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Supplemental Material

MPLEx: A method for simultaneous pathogen inactivation and extraction of samples for multi-omics profiling

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EXPERIMENTAL DETAILS AND RESULTS FOR THE INACTIVATION STUDIES FOR EACH OF THE DIVERSE BACTERIAL AND VIRAL PATHOGENS EVALUATED WITH MPLEx

This results section details both the experimental details and the inactivation studies for each of the diverse bacterial and viral pathogens evaluated with MPLEx. The ability of MPLEx to inactivate pathogens was determined by comparing the presence and abundance of each pathogen in either pre-treatment or positive controls vs. treated samples. Some of the bacterial pathogens and all of the viral pathogens were assessed in infection studies where infected host cells were treated with MPLEx and inactivation was evaluated via inoculation of the resulting cell lysates into fresh cells. For these infection studies, the MPLEx-treated cell lysates were diluted to retain host cell viability, as undiluted samples can induce toxicity. Lastly, since MPLEx facilitates the molecular characterization of infectious samples by enabling concurrent pathogen inactivation and simultaneous extraction of proteins, metabolites, and lipids from a single sample for subsequent mass spectrometry-based proteomics, metabolomics, and lipidomics measurements, we conclude our inactivation studies by demonstrating that our method results in reliable MS-based multi-omics data with respect to infection duration in Calu-3 cells infected with H7N9 wild-type and mutant viral strains. Where subsequent multi-omic analyses were planned, prior to MPLEx, samples were washed with a quenching solution, which arrested enzymatic metabolism.

BACTERIA

***Yersinia pestis* strain CO92:** *Y. pestis* is a Gram-negative bacterial pathogen and a causative agent of bubonic and pneumonic plagues [1]. In this study, we used the fully virulent, whole-genome sequenced *Y. pestis* CO92 (*Orientalis* biovar strain), which is the reference strain in biodefense-related studies[2-5]. **In the current study, *Y. pestis* CO92 was inactivated completely in pure bacterial culture and in infected host macrophages by MPLEx, as no colony forming units (CFUs) were found across the 20 experimental replicates compared to pre-treatment controls.**

Y. pestis in axenic culture: The wild-type *Y. pestis* strain CO92 was plated from glycerol stock onto Heart Infusion Broth (HIB) agar plates and grown for 40-42 h at 26°C. The resulting bacteria were suspended in sterile 150 mM ammonium bicarbonate buffer at an OD_{600} of 1, roughly corresponding to $5x10^8$ CFUs per mL. The exact bacterial concentration was calculated by making 10-fold dilutions and plating onto HIB agar. Twenty MPLEx-treated experimental replicates were created by transferring 150 µL of the bacterial suspension containing the highest bacterial concentration, 6.3×10^7 CFUs (the pre-treatment control), to 1.5 mL microcentrifuge tubes and adding 600 µL of MPLEx solution. For the 20 MPLEx-treated experimental replicates, 100 µL of each solution was plated on HIB. The plates were evaluated for CFUs to quantify inactivation of *Y. pestis* after 4 d at 26°C. No individual colonies formed on the agar plate, indicating that all *Y. pestis* bacteria were inactivated by MPLEx (**Figure 1**). A second member of the genus *Yersinia*, *Y. enterocolitica,* was also completely inactivated by MPLEx (**Table S1**).

Y. pestis infection in macrophages: The mouse macrophage cell line RAW 264.7 was grown in DMEM High Glucose medium supplemented with 1 mM sodium pyruvate and 10% Fetal Bovine Serum (FBS). Macrophages were plated at 2×10^5 cells per well (70-80% confluence) and were infected with *Y.*

pestis strain CO92 at a multiplicity of infection (MOI) of 100. The plate was incubated for 2 h at 37°C in 5% CO_2 to allow macrophages to engulf *Y. pestis* bacteria. The macrophages were then lysed with sterile 0.1% sodium deoxycholate. To create 20 pre-treatment controls, the parent lysate was aliquoted 20 times by diluting 10-fold in buffer and plating on agar. The average bacterial concentration of the 20 pre-treatment controls was 5.5±0.6 x 10^7 (max, 1.1 x 10^8; min, 2.0 x 10^7) CFUs per mL. Twenty MPLEx-treated experimental replicates were created by transferring 150 µl of each pre-treatment lysate to 1.5 mL microcentrifuge tubes and adding the MPLEx solution as described in the *Y. pesti*s pure culture experiment. Aliquots (100 µL) of each treated extract were plated on agar and evaluated after 4 d for bacterial growth. All *Y. pestis* bacteria from this macrophage infection study were inactivated by MPLEx (**Figure 1**).

