

1 **Laboratory Validation of a Clinical Metagenomic Sequencing Assay for Pathogen**

2 **Detection in Cerebrospinal Fluid**

3 Miller S^{#1,2}, Naccache SN^{1,2,3#}, Samayoa E¹, Messacar K⁴, Arevalo S^{1,2}, Federman S^{1,2}, Stryke
4 D^{1,2}, Pham E¹, Fung B¹, Bolosky WJ⁵, Ingebrigtsen D¹, Lorizio W¹, Paff SM¹, Leake JA⁶, Pesano
5 R⁶, DeBiasi RL^{7,8}, Dominguez SR⁴, and CY Chiu^{1,2,9*}

6
7 ¹Department of Laboratory Medicine, University of California, San Francisco, San Francisco,
8 CA, USA

9 ²UCSF-Abbott Viral Diagnostics and Discovery Center, San Francisco, CA, USA

10 ³Department of Pathology and Laboratory Medicine, Children's Hospital Los Angeles, Los
11 Angeles, California, USA

12 ⁴Department of Pediatrics, Children's Hospital Colorado and University of Colorado School of
13 Medicine, Aurora, CO, USA

14 ⁵Microsoft Research, Redmond, WA, USA.

15 ⁶Quest Diagnostics Nichols Institute, San Juan Capistrano, CA, USA.

16 ⁷Department of Pediatrics, Division of Pediatric Infectious Diseases, Children's National Health
17 System, Washington, DC, USA

18 ⁸Department of Pediatrics, Microbiology, Immunology, and Tropical Medicine, The George
19 Washington University School of Medicine, Washington, D.C., USA

20 ⁹Department of Medicine, Division of Infectious Diseases, University of California, San
21 Francisco, San Francisco, CA USA

22

23 [#]These authors contributed equally to the manuscript.

24

25 *Correspondence to:

26 Charles Chiu

27 Department of Laboratory Medicine and Medicine, Division of Infectious Diseases

28 University of California, San Francisco, San Francisco, CA

29 e-mail: charles.chiu@ucsf.edu

30 tel: (415) 514-8129

31

32 **RUNNING TITLE:** Clinical mNGS Assay for Neurological Infections

33

34 **KEYWORDS:** metagenomic next-generation sequencing, clinical assay validation, SURPI+
35 bioinformatics pipeline, diagnosis of neurological infections, meningitis and encephalitis

36 **ABSTRACT**

37 Metagenomic next-generation sequencing (mNGS) for pan-pathogen detection has been
38 successfully tested in proof-of-concept case studies in patients with acute illness of unknown
39 etiology, but to date has been largely confined to research settings. Here we developed and
40 validated an mNGS assay for diagnosis of infectious causes of meningitis and encephalitis from
41 cerebrospinal fluid (CSF) in a licensed clinical laboratory. A clinical bioinformatics pipeline,
42 SURPI+, was developed to rapidly analyze mNGS data, automatically report detected
43 pathogens, and provide a graphical user interface for evaluating and interpreting results. We
44 established quality metrics, threshold values, and limits of detection of between 0.16 – 313
45 genomic copies or colony forming units per milliliter for each representative organism type.
46 Gross hemolysis and excess host nucleic acid reduced assay sensitivity; however, a spiked
47 phage used as an internal control was a reliable indicator of sensitivity loss. Diagnostic test
48 accuracy was evaluated by blinded mNGS testing of 95 patient samples, revealing 73%
49 sensitivity and 99% specificity compared to original clinical test results, with 81% positive
50 percent agreement and 99% negative percent agreement after discrepancy analysis.
51 Subsequent mNGS challenge testing of 20 positive CSF samples prospectively collected from a
52 cohort of pediatric patients hospitalized with meningitis, myelitis, and/or encephalitis showed
53 92% sensitivity and 96% specificity relative to conventional microbiological testing of CSF in
54 identifying the causative pathogen. These results demonstrate the analytic performance of a
55 laboratory-validated mNGS assay for pan-pathogen detection, to be used clinically for diagnosis
56 of neurological infections from CSF.

57

58

59 **INTRODUCTION**

60 Metagenomic next-generation sequencing (mNGS) provides a comprehensive method
61 by which nearly all potential pathogens – viruses, bacteria, fungi, and parasites – can be
62 accurately identified in a single assay (Goldberg et al. 2015; Chiu and Miller 2016). This
63 approach is attractive for diagnosis of infectious diseases, as pathogens that cause an
64 infectious syndrome commonly have non-specific, overlapping clinical presentations
65 (Washington 1996). Recent advances in sequencing technology and the development of rapid
66 bioinformatics pipelines have enabled mNGS testing to be performed within a clinically
67 actionable time frame (Cazanave et al. 2013; Naccache et al. 2014; Wilson et al. 2014;
68 Fremond et al. 2015; Greninger et al. 2015; Naccache et al. 2015; Salzberg et al. 2016;
69 Mongkolrattanothai et al. 2017; Parize et al. 2017; Schlaberg et al. 2017b). However, numerous
70 challenges remain with migrating mNGS testing into the clinical microbiology laboratory. These
71 include (1) lack of an established blueprint for mNGS clinical validation, (2) difficulty in
72 discriminating pathogens from colonizing microorganisms or contaminants, (3) paucity of
73 bioinformatics software customized for clinical diagnostic use, (4) concern over quality and
74 comprehensiveness of available reference databases, and (5) requirement for regulatory
75 compliance inherent to patient diagnostic testing in a CLIA (Clinical Laboratory Improvement
76 Amendments) environment.

77 Acute neurological illnesses such as meningitis and encephalitis are devastating
78 syndromes, remaining undiagnosed in a majority of cases (Glaser et al. 2003; Glaser et al.
79 2006; Granerod et al. 2010). The diagnostic workup for many patients requires extensive, and
80 often negative, serial testing that typically utilizes a combination of culture, antigen, serologic,
81 and molecular methods, resulting in delayed or missed diagnoses and increased costs. Given
82 the high burden of encephalitis-associated hospitalizations in the United States (Khetsuriani et

83 al. 2002), there is a large unmet clinical need for better and more timely diagnostics for this
84 syndrome, both to identify and to exclude infectious etiologies.

85 Here we present the development and validation of an mNGS assay for comprehensive
86 diagnosis of infectious causes of meningitis and encephalitis from CSF, expanding on summary
87 data presented in a previously published review (Schlaberg et al. 2017a). The analytic
88 performance of the mNGS assay was compared to results from conventional clinical
89 microbiological testing performed in hospital or commercial diagnostic laboratories. We also
90 tested the assay by blinded analysis of a challenge set of 20 CSF samples prospectively
91 collected from patients with diagnosed neurological infections at a single pediatric tertiary care
92 hospital.

