Hazard Analysis of Wet-Lab Materials

ICHNOS · Patras Medicine iGEM 2026

Part of our Safety and Security work

October 2026

Scope: Biological materials, chemicals, commercial kits and consumables used in the ICHNOS wet-lab workflow, assessed for hazard, exposure and control.

Status: Experimental work for the Proof of Concept is complete. This assessment covers the materials and procedures used in that work.

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How to read this document

Where a statement rests on something we checked ourselves rather than on our project records, we say so and give the method.

We distinguish throughout between **hazard** (a material's intrinsic capacity to cause harm) and **risk** (the probability of harm given the task, concentration, quantity, exposure route and controls in place). A reagent with a severe classification used once, in microlitres, at a thousandfold dilution, behind a fume sash, is a different risk from the same reagent in bulk. Where this distinction matters we assess stock preparation separately from experimental use.

Terminology. Risk Group, Biosafety Level and the EU contained-use class under Directive 2009/41/EC are three distinct concepts and we do not use them interchangeably. Risk Group is a property of the organism. Biosafety Level describes the facility and practices. Contained-use class is a regulatory determination made by the institution for a specific activity. This document states the Risk Group of our chassis and describes our practices. The formal containment classification for our work is the responsibility of our host institution.

1. Purpose

This document reflects the safety culture our team built throughout the year: identifying hazards, questioning our choices and documenting how we reduced risk was part of how we worked from the start, not a step added at the end. It covers three questions:

- **Biological:** what is our chassis, what did we put into it and what could the engineered strain do outside the flask?
- **Chemical:** what are we handling, how are we exposed to it and what reduces that exposure?
- **Procedural and security:** waste streams, chemical incompatibilities and the dual-use question.

Our guiding principle is that identifying a hazard is not the same as eliminating it. Several reagents in our workflow carry serious classifications and have no practical substitute for the experiments we needed to run. For those, we state what the hazard is, why the reagent was necessary and what specific measures reduced the residual risk. Where we made a compromise we say so. Section 7 on ethidium bromide is the clearest case and we have not written it to flatter ourselves.

2. Biological risk: the chassis

2.1 *Saccharomyces cerevisiae* CEN.PK2-1C

Our chassis is *S. cerevisiae* CEN.PK2-1C, obtained from EUROSCARF, genotype MATa; *his3Δ1*; *leu2-3,112*; *ura3-52*; *trp1-289*; MAL2-8c; SUC2.

Risk Group. *S. cerevisiae* is a **Risk Group 1** organism, not a pathogen for healthy humans, animals or plants. The US EPA's risk assessment of the species concluded that it presents no hazard to healthy individuals, citing centuries of safe use in baking and fermentation, its ubiquity on food crops and in the environment and its designation as a Class 1 containment agent under the NIH Guidelines. The same assessment records that *S. cerevisiae* accounts for under 1% of yeast infections in surveyed hospitals.

The caveat we do not skip. *S. cerevisiae* can cause opportunistic infection. The documented cases occurred in severely immunocompromised patients, with underlying disease and exposures far beyond laboratory or

dietary contact. The status of a species as "baker's yeast" does not by itself establish that every strain and every engineered derivative is harmless to every person. For this reason, we did not treat our strains as harmless by default: all work followed BSL-1 practice, as described below.

Exposure routes. Splashes, aerosols from vortexing and vigorous pipetting, broken vessels and contaminated surfaces. Cultures are opened in the designated microbiology area, vessels are closed during transport, centrifuge loads are balanced and breakages investigated before the rotor is reopened, hand hygiene is observed and no food or drink is allowed in the work area.

2.2 Containment: an honest account of what the auxotrophy does and does not do

Two points here must be stated precisely, because both are easy to overstate. Our containment argument depends on getting them right:

- **The engineered strain is not quadruply auxotrophic.** Our cassette supplies a functional *HIS3*, which complements *his3Δ1*. The transformed strain synthesises its own histidine, which is precisely the basis of our selection. It retains **three** auxotrophies (*leu2*, *ura3*, *trp1*), not four.
- **Auxotrophy is not environmental containment.** Growth on histidine-free medium demonstrates a **selectable phenotype**, which is what we used it for. It does not demonstrate that the strain cannot survive outside the laboratory. Nutrient-rich environments supply amino acids, cross-feeding from other microorganisms supplies them, decaying organic material supplies them. Auxotrophy constrains growth in a *defined minimal* medium, which is not the environment. In practice, environments that simultaneously supply uracil, tryptophan and leucine in usable amounts are uncommon, which makes establishment unlikely, but that is a statement about probability, not about containment.

What we can legitimately claim. The auxotrophic chassis was a deliberate choice over a prototrophic one and that choice has genuine safety value as **one layer among several**. Stated accurately, our containment rests on:

1. **A Risk Group 1 chassis** with no pathogenicity in healthy individuals. This is the largest single contributor to low biological risk.
2. **Three auxotrophies**, which impose a real metabolic constraint on growth in nutrient-poor environments, while not being an absolute barrier.
3. **No antibiotic resistance marker in the yeast strain.** Selection is by histidine prototrophy and no antibiotic is used anywhere in our yeast workflow.
4. **Chromosomal integration rather than episomal maintenance**, so the construct is not carried on a mobilisable element (see Section 3).
5. **Inactivation by autoclaving** of all biological waste, on the host laboratory's standard cycle of 121 °C under 1–1.5 atm for 15 minutes. This is the measure that actually prevents release and we want to be clear that it, not the genotype, is the primary containment step.

On presenting auxotrophy as a safeguard. Auxotrophic selection is widely and legitimately described as a biosafety feature and we describe it as such. What we do not do is describe it as a kill switch or as an engineered containment device. We did not build one, in part to avoid adding further metabolic burden to the strain. We used a marker system whose containment value is real but partial. Describing a parent-strain genotype as a designed safeguard would misrepresent our engineering. The accurate claim is strong enough on its own: a Risk Group 1 organism, with no antibiotic resistance introduced, integrated rather than episomal, metabolically constrained and autoclaved before disposal.

What we did not test. We did not perform an environmental survival or persistence experiment. Our containment assessment is therefore based on the properties of the chassis and construct and on our waste-inactivation procedure, not on measured survival data.