***Salmonella enterica* subsp. *enterica* serovar Typhimurium:** *S.* Typhimurium is a Gram-negative bacterial pathogen and is a leading cause of gastroenteritis in humans. In addition, it is used as a mouse model of human typhoid fever[6, 7]. **In this study, *S.* Typhimurium was inactivated completely by MPLEx since no CFUs were detected across the 20 experimental replicates.**

S. Typhimurium *in axenic culture: Salmonella* Typhimurium 14028 was spread onto a Luria broth (LB) agar plate; 20 individual colonies were randomly selected, inoculated into LB broth, and grown overnight at 37°C while shaking at 200 rpm. Serial dilutions of the cultures quantified the average pre-treatment CFUs for the 20 colonies to be 3.2±0.4 x 10^8 (max, 8.0 x 10^8; min, 1.0 x 10^8) per mL. Bacteria from each of the 20 overnight cultures were pelleted, washed with sterile H_2O prior to the rapid quenching protocol, and MPLEx solution was added. Aliquots (100 µL) of each MPLEx-treated sample were plated on agar at 37°C overnight and evaluated for bacterial growth. No viable *S.* Typhimurium was found after MPLEx (**Figure 1**). In addition, all *S.* Typhimurium from a human epithelial cell (HeLa cells) infection study were inactivated by MPLEx (**Table S1).**

***Campylobacter jejuni*:** *C. jejuni* is a Gram-negative bacterial pathogen and one of the leading cause of bacterial food-borne diarrheal disease throughout the world[8]. In addition, a neuromuscular paralysis known as Guillain-Barré syndrome is a rare but serious complication of *C. jejuni* infection[9]. **In this study, *C. jejuni* was inactivated completely in pure bacterial culture and in host HeLa (ATCC CCL2) cells by MPLEx since no CFUs were detected across the 20 experimental replicates.**

C. jejuni in axenic culture: Campylobacter jejuni F38011 clinical isolate was grown on Mueller-Hinton Blood (MHB) agar supplemented with 5% bovine blood and incubated at 37°C under microaerobic conditions. Twenty cultures were suspended in PBS to an OD_{540} of 0.15 and 1 mL of the suspension was pelleted and treated with quenching solution. Bacteria were pelleted, suspended in 150 µL of 150 mM ammonium bicarbonate buffer and then mixed with 600 µL of MPLEx solution. Aliquots (100 µL) of the 20 MPLEx-treated samples were plated on agar at 37°C in a microaerobic environment and evaluated for bacterial growth after 3 d. No viable *C. jejuni* was detected after MPLEx (**Figure 1**). For positive controls (n = 20), phosphate buffered saline was substituted for all buffers and reagents. The average bacterial concentration in positive controls was 5.3±0.9 x 10^8 (max, 2.0 x 10^9; min, 1.0 x 10^8) CFU per mL.

C. jejuni infection in HeLa cells: Intracellular *C. jejuni* was recovered using the gentamicin protection assay[10]; the antibiotic gentamicin cannot penetrate host cells and therefore only kills

extracellular bacteria. Briefly, HeLa cells were infected with *C. jejuni* at a MOI of 200 and incubated for 3 h, followed by 3 h of incubation with media containing 250 µg/mL gentamicin. To quantify intracellular *C. jejuni*, HeLa cells were washed twice to remove gentamicin, permeabilized with 0.1% Triton X-100 and the resulting lysates serially diluted and plated on MHB agar. The average pre-treatment bacterial concentration was 1.1±0.1 x 10^5 (max, 1.7 x 10^5; min, 5.0 x 10^4) CFU per mL across the 20 infection replicates. To evaluate MPLEx inactivation, infected HeLa cells were treated with quenching solution, suspended in 150 µL of 150 mM ammonium bicarbonate buffer and then mixed with 600 µL of MPLEx solution. Aliquots (100 µL) of each treated lysate were plated on agar and assessed for CFUs. No infected HeLa cells showed bacterial growth after treatment with MPLEx.

