Supporting Information

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SI Materials and Methods

Single Nucleotide Extension Assay for dGTP, dCTP, and dATP Levels. The levels of dGTP, dCTP, and dATP were determined as described for dUTP and dTTP (*Materials and Methods*) with the following modifications. The dUTPase reaction was not performed, and the extension reaction was performed in 50 mM Tris-HCl, pH 8.0, 1 mM $MgCl_2$, 0.5 mM β-mercaptoethanol (β-ME), and 0.1% BSA. The one base overhang on the template used was the canonical base that would pair with the base being interrogated. Analysis was performed as previously described (1).

Neutral Red Cytotoxicity Assay. HT29 cells were plated at a density of 1×10^4 cells in reduced folate medium (RFM) in a 96-well plate and allowed to adhere overnight. Raltitrexed (RTX) was added to the experimental wells and allowed to incubate for 6 h. After drug treatment, media were removed and replaced with 200 μL fresh RFM. At 24 or 48 h, media were removed and replaced with media containing 40 μg/mL Neutral Red (Sigma) and incubated for 2 h. At the end of incubation, the Neutral Red solution was removed, and cells were washed once with PBS. The Neutral Red dye was extracted from cells with 150 μL of destain solution [1% glacial acetic acid, 49% (vol/vol) ddH_20, and 50% (vol/vol) EtOH], and absorbance was measured at 544 nm. Viability was determined to be the percentage of fluorescence of experimental wells relative to the fluorescence of DMSO only control wells.

Western Blots. Global protein expression after RTX treatment was assessed by blotting HT29 extracts for α-tubulin. Protein extracts were prepared from HT29 cells that had been treated with 150 nM RTX for 6 h, washed, and incubated for an additional 48 h. Protein extraction was performed using CelLytic M reagent according to the manufacturer's instructions. Protein concentration was determined by the Bradford assay (BioRad). Ten micrograms of each sample was run on an SDS/PAGE gel and transferred to a PVDF membrane. The primary antibody for α-tubulin used was a rabbit polyclonal antibody from Abcam (ab15246), and the secondary antibody was HRP-conjugated anti-rabbit IgG (ab97080; Abcam).

For detection of virion-associated viral protein R (Vpr), purified viral particles were resuspended directly in SDS loading buffer, run on an SDS/PAGE gel, and transferred to a PVDF membrane. The primary Ab that detected Vpr was a polyclonal antibody from rabbits inoculated against residues 1–50 of Vpr (AIDS Research and Reference Reagents Program Catalog No. 11836). The secondary antibody was the HRP-conjugated anti-rabbit IgG described above. For an internal control, the same membrane was also blotted for p24 using the mouse monoclonal anti-p24 primary antibody (AIDS Research and Reference Reagent Program Catalog No. 6457) (2, 3). The secondary antibody used was an HRP-conjugated goat anti-mouse IgG (ab97040; Abcam).

qPCR (Early and Intermediate cDNA Synthesis, β-Globin). Real-time PCR analysis was performed on DNA extracted from cells 6 h after infection. The primers and probe for the early qPCR are Early-F (5′-GCTAACTAGGGAACCCACTGCTT-3′), Early-R (5′-CAACAGACGGGCACACACTGCTT-3′), and Early-p (5′-/6FAM/AGCCTCAATAAAGCTTGCCTTGAGTGCTTC/TAMRA/-3′). The primers and probe for the intermediate qPCR are Mid-F (5′-GGTGCGAGAGCGTCGGTATTAAG-3′), Mid-R (5′-AGCTCCCTGCTTGCCCATA-3′), and Mid-p (5′-/6FAM/AAAATTCGGTTAAGGCCAGGGGGAAAGAA/TAMRA/-3′). β-globin was amplified from genomic DNA to monitor its uracilation state. Primers and probe for the β-globin PCR were previously described (4).

