

β -D- N^4 -hydroxycytidine Inhibits SARS-CoV-2 Through Lethal Mutagenesis But Is Also Mutagenic To Mammalian Cells

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Mutagenic ribonucleosides can act as broad-based antiviral agents. They are metabolized to the active ribonucleoside triphosphate form and concentrate in genomes of RNA viruses during viral replication. β -D- N^4 -hydroxycytidine (NHC, initial metabolite of molnupiravir) is >100-fold more active than ribavirin or favipiravir against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), with antiviral activity correlated to the level of mutagenesis in virion RNA. However, NHC also displays host mutational activity in an animal cell culture assay, consistent with RNA and DNA precursors sharing a common intermediate of a ribonucleoside diphosphate. These results indicate highly active mutagenic ribonucleosides may hold risk for the host.

Keywords. molnupiravir; mutagenicity; NHC; SARS-CoV-2.

Emerging RNA viruses arising from highly heterogeneous pools of precursor strains are responsible for most recent epidemic and pandemic disease outbreaks in the late 20th and early 21st century. Broad direct-acting antiviral agents represent the most specific way of treating a viral infection, although these are often specific to a group of closely related viruses. Besides remdesivir, which blocks the replication of several coronaviruses, limited therapeutic options are available for treating severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections [1]. β -D- N^4 -hydroxycytidine (NHC, or to designate

its ribonucleoside form rNHC) has the potential to be a broad-based inhibitor as it is very similar in structure to the normal ribonucleoside cytidine, which likely ensures its efficient uptake and metabolism to an active ribonucleotide that can be incorporated into RNA. rNHC has the ability to pair ambiguously as cytidine or uridine, thus introducing an elevated mutation load when incorporated. Once in the ribonucleotide precursor pool, rNHC should concentrate in the viral RNA genome over cellular RNA as viral RNA replication passes through the ribonucleotide pool multiple times for the synthesis of both plus and minus strands, resulting in lethal mutagenesis. The fact that rNHC shows broad antiviral activity against all coronaviruses tested, including SARS-CoV-2, suggests that it is promiscuously incorporated by viral RNA-dependent RNA polymerase [2], similar to its broad antiviral activity against other RNA viruses [2, 3]. We have previously shown that the orally available prodrug form of rNHC, molnupiravir (EIDD-2801 or MK-4482), reduces disease in mouse infection models of SARS-CoV and Middle East respiratory syndrome coronavirus (MERV-CoV), and that rNHC is antiviral through lethal mutagenesis for MERS-CoV [2]. In addition, molnupiravir was active in a lung humanized mouse model of SARS-CoV-2 infection [4]. Molnupiravir has undergone phase 1 safety testing [5] and is currently in phase 2/3 clinical trials to treat SARS-CoV-2 infections.

The ambiguous base-pairing of rNHC after incorporation places it in the class of mutagenic compounds targeting incorporation into viral RNA (along with favipiravir [FAV], a base analog [6], and ribavirin [RBV], a ribonucleoside analog [7]). Here we considered the antiviral activity against SARS-CoV-2 of rNHC, FAV, and RBV in a head-to-head comparison of viral inhibition and the ability to induce mutations in the viral genome. Due to their mechanism of action, mutagenic ribonucleoside analogs could be metabolized by the host cell to the 2'-deoxyribonucleotide form by ribonucleotide reductase and then incorporated into DNA, leading to mutagenesis of the host. Thus, we also examined mutagenesis of host DNA using a modified hypoxanthine phosphoribosyltransferase (*HPRT*) gene mutation assay [8]. We found that rNHC has potent antiviral activity far beyond FAV and RBV but is also mutagenic to the host in the *HPRT* mutagenesis assay.

