MEDLAB BASICS · STUDY SHEET

Gram Stain and Bacterial Identification

Gram stain steps and errors, morphology, primary media, spot tests, identification flowcharts for gram-positive cocci, gram-negative bacilli, fastidious gram-negatives, gram-positive bacilli and anaerobes, MALDI-TOF, and the sentinel lab's rule-out and refer role

31 sources + more detail

medlabbasics.com/sheets/lab-micro-gram-id.html

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1 Gram stain: the steps and why each matters

Times follow the CDC meningitis manual; UK SMI TP 39 (Hucker modification) uses 30 s steps. Follow the laboratory's validated procedure or the manufacturer's.

STEP	REAGENT AND TIME	WHAT IT DOES
1. Smear	Thin, even film from 1-2 drops (centrifuged sediment for CSF); air dry completely	A monolayer lets single cells and their arrangement be seen; a wet or thick smear washes off or reads falsely
2. Fix	95% methanol for at least 2 min, or pass gently through a flame	Sticks cells to the slide; overheating distorts cells and can make gram-positives stain negative
3. Primary stain	Crystal violet, about 1 min (30 s in the Hucker method); rinse	Stains every cell purple
4. Mordant	Gram's (Lugol's) iodine, about 1 min (30 s in Hucker); rinse	Forms a crystal violet-iodine complex that the thick peptidoglycan of gram-positive walls holds
5. Decolorize	95% ethanol (or acetone or acetone-alcohol) until no more color runs, about 5-10 s; rinse at once	The critical step: removes the complex from gram-negative cells; time depends on smear thickness
6. Counterstain	Safranin about 30 s (carbol fuchsin 10-15 s, or neutral red about 2 min); rinse, blot dry	Stains the now colorless gram-negative cells pink-red
7. Read	Oil immersion (100x)	Gram-positive: deep blue-purple. Gram-negative: pink-red. Note cells, arrangement, and host cells (neutrophils, squamous cells)

EXAM TIP Run a control slide with a gram-positive and a gram-negative organism (CDC suggests S. aureus and E. coli) with each batch. If both control organisms are pink, the run was over-decolorized (or the crystal violet or iodine failed): no patient result from that run is valid. Restain the patient smear with the control.

2 Gram morphology at a glance

WHAT YOU SEE	THINK OF
Gram-positive cocci in grape-like clusters (also pairs, tetrads)	Staphylococcus
Gram-positive cocci in chains	Streptococcus, Enterococcus
Gram-positive lancet-shaped diplococci, sometimes short chains	Streptococcus pneumoniae
Gram-negative coffee-bean diplococci, inside or outside neutrophils	Neisseria (N. meningitidis, N. gonorrhoeae)
Small pleomorphic gram-negative rods or coccobacilli, random arrangement	Haemophilus
Small curved gram-negative rods (darting motility in a wet mount)	Vibrio
Pleomorphic gram-negative rods from an abscess	Bacteroides fragilis group
Large gram-positive rods (1-1.5 x 3-5 um)	Bacillus (B. anthracis: capsule, no spores in direct smears); Clostridium
Club-shaped gram-positive rods	Corynebacterium
Short gram-positive rods with rounded ends, may look like diphtheroids or diplococci	Listeria monocytogenes
Beaded, thin, branching gram-positive filaments	Nocardia (check with a modified acid-fast stain)
Tiny, faint gram-negative coccobacilli	Brucella, Francisella (think biothreat; see the last section)
Gram-positive budding yeasts	Candida, Cryptococcus

