Sample preparation for microbial RNA-seq.

For bacterial and eukaryotic microbe (yeast, fungi) systems. RNAlater-based workflow — no LN_2 required.

30M	rRNA depletion	RNAlater	2-8 wks
Recommended read depth	**Library prep required**	**Stabilization reagent**	**Analysis turnaround**
paired-end reads / sample	*no poly-A for bacteria*	*no LN_2 required*	*after we receive your data*

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§ 1

Overview & scope

This protocol covers sample preparation for microbial RNA-seq — bacteria (gram-positive and gram-negative) and eukaryotic microbes (yeast, fungi). It is a companion to our mammalian protocol; if you're working with mammalian cells, use the mammalian document instead.

Auperon does not run sequencing itself, coordinate vendors, or handle sample logistics. You send us data in one of three ways: (1) existing RNA-seq data you already have, (2) new data you generate with the vendor of your choice following the sample prep guidance in this document, or (3) public data (NCBI SRA, GEO). This document covers the sample prep guidance for option 2 — using **RNAlater** stabilization, which is the industry standard for microbial RNA-seq when LN_2 is not available.

If your engagement needs vendor management, sequencing coordination, sample prep guidance beyond this document, or wet-lab work, that's **Van Heron Labs full-service** — the enterprise pathway that handles the pipeline end-to-end.

This document reflects Van Heron Labs' **preferred** microbial RNA-seq setup — the configuration that produces the strongest Auperon output. That said, we can work with virtually any RNA-seq data. If your existing setup, vendor, or read structure deviates from what's below, send it anyway — most of the time we can accommodate. Flag deviations at project description and scoping so we can advise.

Hand this document to your bench team. If any protocol here doesn't fit your setup, email **hello@auperon.io** before capture.

SPECIAL CONSIDERATIONS FOR MICROBIAL RNA-SEQ

- **Bacterial mRNA half-life is short** (typically 2-10 minutes) — capture must be fast, and RNAlater must be added quickly.
- **Growth phase timing is critical** and depends on your study goal — see §3.
- **Bacteria and eukaryotic microbes have different cell-wall lysis requirements** — see §5. Flag your organism to your sequencing vendor at project intake so they use the correct extraction workflow.

§ 2

Sequencing input requirements

Recommended defaults

- **Read depth:** ~30-50M paired-end reads per sample is a comfortable default for most microbial studies. Bacteria often need less (15-25M is sufficient for standard differential expression); eukaryotic microbes benefit from slightly more (30-40M).
- **Library prep: rRNA depletion:** Required for prokaryotes and strongly preferred over polyA for eukaryotic microbes.
- **Format:** paired-end preferred for better alignment accuracy and operon-boundary detection in bacteria.

Confirm your vendor's rRNA depletion approach matches your organism type — some kits are validated for gram-negative or gram-positive specifically.

rRNA depletion for eukaryotic microbes

Eukaryotic microbes (yeast, fungi) DO have polyadenylated mRNA, so poly-A selection will work. However, poly-A prep loses non-coding regulatory transcripts and can shift with cell state, which reduces cross-condition comparison quality. **rRNA depletion is still preferred** for Auperon analyses, but poly-A is acceptable — flag the format at kickoff.

Mitochondrial signal. Yeast and fungi are heavily mitochondria-dependent, and mitochondrial physiology is central to fermentation biology, stress response, and productivity. Mitochondrial mRNAs are polyadenylated but with much shorter poly-A tails than nuclear mRNAs (roughly 25-55 nt vs 150-250 nt). Poly-A selection systematically depletes short-tailed mRNAs — meaning the metabolic signal you want to capture is the signal poly-A selection is most likely to lose. rRNA depletion kits that target both cytoplasmic and mitochondrial rRNA preserve mitochondrial mRNAs in the library.

Depth by use case

- **Baseline characterization / discovery:** 10-20M PE reads sufficient for most bacteria.
- **Deep comparative analysis / differential expression:** 20-30M PE recommended.
- **Long-tail transcript detection / novel biomarker discovery / operon boundary detection:** 30-50M PE recommended.
- **Eukaryotic microbes (larger transcriptome):** add 25-50% to the above depths.