Generation of Vpr Mutant Virus. Plasmids for expressing Vpr mutant viruses were generated via site-directed mutagenesis of pNL4-3-ΔE-eGFP. The first Vpr mutant contained a start mutant codon (ATG→GTG) that prevents initiation of translation of the Vpr ORF (5). The second Vpr mutant contained a point mutation at amino acid 54 that was mutated from tryptophan to glycine. Trp54 has been implicated in the recruitment of the nuclear isoform of human uracil DNA glycosylase (hUNG2) into viral particles (6). Primers used for site-directed mutagenesis were as follows: Vpr W54G sense, 5′-ACTTACGGGGATACTGGAGCAGGAGTGGAAGCC-3′; Vpr W54G antisense, 5′-GGCTTCCACTCCTGCTCCAGTATCCCCGTAAGT-3′; Vpr start mut sense, 5′-GGAAACTGACAGAGGACAGGTGGAACAAGCCCC-3′; Vpr start mut antisense, 5′-GGGGCTTGTTCCACCTGTCCTCTGTCAGTTTCC-3′. Virus was produced as described in *Materials and Methods*.

Sequencing Viral DNA. A 566-bp segement of the NL4-3-ΔE-eGFP viral genome spanning a portion of the N terminus of Env and the eGFP gene that interrupts the Env reading frame was amplified from cells 48 h after infection. Amplification was performed using the dU-insensitive polymerase Pfu C_x (Stratagene) to prevent a bias in amplification. Primers for amplification were as follows: Env-eGFP forward, 5′-GAGTCCTCTAGAAAAGAGCAGAAGACAGTGGCA-3′; Env-eGFP reverse, 5′-ACTGTAAAGCTTAAGTCGATGCCCTTCAGCTCG-3′. Amplicons were cloned into the XbaI and HindIII sites of pUC19 and sequenced.

Cell Cycle Analysis. HT29 cells were plated at a density of 100,000 cells per well of a 96-well plate and were allowed to expand for 2 d. Media was changed to RFM, and RTX or DMSO was added to the wells. After 6 h, the cells were washed once with PBS. Fresh RFM media were added to the wells until their collection time points. At each time point, the cells were trypsinized, spun down, and washed once in PBS. To stain the cells with propidium iodide (PI), the cell pellets were resuspend in 300 μL of staining buffer (50 μg/mL PI, 1 mg/mL RNase A, and 0.3% Nonidet P-40 in PBS) and stored at 4 °C for 30 min. Cells were read immediately on a FACSCalibur.

1. Grogan BC, Parker JB, Guminski AF, Stivers JT (2011) Effect of the thymidylate synthase inhibitors on dUTP and TTP pool levels and the activities of DNA repair glycosylases on uracil and 5-fluorouracil in DNA. *Biochemistry* 50(5):618–627.
2. Fouchier RA, Meyer BE, Simon JH, Fischer U, Malim MH (1997) HIV-1 infection of non-dividing cells: Evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. *EMBO J* 16(15): 4531–4539.
3. Simon JH, et al. (1997) The Vif and Gag proteins of human immunodeficiency virus type 1 colocalize in infected human T cells. *J Virol* 71(7):5259–5267.
4. Liszewski MK, Yu JJ, O'Doherty U (2009) Detecting HIV-1 integration by repetitive-sampling Alu-gag PCR. *Methods* 47(4):254–260.
5. Yao XJ, et al. (1995) Mutagenic analysis of human immunodeficiency virus type 1 Vpr: Role of a predicted N-terminal alpha-helical structure in Vpr nuclear localization and virion incorporation. *J Virol* 69(11):7032–7044.
6. Fenard D, et al. (2009) Uracil DNA glycosylase 2 negatively regulates HIV-1 LTR transcription. *Nucleic Acids Res* 37(18):6008–6018.

