ICHNOS in Biomanufacturing: Safety Protocol

ICHNOS - Patras Medicine iGEM 2026

Part of our Safety and Security work

Prospective safety framework

October 2026

Scope: The conditions under which ICHNOS could be evaluated and eventually used as a stress-recording tool in biological manufacturing. This covers industrial enzymes and proteins, metabolites and fuels, and biopharmaceuticals as a separate and more demanding case.

Status: This is a forward-looking framework rather than an approved method. ICHNOS has not been tested in any production process and nothing here constitutes authorisation. Its purpose is to set out, before anyone attempts such work, what would have to be true for it to be safe.

Revision note: Revised by the team after a further consultation in October 2026, described in Section 2. That consultation did not change the staged structure of this protocol, since the first study it proposed matches the entry route already set out here. It did add a decision that now comes before Gate 0, which is the choice of what area of biomanufacturing the application is for.

Companion documents: *ICHNOS - Mechanism Risk Assessment* (what can go wrong inside the circuit) and *Hazard Analysis of Wet-Lab Materials* (reagents and bench practice). This protocol deals with what changes once the circuit leaves a laboratory flask.

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1. Purpose and three risks that are new at production scale

A monitoring tool in a production environment creates three categories of risk that do not exist at the bench. They are the organising structure of this document and the reason it is written around safety rather than around method.

1.1 It puts a genetically modified organism somewhere new

The volumes differ by orders of magnitude. Containment is engineered rather than procedural, the waste streams are industrial and the people handling the organism are operators rather than researchers. Little of the containment reasoning that applies to a 50 mL flask transfers unchanged.

1.2 It can contaminate the product

Anything living in a production vessel contributes biomass, nucleic acid and protein to what comes out of it. For a product intended for human use these are impurities whose clearance has to be demonstrated.

1.3 It can be believed

A monitoring tool exists to inform decisions. The decision it would inform here is whether to continue a batch or discard it, which has safety consequences once the product reaches a patient. A reading that is wrong in the reassuring direction is the failure mode that matters. That is why this protocol places the burden of proof on the reading rather than on the organism.

2. What the experts told us and what it changed

We consulted specialists in industrial biotechnology, bioprocess engineering, fermentation and protein production. Their input changed the shape of this protocol and we set out how, because several of their points contradicted our initial assumptions.

(Names and affiliations of the consulted experts are recorded in our Human Practices documentation. The attributions below are by area of expertise.)

2.1 The valuable question is diagnosing a failed process, not watching a healthy one

An industrial fermentation expert put this directly. In an ideal bioprocess where everything works there is little to learn. In industry something always breaks: a heat exchanger, a steam generator, an electrode. When a failure of that kind leaves a culture outside its normal operating conditions for hours, the operator has one immediate and expensive question, which is whether the culture took damage. Today the answer comes from waiting a day or two to see whether the culture recovers. A method that answers it in hours would let the batch be stopped early or continued with confidence, saving vessel time and energy.

We adopted this reframing because it is what makes the safety case tractable. The forensic use is retrospective and offline. It does not require ICHNOS to control anything, to operate in real time or to be trusted with a release decision. It is the lowest-authority role a monitoring tool can have and therefore the one with the shortest route to being safe.

2.2 Robust industrial processes are a poor target and sensitive ones are the real need

The same expert noted that industrial biotechnology deliberately uses hardy organisms because sensitivity is expensive. Yeast in particular is robust, well understood after a century of use and often does not need this. The processes where cells are genuinely fragile and a batch is genuinely costly are mammalian cell cultures, such as CHO cells producing monoclonal antibodies, with expensive media, single-use reactors, limited volumes and fermentations lasting many days. That is where the information would be worth most. It is also a

host ICHNOS does not work in. We record this as an open tension rather than resolving it, since the clearest commercial need sits in a system our circuit has never been built for.

2.3 Choosing the area of production comes before everything else

A later consultation with a specialist in bioinnovation and protein production made this his central point. The application is interesting but the field is broad and it cannot be assessed until we state which part of production it is for. Recombinant proteins and antibodies were the two areas he named.

We had treated the choice of host and product as something Gate 0 would record. His point is that it governs everything upstream of Gate 0, because it determines which stressors are relevant, which sampling points exist, what counts as a quality attribute and which regulatory framework applies. We have therefore taken it out of the gate structure and made it the condition for entering that structure at all.