Other formats we handle

Auperon supports formats beyond the recommended bulk rRNA-depleted prep. Note the format in your project description at kickoff:

- **Poly-A prep (eukaryotic microbes only)** — analysis weights differ; some non-coding signal lost.
- **Oxford Nanopore / long-read RNA-seq** — excellent for operon structure and isoform detection.
- **Bacterial small RNA (sRNA) enrichment** — supported for regulatory RNA studies.
- **Dual RNA-seq (host + pathogen)** — supported; scoped differently.
- **Existing published datasets** — supported for discovery-mode and feasibility work.

A NOTE ON SINGLE-CELL MICROBIAL RNA-SEQ

• Single-cell RNA-seq for microbes is emerging (methods like MATQ-seq, PETRI-seq, microSPLiT) but is still much less mature than mammalian scRNA-seq.

• **Ambient RNA contamination applies** — reads from lysed neighbors get incorporated into apparent single-cell profiles, so marker genes from non-target species or subpopulations can appear where they shouldn't.

• For most microbial studies, **bulk RNA-seq is the right choice**. If you have a genuine single-cell microbial application, flag it at scoping — we advise on the current best practices for the specific method.

§ 3

Sample capture — timing by study goal

The right time to capture your microbes depends on what you're trying to optimize. Growth-phase considerations are especially important in microbial systems because physiology shifts dramatically between phases — sometimes within minutes.

For proliferation / growth-rate optimization

Capture cells in **early-to-mid exponential phase (mid-log)**:

- Cells actively dividing at maximum rate.
- **Not lag phase** (adaptation, transcriptionally noisy from environment shift).
- **Not stationary phase** (nutrient depletion, stress response, sporulation for spore-formers).
- For bacteria: typically OD_{600} = 0.4-0.8 depending on species and vessel geometry.
- For yeast: typically OD_{600} = 0.5-1.5 depending on species and doubling time.
- Verify with a growth curve for your specific strain + medium before the RNA-seq run.

Why: proliferation biomarkers and modulator recommendations are only meaningful when cells are actively dividing at steady-state growth kinetics. Lag or stationary captures give you information about the wrong biology.

For titer / production optimization

Capture at **two timepoints — early AND late in the production window**. The precise timing depends on whether your product is growth-associated or non-growth-associated:

- **Growth-associated production** (product accumulates during exponential growth — most primary metabolites, some recombinant proteins): early = early-mid exponential; late = late exponential.
- **Non-growth-associated production** (product accumulates in stationary phase — most secondary metabolites, many recombinant proteins under starvation-induced systems): early = early stationary (production induction); late = mid-to-late stationary (peak accumulation).

- **Induction-dependent production** (IPTG, arabinose, tetracycline, etc.): capture pre-induction (baseline), early post-induction (2-4 hours), and late post-induction (12-24 hours).

Why: production physiology changes dramatically across a production run. Single-timepoint sampling gives you only part of the story. Media and process optimization built from both timepoints supports both production ramp AND production sustain.

For viability / stress response studies

- **Match sampling exactly across conditions** — same OD, same media composition, same handling.
- For stress-response studies, define stress duration precisely and capture at the same duration in every replicate.
- **Capture as quickly as possible after stress induction** — bacterial mRNA half-lives are 2-10 minutes; slow capture loses acute response signal.

For chemically-defined media / feed optimization

- Extended culture in target media before capture (multiple generations).
- Match adaptation time across all conditions being compared.
- Capture in the growth phase relevant to your production goal (see above).

UNIVERSAL RULES FOR MICROBIAL COMPARATIVE STUDIES

- **Match growth phase** (by OD or growth curve) across all conditions.
- **Same media age, same vessel geometry, same temperature, same agitation, same conditions.**.
- **Capture as quickly as possible** — bacterial mRNA turns over in minutes.
- **Take an OD reading at capture** and record it in the sample manifest.
- If any condition can't hit these exactly, flag it at scoping — covariate corrections are sometimes possible.