3 Primary media: selective and differential agents

MEDIUM	SELECTIVE AGENTS	DIFFERENTIAL SYSTEM	USE AND READING
Sheep blood agar (BAP)	None	Hemolysis	General growth; 5% sheep blood (human blood is not acceptable: its antibodies can inhibit growth). Haemophilus does not grow without a V-factor source
Chocolate agar (CHOC)	None	None	Heat-lysed blood releases hemin (X) and NAD (V); grows Haemophilus, Neisseria, HACEK and most other pathogens
MacConkey (MAC)	Bile salts, crystal violet (inhibit gram-positives)	Lactose with neutral red	Lactose fermenters pink-red; nonfermenters colorless (Shigella colorless, 2-3 mm)
Eosin methylene blue (EMB)	Eosin Y and methylene blue (inhibit gram-positives)	Lactose; the dyes also act as the indicator	Lactose fermenters colored (E. coli: flat colonies with dark centers, with or without a metallic sheen); nonfermenters colorless
Hektoen enteric (HE)	Bile salts	Lactose, sucrose, salicin with bromthymol blue and acid fuchsin; thiosulfate and ferric ammonium citrate for H2S	Stool. Salmonella blue-green to blue, with or without black centers (some almost all black); Shigella green; E. coli salmon-orange with a bile precipitate
Xylose lysine desoxycholate (XLD)	Sodium desoxycholate	Xylose, lactose, sucrose and lysine with phenol red; thiosulfate and ferric ammonium citrate for H2S	Stool. Shigella transparent pink-red; Salmonella pink-red with or without black centers (atypical strains yellow); coliforms yellow
Salmonella-Shigella (SS)	Brilliant green among its inhibitors	Lactose and H2S, read like HE and XLD	Stool: Salmonella and Shigella
Columbia CNA	Colistin and nalidixic acid (inhibit gram-negatives)	None	Gram-positives from mixed specimens
Phenylethyl alcohol (PEA) blood agar	Phenylethyl alcohol (inhibits most gram-negatives)	Blood: hemolysis	Gram-positive cocci from mixed flora; stops Proteus swarming
Modified Thayer-Martin (TM)	Vancomycin, colistin, nystatin, trimethoprim on chocolate base	None	Pathogenic Neisseria from nonsterile sites; needs 5-10% CO2
Buffered charcoal yeast extract (BCYE)	Antibiotics in the selective version	None	Legionella: needs L-cysteine and iron in the medium; growth can take up to 14 days. Also supports Nocardia and Francisella
TCBS	Bile (oxgall, sodium cholate)	Sucrose with bromthymol and thymol blue; thiosulfate and ferric citrate	Vibrio: V. cholerae yellow, shiny, 2-4 mm (sucrose fermenter); V. parahaemolyticus green to blue-green. Never autoclave
CIN (Yersinia selective)	Cefsulodin, irgasan, novobiocin, crystal violet, desoxycholate	Mannitol with neutral red	Yersinia enterocolitica from stool, incubated at about 30 C: small (1-2 mm) colonies with a deep red center and sharp border inside a clear, colorless rim (the bull's-eye)
Campylobacter selective (e.g. CCDA)	Antibiotics on a charcoal, blood-free base	None	Campylobacter jejuni: microaerophilic, 42 C
Regan-Lowe	Cephalexin	None	Bordetella pertussis: charcoal agar with 10% horse blood and cephalexin (about 40 ug/mL) against nasopharyngeal flora; examine daily for up to 7 days
Lowenstein-Jensen (LJ)	Malachite green	None	Mycobacteria: egg-based; colonies in 4-8 weeks (see the Mycobacteria page)

ATMOSPHERE OR TEMPERATURE	EXAMPLES
5-10% CO2 (capnophilic)	Neisseria gonorrhoeae, Haemophilus influenzae; S. pneumoniae and N. meningitidis grow best in about 5% CO2 or a candle jar
Microaerophilic (2-10% O2 plus CO2)	Campylobacter jejuni (also grows at 42 C)
Strict anaerobic	Clostridioides difficile and other anaerobes
Cooler than 35 C	Yersinia pestis grows better at 25-28 C; Mycobacterium marinum at 25-30 C; Listeria grows from 4 to 37 C

EXAM TIP A Thayer-Martin plate sent without a CO2 source and put in an ambient-air incubator can stay sterile even when N. gonorrhoeae was present. No growth on BAP but growth on CHOC means the organism needs X and/or V factor: think Haemophilus (and other fastidious gram-negatives).

