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A stabilized respiratory syncytial virus reverse genetics system amenable to recombination-mediated mutagenesis

Anne L. Hotard [a,b], Fyza Y. Shaikh [c], Sujin Lee [a,b], Dan Yan [a,b], Michael N. Teng [e], Richard K. Plemper [a,b], James E. Crowe Jr. [c,d], Martin L. Moore [a,b,*]

[a] Department of Pediatrics, Emory University, Atlanta, GA 30322, USA
[b] Children's Healthcare of Atlanta, Atlanta, GA 30322, USA
[c] Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[d] Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN 37232, USA
[e] Division of Allergy and Immunology, Department of Internal Medicine, University of South Florida College of Medicine, Tampa, FL 33612, USA

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ABSTRACT

We describe the first example of combining bacterial artificial chromosome (BAC) recombination-mediated mutagenesis with reverse genetics for a negative strand RNA virus. A BAC-based respiratory syncytial virus (RSV) rescue system was established. An important advantage of this system is that RSV antigenomic cDNA was stabilized in the BAC vector. The RSV genotype chosen was A2-line19F, a chimeric strain previously shown to recapitulate in mice key features of RSV pathogenesis. We recovered two RSV reporter viruses, one expressing the red fluorescent protein monomeric Katushka 2 (A2-K-line19F) and one expressing *Renilla* luciferase (A2-RL-line19F). As proof of principle, we efficiently generated a RSV gene deletion mutant (A2-line19FΔNS1/NS2) and a point mutant (A2-K-line19F-I557V) by recombination-mediated BAC mutagenesis. Together with sequence-optimized helper expression plasmids, BAC-RSV is a stable, versatile, and efficient reverse genetics platform for generation of a recombinant *Pneumovirus*.

Introduction

Respiratory syncytial virus (RSV) is the most important lower respiratory tract pathogen of infants, but there are no effective vaccines or antivirals in use. RSV is a member of the *Paramyxoviridae* family, genus *Pneumovirinae*, containing a non-segmented, negative-sense RNA genome (Collins and Crowe, 2007). Plasmid-based reverse genetics and virus rescue is a cornerstone of RSV research and vaccinology (Collins et al., 1995; Karron et al., 2005). However, plasmid-based virus rescue systems for the *Pneumovirinae* are plagued by cDNA instability (Biacchesi et al., 2007; Moore et al., 2009). RSV rescue involves co-transfection of an antigenomic cDNA plasmid in addition to four expression plasmids encoding the RSV nucleoprotein (N), phosphoprotein (P), large polymerase (L), and matrix 2-1 protein (M2-1) (Collins et al., 1995; Jin et al., 1998). Current published rescue cDNA plasmids encode antigenome of the reference A2 strain of RSV (Collins et al., 1995; Jin et al., 1998). The proteins expressed from the helper plasmids also are based on the genes from strain A2 (Collins et al., 1995; Jin et al., 1998). We chose RSV strain A2-line19F for reverse genetics because infection with this chimeric strain better recapitulates RSV pathogenesis in the mouse model (Moore et al., 2009). We chose a bacterial artificial chromosome (BAC) as the backbone vector because BACs and other low copy vectors enhance stability of cDNAs that are difficult to clone (Almazan et al., 2000; Fan and Bird, 2008; Hall et al., 2012; Yun et al., 2003; Zhou et al., 2011). Our BAC-RSV was used successfully to directly transfect mammalian cells and recover recombinant virus. We utilized this new BAC-RSV construct for recombination-mediated genetic engineering to generate RSV mutants (Fig. 1). This BAC-RSV mutagenesis system provides an improved platform for RSV reverse genetics that could be applied to other RNA viruses as well.

Results

Sequence-optimized RSV N, P, M2-1, and L genes direct greater RSV transcription than wild-type RSV genes

Efficient recombinant RSV recovery requires co-transfection with helper plasmids encoding the RSV N, P, M2-1, and L proteins

* Corresponding author at: Division of Pediatric Infectious Diseases, Emory University School of Medicine, 2015 Uppergate Dr NE Room 514, Atlanta, GA 30322, USA. Fax: +1 404 727 9223.
E-mail address: martin.moore@emory.edu (M.L. Moore).

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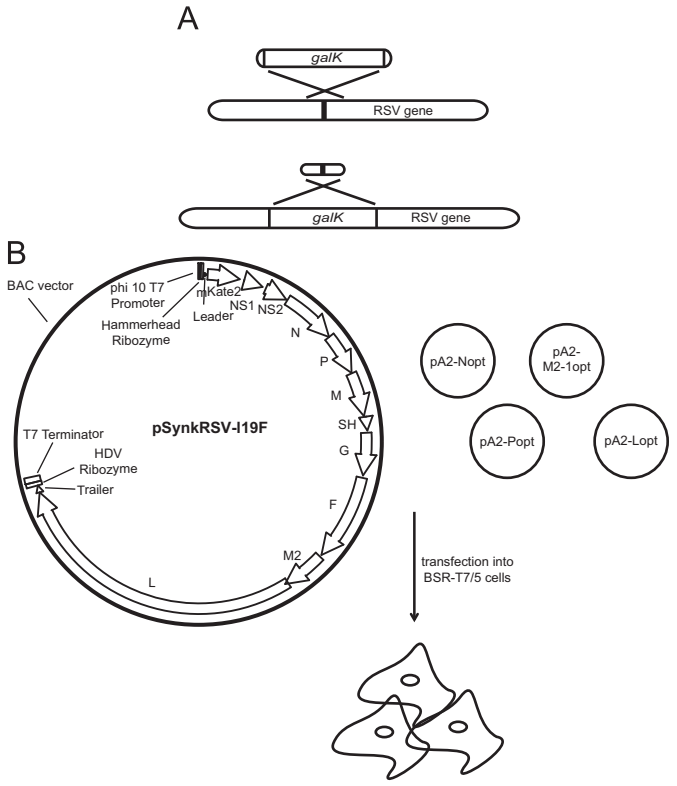

Fig. 1. Model of RSV-BAC recombination-mediated mutagenesis and reverse genetics. (A) First recombination step in which a double stranded PCR product containing the *galK* cassette recombines with RSV antigenome sequence, based on 50 base-pair homology sequence flanking *galK*. In the second step, annealed oligonucleotides containing the desired mutation flanked by the same 50 base-pair homology arms recombines with the RSV antigenome to displace galK. (B) RSV reverse genetics scheme. The RSV-BAC and sequence-optimized helper plasmids encoding RSV N, P, M2-1, and L are transfected into BSR-T7/5 cells, and infectious virus is recovered.