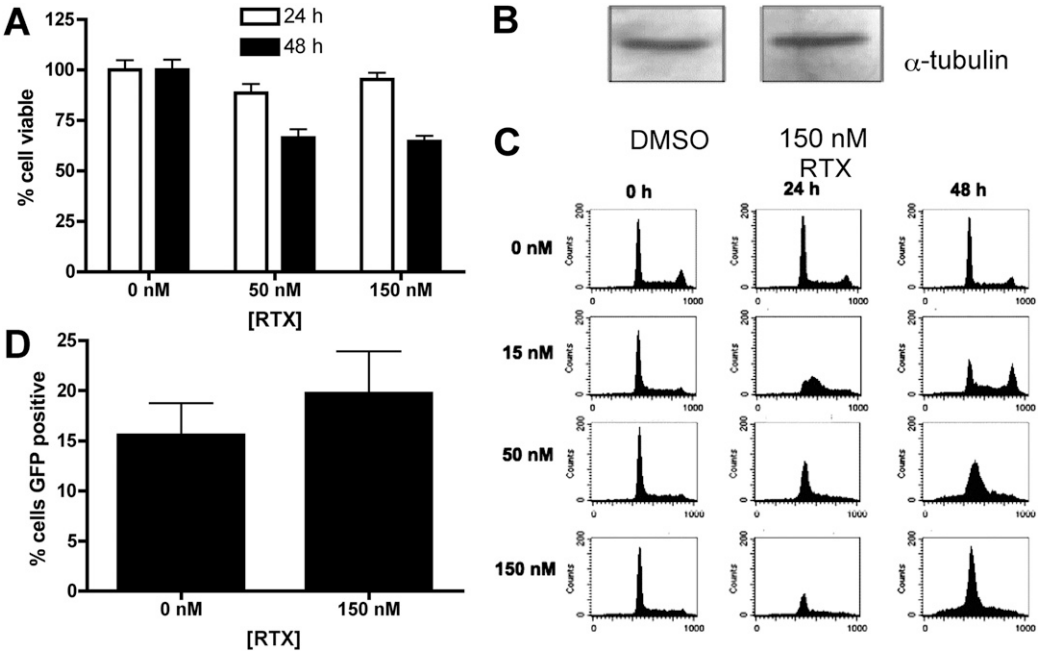

Fig. S1. Pulse treatment with RTX has minimal effects on HT29 cells. HT29 cells were pulse treated with RTX or DMSO vehicle for 6 h and then analyzed for the impact of treatment on cell metabolism at various time points. (*A*) Cell viability was assayed using the Neutral Red reagent at 24 and 48 h after drug washout. $n = 3$ at both time points. (*B*) HT29 cell lysates were collected 48 h after washout and blotted for α-tubulin levels as an indicator of global protein expression. Shown is a representative of $n = 3$ samples. (*C*) Cell cycle progression was analyzed at 0, 24, or 48 h after washout by staining for DNA content via propidium iodide followed by FACS analysis. (*D*) The ability to express protein from the viral LTR promoter was analyzed by nucleofecting the viral plasmid pNL4-3-ΔE-eGFP immediately after drug treatment and analyzing eGFP expression 48 h after nucleofection by FACS. $n = 3$.

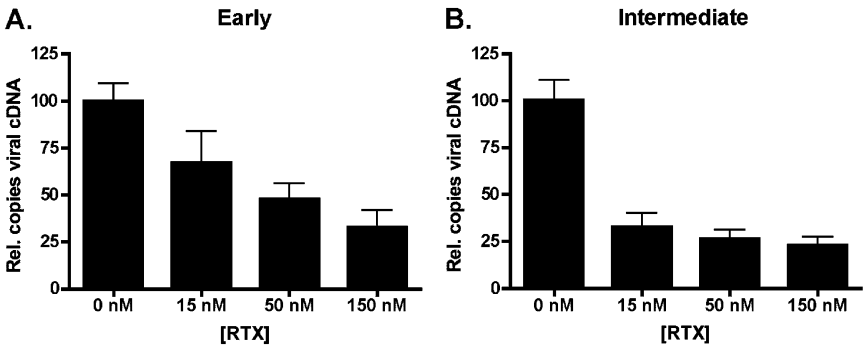

Fig. S2. The inhibition of viral cDNA production at 6 h occurs early in reverse transcription. DNA isolated from infected HT29 cells 6 h after infection was amplified with primer sets that are specific for RU5 (Early-A) and minus strand Gag (Intermediate-B) cDNA products. $n = 3$ for both data sets.