93

94 **METHODS**

95

96 **mNGS Assay**

97 The processing and analysis workflow for the mNGS assay was performed as follows
98 (**Figure 1**), with a more detailed description provided in the **Supplemental Methods**. Briefly,
99 each CSF sample was first subjected to bead-beating to lyse organisms (**Figure 1A**), followed
100 by addition (“spiking”) of DNA T1 and RNA M2 bacteriophages as an internal control (IC). Total
101 nucleic acid was then extracted and split into 2 aliquots for construction of separate DNA and
102 RNA libraries. Microbial sequences were enriched by antibody-based removal of methylated
103 host DNA (for DNA libraries) or DNase treatment (for RNA libraries), followed by transposon-
104 based library construction (**Figure 1B**). Each sequencing run on an Illumina HiSeq instrument
105 included up to 8 samples, along with a negative “no template” control (NTC) consisting of elution
106 buffer, intended to allow for sensitive detection of contamination, and a positive control (PC)
107 consisting of a mixture of 7 representative organisms (RNA virus, DNA virus, Gram-positive

108 bacterium, Gram-negative bacterium, fungus, mold, and parasite). Some of the early
109 sequencing data used for validation was generated on an Illumina MiSeq instrument. Sequence
110 analysis (**Figure 1C**) using the SURPI+ computational pipeline (Naccache et al. 2014) consisted
111 of (1) pre-processing for trimming of adapters and removal of low-complexity and low-quality
112 reads, (2) human host background subtraction, (3) alignment to the National Center for
113 Biotechnology Information (NCBI) GenBank NT (nucleotide) reference database for microbial
114 identification, (4) taxonomic classification of aligned reads, and (5) visualization and
115 interpretation of sequencing data. Receiver-operator curve (ROC) analyses were performed to
116 determine optimal threshold values for organism detection based on mNGS data output. Each
117 mNGS run was analyzed by experienced laboratory physicians (SM and CYC), and results were
118 generated for 5 categories per sample (RNA virus, DNA virus, bacteria, fungi and parasite). Run
119 quality control (QC) metrics included a minimum of 5 million reads per library, ≥ 100 reads per
120 million (RPM) for the IC T1 and MS2 phages in the DNA and RNA libraries, respectively, and
121 positive qualitative detection of each of the 7 microorganisms in the PC using pre-designated
122 thresholds, as described below.

123

124 **Evaluation of mNGS Analytical Performance Characteristics**

125 A detailed description of the methods used to evaluate mNGS analytical performance
126 characteristics is provided in the **Supplemental Methods**. Briefly, limits of detection (LOD)
127 were determined for each of the 7 representative organisms in the PC by analyzing a series of
128 10-fold dilutions for qualitative detection. The LOD was determined using probit analysis,
129 defined as the concentration at which 95% of replicates would be detected, with at least 3
130 replicates performed for concentrations above and below this level. Precision was determined
131 using repeat analysis of the PC and NTC over 20 consecutive sequencing runs (inter-assay
132 reproducibility) and 3 sets of separately PC and NTC controls processed in parallel on the same
133 run (intra-assay reproducibility). Test stability was determined using control samples held at

134 various temperatures and subject to multiple freeze/thaw cycles. Interference was determined
135 using PC spiked with known amounts of human DNA or RNA material. Results were assessed
136 for qualitative detection of organisms in the PC.

137 Accuracy was determined using 95 clinical CSF samples (**Supplemental Figure S1**),
138 with up to 5 potential result categories per sample (RNA virus, DNA virus, bacteria, fungus and
139 parasite). Samples were obtained from patients at University of California, San Francisco
140 (UCSF) (n=59), Children's National Medical Center (n=19), Children's Hospital Colorado
141 (CHCO) (n=1), and Quest Diagnostics (n=16). Due to the varying number of clinical tests
142 performed per sample and limited residual CSF volume, we generated 3 composite reference
143 standards for purposes of comparison. The first composite standard consisted of the original
144 conventional clinical test results (both positive and negative) available prior to mNGS analysis,
145 providing an assessment of mNGS sensitivity and specificity relative to clinical testing. If mNGS
146 detected an organism that had not been tested for clinically, the result was considered
147 "reference untested", and that result was excluded from the comparison. Negative mNGS
148 results corresponding to a given category were also excluded if no clinical testing for pathogens
149 within that category had been performed. A second composite standard consisted of combined
150 results from the original clinical testing and additional molecular testing of CSF samples (volume
151 permitting), either when clinical and mNGS results were discrepant (n=8) or when mNGS
152 detected an organism that had not been included in the original testing (n=10). Finally, a third
153 composite standard was generated, which excluded samples with high human sequence
154 background (n=26), defined as samples with phage IC sequence recovery below a pre-
155 designated 100 RPM threshold. The second and third comparisons are described as positive
156 percent agreement (PPA) and negative percent agreement (NPA), as selective discrepancy
157 testing can bias estimates of test sensitivity and specificity (Meiser 2002). To evaluate mNGS
158 detection performance for additional organism types not readily available from clinical CSF
159 samples, the accuracy study also included contrived samples of 5 known organisms (*N.*

160 *meningitidis*, *S. agalactiae*, *C. albicans*, *M. fortuitum*, *M. abscessus*) spiked into negative CSF at
161 defined concentrations.

162

163 **Challenge study**

164 The Aseptic Meningitis and Encephalitis Study (AMES) is a prospective cohort study
165 enrolling children presenting to CHCO with culture-negative meningitis and encephalitis since
166 2012. Ethical approval for the study was obtained from the Colorado Multiple Institutional
167 Review Board (protocol #12-0745), and all subjects provided informed consent for specimen
168 collection and testing. A subset of CSF specimens (n=20) with sufficient residual volume (600
169 uL) from subjects with known and unknown etiologies was coded for mNGS testing as a
170 challenge set. Samples were processed in a blinded fashion at UCSF, and results discussed in
171 clinical context with site investigators at CHCO over web-based teleconferencing. Results from
172 the mNGS assay and conventional clinical testing were compared for up to 5 result categories
173 (RNA virus, DNA virus, bacterium, fungus, and parasite) per sample.

174

175 **Accession numbers**

176 Microbial sequences with human reads removed have been deposited in the NCBI
177 Sequence Read Archive (BioProject accession number PRJNA234047). Sequences
178 corresponding to the HIV-1 and CMV controls in the PC and the MS2 (RNA) phage and T1
179 (DNA) phage spiked IC samples have been deposited in GenBank (accession numbers
180 pending).