This sharpens the tension in 2.2 rather than resolving it. Of the two areas he named, antibody production is overwhelmingly mammalian, so choosing it would mean taking on the host gap as the first technical problem. Recombinant protein production in yeast is the option consistent with the system that exists today. We record that as the realistic choice without treating it as made, because it is a scientific decision for the team rather than a safety one.

2.4 The stress that matters depends on the product

A second expert observed that the literature on yeast stress in industry divides by product class, covering ethanol and biofuels, heterologous proteins and terpenes. Each biosynthesis imposes its own metabolic burden on top of the process stresses. There is no universal stress profile to monitor. The relevant stressors have to be defined per host, per product and per process and a result in one combination says nothing about another.

One expert also noted that yeast platforms are used in vaccine manufacture. That anchors the biopharmaceutical case in a real precedent rather than a hypothetical one.

2.5 The vulnerable points are transitions rather than steady states

Several experts converged here. The adaptation phase after inoculation is the classic stress point and so is the transfer from seed culture to scale-up and into the bioreactor, mainly because of the change in volume and the shift to vigorous agitation and forced aeration. Written input from a microbiology expert made the mechanism explicit. A change in conditions mid-growth pushes cells back into an extended lag phase and depending on severity growth may not resume at all.

2.6 Start outside the production line and start with a responsiveness check

Asked where ICHNOS should be integrated, the experts converged on a parallel research tool first. One added that integration into a reactor immediately raises the question of whether it would even be permitted. One expert, speaking as a non-specialist in fermentation, thought scale-up and bioreactor operation would be the most informative points, which is consistent, since informative and permissible are different questions.

The October consultation proposed a specific first experiment. Expose our cells in the laboratory to conditions representative of bioreactor operation or to changes of the kind that occur inside one and establish whether such conditions produce a stress response our system detects at all. We note carefully what this is. It is not a scale-down model of a particular process and it is not evidence about any producer strain. It is the prior question of whether the circuit responds to this class of condition, which the protocol takes for granted everywhere ahead of Gate 1. It needs no production equipment, no product contact and no new containment case, which is why it sits inside C1 and ahead of Gate 1.

2.7 For biopharmaceuticals the obstacle is regulatory

This was stated plainly by one expert. The hardest obstacle for medicines is regulatory compliance rather than safety or interpretation. We have given it its own section, because it is the point most likely to stop this application regardless of how well the biology performs.

2.8 What to define first and what would count as evidence

Asked what to specify before anything else, the written input identified the stress types and the sampling points, ahead of quality indicators and acceptance criteria. Another expert agreed the order was sensible but added that all of them need work, so the team should start wherever it is easiest.

On evidence, the answer was to prove the method on well-characterised model processes and then test the purity of the final product. That is the backbone of Gates 1 and 2. The same expert added that progress beyond that point is a matter of repeating such assays and then of commercial and public-relations planning rather than of further biology. Two further points were stated directly. Where the product is consumed by humans, safety comes before every other consideration, which is the reason Section 7 exists. Reproducibility over time is the acceptance criterion rather than a single successful demonstration.

2.9 The challenge we could not answer

A bioprocess expert pressed us on the sentinel configuration, in which ICHNOS-bearing cells are co-cultured alongside producer cells as an environmental witness. He asked whether the sentinel's experience actually represents the producer's. We answered that in theory they share conditions and conceded that we cannot confirm it experimentally. He also pointed out that per-cell information requires flow cytometry, which we do not currently have. Both points are carried into this protocol as requirements rather than assumptions and the sentinel configuration is treated below as the option needing the most evidence rather than the least.

3. Deployment configurations ranked by risk

This is the central decision of the protocol. The configuration determines both how much biological risk is introduced and what the measurement actually means. The two are inversely related, which is the uncomfortable fact this section exists to make visible. The configurations that tell you most about the producer are the ones that put the most into the vessel.

A configuration must be selected, recorded and approved before any work begins. **C1 is the default entry route. Nothing may begin at C3 or C4.**

3.1 C1: scale-down parallel model (default)

ICHNOS-bearing cells are cultured in separate laboratory equipment, under conditions engineered to mirror the production process and are never introduced into the production line.

Biosafety. Contained laboratory work at the scale the companion documents already cover. No engineered organism enters production equipment, there is no product contact and no new waste stream. This is why it is the default.

The entry step within C1, stated separately because it is where work should begin. Before any attempt to mirror a named process, reporter cells are exposed to conditions representative of bioreactor operation and to imposed changes in them, to establish whether the circuit responds to this class of condition and over what range. A negative result stops the application at the cheapest possible point, which is the reason to do it first. A positive result establishes responsiveness only. It is not a scale-down claim and not a statement about any producer. Gate 1 then applies to the process model proper.