3. Biological risk: the genetic constructs

3.1 What was built

Three integrative constructs, each carrying a *HIS3* marker. They were designed as integrative plasmids, ordered from IDT as linear synthetic fragments (gBlocks, plus one custom gene in a plasmid vector for pSynOS4(alt1)) and assembled in the yeast by homologous recombination. The designed plasmid sizes were:

- **Plasmid 1 - pCUP1-HIS3**, 8,421 bp - copper-inducible orthogonality control
- **Plasmid 2 - pKAR2-HIS3**, 8,957 bp - ER-homeostasis sensing module
- **Plasmid 3 - pSynOS_4alt1-HIS3**, 8,128 bp - oxidative-stress sensing module

The functional payload is: a stress-responsive or inducible promoter, the TIP peptide, the TetR repressor with its cognate pGAL1-D12 promoter and an sfGFP–mCherry tandem fluorescent timer.

No part encodes a toxin, virulence factor or pathogenicity determinant. Several parts are bacterial or viral in origin and we say so rather than leave it to be assumed otherwise. The TetR repressor is the tetracycline repressor of the bacterial transposon Tn10, routinely propagated in laboratory *E. coli*. The GB1 solubility domain is a 54-residue fragment of streptococcal protein G and the SV40 nuclear localization signal is a seven-residue viral peptide. The tet operator sites in the pGAL1-D12 promoter come from the same Tn10 system, and the short attB linker sequences between parts in the assembled cassettes derive from bacteriophage lambda. Neither of these two non-coding elements encodes a protein. The *bla* marker and pUC/pMB1 origin of the plasmid vector that carried one of the custom genes (Fragment 5, Section 3.3) are bacterial too. The claim that carries weight is functional rather than one of provenance: TetR is a DNA-binding transcriptional repressor, not a virulence determinant. The laboratory *E. coli* lineages from which such parts are drawn are Risk Group 1. Bacterial origin says nothing on its own about whether a part causes harm, since a large share of the standard parts in synthetic biology are bacterial. The functional point, therefore, is the one we make and the only one we make: none of our parts encodes a toxin or virulence factor, none confers a fitness advantage outside selective medium and the system's output is fluorescence, which has no biological activity of its own. We state the limit of this claim explicitly: it is an interpretation of the intended design, not the result of a comprehensive sequence screen against toxin and pathogenicity databases.

3.2 Sequence-level safety check

What the design guarantees. We examined the sequence files of all three constructs *in silico* and confirmed that no coding sequence is present beyond the designed payload and the native yeast genomic sequence contained in the promoter and flank fragments. The overlaps between the fragments are designed to permit only one arrangement, but assembly in vivo can also give other products, so the construct that formed has not been shown to be the intended one. The three plasmid designs carry a *bla* (ampicillin resistance) gene and a bacterial pUC/pMB1 origin, both of which serve plasmid propagation in *E. coli* only. Both were removed from the ordered fragments, and Section 3.3 sets out why neither is expected to enter the yeast strain. No yeast origin of replication (2μ, CEN or ARS) is present, consistent with the integrative design. The sequences of the three assembled cassettes are given in the iGEM Registry entries BBa_260JV63R (pCUP1), BBa_26VMYDOK (pKAR2) and BBa_26K3L3KY (pSynOS4(alt1)).

What we did not verify. Colony PCR on the transformants did not yield a product and we did not sequence the integrated constructs. Our evidence for successful integration is therefore functional: transformants grow on histidine-free medium, which requires a functional *HIS3* gene. That is consistent with integration but does not show that the construct sits at the *HO* locus, since *HIS3* sequence could also act at the native *his3Δ1* locus, and it does not exclude a partial or rearranged event at the insertion site. We record it as a limit of our characterisation rather than resolve it here. Sequencing across the integration locus remains the step that would settle it.

3.3 Antibiotic resistance: disclosed and why it does not reach the yeast

The plasmid designs carry *bla* (β-lactamase, AmpR), and the plasmid vector delivered with one custom gene carries an ampicillin resistance marker as well. Three points bound the risk:

1. *bla* exists to permit plasmid propagation in *E. coli* during commercial synthesis and amplification. It has no function in yeast.
2. Yeast transformation used linear PCR fragments, not circular plasmid. Five or six overlapping fragments per strain, amplified by PCR from the synthetic DNA, were assembled in the yeast by homologous recombination into a cassette that spans promoter–payload–terminator–*HIS3* with its genomic homology arms. The fragments do not include *bla* or the bacterial origin. One fragment (Fragment 5, pSynOS4(alt1) only) was amplified from a custom gene delivered in an ampicillin-resistance plasmid vector. The column clean-up does not remove template plasmid, so trace carry-over into that transformation cannot be excluded. The vector has no yeast origin of replication, so it cannot be maintained in yeast.
3. Selection was by **histidine prototrophy**. No antibiotic was used at any point in the yeast workflow, removing both the selective pressure for resistance and the reagent hazard of handling antibiotics.

3.4 Burden and unintended effects

Expression of the circuit may impose a metabolic burden, alter growth rate or perturb the stress responses the system is designed to report, which would affect the interpretation of fluorescence as well as strain behaviour. Integration can also disrupt the target locus or nearby regulation. The starting OD600 of every culture was recorded, but growth and viability were not measured alongside the dose-response curves. Unmodified parental cells were exposed to the same inducers, and no-inducer controls were run in the same experiments.

3.5 Dual-use and security

Assessment: low dual-use potential, for structural reasons rather than incidental ones. ICHNOS is a **recording** device, not an effector. It senses a cellular state and reports it as a fluorescence ratio. It secretes no product, acts on nothing outside the cell that carries it and redirects no metabolism toward a product. Its components (TetR, the TIP peptide fusion and two fluorescent proteins) act only inside the cell, as part of the circuit, and the circuit confers no capability on the chassis beyond making an internal state externally visible.

The modularity that is our central engineering claim deserves specific scrutiny, since a plug-and-play architecture is by definition repurposable. The boundary is this: swapping the **sensing** promoter changes what the system detects and every plausible substitution yields another sensor. Producing harm would require replacing the **reporting** module with an effector payload, not a modification of ICHNOS but a replacement of the part that makes it ICHNOS. Our published design gives no meaningful uplift toward that, because the recording architecture is not the difficult component of such a project.