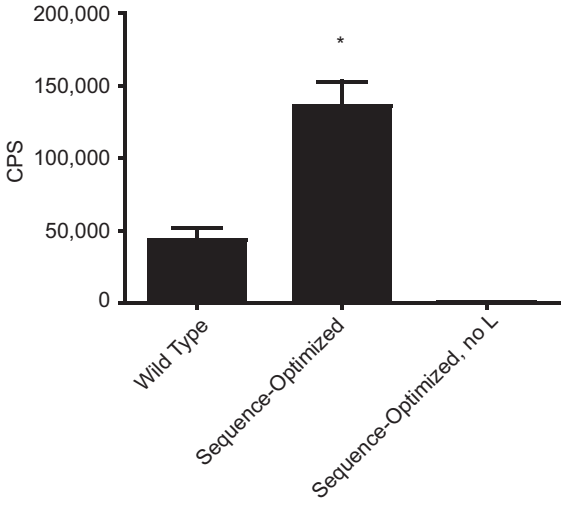

Fig. 2. Sequence-optimized RSV helper plasmids drive more minigenome activity than wild-type RSV helper plasmids. Minigenome activity driven by wild-type or sequence-optimized helper plasmids. Results are from transfection using 0.8, 0.4, 0.4, 0.4, and 0.2 μg of minigenome, N, P, M2-1, and L respectively. Data are shown as mean ± SEM. $P < 0.05$ by ANOVA of sequence-optimized relative to other conditions.

(Buchholz et al., 1999; Collins et al., 1995; Jin et al., 1998). Sequence optimization of RSV cDNA for human codon bias and other optimal features enhances cDNA expression in mammalian cells (Huang et al., 2010). Plasmids encoding the wild-type (non-optimized) N, P, M2-1, and L genes have been used previously to drive minigenome expression (Grosfeld et al., 1995). Using an RSV-luciferase minigenome assay in BSR-T7/5 cells (Dochow et al., 2012), we compared the activity of these wild-type helper plasmids to the activity of plasmids that encode sequence-optimized N, P, M2-1, and L genes (Dochow et al., 2012). Compared to wild-type RSV cDNAs, the sequence-optimized helper plasmids yielded approximately three fold higher polymerase activity, based on a RSV minigenome containing a luciferase reporter gene (Fig. 2).

Generation of BAC-RSV reporter viruses

Antigenomic cDNA of A2-line19F was synthesized in three segments. We cloned the three segments sequentially into the BAC vector pKBS2 to generate the plasmid pSynkRSV-line19F. An additional gene was included in the RSV antigenomic cDNA to enhance detection of infection. The gene for the far-red fluorescent protein monomeric Katushka 2 (*mKate2*) lies in the first gene position flanked by RSV regulatory elements (Shcherbo et al., 2009). The features of pSynkRSV-line19F are delineated by nucleotide position in Table 1. We compared recovery efficiency of the previously published RSV strain A2 antigenomic clone D46/6120 with recovery of pSynkRSV-line19F, and there was no difference in the recovery efficiency as measured by time (in days) to rescue (data not shown). We also compared the efficiency of pSynkRSV-line19F rescue using wild-type or sequence-optimized helper plasmids. In replicate experiments, sequence-optimized helpers yielded two-fold higher levels (0.5% of cells compared to 0.2%) of mKate2 fluorescence three days after transfection, but there was no significant difference in time to rescue between wild-type and sequence-optimized helpers. We used the sequence-optimized helper plasmids and pSynkRSV-line19F to recover recombinant virus A2-K-line19F. Virus-encoded mKate2 serves as a marker for infected cells and provides means to assay infected cells by fluorescence (Fig. 3A and B). HEp-2 cell monolayer cultures were infected with RSV A2-line19F or A2-K-line19F then fixed and immunostained 48 h post-infection (Fig. 3A). In addition, the percentage of cells expressing RSV F (fusion protein) and mKate2 was quantified using flow cytometric analysis at 24 h post-infection (Fig. 3B). F protein positive cells were detected by indirect immunofluorescence using a monoclonal antibody to RSV F, while mKate2 fluorescence was directly detected in infected cells. mKate2 is a rapid and sensitive marker of infected cells, and is detectable using fluorescence microscopy and flow cytometry (Fig. 3).

Introduction of foreign genes into the RSV genome can attenuate RSV growth (Bukreyev et al., 1996). We performed virus high and low multiplicity replication analyses of A2-K-line19F and A2-line19F. A2-K-line19F grew to similar titers as A2-line19F in HEp-2 cell monolayer cultures (Fig. 4A and B). We inserted the *Renilla* luciferase gene into pSynkRSV-line19F in place of the gene for mKate2 and recovered A2-RL-line19F virus. Multi-step replication analysis in HEp-2 cell monolayer cultures showed that A2-RL-line19F grew to similar titers as A2-K-line19F in this cell line (Fig. 4C). We then used the A2-RL-line19F virus in a replication assay to determine how the titer of live virus detected as plaque forming units correlated with luciferase activity. The lowest levels of luciferase activity correlated with the lowest viral titers, and the highest levels of luciferase activity correlated with the peak viral titers (Fig. 4C). Virus-directed protein expression occurs prior to virus particle assembly, consequently, luciferase activity neared its maximum levels at an earlier time than virus titer (Fig. 4C). We next compared the lung

Table 1
Nucleotide sequence positions of features in pSynkRSV-line19F.

