Respiratory Syncytial Virus (RSV) A2 Nucleoprotein (N) Helper Plasmid, pA2-Nopt

Catalog No. NR-36462

For research use only. Not for human use.

Contributor:
BEI Resources

Manufacturer:
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Product Description:
NR-36462 is a component of a bacterial artificial chromosome (BAC)-based RSV rescue system that allows RSV infection to be monitored by fluorescence and is an important tool in RSV vaccine research and mutagenesis studies. Please refer to Appendix I for the manufacturer's RSV rescue protocol.

The N helper plasmid was constructed from codon-optimized RSV A2 N sequences. The codon-optimized cDNA sequences were synthesized and cloned into the pcDNA™3.1$^{(+)}$ mammalian expression plasmid (Life Technologies™ Invitrogen™).[1,2] The plasmid was produced in *Escherichia coli*, strain 10-beta (a DH10B derivative, New England BioLabs®) and extracted using a Endo-Free Plasmid Maxi Kit (Qiagen).[2] The complete sequence for pA2-Nopt is reported in Appendix II.

Material Provided:
Each vial contains 0.5 µg of plasmid DNA in RNase/DNase-free 10 mM Tris-HCl, 1 mM EDTA buffer (pH 8). The concentration is shown on the Certificate of Analysis. The vial should be centrifuged prior to opening.

Packaging/Storage:
NR-36462 was packaged aseptically in screw-capped plastic cryovials. The product is provided frozen on dry ice and should be stored at -80°C or colder immediately upon arrival. Freeze-thaw cycles should be minimized.

Functional Activity:
Recombinant RSV was produced by co-transfection of BHK-21 clone BSR T7/5 cells[3] with pSynkRSV-I19F, a BAC plasmid containing RSV A2-line19F antigenomic DNA and the gene for the far-red fluorescent protein monomeric Katushka 2 (mKate2) to enable detection of infection through fluorescence, (NR-36460) and four helper plasmids encoding sequence-optimized genes from RSV strain A2: large polymerase (L) (NR-36461), nucleoprotein (N) (NR-36462), phosphoprotein (P) (NR-36463) and matrix 2-1 protein (M2-1) (NR-36464). RSV rescue and infection could be detected by red fluorescent syncytia.

Citation:
Acknowledgment for publications should read "The following reagent was obtained through BEI Resources, NIAID, NIH: Respiratory Syncytial Virus (RSV) A2 Nucleoprotein (N) Helper Plasmid, pA2-Nopt, NR-36462."

Biosafety Level: 1
Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, and National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. 5th ed. Washington, DC: U.S. Government Printing Office, 2009; see www.cdc.gov/biosafety/publications/bmbl5/index.htm.

Disclaimers:
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References:
1. Hotard, A. L., et al. "A Stabilized Respiratory Syncytial Virus Reverse Genetics System Amendable to Recombination-Mediated Mutagenesis." Virology 434 (2012): 129-136. PubMed: 23062737.

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2. M. L. Moore, Personnel Communication.
3. Buchholz, U. J., S. Finke and K. -K. Conzelmann. "Generation of Bovine Respiratory Syncytial Virus (BRSV) from cDNA: BRSV NS2 Is Not Essential for Virus Replication in Tissue Culture, and Human RSV Leader Region Acts as a Functional BRSV Genome Promoter." J. Virol. 73 (1999): 251-259. PubMed: 9847328.

Support Provided by NIAID

Appendix I

Transfection Procedure for Virus Recovery of Recombinant Respiratory Syncytial Virus

Materials (Suggested suppliers and catalog numbers are indicated):