Change of context requires a new assessment. Any change of host organism, promoter, cargo, scale or application invalidates this assessment and requires a fresh one.

Physical and information security. Strains and plasmid stocks are held in the host laboratory's freezers and are labelled with a strain ID, the construct they carry and the date. Engineered yeast is not used in public demonstrations and is not released outside the laboratory. All part sequences will be published consistent with iGEM's openness principles. We identified nothing warranting restriction.

4. Consolidated hazard summary

Reagents carrying the most serious classifications, in descending order of concern:

1. **Ethidium bromide** - H330 (fatal if inhaled, powder), H341 (suspected genetic defects), H302. Section 7.
2. **Glacial acetic acid** - H314, H226. Concentrated stock only.
3. **Hydrogen peroxide, 30% stock** - H318 (serious eye damage), H302 and H332 (harmful if swallowed or if inhaled), signal word Danger, per the SDS for the bottle we use. Fume hood and splash goggles for the concentrate. H271 and H314 belong to concentrated peroxide rather than to a 30% solution.
4. **Copper(II) sulfate** - **H302** (harmful if swallowed), **H318** (serious eye damage), **H410** (very toxic to aquatic life).
5. **Dithiothreitol (DTT)** - H319 (serious eye irritation), H302, H315, signal word Warning, per the SDS for our product (Biotium 91050). Classification varies between suppliers.
6. **Concentrated NaOH stock** - H314, H318, H290. Working dilution unclassified.
7. **Guanidinium kit buffers (NTI, RA1, RAW2)** - H302, H412, H226. Absolute bleach prohibition.
8. **Tris base, solid** - H315, H319, H335.
9. **Lithium acetate** - H319.
10. Ethanol, 99.8% - H225 (highly flammable liquid and vapour), H319. Held neat only to prepare the 70% working disinfectant. Incompatible with the 30% peroxide.

5. Assessment method for chemicals

Classifications follow the GHS as implemented in the EU by **Regulation (EC) No 1272/2008 (CLP)**, cited from supplier Safety Data Sheets. Two conventions matter for reading what follows:

Physical form matters. A classification derived from a powder SDS does not transfer automatically to a supplied solution of the same substance. This is most consequential for ethidium bromide, whose H330 (fatal if inhaled) classification attaches to the powder. We use only pre-made solution, which is why that route is controlled at source rather than by procedure.

Concentration matters. Where a reagent is used dilute, we state whether it falls below the CLP concentration limit at which the classification applies. Attaching a corrosive classification to a millimolar solution would be inaccurate, and inaccurate hazard labelling is itself a safety problem because it trains people to disregard warnings.

A limit of this review. A classification belongs to the product in the bottle rather than to the substance in the abstract, so a hazard statement is only as good as the sheet it came from. For two reagents this is settled against the containers actually on our bench: the hydrogen peroxide (Carl Roth, 30% w/w) and the DTT (Biotium 91050). For the remaining reagents the classifications are drawn from representative supplier sheets and where our actual supplier differs the specific product SDS governs, since formulations do vary. The supplier's sheet for the product in hand, not this document, is the operative record.

6. Chemical risk by workflow stage

6.1 Media preparation and culture

This stage carries the highest cumulative handling time and the lowest intrinsic hazard. Risks here are chronic rather than acute.

1. YPD components (soy peptone, yeast extract, dextrose), YNB, CSM-His dropout, histidine, agar, galactose. None carries a harmonised GHS classification. The real hazard is **inhalation of fine organic powder** during weighing. Peptone dust is a recognised occupational sensitiser in industrial settings.

Powders were weighed on a balance that sits inside a fume hood, with a dust mask, and were not poured from height, since that is what sends dust into the air.

YNB formulations contain trace micronutrients including boric acid at microgram-per-litre concentrations - orders of magnitude below any level at which boric acid's reproductive toxicity classification applies.

2. Molten agar and agarose are scald hazards. Superheated agar from an autoclave or microwave boils over violently.

It was cooled to approximately 50 °C before pouring, hot bottles were not handled bare-handed, bottles were never sealed while hot and microwaving was done in short bursts with swirling.

6.2 Transformation and genome integration

1. Lithium acetate dihydrate. CAS 6108-17-4. **Eye Irritation Category 2**, signal word Warning: **H319, causes serious eye irritation**. The exposure route is a splash during stock preparation and during the transformation mix.

Safety glasses were mandatory, stock was prepared once in a fume hood and labelled, spills were wiped immediately rather than left to dry into a powder that can later become airborne.

2. PEG 3350, 50% w/v. Not classified. The hazard is physical: a 50% PEG solution is viscous and extremely slippery, making glassware hard to grip, and a floor spill is a genuine slip risk. Spills were cleaned up immediately. We record this because a purely toxicological assessment misses it entirely.

3. Salmon sperm carrier DNA. Not classified. Supplied sterile and denatured. Purified nucleic acid preparations of animal origin present negligible adventitious agent risk. Standard gloves were worn.

4. NucleoSpin Gel and PCR Clean-up kit (MACHEREY-NAGEL). One of the two most serious chemical incompatibilities in our workflow:

- *Buffer NTI* contains **guanidinium thiocyanate 30-45%**: signal word **Warning**, **H302 - Harmful if swallowed**, **H412 - Harmful to aquatic life with long lasting effects** (P264, P273, P301+P312, P330). Guanidinium salts are chaotropic protein denaturants. Beyond the formal classification, skin contact should be avoided because that is the mechanism of action.
- *Buffer NT3* is a concentrate requiring 96–100% ethanol before use. The working solution is therefore flammable.
- *Buffer NE* (5 mM Tris/HCl pH 8.5) is not classified.

The critical incompatibility. The manufacturer states that waste containing chaotropic salts **must not** receive bleach or acid, because guanidinium salts react with sodium hypochlorite to form highly reactive compounds - in practice, liberation of cyanide- and chlorine-containing species. This matters in any laboratory where bleach is the default disinfectant, which is most of them.

Kit waste was collected in a separate, clearly labelled container that never receives bleach and this specific incompatibility is a named item in our safety induction rather than a line in a manual.

6.3 Colony PCR

1. Taq polymerase, PCR buffer, dNTPs, primers. Not classified at working concentrations. **$MgCl_2$** solid is classified by some suppliers as a mild skin and eye irritant (H315, H319). PCR-concentration solutions fall below thresholds.

