ID Molecular Testing Guidance

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Plasma Metagenomic Next-Generation Sequencing[a]

Note that this is the current recommendation of the infectious disease division in an evolving field. This should not replace clinical judgement; please discuss with ID consults if questions.

What it is: Target-agnostic DNA sequencing of a plasma sample for broad-based detection of DNA viruses, bacteria, fungi, and parasites[1]. Does not detect RNA pathogens.

Specimen: Plasma only.

Use Cases (citations to major papers provided):
First-line test (send at time of initial ID consultation):

- **Severely immunocompromised[b] patients with pneumonia[2] particularly if:**
  - Concern for atypical infection such as invasive fungal infection,[3] P*neumocystis jirovecii* pneumonia (PCP), Nocardia, Legionella, etc
  - Not responding to standard care.
- **Fulminant CNS infection and CNS/CSF sampling is not** feasible and not likely to become so (uncommon) *OR* high concern that fulminant CNS infection represents multi-organ process.
- **Fulminant infection and strong epidemiologic concern for atypical infection** such as (Mycobacterium tuberculosis complex, Nocardia sp., Legionella sp., Bartonella quintana, invasive fungal infection, Coxiella burnetii (Q fever), Brucella sp., and Francisella tularensis (tularemia))

Second line test (send if initial tests are negative as detailed as below):

- **Severely immunocompromised[c] patient with persistent febrile neutropenia[5, 6]** and negative first-line work-up (blood cultures, CT C/A/P)
- **Culture-negative endocarditis or other intra-vascular infection[7-9]** plus:
  - Negative first-line workup (blood cultures and appropriate serologies).
- **Fever of unknown origin** plus:
  - Negative first-line workup (blood cultures, CT C/A/P, any other tests clinically indicated).
- **Deep-seated abscesses / lesions** plus:
  - Negative first-line workup (biopsy and cultures, any other tests clinically indicated).

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[a] currently commercially available through Karius; in-house plasma mNGS testing at UCSF in development

[b] Severe immunocompromise is defined as any of the following: bone marrow transplant <1 year for transplant, solid organ transplant <1 year from transplant, primary immunodeficiency, HIV with CD4<200, or B-cell depleting therapy specifically (rituximab)

Repeating tests:

Repeat for diagnostic purposes, consider if:

- Initial test was negative and ongoing concern for infectious syndrome that fits above use cases AND it has been at least 1-2 weeks
- Other testing is non-revealing
- New infectious syndrome that fits above use cases

Do **not** repeat testing to guide end of therapy given current lack of data at this time.

- Some evidence suggests mNGS positivity correlates with metastatic infection in *S. aureus* and gram-negative bacteremia[10], but more data is needed to know how to interpret this.

Logistics:

Ordering:

- Order details: EDTA whole blood (lavender or pearl top) centrifuged and plasma separated from RBCs within 6 hours
- Minimum volume 1 mL (preferred 2 mL)
- Recommend collection of a dedicated tube (avoid add-ons if possible)
- Aliquot if needed under sterile conditions
- Freeze at -70 C (20C acceptable) within 5 days of collection, recommend within 24 hours.

Challenges: at the moment substantial % of tests are (a) not sent, or (b) sent incorrectly and rejected.