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Gram Stain and Bacterial Identification

4 Spot tests

TEST	PRINCIPLE AND READING	PITFALLS
Catalase	3% hydrogen peroxide on a colony: bubbles = positive. Staphylococcus +, Streptococcus and Enterococcus -	Picking up blood agar gives a false positive (red cells contain catalase). Control: S. aureus +, a streptococcus -. Makes aerosols: do it covered or in a BSC if a biothreat is possible
Oxidase	1% tetramethyl-p-phenylenediamine on filter paper: dark purple within 10 s = positive; ignore color after 10 s	Nichrome loops give false positives (use platinum, plastic or wood). Do not test growth from TCBS or carbohydrate media. Enterobacterales are oxidase-negative, except Plesiomonas shigelloides (oxidase positive, now placed in the order)
Tube coagulase	Free coagulase clots plasma: separates S. aureus (and other coagulase-positive staphylococci) from coagulase-negative staphylococci	Slow; slide (clumping factor) test is obsolete because of poor sensitivity and specificity
Latex or hemagglutination for S. aureus	Detects clumping factor, protein A and capsule antigens; rapid	False positives with S. lugdunensis and some other coagulase-negative staphylococci
Spot indole	Tryptophan to indole (E. coli positive)	Cinnamaldehyde reagent turns blue (preferred), benzaldehyde pink; Kovac's reagent is used on SIM tubes
Urease	Urea agar or broth turns pink-red (alkaline)	Over-incubation gives nonspecific results
Motility	Semisolid agar stabbed 1-2 cm: growth spreading from the stab line = motile; TTC dye turns red where bacteria grow	A wet surface lets nonmotile organisms creep down the side. Avoid wet mounts for suspected biothreat agents
PYR	Detects pyrrolidonyl arylamidase: positive for S. pyogenes and Enterococcus	Read by the kit's instructions

5 Gram-positive cocci: the flowchart as a table

Hemolysis does not separate S. pneumoniae from viridans streptococci: both are alpha. Pneumococcal colonies flatten and dip in the center after 24-48 h; viridans colonies stay raised.

STEP	TEST	RESULT	POINTS TO
1	Catalase	Positive	Staphylococcus (go to 2)
1	Catalase	Negative	Streptococcus or Enterococcus (go to 4)
2	Tube coagulase	Positive	S. aureus
2	Tube coagulase	Negative	Coagulase-negative staphylococci (CoNS); from urine go to 3
3	Novobiocin 5 ug disk	Resistant	S. saprophyticus (urinary tract infection in young women)
3	Novobiocin 5 ug disk	Susceptible	S. epidermidis group
4	Hemolysis on sheep blood agar	Beta	Go to 5
4	Hemolysis	Alpha	Go to 6
4	Hemolysis	Alpha or none, optochin resistant	Go to 7
5	Bacitracin and PYR	Bacitracin susceptible, PYR positive	S. pyogenes (group A)
5	CAMP and hippurate	Both positive (bacitracin resistant, PYR negative)	S. agalactiae (group B)
5	Lancefield grouping	Group C or G, PYR and CAMP negative	S. dysgalactiae subsp. equisimilis, or the S. anginosus group (VP positive)
6	Optochin (5 ug, 6 mm disk, CO2)	Zone 14 mm or more	S. pneumoniae
6	Optochin	Zone under 14 mm or none	Do bile solubility
6	Bile solubility (2% deoxycholate)	Suspension clears (saline control does not)	S. pneumoniae; partial clearing is not positive
6	Bile solubility	No clearing	Viridans streptococci
7	Bile esculin and 6.5% NaCl	Both positive (PYR positive)	Enterococcus
7	Bile esculin and 6.5% NaCl	Bile esculin positive, NaCl negative	S. bovis group (S. gallolyticus and relatives)
7	Bile esculin and 6.5% NaCl	Both negative	Viridans streptococci

6 Gram-negative bacilli: TSI or KIA

KIA has glucose and lactose; TSI adds sucrose. Stab the butt and streak the slant from the center of one colony; loosen caps; read at 18-24 h at 35-37 C. Tight caps or a shallow butt give misleading reactions.

