 cells/mouse S.C. in the flank. TC1 cells growing in vitro culture, were trypsinised and washed two times in serum-free medium and injected. 5 mice used in each group were monitored twice a week for tumour growth.

[0416] **FIG. 30** shows that vaccination with the E7 protein in the SB62 QS21, 3D-MPL adjuvant protect the mice against the development of tumour (only one animal/5 has a very small and stable tumour) in all the other groups, that received the E7 protein without the adjuvant or the adjuvant alone developed growing tumours.

[0417] Immunological Read Out

[0418] Three weeks after the latest vaccination, before the tumour challenge 5 mice in each group were sacrified for immunological read out.

[0419] Proliferation Assay

[0420] For in vitro assay, Lymphocytes were prepared as described above from the spleen and from the popliteal draining lymph nodes. An aliquot of 2×10e5 cells was plated in triplicate in 96 well plates with decreasing concentrations (10, 1, 0.1 μg/ml) of protD 1/3 E7 His coated or not onto latex microbeads (Sigma) to restimulate the cells in vitro (72 Hrs). T cell proliferation was measured by 3H thymidine incorporation.

[0421] **FIGS. 31 and 32** show repectively that, both with splenocytes or popliteal lymph node cells, as it was observed in the therapeutic settings, a better lymphoproliferative activity was obtained for the mice that received the E7 protein in the SB62 QS21, 3D-MPL adjuvant antibody response.

[0422] **FIG. 33** shows that as in the therapeutic settings, a better antibody response was observed in the serum of mice vaccinated with the ProtD1/3 E7 protein formulated in the 3D-MPL, QS21 O/W adjuvant. A mixed antibody response was triggered, as all the IgG subclass tested (IgG 2a, IgG2b, IgG1), in this case also, IgG2b was the predominant isotype found, representing 75% of the total IgG.

EXAMPLE XXI

Vaccination Experiments with Prot D1/3 E7 (HPV 18)

[0423] Mice were vaccinated twice, 2 weeks apart, with 5 μg in 100 μl of protD 1/3 18 E7 His intra foot pad (50 μl/foot pad) in PBS or QS21, 3D-MPL and SB62, DQ MPL as described in WO96/33739 or DQ alum MPL as described in WO98/15827. Eight 6-8 weeks old Balb/c mice (Iffa Credo) were used in each group.

[0424] 14 days post II, the spleen and lymph nodes were taken for immunological read out and blood sampling for serology.

[0425] Immunological Read Out:

[0426] Proliferation Assay:

[0427] For in vitro assay, lymphocytes were prepared by crushing the spleen or the popliteal lymph nodes from the vaccinated mice at day 28

[0428] An aliquot of 2×10e5 cells was plated in triplicate in 96 well plates with decreasing concentrations (10, 1, 0.1, 0.01 μg/ml) of protD 1/3 18 E7 His to restimulate the cells in vitro (72 Hrs). T cell proliferation was measured by 3H thymidine incorporation. The results are expressed as stimulation index (cpm sample/cpm baseline)

[0429] **FIGS. 34 and 35** compares the ability of protD 1/3 18 E7 to stimulate the proliferation of splenocytes or lymph node cells primed in vivo either by ProtD1/3 18 E7 His or Prot D1/3 18 E7 His+adjuvant and shows that only a basal lymphoproliferation is seen in mice that received the protein alone, on the contrary, high proliferative responses in spleen and very high responses in lymph nodes were detected in mice immunised with protD1/3 18 E7 His in adjuvant.

[0430] Cytokine Production

[0431] The cytokines (IL-5 and IFNg) produced in the culture supernatant after a 96 Hrs period of in vitro re-stimulation of spleen or lymph node cells, with medium or with the ProtD1/3 18E7 (1 or 3 μg/ml) was measured by ELISA as described:

[0432] IFNg (Genzyme)

[0433] Quantitation of IFNγ was performed by Elisa using reagents from Genzyme. Samples and antibody solutions were used at 50 μl per well. 96-well microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark) were coated overnight at 4° c. with 50 μl of hamster anti-mouse IFNg diluted at 1.5 μg/ml in carbonate buffer pH 9.5. Plates were then incubated for lhr at 37° c. with 100 μl of PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation

buffer). Two-fold dilutions of supernatant from in vitro stimulation (starting at ½) in saturation buffer were added to the anti-IFNg-coated plates and incubated for 1 hr 30 at 37° c. The plates were washed 4 times with PBS Tween 0.1% (wash buffer) and biotin-conjugated goat anti-mouse IFNg diluted in saturation buffer at a final concentration of 0.5 g/ml was added to each well and incubated for 1 hr at 37° c. After a washing step, AMDEX conjugate (Amersham) diluted 1/10000 in saturation buffer was added for 30 min at 37° c. Plates were washed as above and incubated with 50 μl of TMB (Biorad) for 15 min. The reaction was stopped with H_2SO_4 0.4N and read at 450 nm. Concentrations were calculated using a standard curve (mouse IFNγ standard) by SoftmaxPro (four parameters equation) and expressed in pg/ml.