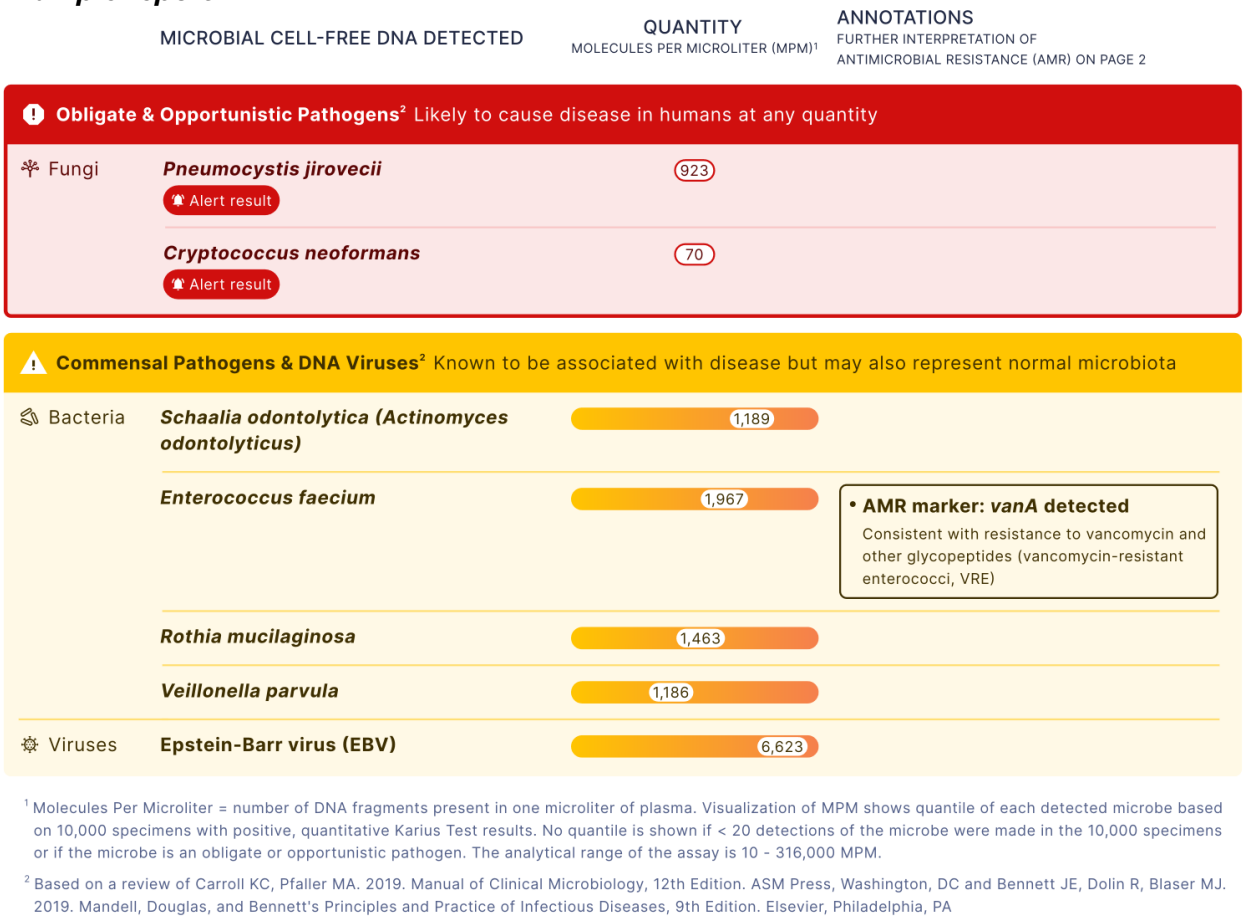
- If mNGS is likely to be a critical diagnostic, recommend ID team check in with the team daily to ensure test is correctly sent.
- For team, advise them to talk directly with phlebotomy or bedside RN daily to ensure test is sent.

Early Reporting and Application Access:

- Call (866) 452-7487 for urgent patient updates
- If interested in getting more systemic access, do the following:
    - Send email to Karius (https://kariusdx.com/contact) requesting access to their test logs. They will respond with name/login, and so you can see all UCSF test results in real time before micro lab updates it.
    - There is also a Karius app for Iphones.

Result Interpretation

Example report:



Result Components.

- Microbes Detected:
  - Obligate/Opportunistic pathogens: Include highly pathogenic fungi (*Rhizopus*), mycobacteria, highly pathogenic bacteria (*Legionella*).
    - Interpretation: always assume true pathogen.

  - Commensal pathogens and DNA viruses: Include bacteria (*S epidermidis*, *C acnes, E faecalis*, *Rothia* etc.) that may be skin or gut flora. Include DNA viruses.
    - Interpretation:
      - *If in doubt*, send orthogonal test (blood cultures for bacteria, CMV PCR for CMV, etc). **Do not treat bacterial detection as equivalent to bacteremia.**
      - *If orthogonal tests negative:* Our practice has been to not treat bacteria on mNGS without additional evidence of infection (e.g. positive blood cultures)

- Quantity (molecules per microliter, MPM): rough estimate of how much pathogen is present.

- Some evidence that MPM correlates well with conventional PCRs for some pathogens such as CMV[11].
- Likely cannot compare MPM between different species and especially between different kingdoms (DNA levels from fungi/bacteria/viruses may be very different).
- May be able to compare to range of recent detections for the same organism in other patients with positive testing at Karius to understand trend of disease burden.
- Prognosis: some evidence that more microbial cell-free DNA is associated with worse prognosis[12].