181

182 **RESULTS**

183 **Sample processing and bioinformatics analysis**

184 We developed an mNGS assay for pathogen identification from CSF consisting of library
185 preparation, sequencing, and bioinformatics analysis for pathogen detection (**Figure 1**), and
186 validated the performance of the assay in a CLIA-certified laboratory. Wet bench protocols and
187 sequencing runs for the study were performed by state-licensed clinical laboratory scientists.
188 For each sequencing run, NTC (“no template” control), PC (positive control), and up to 8 patient
189 CSF samples were processed by DNA/RNA enrichment, nucleic acid extraction, construction of
190 DNA and RNA libraries, and sequencing on an Illumina HiSeq instrument in rapid run mode,
191 targeting a total of 5 to 20 million sequences per library (**Figure 1A and B**). Raw mNGS
192 sequence data were analyzed using SURPI+, a bioinformatics analysis pipeline for pathogen
193 identification from mNGS sequence data (Naccache et al. 2014) that was modified for clinical
194 use. Specifically, the SURPI+ pipeline included filtering algorithms for exclusion of false-
195 positive hits from database misannotations, taxonomic classification for accurate species-level
196 identification, automated report generation implementing *a priori* established thresholds for
197 pathogen detection, and a web-based graphical user interface to facilitate laboratory director
198 review and confirmation of mNGS findings (**Figures 1C and Supplemental Figure S2**).
199

200 **Establishing thresholds for reporting detected pathogens**

201 To minimize the potential for false positive results from low-level background
202 contamination, threshold criteria were established for organism detection (**Figure 1C**). For
203 viruses, we developed threshold criteria based on the detection of nonoverlapping reads from at
204 least 3 distinct genomic regions, taking into consideration viruses incidentally detected in the
205 NTC sample that were potential background contaminants. Viruses comprising known flora,
206 such as anelloviruses and papillomaviruses, or laboratory reagent contaminants, such as
207 murine gammaretroviruses, were not reported. For identification of bacteria, fungi, and
208 parasites, we developed a reads per million (RPM) ratio metric, or RPM-r, defined as $RPM-r =$
209 RPM_{sample} / RPM_{NTC} (with the minimum RPM_{NTC} set to 1). This metric accounted for background

210 contamination by normalizing the RPM of detected pathogen reads assigned to a given
211 taxonomic classification (family, genus, or species) with respect to the RPM in the NTC. To
212 determine the optimal threshold value for RPM-r, we plotted receiver operating characteristic
213 (ROC) curves at varying ratios corresponding to mNGS analysis of 95 clinical CSF samples that
214 were included in the accuracy evaluation (**Supplemental Figure S3**; also see below). The
215 ROC curve analysis showed that an RPM-r of 10 maximized accuracy for organism detection.
216 Thus, we designated a minimum threshold of 10 RPM-r for reporting the detection of a
217 bacterium, fungus, or parasite. Occasionally, multiple bacterial genera (≥ 2) from environmental
218 and/or skin flora were detected in a CSF sample above the 10 RPM-r threshold, and attributed
219 to contamination. In these cases (n=7), mNGS results were reported as “multiple bacterial
220 genera detected” (with an interpretive comment indicating likely sample contamination), and
221 were considered as negative for bacterial detection by mNGS.

222

223 **Limits of detection**

224 To calculate the 95% limits of detection (LOD), defined as the lowest concentration at
225 which 95% of positive samples are detected, multiple replicates of the PC at serial dilutions near
226 the estimated detection limit were tested by mNGS. Using probit analysis, a 95% limit of
227 detection was determined for each of the 7 representative organisms in the PC (**Table 1**). The
228 final working PC consisted of the 7 organisms spiked at concentrations ranging from 0.5-2 log
229 above the 95% limit of detection. A linear correlation was observed between the input
230 concentration and number of reads / genome coverage for viruses (R^2 values 0.9083-0.9911),
231 and between the input concentration and RPM-r for bacteria, fungi, and parasites (R^2 values
232 .09856-.09996) (**Supplemental Figure S4**).

233

234 **Precision**

235

236 We demonstrated inter-assay reproducibility by mNGS testing of the NTC and PC
237 across 20 consecutive sequencing runs, and intra-assay reproducibility by testing of
238 3 independently generated sets of NTC and PC on the same run. Internal spiked phage controls
239 passed QC for every run, and only one PC RNA library (out of 46 total DNA and RNA libraries)
240 had fewer than the minimum designated cutoff of 5 million reads. All 7 organisms were detected
241 using pre-established threshold criteria for the intra-assay run and each replicate inter-assay run
242 (**Table 1**).

243

244 **Accuracy**

245 For evaluation of accuracy, a total of 95 CSF patient samples (73 positive and 22
246 negative for a pathogen by conventional clinical testing) were tested using the mNGS assay.
247 There were 5 categories of results for each sample, corresponding to 5 different pathogen types
248 (bacteria, fungi, parasites, DNA viruses, and RNA viruses). mNGS results were compared to (1)
249 original clinical test results, (2) results after discrepancy testing, and (3) results after
250 discrepancy testing and exclusion of samples with high host background (**Table 1 and**
251 **Supplemental Table S1**).

252 Overall, the mNGS assay showed 73% sensitivity and 99% specificity compared to
253 original clinical test results. 21 mNGS results were considered false-negatives, including 4 RNA
254 viruses (1 enterovirus and 3 West Nile virus (WNV) diagnosed by CSF serology) , 4 DNA
255 viruses (2 VZV, 1 HSV-2, 1 EBV, diagnosed by PCR) , 9 bacteria (diagnosed by culture), and 4
256 fungi (diagnosed by culture and/or antigen testing) (**Supplemental Table S1**). One mNGS
257 result was considered to be false-positive for *Bacillus* sp. detection in a culture-negative CSF.
258 All 5 organisms spiked into negative CSF matrix as part of the accuracy study were correctly
259 detected (*N. meningitidis*, *S. agalactiae*, *C. albicans*, *M. fortuitum*, *M. abscessus*).

260 Discrepancy analysis using targeted clinical PCR was performed on 18 samples with
261 sufficient volume available for testing. For organisms detected by mNGS but not tested for

262 clinically (n=10), discrepancy tests confirmed the mNGS results in all 10 cases (5 HIV, 1 CMV, 2
263 EBV, 1 HSV, 1 HHV6). In 8 cases, mNGS results were initially considered to be false negatives
264 but enough sample was available for discrepancy testing using molecular methods. Overall,
265 discrepancy testing using molecular testing failed to detect the causative organism in 5 of the 8
266 cases with negative mNGS results. Two cases of WNV diagnosed by positive CSF IgM serology
267 (1 case also had cross-reactive IgM antibodies to Japanese Encephalitis Virus (JEV) in blood)
268 were negative by WNV PCR testing, concordant with the mNGS results. Two culture-positive
269 bacterial cases with negative mNGS results underwent orthogonal testing using 16S rRNA
270 bacterial PCR, and one was not detected (*P. mirabilis*), whereas one was positive (*E.*
271 *galinarum*). Four culture-positive samples with negative mNGS results for fungi were tested
272 using 18S internal transcribed spacer (ITS) PCR, and 2 were negative (*C. parapsilosis* and *C.*
273 *neoformans*) whereas the other 2 were positive (*A. fumigatus* and *S. schenckii*).

