Respiratory Syncytial Virus (RSV) A2 Phosphoprotein (P) Helper Plasmid, pA2-Popt

Catalog No. NR-36463

For research use only. Not for human use.

Contributor:
BEI Resources

Manufacturer:
Martin L. Moore, Assistant Professor, Department of Pediatrics, Division of Infectious Diseases, Emory University School of Medicine, Atlanta, Georgia, USA

Product Description:
NR-36463 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 P helper plasmid was constructed from codon-optimized RSV A2 P 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-Popt 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-36463 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 Phosphoprotein (P) Helper Plasmid, pA2-Popt, NR-36463."

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-Popt 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 phosphoprotein (929,1654)
                                |        |
901    GTTTAAACTTAAGCTTGGTACCGCCACCATGGAAAAGTTCGCCCCCGAGTTCCACGGCGA 960
       CAAATTTGAATTCGAACCATGGCGGTGGTACCTTTTCAAGCGGGGGCTCAAGGTGCCGCT
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961   GGACGCCAACAACCGGGCCACCAAGTTTCTGGAATCCATCAAGGGCAAGTTCACCAGCCC 1020
      CCTGCGGTTGTTGGCCCGGTGGTTCAAAGACCTTAGGTAGTTCCCGTTCAAGTGGTCGGG

1021  CAAGGACCCCAAGAAGAAGGACAGCATCATCAGCGTGAACAGCATCGACATCGAAGTGAC 1080
      GTTCCTGGGGTTCTTCTTCCTGTCGTAGTAGTCGCACTTGTCGTAGCTGTAGCTTCACTG

1081  CAAAGAGAGCCCCATCACCAGCAACAGCACCATCATCAACCCCACCAACGAGACAGACGA 1140
      GTTTCTCTCGGGGTAGTGGTCGTTGTCGTGGTAGTAGTTGGGGTGGTTGCTCTGTCTGCT

1141  CACCGCCGGCAACAAGCCCAACTACCAGCGGAAGCCCCTGGTGTCCTTCAAAGAGGACCC 1200
      GTGGCGGCCGTTGTTCGGGTTGATGGTCGCCTTCGGGGACCACAGGAAGTTTCTCCTGGG

1201  CACCCCCAGCGACAACCCCTTCAGCAAGCTGTACAAAGAGACAATCGAGACATTCGACAA 1260
      GTGGGGGTCGCTGTTGGGGAAGTCGTTCGACATGTTTCTCTGTTAGCTCTGTAAGCTGTT

1261  CAACGAGGAAGAGAGCAGCTACAGCTACGAGGAAATCAACGACCAGACCAACGACAACAT 1320
      GTTGCTCCTTCTCTCGTCGATGTCGATGCTCCTTTAGTTGCTGGTCTGGTTGCTGTTGTA

1321  CACCGCCAGACTGGACCGGATCGACGAGAAGCTGAGCGAGATCCTGGGCATGCTGCACAC 1380
      GTGGCGGTCTGACCTGGCCTAGCTGCTCTTCGACTCGCTCTAGGACCCGTACGACGTGTG

1381  CCTGGTGGTGGCCTCTGCCGGCCCTACAAGCGCCAGAGATGGCATCCGGGACGCCATGAT 1440
      GGACCACCACCGGAGACGGCCGGGATGTTCGCGGTCTCTACCGTAGGCCCTGCGGTACTA

1441  CGGCCTGCGGGAAGAGATGATCGAGAAGATCCGGACCGAGGCCCTGATGACCAACGACCG 1500
      GCCGGACGCCCTTCTCTACTAGCTCTTCTAGGCCTGGCTCCGGGACTACTGGTTGCTGGC

1501  GCTGGAAGCCATGGCCCGGCTGCGGAACGAGGAATCCGAGAAGATGGCCAAGGACACCAG 1560
      CGACCTTCGGTACCGGGCCGACGCCTTGCTCCTTAGGCTCTTCTACCGGTTCCTGTGGTC

1561  CGACGAGGTGTCCCTGAACCCCACCTCTGAGAAGCTGAACAACCTGCTGGAAGGCAACGA 1620
      GCTGCTCCACAGGGACTTGGGGTGGAGACTCTTCGACTTGTTGGACGACCTTCCGTTGCT
                                        XhoI
                                         |
1621  CAGCGACAACGACCTGAGCCTGGAAGATTTCTGACTCGAGTCTAGAGGGCCCGTTTAAAC 1680
      GTCGCTGTTGCTGGACTCGGACCTTCTAAAGACTGAGCTCAGATCTCCCGGGCAAATTTG