Methicillin-resistant *Staphylococcus aureus* USA300 (CA-MRSA): Community-associated methicillin-resistant *S. aureus* (CA-MRSA) isolate USA300 is a Gram-positive bacterial pathogen that readily acquires resistance against most classes of antibiotics through mutation of an existing bacterial gene or horizontal transfer of a resistance gene from another bacterium[11]. CA-MRSA strains are now endemic in the United States causing tissue-destructive infections, such as necrotizing fasciitis, fulminant, necrotizing pneumonia, and endocarditis, bacteremia, and sepsis[12-14]. **In this study, MPLEx treatment of 20 MRSA colonies with an average concentration of 4.9 x 10^8 CFUs per mL resulted in complete inactivation; in comparison, MPLEx treatment of 20 MRSA colonies with a magnitude higher average concentration of 1.9 x 10^9 CFUs per mL resulted in in slightly less successful inactivation with a >99.9% reduction of viable bacteria after treatment with an average concentration of 1 CFU remaining in 2 of the treated samples.**

MRSA in axenic culture: For details on the growth and treatment of MRSA see *S.* typhimurium experimental details. For evaluating MPLEx inactivation of MRSA, serial dilutions were prepared from the resulting 20 MPLEx-treated lysates from each study. Aliquots (100 µL) of each treated sample were plated on LB agar and CFUs were counted after incubation at 37°C overnight. MRSA was completely inactivated by MPLEx in the first study with an average pre-treatment CFU of 4.9±0.9 x 10^8 (max, 1.5 x 10^9; min, 1.0 x 10^8) per mL. MRSA was decreased to 1 CFU in 2 of the 20 replicates (the other 18 replicates showed complete inactivation) following MPLEx in the second study with an average pre-treatment CFU of 1.9±0.2 x 10^9 (max, 4.4 x 10^9; min, 8.2 x 10^8) per mL.

Clostridium difficile* strain 630 spores*: *C. difficile* is a Gram-positive, anaerobic, spore-forming, nosocomial intestinal pathogen. The genome of the toxigenic and multidrug-resistant strain, designated 630, contains an extensive array of genes involved in antimicrobial resistance, virulence, host interaction and the production of surface structures[15]. When the normal intestinal microbiota is disturbed (e.g., via antibiotic treatment) opportunistic *C. difficile* can overgrow; the severity of *C. difficile* infections can escalate to life threatening toxic megacolon, with an overall mortality rate of ~6% across all populations and higher than 13% in advanced age populations (> 80 years of age)[16]. **In this study, MPLEx decreased *C. difficile* concentrations in aerobic and anaerobic experiments; longer exposure time increased the magnitude of this decrease.**

C. difficile spores: *Clostridium difficile* strain 630 was grown in an anaerobic chamber (90% N_2, 5% CO_2, 5% H_2) overnight in brain heart infusion medium broth containing yeast extract. After 24 h, an aliquot (100 µL) of the culture was spread onto plates containing the same medium and incubated for 3

d anaerobically. From this culture, 20 individual colonies were randomly selected and re-streaked onto fresh plates. The plated colonies were allowed to grow for 7 d before being removed from the anaerobic chamber and subjected to oxygen overnight to kill vegetative *C. difficile* and to result in spore formation. Plates were flooded with cold water and spores were removed by scraping. The suspension was then heated at 60°C for 20 min to kill any remaining vegetative forms and stored at 4°C in sterile water. The presence of spores was confirmed using phase contrast microscopy. To determine if MPLEx inactivates spores, the suspensions were titered by spot-plating a dilution series on plates containing tauro-cholate (0.01%) and D-cycloserine (1%) prior to, and after treatment with the solvent for 5, 20 and 120 min. Plates were then placed in the anaerobic chamber at 37°C for germination and colonies counted. Although spores were not completely inactivated by MPLEx, longer exposure times had the greatest impact on decreasing CFUs ($4.5 \pm 1.9 \times 10^4$ [max, 3.3×10^5; min, 1.7×10^2] at 5 min**,** $2.7 \pm 0.8 \times 10^4$ [max, 1.0×10^5; min, 6.7×10^2] at 20 min, $1.6 \pm 0.6 \times 10^4$ [max, 1.0×10^5; min, 2.7×10^2] at 120 min) compared to the pre-treatment control ($3.7 \pm 2.3 \times 10^6$ [max, 3.3×10^7; min, 3.3×10^4]). The shortest 5 min exposure eliminated 98.8% of spores while the 20 and 120 min exposures eliminated 99.3% and 99.6%, respectively.