274 Among the 3 remaining *bona fide* false-negative mNGS cases (*E. galinarum*, *A.*
275 *fumigatus*, and *S. schenckii*), the first 2 cases were only weakly positive by original clinical testing
276 (the *E. galinarum* grew from broth only, whereas the *A. fumigatus* was galactomannan-positive
277 and fungal culture negative). Both were also high-background samples (**Supplemental Table**
278 **S1**), and thus the number of identified pathogen reads did not meet thresholds for reporting. The
279 third case (*S. schenckii*) was likely missed by mNGS testing because the ~32 Mb genome of *S.*
280 *schenckii*, while publicly available (Cuomo et al. 2014), is not part of the GenBank NT reference
281 database used by the SURPI+ computational pipeline. This resulted in only 33 reads in the
282 sample (RPM-r 1.93) being identified as *S. schenckii*, also below reporting thresholds. Adjusting
283 the results comparison on the basis of the discrepancy testing results yielded 81% positive
284 percent agreement and 99% negative percent agreement for the mNGS assay relative to
285 combined original and discrepancy testing results.

286 There were additional incidental organism detections by mNGS (n=12) where sample
287 volume was insufficient to perform confirmatory molecular testing. These included HIV (n=4),

288 WNV (n=1), rotavirus (n=1), rhinovirus (n=1), HCV (n=1), parvovirus B19 (n=2), human
289 herpesvirus 7 (n=1) and *Bacillus* spp. (n=1). With the exception of *Bacillus* spp. which might
290 have been recovered in CSF cultures, the presence of these organisms could not be
291 independently confirmed by molecular testing and thus these mNGS results were excluded from
292 the comparisons, as it could not be determined whether a given additional detection was a true
293 or false positive.

294 A third comparison was performed after exclusion of results from CSF samples with an
295 IC RPM of <100, indicating potential decreased sensitivity for the mNGS assay due to high
296 human host background (see “Interference”, below). A total of 26 samples had high background
297 (1 RNA virus, 3 DNA virus, 19 bacteria, 2 fungi, 1 negative), and exclusion of these yielded 91%
298 positive percent agreement and 99% negative percent agreement for the mNGS assay overall.
299 Notably, the 19 bacterial samples with high background comprised 70.4% of the total number of
300 culture-positive bacterial cases (n=27), consistent with the relatively high leukocyte levels seen
301 in typical cases of bacterial meningitis.

302

303 **Interference**

304 We evaluated the effects of interference with human DNA and RNA, red blood cell
305 hemolysis, and mixtures of related species in the same genus (*Staphylococcus aureus* and
306 *Staphylococcus epidermidis*) on mNGS assay performance (**Table 1**). Addition of human DNA
307 at a level equivalent to 1×10^6 cells/mL resulted in complete failure to detect spiked DNA
308 pathogens in the PC, whereas addition of exogenous RNA and DNA at lower levels ($\leq 1 \times 10^4$
309 cells/mL) did not impact qualitative detection. The number of sequenced IC phage reads was
310 found to be linearly correlated with the amount of added exogenous DNA ($R^2 = 0.999$). Based
311 on the interference results, an RPM threshold of 100 was chosen for the IC phage reads, with
312 RPM values below this level indicating that the sample had high human DNA and/or RNA
313 background (**Supplemental Figure S5**). For mNGS reporting, these high-background samples

314 included a comment that the assay had decreased sensitivity for detection of RNA viruses (from
315 RNA libraries) or DNA viruses, bacteria, fungi and parasites (from DNA libraries).

316 Available data from 55 CSF samples in the accuracy study with recorded white blood
317 cell (WBC) counts were used to evaluate the effect of WBC count, related to the amount of
318 human nucleic acid background, on recovery of IC phage sequences. Among 26 samples with
319 IC DNA phage counts of <100 RPM, indicating high human background, the average WBC was
320 5,896 cells/mm³, while 29 samples with IC counts of >100 RPM had an average WBC count of
321 27 cells/ mm³ (p = 0.0498 by two-tailed t-test).

322 Gross hemolysis (dark red CSF) resulted in decreased sensitivity for RNA virus
323 detection (HIV-1 in the PC) by mNGS, but did not affect detection sensitivity for DNA pathogens.
324 Moderate to low levels of hemolysis (pink to light red CSF) did not affect detect sensitivity for
325 any of the PC organisms. Analysis of spiked samples containing *S. aureus* and *S. epidermidis*
326 with equivalent RPM-r values at baseline demonstrated accurate discrimination of species
327 within the same genus when mixed at 1:1, 4:1, and 1:4 ratios, as both species were correctly
328 identified and calculated RPM-r values were within 7% of that expected on the basis of the
329 spiked amounts.

330

331 **Stability**

332 Analysis of replicates of the PC held at 4°C for 0, 2, 5, and 6 days and subjected to 3
333 freeze-thaw cycles demonstrated detection of all organisms (**Table 1**).

334

335 **Challenge study**

336 We blindly evaluated the performance of the mNGS assay on a set of 20 prospectively
337 collected CSF samples from pediatric patients hospitalized at CHCO with meningitis,
338 encephalitis, and/or myelitis (**Supplemental Table S2**). Comparison of the results assembled
339 from each of the 5 organism categories yielded a sensitivity of 92% and specificity of 96% for

340 mNGS testing relative to conventional microbiological testing of CSF (culture, PCR, antigen,
341 and serological testing). The assay correctly identified the causative pathogen in 11 of 12 cases
342 that were previously positive for direct organism detection or serology from CSF, including
343 cases of enterovirus (n=8), HSV-1 (n=1), HIV-1 (n=1) and WNV (n=1) in a patient with positive
344 IgM serology from CSF. The mNGS assay failed to detect WNV in a second patient with
345 positive CSF IgM serology. Three additional organisms (*Enterobacter* sp. *Corynebacterium* sp.,
346 and EBV) were detected by mNGS, each from a different sample. The detection of *Enterobacter*
347 sp. and *Corynebacterium* sp. were considered to be mNGS false-positives, since the samples
348 had previously tested negative by culture. mNGS also identified EBV in a CSF sample from a
349 patient with positive testing for EBV IgG antibodies in blood; this finding was excluded from the
350 comparison due to the lack of confirmatory testing from CSF. In addition, mNGS failed to detect
351 organisms in 4 cases: 1 case of *Borrelia burgdorferi* diagnosed using peripheral blood serology
352 only, 2 cases of presumptive *Mycoplasma* encephalitis with PCR-positive respiratory but not
353 CSF samples, and 1 case of presumptive enterovirus 71 infection with a positive viral culture
354 from rectal swab but negative CSF PCR. Since the diagnoses in these 4 cases were not made
355 directly from CSF, these results were also excluded from the comparison. Negative mNGS
356 testing in 4 undiagnosed cases was concordant with negative conventional microbiological
357 testing, including 1 case of culture-negative, presumptive bacterial meningitis and 3 cases of
358 idiopathic encephalitis.