[0434] IL5 (Pharmingen)

[0435] Quantitation of IL5 was performed by Elisa using reagents from Pharmingen. Samples and antibody solutions were used at 50 μl per well. 96-well microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark) were coated overnight at 4° c. with 50 μl of rat anti-mouse IL5 diluted at 1 μg/ml in carbonate buffer pH 9.5. Plates were then incubated for lhr at 37° c. with 100 μl PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Two-fold dilutions of supernatant from in vitro stimulation (starting at ½) in saturation buffer were added to the anti-IFNg-coated plates and incubated for 1 hr 30 at 37° c. The plates were washed 4 times with PBS Tween 0.1% (wash buffer) and biotin-conjugated rat anti-mouse IL5 diluted in saturation buffer at a final concentration of 1 μg/ml was added to each well and incubated for 1 hr at 37° c. After a washing step, AMDEX conjugate (Amersham) diluted 1/10000 in saturation buffer was added for 30 min at 37° c. Plates were washed as above and incubated with 50 μl of TMB (Biorad) for 15 min. The reaction was stopped with H_2SO_4 0.4N and read at 450 nm. Concentrations were calculated using a standard curve (recombinant mouse IL5) by SoftmaxPro (four parameters equation) and expressed in pg/ml.

[0436] Starting with spleen cells, no IL-5 could be detected whatever the group tested, on the contrary, a very high production of IFNg production was observed in all groups, with only a slight increase in the group of mice that received the SBAS1c adjuvanted protein compared to the other groups. This suggest the induction of a TH1 type of immune response.

[0437] Regarding lymph node cells, a very weak IFNg production was obtained in the group of mice that received the protein alone and a 5-10 fold increase is observed with the adjuvanted protein. IL5 could only be detected in the group of mice receiving the SBAS2 adjuvanted protein.

[0438] **FIGS. 36 and 37** compares the ability of ProtD1/3 18 E7 His to stimulate the production of cytokines 'IFNg and IL5) after in vitro re-stimulation of spleen or lymph node cells respectively.

[0439] Antibody Response

[0440] Individual serum were taken at the same time as the organs and submitted to indirect ELISAs.

[0441] 2.5 μg/ml of purified of protD1/3 18E7 protein HPV 18 was used as coated antigen. After saturation in PBS+1% newborn calf serum 1 Hr at 37° C., the sera were serially diluted (starting at 1/100) in the saturation buffer and incubated O/N at 4° C. or 90 min at 37° C. After washing in PBS Tween 20 0.1%, biotinylated goat Anti mouse Ig (1/1000) or goat anti mouse Ig subclass (total IgG, IgGI, IgG2a, IgG2b) antisera (1/5000) were used as second antibodies, after an incubation of 90 min at 37° C., streptavidin coupled to peroxydase was added and TMB (tetra-methyl-benzidine/peroxide) was used as substrate, after 10 min. the reaction was stopped with H2SO4 0.5 M and the O.D.450 was determined.

[0442] A very weak antibody response is triggered with 2 injections of ProtD 1/3 18 E7 alone. The total IgG level was greatly increased by the addition of adjuvants to the protein vaccine.

[0443] The analysis of the concentrations of the different IgG subclass show that when the protein was injected in the presence of adjuvants, DQS21 3D-MPL or SB62, QS21/3D-MPL, a slight increase of the IgG2a subtype percentage was obtained: 28% IgG1, 48% IgG2a and 43% IgG1, 44% IgG2a respectively, compared to 46% of IgG1, 32% of IgG2a with the non adjuvanted protein. The strongest antibody response is obtained with the protein formulated in DQ alum with a clear shift in the isotype concentration (80% IgG1, 8% IgG2a). As the IgG2a isotype in Balb/c mice is generally conidered to be associated with the induction of a TH1 type of immune response, these results suggested that the DQS21, 3D-MPL and SB62 QS21/3D-MPL adjuvants tend toincrease the THI type profile of the humoral response while SBAS5 induce a clear TH2 type of response.

[0444] **FIG. 38**. The comparison of the midpoint dilution of the serum and relative percentage of the different isotypes elicited by the vaccinations in the different groups of mice are shown.

[0445] Conclusion:

[0446] We have demonstrated that the fused protein: ⅓ Prot D and early protein E7 of HPV 16 induced a potent systemic antitumour immunity and the fusion protein ProtD1/3 and E7 of HPV18 has also been showed to be immunogenic in mice Vaccination with the prot D1/3 E7 HPV16 fusion protein protected the mice from a tumour challenge with E7 expressing tumour cells and eliminated small pre-established tumours expressing the E7 of HPV16 injected at a distant site from the vaccination site.

[0447] We have demonstrated that the ProtD1/3 E7 HPV16 protein in adjuvant is capable of enhancing helper T cell proliferation suggesting that the antitumour immune response induced by this vaccine is at least in part associated with a CD4+ T cell response.

[0448] We have also demonstrated that a better antibody response was triggered by the vaccination with the ProtD1/3 E7 in the presence of the 3D-MPL containing adjuvant. The predominant isotype found in the serum of C57BL/6 mice being IgG2b suggesting that a THI type immune response was raised.