1681  CCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCC 1740
      GGCGACTAGTCGGAGCTGACACGGAAGATCAACGGTCGGTAGACAACAAACGGGGAGGGG

1741  CGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGA 1800
      GCACGGAAGGAACTGGGACCTTCCACGGTGAGGGTGACAGGAAAGGATTATTTTACTCCT

1801  AATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGA 1860
      TTAACGTAGCGTAACAGACTCATCCACAGTAAGATAAGACCCCCCACCCCACCCCGTCCT

1861  CAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTAT 1920
      GTCGTTCCCCCTCCTAACCCTTCTGTTATCGTCCGTACGACCCCTACGCCACCCGAGATA

1921  GGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCCACGCGCCCTGTAG 1980
      CCGAAGACTCCGCCTTTCTTGGTCGACCCCGAGATCCCCCATAGGGGTGCGCGGGACATC

         f1 origin (1985,2291)
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        |
1981  CGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAG 2040
      GCCGCGTAATTCGCGCCGCCCACACCACCAATGCGCGTCGCACTGGCGATGTGAACGGTC

2041  CGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTT 2100
      GCGGGATCGCGGGCGAGGAAAGCGAAAGAAGGGAAGGAAAGAGCGGTGCAAGCGGCCGAA

2101  TCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCA 2160
      AGGGGCAGTTCGAGATTTAGCCCCCGAGGGAAATCCCAAGGCTAAATCACGAAATGCCGT

2161  CCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATA 2220
      GGAGCTGGGGTTTTTTGAACTAATCCCACTACCAAGTGCATCACCCGGTAGCGGGACTAT

2221  GACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCA 2280
      CTGCCAAAAAGCGGGAAACTGCAACCTCAGGTGCAAGAAATTATCACCTGAGAACAAGGT

2281  AACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCC 2340
      TTGACCTTGTTGTGAGTTGGGATAGAGCCAGATAAGAAAACTAAATATTCCCTAAAACGG

2341  GATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTAATT 2400
      CTAAAGCCGGATAACCAATTTTTTACTCGACTAAATTGTTTTTAAATTGCGCTTAATTAA
                        SV40 promoter (2423,2744)
                              |
2401  CTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGT 2460
      GACACCTTACACACAGTCAATCCCACACCTTTCAGGGGTCCGAGGGGTCGTCCGTCTTCA

2461  ATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCA 2520
      TACGTTTCGTACGTAGAGTTAATCAGTCGTTGGTCCACACCTTTCAGGGGTCCGAGGGGT

2521  GCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTA 2580
      CGTCCGTCTTCATACGTTTCGTACGTAGAGTTAATCAGTCGTTGGTATCAGGGCGGGGAT
                SV40 origin (2590,2667)
                |
2581  ACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGA 2640
      TGAGGCGGGTAGGGCGGGGATTGAGGCGGGTCAAGGCGGGTAAGAGGCGGGGTACCGACT

2641  CTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGCCTCTGAGCTATTCCAGAAG 2700
      GATTAAAAAAAATAAATACGTCTCCGGCTCCGGCGGAGACGGAGACTCGATAAGGTCTTC

2701  TAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTCCCGGGAGCTTGTATA 2760
      ATCACTCCTCCGAAAAAACCTCCGGATCCGAAAACGTTTTTCGAGGGCCCTCGAACATAT
                                                  neomycin^R (2806,3600)
                                                    |
2761  TCCATTTTCGGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGAT 2820
      AGGTAAAAGCCTAGACTAGTTCTCTGTCCTACTCCTAGCAAAGCGTACTAACTTGTTCTA

2821  GGATTGCACGCAGGTTCTCCGGCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCA 2880
      CCTAACGTGCGTCCAAGAGGCCGGCGAACCCACCTCTCCGATAAGCCGATACTGACCCGT

2881  CAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCG 2940
      GTTGTCTGTTAGCCGACGAGACTACGGCGGCACAAGGCCGACAGTCGCGTCCCCGCGGGC
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2941  GTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCG 3000
      CAAGAAAAACAGTTCTGGCTGGACAGGCCACGGGACTTACTTGACGTCCTGCTCCGTCGC

3001  CGGCTATCGTGGCTGGCCACGACGGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACT 3060
      GCCGATAGCACCGACCGGTGCTGCCCGCAAGGAACGCGTCGACACGAGCTGCAACAGTGA