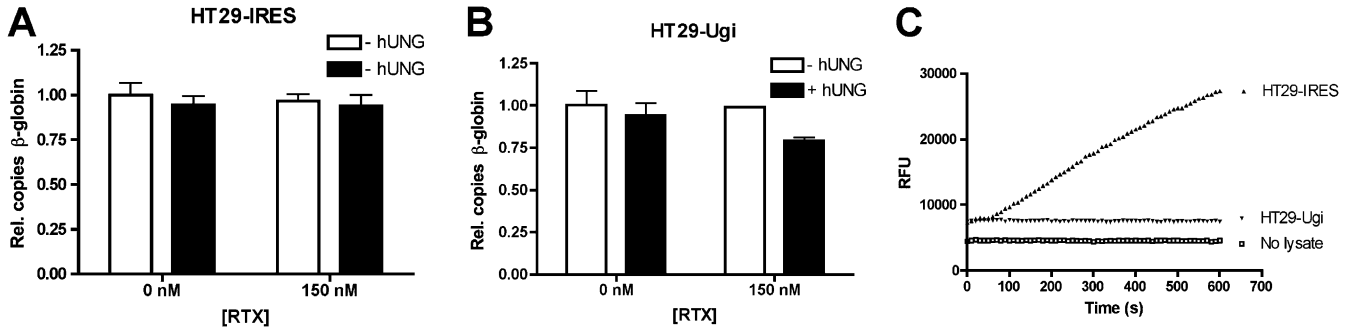

Fig. S3. Pulse treatment with RTX does not lead to genomic uracilation in HT29-IRES cells, but slight uracilation in HT29-UGI cells. HT29-IRES (*A*) and HT29-Ugi cells (*B*) were treated with RTX for 6 h, and genomic DNA was obtained 48 h after washout. The copy number for β-globin was determined by qPCR. To determine the level of uracilation, template DNA was either subjected to a mock reaction (white bars) or hUNG/APE1 reaction (black bars) before PCR. For all qPCRs, $n = 3$. (*C*) To confirm that UNG activity is inhibited in HT29-Ugi cells, 10 μg of cell lysate from HT29-IRES and HT29-Ugi cells was subjected to the fluorescence UNG activity assay (*Materials and Methods*). HT29-Ugi lysate failed to induce an increase in fluorescence over the time period where HT29-IRES cells induced a nearly fourfold increase.