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ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt    300

caaagtttag aaatgacaga aaactttgaa accatggcca tgcatggaga tacacctaca    360

ttgcatgaat atatgttaga tttgcaacca gagacaactg atctctactg ttatgagcaa    420
```

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ttaaatgaca gctcagagga ggaggatgaa atagatggtc cagctggaca agcagaaccg 480

gacagagccc attacaatat tgtaaccttt tgttgcaagt gtgactctac gcttcggttg 540

tgcgtacaaa gcacacacgt agacattcgt actttggaag acctgttaat gggcacacta 600

ggaattgtgt gccccatctg ttctcagaaa ccaactagtg gccaccatca ccatcaccat 660

taa 663

<210> SEQ ID NO 3
<211> LENGTH: 822
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 3

atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc 60

attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca 120

cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt 180

cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc 240

ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt 300

caaagtttag aaatgacaga aaactttgaa accatggcca tgtttcagga cccacaggag 360

cgacccagaa agttaccaca gttatgcaca gagctgcaaa caactataca tgatataata 420

ttagaatgtg tgtactgcaa gcaacagtta ctgcgacgtg aggtatatga ctttgctttt 480

cgggatttat gcatagtata tagagatggg aatccatatg ctgtatgtga taaatgttta 540

aagttttatt ctaaaattag tgagtataga cattattgtt atagtttgta tggaacaaca 600

ttagaacagc aatacaacaa accgttgtgt gatttgttaa ttaggtgtat taactgtcaa 660

aagccactgt gtcctgaaga aaagcaaaga catctggaca aaaagcaaag attccataat 720

ataaggggtc ggtggaccgg tcgatgtatg tcttgttgca gatcatcaag aacacgtaga 780

gaaacccagc tgactagtgg ccaccatcac catcaccatt aa 822

<210> SEQ ID NO 4
<211> LENGTH: 273
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 4

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110

Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu

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        115                 120                 125

Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu Glu Cys Val
    130                 135                 140

Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp Phe Ala Phe
145                 150                 155                 160

Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr Ala Val Cys
                165                 170                 175

Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr Arg His Tyr
            180                 185                 190

Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr Asn Lys Pro
        195                 200                 205

Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys Pro Leu Cys
    210                 215                 220

Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg Phe His Asn
225                 230                 235                 240

Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys Arg Ser Ser
                245                 250                 255

Arg Thr Arg Arg Glu Thr Gln Leu Thr Ser Gly His His His His His
            260                 265                 270

His
```

<210> SEQ ID NO 5
<211> LENGTH: 1116
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 5

```
atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc     60

attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca    120

cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt    180

cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc    240

ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt    300

caaagtttag aaatgacaga aaactttgaa accatggcca tgtttcagga cccacaggag    360

cgacccagaa agttaccaca gttatgcaca gagctgcaaa caactataca tgatataata    420

ttagaatgtg tgtactgcaa gcaacagtta ctgcgacgtg aggtatatga ctttgctttt    480

cgggatttat gcatagtata tagagatggg aatccatatg ctgtatgtga taaatgttta    540

aagttttatt ctaaaattag tgagtataga cattattgtt atagtttgta tggaacaaca    600

ttagaacagc aatacaacaa accgttgtgt gatttgttaa ttaggtgtat taactgtcaa    660

aagccactgt gtcctgaaga aaagcaaaga catctggaca aaaagcaaag attccataat    720

ataaggggtc ggtggaccgg tcgatgtatg tcttgttgca gatcatcaag aacacgtaga    780

gaaacccagc tgatgcatgg agatacacct acattgcatg aatatatgtt agatttgcaa    840

ccagagacaa ctgatctcta ctgttatgag caattaaatg acagctcaga ggaggaggat    900

gaaatagatg gtccagctgg acaagcagaa ccggacagag cccattacaa tattgtaacc    960

ttttgttgca agtgtgactc tacgcttcgg ttgtgcgtac aaagcacaca cgtagacatt   1020

cgtactttgg aagacctgtt aatgggcaca ctaggaattg tgtgccccat ctgttctcag   1080

aaaccaacta gtggccacca tcaccatcac cattaa                              1116
```

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<210> SEQ ID NO 6
<211> LENGTH: 371
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 6

```
Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110

Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu Pro Gln Leu
        115                 120                 125

Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu Glu Cys Val
    130                 135                 140

Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp Phe Ala Phe
145                 150                 155                 160

Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr Ala Val Cys
                165                 170                 175

Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr Arg His Tyr
            180                 185                 190

Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr Asn Lys Pro
        195                 200                 205

Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys Pro Leu Cys
    210                 215                 220

Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg Phe His Asn
225                 230                 235                 240

Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys Arg Ser Ser
                245                 250                 255

Arg Thr Arg Arg Glu Thr Gln Leu Met His Gly Asp Thr Pro Thr Leu
            260                 265                 270

His Glu Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr Asp Leu Tyr Cys
        275                 280                 285

Tyr Glu Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu Ile Asp Gly
    290                 295                 300

Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr
305                 310                 315                 320

Phe Cys Cys Lys Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr
                325                 330                 335

His Val Asp Ile Arg Thr Leu Glu Asp Leu Leu Met Gly Thr Leu Gly
            340                 345                 350

Ile Val Cys Pro Ile Cys Ser Gln Lys Pro Thr Ser Gly His His His
```

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        355                 360                 365