BHK-21 clone BSR T7/5 cell cultures or alternative cells [BHK21 cells (ATCC® CCL10™) transfected with phage T7 polymerase from Modified Vaccinia Ankara (MVA)] Note: This protocol is optimized for use with BHK-21 clone BSR T7/5 cells. Use of alternative cells may result in decreased recovery of RSV.
Opti-MEM (serum-free) (Gibco/Life Technologies catalog #11058-021)
GMEM [Glasgow's MEM (Gibco/Life Technologies catalog #11710-035)] + 3% FBS
MEM non-essential amino acids (NEAA) 100X solution (Gibco/Life Technologies catalog #11140-050)
G418 sulfate, 50 mg/mL solution (500X) (Agilent Technologies Genomics catalog # 200049)
Trypsin-EDTA (0.25%) (Gibco/Life Technologies catalog #25200-072)
Antibiotic-Antimycotic solution, penicillin/streptomycin/amphotericin (100X) (Corning cellgro® catalog #30-004-Cl) or equivalent
Plasmid with RSV antigenome (NR-36460) each vial contains 0.5 µg in 5 µL total volume (Note: This protocol requires 0.8 µg of pSynkRSV-I19F; thus 2 vials of NR-36460 are required per transfection.)
Helper Plasmids – (all codon optimized) each vial contains 0.5 µg in 5 µL total volume:
pA2-Lopt, L protein (NR-36461)
pA2-Nopt, N protein (NR-36462)
pA2-Popt, P protein (NR-36463)
pA2-M2-1opt, M2-1 protein (NR-36464)
Lipofectamine 2000 transfection reagent (Gibco/Life Technologies catalog #11668-019)
Phosphate buffered saline pH 7.2 (Gibco/Life Technologies catalog #20012027)
6-well tissue culture plates
25 cm^2 tissue culture flasks
Shaker/rocker plate
Tissue culture humidified incubator with 3% to 5% CO_2
Assorted sterile pipettes and tips

Procedure:

Note: This protocol assumes the user is familiar with cell culture techniques and transfection procedures.

1. Initial cell culture:
   a. For routine sub-passage of BSR T7/5 cells, prepare new 25 cm^2 cultures at a ratio of one donor culture to three new cultures, based on surface area of the culture flasks (1:3 passage ratio). Use GMEM with 3% FBS + 1X NEAA + 1X antibiotics as growth medium, 5 mL per flask. When maintaining donor cultures, add 1X G418 to the growth medium every other passage.
   b. For transfections, sub-pass BSR T7/5 cells from "donor" cultures into 6 well plates so they will be 100% confluent at time of transfection. Use one 25 cm^2 culture to prepare one 6 well plate (1:2.5 passage ratio).

2. Prepare 6 well plates for transfection from 25 cm^2 donor cultures. Determine how many plates will be required and use the corresponding number of flasks. Aspirate the growth medium from the flasks, and then add 0.25 mL of warm trypsin-EDTA per 25 cm^2 flask. Rock flasks to distribute the trypsin-EDTA and incubate at 37°C for 5 to10 minutes. When cells start to dislodge from the flask, add 12 mL of GMEM with 3% FBS to each flask and use a pipet to suspend the cells in this growth medium. Add 2 mL of the cell suspension to each well in the 6 well plates. Incubate the plates at 37°C in the tissue culture incubator until the cell sheets are confluent and ready for transfection.

3. Prepare the reagents for the transfection procedure. Transfection will be done using Lipofectamine 2000 as the transfection reagent. Additionally, it is important to include control transfections (Lipofectamine only/wild type virus for mutants etc.)
   a. Use a 3:1 ratio of Lipofectamine (µL) to plasmid/helper plasmid (µg). Dilute each component with Opti-MEM to make 100 µL of each. After dilution, allow each dilution to sit at room temperature for 5 minutes.
   b. Use the following amounts of each component per transfection:
      i. RSV antigenome (NR-36460) 0.8 µg (8 µL of 0.1 µg/µL) + 92 µL Opti-MEM (2 vials of NR-36460 are required per transfection.)
      ii. pA2-Lopt, L protein (NR-36461) 0.2 µg (2 µL of 0.1 µg/µL) + 98 µL Opti-MEM
      iii. pA2-Nopt, N protein (NR-36462) 0.4 µg (4 µL of 0.1 µg/µL) + 96 µL Opti-MEM
      iv. pA2-Popt, P protein (NR-36463) 0.4 µg (4 µL of 0.1 µg/µL) + 96 µL Opti-MEM
      v. pA2-M2-1opt, M2-1 protein (NR-36464) 0.4 µg (4 µL of 0.1 µg/µL) + 96 µL Opti-MEM

vi. Lipofectamine 2000 6.6 µL + 93.4 µL Opti-MEM

Note: For multiple transfections increase the above quantities proportionally.