To evaluate inactivation of vegetative cells, the same experiment was performed within the anaerobic chamber to prevent sporulation. Two hours of MPLEx exposure decreased *C. difficile* CFUs by 99.9% ($2.0 \pm 0.8 \times 10^2$ [max, 1.7×10^3; min, 0]) versus the pre-treatment control ($1.9 \pm 0.4 \times 10^5$ [max, 6.7×10^5; min, 3.3×10^4]).

VIRUSES

West Nile virus (WNV) New York 1999 strain: WNV is a member of the *Flaviviridae* family of positive-sense single-stranded RNA viruses. WNV is transmitted between mosquitoes and birds, but can also infect humans and other vertebrate animals. It is endemic to Africa, Asia, and parts of Europe, and was introduced into North America in 1999. Seroprevalence studies suggest that ~20% of infected individuals develop clinical manifestations[17], and that ~1/150 infected people develop neuroinvasive disease[18, 19]; of those, ~10% succumb to the infection[20]. No specific treatment options exist, and no vaccines are approved for use in humans. **In this study, after MPLEx treatment, infectious WNV was not detected in primary mouse cortical neurons (CN), bone marrow derived dendritic cells (DC), or granule cells neurons (GCN).**

WNV infection in primary mouse CN, DC, and GCN: 2-5 x 10^5 CN, DC, and GCN, were inoculated (MOI 250-500) with an infectious clone-derived virulent WNV NY99 strain[21]. Twenty-one samples were generated for each cell type and an anti-WNV antibody quantified one pre-treatment virus titer 24 h after inoculation[22] as follows: 4 x 10^7 focus forming units (FFU) (CN), 8 x 10^7 FFU (DC), 7 x 10^8 FFU (GCN). Treated cells (trypsin removed adherent CN and GCN cells) were washed in PBS, resuspended in 300 µL of 150 mM ammonium bicarbonate buffer and then mixed with 1,200 µL of MPLEx solution. Solvent treated, infected CN, DC, and GCN were then serially diluted (1:10 – 1:100,000,000,000) in duplicate and 100 µL of each dilution was incubated on Vero cells (non-human primate kidney epithelial cells)[22]. Vero cells were then fixed and FFU per mL detected [22]. While the 1:10 dilution of MPLEx-treated sample was toxic to Vero cells, no FFU were detected in viable Vero cells incubated with the 1:100 dilution or any other further dilution across the 20 replicate samples from each infected cell type resulting in a minimum of a 5-log reduction in infectious WNV after MPLEx.

Middle East Respiratory Syndrome Coronavirus (MERS-CoV): The newly emerged highly pathogenic MERS-CoV contains a positive-sense, single stranded RNA genome and causes severe pulmonary disease in humans[23, 24]. The current MERS-CoV outbreak began in 2012 and has resulted in >1,600 laboratory-confirmed cases and >580 deaths in 26 countries[25]. **In this study, cells that were infected with MERS-CoV but not treated with MPLEx had average viral titers of 6.6 x 10^5 plaque forming units (PFU per mL) whereas MERS-CoV infected MPLEx-treated samples had no detectable plaques in 20 replicate samples.**

MERS-CoV infection in human lung epithelial (Calu-3 2B4) cells: Calu-3 2B4 cells were inoculated (MOI 5) with MERS-CoV for 18 h (n=20). Cells were washed once with 3 mL rapid quenching solution, scraped into 150 µL of 150 mM ammonium bicarbonate buffer, and mixed with 600 µL of MPLEx solution. The samples were vortexed and 100 µL aliquots of the MPLEx-treated samples were serially diluted 1:10 through 1:1000. Plaque assays were performed on Vero81 cells inoculated with serial dilutions (through 1:1000) from the 20 treated samples and 20 pre-treated controls (media from MERS-CoV Calu-3 2B4 infected wells) were plated in parallel and incubated for 72 h. From viral plaque assays, the 20 pre-treated samples showed 6.6±1.3 x 10^5 [max, 2.0 x 10^6; min, 1.5 x 10^5] PFUs per mL, and the 20 replicates of MPLEx-treated samples had no detectable plaques or were below the limit of detection.