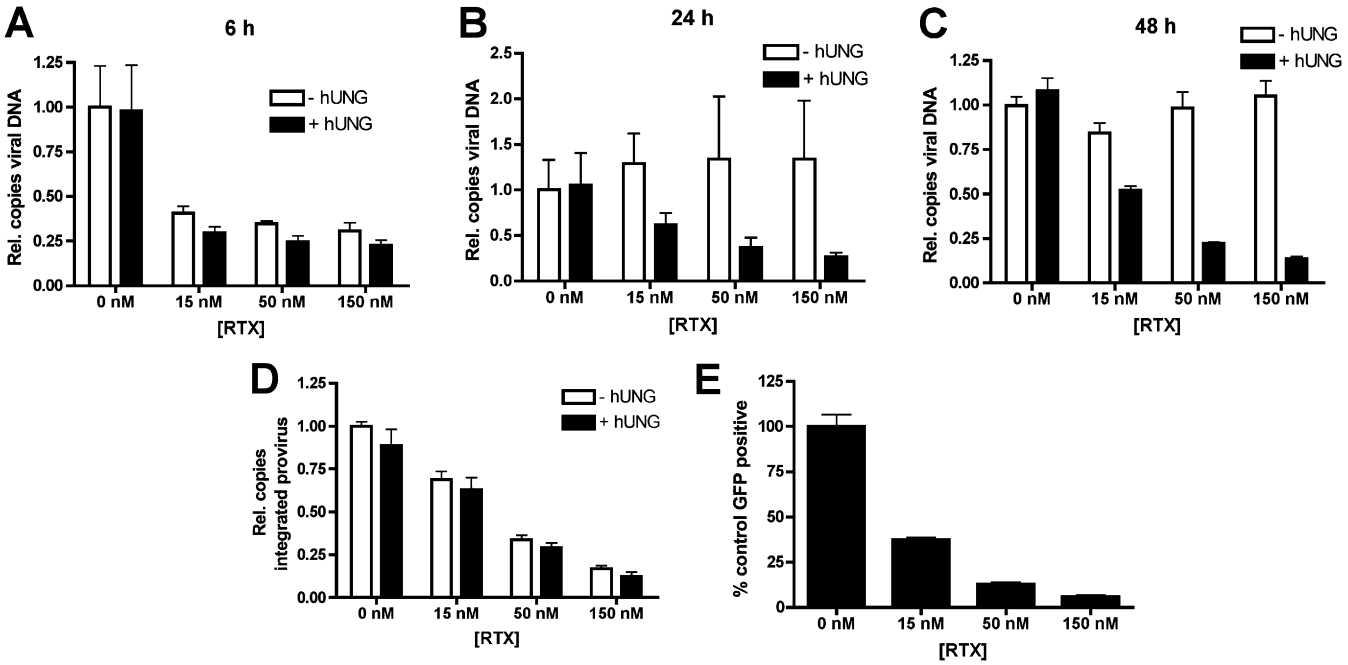

Fig. S4. RTX mediates dUTP incorporation and inhibition of integration and viral protein expression in HT29-IRES cells. HIV-1 cDNA levels were measured in RTX-treated HT29-IRES cells at 6 (*A*), 24 (*B*), and 48 (*C*) h after infection. (*D*) Integrated proviruses were measured 48 h after infection using the Alu-Gag PCR as previously described. DNA uracilation was determined by pretreating template DNA with hUNG and APE1 (black bars) or mock treatment (white bars). (*E*) Expression of virally encoded eGFP 48 h after infection as analyzed by FACS. See *Materials and Methods* for qRT-PCR methods used. For all experiments, $n = 3$.

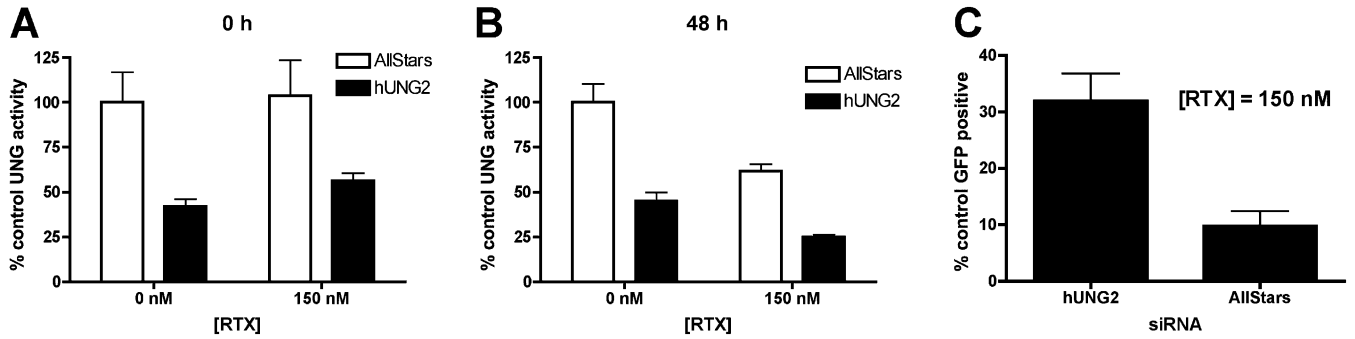

Fig. S5. hUNG2 siRNA reduces UNG activity in HT29 cells and protects against the antiviral effects of RTX. HT29 cells were nucleofected with hUNG2 or AllStars siRNAs (*Materials and Methods*). After a 14-h incubation, cells were treated with 150 nM RTX for 6 h. In *A* and *B*, cells were washed and incubated for an additional 4 or 52 h, respectively, and then analyzed for UNG activity. These time points correlate with 0 and 48 h after infection. (*C*) After drug treatment, cells were spin infected and incubated for 48 h and then analyzed for virally encoded eGFP expression by FACS ($n = 3$).

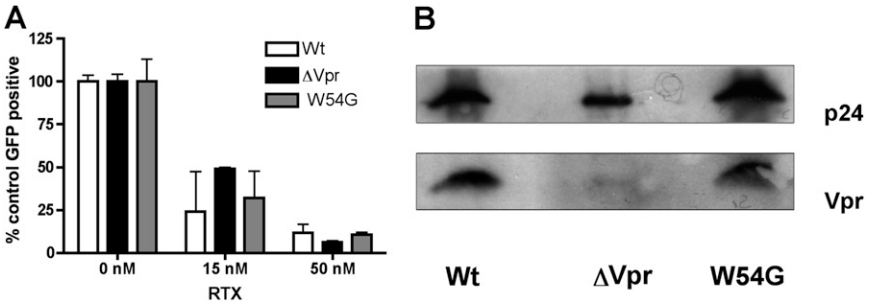

Fig. S6. The antiviral effect of RTX is independent of Vpr status. (*A*) HT29 cells were treated with RTX and infected with NL4-3-ΔE-eGFP virus containing WT Vpr (Wt), a start codon mutation Vpr (ΔVpr), or a Vpr with a point mutation (W54G) that is implicated in binding with UNG2 ($n = 3$). (*B*) Purified virions were blotted for p24 and Vpr to confirm that the start mutant virus had significantly reduced Vpr levels.