359

360 **DISCUSSION**

361 Here we developed and validated a clinical mNGS assay intended to diagnose infectious
362 etiologies of meningitis, encephalitis, and myelitis from CSF, followed by blinded evaluation of
363 mNGS performance using a set of 20 prospectively collected CSF samples from pediatric
364 patients admitted to a tertiary care hospital. As CSF is considered a normally sterile site, we

365 postulated that mNGS data generated from testing of this body fluid type would be more
366 straightforward to interpret than data from more “environmental” samples such as respiratory
367 secretions and stool. However, numerous challenges had to be overcome for successful
368 implementation of mNGS in the clinical laboratory. First, a universal sequencing library
369 preparation protocol was required that was robust across the wide range of potential nucleic
370 acid concentrations in patient CSF (0 – 10^8 cells/mm³). This ultimately required a protocol
371 incorporating two PCR steps, an initial step for library amplification and a recovery amplification
372 step to ensure robust library construction from relatively acellular CSF samples or the NTC
373 buffer control, both containing little to no human host background. Second, the mNGS assay
374 had to be capable of simultaneously detecting a broad spectrum of pathogens, including viruses
375 (both single- and double-stranded RNA and DNA genomes), bacteria, fungi, and parasites.
376 Thus, the mNGS protocol incorporated (1) a bead-beating step for lysis of microbial cell walls,
377 (2) separate construction of RNA and DNA libraries from nucleic acid extracts for detection of
378 RNA viruses and DNA pathogens, respectively, and (3) bioinformatics analysis using the
379 entirety of NCBI GenBank NT database as a comprehensive reference database. Finally,
380 reproducible threshold metrics needed to be developed and evaluated using ROC curve
381 analysis to enable correct identification of pathogens from mNGS data above background noise.

382 We developed quality control materials and metrics for the CSF mNGS assay, including
383 acceptable criteria for the performance of external positive and negative controls, as well as
384 spiked internal controls. Given the untargeted nature of mNGS, a key limitation of the approach
385 for infectious disease diagnostics is background interference, generally from human host DNA.
386 The use of a spiked phage IC was found to be useful for assessing whether high background
387 was present, indicating decreased sensitivity of pathogen detection by mNGS (Schlaberg et al.
388 2017a). Overall, 27.4% of DNA libraries and 6.3% of RNA libraries in the accuracy study had
389 fewer than 100 RPM IC phage reads recovered, making background interference a fairly

390 common limitation. Thus, in high background samples, negative mNGS findings may be less
391 useful for excluding infection, and other diagnostic tests that may be less sensitive to
392 background should be considered, such as 16S rRNA bacterial PCR (Salipante et al. 2013) and
393 ITS fungal PCR (Pryce et al. 2006). This is especially relevant in cases of bacterial meningitis
394 with high leukocyte counts in CSF. However, despite this limitation, mNGS was still able to
395 detect bacterial pathogens in 12 of 19 culture-positive samples in the accuracy study with high
396 host background.

397 The overall accuracy of the mNGS assay for pathogen detection over 5 categories of
398 microorganisms as compared to initial conventional microbiological testing was 90%, with 73%
399 sensitivity and 99% specificity. Positive percent agreement rose to 81% after discrepancy
400 testing of samples with sufficient volume, and exclusion of samples with high host background
401 increased this further to 91%. Only a fraction of all possible diagnostic tests for pathogens are
402 performed in clinical microbiology laboratories given limited CSF sample volume. Thus, we
403 decided to exclude organism detections by mNGS for which independent testing had not been
404 performed in assessment of initial test performance (n=22), as no reference result was
405 available. However, in 10 cases with sufficient CSF volume for orthogonal confirmatory testing,
406 all 10 were found to be analytical true positives. Furthermore, in 5 of 8 (62.5%) cases where
407 mNGS failed to detect an organism that was found with initial clinical testing (using culture
408 and/or serology), confirmatory orthogonal PCR testing for this organism was negative, indicating
409 that the sample may have degraded over time or that the original clinical result may have been
410 incorrect.

411 As with any diagnostic assay, mNGS testing is prone to contamination. There is the
412 potential to detect colonizing organisms that constitute normal human body flora (e.g.
413 anelloviruses in CSF and blood (Maggi and Bendinelli 2010; Moustafa et al. 2017)), as well as
414 exogenous (Strong et al. 2014) or cross-contamination. Often, the identity of the species

415 detected can provide clues as to the contamination source such as skin flora (e.g. *S.*
416 *epidermidis*, papillomaviruses), laboratory reagents (murine gammaretroviruses, *E. coli*, insect
417 viruses), body flora (e.g. anelloviruses), or environmental flora (e.g. *Thermus* sp., *Bacillus* sp.).
418 Cross-contamination in particular is a major concern given that the mNGS protocol involves
419 PCR amplification. Strict processing controls to minimize contamination are essential and
420 include unidirectional workflow, positive pressure ventilation in pre-amplification areas, and
421 workspace separation for different assay steps. New reagent lots must undergo QC testing with
422 mNGS of a reference standard, such as a previously run sample, before reagents can be put
423 into clinical use. Background contamination is also continually monitored by keeping track of
424 contaminants seen in the NTC or PC, and conservative threshold criteria are used to minimize
425 the reporting of false-positive results. Additionally, periodic swipe tests of instruments and lab
426 sources followed by mNGS of the swabs can facilitate targeted cleaning to ensure absence of
427 laboratory contamination. However, despite use of contamination controls for the mNGS assay,
428 7.4% of clinical samples in the accuracy study had multiple bacteria genera detected above
429 thresholds, generally consisting of environmental or skin flora. Rarely are bacterial co-infections
430 causative for cases of meningitis / encephalitis (with the possible exception of brain abscesses
431 communicating with CSF), so these findings were noted as indicating probable sample
432 contamination, and were considered as negative for bacterial pathogen detection in this
433 analysis. It is likely that bacterial DNA is introduced from *bona fide* uncultivable organisms
434 during sample collection or via reagents / tubes that are sterile but not DNA-free, and mNGS
435 analysis and interpretation must be able to deal with these contamination risks.

436 The challenge study demonstrated that mNGS detected the same organism identified
437 through conventional direct organism detection methods or serology from CSF in 11 of 12
438 (91.7%) of cases. One case of WNV, diagnosed serologically, was missed by mNGS (false-
439 negative). There were 2 mNGS false-positives (*Corynebacterium* sp. and *Enterobacter* sp.),
440 likely due to contamination introduced during sample collection, handling, or the assay

441 procedure. mNGS of CSF failed to identify the pathogen in 4 patients who had a presumptive
442 infectious diagnosis from peripheral microbiological testing (serology, culture, and/or PCR) done
443 from sites other than CSF. We found that the sensitivity of mNGS is critically dependent on
444 whether the organism (or nucleic acid from the organism) is present at the time of sample
445 collection. It is thus not unexpected that mNGS testing missed a number of infections that are
446 most often diagnosed by serology because the pathogen is absent or only transiently present in
447 CSF (e.g. neurosyphilis, Lyme neuroborreliosis, and WNV). For these cases, direct detection
448 testing approaches such as PCR and mNGS may be inappropriate and there should be a low
449 threshold for ordering antibody-based serologic testing or performing microbial analysis from
450 other body sites to establish the diagnosis if there is clinical suspicion (Debiasi and Tyler 2004).