Ebola virus: Ebola is a negative-sense, single stranded RNA virus that is highly pathogenic in humans, causing severe hemorrhagic fever with fatality rates of 50–70%[26]. The 2014-2015 EBOV outbreak in western Africa began in March 2014 and resulted in >28,000 suspected cases (>15,000 laboratory-confirmed) and >11,000 deaths[27]. **In this study, after MPLEx treatment, Ebola was inactivated in human hepatocyte (IHH) cells and human serum since the virus was undetectable across the 20 experimental replicates of each experiment.**

Ebola infection in IHH cells: IHH cells infected with Ebola virus (3 x 10^6 particles; Ebola-Zaire delta-VP30 [BSL3 version]) were washed with rapid quenching solution and collected in 150 µL of 150 mM ammonium bicarbonate and then mixed with 600 µL MPLEx solution. All phases (protein, metabolite, and lipid) were evaluated for viral activity. After samples were vortexed, the protein, metabolite, and lipid fractions were obtained after centrifugation. The individual fractions were dried down and the lyophilized material was resuspended in 2 mL of growth medium. Confluent Vero cells were then inoculated for 1 h with 100 µL of the resuspended fractions for a total of 20 wells of cells per fraction (i.e., protein, metabolite, lipid) and an additional 900 µL of medium was added. As a positive control, non-inactivated virus was used to infect Vero cells. Cells were then incubated at 37°C for 12 days. After 12 days, cells were fixed and stained for viral antigen. No virus-induced or chemical-induced cytotoxicity or viral antigen was detectable compared to mock-infected cells. No virus was detected in the MPLEx-treated samples while infectious virus was detected in the positive control (2.5x10^6 FFU/mL). To test if MPLEx-treatment inactivated Ebola in human serum, 10 µL of Ebola-Zaire delta-VP30 virus (~9.5x10^6 virus particles) were spiked into 140 µL of human serum (n=20) and then mixed with 600 µL MPLEx solution. As described above, Vero cells were inoculated with the resulting protein, lipid, and metabolite fractions for each replicate experiment. As a positive control, the same amounts of virus and serum were vortexed with 600 µL of PBS; this mixture was diluted 10-fold and used to infect Vero cells in order to determine virus titer. No virus was detected in the MPLEx-treated samples, while infectious

virus was detected in the positive control ($6x10^7$ FFU/mL). Thus, MPLEx treatment is also a reliable inactivation method for Ebola virus in human serum.

Avian Influenza (H7N9) virus: The novel H7N9 virus is a negative-sense, single-stranded, segmented RNA virus. The first human infected with H7N9 was reported in China in March 2013 and the seasonal re-emergence of this enzootic virus highlights its potential to become a long-term threat to public health[28]. **In this study, after MPLEx treatment H7N9 was inactivated in Calu-3 cells and mouse lung tissue since the virus was undetectable across the 20 experimental replicates.**

H7N9 infection in Calu-3 cells: Calu-3 cells were infected with influenza A/Anhui/1/2013 (H7N9), washed with rapid quenching solution, scraped into 150 µL of 150 mM ammonium bicarbonate, and mixed with 600 µL of MPLEx solution. The sample was vortexed and diluted 1:10 with cell culture medium (in a 750 µL final volume), and the resultant mixture was equally distributed into 20 T25 tissue culture flasks containing confluent monolayers of Madin-Darby canine kidney (MDCK) cells and 5 mL of virus growth medium (~37.5 µL of the diluted, MPLEx-treated sample was added to each flask). The sample was vortexed before each flask inoculation to prevent its separation into polar and non-polar phases. The 1:10 dilution of the MPLEx-treated cell lysate was necessary to retain MDCK viability, as undiluted and lesser diluted samples (1:2 and 1:5) induced significant toxicity to the cell culture within 20 min of inoculation (as determined in a preliminary experiment). Following MDCK culture inoculation, cells were incubated at 37°C for 48 h. At 48 h post-inoculation, all 20 MDCK cultures were observed microscopically for signs of virus-induced CPE. Influenza hemagglutination (HA) assays were performed on supernatants from the pre-treated Calu-3 culture (256 HA units) and the MPLEx-treated MDCK cultures which showed complete inactivation with no Hemagglutinin (HA) detected across all 20 replicates.