§ 4

Sample preparation — RNAlater workflow

RNAlater (Thermo Fisher) is our recommended stabilization reagent for microbial RNA-seq. It's a proprietary solution containing high concentrations of quaternary ammonium sulfates that denatures RNases on contact, stabilizing RNA expression profiles without requiring liquid nitrogen.

RNAlater has been validated for both gram-positive and gram-negative bacteria (*E. coli, P. aeruginosa, C. fetus, B. subtilis, S. aureus*) and for eukaryotic microbes (yeast). RNA integrity (RIN > 7) is preserved for up to a week at 25°C and longer at 4°C or -20°C.

BEFORE YOU BEGIN

- **Pre-purchase RNAlater** (Thermo Fisher, cat. AM7020 or equivalent). Have enough on hand for all samples plus ~30% margin.

- **Confirm with your sequencing vendor** that they accept RNAlater-preserved samples. Most do; a few do not.
- **Work sterilely** — cross-contamination between samples is a real risk with microbial cultures and shows up in the RNA-seq data.
- **Verify your capture window** (see §3) with a growth curve before the RNA-seq run itself.

WHO HANDLES RNA EXTRACTION?

- **We generally recommend letting your sequencing vendor perform the RNA extraction.** Ship RNAlater-preserved samples; let the vendor extract on their end. This is the default in most Auperon microbial engagements.
- **Why:**
- **Eliminates a variation source** — one protocol, one lab, one operator across all your samples meaningfully reduces batch effects vs multiple in-house extractions.
- **Standardized with downstream library prep** — vendor extraction is validated against their library prep workflow, so you get more consistent library yields.
- **Cost** — bacterial and fungal extraction kits (Qiagen RNeasy, Zymo Direct-zol, MasterPure, etc.) plus specialized lysis reagents (lysozyme, zymolyase) run $200-500+ per kit; bundling extraction into the sequencing quote is usually cheaper.
- **Vendor knows the lysis** — they handle gram-positive vs gram-negative bacteria, yeast, and fungi differently (see §5); their standard workflows are already tuned to your organism type.
- **Upstream QC** — if extraction fails, the vendor knows first and can flag it before library prep.
- **When to extract in-house instead:**
- **The preferred vendor does not accept RNAlater preserved samples.**
- **Very hard-to-lyse organisms** where you have a specialized in-house protocol (mycobacteria with extended bead beating, some spore-formers, biofilm-embedded cells).
- **BSL2+ material** without vendor biosafety approval. See §8.
- **Regulatory / GMP / chain-of-custody constraints** — extract in-house under your quality system if required.
- **Extremely time-sensitive stability studies** where downstream shipping delay would be a confound.
- If in doubt, email **hello@auperon.io** before capture — we advise based on your specific organism and system.

Sample capture protocol

For each sample, at the capture time you determined in §3:

1. **Cool centrifuge to 3°C.** Prepare an ice bucket filled with ice.
2. **Label sterile microcentrifuge tubes** (lock-cap or screw-cap preferred — see §7). Place tubes on ice.
3. **Take an OD reading** of your culture at the moment of capture. Record it in the sample manifest.
4. **Transfer 1 mL of culture** to each labeled tube (multiple tubes per sample if desired — you can pool later if needed/desired).
5. **Spin at 5,000 RPM for 6 minutes at 3°C.** This forms a tight cell pellet.

6. **Discard the supernatant** carefully — do not disturb the pellet.
7. **Wash: resuspend the pellet in 100 µL RNAlater** by pipetting up and down.
8. **Spin again** at 5,000 RPM for 6 minutes at 3°C.
9. **Discard the supernatant.**
10. **Resuspend the pellet in 100 µL fresh RNAlater.** This is the final storage.
11. **Seal each tube with Parafilm** to prevent cap-seal failures during transit.
12. **Store at 4°C overnight** to allow RNAlater to fully penetrate the cells.
13. **Transfer to -20°C** the following day. Cells can be stored at -20°C for months if needed.