Nucleotide position	Feature	Reference
1–26	phi 10 T7 promoter	Studier et al., (1990)
30–77	Hammerhead ribozyme	Kwilas et al. (2010), Walker et al. (2003)
78–121	RSV leader	Mink et al. (1991)
122–131	NS1 gene start	Collins and Wertz (1985)
132–163	NS1 non-coding	Collins and Wertz (1985)
164–874	mKate2 gene	Shcherbo et al. (2009)
875–886	L noncoding	Stec et al. (1991)
887–898	L gene end	Stec et al. (1991)
899–917	NS1/NS2 intergenic	Collins et al. (1987)
918–1391	NS1 gene	Collins and Wertz (1985)
1392–1468	NS1/NS2 intergenic	Collins et al., 1987
1469–1971	NS2 gene	Collins and Wertz (1985)
1972–1996	NS2/N intergenic	Collins et al. (1995)
1997–3200	N gene	Collins et al. (1987)
3201–4115	P gene	Collins et al. (1987)
4116–4124	P/M intergenic	Moudy et al. (2004)
4125–5081	M gene	Collins et al. (1987)
5082–5100	M/SH intergenic	Collins et al. (1987)
5101–5399	SH gene	Collins et al. (1990)
5400–5445	SH/G intergenic	Collins et al. (1987)
5446–6368	G gene	Collins et al. (1987)
6369–6419	G/F intergenic	Collins et al. (1987)
6420–8322	F gene	Moore et al. (2009)
8323–8368	F/M2 intergenic	Johnson and Collins (1988)
8369–9329	M2 gene	Collins et al. (1987)
9261–15839	L gene	Stec et al. (1991)
15840–15993	RSV trailer	Kwilas et al. (2010), Mink et al. (1991)
15994–16078	Hepatitis delta virus ribozyme	Perrotta and Been (1991)
16079–16201	T7 terminator	Studier et al. (1990)

viral loads of A2-K-line19F, A2-RL-line19F, and A2-line19F in BALB/c mice. The lung viral load of A2-K-line19F was similar to that of A2-line19F at day 4 post-infection, while lung viral load of A2-RL-line19F was significantly lower than both A2-line19F and A2-K-line19F (Fig. 4D). These results indicate that mKate2 did not cause growth restriction in vitro or in vivo. In addition, the luciferase encoded by A2-RL-line19F provides a useful tool for detecting virus infection in vitro, even though the A2-RL-line19F was slightly restricted in replication in BALB/c mice.

BAC-RSV is a stable platform for subcloning and for recombination-mediated genetic engineering

We determined the efficiency of cloning for an unstable fragment of RSV cDNA. A high-copy number cloning vector containing RSV cDNA including part of the small hydrophobic (SH) gene, the attachment glycoprotein (G), F, and M2 genes, and part of the L gene was transformed into two competent *E. coli* cell strains. None of the transformants selected contained the correct vector/insert. In contrast, when the same cDNA fragment of RSV cDNA was cloned into BAC vector pKBS2, all of the transformant colonies analyzed contained the correct vector/insert DNA. The enhanced stability of RSV cDNA provided by the BAC allowed for more efficient cloning.

We sought to develop recombination-mediated mutagenesis for RSV using a method relying on positive and negative selection of the *galK* cassette (Warming et al., 2005). To demonstrate its utility for RSV reverse genetics, we used this method to delete the genes for the RSV nonstructural proteins NS1 and NS2. The gene deletions were confirmed by nucleotide sequence determination, and lack of NS1 and NS2 production was confirmed by Western blot analysis of infected cell lysates (Fig. 5). We also used this recombination-based mutagenesis to generate a point mutation in the F protein of pSynkRSV-line19F, creating mutant I557V. The sequence of the F gene was confirmed to contain the correct mutation, demonstrating that recombination-mediated mutagenesis is a powerful tool for reverse genetic manipulations.

Discussion

We have assembled the first BAC recombination-mediated mutagenesis system for reverse genetics of a negative strand RNA virus. pSynkRSV-line19F is a RSV cDNA-containing BAC that is highly stable and enhances the efficiency of RSV reverse genetics. Recombination-mediated genetic engineering is commonly used for large BAC clones and reverse genetics of DNA viruses (Donofrio et al., 2007; Moyer et al., 2011). We demonstrated the utility of the BAC-RSV mutagenesis system by generating viruses with either gene deletions or a point mutation. We demonstrated the advantage of using sequence-optimized helper plasmids for virus recovery. These aspects of our reverse genetics system are likely to enhance rescue of attenuated mutants. We recovered two reporter viruses, A2-K-line19F and A2-RL-line19F, which express mKate2 and *Renilla* luciferase, respectively. Although these recombinant viruses encoding reporter genes were attenuated in mice, we were able to demonstrate their utility in vitro.

Stable and efficient mutagenesis of the RSV-BAC construct will facilitate RSV research and vaccine development. Despite the high clinical significance of RSV, reverse genetics reagents for this virus are not in widespread use relative to other members of the *Mononegavirales*, potentially due to cloning obstacles. BAC-RSV cloning alleviates cDNA instability, enhances the efficiency of traditional cloning, and enables recombination-mediated mutagenesis. This is particularly efficient for generating point mutations and deletions in the full-length antigenomic cDNA where convenient restriction enzyme sites are not available. This system will be useful for manipulation of RSV and may be useful for rescue and mutagenesis of *Pneumovirinae* or other RNA viruses with cDNA that is difficult to clone.

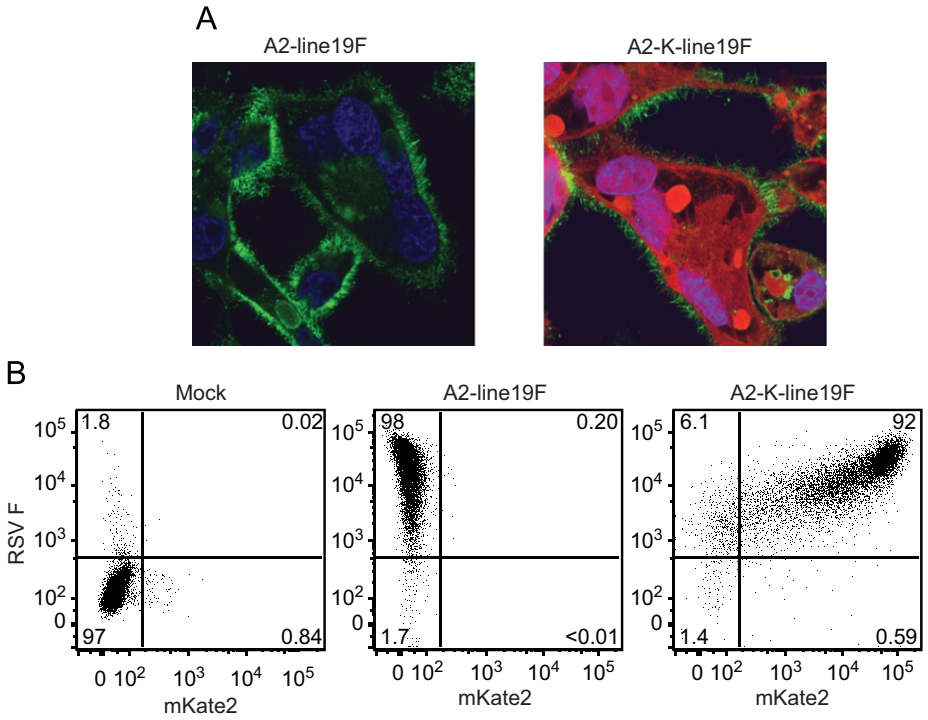

Fig. 3. A2-K-line19F encoded mKate2 serves as a marker for RSV infected cells. (A) HEp-2 cell monolayer cultures were infected with indicated virus at MOI 1.0 and incubated for 48 h. RSV F protein was detected using indirect immunostaining and cell nuclei were detected using TO-PRO-3 iodide stain. RSV F is in green, mKate2 in red, and nuclei in blue. (B) HEp-2 cell culture monolayers were inoculated with indicated virus at MOI 3.0 and incubated for 24 h. Panels show scatter plots from representative samples with a percentage of total cells detected as indicated for each quadrant.