H7N9 in vivo infection of mouse lung tissue: Mouse lungs infected with influenza A/Anhui/1/2013 (H7N9) were homogenized in 300 µL of 150 mM ammonium bicarbonate. The homogenate was then centrifuged to remove debris, and the resultant supernatant was mixed with 1200 µL of MPLEx solution. MDCK infection studies were performed as described above for the H7N9 infection in Calu-3 cells experiment. HA assays were performed on supernatants from the pre-treated infected mouse lung (128 HA units) and the MPLEx-treated MDCK cultures which showed complete inactivation with no HA detected across all 20 replicates.

Adenovirus type 5 (Ad5) virus: Ad5 does not contain an outer lipid bilayer; instead, the viral double-stranded DNA is contained by a rigid icosahedral capsid shell[29]. **In this study, MPLEx treatment resulted in >99% reduction in Ad5, where low levels of pathogenic virus was still detected in 1 out of 8 metabolite fractions and 4 out of 8 protein fractions after treatment across our two replicate experiments. Figure 2 depicts inactivation levels of the Ad5 in the 4 MPLEx derived protein samples in one of our two replicate experiments.**

Ad5 infection in human alveolar epithelial (A549) adenocarcinoma cells: A549 cells were infected with Ad5 (McEwan strain provided by P. Kinchington at the University of Pittsburgh) at a MOI of 3 PFU per cell. At 2 days post-infection, we harvested 2 replicates from infected cells and 1 mock-infected replicate (based on previous studies, our pre-treatment samples were estimated to be 1×10^7-1×10^8 PFU/mL). Cells were scraped into 150 µL of 150 mM ammonium bicarbonate, and mixed with 600 µL of

MPLEx solution. Dried samples were reconstituted in 1 mL of DMEM/F12 medium (with 10% FBS and pen/strep). Each replicate derived from Ad5 infections were divided into 4 equal aliquots for inoculation of T25 flasks of nearly confluent A549 cells. Samples derived from mock infections were inoculated in entirety into a single T25 culture. To provide positive controls to assess the amount of virus left in any samples, we inoculated T25 cultures with a known amount of Ad5 (100, 1,000 or 10,000 PFU). At 5 d post-infection, a significant cytopathic effect (CPE) was observed in all infected control samples. No CPE was observed in any of the other cultures. All cultures were harvested by scraping monolayers into the medium, cultures were subjected to 3 freeze thaw cycles to release the virus, and the samples were cleared of debris by centrifugation. The entire volume of the cleared samples from the previous step were used to inoculate T75 flasks of nearly confluent A549 cells (this is the second passage, used to amplify any virus that is present in the sample). At 5 d post-inoculation, we again observed significant CPE in the three infected control samples, but not in other samples. Cultures were harvested, freeze-thawed and cleared as previously described. Plaque assay detected no virus in in any of the mock-infected samples and all 3 infected control samples had titers >1 x 10^8 PFU/mL. For the MPLEx-treated samples, in replicate 1 (depicted in **Figure 2**); 0 of 4 lipid samples, 1 (1.3 x 10^4 PFU/mL) of 4 metabolite samples, and 3 (1.5 x 10^5, 3.9 x 10^4, and 2.5 x 10^4 PFU/mL [**Fig. 2**]) of 4 protein samples were Ad5 positive. In replicate 2; 0 of 4 lipid samples, 0 of 4 metabolite samples, and 1 (3.0 x10^3 PFU/mL) of 4 protein samples were positive for Ad5 (**Table S1**).