The dominant hazards here are not chemical. The thermocycler lid operates above 100 °C and is a silent, unmarked burn hazard when loading. Multi-plate setup involves sustained repetitive pipetting. Breaks are scheduled into long sessions.

2. Sodium hydroxide, 20 mM. CAS 1310-73-2. Used to lyse colonies before PCR, heated at 95 °C for 10 minutes. Concentrated NaOH is **Skin Corr. 1A**, **Eye Dam. 1**, **Met. Corr. 1**: H290, H314, H318, signal word Danger. The CLP concentration limits are decisive: Skin Corr. 1A at C ≥ 5%, Skin Corr. 1B at 2 to 5%, Skin Irrit. 2 at 0.5 to 2%. **Our 20 mM working solution is approximately 0.08% w/v - below the 0.5% threshold at which any classification applies** and is therefore not classified. The concentrated stock from which it is prepared *is* fully corrosive and is handled in a fume hood with chemical-resistant gloves, goggles and lab coat, while the working dilution is conducted with routine gloves and eye protection.

What the assay reads. Our colony PCR is scored as the presence or absence of a single expected product: a band was expected in correct integrants, while the untransformed parent gives no band at all. It does **not** resolve a size difference between integrant and wild type. The distinction has a practical consequence. Because a negative result is an absent band rather than a shifted one, a faint product is the failure mode that matters, which is why gel sensitivity and not convenience is what the staining decision in Section 7 turns on. In the event, the reaction yielded no product, which Section 3.2 records.

6.4 Gel electrophoresis

1. Agarose. Not classified. Powder and scald precautions were followed, as mentioned above.

2. Tris base. CAS 77-86-1. **H315, H319, H335** (Skin Irrit. 2, Eye Irrit. 2A, STOT-SE 3), signal word Warning. Applies to the solid during weighing. The 1× TBE working buffer is far below thresholds.

It was weighed in a fume hood with gloves and eye protection, addressing H335 at source.

3. Acetic acid. CAS 64-19-7. Glacial: **Flam. Liq. 3** and **Skin Corr. 1A** - **H226**, **H314, causes severe skin burns and eye damage**. A serious classification applying to the concentrated stock, not the buffer.

It was dispensed only in a fume hood with gloves, safety glasses and lab coat. It is added to water, never the reverse. It is stored in a dedicated acid cabinet.

4. EDTA. Disodium EDTA classified as an eye irritant (H319), with some suppliers additionally classifying the dust H332. The classification applies to solid handling only.

5. Bromophenol blue loading buffer, 2-Log DNA ladder. Not classified.

6. Electrical hazard. Gel tanks run at voltages and currents capable of serious injury, in an apparatus full of conductive buffer.

The lid was interlocked and in place before power on and hands were never in the tank while connected. Power was off and leads were disconnected before the gel was removed.

7. UV transilluminator. UV-B/UV-C exposure causes erythema and carries long-term skin cancer risk.

The protective lid was closed wherever possible. Where direct access was needed, a UV face shield or UV-blocking glasses were worn, with a buttoned lab coat and gloves.

8. Ethidium bromide. Section 7.

6.5 Dose-response assays and stress induction

The inducers are the reagents we could not design around: ICHNOS is a stress sensor, so applying stressors is the experiment. The argument is entirely about dose, volume and containment.

The series used. H_2O_2 at 20, 75, 300 and 600 µM. DTT at 100, 250, 500, 1,000 and 2,000 µM. $CuSO_4$ at 10, 25, 50 and 100 µM. Each series was run against an untreated control. These are the figures the dilution factors below are calculated from.

Hydrogen peroxide. CAS 7722-84-0. **We used 30% w/w stock solution**, diluting it to working concentration ourselves. The SDS for our bottle classifies the solution as supplied: **Acute Tox. 4 - H302**, Harmful if swallowed, **Acute Tox. 4 - H332**, Harmful if inhaled, with **Eye Dam. 1 - H318**, Causes serious eye damage, signal word **Danger**.

What the classification does and does not cover. Oxidising Liquid 1 (H271) and Skin Corr. 1A (H314) are often quoted for hydrogen peroxide. They are the statements for the concentrated substance at the high end of the scale and the sheet for a 30% w/w solution carries neither, so we do not attach them to our bottle. Section 5 gives the reason this matters: a classification inflated beyond its source is itself a safety problem, because it trains people to disregard warnings. What governs handling at 30% is the eye. H318 means irreversible damage and a splash is the credible accident in our workflow. Prolonged skin contact still irritates and can blanch the skin. The solution remains an oxidiser in the chemical sense that drives the incompatibilities in Section 8. One point is worth stating plainly for a student team: an accidental contact that produced no visible injury is not evidence that a reagent is mild. It is one observation, at one duration, on one person's skin. The safety data sheet sets the controls and an uneventful exposure does not relax them.

Concentration and dilution factor. The bottle is labelled 30% w/w, confirmed against the supplier SDS. A 30% w/w solution at density 1.11 g/mL is 333 g/L, which at M = 34.01 g/mol is **9.79 M** - 9.79×10^6 µM. Against the series above, the dilutions are therefore **roughly 16,000-fold to reach 600 µM and 490,000-fold to reach 20 µM**. A dilution of this magnitude is not practical in a single step and was performed as a serial dilution, which matters for data quality as well as safety, since pipetting error compounds across steps and propagates into the dose-response curve.

Necessity and control. H_2O_2 is the standard, literature-validated inducer of the Yap1-dependent oxidative stress response that pSynOS4(alt1) reports and the series of 20 to 600 µM was derived from that literature as our calibration baseline. A literature-derived experimental range is not an occupational exposure limit and carries no implication of safety for the handler.

Because the concentrated stock is handled, the controls are procedural rather than substitutional. The stock bottle is opened and dispensed **only in a fume hood**, with **chemical-resistant gloves and splash goggles** and a buttoned lab coat. Safety glasses alone are not adequate against an H318 liquid. Serial dilutions are prepared in the fume hood and the concentrated stock is returned to storage before the dilute working solutions go to the bench. The stock is stored upright in its original container, away from organics, ethanol in particular, metals - the SDS names lead, iron, copper, bronze, brass, silver, zinc and chromium, with our own copper sulfate on that list - reducing agents including our DTT and heat, because 30% H_2O_2 decomposes to oxygen and water and will pressurise a sealed vessel. Dispensed material is never returned to the stock bottle. Spills of concentrated peroxide are flooded with water rather than wiped, since absorbent paper saturated with a concentrated oxidiser is itself a fire hazard and ethanol is never used to wipe one.