451 While mNGS testing can provide broad-spectrum pathogen identification, assessment of
452 the clinical significance of the findings requires interpretation. Thus, mNGS results are
453 submitted to the patient electronic medical record as an interpretive report by pathologists with
454 expertise in microbiology and genomics, after reviewing and citing of relevant literature. In
455 addition to the submitted report, direct discussion or teleconferences can also be set up
456 between pathologists and providers to clarify and review mNGS results in clinical context. These
457 forums can also be used to communicate results of supplementary analyses of mNGS data,
458 including (1) genome assembly for characterization of predicted antibiotic or antiviral resistance
459 mutations, (2) phylogenetic analysis for genotyping and strain-level identification, and (3)
460 disclosure of reads from potential pathogens below formal reporting thresholds. Our finding of a
461 linear correlation between the number of reads or genome coverage and pathogen titer (as
462 previously noted for influenza virus in nasal swabs (Greninger et al. 2010)) also raises the
463 prospect of extracting quantitative information from metagenomic sequence data. The
464 metagenomic analyses performed here were facilitated by the use of SURPI+ software, as it
465 provides summaries and graphical visualization tools tailored for evaluation and reporting of
466 mNGS results. Thus, the clinical relevance of mNGS findings can be efficiently communicated

467 to physicians, potentially informing the next steps in diagnosis, management, and treatment of
468 the patient, and may also prove informative for public health surveillance and outbreak
469 investigation (Chiu et al. 2017).

470

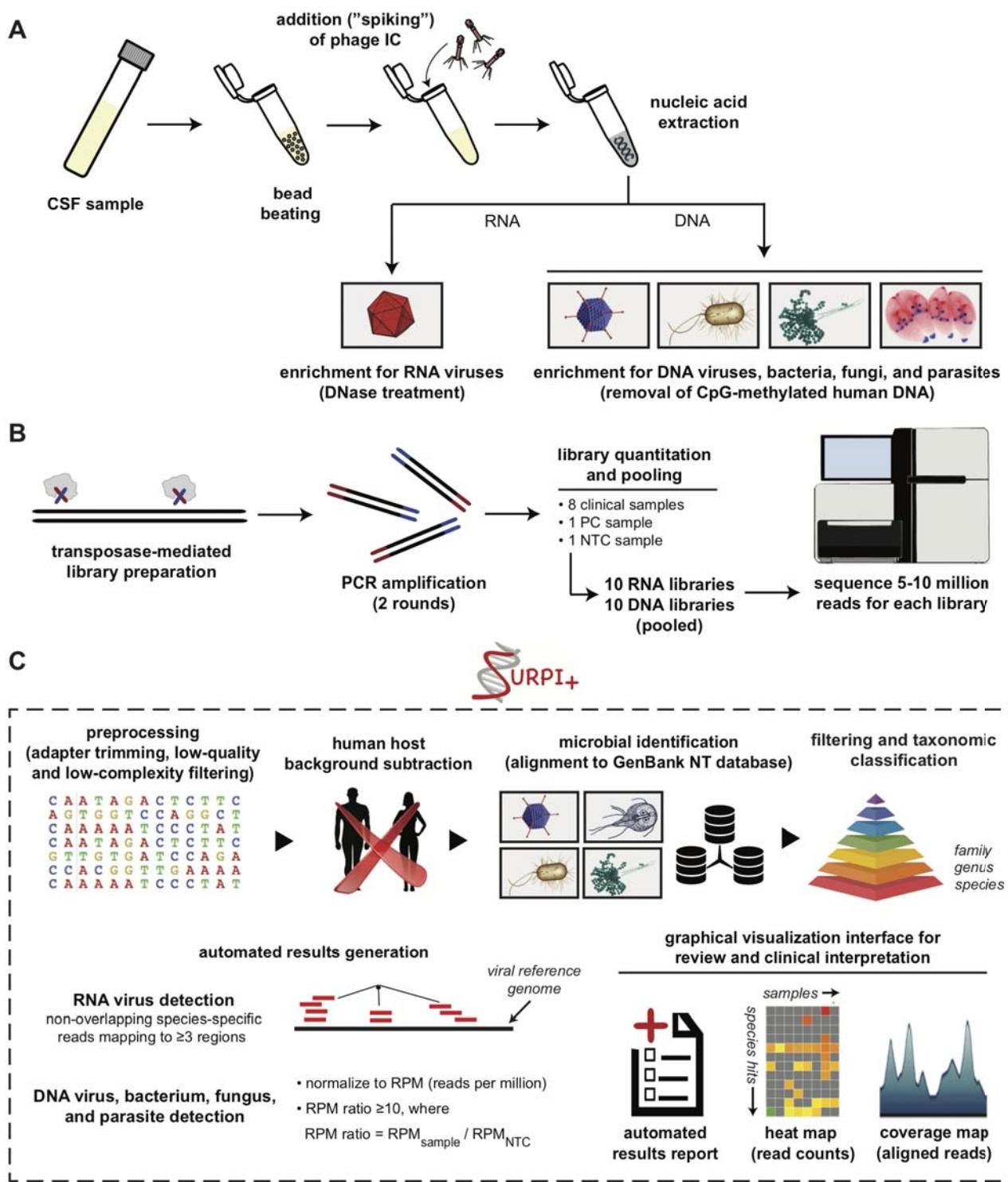
Performance Metric	Method	Results						
Limits of Detection (LoD)^a	Qualitative detection of PC dilution replicates by probit analysis							
	Pathogen type	Representative organism	LoD					
	DNA virus	CMV	14 copies/mL					
	RNA virus	HIV	313 copies/mL					
	Bacterium, gram-positive	<i>Streptococcus agalactiae</i>	10 CFU/mL					
	Bacterium, gram-negative	<i>Klebsiella pneumoniae</i>	8 CFU/mL					
	Fungus, mold	<i>Aspergillus niger</i>	220 CFU/mL					
	Fungus, yeast	<i>Cryptococcus neoformans</i>	0.2 CFU/mL					
	Parasite	<i>Toxoplasma gondii</i>	81 organisms/mL					
Precision^a	Qualitative detection over 20 consecutive PC runs (intra-assay)	100% concordance						
	Qualitative detection of 3 PC samples on the same run (inter-assay)	100% concordance						
Stability^a	Qualitative detection of PC held at 4°C for 0, 2, 5, and 6 days	100% concordance						
	Qualitative detection of PC subjected to 1, 2, and 3 freeze-thaw cycles	100% concordance						
Interference^a	Qualitative detection of PC with spiked DNA (low, medium, high concentration)	DNA	All spiked ICs and PC organisms (DNA viruses, bacteria, fungi, parasites) detected above QC thresholds					
	Qualitative detection of PC with spiked RNA (low, medium, high concentration)	RNA	All spiked ICs and PC organisms (RNA viruses) detected					
	Qualitative detection of PC spiked with hemolytic blood (low, medium, high concentration)	DNA	All spiked ICs and PC organisms (DNA viruses, bacteria, fungi, parasites) detected except for hemolytic blood spiked into the PC at high concentration					
	Qualitative detection of PC spiked with hemolytic blood (low, medium, high concentration)	RNA	All spiked ICs and PC organisms (RNA viruses) detected					
Accuracy	95 clinical CSF samples, results comparison		Original clinical testing (n=216 results)	After discrepancy testing (n=217 results) ^b		Excluding high background (n=168 results) ^c		
		Pathogen type	Sens	Spec	PPA	NPA	PPA	NPA
		RNA virus	67	100	92	100	91	100
		DNA virus	85	100	87	100	93	100
		Bacterium	64	98	67	98	80	98
		Fungus	71	100	83	100	90	100
		Parasite	100	100	100	100	100	100
		Overall	73	99	81	99	91	99