Reproducible proteomic, metabolomic, and lipidomic results from Calu-3 cells infected with wild-type and mutant H7N9: Here we demonstrate that the MPLEx method for concurrent pathogen inactivation and simultaneous protein, metabolite, and lipid extraction of each Calu-3 cell sample infected with either H7N9 wild-type, A/Anhui/1/2013 (AH1), or mutant AH1-NS1-L103F/I106M (FM) viral strains, resulted in reliable mass spectrometry-based multi-omics data with respect to infection duration. In mouse studies, compared to wild-type AH1, the FM viral mutant resulted in reduced overall pathogenicity (as indicated by MLD50), weight loss and lung titers. Quantitative protein, metabolite and lipid changes due to infection duration were obtained by comparing infected samples to mockulum treated controls for each of the 6 timepoints; 0, 3, 7, 12, 18 and 24 h, using 5 biological replicates for sample type (treatment and timepoint). To assess reproducibility, we calculated the coefficient of variation (CV) of the 5 biological replicates within each of the datatypes (protein, metabolite, and lipid). For proteomics, metabolomics, negative ion lipidomics, and positive ion lipidomics, the maximum CV across features within each set of biological replicates was <0.24, < 0.17, <0.22, and <0.19, respectively (**Table S2**). To visually depict the high reproducibility of our omic data, **Figure 3** shows viral peptides, ribosomal proteins, metabolites and lipids significantly increasing in infected samples compared to matched mock controls due to infection duration across both viral strains (**Table S2**). The top of **Figure 3** depicts the 123 viral peptides belonging to viral proteins; hemagglutinin (HA), matrix protein 1 (M1), neuraminidase (NA), nucleoprotein (NP), NS1, NS2, and RNA polymerase complex (PA, PB1, and PB2) significantly increased by G-test (only observed in infected samples) as early as 7 h with the number of significant peptides increasing from 7-24 h. Statistical analysis of our proteomic data also found significant quantitative increases in the relative abundance of ribosomal subunits 40S and 60S with infection duration across both viral strains. Free fatty acid metabolites myristic acid (14:0) and oleic acid (18:1) significantly increased at 18 h post infection across all 3 viral strains. Ceramides (Cer) containing

stearic acid (18:0) and tetracosanoic acid (24:0) significantly increased at 18 and 24 h in both AH1 and FM and at 12 h in AH1; likewise, Cer containing stearic acid (18:0) and nervonic acid (24:1) significantly increased at 18 h in both AH1 and FM and at 24 h in FM.

METHODOLOGICAL DETAILS FOR THE PROTEOMIC, METABOLOMIC, AND LIPIDOMIC STUDIES OF CALU-3 CELLS INFECTED WITH INFLUENZA H7N9 WILD-TYPE AND MUTANT VIRAL STRAINS

For proteomic experiments, identification and quantification of the detected peptide peaks was performed using the accurate mass and time (AMT) tag approach[30]. Peptide reference database generation utilized identifications from tandem mass spectrometry (MS/MS) analyses[31, 32] of pooled fractionated samples[33]. Multiple in-house developed[34, 35] informatics tools were used to process the quantitative proteomics data obtained from subsequent liquid chromatography-mass spectrometry (LC-MS) analyses of individual samples and to match the resulting LC-MS peptide features to the AMT tag reference database containing LC elution times and accurate mass information for each identified peptide.

For analysis of polar metabolites, extracted metabolites were chemically derivatized to their trimethylsilyl ester forms as previously described, followed by analysis using gas chromatography-mass spectrometry[36]. Metabolomics data analysis was performed using MetaboliteDetector, including chromatographic alignment of all datasets[37] and matching detected metabolites against entries in the Agilent Fiehn Metabolomics Retention Time Locked Library[38] based on both mass spectra and retention index. Manual validation of the spectra was performed to avoid erroneous identifications of metabolites. The NIST08 GC-MS library aided the manual validation process by cross-checking similarities of fragmented metabolite MS spectra.

For LC-ESI-MS/MS-based lipidomics analyses, a Waters NanoAquity UPLC system interfaced with a Velos-ETD Orbitrap mass spectrometer (Thermo Scientific, San Jose, CA) was used. Lipid extracts were reconstituted in methanol and lipids were separated over a 90 min gradient elution (mobile phase A: ACN/H_2O (40:60) containing 10 mM ammonium acetate; mobile phase B: ACN/IPA (10:90) containing 10 mM ammonium acetate) at a flow rate of 30 µl/min. Samples were analyzed in both positive and negative ionization using HCD (higher-energy collision dissociation) and CID (collision-induced dissociation) to obtain high coverage of the lipidome. Confident lipid identifications were determined by examining the tandem mass spectra for diagnostic ion fragments along with associated chain fragment information. In addition, the isotopic profile, extracted ion chromatogram (XIC), and mass error of measured precursor ions were examined for each lipid species.

Peptide, metabolite, and lipid statistics were performed by comparing data from matched virus and mockulum treated samples using Analysis of Variance (ANOVA) with a Dunnett multiple test correction within time point and for qualitative changes via a G-test with a Bonferroni multiple test correction within time point[39]. Peptides, metabolites, and lipids with a p-value less than 0.05 were identified as significantly different. Proteins were quantified using a signature-based approach[40]. Proteins in infected samples were then evaluated in the same manner as the peptides for changes in expression from the time matched mock either via Dunnett test or G-test.