- AMR reports
  - Available only for the most common bacterial pathogens (gram negatives, S*. aureus, Enterococcus* spp*).*
  - For GPCs: consider broadening therapy if resistance is found in gram positives (*S. aureus*, *Enterococcus* spp. Would not narrow based on an absence of identified resistance markers.
  - For GNRs; both sensitivity and specificity are unknown, would not change management based on results (either positive or negative).

Limitations:

- **Sensitivity issues (false negatives):** False negative rates by pathogen/syndrome are largely unknown.
  - Negative mNGS alone should not be used to de-escalate antimicrobials.
- **Specificity issues (false positives):**
  - Technical false positives: May have detection of environmental bacteria, including *Delftia, E coli, Burkholderia,* etc.
  - Commensal flora or DNA viruses of unclear significance: Frequently detects DNA viruses and/or oral or gut bacteria in patients who are immunocompromised or critically ill. See Commensal Pathogens. When in doubt, use an orthogonal test such as blood culture to guide management.

Turn-around time: Variable. Usually 3-4 days.

Cost: $2000/sample. May have inconsistent outpatient coverage.

CSF mNGS[d]

What it is**:** Target-agnostic DNA **and RNA** sequencing of a CSF sample for broad-based detection of DNA viruses, RNA viruses, bacteria, fungi, and parasites[1].

Specimen: CSF only.

Use Cases (citations to major papers provided):
Note that this is the current recommendation of the infectious disease division in an evolving field. This should not replace clinical judgement; please discuss with ID consults if questions.
First line test:

- **Meningitis, encephalitis, or myelitis of unclear etiology AND CSF pleocytosis, if patient is immunocompetent [13, 14]**:
    - Recommend canceling if bacterial culture or HSV or VZV PCR return positive.
- **Abnormal MRI imaging showing discrete CNS abscesses or lesions for which sampling is not feasible**
    - Expect direct sampling of a lesion to be higher yield if technically feasible (and can send universal PCR or other orthogonal tests).

When not to send CSF mNGS

- **Meningitis associated with indwelling hardware (**Shunt, other CNS hardware) as this is much more likely to be a typical bacterial organism and diagnostic yield is low.

Repeating tests:

- Consider if initial test negative or non-diagnostic due to high host background, and patient clinically worsening and one of above use cases.
- If CSF mNGS results are negative but show decreased sensitivity from high host background, consider ordering universal PCR as a follow up diagnostic test.

Logistics
Ordering:

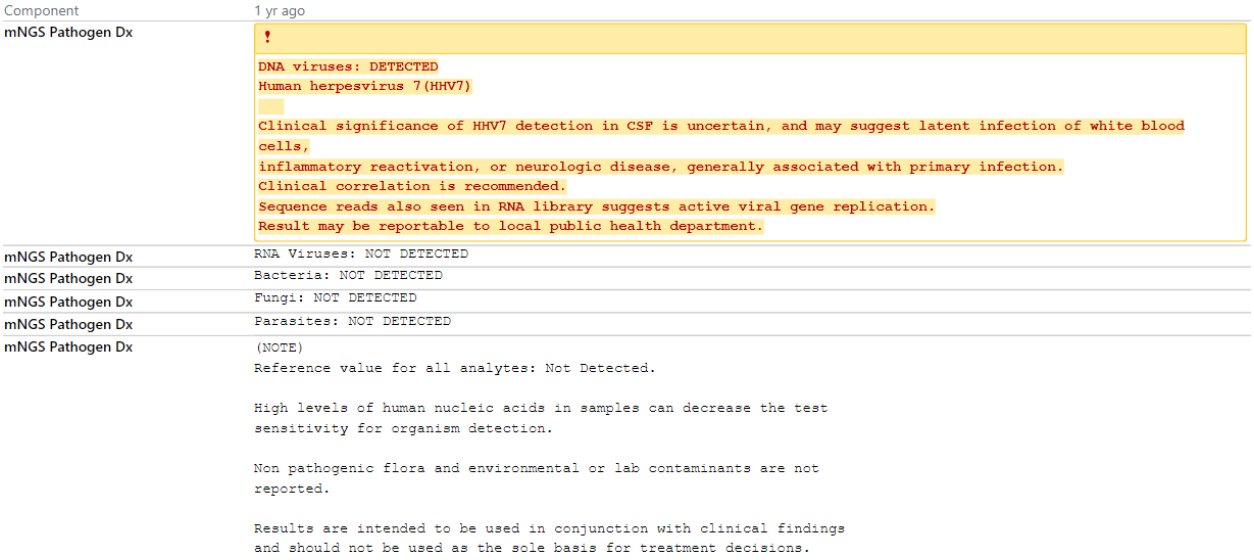
- Order details: CSF, unspun
- Minimum volume 1 mL (preferred 2 mL)
- Aliquot if needed under sterile conditions
- Freeze at -70 C (20C acceptable) within 5 days of collection, recommend within 24 hours.