METHODS

We measured SARS-CoV-2 antiviral activity in the presence of a panel of compounds (rNHC, RBV, and FAV) using the A549-hACE2 cell model [9, 10]. Sequence analysis to detect the mutation load was done using the previously published multiplex Primer ID approach to sequence several regions of the

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SARS-CoV-2 genomic RNA extracted from cell culture supernatants [2, 10]. We used a modified *HPRT* assay to measure mutagenesis of host DNA in the presence of test compounds as mutation of the *HPRT* gene to create resistance to the toxic compound 6-thioguanine (6-TG). Detailed methods can be found in the [Supplementary Material](#) and [Supplementary Figure 1](#).

RESULTS

rNHC Inhibits SARS-CoV-2 Replication in Cell Culture Through Lethal Mutagenesis

rNHC showed a significant dose-dependent antiviral effect on SARS-CoV-2 during viral replication in cell culture. At $\geq 3 \mu\text{M}$ rNHC, virus growth was almost completely inhibited, with a 50% maximum effective concentration (EC_{50}) of $0.3 \mu\text{M}$ ([Figure 1A](#)). Sequence analysis of supernatant viral RNA showed that rNHC treatment increased the mutation rate, especially transitions, in a dose-dependent manner ([Figure 1B](#) and [1C](#) for the *nsp12* region, and [Supplementary Figure 2](#) for the S protein RBD coding domain), starting with the culture exposed to $0.3 \mu\text{M}$ (detectable in the spike domain; [Supplementary Figure 2](#)) to

$10 \mu\text{M}$ rNHC ([Figure 1B](#) and [1C](#) and [Supplementary Figure 2](#)). The antiviral effect was correlated with the increase of the mutation load in viral RNA ([Figure 1D](#)), consistent with rNHC acting through lethal mutagenesis as we previously observed with MERS-CoV [2].

FAV and RBV inhibited SARS-CoV-2 replication at the highest concentrations tested (with an EC_{50} of $110 \mu\text{M}$ for FAV and $195 \mu\text{M}$ for RBV), similar to a recent report [11]. We observed an unexplained increase in the SARS-CoV-2 production for both FAV and RBV in cells treated with concentrations between $3 \mu\text{M}$ and $30 \mu\text{M}$. Sequence analysis of viral RNA showed a slight increase in the overall mutation rate and the C-to-U mutation rate in RBV at $100 \mu\text{M}$ and $300 \mu\text{M}$, corresponding to a 10-fold drop in virus titer ([Figure 1A](#)); we failed to detect any mutagenesis above background in the FAV-treated cultures. We also examined toxicity of these analogs in the target A549-hACE-2 cells ([Supplementary Figure 3](#)), measuring cell growth rate over a 4-day period. rNHC reduced the rate of cell growth at $10 \mu\text{M}$ and higher (50% cytotoxic concentration [CC_{50}] of $12 \mu\text{M}$), FAV did not inhibit cell growth at any concentration