His His His
    370


<210> SEQ ID NO 7
<211> LENGTH: 663
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 7

atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc     60

attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca    120

cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt    180

cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc    240

ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt    300

caaagtttag aaatgacaga aaactttgaa accatggcca tgcatggaga tacacctaca    360

ttgcatgaat atatgttaga tttgcaacca gagacaactg atctctacgg ttatcagcaa    420

ttaaatgaca gctcagagga ggaggatgaa atagatggtc cagctggaca agcagaaccg    480

gacagagccc attacaatat tgtaaccttt tgttgcaagt gtgactctac gcttcggttg    540

tgcgtacaaa gcacacacgt agacattcgt actttggaag acctgttaat gggcacacta    600

ggaattgtgt gccccatctg ttctcagaaa ccaactagtg gccaccatca ccatcaccat    660

taa                                                                  663


<210> SEQ ID NO 8
<211> LENGTH: 220
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 8

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110

Ala Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met Leu Asp Leu
        115                 120                 125

Gln Pro Glu Thr Thr Asp Leu Tyr Gly Tyr Gln Gln Leu Asn Asp Ser
    130                 135                 140

Ser Glu Glu Glu Asp Glu Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro
145                 150                 155                 160

Asp Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys Cys Asp Ser
```

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```
                165                 170                 175

Thr Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile Arg Thr Leu
            180                 185                 190

Glu Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro Ile Cys Ser
        195                 200                 205

Gln Lys Pro Thr Ser Gly His His His His His His
    210                 215                 220


<210> SEQ ID NO 9
<211> LENGTH: 879
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 9

atgaaagggg gaattgtaca ttcagacggc tcttatccaa aagacaagtt tgagaaaatc     60

aatggcactt ggtactactt tgacagttca ggctatatgc ttgcagaccg ctggaggaag    120

cacacagacg gcaactggta ctggttcgac aactcaggcg aaatggctac aggctggaag    180

aaaatcgctg ataagtggta ctatttcaac gaagaaggtg ccatgaagac aggctgggtc    240

aagtacaagg acacttggta ctacttagac gctaaagaag gcgccatggt atcaaatgcc    300

tttatccagt cagcggacgg aacaggctgg tactacctca aaccagacgg aacactggca    360

gacaggccag aattggccag catgctggac atggccatgt ttcaggaccc acaggagcga    420

cccagaaagt taccacagtt atgcacagag ctgcaaacaa ctatacatga tataatatta    480

gaatgtgtgt actgcaagca acagttactg cgacgtgagg tatatgactt tgcttttcgg    540

gatttatgca tagtatatag agatgggaat ccatatgctg tatgtgataa atgtttaaag    600

ttttattcta aaattagtga gtatagacat tattgttata gtttgtatgg aacaacatta    660

gaacagcaat acaacaaacc gttgtgtgat ttgttaatta ggtgtattaa ctgtcaaaag    720

ccactgtgtc ctgaagaaaa gcaaagacat ctggacaaaa agcaaagatt ccataatata    780

aggggtcggt ggaccggtcg atgtatgtct tgttgcagat catcaagaac acgtagagaa    840

acccagctga ctagtggcca ccatcaccat caccattaa                           879


<210> SEQ ID NO 10
<211> LENGTH: 292
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 10

Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
 1               5                  10                  15

Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
            20                  25                  30

Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
        35                  40                  45

Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
    50                  55                  60

Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
65                  70                  75                  80

Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met
                85                  90                  95

Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
```

-continued

```
            100                 105                 110

Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
        115                 120                 125

Leu Asp Met Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu
    130                 135                 140

Pro Gln Leu Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu
145                 150                 155                 160

Glu Cys Val Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp
                165                 170                 175

Phe Ala Phe Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr
            180                 185                 190

Ala Val Cys Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr
        195                 200                 205

Arg His Tyr Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr
    210                 215                 220

Asn Lys Pro Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys
225                 230                 235                 240

Pro Leu Cys Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg
                245                 250                 255

Phe His Asn Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys
            260                 265                 270

Arg Ser Ser Arg Thr Arg Arg Glu Thr Gln Leu Thr Ser Gly His His
        275                 280                 285

His His His His
    290
```

<210> SEQ ID NO 11
<211> LENGTH: 720
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 11

```
atgaaagggg gaattgtaca ttcagacggc tcttatccaa aagacaagtt tgagaaaatc     60

aatggcactt ggtactactt tgacagttca ggctatatgc ttgcagaccg ctggaggaag    120

cacacagacg gcaactggta ctggttcgac aactcaggcg aaatggctac aggctggaag    180

aaaatcgctg ataagtggta ctatttcaac gaagaaggtg ccatgaagac aggctgggtc    240

aagtacaagg acacttggta ctacttagac gctaaagaag gcgccatggt atcaaatgcc    300

tttatccagt cagcggacgg aacaggctgg tactacctca aaccagacgg aacactggca    360

gacaggccag aattggccag catgctggac atggccatgc atggagatac acctacattg    420

catgaatata tgttagattt gcaaccagag acaactgatc tctactgtta tgagcaatta    480

aatgacagct cagaggagga ggatgaaata gatggtccag ctggacaagc agaaccggac    540

agagcccatt acaatattgt aacctttttgt tgcaagtgtg actctacgct tcggttgtgc    600

gtacaaagca cacacgtaga cattcgtact ttggaagacc tgttaatggg cacactagga    660

attgtgtgcc ccatctgttc tcagaaacca actagtggcc accatcacca tcaccattaa    720
```

<210> SEQ ID NO 12
<211> LENGTH: 239
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