For early reporting or technical questions: contact the microbiology lab.

[d]available through UCSF

Result Interpretation

Example report



Component	1 yr ago
mNGS Pathogen Dx	! DNA viruses: DETECTED Human herpesvirus 7(HHV7) Clinical significance of HHV7 detection in CSF is uncertain, and may suggest latent infection of white blood cells, inflammatory reactivation, or neurologic disease, generally associated with primary infection. Clinical correlation is recommended. Sequence reads also seen in RNA library suggests active viral gene replication. Result may be reportable to local public health department.
mNGS Pathogen Dx	RNA Viruses: NOT DETECTED
mNGS Pathogen Dx	Bacteria: NOT DETECTED
mNGS Pathogen Dx	Fungi: NOT DETECTED
mNGS Pathogen Dx	Parasites: NOT DETECTED
mNGS Pathogen Dx	(NOTE) Reference value for all analytes: Not Detected. High levels of human nucleic acids in samples can decrease the test sensitivity for organism detection. Non pathogenic flora and environmental or lab contaminants are not reported. Results are intended to be used in conjunction with clinical findings and should not be used as the sole basis for treatment decisions.

Result Components

- mNGS Pathogen Dx: Summary of microbe[s] detected.
- RNA viruses, bacteria, fungi, parasites: line by line.
- May have note indicating that a result is of unclear significance (as above).
- May have a note saying that high levels of human nucleic acids interfere with results.

Highlighted Studies:

First clinical mNGS test for pathogen detection validated in the UCSF Clinical Microbiology Laboratory[15]

- Outline: Study describes the development and validation of the first clinical mNGS test for CNS infections at UCSF. A cloud computing-based pipeline, UCSF SURPI+, was used to analyze mNGS data from CSF for detection of both DNA and RNA pathogens. Analysis of a prospective pilot cohort showed 92% sensitivity and 96% specificity for pathogen detection compared to conventional microbiologic testing.
- Largest study of mNGS for use for diagnosing meningitis or encephalitis is from UCSF[13]
- Outline**:** Study enrolled 204 participants with concern for meningitis or encephalitis. 57 were diagnosed with infection[s]. mNGS made 13 diagnoses not identified by conventional testing and concurrently diagnosed 19 patients also diagnosed by conventional testing. The 13 diagnosed with mNGS alone usually had viruses not conventionally tested for, or had bacteria or fungi that did not culture.

7-year longitudinal study of the diagnostic yield and clinical utility of CSF mNGS testing done at UCSF for UCSF patients and non-UCSF patients nationwide (Benoit, et al., 2024, in press at *Nature Medicine*, medRxiv preprint at https://www.medrxiv.org/content/10.1101/2024.03.14.24304139v1)

- Outline: This study describes UCSF CSF mNGS analysis of 4,828 samples over 7 years, of which 1,164 were from UCSF patients. 14.4% of CSF mNGS tests overall were positive for a microorganism. The sensitivity, specificity, and accuracy of mNGS testing for CNS infections were 63.1%, 99.6%, and 92.9%, respectively. When only considering diagnoses made by CSF direct detection testing, the sensitivity of mNGS testing increased to 86%.