2. Dithiothreitol (DTT). CAS 3483-12-3, M = 154.2 g/mol. Our product is **Biotium 91050** and its SDS classifies it signal word **Warning**: **H302** - Harmful if swallowed, **H315** - Causes skin irritation, **H319** - Causes serious eye irritation.

Why the supplier matters here. DTT is classified differently by different suppliers. H314, H318 and H412 all appear on DTT sheets somewhere. We therefore work from the sheet for the product we actually hold rather than from a generic one and ours is Eye Irrit. 2 rather than Eye Dam. 1. The milder classification changes nothing operationally: eye protection is mandatory at every concentration, because H319 still describes serious injury and because powder reaching an eye is the exposure being guarded against either way.

Necessity and control. DTT is the standard inducer of the Unfolded Protein Response via Ire1/Hac1 signaling, which pKAR2 reports. The working series is 100 µM to 2 mM, taken from the literature.

Powder was weighed in a fume hood with a dust mask, because H319 applies to dust reaching the eye as much as to solution. Safety glasses are mandatory at every concentration, without exception. Stocks were aliquoted and frozen, with the product stored at -35 to -10 °C with desiccant as its label requires, which reduced powder-handling events across the project to one. Reducing-agent waste was kept apart from oxidisers, including the peroxide. Stock and working dilutions were labelled separately.

3. Copper(II) sulfate pentahydrate. CAS 7758-99-8. **Acute Tox. 4** (oral), **Eye Dam. 1**, **Aquatic Chronic 1**, signal word **Danger**: **H302**, harmful if swallowed, **H318**, causes serious eye damage, **H410**, very toxic to aquatic life with long lasting effects. Some supplier sheets also list Skin Irrit. 2 (H315).

Two features deserve emphasis. **H318** means serious eye damage, so goggles and not only safety glasses are needed for the solid and the concentrated solution. Copper salts are also harmful by ingestion (**H302**). **H410** means copper must never enter the drain. Copper is acutely toxic to aquatic organisms at very low concentrations and does not degrade.

Necessity and control. $CuSO_4$ induces pCUP1 in our Strain 3 orthogonality control, the experiment demonstrating that the reporting module functions independently of stress signalling, which is our central modularity claim. Our design documentation identifies copper toxicity to yeast above 200 µM and sets our working series deliberately at 10–100 µM, below that threshold.

Everything was weighed in a fume hood with gloves, safety glasses and dust mask. All copper-containing waste, including spent culture medium from induction experiments, was collected as heavy-metal waste and never poured down the sink. Because that spent medium is also a genetically modified culture it is autoclaved first and consigned as chemical waste second, in the order set out in Section 8. Stocks were labelled with the H410 hazard so the disposal requirement travels with the bottle.

6.6 RNA extraction and RT-qPCR

1. NucleoSpin RNA kit (MACHEREY-NAGEL). The second source of the guanidinium–bleach incompatibility.

- *Buffer RA1* contains **guanidinium thiocyanate 45–60%**, a higher proportion than the NTI of the PCR clean-up kit. Signal word **Warning**: **H302**, **H412** (P264, P273, P301+P312, P330).
- *Buffer RAW2* contains **guanidinium hydrochloride 24–36% and ethanol 20–35%**: **H226**, **H302** (P210, P264, P301+P312, P330).
- *Buffer MDB* contains 5–20% ethanol: **H226**.
- *Buffer RA3* is a concentrate requiring ethanol addition, so the prepared solution is flammable.

The same absolute bleach prohibition applies, for the same reason. All RNA kit waste goes to the dedicated chaotrope container, kept closed and away from ignition sources because several buffers are ethanolic.

2. Reducing agent in lysis buffer. Where the protocol requires a reducing agent in Buffer RA1, we used DTT rather than β-mercaptoethanol. This was a deliberate substitution on safety grounds, since β-mercaptoethanol is acutely toxic with a severe odour and strict fume-hood requirement, while DTT achieves the same reduction at a substantially lower hazard. The H319 handling requirement above applies.

3. RT-qPCR reagents. Reverse transcriptase and master mix components are not classified. The kit used, the Power SYBR Green RNA-to-CT 1-Step Kit, contains the intercalating dye SYBR Green, which was handled with gloves and no skin contact, since intercalating agents as a class have potential genotoxicity. Reaction set-up is carried out in a fume hood throughout.

6.7 Consumables, plasticware and equipment

No chemical classification, but this is where most laboratory injuries actually happen.

1. Petri dishes, tips, Eppendorf and Falcon tubes, 96-well plates, spreaders, loops. The risks are **contaminated sharps** (cracked plastic and broken glass cause cuts that breach the primary barrier against biological exposure), **aerosol generation** during pipetting, spreading and vortexing, together with autoclave burns.

Plastic was preferred over glass throughout, with the exception of microscope slides and coverslips (Section 6.8). Broken plastic was disposed of in a sharps container, never in a bin bag. Plates were spread gently and were not agitated while open, so that aerosols were not generated at the point where cultures are most exposed. Tubes were allowed to settle after centrifugation before they were opened. Centrifuges were never opened while the rotor was still spinning. All biological waste was autoclaved before disposal on the cycle defined in Section 8.

2. Micropipettes. Sustained pipetting during dose-response and 96-well work carries a repetitive strain risk. Work was shared between team members and long sessions were broken up.

3. Gloves. Nitrile gloves were worn throughout. They were changed immediately after contact with any classified reagent rather than worn until visibly soiled. They were removed before touching door handles, keyboards, phones or freezer handles (the principal route by which contamination leaves the bench).

6.8 Fluorescence microscopy and the imaging room

The read-out of ICHNOS is fluorescence, so microscopy is the one place where the engineered strain, a UV source and glass meet on the same bench. Three hazards belong here and none of them is a chemical classification.