c. After allowing the diluted components to sit at room temperature for 5 minutes, combine all six components in one vial, mix gently and incubate the transfection mixture at room temperature for 20 minutes.
d. Transfection mixtures should be 600 µL total (Opti-MEM, Lipofectin, and DNA)
e. Aspirate the media from the BSR T7/5 cell culture plate, wash cells twice with 1 mL warm Opti-MEM for each wash, and aspirate the final wash.
f. Add 600 µL transfection mixture to each well and incubate the plate 2 hours at room temperature on a shaker/rocker plate set at low speed.
g. After 2 hours, add an additional 600 µL warm Opti-MEM per well and place plate in a 37°C tissue culture incubator overnight (8-12 hours).

4. After incubation, aspirate and discard the transfection mixture from the wells, wash each well once with 1 mL warm sterile PBS, aspirate the PBS and replace with 2 mL of warm GMEM with 3% FBS per well. Continue incubating at 37°C in the tissue culture incubator overnight.

5. Day 2 post transfection, sub-pass the cells into 25 cm^2 flasks using the trypsin-EDTA procedure described above. Pass at a 1:3 surface area ratio unless cell morphology appears weak, in which case the ratio should be decreased accordingly up to an even 1:1 ratio. (Note: surface area of each well in the 6 well plate is 10 cm^2). Cells should remain in GMEM with 3% FBS throughout the rest of recovery.

6. Monitor flasks for cytopathic effect (CPE) and sub-pass at 1:3 ratio into new 25 cm^2 flasks as needed (approximately every 48 hours). CPE shows first as mini-syncytia and then grows into rounded up clumps of cells.

7. When CPE is evident throughout the flask, scrape the cells into the growth media and aliquot into cryovials. Freeze at -80°C or colder.

Appendix 2: pA2-Nopt Sequence

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1      GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATG 60
       CTGCCTAGCCCTCTAGAGGGCTAGGGGATACCACGTGAGAGTCATGTTAGACGAGACTAC

61     CCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCG 120
       GGCGTATCAATTCGGTCATAGACGAGGGACGAACACACAACCTCCAGCGACTCATCACGC

121    CGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGC 180
       GCTCGTTTTAAATTCGATGTTGTTCCGTTCCGAACTGGCTGTTAACGTACTTCTTAGACG

181    TTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATT 240
       AATCCCAATCCGCAAAACGCGACGAAGCGCTACATGCCCGGTCTATATGCGCAACTGTAA

241    GATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATA 300
       CTAATAACTGATCAATAATTATCATTAGTTAATGCCCCAGTAATCAAGTATCGGGTATAT

301    TGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACC 360
       ACCTCAAGGCGCAATGTATTGAATGCCATTTACCGGGCGGACCGACTGGCGGGTTGCTGG

361    CCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCC 420
       GGGCGGGTAACTGCAGTTATTACTGCATACAAGGGTATCATTGCGGTTATCCCTGAAAGG

421    ATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGT 480
       TAACTGCAGTTACCCACCTCATAAATGCCATTTGACGGGTGAACCGTCATGTAGTTCACA

481    ATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATT 540
       TAGTATACGGTTCATGCGGGGGATAACTGCAGTTACTGCCATTTACCGGGCGGACCGTAA

541    ATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCA 600
       TACGGGTCATGTACTGGAATACCCTGAAAGGATGAACCGTCATGTAGATGCATAATCAGT

601    TCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTG 660
       AGCGATAATGGTACCACTACGCCAAAACCGTCATGTAGTTACCCGCACCTATCGCCAAAC

661    ACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACC 720
       TGAGTGCCCCTAAAGGTTCAGAGGTGGGGTAACTGCAGTTACCCTCAAACAAAACCGTGG