Experimental procedures

Plasmids

Using published RSV sequences, we designed an antigenomic cDNA of a chimeric RSV strain A2 harboring the F gene of RSV strain line 19 (Table 1) (Moore et al., 2009). The cDNA was synthesized (GeneArt, Regensberg, Germany) in three separate fragments. In order 5′ to 3′, the first fragment contains a T7 promoter, a hammerhead ribozyme, RSV leader sequence, and genes encoding monomeric Katushka 2 (a red fluorescent protein mKate2) (Shcherbo et al., 2009), RSV NS1, NS2, N, P, M, and a portion of SH flanked by appropriate RSV gene start, intergenic, and gene end sequences. This fragment is flanked by restriction sites BstBI and SacI. The second segment is flanked by restriction sites SacI and ClaI, and it contains the continuation of the SH gene followed by the RSV G, RSV line 19 strain F, and M2 genes, as well as a portion of the L gene. The third fragment contains the rest of the L gene followed by the RSV A2 trailer sequence, the hepatitis delta virus ribozyme, and a T7 terminator. This segment is flanked by ClaI and MluI restriction enzyme sites. We obtained a BAC vector (pKBS2) harboring the mouse adenovirus type 1 (MAV-1) genome (Katherine Spindler, University of Michigan). The MAV-1 DNA was removed, and the three RSV cDNA fragments were inserted sequentially in the BAC vector. This synthetic, mKate2-expressing BAC-RSV construct was termed pSynkRSV-line19F. The nucleotide features contained in pSynkRSV-line19F are shown in Table 1 with references.

We developed mammalian expression vectors for the RSV N, P, M2-1, and L proteins (Dochow et al., 2012). Briefly, the sequences of the wild-type genes encoding these proteins in the RSV strain A2 were obtained from GenBank. We optimized the sequences for expression using computational algorithms to reduce the use of rare codons, occult splice sites, mRNA instability sequences, and RNA secondary structural elements. The optimized cDNA sequences were synthesized (GeneArt) and then cloned into the pcDNA3.1 mammalian expression plasmid vector (Invitrogen, Grand Island, New York, USA). Plasmids were used to transfect 293F cells, then cells were fixed and permeabilized for immunofluorescence staining or lysed for analysis of expression by SDS-PAGE and Western blot. Each of the proteins were expressed in mammalian cells with the predicted molecular weight, as detected using polyclonal antisera to RSV (data not shown). The four helper plasmids pTM1-N, pTM1-P, pTM1-M2-1, and pTM1-L, each containing the gene under control of a T7 promoter, were obtained from Peter Collins (Collins et al., 1995).

cDNA stability

To determine the stability of RSV antigenomic cDNA in cloning plasmids, a high-copy number vector (pMA, GeneArt) or single-copy number (pKBS2) vector containing the same piece of RSV antigenomic cDNA (corresponding to nucleotides 5382–9949 in pSynkRSV-line19F) were transformed into NEB-10β (New England BioLabs, Ipswitch, Massachusetts, USA) or One Shot Stbl-3 (Invitrogen) *E. coli* following manufacturer's protocols. *E. coli* were cultured at 32° C. The high-copy number vector was the GeneArt vector pMA, while the single-copy vector was pKBS2, as described above. pMA has a ColE1 origin of replication and carries an ampicillin resistance gene. Ten transformants from each were selected at random, and restriction digests were used to determine if plasmids contained in the transformants displayed the expected digestion pattern.

RSV-BAC mutations

We used recombination-mediated mutagenesis to generate a point mutation in the F gene of pSynkRSV-line19F. Our protocol was based a method using positive and negative selection of the galactokinase expression cassette (*galK*) (Warming et al., 2005). The *galK* cassette was amplified from plasmid pgalK using primers