-continued

<400> SEQUENCE: 12

Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
1 5 10 15

Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
20 25 30

Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
35 40 45

Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
50 55 60

Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
65 70 75 80

Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met
85 90 95

Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
100 105 110

Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
115 120 125

Leu Asp Met Ala Met His Gly Asp Thr Pro Thr Leu His Glu Tyr Met
130 135 140

Leu Asp Leu Gln Pro Glu Thr Thr Asp Leu Tyr Cys Tyr Glu Gln Leu
145 150 155 160

Asn Asp Ser Ser Glu Glu Glu Asp Glu Ile Asp Gly Pro Ala Gly Gln
165 170 175

Ala Glu Pro Asp Arg Ala His Tyr Asn Ile Val Thr Phe Cys Cys Lys
180 185 190

Cys Asp Ser Thr Leu Arg Leu Cys Val Gln Ser Thr His Val Asp Ile
195 200 205

Arg Thr Leu Glu Asp Leu Leu Met Gly Thr Leu Gly Ile Val Cys Pro
210 215 220

Ile Cys Ser Gln Lys Pro Thr Ser Gly His His His His His His
225 230 235

<210> SEQ ID NO 13
<211> LENGTH: 1173
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 13

atgaaagggg gaattgtaca ttcagacggc tcttatccaa aagacaagtt tgagaaaatc 60

aatggcactt ggtactactt tgacagttca ggctatatgc ttgcagaccg ctggaggaag 120

cacacagacg gcaactggta ctggttcgac aactcaggcg aaatggctac aggctggaag 180

aaaatcgctg ataagtggta ctatttcaac gaagaaggtg ccatgaagac aggctgggtc 240

aagtacaagg acacttggta ctacttagac gctaaagaag gcgccatggt atcaaatgcc 300

tttatccagt cagcggacgg aacaggctgg tactacctca aaccagacgg aacactggca 360

gacaggccag aattggccag catgctggac atggccatgt ttcaggaccc acaggagcga 420

cccagaaagt taccacagtt atgcacagag ctgcaaacaa ctatacatga tataatatta 480

gaatgtgtgt actgcaagca acagttactg cgacgtgagg tatatgactt tgcttttcgg 540

gatttatgca tagtatatag agatgggaat ccatatgctg tatgtgataa atgtttaaag 600

ttttattcta aaattagtga gtatagacat tattgttata gtttgtatgg aacaacatta 660

-continued

gaacagcaat acaacaaacc gttgtgtgat ttgttaatta ggtgtattaa ctgtcaaaag 720

ccactgtgtc ctgaagaaaa gcaaagacat ctggacaaaa agcaaagatt ccataatata 780

aggggtcggt ggaccggtcg atgtatgtct tgttgcagat catcaagaac acgtagagaa 840

acccagctga tgcatggaga tacacctaca ttgcatgaat atatgttaga tttgcaacca 900

gagacaactg atctctactg ttatgagcaa ttaaatgaca gctcagagga ggaggatgaa 960

atagatggtc cagctggaca agcagaaccg gacagagccc attacaatat tgtaaccttt 1020

tgttgcaagt gtgactctac gcttcggttg tgcgtacaaa gcacacacgt agacattcgt 1080

actttggaag acctgttaat gggcacacta ggaattgtgt gccccatctg ttctcagaaa 1140

ccaactagtg gccaccatca ccatcaccat taa 1173

<210> SEQ ID NO 14
<211> LENGTH: 390
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 14

Met Lys Gly Gly Ile Val His Ser Asp Gly Ser Tyr Pro Lys Asp Lys
 1   5   10   15

Phe Glu Lys Ile Asn Gly Thr Trp Tyr Tyr Phe Asp Ser Ser Gly Tyr
            20   25   30

Met Leu Ala Asp Arg Trp Arg Lys His Thr Asp Gly Asn Trp Tyr Trp
        35   40   45

Phe Asp Asn Ser Gly Glu Met Ala Thr Gly Trp Lys Lys Ile Ala Asp
    50   55   60

Lys Trp Tyr Tyr Phe Asn Glu Glu Gly Ala Met Lys Thr Gly Trp Val
65   70   75   80

Lys Tyr Lys Asp Thr Trp Tyr Tyr Leu Asp Ala Lys Glu Gly Ala Met
                85   90   95

Val Ser Asn Ala Phe Ile Gln Ser Ala Asp Gly Thr Gly Trp Tyr Tyr
            100   105   110

Leu Lys Pro Asp Gly Thr Leu Ala Asp Arg Pro Glu Leu Ala Ser Met
        115   120   125

Leu Asp Met Ala Met Phe Gln Asp Pro Gln Glu Arg Pro Arg Lys Leu
    130   135   140

Pro Gln Leu Cys Thr Glu Leu Gln Thr Thr Ile His Asp Ile Ile Leu
145   150   155   160

Glu Cys Val Tyr Cys Lys Gln Gln Leu Leu Arg Arg Glu Val Tyr Asp
                165   170   175

Phe Ala Phe Arg Asp Leu Cys Ile Val Tyr Arg Asp Gly Asn Pro Tyr
            180   185   190

Ala Val Cys Asp Lys Cys Leu Lys Phe Tyr Ser Lys Ile Ser Glu Tyr
        195   200   205

Arg His Tyr Cys Tyr Ser Leu Tyr Gly Thr Thr Leu Glu Gln Gln Tyr
    210   215   220