721    AAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCG 780
       TTTTAGTTGCCCTGAAAGGTTTTACAGCATTGTTGAGGCGGGGTAACTGCGTTTACCCGC

781    GTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCA 840
       CATCCGCACATGCCACCCTCCAGATATATTCGTCTCGAGAGACCGATTGATCTCTTGGGT
                          T7 promoter (863,881)
                                  |
841    CTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGCTAGC 900
       GACGAATGACCGAATAGCTTTAATTATGCTGAGTGATATCCCTCTGGGTTCGACCGATCG
                               KpnI    RSV nucleoprotein(929,2104)
                                |       |
901    GTTTAAACTTAAGCTTGGTACCGCCACCATGGCCCTGAGCAAAGTGAAGCTGAACGACAC 960
       CAAATTTGAATTCGAACCATGGCGGTGGTACCGGGACTCGTTTCACTTCGACTTGCTGTG
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961   CCTGAACAAGGATCAGCTGCTGAGCAGCAGCAAGTACACCATCCAGCGGAGCACCGGCGA 1020
      GGACTTGTTCCTAGTCGACGACTCGTCGTCGTTCATGTGGTAGGTCGCCTCGTGGCCGCT

1021  CAGCATCGACACCCCCAACTACGACGTGCAGAAGCACATCAACAAGCTGTGCGGCATGCT 1080
      GTCGTAGCTGTGGGGGGTTGATGCTGCACGTCTTCGTGTAGTTGTTCGACACGCCGTACGA

1081  GCTGATCACCGAGGACGCCAACCACAAGTTCACCGGCCTGATCGGCATGCTGTACGCCAT 1140
      CGACTAGTGGCTCCTGCGGTTGGTGTTCAAGTGGCCGGACTAGCCGTACGACATGCGGTA

1141  GAGCAGACTGGGACGCGAGGACACCATCAAGATCCTGCGGGACGCCGGCTACCACGTGAA 1200
      CTCGTCTGACCCTGCGCTCCTGTGGTAGTTCTAGGACGCCCTGCGGCCGATGGTGCACTT

1201  GGCCAATGGCGTGGACGTGACCACCCACCGGCAGGACATCAACGGCAAAGAAATGAAGTT 1260
      CCGGTTACCGCACCTGCACTGGTGGGTGGCCGTCCTGTAGTTGCCGTTTCTTTACTTCAA

1261  CGAGGTGCTGACCCTGGCCAGCCTGACCACCGAGATCCAGATCAACATCGAGATCGAGAG 1320
      GCTCCACGACTGGGACCGGTCGGACTGGTGGCTCTAGGTCTAGTTGTAGCTCTAGCTCTC

1321  CCGGAAGTCCTACAAGAAAATGCTGAAAGAAATGGGCGAGGTGGCCCCCGAGTACCGGCA 1380
      GGCCTTCAGGATGTTCTTTTACGACTTTCTTTACCCGCTCCACCGGGGGCTCATGGCCGT

1381  CGATAGCCCCGACTGCGGCATGATCATCCTGTGTATCGCTGCCCTGGTGATCACAAAGCT 1440
      GCTATCGGGGCTGACGCCGTACTAGTAGGACACATAGCGACGGGACCACTAGTGTTTCGA

1441  GGCCGCTGGCGACAGATCCGGACTGACCGCTGTGATCAGACGGGCCAACAACGTGCTGAA 1500
      CCGGCGACCGCTGTCTAGGCCTGACTGGCGACACTAGTCTGCCCGGTTGTTGCACGACTT

1501  GAACGAGATGAAGCGGTACAAGGGCCTGCTGCCCAAGGATATCGCCAACAGCTTCTACGA 1560
      CTTGCTCTACTTCGCCATGTTCCCGGACGACGGGTTCCTATAGCGGTTGTCGAAGATGCT

1561  GGTGTTCGAGAAGCACCCCCACTTCATCGACGTGTTCGTGCACTTCGGAATCGCCCAGAG 1620
      CCACAAGCTCTTCGTGGGGGTGAAGTAGCTGCACAAGCACGTGAAGCCTTAGCGGGTCTC