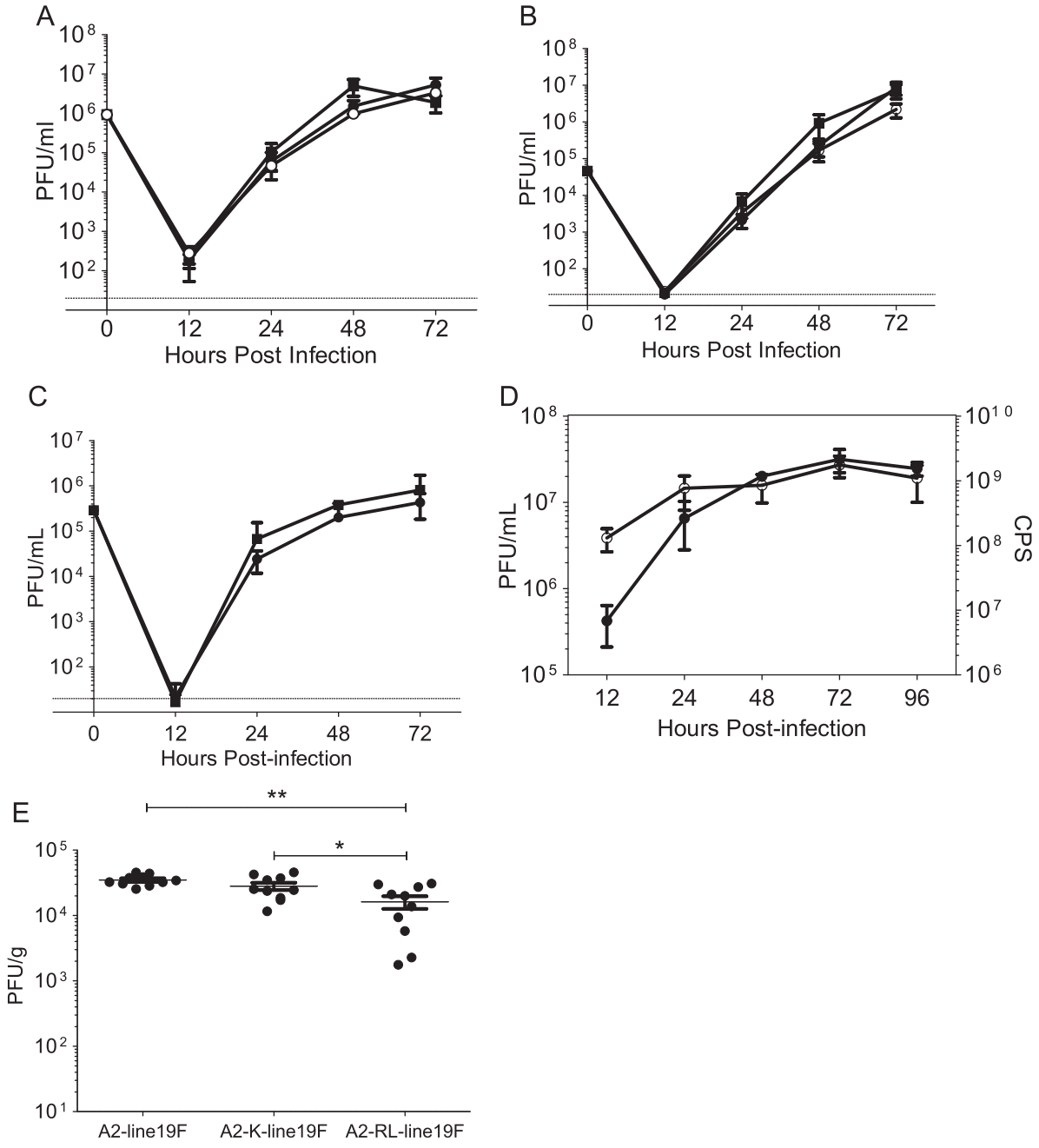

Fig. 4. In vitro and in vivo growth characteristics of A2-K-line19F and A2-RL-line19F. (A and B) Growth curves of A2 (■), A2-line19F (●), and A2-K-line19F (○) in HEp-2 cell monolayer cultures infected at MOI 2.0 (A) or MOI 0.1 (B). (C) Growth curve of A2-K-line19F (■) and A2-RL-line19F (●) in HEp-2 cell monolayer cultures infected at MOI 0.5. (D) Viral titers and luciferase activity in HEp-2 cell monolayer cultures infected with A2-RL-line19F at an MOI of 0.5. Titers are indicated by plaque forming units per mL (●) and luciferase activity is indicated by counts per second (○). Data for panels A–D are indicated as mean ± SEM. (E) BALB/c mice were infected with 5×10^5 PFU/mouse (5 mice per group) of A2-line19F, A2-K-line19F, or A2-RL-line19F and viral load was determined day 4 post-infection via immunodetection plaque assay. $^{*}P=0.05$, $^{**}P=0.01$ by ANOVA.

557galK F 5′-**CATTAATTGCTGTTGGACTGCTCCTATACTGTAAGGCCA-GAAGCACACCA***CCTGTTGACAATTAATCATCGGCA*-3′ and 557galK R 5′-**ACTAAATGCAATATTATTTATACCACTCAGTTGATCCTTGCTTAGT-GTGA***TCAGCACTGTCCTGCTCCTT*-3′. pSynkRSV-line19F homology arms are in bold and *galK* sequence is italicized. The PCR conditions were 94 °C denaturation for 30 s followed by 30 cycles (94 °C for 15 s, 60 °C for 30 s, and 72 °C for 1 min) and a final 6 min, 72 °C extension. The resulting double stranded product was the *galK* cassette flanked by RSV homology arms. Upon recombination, the *galK* cassette replaced the nucleotide to be changed in pSynkRSV-line19F. For the second recombination step to replace *galK* with the desired mutation, we used annealed oligonucleotide adapters with the following sequences: 557inF 5′-CATTAATTGCTGTTGGACTGCTCCTATACTGTAAGGCCAGAAGCA-CACCA**G**TCACACTAAGCAAGGATCAACTGAGTGGTATAAATAATAT-TGCATTTAGT-3′ and 557inR 5′-ACTAAATGCAATATTATTTATAC-CACTCAGTTGATCCTTGCTTAGTGTGA**C**TGGTGTGCTTCTGGCCTTA-CAGTATAGGAGCAGTCCAACAGCAATTAATG-3′. 557inF and 557inR are entirely homologous to pSynkRSV-line19F except for the bold nucleotide, which designates the mutation.

The NS1 and NS2 genes were deleted from a version of the BAC-RSV without the gene for mKate2. The primers for *galK* amplification from pgalK were NS1startF 5′–**ATAAGAATTTGATAAGTAC-CACTTAAATTTAACTCCCTTGGTTAGAGATG***CCTGTTGACAATTAATCA-TCGGCA*-3′ and NS2stopR 5′-**TATGCATAGAGTTGTTGTTTTAGATT-GTGTGAATATTGTGTTGAAATTTA***TCAGCACTGTCCTGCTCCTT-3′*. RSV

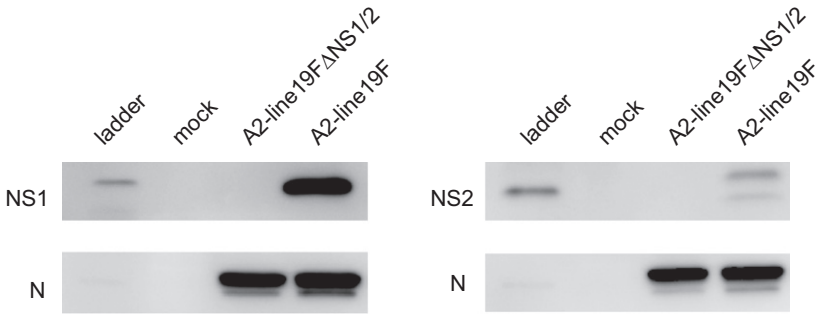

Fig. 5. Recombination-mediated mutagenesis derived mutant viruses. Western blots of cell lysates from Vero cell monolayer cultures infected with A2-line19F or A2-line19FΔNS1/NS2. Polyclonal antisera for NS1 or NS2 were used to detect protein expression. Anti-RSV N was used to detect RSV N as a loading control.

antigenome homology arms are in bold and *galK* sequence is italicized. The PCR conditions were the same as described above. To complete the NS1/NS2 deletion and replace *galK*, the following oligonucleotides were annealed and used for recombination. dNS12F 5′-ATAAGAATTTGATAAGTACCACTTAAATTTAACTCCCTTGGTTAGAGATGGCTCTTAGCAAAGTCAAGTTGAATGATACACTCAACAAAGATCAACTTCT-3′ and dNS12R 5′-AGAAGTTGATCTTTGTTGAGTGTATCATTCAACTTGACTTTGCTAAGAGCCATCTCTAACCAAGGGAGTTAAATTTAAGTGGTACTTATCAAATTCTTAT-3′. dNS12F and dNS12R are entirely homologous sequences to the RSV antigenome.