1. Glass slides and coverslips. Plastic was preferred everywhere else in the workflow, but imaging requires glass. Coverslips are thin, brittle and sharp. A cut taken while handling a slide that carries an engineered culture breaches the primary barrier against biological exposure at the one moment when there is something to be exposed to. Slides and coverslips are handled by the edges and never pressed down with a bare finger, breakages go directly to the sharps container and never into a bin bag or onto the bench. A cut sustained during imaging is treated as a biological exposure incident rather than as a cut: wash, report to the safety officer, document.

2. Cleaning with 70% ethanol. Slides and coverslips are cleaned with 70% ethanol before and after each use. The 70% working solution is prepared in-house from 99.8% ethanol. It is the neat ethanol that carries the hazard: **Flam. Liq. 2 - H225**, Highly flammable liquid and vapour, with Eye Irrit. 2 **- H319**, signal word Danger. The neat stock is kept closed and away from ignition sources and away from the 30% peroxide (Section 8), only the working dilution is taken to the microscope and ethanol-soaked tissues are not left on the bench or near a warm lamp housing. The quantity is small and the hazard is correspondingly modest, but it is

the one flammable liquid in routine daily use and it sits in the same room as a hot arc lamp, which is why it is named here rather than treated as background.

3. The fluorescence lamp. The excitation source is a hazard in its own right, independently of the microscope and of anything on the slide. An arc lamp emits in the UV as well as the visible, at an intensity that can injure the retina and burn skin at short range. The housing runs hot. A lamp under pressure can fail violently if it is handled hot or run beyond its rated hours. A mercury-containing lamp is hazardous waste rather than general waste. The lamp is switched on with the light path closed, no one looks into the beam or at the lamp directly or through an open port, the housing is left to cool fully before anyone handles it and a spent lamp goes out through the host institution's hazardous-waste route rather than into general waste. The UV transilluminator in Section 6.4 is governed by the same principle: the control is to keep the source enclosed, not to rely on personal protection against an exposed one.

4. Moving engineered cultures to the imaging room. Microscopy happens outside the designated microbiology area, so cultures carrying the construct - and, in the dose-response experiments, an inducer as well - are carried there deliberately and that transfer is part of the workflow, not an exception to it. Vessels are closed for transport and carried in a secondary container, so that a broken tube is caught rather than spread. Disinfectant is kept in the imaging room as well as at the bench. The stage and the surrounding surfaces are disinfected after each session. Every slide, coverslip, tip and tissue that has touched an engineered culture returns to the biological waste stream and is autoclaved on the cycle in Section 8. Where the culture also contains copper, the liquid follows the heavy-metal route after autoclaving, for the reason given there. A spill in this room is handled by the procedure under Emergency response in Section 8.

7. Ethidium bromide: a necessary compromise, stated honestly

This is the most seriously classified substance we handle. It is also the one where the distance between what was available and what we used is widest, so we set the decision out in full.

7.1 The hazard

Ethidium bromide, CAS 1239-45-8, signal word **Danger**:

- **Acute Tox. Inhalation 2 - H330, fatal if inhaled.** LC_{50} (rat, 1 h) 0.0118–0.134 mg/L. **This classification derives from the powder**, which is why our first control is never to handle it in solid form.
- **Germ Cell Mutagenicity 2 - H341, Suspected of causing genetic defects.** EtBr is a DNA intercalator, which is why it works as a stain and why it is mutagenic. It is strongly mutagenic in the Ames test with S9 metabolic activation.
- **Acute Tox. Oral 4 - H302, Harmful if swallowed.** LD_{50} (rat, oral) 1,503–2,177 mg/kg.

Two points of precision, because an inflated hazard assessment is as unhelpful as a minimised one. **Ethidium bromide is not classified as a carcinogen** by IARC, ACGIH, NTP or OSHA, though a suspected mutagen is a serious but different claim. All the classifications above are drawn from powder SDS documents. They do not transfer automatically to the 10 mg/mL solution we actually use. The stain is added to the molten agarose and TBE before casting, at 1 µL of the 10 mg/mL solution per 60 mL of gel. That is 10 µg of ethidium bromide in 60 mL, a final concentration of about 0.17 µg/mL.

7.2 Why we did not substitute: the honest account

Safer alternatives **exist and are well documented**. Biotium has published Ames data for GelRed and GelGreen. GelGreen tested non-mutagenic across the whole range reported. GelRed tested non-mutagenic in every condition but one, a weak response in TA98 with S9 at the top dose of 50 µg per plate, which is far above

the concentration at which it is used. Ethidium bromide in the same assay is strongly mutagenic with S9. We will not claim no alternative was available, because that claim is false and any reviewer familiar with the field would know it. We also note that "safer" is not "hazard free", since an alternative stain would carry its own SDS and its own controls.

Our reasons were practical constraints, presented as such:

The stain was already established in our host laboratory. We changed laboratory partway through the project, for reasons set out elsewhere in our documentation, and began experimental work in the new host. That laboratory stocks ethidium bromide, with an established waste-handling and decontamination protocol, dedicated equipment, a fume hood kept for it and staff trained in its use. A different stain would mean introducing a reagent the host does not stock into a workflow whose safety infrastructure is built around the one it does.

The detection system was matched to the stain. The available transilluminator and filter set are configured for EtBr. GelRed is broadly compatible in this respect, but confirming equivalent sensitivity for our specific application would have required re-validation on our own samples. Our colony PCR scores integration as the presence or absence of a single expected product, as Section 6.3 sets out, so the property that would have to be re-established for a new stain is sensitivity at the faint end of the range, which is the harder thing to demonstrate rather than the easier.

Time and budget. Re-validating a new stain would have had to fit inside the integration verification schedule, which a student team working to a fixed competition deadline could not absorb. Nothing in our results depends on which stain was used, so a team reproducing this work should take the non-mutagenic alternative rather than follow us here.

What we are not claiming. We are not claiming this was the safest possible choice. It was the choice available under real constraints, made with full knowledge of the hazard rather than by default. We record it so the decision is visible and reviewable. Scientific usefulness alone does not make a risk acceptable. What makes the residual risk acceptable is the control set below and we would not have proceeded without it.

7.3 Controls applied

1. Form. Used **only as pre-made 10 mg/mL solution, never powder.** The powder never came into the laboratory, which removes the H330 inhalation route, the most severe classification, at source.