1621  CAGCACCAGAGGCGGCAGCCGGGTGGAAGGCATCTTCGCCGGCCTGTTCATGAACGCCTA 1680
      GTCGTGGTCTCCGCCGTCGGCCCACCTTCCGTAGAAGCGGCCGGACAAGTACTTGCGGAT

1681  CGGCGCTGGCCAGGTGATGCTGAGATGGGGCGTGCTGGCCAAGAGCGTGAAGAACATCAT 1740
      GCCGCGACCGGTCCACTACGACTCTACCCCGCACGACCGGTTCTCGCACTTCTTGTAGTA

1741  GCTGGGCCACGCCAGCGTGCAGGCCGAGATGGAACAGGTGGTGGAAGTGTACGAGTACGC 1800
      CGACCCGGTGCGGTCGCACGTCCGGCTCTACCTTGTCCACCACCTTCACATGCTCATGCG

1801  CCAGAAGCTGGGCGGCGAGGCCGGCTTCTACCACATCCTGAACAACCCCAAGGCCAGCCT 1860
      GGTCTTCGACCCGCCGCTCCGGCCGAAGATGGTGTAGGACTTGTTGGGGTTCCGGTCGGA

1861  GCTGTCCCTGACCCAGTTCCCCCACTTCTCCAGCGTGGTGCTGGGCAATGCCGCCGGACT 1920
      CGACAGGGACTGGGTCAAGGGGGTGAAGAGGTCGCACCACGACCCGTTACGGCGGCCTGA

1921  GGGCATCATGGGCGAGTACAGAGGCACCCCCCGGAACCAGGACCTGTACGACGCCGCCAA 1980
      CCCGTAGTACCCGCTCATGTCTCCGTGGGGGGCCTTGGTCCTGGACATGCTGCGGCGGTT

1981  GGCCTACGCCGAGCAGCTGAAAGAAAACGGCGTGATCAACTACAGCGTGCTGGATCTGAC 2040
      CCGGATGCGGCTCGTCGACTTTCTTTTGCCGCACTAGTTGATGTCGCACGACCTAGACTG
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2041  CGCCGAGGAACTGGAAGCCATCAAGCACCAGCTGAACCCCAAGGACAACGACGTGGAACT 2100
      GCGGCTCCTTGACCTTCGGTAGTTCGTGGTCGACTTGGGGTTCCTGTTGCTGCACCTTGA
           XhoI
           |
2101  GTGACTCGAGTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAG 2160
      CACTGAGCTCAGATCTCCCGGGCAAATTTGGGCGACTAGTCGGAGCTGACACGGAAGATC

2161  TTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCAC 2220
      AACGGTCGGTAGACAACAAACGGGGAGGGGGCACGGAAGGAACTGGGACCTTCCACGGTG

2221  TCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCA 2280
      AGGGTGACAGGAAAGGATTATTTTACTCCTTTAACGTAGCGTAACAGACTCATCCACAGT

2281  TTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAG 2340
      AAGATAAGACCCCCCACCCCACCCCGTCCTGTCGTTCCCCCTCCTAACCCTTCTGTTATC

2341  CAGGCATGCTGGGGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGG 2400
      GTCCGTACGACCCCTACGCCACCCGAGATACCGAAGACTCCGCCTTTCTTGGTCGACCCC
                                  f1 origin(2435,2741)
                                  |
2401  CTCTAGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGT 2460
      GAGATCCCCCATAGGGGTGCGCGGGACATCGCCGCGTAATTCGCGCCGCCCACACCACCA

2461  TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTT 2520
      ATGCGCGTCGCACTGGCGATGTGAACGGTCGCGGGATCGCGGGCGAGGAAAGCGAAAGAA

2521  CCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCC 2580
      GGGAAGGAAAGAGCGGTGCAAGCGGCCGAAAGGGGCAGTTCGAGATTTAGCCCCCGAGGG

2581  TTTAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGA 2640
      AAATCCCAAGGCTAAATCACGAAATGCCGTGGAGCTGGGGTTTTTTGAACTAATCCCACT