REACTION (SLANT/BUTT)	MEANING	EXAMPLES
K/K (red/red) or no change	No fermentation	Pseudomonas aeruginosa, Acinetobacter (nonfermenters)
K/A (red/yellow)	Glucose only fermented	Shigella, Salmonella, Proteus mirabilis
A/A (yellow/yellow)	Glucose plus lactose (KIA) or lactose and/or sucrose (TSI)	E. coli, Klebsiella, Enterobacter; V. cholerae is A/A in TSI (sucrose) but K/A in KIA
Black butt	H2S	Salmonella, Proteus, Citrobacter
Cracks or lifted agar	Gas from glucose	E. coli (Shigella little or none)

+ positive, - negative, v variable. Serotype from KIA, TSI or a nonselective plate, never from MAC or XLD (false negatives).

ORGANISM	LACTOSE	H2S	INDOLE	UREASE	MOTILITY	OTHER CLUES
Escherichia coli	+	-	+	-	+	Citrate -, MR +, VP -, gas +
Klebsiella pneumoniae	+	-	-	+	-	Citrate +, VP +; heavily encapsulated
Enterobacter cloacae	+	-	-	-	+	Citrate +, VP +
Citrobacter freundii	+	+	-	v	+	Citrate +; H2S-positive lactose fermenter
Proteus mirabilis	-	+	-	+	+	Red slant on LIA (deamination); swarms (PEA stops it)
Morganella morganii	-	-	+	+	+	Indole and urease positive lactose nonfermenter
Salmonella (non-Typhi)	-	+	-	-	+	Citrate usually +, LDC +
Salmonella Typhi	-	+	-	-	+	No gas, citrate -
Shigella	-	-	v	-	-	K/A with little or no gas; LIA K/A (LDC -); confirm with antisera

ORGANISM	KEY REACTIONS
Pseudomonas aeruginosa	Oxidase +, nonfermenter (K/K), motile, nitrate reduced, often blue-green pyocyanin; grows on cetrimide agar
Acinetobacter baumannii	Oxidase -, nonfermenter, nonmotile
Vibrio cholerae	Oxidase +, string test +, yellow on TCBS, TSI A/A, LIA K/K (LDC +)
Aeromonas	Oxidase +, string test usually -
Campylobacter jejuni	Oxidase +, hippurate +, motile, microaerophilic at 42 C
Plesiomonas, Alcaligenes	Oxidase +

EXAM TIP Decarboxylases (LDC, ODC, ADH) are read in a deep, anaerobic butt: purple (alkaline) = amino acid decarboxylated; yellow (acid) = negative. Always read against a base tube without the amino acid. On LIA a red slant means deamination (Proteus, Providencia, Morganella).

7 Fastidious gram-negatives

CTA sugars: positive = turbidity plus yellow in the upper medium (yellow without growth is not positive). Hold 72 h, up to 5 days, before calling negative. Neisseria are oxidase-positive gram-negative diplococci.

ORGANISM	GLUCOSE	MALTOSE	LACTOSE	SUCROSE
N. meningitidis	+	+	-	-
N. gonorrhoeae	+ (some weak)	-	-	-
N. lactamica	+	+	+	-
N. sicca	+	+	-	+
Moraxella catarrhalis	-	-	-	-

Disks or a Quad plate on a medium without X or V: H. influenzae grows only around the X+V disk. Use a light inoculum with no agar carried over. H. aphrophilus and H. paraphrophilus (V-dependent, X-independent) are now one species, Aggregatibacter aphrophilus, so its factor pattern varies. Satellitism: H. influenzae grows as tiny colonies only beside a streak of S. aureus on BAP, which supplies V factor.

ORGANISM	NEEDS X (HEMIN)	NEEDS V (NAD)	BETA-HEMOLYSIS ON HORSE BLOOD
H. influenzae	Yes	Yes	No
H. haemolyticus	Yes	Yes	Yes
H. parainfluenzae	No	Yes	No
H. parahaemolyticus	No	Yes	Yes
H. aphrophilus (now Aggregatibacter aphrophilus)	Yes	No	No

- HACEK: Haemophilus (H. parainfluenzae), Aggregatibacter, Cardiobacterium, Eikenella corrodens, Kingella kingae. Oral and upper respiratory flora; slow-growing fastidious gram-negatives that cause endocarditis, often after a delay or with 'culture-negative' blood cultures.
- They grow better on subculture to sheep blood and chocolate agar at 37 C in 5-10% CO2. MALDI-TOF identifies them well.
- Kingella kingae: oxidase +, catalase -.

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