What it measures and the limit. The model's own stress history. Transfer of that conclusion to the producer is a claim requiring evidence rather than an assumption. A small vessel does not reproduce the mixing and oxygen gradients of a large one and similar average sensor readings do not mean the cells experience the same local conditions. The expert point about gradients applies directly here.

The condition the timer needs and a bioreactor does not provide. The mechanism risk assessment records that the ratio reflects how fast the existing reporter population is replaced, that dilution by division dominates that replacement in a growing culture and that valid temporal comparisons are therefore restricted to matched growth conditions. A production culture does not hold growth rate constant. It varies by phase, by feed strategy and under the very stress the method is meant to record. Any temporal claim consequently requires growth rate recorded alongside every fluorescence measurement, plus a calibration carried out at deliberately varied growth rates. Until that calibration exists, the output of a parallel model states whether a response occurred rather than when it occurred.

Required evidence. Documented differences between model and production in host, medium and operating conditions. Demonstration that the conditions of interest are actually reproduced. Independent process records to anchor the comparison.

3.2 C2: offline sample-exposure assay

Reporter cells are exposed, in the laboratory, to material withdrawn from the process.

Biosafety. As for C1. Sampled material is not returned to the production vessel under any circumstance.

The limit, stated carefully because it is easy to get wrong. This measures how reporter cells respond to the sample **as it is now**. It does not recover the producer cells' earlier stress history. A circuit can only record an event it was present for. An assay performed after the fact on withdrawn material is a sample-chemistry assay and should be named and validated as one. It is useful, but it is a different measurement from the forensic question that motivates this protocol.

3.3 C3: sentinel co-culture

ICHNOS-bearing cells are cultured in the production vessel alongside the producer as an environmental witness, with the circuit absent from the producing strain itself.

Why it is attractive. It leaves the production strain genetically untouched, which avoids the whole question of whether the circuit changes productivity.

Why it carries the most unresolved risk. A second organism is in the vessel. That introduces competition for nutrients, possible cross-feeding, drift in the population ratio over a multi-day fermentation and a second set of process-related impurities, namely sentinel biomass, sentinel DNA and fluorescent proteins, whose clearance downstream processing must be shown to achieve. It also requires a reliable way to distinguish and remove sentinel cells and a demonstration that their presence does not alter the producer's behaviour or the product.

The validity question, which is biological rather than procedural. A sentinel is a different organism with a different metabolic load, occupying a different position in the vessel's gradients. Whether its recorded stress represents the producer's is a hypothesis. It was put to us directly by a bioprocess expert and we could not demonstrate it. **It must be demonstrated before this configuration is used for any decision**, by parallel measurement in both populations under known imposed conditions.

Prohibited contexts. Any product intended for human or animal consumption and any regulated manufacture, unless and until clearance of sentinel-derived material is validated and the change is accepted under the applicable quality system.

3.4 C4: circuit integrated into the production strain

This is the highest information and the highest barrier. The recording happens in the cells whose history is in question, which is the only configuration that fully answers the forensic use case.

Risks specific to it. The circuit imposes expression burden on a strain selected for productivity, so growth, yield, culture duration and product quality all have to be shown unchanged against the parental producer under matched conditions. Genetic stability over a full production campaign becomes a requirement rather than a research question, since drift is toward signal loss. The producing organism is now a different modified organism, which reopens its containment classification, its registration and, for a licensed product, its approved process.

A risk that belongs to this configuration alone. The mechanism risk assessment notes that expressing the circuit consumes transcriptional, translational and folding capacity. Load of that kind is itself the sort of stress the ER module is built to detect, so in principle the circuit can report its own presence. In a production strain this stops being a theoretical point, because the reading would be used to judge a batch. Uninduced engineered cells have to be compared against the parental strain and against a marker-matched strain, with growth rate and viability recorded in the same experiment. An elevated baseline in uninduced cells disqualifies the configuration until it is explained.

Not an entry route. C4 requires completed Gates 1 to 3 in C1 and acceptance under the site's change-control system.

3.5 Configuration selection record

Record for each proposed use: the configuration, the host and product, the production stage, the specific question to be answered, who approved it and the evidence relied upon. **"Monitoring stress" is not a question.** "Did the thermal excursion between 14:00 and 19:00 damage this culture enough to abandon the batch" is one.