Abbreviations: PC, positive control mix of 7 representative organisms; IC, spiked internal control consisting of a DNA T1 phage and RNA M2 phage; mNGS, metagenomic next-generation sequencing; QC, quality control; Sens, sensitivity; Spec, specificity; PPA, positive percent agreement; NPA, negative percent agreement

^a Pre-designated QC thresholds included >5 million reads per library, >100 RPM for the spiked ICs, >3 nonoverlapping gene regions for the viruses in the PC, and >10 RPM for the non-viral pathogens in the PC

^b Discrepancy testing is performed on remaining CSF sample, if available, using molecular methods (i.e. PCR)

^c High background is defined as samples with IC RPM <100



474 **Figure 1. Schematic of the mNGS Assay Workflow. (A)** CSF is extracted after lysis by bead-
475 beating and internal control addition to allow viral, bacterial, fungal and parasite nucleic acid
476 retrieval. Total nucleic acid extracts are enriched for pathogen DNA by removal of methylated
477 DNA (DNA libraries) and treatment with DNase (RNA libraries). **(B)** Libraries are generated
478 using the Nextera XT protocol and amplified using 2 rounds of PCR. Libraries are quantified,
479 pooled, and loaded onto the sequencer. **(C)** Sequences are processed using SURPI+ software
480 for alignment and classification. Reads are preprocessed by trimming of adapters and removal
481 of low-quality / low-complexity sequences, followed by computational subtraction of human
482 reads and taxonomic classification of remaining microbial reads to family, genus, or species. For
483 viruses, reads are mapped to the closest matched genome to identify nonoverlapping regions;
484 for bacteria, fungi, and parasites, a read per million (RPM) ratio (RPM-r) metric is calculated,
485 defined as $RPM-r = RPM_{sample} / RPM_{NTC}$. To aid in analysis, result reports, heat maps of raw /
486 normalized read counts, and coverage maps are automatically generated for use in review and
487 clinical interpretation.

488

489 **COMPETING INTERESTS**

490 CYC is the director of the UCSF-Abbott Viral Diagnostics and Discovery Center (VDDC)
491 and receives research support from Abbott Laboratories, Inc. CYC SA DS SF and SM are
492 inventors on a patent application on algorithms related to SURPI+ software titled “Pathogen
493 Detection using Next-Generation Sequencing”) (PCT/US/16/52912).

494

495 **AUTHOR CONTRIBUTIONS**

496 CYC SM ES and SNN developed the project. ES EP SA BF WL generated libraries.
497 SNN SM SM CYC analyzed data for clinical validation. SF DS CYC developed SURPI+ software
498 and graphical user interface for clinical use. WB modified the SNAP algorithm to facilitate
499 taxonomic classification by SURPI+, SP banked CSF samples. BG JAL SD KM SM CYC
500 provided clinical specimens. KM SD SM CYC JAL SNN SP CYC SM conducted chart review. DI
501 BF SA conducted discrepancy testing. SM SNN and CYC wrote the manuscript with
502 contributions from all authors.

503

504 **ACKNOWLEDGEMENTS**

505 We thank the staff of the Clinical Immunology lab for their help in discrepancy testing,
506 and Gail Cunningham for her expert help in obtaining AFB organisms. We would also like to
507 thank Brittany Goldberg for providing clinical samples for use in the validation.

508 This work is supported by NIH grants R01 HL105704 (CYC), a UC Center for
509 Accelerated Innovation grant funded by NIH grants U54 HL119893 and NCATS UCSF-CTSI
510 grant UL1 TR000004 (CYC), the California Initiative to Advance Precision Medicine (CYC and

511 SM), research support from Abbott Laboratories, Inc (CYC), and supplemental funding from the
512 UCSF Medical Center (CYC and SM).

513

514

515 **REFERENCES**

516 Cazanave C, Greenwood-Quaintance KE, Hanssen AD, Karau MJ, Schmidt SM, Gomez Urena
517 EO, Mandrekar JN, Osmon DR, Lough LE, Pritt BS et al. 2013. Rapid molecular
518 microbiologic diagnosis of prosthetic joint infection. *J Clin Microbiol* **51**: 2280-2287.

519 Chiu C, Miller S. 2016. Next-Generation Sequencing. In *Molecular Microbiology: Diagnostic*
520 *Principles and Practice, 3rd Edition*, (ed. DH Persing, et al.), pp. 68-79. ASM Press,
521 Washington, DC.

522 Chiu CY, Coffey LL, Murkey J, Symmes K, Sample HA, Wilson MR, Naccache SN, Arevalo S,
523 Somasekar S, Federman S et al. 2017. Diagnosis of Fatal Human Case of St. Louis
524 Encephalitis Virus Infection by Metagenomic Sequencing, California, 2016. *Emerg Infect*
525 *Dis* **23**: 1964-1968.

526 Cuomo CA, Rodriguez-Del Valle N, Perez-Sanchez L, Abouelleil A, Goldberg J, Young S, Zeng
527 Q, Birren BW. 2014. Genome Sequence of the Pathogenic Fungus *Sporothrix schenckii*
528 (ATCC 58251). *Genome Announc* **2**.

529 Debiasi RL, Tyler KL. 2004. Molecular methods for diagnosis of viral encephalitis. *Clin Microbiol*
530 *Rev* **17**: 903-925, table of contents.

531 Fremond ML, Perot P, Muth E, Cros G, Dumarest M, Mahlaoui N, Seilhean D, Desguerre I,
532 Hebert C, Corre-Catelin N et al. 2015. Next-Generation Sequencing for Diagnosis and
533 Tailored Therapy: A Case Report of Astrovirus-Associated Progressive Encephalitis. *J*
534 *Pediatric Infect Dis Soc* **4**: e53-57.

535 Glaser CA, Gilliam S, Schnurr D, Forghani B, Honarmand S, Khetsuriani N, Fischer M, Cossen
536 CK, Anderson LJ, California Encephalitis P. 2003. In search of encephalitis etiologies:
537 diagnostic challenges in the California Encephalitis Project, 1998-2000. *Clin Infect Dis*
538 **36**: 731-742.