A double recombination-PCR strategy was employed to generate cDNA copies of pSynkRSV-Luc$_{Ren}$ in pSynkRSV-line19F. First, individual segments of pSynkRSV-line19F flanking the luciferase insertion site and harboring unique BstBI and AvrII restrictions sites were amplified using oligonucleotide primer pairs 5′–CCCAGGCCGT GCCGGC/5′–GCTAAGCAAGGGAGTTAAATTTAAGTGG. The *Renilla* luciferase gene was amplified from pGL4.74[*hRluc*/TK] (Promega, Madison, Wisconsin, USA) with oligonucleotide primer pairs 5′–**CCACTTAAATTTAA CTCCCTTGCTTAGC**ATGGCTTCCAAGGTGTACG/5′–**CTGTTAAGTTTTTAATAACTAT AATTGAATACTCA**CTGCTCGTTCTTCAGCACGC (the bold nucleotides are complementary to the RSV genomic sequences flanking the insertion site). In two consecutive PCR reactions, the luciferase-encoding amplicon was then recombined with the pSynkRSV-line19F-derived segments, and the final amplicon subcloned into the pCR2.1-TOPO (Invitrogen) vector. Transfer of BstBI/AvrII segments harboring the luciferase gene into the similarly opened pSynkRSV-line19F produced pSynkRSV-Luc$_{Ren}$.

We modified the protocol from Warming et al. for our recombination-mediated mutagenesis procedure (Warming et al., 2005). Most notably, we used 50 μL electrocompetent *E. coli* and 100–200 ng of PCR product or annealed oligonucleotides for each electroporation event. In addition, *E. coli* containing pSynkRSV-line19F routinely took 4–5 days to form colonies on both positive and negative *galK* selection plates.

Recombinant virus recovery

Recombinant viruses were recovered by modifying the protocol from previous reports (Buchholz et al., 1999; Collins et al., 1995). BHK cells constitutively expressing the T7 polymerase (BSR-T7/5 cells) were transfected with BAC-RSV plasmids as well as the sequence-optimized helper plasmids encoding N, P, M2-1, and L, all under T7 control (Buchholz et al., 1999). Using Lipofectamine 2000 (Invitrogen), the plasmids were transfected at concentrations of 0.8 μg, 0.4 μg, 0.4 μg, 0.4 μg, and 0.2 μg, respectively. Prior to transfection, BSR-T7/5 cells were maintained in Glasgow's minimal essential media (GMEM, Invitrogen) supplemented with 10% FBS, 1% penicillin, streptomycin sulfate, amphotericin B solution (Invitrogen), and 2% minimal essential amino acids (Invitrogen), with 1 mg/mL Geneticin (Invitrogen) added every other passage. Cells were plated to be confluent in 6 well plates at the time of transfection. Cells were incubated with transfection complexes of 500 μL at room temperature for 2 h on a rocking platform. Following this incubation, 500 μL of GMEM supplemented as described above, except that FBS concentration was 3%, was added to each well and the cells were incubated overnight at 37 °C. The next morning, the transfection complexes were removed from the wells and replaced with 2 mL of GMEM+3% FBS. Cells were passed into 25 cm^2 flasks 2 days post-transfection, and every 2–3 days following until cytopathic effect was seen, at which time the cells were scraped from the flasks, aliquoted, and frozen. After thawing to release to virus, the cells and cell debris were pelleted at 1800 × *g* for 5 min at 4 °C, and the supernatant was used to infect subconfluent HEp-2 cell monolayer cultures in order to propagate recovered virus.

RSV minigenome luciferase reporter assay

Construction of the RSV minigenome reporter plasmid has been described (Dochow et al., 2012). BSR-T7/5 cells were transfected with the RSV minigenome reporter and the helper plasmids encoding N, P, M2-1, and L in either sequence-optimized or non-optimized forms. Using Lipofectamine 2000, the plasmids were transfected in the following quantities: 0.8 μg minigenome, 0.4 μg N, P, and M2-1, and 0.2 μg L. The transfection procedure was identical to that for recombinant virus recovery, except that two hours post-transfection 1.5 ml of GMEM supplemented with 3% FBS was added to cells. Approximately 24 h post-transfection, the supernatant was removed, the cells washed once in phosphate buffered saline (PBS), and lysed in 300 μL 1 × reporter lysis buffer (Promega). Samples were diluted 1:100 and luciferase activity was determined using a Perkin Elmer Top Count scintillation and luminescence counter.

Virus growth curves and quantification of lung viral load

Subconfluent HEp-2 cell monolayer cultures in 6-well plates were infected in triplicate with RSV A2, A2-line19F, or A2-K-line19F at an MOI of 0.1 or 2.0. The virus was adsorbed for one hour at room temperature with rocking. Unbound inoculum was washed off with PBS and EMEM supplemented with 10% FBS and 1% penicillin, streptomycin sulfate, amphotericin B solution (Invitrogen) was added to the cells. Samples of the supernatant were taken at 12, 24, 48, and 72 h post-infection and frozen until plaque titration by immunodetection plaque assay (Stokes et al., 2011). For the A2-RL-line19F growth curve, HEp-2 cell monolayer cultures were infected at an MOI of 0.5. 12, 24, 48, 72, and 96 h post-infection, cells were washed in PBS and either 1 ml of MEM or 200 μL of Renilla-Glo lysis buffer (Promega) was added. The cells in MEM were scraped and frozen until plaque titration.

The cells in lysis buffer were scraped and frozen until luciferase activity was determined using the Renilla-Glo system (Promega).