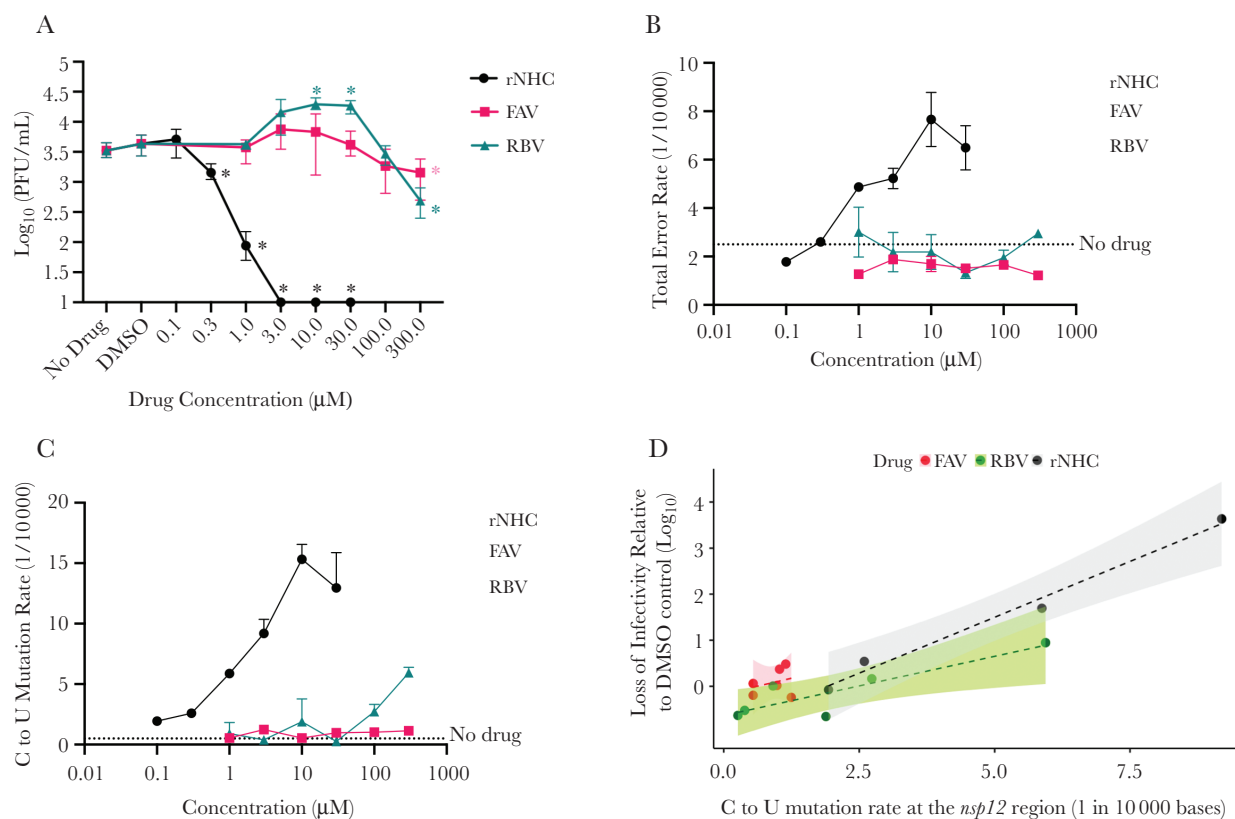


Figure 1. Antiviral and mutagenic effect of rNHC, RBV, and FAV on SARS-CoV-2 in the A549-hACE2 cell model. *A*, Antiviral effect of rNHC, RBV, and FAV on SARS-CoV-2. Significance compared to DMSO control (* $P < .05$) was determined using a 2-stage linear t test with step-up procedure of Benjamini, Krieger, and Yekutieli through the GraphPad Prism version 8.3.0 built-in function. *B*, Total nucleotide substitution rate in the SARS-CoV-2 viral genome within the *nsp12* coding region. *C*, Cytidine to uridine substitution rate of the SARS-CoV-2 viral genome within the *nsp12* region. *D*, Correlation of the cytidine to uridine mutation level within the *nsp12* region to the loss of infectivity relative to the DMSO control. Linear regression lines are shown as dashed lines for each drug group, and the shadows indicate the confidence intervals of the regressions. Abbreviations: DMSO, dimethyl sulfoxide; FAV, favipiravir; PFU, plaque-forming unit; RBV, ribavirin; rNHC, β -D-*N*-hydroxycytidine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

up to 300 μM , and RBV showed significant toxicity starting at 30 μM (CC_{50} of 24 μM). Thus, an antiviral effect of rNHC could be observed at subtoxic concentrations (0.3–3 μM), but the antiviral and mutagenic effects of RBV were observed only under conditions that were severely toxic to the host cell, while FAV demonstrated modest antiviral activity at high concentration but with undetectable mutagenic effect and with no cell toxicity at the highest concentrations tested (300 μM).