2641  TGGTTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTC 2700
      ACCAAGTGCATCACCCGGTAGCGGGACTATCTGCCAAAAAGCGGGAAACTGCAACCTCAG

2701  CACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGT 2760
      GTGCAAGAAATTATCACCTGAGAACAAGGTTTGACCTTGTTGTGAGTTGGGATAGAGCCA

2761  CTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCT 2820
      GATAAGAAAACTAAATATTCCCTAAAACGGCTAAAGCCGGATAACCAATTTTTTACTCGA
                                                SV40 promoter(2873,3194)
                                                |
2821  GATTTAACAAAAATTTAACGCGAATTAATTCTGTGGAATGTGTGTCAGTTAGGGTGTGGA 2880
      CTAAATTGTTTTTAAATTGCGCTTAATTAAGACACCTTACACACAGTCAATCCCACACCT

2881  AAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCA 2940
      TTCAGGGGTCCGAGGGGTCGTCCGTCTTCATACGTTTCGTACGTAGAGTTAATCAGTCGT

2941  ACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTC 3000
      TGGTCCACACCTTTCAGGGGTCCGAGGGGTCGTCCGTCTTCATACGTTTCGTACGTAGAG
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SV40 origin(3040,3117)

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3001  AATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCC 3060
      TTAATCAGTCGTTGGTATCAGGGCGGGGATTGAGGCGGGTAGGGCGGGGATTGAGGCGGG

3061  AGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAG 3120
      TCAAGGCGGGTAAGAGGCGGGGTACCGACTGATTAAAAAAAATAAATACGTCTCCGGCTC

3121  GCCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGC 3180
      CGGCGGAGACGGAGACTCGATAAGGTCTTCATCACTCCTCCGAAAAAACCTCCGGATCCG