2. A hood of its own. All EtBr work (adding the stain, gel handling, imaging) is done in a **fume hood used for ethidium bromide and nothing else**. The pipettes and consumables used for it are kept for that purpose only and do not go back into general use. The balance used for these preparations sits inside the same hood.

3. Exposure minimisation by time. Dose absorbed is a function of concentration *and* duration. Gels are handled for the minimum time necessary. Imaging is planned before the gel leaves the tank, so no one stands over an exposed stained gel deciding what to do next. The stain is cast into the gel rather than applied in a post-electrophoresis bath. The handling step that carries the exposure is the addition of stock to the molten agarose, done once per gel inside the dedicated hood. There is no open staining tray and no staining solution held between runs, so the contaminated materials are the gel itself and the tip used to add the stock.

4. PPE. Double nitrile gloves were worn for all direct handling and were changed immediately after each contact and before any shared surface was touched. A buttoned lab coat, safety glasses, full-length clothing and closed shoes were worn throughout. **Glove discipline is one of the most critical controls and the most commonly failed one**. The characteristic contamination pattern in teaching laboratories is fluorescent fingerprints on door handles, freezer latches and pipette barrels.

5. Waste. Because the stain is cast into the gel, the waste is solid: the gels themselves, with the gloves and tips from the same work. These go into a dedicated container marked for ethidium bromide waste only, which takes nothing else, and leave through the host institution's licensed waste route. Ethidium bromide is never put down the drain at any dilution, because dilution removes neither the mutagenic hazard nor the environmental persistence. Autoclaving and routine bleach treatment do not eliminate the chemical hazard and were not used as decontamination.

6. Training and access. No team member handles EtBr before a specific briefing covering the three hazard statements, the rules for the dedicated hood, glove discipline and the waste container. The briefing was given by the host laboratory's responsible scientist and by the technical staff who handle this reagent routinely, rather than by the team to itself.

7. Spill response. Contain the spill, absorb it, decontaminate the surface and put everything used into the ethidium bromide waste container. The spill is reported to the safety officer and recorded.

7.4 Documented recommendation for future teams

Future teams should budget for GelRed or an equivalent non-mutagenic stain from the outset and validate it during protocol development, before the experimental timeline creates pressure to use whatever is already on the shelf. The published Ames data support the substitution and the disposal burden is substantially lower. We are also raising this with our host laboratory as a suggestion for their general practice.

8. Waste, incompatibilities and emergency response

We operate **five separate waste streams**. The separation is deliberate: the most serious chemical accidents in student laboratories come from mixing incompatible wastes in one container.

1. **Biological.** All cultures, plates, contaminated plasticware, DNA-containing material, plus all slides and coverslips from Section 6.8. **Autoclaved before disposal** at 121 °C under 1–1.5 atm for 15 minutes, the primary containment measure for the organism.
2. **Chaotropic salt (guanidinium).** All buffers and flow-through from both MACHEREY-NAGEL kits. **No bleach, no acid.** Labelled with the specific incompatibility, not merely “chemical waste.”
3. **Ethidium bromide.** Solid and liquid, per Section 7.3. Never mixed with any other stream.
4. **Heavy metal.** All copper sulfate solutions and spent copper-induction culture medium, on the basis of H410. Never to drain. Order matters here, because this stream is both biological and chemical: the spent medium is a genetically modified culture, so it is **autoclaved first**, on the cycle above, then **consigned as heavy-metal waste**. The autoclave inactivates the organism and does nothing whatever to the copper. The chemical route handles the copper and would never have addressed the organism. Taking them in that order is what satisfies both requirements and it is the sequence written into the protocol rather than left to be worked out at the bin.
5. **General chemical.** Remainder, segregated by compatibility, via the host institution’s licensed contractor.

Mixed streams. Biological inactivation must be compatible with chemical contaminants. Culture medium containing copper and kit waste containing chaotropic salts cannot simply be autoclaved and discarded. These require the routes above. Where a waste stream is both biological and chemical, the chemical route governs for disposal, while any necessary inactivation is done first: copper-containing spent medium is autoclaved and then consigned as heavy-metal waste, whereas guanidinium kit waste is never autoclaved at all, since the chaotrope has already lysed and inactivated the cells chemically and heating a thiocyanate buffer is the last thing anyone should do. In any case of doubt the host laboratory’s advice is sought.

8.1 Incompatibilities we train on by name

- **Guanidinium + bleach** → highly reactive compounds including cyanide and chlorine species. The single most dangerous plausible error in our workflow, precisely because bleach is the default disinfectant.
- **Guanidinium + acid** → the same absolute prohibition as bleach, for a related reason: acidifying a thiocyanate-containing buffer liberates hydrogen cyanide. The chaotrope container therefore receives neither bleach nor acid. The acid in the room is the glacial acetic acid of Section 6.4, which is why the two are named together in the induction.
- 30% hydrogen peroxide + organics, metals (the SDS names lead, iron, copper, bronze, brass, silver, zinc and chromium, our own copper sulfate among them), reducing agents (including DTT) → oxidation, heat, potential ignition. Stored separately and vented.
- **30% hydrogen peroxide + ethanol** → an oxidiser and a fuel. We keep both on the bench: peroxide as an inducer and 99.8% ethanol for the 70% disinfectant. Dilute mixtures are stable and the pairing is not exotic, which is precisely the risk, since it invites being treated as harmless. Concentrated peroxide with ethanol is a recognised fire and explosion hazard. Organic peroxides can form. They are stored apart and never mixed. Ethanol is not used to clean up a peroxide spill.
- **Concentrated acetic acid + bases, oxidisers** → violent reaction. Dedicated acid cabinet.
- **Copper sulfate + drain** → not a reaction, but the incompatibility that matters most environmentally.

8.2 Emergency response

Eye exposure: immediate 15-minute eyewash irrigation and medical evaluation. This is the reason eye protection is non-negotiable for DTT (H319), the 30% hydrogen peroxide (H318) and the concentrated reagents.

Skin contact: immediate washing, with the safety shower for corrosives.

Ingestion: do not induce vomiting. Seek immediate medical attention with the SDS to hand. All incidents including near-misses and minor spills are reported to the safety officer and documented, because an unrecorded near-miss is a hazard the next person meets unwarned.