Asn Lys Pro Leu Cys Asp Leu Leu Ile Arg Cys Ile Asn Cys Gln Lys
225   230   235   240

Pro Leu Cys Pro Glu Glu Lys Gln Arg His Leu Asp Lys Lys Gln Arg
                245   250   255

Phe His Asn Ile Arg Gly Arg Trp Thr Gly Arg Cys Met Ser Cys Cys

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            260                 265                 270

Arg Ser Ser Arg Thr Arg Arg Glu Thr Gln Leu Met His Gly Asp Thr
        275                 280                 285

Pro Thr Leu His Glu Tyr Met Leu Asp Leu Gln Pro Glu Thr Thr Asp
    290                 295                 300

Leu Tyr Cys Tyr Glu Gln Leu Asn Asp Ser Ser Glu Glu Glu Asp Glu
305                 310                 315                 320

Ile Asp Gly Pro Ala Gly Gln Ala Glu Pro Asp Arg Ala His Tyr Asn
                325                 330                 335

Ile Val Thr Phe Cys Cys Lys Cys Asp Ser Thr Leu Arg Leu Cys Val
            340                 345                 350

Gln Ser Thr His Val Asp Ile Arg Thr Leu Glu Asp Leu Leu Met Gly
        355                 360                 365

Thr Leu Gly Ile Val Cys Pro Ile Cys Ser Gln Lys Pro Thr Ser Gly
    370                 375                 380

His His His His His His
385                 390


<210> SEQ ID NO 15
<211> LENGTH: 684
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 15

atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc     60

attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca    120

cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt    180

cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc    240

ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt    300

caaagtttag aaatgacaga aaactttgaa accatggcca tgcatggacc taaggcaaca    360

ttgcaagaca ttgtattgca tttagagccc caaaatgaaa ttccggttga ccttctatgt    420

cacgagcaat taagcgactc agaggaagaa aacgatgaaa tagatgaagt taatcatcaa    480

catttaccag cccgacgagc cgaaccacaa cgtcacacaa tgttgtgtat gtgttgtaag    540

tgtgaagcca gaattgagct agtagtagaa agctcagcag acgaccttcg agcattccag    600

cagctgtttc tgaacaccct gtcctttgtg tgtccgtggt gtgcatccca gcagactagt    660

ggccaccatc accatcacca ttaa                                           684


<210> SEQ ID NO 16
<211> LENGTH: 227
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 16