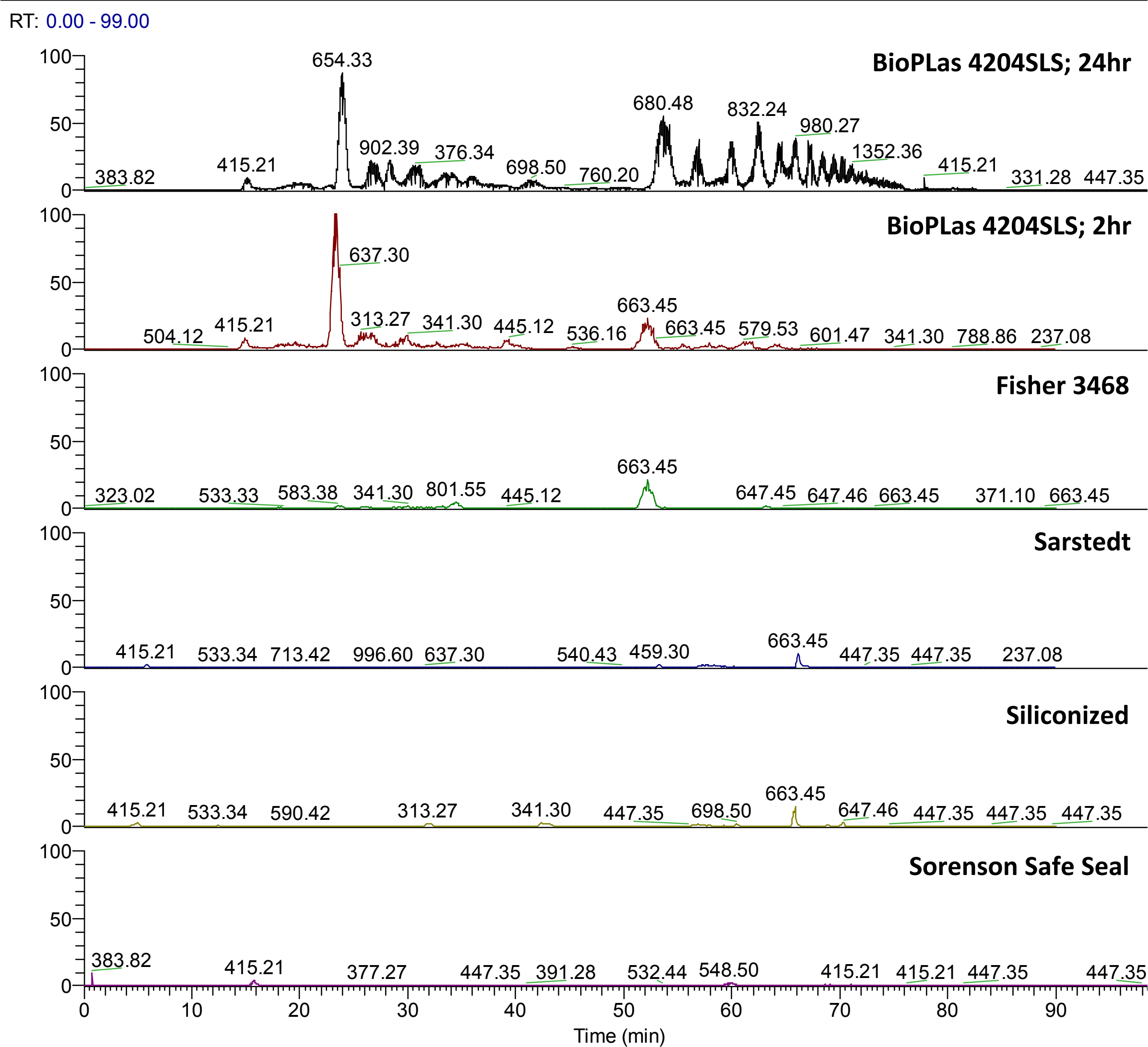

Figure S1: Polymer contaminants associated with the exposure of different commercially available tubes to chloroform. All extractions were performed in SafeSeal^{TM} microcentrifuge tubes from Sorenson BioScience, Inc. (Salt Lake City, Utah) since they showed limited leaching of polymers due to chloroform exposure.

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