rNHC Is Mutagenic in a Mammalian Cell Assay

Mutagenic ribonucleoside analogs must be metabolized through a 5'-diphosphate precursor, which for the normal ribonucleotides is the substrate for ribonucleotide reductase (RNR) in the biosynthetic pathway for the synthesis of 2'-deoxyribonucleotide DNA precursors [12]. As a DNA precursor, dNHC (2'-deoxyribose form of NHC) would have the potential to be a mutagen to host cell DNA. We examined this possibility using a genetic selection system that records loss of gene function. CHO-K1 cells are functionally haploid for the *HPRT* gene, which when expressed confers sensitivity to the toxic base analog 6-TG, with resistance to 6-TG being conferred by mutations that inactivate the *HPRT* gene/protein function. We used loss of *HPRT* gene function in CHO-K1 cells to test for DNA mutagenesis activity of rNHC, FAV, and RBV. To increase the sensitivity of detection of mutagenic activity, we exposed the cells to these analogs for 32 days before selecting for *HPRT* knockout with 6-TG. As a positive control we briefly exposed cells to UV light. In an initial experiment we found rNHC was mutagenic to the cells in a dose-dependent manner (up to 3 μM), presumably through conversion to dNHC (Figure 2A). In a second experiment we again tested rNHC up to 3 μM and in addition tested FAV and RBV at 10 μM . rNHC again conferred 6-TG resistance in a dose-dependent fashion, while RBV showed little to no activity and FAV at 10 μM exhibited a modest yet significant increase in the number of 6-TG-resistant colonies (Figure 2A). rNHC and FAV did not inhibit cell growth at the concentrations tested for mutagenicity, while RBV inhibited cell growth even at 3 μM as well as the 10 μM tested (Supplementary Figure 4). For this second experiment, we also tested 3 anti-human immunodeficiency virus-1 (HIV-1) nucleoside analogs as an additional control for mutagenic activity. Zidovudine (AZT), lamivudine (3TC), and tenofovir (TDF) showed no mutagenic activity when tested at 10 μM (Figure 2B), a concentration that did not inhibit cell growth (Supplementary Figure 4) even though this concentration was able to inhibit HIV-1 infectivity (with EC_{50} s between 1 and 10 μM) in this cell type, demonstrating uptake and metabolism (Supplementary Figure 5). Thus, rNHC induced 6-TG resistance at concentrations that did not inhibit cellular growth, consistent with rNHC-diphosphate being a substrate both for the synthesis of rNHC-triphosphate for incorporation into RNA and for RNR on the pathway to synthesize dNHC-triphosphate for incorporation into DNA.

We next extracted RNA from individual colonies of cells, then amplified and sequenced most of the *HPRT* mRNA to document the presence of mutations in 6-TG-resistant colonies. A total of 42 colonies were sequenced and 32 (76%) of them had missense substitutions or frame shifts from deletions within the partial gene region sequenced (Figure 2B). In the 3 μM rNHC culture, 80% of the sequences contained different lesions, with 20% likely representing clones that expanded and resampled before selection. This analysis shows that the *HPRT* target is capable of recording many different mutations but also that in this format the assay is semiquantitative in that there is a possibility for 1 mutation to give rise to more than 1 resistant colony with the same mutation.

DISCUSSION

Here we carried out a head-to-head comparison of 3 mutagenic ribonucleoside/base analogs for antiviral and mutagenic activity against SARS-CoV-2. We found that rNHC was potentially antiviral and mutagenic against SARS-CoV-2 starting at concentrations as low as 0.3 μM (Figure 1 and Supplementary Figure 2); however, FAV showed no detectable mutagenic activity up to a concentration of 300 μM with modest antiviral activity starting at 100 μM , while RBV showed mutagenic activity at 300-fold the concentration of the comparable mutagenic activity of rNHC. Also, RBV antiviral activity and mutagenesis were observed only in the face of significant cellular toxicity. Thus, rNHC has a markedly different antiviral profile against coronavirus in the ability to induce lethal mutagenesis in the viral population compared to FAV and RBV. It is possible that the greater than 100-fold activity of rNHC compared to FAV and RBV is due to the virtual identity of rNHC to the normal nucleoside cytidine, allowing for its efficient metabolism inside of the cell.