For eukaryotic microbes (yeast, fungi)

The protocol above works for yeast and most fungal cells at typical culture densities. For denser cultures or larger cells, consider:

- Scaling up to 2-5 mL per sample if OD is low.
- Using a longer spin (7-8 min) to ensure a tight pellet for larger cells.
- Extended RNAlater incubation at 4°C (24 hours) for full cell wall penetration.

§ 5

Bacterial vs eukaryotic microbe considerations

Bacteria and eukaryotic microbes have important biological differences that affect both wet-lab prep and downstream sequencing choices. Flag your organism type — and ideally the specific species — to your sequencing vendor at project intake so they use the correct extraction workflow.

Cell wall lysis

- **Gram-negative bacteria:** lysozyme + physical (bead beating or sonication) — most vendors' standard workflow.
- **Gram-positive bacteria:** may need stronger lysis (lysostaphin for Staphylococcus, mutanolysin for streptococci, extended bead beating for spore-formers).
- **Yeast (S. cerevisiae, P. pastoris, etc.):** zymolyase or lyticase + bead beating.
- **Filamentous fungi:** bead beating with ceramic or glass beads; may need cryo-grinding depending on species.
- **Mycobacteria:** require extended bead beating with silica beads and specialized lysis buffers.

You typically won't need to do the lysis yourself — the sequencing vendor handles extraction after RNAlater-preserved samples arrive. But **tell them your organism** so they choose the right protocol.

mRNA half-life and capture speed

- **Bacteria:** mRNA half-life is 2-10 minutes typically. Capture (culture → spin → RNAlater) must be as fast as possible. Any delay above 5-10 minutes will lose acute regulatory signal.
- **Yeast and fungi:** mRNA half-life is 10-30 minutes (S. cerevisiae) up to a few hours (filamentous fungi). Less time-critical, but still capture promptly.

Volume and OD considerations

- **Bacteria at OD_{600} 0.5-1.0:** 1 mL per sample is sufficient (yields ~10^9 cells).
- **Bacteria at lower OD or during induction:** scale up to 2-5 mL.
- **Yeast at OD_{600} 0.5-2:** 1-2 mL per sample.

- **Filamentous fungi:** discuss with vendor — mycelium collection differs from single-cell suspension work.

Genome reference availability

For accurate read mapping, the sequencing vendor and Auperon will need access to a reference genome for your organism. Common industrial and research strains are in NCBI or Ensembl. If you're working with a proprietary strain, engineered variant, or under-characterized isolate, **flag this at scoping** — we'll need either the reference assembly or the parent strain identifier for accurate mapping.

§ 6

Sample labeling & naming

Most sequencing vendors have naming constraints, and the rules below are broadly compatible with common vendors including Novogene, Genewiz/Azenta, and most academic cores. Sample names on tubes **must match exactly** with your vendor's Sample Information Form (SIF) or equivalent intake document.

Naming best practices

- Begin with a letter of the alphabet.
- Contain a maximum of **12 alphanumeric characters or underscores**.
- Contain no hyphens, spaces, or special characters.
- Match exactly between tube labels and the intake document you submit to your vendor.

Example scheme for microbial samples

Encode strain, condition, timepoint, and replicate. Example: `Ec_ctrl_T1_1` = *E. coli*, control condition, timepoint 1, biological rep 1. Or `Sc_gluc_LP_2` = *S. cerevisiae*, glucose condition, late production, biological rep 2. Keep the scheme within **≤12 characters**.

Physical labeling

Label tubes on the side, not cap-only. Write name with cryo-marker and cover with clear tape. Cap-top labels rub off in transit or storage.

INCLUDE AN OD READING IN THE MANIFEST

• For microbial samples, include the **OD_{600} at time of capture** in the sample manifest — this is important context for interpreting expression patterns and often makes the difference in correctly attributing signal to biology vs growth phase.