To determine lung viral load, 6–8 week old BALB/c mice (The Jackson Laboratory, Bar Harbor, Maine, USA) were infected intranasally with 5×10^5 pfu of RSV per mouse as described (Moore et al., 2009). On day four post-infection, the left lung was harvested and viral load was determined as described previously (Stokes et al., 2011). The procedures for all experiments involving mice were approved by the Emory University Institutional Animal Care and Use Committee.

NS1/NS2 Western blotting

Vero cells maintained in EMEM supplemented with 10% FBS and antibiotic/antimycotic were infected with A2-line19F and A2-line19FΔNS1/NS2 and infected cell lysates were harvested 24 h post-infection in RIPA buffer (Sigma-Aldrich, St. Louis, Missouri, USA). Lysates were mixed 1:1 with Laemmli sample buffer prior to loading on SDS 4%–20% polyacrylamide gels (Bio-Rad, Hercules, California, USA). Proteins were transferred to polyvinylidene fluoride membranes. NS1 and NS2 were detected using polyclonal rabbit sera raised to recombinant NS1 or NS2 proteins. The NS2 antisera slightly cross-react with NS1. Secondary incubation with horse radish peroxidase (HRP) coupled goat anti-rabbit (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania, USA) IgG antibodies followed by chemiluminescent detection using SuperSignal West Femto Substrate (Thermo Scientific, Rockford, Illinois, USA) on a ChemiDoc imaging system (Bio-Rad) enabled visualization of proteins. Antibodies were stripped from membranes using Restore Western Blot Stripping Buffer (Thermo Scientific) and re-probed using an anti-N RSV antibody (clone D14, a gift from Dr. Ed Walsh) and HRP-coupled donkey anti-mouse secondary (Jackson Immunoresearch Laboratories) (Walsh and Hruska, 1983).

Immunofluorescence microscopy

HEp-2 cell monolayer cultures on 6-well plates were inoculated with RSV at MOI 1.0. 48 h later cells were fixed with 3.7% (w/v) paraformaldehyde in PBS for 10 min then permeabilized with 0.3% (w/v) Triton X-100 and 3.7% paraformaldehyde in PBS for 10 min at RT. After fixation, cells were blocked in 3% (w/v) BSA in PBS for 60 min followed by addition of primary antibody in the blocking solution for 60 min. Cells then were washed three times in PBS, and species-specific anti-IgG Alexa Fluor conjugated antibodies (Invitrogen) were added at a dilution of 1:1000 in block solution for 60 min to detect primary antibodies. Cells were washed three times in PBS and fixed on glass slides using Prolong Antifade kit (Invitrogen). All steps were performed at room temperature. Images were obtained on a Zeiss inverted LSM510 confocal microscope using a $63 \times /1.40$ Plan-Apochromat oil lens. Anti-RSV F protein humanized mouse monoclonal antibody (palivizumab; MedImmune) was obtained from the Vanderbilt Pharmacy, and TO-PRO-3 iodide stain (Invitrogen) was used to visualize the nucleus.

Flow cytometric assay for quantitative surface expression of RSV F protein

HEp-2 cell monolayer cultures on 6-well plates were inoculated with RSV at MOI 3.0. After 24 h, cells were treated with 20 mM EDTA in PBS to form a single cell suspension. Cells were washed twice in wash buffer (2% FBS in PBS) and then incubated with RSV F-specific monoclonal antibody (palivizumab) at 1 μg/mL for 30 min at RT. Cells again were washed twice with wash buffer and immunostained with an Alexa Fluor 488 labeled goat anti-mouse secondary antibodies at a final concentration of 2 μg/mL. Cells were washed twice in wash buffer and analyzed on a 5-laser LSRFortessa™ flow cytometer (Becton Dickinson, Franklin Lakes, New Jersey, USA) in the Vanderbilt Medical Center Flow Cytometry Shared Resource. Data analysis was performed using FlowJo version 7.6.5 (Treestar Inc., Ashland, Oregon, USA).

Statistical analyses

Groups were compared by one way analysis of variance (ANOVA) and Tukey multiple comparison tests unless otherwise noted. $P < 0.05$ was considered significant.

Disclosure statement

M. Moore and A. Hotard disclose that they are listed as inventors on a patent application for the RSV recombination-mediated mutagenesis system, submitted by Emory University.

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References

Almazan, F., Gonzalez, J.M., Penzes, Z., Izeta, A., Calvo, E., Plana-Duran, J., Enjuanes, L., 2000. Engineering the largest RNA virus genome as an infectious bacterial artificial chromosome. Proc. Natl. Acad. Sci. USA 97 (10), 5516–5521.

Biacchesi, S., Murphy, B.R., Collins, P.L., Buchholz, U.J., 2007. Frequent frameshift and point mutations in the SH gene of human metapneumovirus passaged in vitro. J. Virol. 81 (11), 6057–6067.

Buchholz, U.J., Finke, S., Conzelmann, K.K., 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. 73 (1), 251–259.

Bukreyev, A., Camargo, E., Collins, P.L., 1996. Recovery of infectious respiratory syncytial virus expressing an additional, foreign gene. J. Virol. 70 (10), 6634–6641.

Collins, P.L., Crowe Jr., J.E., 2007. Respiratory syncytial virus and metapneumovirus. In: Knipe, D.M., Howley, P.M. (Eds.), Fields Virology, vol. 2. Lippincott, Williams, and Wilkins, Philadelphia, PA, pp. 1601–1646. (2 vols.).

Collins, P.L., Wertz, G.W., 1985. Nucleotide sequences of the 1B and 1C non-structural protein mRNAs of human respiratory syncytial virus. Virology 143 (2), 442–451.

Collins, P.L., Olmsted, R.A., Johnson, P.R., 1990. The small hydrophobic protein of human respiratory syncytial virus: comparison between antigenic subgroups A and B. J. Gen. Virol. 71 (7), 1571–1576.

Collins, P.L., Olmsted, R.A., Spriggs, M.K., Johnson, P.R., Buckler-White, A.J., 1987. Gene overlap and site-specific attenuation of transcription of the viral polymerase L gene of human respiratory syncytial virus. Proc. Natl. Acad. Sci. USA 84 (15), 5134–5138.

Collins, P.L., Hill, M.G., Camargo, E., Grosfeld, H., Chanock, R.M., Murphy, B.R., 1995. Production of infectious human respiratory syncytial virus from cloned cDNA confirms an essential role for the transcription elongation factor from the 5′ proximal open reading frame of the M2 mRNA in gene expression and provides a capability for vaccine development. Proc. Natl. Acad. Sci. USA 92 (25), 11563–11567.