3181  TTTTGCAAAAAGCTCCCGGGAGCTTGTATATCCATTTTCGGATCTGATCAAGAGACAGGA 3240
      AAAACGTTTTTCGAGGGCCCTCGAACATATAGGTAAAAGCCTAGACTAGTTCTCTGTCCT
```

Neomycin^{R} (3256,4050)

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3241  TGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGG 3300
      ACTCCTAGCAAAGCGTACTAACTTGTTCTACCTAACGTGCGTCCAAGAGGCCGGCGAACC

3301  GTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCC 3360
      CACCTCTCCGATAAGCCGATACTGACCCGTGTTGTCTGTTAGCCGACGAGACTACGGCGG

3361  GTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGT 3420
      CACAAGGCCGACAGTCGCGTCCCCGCGGGCCAAGAAAAACAGTTCTGGCTGGACAGGCCA

3421  GCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGCGTT 3480
      CGGGACTTACTTGACGTCCTGCTCCGTCGCGCCGATAGCACCGACCGGTGCTGCCCGCAA

3481  CCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGGC 3540
      GGAACGCGTCGACACGAGCTGCAACAGTGACTTCGCCCTTCCCTGACCGACGATAACCCG

3541  GAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATC 3600
      CTTCACGGCCCCGTCCTAGAGGACAGTAGAGTGGAACGAGGACGGCTCTTTCATAGGTAG

3601  ATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCAC 3660
      TACCGACTACGTTACGCCGCCGACGTATGCGAACTAGGCCGATGGACGGGTAAGCTGGTG

3661  CAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAG 3720
      GTTCGCTTTGTAGCGTAGCTCGCTCGTGCATGAGCCTACCTTCGGCCAGAACAGCTAGTC

3721  GATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTCGCCAGGCTCAAG 3780
      CTACTAGACCTGCTTCTCGTAGTCCCCGAGCGCGGTCGGCTTGACAAGCGGTCCGAGTTC

3781  GCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAAT 3840
      CGCGCGTACGGGCTGCCGCTCCTAGAGCAGCACTGGGTACCGCTACGGACGAACGGCTTA

3841  ATCATGGTGGAAAATGGCCGCTTTTCTGGATTCATCGACTGTGGCCGGCTGGGTGTGGCG 3900
      TAGTACCACCTTTTACCGGCGAAAAGACCTAAGTAGCTGACACCGGCCGACCCACACCGC

3901  GACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAA 3960
      CTGGCGATAGTCCTGTATCGCAACCGATGGGCACTATAACGACTTCTCGAACCGCCGCTT

3961  TGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTCGCAGCGCATCGCC 4020
      ACCCGACTGGCGAAGGAGCACGAAATGCCATAGCGGCGAGGGCTAAGCGTCGCGTAGCGG
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4021  TTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACC 4080
      AAGATAGCGGAAGAACTGCTCAAGAAGACTCGCCCTGAGACCCCAAGCTTTACTGGCTGG

4081  AAGCGACGCCCAACCTGCCATCACGAGATTTCGATTCCACCGCCGCCTTCTATGAAAGGT 4140
      TTCGCTGCGGGTTGGACGGTAGTGCTCTAAAGCTAAGGTGGCGGCGGAAGATACTTTCCA

4141  TGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCA 4200
      ACCCGAAGCCTTAGCAAAAGGCCCTGCGGCCGACCTACTAGGAGGTCGCGCCCCTAGAGT

4201  TGCTGGAGTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAA 4260
      ACGACCTCAAGAAGCGGGTGGGGTTGAACAAATAACGTCGAATATTACCAATGTTTATTT

4261  GCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTT 4320
      CGTTATCGTAGTGTTTAAAGTGTTTATTTCGTAAAAAAAGTGACGTAAGATCAACACCAA

4321  TGTCCAAACTCATCAATGTATCTTATCATGTCTGTATACCGTCGACCTCTAGCTAGAGCT 4380
      ACAGGTTTGAGTAGTTACATAGAATAGTACAGACATATGGCAGCTGGAGATCGATCTCGA

4381  TGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCAC 4440
      ACCGCATTAGTACCAGTATCGACAAAGGACACACTTTAACAATAGGCGAGTGTTAAGGTG
    lac promoter(4443,4472)
       |
4441  ACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAAC 4500
      TGTTGTATGCTCGGCCTTCGTATTTCACATTTCGGACCCCACGGATTACTCACTCGATTG

4501  TCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC 4560
      AGTGTAATTAACGCAACGCGAGTGACGGGCGAAAGGTCAGCCCTTTGGACAGCACGGTCG

4561  TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCG 4620
      ACGTAATTACTTAGCCGGTTGCGCGCCCCTCTCCGCCAAACGCATAACCCGCGAGAAGGC

4621  CTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTC 4680
      GAAGGAGCGAGTGACTGAGCGACGCGAGCCAGCAAGCCGACGCCGCTCGCCATAGTCGAG

4681  ACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGT 4740
      TGAGTTTCCGCCATTATGCCAATAGGTGTCTTAGTCCCCTATTGCGTCCTTTCTTGTACA
                                              pBR322 origin(4781,5397)
                                                      |
4741  GAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC 4800
      CTCGTTTTCCGGTCGTTTTCCGGTCCTTGGCATTTTTCCGGCGCAACGACCGCAAAAAGG

4801  ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAA 4860
      TATCCGAGGCGGGGGGACTGCTCGTAGTGTTTTTAGCTGCGAGTTCAGTCTCCACCGCTT