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1               5                  10                  15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
```

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```
    50                  55                  60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110

Ala Met His Gly Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu
        115                 120                 125

Glu Pro Gln Asn Glu Ile Pro Val Asp Leu Leu Cys His Glu Gln Leu
    130                 135                 140

Ser Asp Ser Glu Glu Glu Asn Asp Glu Ile Asp Glu Val Asn His Gln
145                 150                 155                 160

His Leu Pro Ala Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys
                165                 170                 175

Met Cys Cys Lys Cys Glu Ala Arg Ile Glu Leu Val Val Glu Ser Ser
            180                 185                 190

Ala Asp Asp Leu Arg Ala Phe Gln Gln Leu Phe Leu Asn Thr Leu Ser
        195                 200                 205

Phe Val Cys Pro Trp Cys Ala Ser Gln Gln Thr Ser Gly His His His
    210                 215                 220

His His His
225


<210> SEQ ID NO 17
<211> LENGTH: 109
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 17

Met Ser Asp Lys Ile Ile His Leu Thr Asp Asp Ser Phe Asp Thr Asp
 1               5                  10                  15

Val Leu Lys Ala Asp Gly Ala Ile Leu Val Asp Phe Trp Ala Glu Trp
            20                  25                  30

Cys Gly Pro Cys Lys Met Ile Ala Pro Ile Leu Asp Glu Ile Ala Asp
        35                  40                  45

Glu Tyr Gln Gly Lys Leu Thr Val Ala Lys Leu Asn Ile Asp Gln Asn
    50                  55                  60

Pro Gly Thr Ala Pro Lys Tyr Gly Ile Arg Gly Ile Pro Thr Leu Leu
65                  70                  75                  80

Leu Phe Lys Asn Gly Glu Val Ala Ala Thr Lys Val Gly Ala Leu Ser
                85                  90                  95

Lys Gly Gln Leu Lys Glu Phe Leu Asp Ala Asn Leu Ala
            100                 105


<210> SEQ ID NO 18
<211> LENGTH: 684
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 18

atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc     60

attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca    120
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cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt 180

cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc 240

ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt 300

caaagtttag aaatgacaga aaactttgaa accatggcca tgcatggacc taaggcaaca 360

ttgcaagaca ttgtattgca tttagagccc caaaatgaaa ttccggttga ccttctaggt 420

caccagcaat taagcgactc agaggaagaa aacgatgaaa tagatggagt taatcatcaa 480

catttaccag cccgacgagc cgaaccacaa cgtcacacaa tgttgtgtat gtgttgtaag 540

tgtgaagcca gaattgagct agtagtagaa agctcagcag acgaccttcg agcattccag 600

cagctgtttc tgaacaccct gtcctttgtg tgtccgtggt gtgcatccca gcagactagt 660

ggccaccatc accatcacca ttaa 684

<210> SEQ ID NO 19
<211> LENGTH: 227
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 19

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110

Ala Met His Gly Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu
115 120 125

Glu Pro Gln Asn Glu Ile Pro Val Asp Leu Leu Gly His Gln Gln Leu
130 135 140

Ser Asp Ser Glu Glu Glu Asn Asp Glu Ile Asp Gly Val Asn His Gln
145 150 155 160

His Leu Pro Ala Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys
165 170 175

Met Cys Cys Lys Cys Glu Ala Arg Ile Glu Leu Val Val Glu Ser Ser
180 185 190

Ala Asp Asp Leu Arg Ala Phe Gln Gln Leu Phe Leu Asn Thr Leu Ser
195 200 205

Phe Val Cys Pro Trp Cys Ala Ser Gln Gln Thr Ser Gly His His His
210 215 220

His His His
225

<210> SEQ ID NO 20
<211> LENGTH: 837

-continued

<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 20

atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc 60

attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca 120

cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt 180

cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc 240

ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt 300

caaagtttag aaatgacaga aaactttgaa accatggcgc gctttgagga tccaacacgg 360

cgaccctaca agctacctga tctgtgcacg gaactgaaca cttcactgca agacatagaa 420

ataacctgtg tatattgcaa gacagtattg gaacttacag aggtatttga atttgcattt 480

aaagatttat ttgtggtgta tagagacagt ataccgcatg ctgcatgcca taaatgtata 540

gatttttatt ctagaattag agaattaaga cattattcag actctgtgta tggagacaca 600

ttggaaaaac taactaacac tgggttatac aatttattaa taaggtgcct gcggtgccag 660

aaaccgttga atccagcaga aaaacttaga caccttaatg aaaaacgacg atttcacaac 720

atagctgggc actatagagg ccagtgccat tcgtgctgca accgagcacg acaggaacga 780

ctccaacgac gcagagaaac acaagtaact agtggccacc atcaccatca ccattaa 837

<210> SEQ ID NO 21
<211> LENGTH: 278
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 21

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
1 5 10 15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
20 25 30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35 40 45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
50 55 60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65 70 75 80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
85 90 95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
100 105 110

Ala Arg Phe Glu Asp Pro Thr Arg Arg Pro Tyr Lys Leu Pro Asp Leu
115 120 125

Cys Thr Glu Leu Asn Thr Ser Leu Gln Asp Ile Glu Ile Thr Cys Val
130 135 140

Tyr Cys Lys Thr Val Leu Glu Leu Thr Glu Val Phe Glu Phe Ala Phe
145 150 155 160

Lys Asp Leu Phe Val Val Tyr Arg Asp Ser Ile Pro His Ala Ala Cys
165 170 175

His Lys Cys Ile Asp Phe Tyr Ser Arg Ile Arg Glu Leu Arg His Tyr
180 185 190

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Ser Asp Ser Val Tyr Gly Asp Thr Leu Glu Lys Leu Thr Asn Thr Gly
        195                 200                 205

Leu Tyr Asn Leu Leu Ile Arg Cys Leu Arg Cys Gln Lys Pro Leu Asn
    210                 215                 220

Pro Ala Glu Lys Leu Arg His Leu Asn Glu Lys Arg Arg Phe His Asn
225                 230                 235                 240

Ile Ala Gly His Tyr Arg Gly Gln Cys His Ser Cys Cys Asn Arg Ala
                245                 250                 255

Arg Gln Glu Arg Leu Gln Arg Arg Arg Glu Thr Gln Val Thr Ser Gly
            260                 265                 270

His His His His His His
        275
```

<210> SEQ ID NO 22
<211> LENGTH: 1152
<212> TYPE: DNA
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 22

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atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc     60

attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca    120

cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt    180

cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc    240

ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt    300

caaagtttag aaatgacaga aaactttgaa accatggcgc gctttgagga tccaacacgg    360

cgaccctaca agctacctga tctgtgcacg gaactgaaca cttcactgca agacatagaa    420

ataacctgtg tatattgcaa gacagtattg gaacttacag aggtatttga atttgcattt    480

aaagatttat ttgtggtgta tagagacagt ataccgcatg ctgcatgcca taaatgtata    540

gatttttatt ctagaattag agaattaaga cattattcag actctgtgta tggagacaca    600

ttggaaaaac taactaacac tgggttatac aatttattaa taaggtgcct gcggtgccag    660

aaaccgttga atccagcaga aaaacttaga caccttaatg aaaaacgacg atttcacaac    720

atagctgggc actatagagg ccagtgccat tcgtgctgca accgagcacg acaggaacga    780

ctccaacgac gcagagaaac acaagtaatg catggaccta aggcaacatt gcaagacatt    840

gtattgcatt tagagcccca aaatgaaatt ccggttgacc ttctatgtca cgagcaatta    900

agcgactcag aggaagaaaa cgatgaaata gatggagtta atcatcaaca tttaccagcc    960

cgacgagccg aaccacaacg tcacacaatg ttgtgtatgt gttgtaagtg tgaagccaga   1020

attgagctag tagtagaaag ctcagcagac gaccttcgag cattccagca gctgtttctg   1080

aacaccctgt cctttgtgtg tccgtggtgt gcatcccagc agactagtgg ccaccatcac   1140

catcaccatt aa                                                       1152
```

<210> SEQ ID NO 23
<211> LENGTH: 383
<212> TYPE: PRT
<213> ORGANISM: Homo sapien

<400> SEQUENCE: 23

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Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
```