Spill of engineered culture. The area is cleared and access restricted. The spill is covered with absorbent material worked from the outside in, so that nothing is spread outwards. Disinfectant is applied and **left for its full contact time** rather than wiped away immediately, because the contact time is what achieves inactivation and wiping early is the common way to produce a surface that looks clean and is not. The absorbent and all contaminated material are then collected into an autoclave bag and autoclaved on the 121 °C cycle before disposal. Gloves and lab coat are changed afterwards, hands washed, the surface disinfected a second time and the incident recorded. Where the spilled culture also contains an inducer, copper above all, the liquid is handled as both a biological and a chemical waste by the rule set out above.

Numbers to have at hand. These are the numbers to reach for in an emergency, because a number that has to be looked up is a number nobody has.

1. **European emergency number - 112.** Fire, ambulance and police, anywhere in the EU, in Greek or English.
2. **Ambulance, EKAB - 166.**
3. **Fire service - 199.**
4. **Police - 100.**

5. **Poison Information Centre (Κέντρο Δηλητηριάσεων), Athens - 210 779 3777**, 24 hours. Called for any ingestion, inhalation or significant exposure, with the SDS in hand so that the product and its concentration can be read out rather than described.

What the caller says. The substance name as it appears on the bottle, the concentration, the route and the time of exposure and the Section 4 wording of the SDS if it is to hand. For an exposure involving the engineered organism the facts a clinician will ask for are that it is *Saccharomyces cerevisiae*, Risk Group 1, carrying a fluorescent reporter construct that encodes no toxin. Nobody should have to reconstruct that under pressure.

9. PPE and training

Baseline for all bench work: buttoned lab coat, nitrile gloves, safety glasses, closed shoes, long trousers or equivalent, long hair tied back. No food, drink, cosmetics or contact lens handling in the laboratory.

Task	Additional PPE
Weighing any powder	Dust mask, fume hood
Glacial acetic acid, concentrated NaOH, 30% H_2O_2	Fume hood, chemical-resistant gloves, splash goggles
DTT, all steps	Mandatory safety glasses, dust mask for powder
Copper sulfate	Dust mask, goggles
Ethidium bromide	Double gloves, dedicated fume hood (Section 7.3)
UV transilluminator	UV face shield or UV-blocking glasses, buttoned lab coat, gloves
Molten agar/agarose	Cooled to approximately 50 °C before pouring, hand protection for hot bottles
Weighing or handling 99.8% ethanol	Gloves, no ignition sources, kept away from the peroxide
Microscope slides and coverslips	Gloves, handling by the edges, sharps container for breakages
Fluorescence microscopy	Light path closed before illumination, no direct viewing of the lamp or an open port, lamp cooled before handling
Carrying engineered cultures outside the microbiology area	Closed vessels in a secondary container, disinfectant at the destination
Unloading the autoclave	Hand protection, face protection, load left to depressurise before opening

Training. Before independent bench work, every team member completes the host laboratory's general induction plus our project-specific briefing: the Risk Group 1 status and containment logic of the chassis, the guanidinium–bleach incompatibility by name, the ethidium bromide hood rules, the five waste streams, the autoclave cycle, the fluorescence-lamp and imaging-room rules of Section 6.8, spill and emergency procedures and the location of the eyewash station, safety shower, extinguishers and first aid kit, together with the emergency numbers above.

Procedures. Every step described in this document was carried out under the written protocols in force in the host laboratory and under the established protocols of the techniques themselves. Where this assessment

names a control - a fume hood, a contact time, a cycle temperature, a waste route - it is describing what the protocol requires and what was done, not an aspiration. Departing from a protocol is not a matter of individual discretion: it is raised with the host laboratory's responsible scientist and with our safety officer before the work is done and it is recorded.

10. Residual risk and conclusions

Biological: low. A Risk Group 1 chassis with no pathogenicity in healthy individuals and an extensive history of safe use. The construct encodes no toxin, virulence factor or effector, though several of its parts are bacterial in origin and we say so in Section 3.1. It carries no antibiotic resistance gene in the assembled construct, because transformation used linear PCR fragments, and selection was by histidine prototrophy. Trace carry-over of one template plasmid cannot be excluded (Section 3.3), but that plasmid has no yeast origin and cannot be maintained in yeast. Three auxotrophies remain after HIS3 complementation and impose a real but partial metabolic constraint. The measure that actually prevents release is autoclaving of all biological waste at 121 °C under 1–1.5 atm for 15 minutes. The residual biological consideration is the opportunistic infection risk this species carries for severely immunocompromised individuals, which is why the strain is handled under BSL-1 practice rather than treated as harmless.

We state plainly what is not established: we did not perform environmental survival testing and auxotrophy is a selectable phenotype rather than a containment device. Our containment claim rests on chassis properties, construct design and the defined autoclave cycle described above, not on a genetic safeguard we did not build.

Chemical: low to moderate, concentrated in a small number of reagents. Most of the workflow is unclassified or mildly classified. The meaningful risks are ethidium bromide, the guanidinium kit buffers and the concentrated stocks of acetic acid, NaOH and copper sulfate. Each is controlled by a specific named measure rather than by general caution.

Security: low. ICHNOS is a recording system whose output is fluorescence. It confers no harmful capability and its modularity extends to what it can sense rather than to what it can do. Any change of host, cargo, scale or application requires a new assessment.

What we would change. One item is recorded as improvement rather than solved problem: substituting ethidium bromide with a non-mutagenic stain, which we recommend to future teams as a planning decision made early rather than a mid-project one.

A safety analysis is only useful if it is as willing to mark its own limits as to list its controls. Four things here are stated as unsettled rather than settled. We have not tested environmental survival. We did not confirm the integration by sequencing. We have not run a comprehensive sequence screen against toxin and pathogenicity databases. For most reagents the classifications come from representative supplier sheets rather than from the container on our own shelf. One practice behind this assessment is worth naming for the teams who read it after us: for the two reagents where we could do it, the hydrogen peroxide and the DTT, the hazard statements were checked against the supplier sheet for the product in the laboratory and not against generic sources alone. The product in hand is where the discrepancies turn up.

Sources

- Safety data sheet, ethidium bromide (Sigma-Aldrich E7637). https://westliberty.edu/health-and-safety/files/2017/07/Ethidium-bromide.pdf
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