3061  GAAGCGGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCT 3120
      CTTCGCCCTTCCCTGACCGACGATAACCCGCTTCACGGCCCCGTCCTAGAGGACAGTAGA

3121  CACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACG 3180
      GTGGAACGAGGACGGCTCTTTCATAGGTAGTACCGACTACGTTACGCCGCCGACGTATGC

3181  CTTGATCCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGT 3240
      GAACTAGGCCGATGGACGGGTAAGCTGGTGGTTCGCTTTGTAGCGTAGCTCGCTCGTGCA

3241  ACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTC 3300
      TGAGCCTACCTTCGGCCAGAACAGCTAGTCCTACTAGACCTGCTTCTCGTAGTCCCCGAG

3301  GCGCCAGCCGAACTGTTCGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTC 3360
      CGCGGTCGGCTTGACAAGCGGTCCGAGTTCCGCGCGTACGGGCTGCCGCTCCTAGAGCAG

3361  GTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCCGCTTTTCTGGA 3420
      CACTGGGTACCGCTACGGACGAACGGCTTATAGTACCACCTTTTACCGGCGAAAAGACCT

3421  TTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACC 3480
      AAGTAGCTGACACCGGCCGACCCACACCGCCTGGCGATAGTCCTGTATCGCAACCGATGG

3481  CGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGT 3540
      GCACTATAACGACTTCTCGAACCGCCGCTTACCCGACTGGCGAAGGAGCACGAAATGCCA

3541  ATCGCCGCTCCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGA 3600
      TAGCGGCGAGGGCTAAGCGTCGCGTAGCGGAAGATAGCGGAAGAACTGCTCAAGAAGACT

3601  GCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGAGATT 3660
      CGCCCTGAGACCCCAAGCTTTACTGGCTGGTTCGCTGCGGGTTGGACGGTAGTGCTCTAA

3661  TCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCG 3720
      AGCTAAGGTGGCGGCGGAAGATACTTTCCAACCCGAAGCCTTAGCAAAAGGCCCTGCGGC

3721  GCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCAACTTGT 3780
      CGACCTACTAGGAGGTCGCGCCCCTAGAGTACGACCTCAAGAAGCGGGTGGGGTTGAACA

3781  TTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAG 3840
      AATAACGTCGAATATTACCAATGTTTATTTCGTTATCGTAGTGTTTAAAGTGTTTATTTC

3841  CATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTATCATG 3900
      GTAAAAAAAGTGACGTAAGATCAACACCAAACAGGTTTGAGTAGTTACATAGAATAGTAC

3901  TCTGTATACCGTCGACCTCTAGCTAGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTG 3960
      AGACATATGGCAGCTGGAGATCGATCTCGAACCGCATTAGTACCAGTATCGACAAAGGAC
                                    lac promoter (3993,4022)
                                     |
3961  TGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTA 4020
      ACACTTTAACAATAGGCGAGTGTTAAGGTGTGTTGTATGCTCGGCCTTCGTATTTCACAT
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4021  AAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCG 4080
      TTCGGACCCCACGGATTACTCACTCGATTGAGTGTAATTAACGCAACGCGAGTGACGGGC

4081  CTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGA 4140
      GAAAGGTCAGCCCTTTGGACAGCACGGTCGACGTAATTACTTAGCCGGTTGCGCGCCCCT

4141  GAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGG 4200
      CTCCGCCAAACGCATAACCCGCGAGAAGGCGAAGGAGCGAGTGACTGAGCGACGCGAGCC

4201  TCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAG 4260
      AGCAAGCCGACGCCGCTCGCCATAGTCGAGTGAGTTTCCGCCATTATGCCAATAGGTGTC

4261  AATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACC 4320
      TTAGTCCCCTATTGCGTCCTTTCTTGTACACTCGTTTTCCGGTCGTTTTCCGGTCCTTGG
              pBR322 origin (4331,4947)
                |
4321  GTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACA 4380
      CATTTTTCCGGCGCAACGACCGCAAAAAGGTATCCGAGGCGGGGGGACTGCTCGTAGTGT

4381  AAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGT 4440
      TTTTAGCTGCGAGTTCAGTCTCCACCGCTTTGGGCTGTCCTGATATTTCTATGGTCCGCA

4441  TTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACC 4500
      AAGGGGGACCTTCGAGGGAGCACGCGAGAGGACAAGGCTGGGACGGCGAATGGCCTATGG