Dochow, M., Krumm, S.A., Crowe Jr., J.E., Moore, M.L., Plemper, R.K., 2012. Independent structural domains in the paramyxovirus polymerase protein. J. Biol. Chem. 287 (9), 6878–6891.

Donofrio, G., Sartori, C., Ravanetti, L., Cavirani, S., Gillet, L., Vanderplasschen, A., Taddei, S., Flammini, C.F., 2007. Establishment of a bovine herpesvirus 4 based

vector expressing a secreted form of the bovine viral diarrhoea virus structural glycoprotein E2 for immunization purposes. BMC Biotechnol. 7, e68.
Fan, Z.C., Bird, R.C., 2008. An improved reverse genetics system for generation of bovine viral diarrhea virus as a BAC cDNA. J. Virol. Methods 149 (2), 309–315.
Grosfeld, H., Hill, M.G., Collins, P.L., 1995. RNA replication by respiratory syncytial virus (RSV) is directed by the N, P, and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. J. Virol. 69 (9), 5677–5686.
Hall, R.N., Meers, J., Fowler, E., Mahony, T., 2012. Back to BAC: the use of infectious clone technologies for viral mutagenesis. Viruses 4 (2), 211–235.
Huang, K., Lawlor, H., Tang, R., MacGill, R.S., Ulbrandt, N.D., Wu, H., 2010. Recombinant respiratory syncytial virus F protein expression is hindered by inefficient nuclear export and mRNA processing. Virus Genes. 40 (2), 212–221.
Jin, H., Clarke, D., Zhou, H.Z., Cheng, X., Coelingh, K., Bryant, M., Li, S., 1998. Recombinant human respiratory syncytial virus (RSV) from cDNA and construction of subgroup A and B chimeric RSV. Virology 251 (1), 206–214.
Johnson, P.R., Collins, P.L., 1988. The fusion glycoproteins of human respiratory syncytial virus of subgroups A and B: sequence conservation provides a structural basis for antigenic relatedness. J. Gen. Virol. 69 (10), 2623–2628.
Karron, R.A., Wright, P.F., Belshe, R.B., Thumar, B., Casey, R., Newman, F., Polack, F.P., Randolph, V.B., Deatly, A., Hackell, J., Gruber, W., Murphy, B.R., Collins, P.L., 2005. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. J. Infect. Dis. 191 (7), 1093–1104.
Kwilas, A.R., Yednak, M.A., Zhang, L., Liesman, R., Collins, P.L., Pickles, R.J., Peeples, M.E., 2010. Respiratory syncytial virus engineered to express the cystic fibrosis transmembrane conductance regulator corrects the bioelectric phenotype of human cystic fibrosis airway epithelium in vitro. J. Virol. 84 (15), 7770–7781.
Mink, M.A., Stec, D.S., Collins, P.L., 1991. Nucleotide sequences of the 3′ leader and 5′ trailer regions of human respiratory syncytial virus genomic RNA. Virology 185 (2), 615–624.
Moore, M.L., Chi, M.H., Luongo, C., Lukacs, N.W., Polosukhin, V.V., Huckabee, M.M., Newcomb, D.C., Buchholz, U.J., Crowe Jr., J.E., Goleniewska, K., Williams, J.V., Collins, P.L., Peebles Jr., R.S., 2009. A chimeric A2 strain of respiratory syncytial virus (RSV) with the fusion protein of RSV strain line 19 exhibits enhanced viral load, mucus, and airway dysfunction. J. Virol. 83 (9), 4185–4194.
Moudy, R.M., Sullender, W.M., Wertz, G.W., 2004. Variations in intergenic region sequences of human respiratory syncytial virus clinical isolates: analysis of effects on transcriptional regulation. Virology 327 (1), 121–133.
Moyer, C.L., Wiethoff, C.M., Maier, O., Smith, J.G., Nemerow, G.R., 2011. Functional genetic and biophysical analyses of membrane disruption by human adenovirus. J. Virol. 85 (6), 2631–2641.
Perrotta, A.T., Been, M.D., 1991. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta virus RNA. Nature 350 (6317), 434–436.
Shcherbo, D., Murphy, C.S., Ermakova, G.V., Solovieva, E.A., Chepurnykh, T.V., Shcheglov, A.S., Verkhusha, V.V., Pletnev, V.Z., Hazelwood, K.L., Roche, P.M., Lukyanov, S., Zaraisky, A.G., Davidson, M.W., Chudakov, D.M., 2009. Far-red fluorescent tags for protein imaging in living tissues. Biochem. J. 418 (3), 567–574.
Stec, D.S., Hill 3rd, M.G., Collins, P.L., 1991. Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of nonsegmented negative-strand viruses. Virology 183 (1), 273–287.
Stokes, K.L., Chi, M.H., Sakamoto, K., Newcomb, D.C., Currier, M.G., Huckabee, M.M., Lee, S., Goleniewska, K., Pretto, C., Williams, J.V., Hotard, A., Sherrill, T.P., Peebles Jr., R.S., Moore, M.L., 2011. Differential pathogenesis of respiratory syncytial virus (RSV) clinical isolates in BALB/c mice. J. Virol. 85 (12), 5782–5793.
Studier, F.W., Rosenberg, A.H., Dunn, J.J., Dubendorff, J.W., 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. 185, 60–89.
Walker, S.C., Avis, J.M., Conn, G.L., 2003. General plasmids for producing RNA in vitro transcripts with homogeneous ends. Nucleic Acids Res. 31 (15), e82.
Walsh, E.E., Hruska, J.F., 1983. Identification of the virus-specific proteins of respiratory syncytial virus temperature-sensitive mutants by immunoprecipitation. Proc. Soc. Exp. Biol. Med. 172 (2), 202–206.
Warming, S., Costantino, N., Court, D.L., Jenkins, N.A., Copeland, N.G., 2005. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res. 33 (4), e36.
Yun, S.I., Kim, S.Y., Rice, C.M., Lee, Y.M., 2003. Development and application of a reverse genetics system for Japanese encephalitis virus. J. Virol. 77 (11), 6450–6465.
Zhou, B., Jerzak, G., Scholes, D.T., Donnelly, M.E., Li, Y., Wentworth, D.E., 2011. Reverse genetics plasmid for cloning unstable influenza A virus gene segments. J. Virol. Methods 173 (2), 378–383.