4. What changes with scale

A common error in extending a laboratory method to manufacturing is assuming that containment evidence scales. It does not. The following are safety requirements rather than good practice.

Inactivation must be validated at the operating scale, matrix and load. Autoclave validation for 1 L of laboratory culture is not evidence for a kill tank handling cubic metres of spent broth. Thermal penetration, solids content, viscosity, foam and residence time all differ. The site has to demonstrate inactivation for the actual waste stream and specify how a failed cycle is detected.

Cleaning is not inactivation. A CIP cycle is designed to remove residue. Elimination of viable engineered cells is a separate claim and cleaning may not be substituted for it.

Residual viable organisms and residual DNA are different endpoints. They have different acceptance criteria and different methods and the required answer depends on where the stream goes.

Aerosol and release points multiply. Sampling ports, exhaust gas and condensate, foam overflow, seals, transfer lines, pressure relief and vessel opening for maintenance are all potential release points with no laboratory equivalent. Each needs assessment for the specific installation.

Spill response changes in kind. A spill at production scale is a facility event rather than a bench event. The site's emergency procedures govern and the engineered strain has to be included in them before work begins rather than after an incident.

Operators are not researchers. Procedures must be written for people who did not build the circuit, with the interpretation limits in Section 8 stated on the method itself rather than held in the heads of the team that developed it.

5. Gates

Each gate is a stop. Work does not continue until the stated evidence exists and the named reviewer has accepted it.

Gate 0. Authorisation and definition. Documented before anything starts: the production area selected within biomanufacturing, which is the decision identified in consultation as preceding all others and which determines the relevance of everything else in this gate. Then the host, product, process stage and exact question. The selected configuration. The stressors of interest and the sampling points, which are the two items the experts identified as needing definition first. The local biosafety assessment covering host, construct, scale, exposure routes and waste. Verification of the working clone, meaning sequencing across the integration locus together with copy-number determination. The mechanism risk assessment records this as its highest-value outstanding measurement. Copy number is a circuit parameter rather than a housekeeping detail, because the reporter and the feedback copy share a promoter, so additional copies change loop gain instead of simply scaling output. The institutional contained-use classification and any notification requirement. The approved disposal pathway. Unresolved authorisation stops the work.

Gate 1. The reading is reliable in a model process. Evaluated in a well-characterised model with independently recorded exposures, as recommended in consultation. Required: baselines and controls, including parental host and unstressed ICHNOS cells. Demonstrated response to the intended stressor, with assessment of cross-response to other plausible process conditions. Temporal behaviour under known transient, repeated and prolonged events, with the interpretable window defined. Explicit assessment of the confounders identified in the mechanism risk assessment, above all growth-rate dilution, since stress slows growth and growth rate drives the timer ratio, together with fluorophore maturation, oxygen availability, temperature, pH, autofluorescence and handling delay. Identifiability is assessed within this gate. One ratio value can arise from more than one history, since a recent mild episode and an older severe one can converge, as can a single pulse and a series of smaller ones. Known pulsed, sustained and repeated inputs are delivered to establish which histories the readout separates. Histories it cannot separate are reported as ambiguous rather than resolved. Reproducibility across independent runs, operators and days is the acceptance criterion rather than a single successful demonstration.

Gate 2. Non-interference and product comparability. For C4 the engineered producer is compared with its parental strain under matched conditions, at the intended operating point and at relevant deviations, covering growth, viability, culture duration, substrate use, productivity, product identity, purity, activity and product-specific attributes. For C3 the same comparison is made for the producer in the presence and absence of sentinel cells. Independent biological runs are required, with the analysis and equivalence margins predefined. A non-significant difference is not evidence of equivalence. Any new impurity, whether reporter protein, engineered-cell biomass or residual DNA, must have demonstrated downstream clearance.

Gate 3. Scale and sampling integrity. A justified scale-down or pilot study reassessing mixing and oxygen gradients, biomass, pressure, aerosols, cleaning and waste capacity at the intended scale. The sampling workflow is validated end to end: authorised sampling point and aseptic method, recorded timestamp and process conditions, controlled transport and maximum delay, defined preparation, retained raw data with instrument settings and approved disposal. Sampled material never returns to the vessel. Representativeness is assessed explicitly, since a single sample may miss a localised or short-lived event and where single-cell resolution matters the method has to deliver it. A field-average fluorescence measurement cannot distinguish a uniformly mildly stressed population from a mixture of severely stressed and unaffected cells, which is the distinction the flow-cytometry requirement raised in consultation exists to resolve.