§ 7

Tubes, containers & handling

Tube specifications

- **DNase-/RNase-free 1.5 or 2.0 mL microcentrifuge tubes.**
- **Lock-cap or screw-cap preferred** over standard snap-cap — much less risk of cap failure in RNAlater or during shipping.
- **Do not use 96-well plates** or non-standard formats — risk of vendor handling fees or rejection.

- **Seal each tube with Parafilm** before packaging for shipment.

Storage before shipment

- **4°C overnight** after RNAlater is added (to allow penetration).
- **-20°C storage** the next day.
- Samples can be stored at -20°C for weeks-to-months if shipment is delayed.
- **No LN_2 required.** RNAlater eliminates the need for liquid nitrogen storage — one of its main advantages for microbial workflows.

§ 8

Shipping — packaging, courier, timing

General best practices for shipping RNAlater-preserved samples to any sequencing vendor. Always confirm your specific vendor's requirements, deadlines, and biosafety policies before shipment.

Courier & timing

- **Ship on dry ice, overnight.**
- **FedEx overnight** or DHL international express also widely accepted.
- Book shipment at least **48 hours in advance** via your vendor's shipping booking form or portal.
- **Ship Monday-Wednesday** to avoid weekend transit.
- Confirm holiday closures and current lead times with your vendor before booking.

Packaging

- **Dry ice is regulated** — outer box must carry the approved sticker (from your dry-ice supplier).
- Seal each tube with Parafilm.
- Place tubes **upright** in a container (50 mL tube or cryobox) with interior racks or supports — **never loose in bags**.
- Use cotton or absorbent paper to prevent tubes from being jostled by dry ice.
- Cover the polystyrene dry-ice box with an outer cardboard box.

Documents to include inside the box

- Copy of the vendor's Sample Information Form (SIF) or equivalent intake document.
- The Waybill.
- A printed sample manifest matching the tube labels, with OD_{600} at capture recorded per sample.
- Also place a copy of the manifest in the outer cardboard box (in case the inner is damaged).

Biosafety

BSL2+ MATERIAL REQUIRES PRIOR WRITTEN APPROVAL

• **Most sequencing vendors do not accept Biosafety Level 2+ material without prior written approval.** This includes pathogenic bacteria, purified viral genomes, and other BSL2+ agents.

• If your samples fall into this category, secure vendor approval before shipment. Any BSL2+ sample submitted without prior approval may be immediately destroyed or returned by the vendor.

§ 9

Quality standards

Auperon's output is only as good as the data it's built on. Every dataset goes through internal QC before we begin the full analysis pipeline.

Standard incoming-data checks

- Read quality distribution (base-level quality across read length).
- Alignment rate against the reference genome for your organism.
- Read count per sample vs the depth target for your engagement tier.
- RNA integrity indicators (RIN where available — RNAlater-preserved samples typically show RIN > 7).
- **rRNA depletion efficiency** — should be > 90% for a well-executed rRNA depletion prep. Lower than this and the effective sequencing depth is severely reduced.
- Library complexity and duplication rates.
- Comparability across samples in the experimental design.

If a specific sample fails, we flag it early. You may still get meaningful analysis on the remaining samples, or we may recommend re-sequencing that specific sample.

Refund policy for failed sequencing

IF SEQUENCING DATA DOESN'T MEET OUR INTERNAL QC

• If sequencing data you provide **fails our internal QC — meaning it's uninterpretable at the level required to produce mechanism-backed output — we refund the engagement in full** and provide our recommendations on experimental design for a future run.

• This protects both of us: you're not paying for output we can't confidently deliver, and we're not producing recommendations from data we don't trust.

§ 10

Final considerations

Always default to vendor and kit instructions where appropriate. This document aims to serve as a guidelines for best practices as we see them. Happy sequencing!

§ 11

Contact & support

This protocol is a starting point. Every microbial system has quirks. If any step here doesn't fit your organism, your process, or your vendor, email us before capture — we'll adapt.

hello@auperon.io

Response typically within one business day for engaged customers

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Case studies, pricing, and to start an engagement