4861  ACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTC 4920
      TGGGCTGTCCTGATATTTCTATGGTCCGCAAAGGGGGACCTTCGAGGGAGCACGCGAGAG

4921  CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGG 4980
      GACAAGGCTGGGACGGCGAATGGCCTATGGACAGGCGGAAAGAGGGAAGCCCTTCGCACC

4981  CGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGC 5040
      GCGAAAGAGTATCGAGTGCGACATCCATAGAGTCAAGCCACATCCAGCAAGCGAGGTTCG
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5041  TGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATC 5100
      ACCCGACACACGTGCTTGGGGGGCAAGTCGGGCTGGCGACGCGGAATAGGCCATTGATAG

5101  GTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACA 5160
      CAGAACTCAGGTTGGGCCATTCTGTGCTGAATAGCGGTGACCGTCGTCGGTGACCATTGT

5161  GGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACT 5220
      CCTAATCGTCTCGCTCCATACATCCGCCACGATGTCTCAAGAACTTCACCACCGGATTGA

5221  ACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCG 5280
      TGCCGATGTGATCTTCTTGTCATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGC

5281  GAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTTTTTTTG 5340
      CTTTTTCTCAACCATCGAGAACTAGGCCGTTTGTTTGGTGGCGACCATCGCCAAAAAAAC

5341  TTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTT 5400
      AAACGTTCGTCGTCTAATGCGCGTCTTTTTTTCCTAGAGTTCTTCTAGGAAACTAGAAAA

5401  CTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGAT 5460
      GATGCCCCAGACTGCGAGTCACCTTGCTTTTGAGTGCAATTCCCTAAAACCAGTACTCTA

5461  TATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCT 5520
      ATAGTTTTTCCTAGAAGTGGATCTAGGAAAATTTAATTTTTACTTCAAAATTTAGTTAGA
                                Ampillin^R (5552,6412)
                                        |
5521  AAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTA 5580
      TTTCATATATACTCATTTGAACCAGACTGTCAATGGTTACGAATTAGTCACTCCGTGGAT

5581  TCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAA 5640
      AGAGTCGCTAGACAGATAAAGCAAGTAGGTATCAACGGACTGAGGGGCAGCACATCTATT

5641  CTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCAC 5700
      GATGCTATGCCCTCCCGAATGGTAGACCGGGGTCACGACGTTACTATGGCGCTCTGGGTG

5701  GCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAA 5760
      CGAGTGGCCGAGGTCTAAATAGTCGTTATTTGGTCGGTCGGCCTTCCCGGCTCGCGTCTT

5761  GTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAG 5820
      CACCAGGACGTTGAAATAGGCGGAGGTAGGTCAGATAATTAACAACGGCCCTTCGATCTC

5821  TAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGG 5880
      ATTCATCAAGCGGTCAATTATCAAACGCGTTGCAACAACGGTAACGATGTCCGTAGCACC

5881  TGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAG 5940
      ACAGTGCGAGCAGCAAACCATACCGAAGTAAGTCGAGGCCAAGGGTTGCTAGTTCCGCTC

5941  TTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTG 6000
      AATGTACTAGGGGGTACAACACGTTTTTTCGCCAATCGAGGAAGCCAGGAGGCTAGCAAC

6001  TCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTC 6060
      AGTCTTCATTCAACCGGCGTCACAATAGTGAGTACCAATACCGTCGTGACGTATTAAGAG
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6061  TTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCAT 6120
      AATGACAGTACGGTAGGCATTCTACGAAAAGACACTGACCACTCATGAGTTGGTTCAGTA

6121  TCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATA 6180
      AGACTCTTATCACATACGCCGCTGGCTCAACGAGAACGGGCCGCAGTTATGCCCTATTAT

6181  CCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAA 6240
      GGCGCGGTGTATCGTCTTGAAATTTTCACGAGTAGTAACCTTTTGCAAGAAGCCCCGCTT

6241  AACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCA 6300
      TTGAGAGTTCCTAGAATGGCGACAACTCTAGGTCAAGCTACATTGGGTGAGCACGTGGGT

6301  ACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGC 6360
      TGACTAGAAGTCGTAGAAAATGAAAGTGGTCGCAAAGACCCACTCGTTTTTGTCCTTCCG

6361  AAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCC 6420
      TTTTACGGCGTTTTTTCCCTTATTCCCGCTGTGCCTTTACAACTTATGAGTATGAGAAGG

6421  TTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTG 6480
      AAAAAGTTATAATAACTTCGTAAATAGTCCCAATAACAGAGTACTCGCCTATGTATAAAC

6481  AATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC 6540
      TTACATAAATCTTTTTATTTGTTTATCCCCAAGGCGCGTGTAAAGGGGCTTTTCACGGTG

6541  CTGACGTC 6548
      GACTGCAG
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