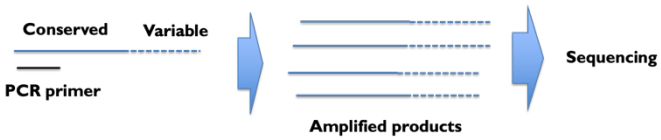
Limitations

- **Sensitivity issues (false negatives):**
    - False negatives: In Wilson et al 2019, 26/57 patients had negative mNGS but were diagnosed by other tests. These included 11 patients diagnosed by serologies (syphilis, dengue, West Nile, *Baylisascaris*, VZV), 7 patients diagnosed by sample other than CSF (usually brain biopsy of an abscess), or 8 where the mNGS had counts too low (*Mycobacterium spp.*, bacteria)
- **Specificity issues (false positives):**
    - Technical false positives: Like serum mNGS, can be subject to low level reagent contamination (*Pantoea, E coli, etc)*
    - Commensal skin flora (pathobionts) or DNA viruses of unclear significance: May detect skin flora (*Corynebacterium*) or viruses of unclear significance (HHV-6, HHV7, etc). If in doubt, consider orthogonal test (culture, viral-specific PCR).
    - Specific issues around HHV-6: HHV-6 reactivation is fairly common in BMT patients and, in <1% of patients, may be chromosomally integrated and present at high levels. HHV-6 should be considered pathogenic only if other causes (infectious and non-infectious) are excluded[16]. Helpful ref is cited here.

Turn-around time: Variable. Usually 10-14 days

Cost: $600/sample (when run in-house for UCSF patients)

Universal/Broad Range PCR from University of Washington

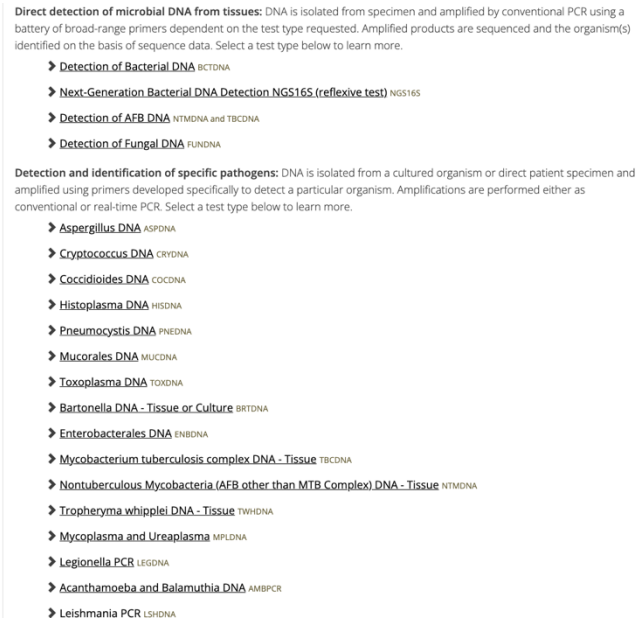


What it is:

- Universal/Broad range PCR: PCR amplification of a broad-range primer that matches to all bacteria, non-tuberculosis mycobacteria, tuberculosis, or fungi, followed by sequencing of a variable region and identification of species present (see diagram). Inherently omits viruses (no conserved region). There is no pan-parasite PCR. There are PCR assays specific to certain parasites (below).
- Other UW PCR: In addition, UW also performs a set of specific pathogen PCRs (see below) that can be added on to samples sent for universal/broad range tests. These are conventional PCRs, not broad range PCR.
    - Reasonable to expect that specific PCRs will have higher sensitivity than broad range PCRs (for example: *Aspergillus* specific PCR would be expected to be more sensitive than fungal PCR).
    - If there is a strong suspicion for a specific infectious etiology but infections from related organisms cannot be excluded, consider ordering a pathogen-specific PCR with reflex to a universal PCR (for example, *Aspergillus*-specific PCR reflexing to fungal PCR if negative).

Specimens Accepted: https://depts.washington.edu/molmicdx/mdx/submit.shtml

- Accept any fresh tissue or fluid samples except blood/plasma. Commonly used for: biopsy samples, abscess samples, joint samples, BAL, etc.
- Accept formalin-fixed tissues, however, sensitivity is expected to be lower.
- For bone specifically, decalcification steps lower sensitivity, so if planning to send bone, ask samples to go directly to micro or ask pathology to hold without decalcification.
- Can also be used for cultured organisms, but we rarely use for this indication.



Direct detection of microbial DNA from tissues: DNA is isolated from specimen and amplified by conventional PCR using a battery of broad-range primers dependent on the test type requested. Amplified products are sequenced and the organism(s) identified on the basis of sequence data. Select a test type below to learn more.