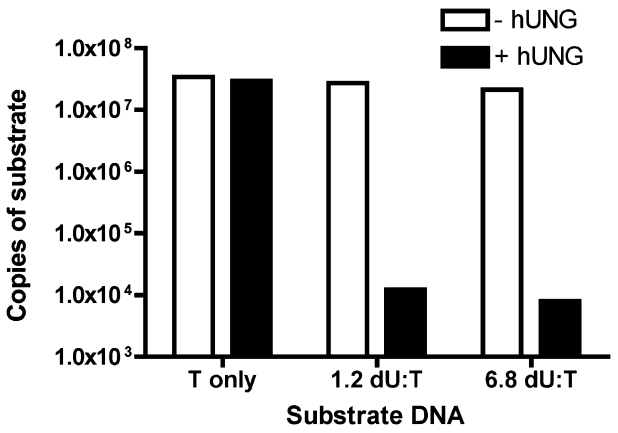

Fig. S7. Substrates PCR amplified in the presence of dUTP are highly uracilated. A 2.5-kb segment of pmaxGFP was PCR amplified at various dUTP:dTTP ratios. qPCR was performed on the substrate using a primer/probe set specific to GFP (*Materials and Methods*). To assess the level of uracilation, a mock reaction (white bars) or a hUNG/APE1 reaction (black bars) was performed before the qPCR.

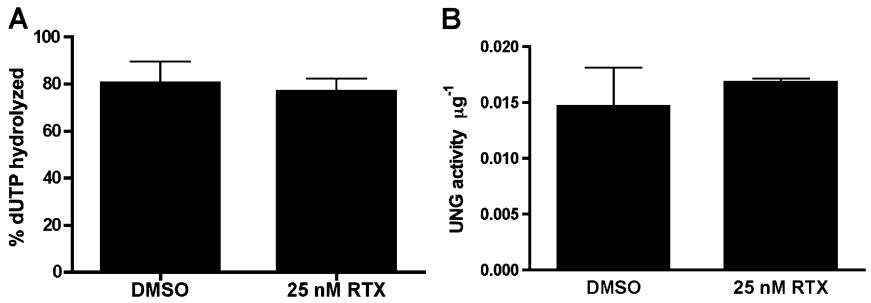

Fig. S8. Pulse RTX treatment does not affect dUTPase or UNG activity in CD4+ T cells. CD4+ T cells were treated with 25 nM RTX or DMSO vehicle for 6 h. Protein lysates were then obtained, and lysates were used in the (*A*) dUTPase and (*B*) UNG activity assays as previously described (*Materials and Methods*; $n = 2$ assayed in duplicate).

Table S1. dNTP levels in HT29 cells after a 6-h pulse treatment with RTX

	pmol/1e6 cells			
dNTP	0 nM	15 nM	50 nM	150 nM
dATP	24.6 ± 0.5	27.2 ± 0.4	27.4 ± 0.8	31.3 ± 0.2
dCTP	16.4 ± 0.1	13.8 ± 0.8	13.3 ± 0.3	13.6 ± 0.6
dGTP	3.3 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	0.9 ± 0.1
dTTP	17.8 ± 0.1	6.3 ± 0.2	5.5 ± 0.1	3.4 ± 0.1

Values are mean ± SD.

Table S2. Mutation frequency of viral DNA isolated from infected HT29-IRES and HT29-Ugi cells after treatment with RTX

								Types of mutations		
Cell line	[RTX], nM	No. of clones	Base pairs sequenced	Percent clones mutated	No. of point mutations	Mutation frequency	Mutations per viral genome	G→A	C→T	Other
HT29-IRES	0	47	26,602	6.4	3	1.1e−4	1.1	1	1	1
	150	63	35,658	7.9	11	3.1e−4	2.9	6	4	1
HT29-Ugi	0	57	32,262	5.3	3	9.3e−5	0.9	3	0	0
	150	53	29,998	7.5	4	1.3e−4	1.3	1	3	0