Cancer therapies often rely on genotoxic agents that disproportionately impact the dividing cells in the tumor, although such treatments can contribute to subsequent tumors [13]. SARS-CoV2 infection results in an age-related acute respiratory disease spectrum that can be life-threatening, especially in the elderly and individuals with select underlying comorbidities [14]. rNHC has the potential to have therapeutic benefit in this setting. However, there are risks for the host in that the same mutagenic activity that impacts viral replication has the potential for incorporation and mutagenesis of host DNA. This risk can be inferred based on the common intermediate of the ribonucleoside diphosphate shared in the synthesis of both ribonucleoside triphosphates and 2'-deoxyribonucleoside triphosphates. The concern would be that mutations in host DNA could contribute to the development of cancer, or cause birth defects either in a developing fetus or through incorporation into sperm precursor cells. To test this inference, we used an *HPRT* gene knockout assay to assess mutagenesis of host DNA and readily demonstrated this activity for rNHC, presumably

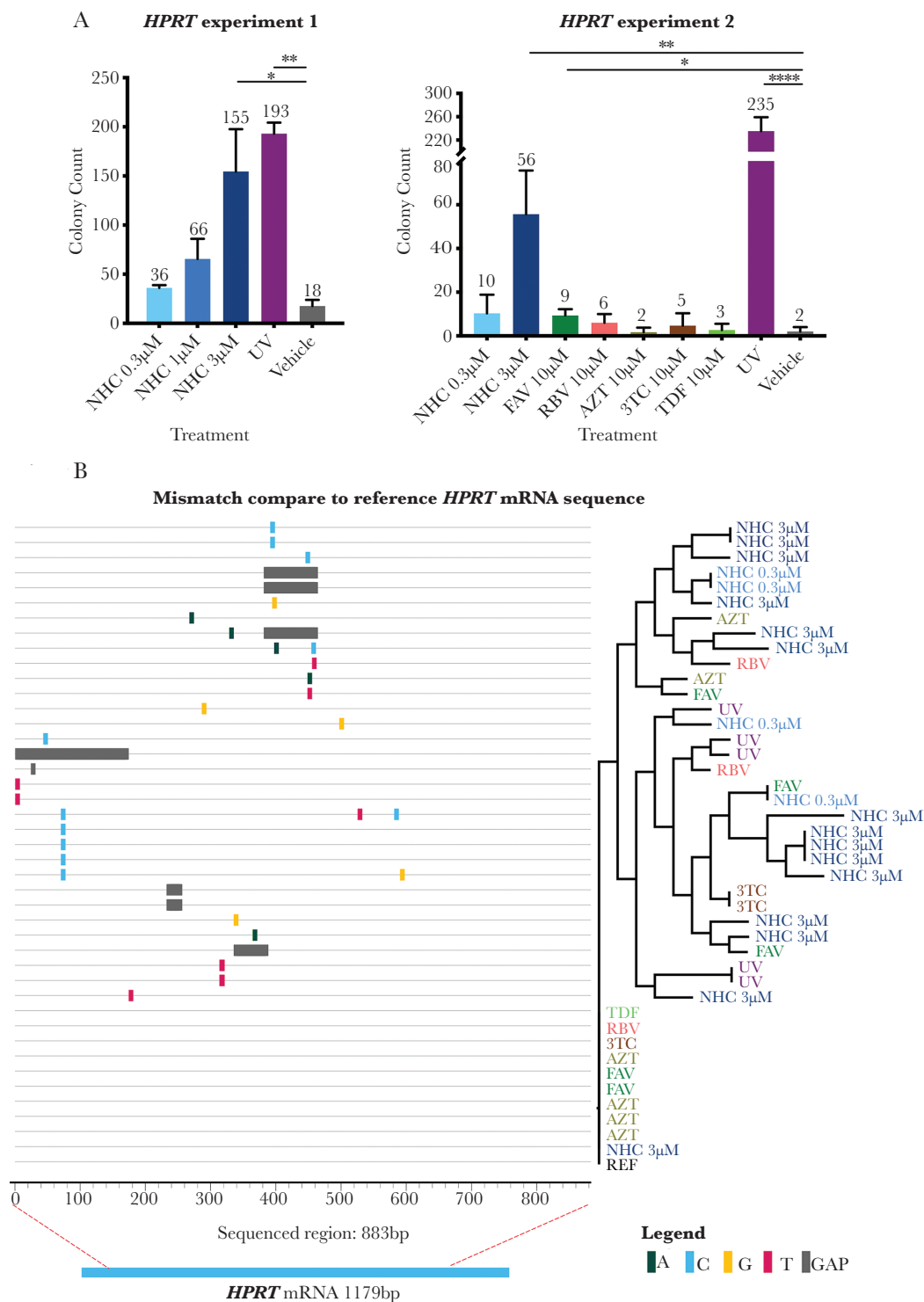


Figure 2. *HPRT* assay to detect genotoxicity of rNHC, RBV, FAV, AZT, 3TC, and TDF in CHO-K1 cells. **A**, 6-thioguanine-resistant colony counts in 2 separate *HPRT* mutagenesis experiments. In the *HPRT* experiment 2, an additional round of initial cleansing for spontaneous *HPRT* mutations was conducted to limit background mutations. Each compound/dose group had 3 replicates. Average numbers of colonies are shown on the top of each bar. Significance compared to vehicle control (* $P = .01-.05$, ** $P = .001-.01$, **** $P < .0001$) was determined using the unpaired *t* test calculated using the GraphPad Prism version 8.3.0 built-in function. **B**, Highlighter plot of the *HPRT* mutation colony sequencing from the second experiment. 6-TG-resistant colonies were scraped from the cell culture dish and transferred into 24-well tissue culture plate wells in complete growth medium with 30 µM 6-TG for an additional 4 days to expand the cells. Cells in each well were collected and total RNA was extracted. We amplified *HPRT* mRNA using 1-step RT-PCR, and sequenced the PCR products with Sanger sequencing. The total sequenced region was 883 bases of the total 1179-base *HPRT* mRNA (XM_007643626.2), with regions on each end of the mRNA not covered by