-continued

```
 1               5                  10                  15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
            20                  25                  30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
        35                  40                  45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
    50                  55                  60

Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
65                  70                  75                  80

Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
                85                  90                  95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
            100                 105                 110

Ala Arg Phe Glu Asp Pro Thr Arg Arg Pro Tyr Lys Leu Pro Asp Leu
        115                 120                 125

Cys Thr Glu Leu Asn Thr Ser Leu Gln Asp Ile Glu Ile Thr Cys Val
    130                 135                 140

Tyr Cys Lys Thr Val Leu Glu Leu Thr Glu Val Phe Glu Phe Ala Phe
145                 150                 155                 160

Lys Asp Leu Phe Val Val Tyr Arg Asp Ser Ile Pro His Ala Ala Cys
                165                 170                 175

His Lys Cys Ile Asp Phe Tyr Ser Arg Ile Arg Glu Leu Arg His Tyr
            180                 185                 190

Ser Asp Ser Val Tyr Gly Asp Thr Leu Glu Lys Leu Thr Asn Thr Gly
        195                 200                 205

Leu Tyr Asn Leu Leu Ile Arg Cys Leu Arg Cys Gln Lys Pro Leu Asn
    210                 215                 220

Pro Ala Glu Lys Leu Arg His Leu Asn Glu Lys Arg Arg Phe His Asn
225                 230                 235                 240

Ile Ala Gly His Tyr Arg Gly Gln Cys His Ser Cys Cys Asn Arg Ala
                245                 250                 255

Arg Gln Glu Arg Leu Gln Arg Arg Arg Glu Thr Gln Val Met His Gly
            260                 265                 270

Pro Lys Ala Thr Leu Gln Asp Ile Val Leu His Leu Glu Pro Gln Asn
        275                 280                 285

Glu Ile Pro Val Asp Leu Leu Cys His Glu Gln Leu Ser Asp Ser Glu
    290                 295                 300

Glu Glu Asn Asp Glu Ile Asp Gly Val Asn His Gln His Leu Pro Ala
305                 310                 315                 320

Arg Arg Ala Glu Pro Gln Arg His Thr Met Leu Cys Met Cys Cys Lys
                325                 330                 335

Cys Glu Ala Arg Ile Glu Leu Val Val Glu Ser Ser Ala Asp Asp Leu
            340                 345                 350

Arg Ala Phe Gln Gln Leu Phe Leu Asn Thr Leu Ser Phe Val Cys Pro
        355                 360                 365

Trp Cys Ala Ser Gln Gln Thr Ser Gly His His His His His His
    370                 375                 380
```

1. An E6 or E7 protein or E6/E7 fusion protein from HPV linked to an immunological fusion partner.

2. A protein as claimed in claim 1 wherein the fusion partner is selected from the group; protein D or a fragment thereof from Heamophilius influenzae B, lipoprotein D or fragment thereof from Heamophilius influenzae B, NS1 or fragment thereof from Influenzae Virus, and LYTA or fragment thereof from *Streptococcus Pneumoniae*.

3. A protein as claimed in claim 1 or **2** wherein the E6 or E7 proteins are derived from HPV16 or HPV18.

4. A protein as claimed in claim 1, **2** or **3** wherein the E7 protein is mutated.

5. A protein as claimed in claim 1, **2** or **3** wherein the E6 protein is mutated.

6. A protein as claimed in any of claims 1 to 5 additionally comprising a hisitidine tag of at least 4 hisitidine residues.

7. A fusion protein comprising a heterologous protein, a hisitidine tag and a C-LYTA tag.

8. A DNA sequence encoding a protein as claimed herein.

9. A vaccine containing a protein as claimed in any of claims 1 to 7 and a pharmaceutically acceptable diluent or excipient.

10. A vaccine as claimed in claim 9 additionally comprising an adjuvant.

11. A vaccine as claimed in claim 9 or **10** wherein the protein is presented in an oil in water emulsion vehicle.

12. A vaccine as claimed in claim 10 or **11** wherein the adjuvant comprises 3D-MPL or QS21 or both.

13. A vaccine as claimed herein comprising an additional HPV antigen.

14. A vaccine as claimed herein for use in medicine.

15. Use of a protein as claimed herein for the manufacture of a vaccine for immunotherapeutically treating a patient suffering from HPV induced tumour lesions (benign or malignant).

16. Use of a protein as claimed herein for the manufacture of a vaccine to prevent HPV viral infection.

17. A vector containing a DNA sequence of claim 8.

18. A vector containing a DNA sequence as claimed in claim 8 and a DNA sequence encoding thioredoxin.

19. A host transformed with a DNA sequence of claim 8.

20. A host transformed with a vector of claim 17 or **18**.

21. A host as claimed in claim 19 additionally transformed with a DNA sequence encoding thioredoxin.

22. A process for the production of a protein as claimed herein comprising transforming a host cell with a DNA sequence of claim 6, expressing said sequence and isolating the desired product.

23. A process for the production of a vaccine as claimed herein, comprising admixing a protein as claimed herein with a suitable adjuvant, diluent or other pharmaceutically acceptable excipient.

* * * * *