539 Glaser CA, Honarmand S, Anderson LJ, Schnurr DP, Forghani B, Cossen CK, Schuster FL,
540 Christie LJ, Tureen JH. 2006. Beyond viruses: clinical profiles and etiologies associated
541 with encephalitis. *Clin Infect Dis* **43**: 1565-1577.

542 Goldberg B, Sichtig H, Geyer C, Leedeboer N, Weinstock GM. 2015. Making the Leap from
543 Research Laboratory to Clinic: Challenges and Opportunities for Next-Generation
544 Sequencing in Infectious Disease Diagnostics. *MBio* **6**: e01888-01815.

545 Granerod J, Ambrose HE, Davies NW, Clewley JP, Walsh AL, Morgan D, Cunningham R,
546 Zuckerman M, Mutton KJ, Solomon T et al. 2010. Causes of encephalitis and differences
547 in their clinical presentations in England: a multicentre, population-based prospective
548 study. *Lancet Infect Dis* **10**: 835-844.

549 Greninger AL, Chen EC, Sittler T, Scheinerman A, Roubinian N, Yu G, Kim E, Pillai DR, Guyard
550 C, Mazzulli T et al. 2010. A metagenomic analysis of pandemic influenza A (2009 H1N1)
551 infection in patients from North America. *PLoS One* **5**: e13381.

552 Greninger AL, Messacar K, Dunnebacke T, Naccache SN, Federman S, Bouquet J, Mirsky D,
553 Nomura Y, Yagi S, Glaser C et al. 2015. Clinical metagenomic identification of
554 *Balamuthia mandrillaris* encephalitis and assembly of the draft genome: the continuing
555 case for reference genome sequencing. *Genome Med* **7**: 113.

556 Khetsuriani N, Holman RC, Anderson LJ. 2002. Burden of encephalitis-associated
557 hospitalizations in the United States, 1988-1997. *Clin Infect Dis* **35**: 175-182.

558 Maggi F, Bendinelli M. 2010. Human anelloviruses and the central nervous system. *Rev Med*
559 *Virol* **20**: 392-407.

560 Meiser KL. 2002. Reporting results from studies evaluating diagnostic tests. In *Clinical*
561 *Microbiology Newsletter*, Vol 24 (ed. FaD Administration), pp. 60-63. Elsevier, Inc.

562 Mongkolrattanothai K, Naccache SN, Bender JM, Samayoa E, Pham E, Yu G, Dien Bard J,
563 Miller S, Aldrovandi G, Chiu CY. 2017. Neurobrucellosis: Unexpected Answer From
564 Metagenomic Next-Generation Sequencing. *J Pediatric Infect Dis Soc* **6**: 393-398.

565 Moustafa A, Xie C, Kirkness E, Biggs W, Wong E, Turpaz Y, Bloom K, Delwart E, Nelson KE,
566 Venter JC et al. 2017. The blood DNA virome in 8,000 humans. *PLoS Pathog* **13**:
567 e1006292.

568 Naccache SN, Federman S, Veeraraghavan N, Zaharia M, Lee D, Samayoa E, Bouquet J,
569 Greninger AL, Luk KC, Enge B et al. 2014. A cloud-compatible bioinformatics pipeline for
570 ultrarapid pathogen identification from next-generation sequencing of clinical samples.
571 *Genome Res* **24**: 1180-1192.

572 Naccache SN, Peggs KS, Mattes FM, Phadke R, Garson JA, Grant P, Samayoa E, Federman
573 S, Miller S, Lunn MP et al. 2015. Diagnosis of neuroinvasive astrovirus infection in an
574 immunocompromised adult with encephalitis by unbiased next-generation sequencing.
575 *Clin Infect Dis* **60**: 919-923.

576 Parize P, Muth E, Richaud C, Gratigny M, Pilms B, Lamamy A, Mainardi JL, Cheval J, de
577 Visser L, Jagorel F et al. 2017. Untargeted next-generation sequencing-based first-line
578 diagnosis of infection in immunocompromised adults: a multicentre, blinded, prospective
579 study. *Clinical Microbiology and Infection* **23**.

580 Pryce TM, Palladino S, Price DM, Gardam DJ, Campbell PB, Christiansen KJ, Murray RJ. 2006.
581 Rapid identification of fungal pathogens in BacT/ALERT, BACTEC, and BBL MGIT
582 media using polymerase chain reaction and DNA sequencing of the internal transcribed
583 spacer regions. *Diagn Microbiol Infect Dis* **54**: 289-297.

584 Salipante SJ, Sengupta DJ, Rosenthal C, Costa G, Spangler J, Sims EH, Jacobs MA, Miller SI,
585 Hoogestraat DR, Cookson BT et al. 2013. Rapid 16S rRNA next-generation sequencing
586 of polymicrobial clinical samples for diagnosis of complex bacterial infections. *PLoS One*
587 **8**: e65226.

588 Salzberg SL, Breitwieser FP, Kumar A, Hao H, Burger P, Rodriguez FJ, Lim M, Quinones-
589 Hinojosa A, Gallia GL, Tornheim JA et al. 2016. Next-generation sequencing in
590 neuropathologic diagnosis of infections of the nervous system. *Neurol Neuroimmunol
591 Neuroinflamm* **3**: e251.

592 Schlaberg R, Chiu CY, Miller S, Procop GW, Weinstock G, Professional Practice C, Committee
593 on Laboratory Practices of the American Society for M, Microbiology Resource
594 Committee of the College of American P. 2017a. Validation of Metagenomic Next-
595 Generation Sequencing Tests for Universal Pathogen Detection. *Arch Pathol Lab Med*
596 **141**: 776-786.

597 Schlaberg R, Queen K, Simmon K, Tardif K, Stockmann C, Flygare S, Kennedy B, Voelkerding
598 K, Bramley A, Zhang J et al. 2017b. Viral Pathogen Detection by Metagenomics and
599 Pan-Viral Group Polymerase Chain Reaction in Children With Pneumonia Lacking
600 Identifiable Etiology. *J Infect Dis* **215**: 1407-1415.

601 Strong MJ, Xu G, Morici L, Splinter Bon-Durant S, Baddoo M, Lin Z, Fewell C, Taylor CM,
602 Flemington EK. 2014. Microbial contamination in next generation sequencing:
603 implications for sequence-based analysis of clinical samples. *PLoS Pathog* **10**:
604 e1004437.

605 Washington JA. 1996. Principles of Diagnosis. In *Medical Microbiology, 4th edition*, (ed. S
606 Baron). University of Texas Medical Branch at Galveston, Galveston, TX.

607 Wilson MR, Naccache SN, Samayoa E, Biagtan M, Bashir H, Yu G, Salamat SM, Somasekar S,
608 Federman S, Miller S et al. 2014. Actionable diagnosis of neuroleptospirosis by next-
609 generation sequencing. *N Engl J Med* **370**: 2408-2417.

610