sequencing. Each colony sequence was compared with the reference mRNA sequence. A total of 42 colonies were sequenced and 32 of them had missense substitutions or frame shifts from deletions within the region sequenced. Most of the mutations were different, while a few colonies contained the identical mutation, consistent with expansion after a mutation early in the treatment cycle. Abbreviations: 3TC, lamivudine; AZT, zidovudine; FAV, favipiravir; RBV, ribavirin; rNHC, β -*D*-*N*⁴-hydroxycytidine; REF, reference; RT-PCR, reverse transcription polymerase chain reaction; TDF, tenofovir.

through a dNHC metabolite. We take this as evidence that in exposing the viral population to mutagenesis in its RNA form, the host is likely to be exposed in its DNA form. It seems unlikely that a short course of therapy would spare the host from this exposure because both RNA precursors that affect the virus and DNA precursors that would affect the host pass through the common ribonucleoside diphosphate intermediate.

Many mutagens damage DNA in dividing or resting cells [13]; in contrast, the mutagenic activity of rNHC in vivo would be limited to dividing cells where the synthesis of DNA precursors is ongoing, conditions analogous to the cell culture assay used here. Thus, an assessment of mutagenic potential to the host in vivo should focus on tissues rich in dividing cells and use sufficiently sensitive assays to detect single nucleotide substitutions.

rNHC has powerful in vitro and in vivo activity against a large number of highly pathogenic emerging RNA viruses and represents a potentially important drug for use in the current and future pandemics. However, the risks for the host may not be zero and these risks need to be carefully evaluated in undertaking therapies that are by their nature designed to change the coding capacity of a virus' genetic material. Evaluating the utility of this drug should be done in those likely to receive the greatest benefit with monitoring provided to assess potential long-term genotoxic side effects. In addition, developing strategies to limit the potential for genotoxicity should be an important goal.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interests. The University of North Carolina is pursuing IP protection for Primer ID and R. S. has received nominal royalties. All other authors report no

potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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