- Detection of Bacterial DNA BCTDNA
- Next-Generation Bacterial DNA Detection NGS16S (reflexive test) NGS16S
- Detection of AFB DNA NTMDNA and TBCDNA
- Detection of Fungal DNA FUNDNA

Detection and identification of specific pathogens: DNA is isolated from a cultured organism or direct patient specimen and amplified using primers developed specifically to detect a particular organism. Amplifications are performed either as conventional or real-time PCR. Select a test type below to learn more.

- Aspergillus DNA ASPDNA
- Cryptococcus DNA CRYDNA
- Coccidioides DNA COCDNA
- Histoplasma DNA HISDNA
- Pneumocystis DNA PNEDNA
- Mucorales DNA MUCDNA
- Toxoplasma DNA TOXDNA
- Bartonella DNA - Tissue or Culture BRTDNA
- Enterobacterales DNA ENBDNA
- Mycobacterium tuberculosis complex DNA - Tissue TBCDNA
- Nontuberculous Mycobacteria (AFB other than MTB Complex) DNA - Tissue NTMDNA
- Tropheryma whipplei DNA - Tissue TWHDNA
- Mycoplasma and Ureaplasma MPLDNA
- Legionella PCR LEGDNA
- Acanthamoeba and Balamuthia DNA AMBPCR
- Leishmania PCR LSHDNA

Use Cases:

- **First line:** any tissue or fluid thought to infected with bacterial, fungal, mycobacteria, AND concern for any of the following:
    - High probability cultures may be negative.
    - High probability extended therapy will be required (large collection, endocarditis, bone or joint involvement)
- Common examples: BAL fluid, joint aspiration, explanted heart valves, brain abscesses.
    - Note: given the lung is not sterile, we do NOT send BAL fluid for bacterial uPCR. Send for fungal only; and/or TB or NTM if specific concerns.
    - Note: Intra-abdominal infections are usually positive for multiple bacteria and so will result in an error report, sending bacterial uPCR for IAI should be uncommon.

        - Note: highest probability of positive result will be from samples with (a) active inflammation, and (B) visualization of organisms on histopathology or microbiology. Absence of these factors portends a probable negative test.
- May consider sending for specific PCRs (as above) in appropriate clinical scenarios (e.g. Bartonella from a heart valve, *T. whipplei* from gut biopsy, *Balamuthia* from brain).
- A note on CSF:
    - mNGS is likely superior to uPCR for diagnosing meningitis/encephalitis[17], likely because many causes of meningitis/encephalitis are viral. Therefore, CSF mNGS has become a more common test.
    - Very uncommon to have a clinical scenario in which universal/broad range PCR was sent on CSF; a rare exception is follow-up universal/broad-range PCR for CSF samples sent for mNGS testing that return as negative with high host background (which reduces sensitivity) but for which the clinical suspicion for infection remains high.
    - There are roles for specific UW PCRs on CSF (pathogen-specific, example: Acanthamoeba/Balamuthia).

Repeating tests:

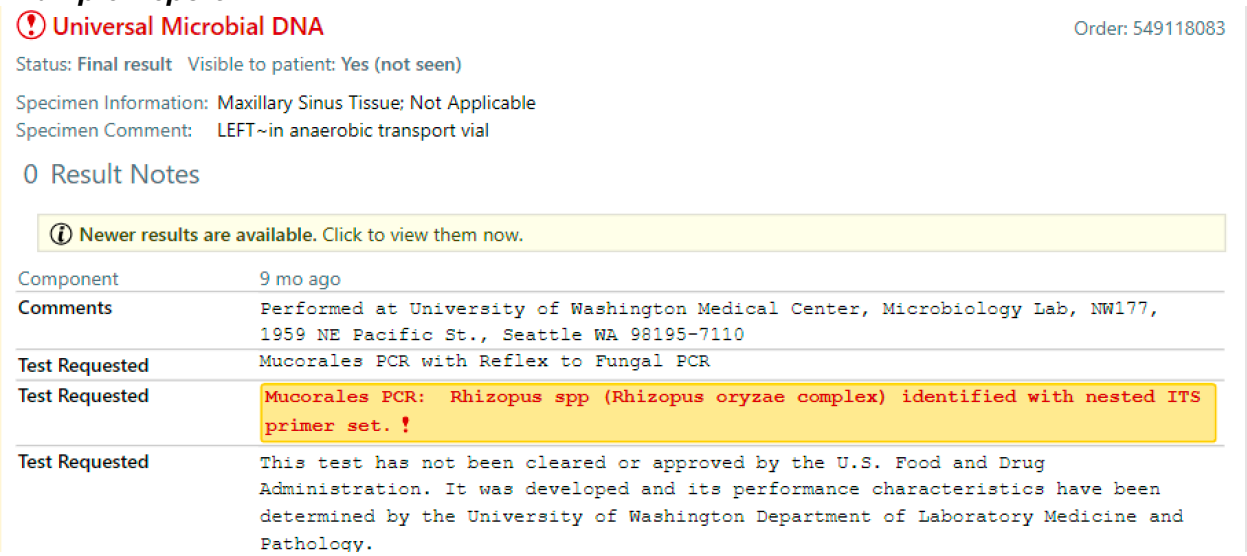
- Consider if initial test negative or non-diagnostic (for example due to interfering templates), and patient clinically worsening and one of above use cases.