4501  TGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATC 4560
      ACAGGCGGAAAGAGGGAAGCCCTTCGCACCGCGAAAGAGTATCGAGTGCGACATCCATAG

4561  TCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGC 4620
      AGTCAAGCCACATCCAGCAAGCGAGGTTCGACCCGACACACGTGCTTGGGGGGCAAGTCG

4621  CCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACT 4680
      GGCTGGCGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTCTGTGCTGA

4681  TATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTG 4740
      ATAGCGGTGACCGTCGTCGGTGACCATTGTCCTAATCGTCTCGCTCCATACATCCGCCAC

4741  CTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTA 4800
      GATGTCTCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTCTTGTCATAAACCAT

4801  TCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCA 4860
      AGACGCGAGACGACTTCGGTCAATGGAAGCCTTTTTCTCAACCATCGAGAACTAGGCCGT

4861  AACAAACCACCGCTGGTAGCGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAA 4920
      TTGTTTGGTGGCGACCATCGCCAAAAAAACAAACGTTCGTCGTCTAATGCGCGTCTTTTT

4921  AAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAA 4980
      TTCCTAGAGTTCTTCTAGGAAACTAGAAAAGATGCCCCAGACTGCGAGTCACCTTGCTTT

4981  ACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTT 5040
      TGAGTGCAATTCCCTAAAACCAGTACTCTAATAGTTTTTCCTAGAAGTGGATCTAGGAAA
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5041  TAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACA 5100
      ATTTAATTTTTACTTCAAAATTTAGTTAGATTTCATATATACTCATTTGAACCAGACTGT
      ampicillin^R (5102,5962)
       |
5101  GTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCA 5160
      CAATGGTTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAAAGCAAGTAGGT

5161  TAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCC 5220
      ATCAACGGACTGAGGGGCAGCACATCTATTGATGCTATGCCCTCCCGAATGGTAGACCGG

5221  CCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAA 5280
      GGTCACGACGTTACTATGGCGCTCTGGGTGCGAGTGGCCGAGGTCTAAATAGTCGTTATT

5281  ACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCC 5340
      TGGTCGGTCGGCCTTCCCGGCTCGCGTCTTCACCAGGACGTTGAAATAGGCGGAGGTAGG

5341  AGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCA 5400
      TCAGATAATTAACAACGGCCCTTCGATCTCATTCATCAAGCGGTCAATTATCAAACGCGT

5401  ACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCAT 5460
      TGCAACAACGGTAACGATGTCCGTAGCACCACAGTGCGAGCAGCAAACCATACCGAAGTA

5461  TCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAG 5520
      AGTCGAGGCCAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGGGTACAACACGTTTTTTC

5521  CGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCAC 5580
      GCCAATCGAGGAAGCCAGGAGGCTAGCAACAGTCTTCATTCAACCGGCGTCACAATAGTG

5581  TCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTT 5640
      AGTACCAATACCGTCGTGACGTATTAAGAGAATGACAGTACGGTAGGCATTCTACGAAAA

5641  CTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTT 5700
      GACACTGACCACTCATGAGTTGGTTCAGTAAGACTCTTATCACATACGCCGCTGGCTCAA

5701  GCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGC 5760
      CGAGAACGGGCCGCAGTTATGCCCTATTATGGCGCGGTGTATCGTCTTGAAATTTTCACG

5761  TCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGAT 5820
      AGTAGTAACCTTTTGCAAGAAGCCCCGCTTTTGAGAGTTCCTAGAATGGCGACAACTCTA

5821  CCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCA 5880
      GGTCAAGCTACATTGGGTGAGCACGTGGGTTGACTAGAAGTCGTAGAAAATGAAAGTGGT

5881  GCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGA 5940
      CGCAAAGACCCACTCGTTTTTGTCCTTCCGTTTTACGGCGTTTTTTCCCTTATTCCCGCT

5941  CACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGG 6000
      GTGCCTTTACAACTTATGAGTATGAGAAGGAAAAAGTTATAATAACTTCGTAAATAGTCC
        ampicillin promoter (6004,6032)
          |
6001  GTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGG 6060
      CAATAACAGAGTACTCGCCTATGTATAAACTTACATAAATCTTTTTATTTGTTTATCCCC
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6061  TTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC 6098
      AAGGCGCGTGTAAAGGGGCTTTTCACGGTGGACTGCAG
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