Logistics:

- See UW link describing sample suitability and preparation: https://depts.washington.edu/molmicdx/mdx/submit.shtml
- Team orders relevant universal PCRs at time of sample collection.
    - Usually only order PCRs relevant to tissue type.
    - For example, on BAL samples we usually send fungal PCR, AFB PCR, and MTB PCR, because there is expected background bacterial DNA from non-sterile site.
    - If adding on to pathology (a common scenario), please place that order as an add on and specify pathology sample. Micro lab then requests the block from pathology.
- Sample is shipped to UW
- May request held tube
- Canceled orders: order may be canceled if an alternative microbiological diagnosis is found.
    - This has rarely resulted in inappropriate cancellations when an unrelated finding happens (example: finding of *S. epidermidis* in culture from sinus leading to fungal PCR cancellation).
- Interfering templates: may get error message especially on AFB broad-range PCR that interfering templates make it uninterpretable. This should be considered a result without meaning and does not change the probability of AFB presence.

Result Interpretation

Example Report:



Universal Microbial DNA

Order: 549118083

Status: **Final result** Visible to patient: **Yes (not seen)**

Specimen Information: Maxillary Sinus Tissue; Not Applicable
Specimen Comment: LEFT~in anaerobic transport vial

0 Result Notes

Newer results are available. Click to view them now.

Component	9 mo ago
Comments	Performed at University of Washington Medical Center, Microbiology Lab, NW177, 1959 NE Pacific St., Seattle WA 98195-7110
Test Requested	Mucorales PCR with Reflex to Fungal PCR
Test Requested	**Mucorales PCR: Rhizopus spp (Rhizopus oryzae complex) identified with nested ITS primer set. !**
Test Requested	This test has not been cleared or approved by the U.S. Food and Drug Administration. It was developed and its performance characteristics have been determined by the University of Washington Department of Laboratory Medicine and Pathology.

Components of Results:

- Test requested: states which test[s] have been run.
- Findings: any positive results.

Highlighted Study:

Major recent paper was from UCSF looking at uPCR usage from 2011-2019, identified 1062 specimens from 864 patients. uPCR was positive in 175/1062 specimens (16.5%) and clinically significant in 10.1% (107/1062)[18]. High difference between different specimen types, with highest yield of clinical relevance in soft tissue specimens (23.4%) and lowest in CSF (3.4%). In general, the highest yield specimens are usually expected to be those with (1) high concentrations of pathogens (heart valves, for example) and (2) high levels of inflammation.

Limitations:

- **Sensitivity issues (false negatives):** False negative rates by pathogen/syndrome are largely unknown.
  - Negative universal PCR alone should very rarely be used to de-escalate antimicrobials.
- **Specificity issues** (**false positives):**
  - Technical false positives: No published data, but institutional experience is that technical false positives do rarely occur, including of high-consequence pathogens (TB, amebas).
  - Commensals/Pathobionts: Samples from a non-sterile or contaminated site (lung samples contaminated by mouth flora, etc, subcutaneous abscess contaminated by skin flora) may PCR positive for expected microbes at that site.

Turn-around time: Variable. Usually around 14 days.

Cost: $300 per PCR per sample. A joint specimen sent for bacterial, fungal, and non-TB mycobacteria would cost $900.

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