Gate 4. Sector-specific acceptance. Section 7.

6. Product safety and downstream burden

Any engineered organism present in a production vessel adds to what must be removed from the product. This section applies to C3 and C4 and is the reason both are gated behind C1.

Process-related impurities. Engineered-cell biomass, host-cell proteins including the fluorescent reporter and residual DNA carrying the construct. For each, the question is not whether it is hazardous in itself, since fluorescent proteins are not, but whether its clearance has been demonstrated for this process to the limits applicable to this product.

The reporter is not inert simply because it is not toxic. sfGFP and mCherry are foreign proteins in the product stream. For a biological medicine, foreign protein is assessed for immunogenicity risk regardless of its function.

Selection markers and auxotrophy. The *HIS3* marker restores histidine prototrophy and is not an antibiotic resistance gene, which removes one common concern. As the companion documents state, it also provides no containment. Media composition in a production process is typically rich, so residual auxotrophies constrain nothing there.

Deliberate stress is sometimes the process. Some production strategies impose stress intentionally to increase a desired metabolite. A signal in such a phase may be exactly what the process intends. Interpretation therefore requires stage-specific baselines. A universal assumption that stress is bad would generate false alarms and damage confidence in the method. This follows from the expert observation that the relevant stress differs by product class.

7. Sector requirements

Industrial enzymes, proteins and technical products. The lowest regulatory barrier and, as the experts noted, often the lowest need, since these processes use robust organisms by design. Focus on functional activity, purity, host-derived impurities and downstream removal of reporter material.

Metabolites, fuels and chemicals. Product identity and composition, yield, unintended metabolites and the distinction between productive stress and harmful deviation.

Food, feed, nutritional and cosmetic applications. A separate authorisation question entirely. Engineered organisms in products entering the food chain are governed by their own framework and suitability for research establishes nothing here. The expert position was unambiguous. Where humans consume the product, safety precedes every other consideration. Our own position is that C3 and C4 should not be pursued in this sector.

Biopharmaceuticals. The precedent is real, since yeast platforms are used in vaccine manufacture and so is the barrier. We record the expert assessment plainly. Regulatory compliance rather than biology is the hardest obstacle here. Introducing a new organism or a genetic modification into a process used to make an authorised medicine is a change to that process. It engages the site's change-control system, the applicable GMP requirements for biological manufacture, qualification and validation and potentially the marketing authorisation itself. Analytical methods entering a pharmaceutical quality system require validation to the applicable standard. None of this is a reason to abandon the application. It is the reason the realistic near-term route is C1, a parallel model generating process understanding, which is not a change to the registered process at all.

8. What ICHNOS may and may not decide

It is advisory. Throughout every gate in this protocol an ICHNOS reading is supplementary information recorded alongside independent process and quality data. It does not release a batch, discard a batch, adjust a feed or trigger any automated action. Any future control function is a separate assessment and a separate validation.

Decision states. A *valid baseline result* is recorded with the independent data and authorises nothing on its own. A *valid unexpected signal* triggers notification of the process and quality leads and a predefined investigation rather than a disposal decision. An *invalid or indeterminate result*, meaning a failed control, a signal below the usable range or compromised sample integrity, is never interpreted as absence of stress and is resolved by investigation and approved resampling. A *containment or contamination concern* follows the site emergency procedure immediately.

The interpretation limits that must appear on the method itself, because they are where a monitoring tool does harm:

- A low or absent signal is not evidence that the culture is healthy. It is equally consistent with cells too damaged to express a reporter, with a reading below the detection limit and with a circuit that has drifted. The mechanism risk assessment sets this out in full.
- A stress signal does not demonstrate contamination, toxicity, cell death or defective product. It demonstrates that the recording cells experienced a condition the circuit responds to.
- The timer reports the age of a reporter protein population. Converting that to time since an event requires demonstration under the relevant conditions and stress itself alters growth rate and therefore the ratio. Report a bounded classification rather than an elapsed time.
- A sentinel or parallel model reports its own experience. Transfer to the producer is a claim requiring evidence.
- The assay is offline. A cellular recording mechanism does not make it a real-time monitor and sampling, preparation and analysis delay the result and may alter the signal.

9. Biosecurity and custody at industrial scale

Transfer of an engineered strain from a university team to an industrial site changes who holds it and who is accountable for it.

- Material transfer under a written agreement recording the recipient, the intended use, the approved configuration and the containment conditions. Transfer for one configuration does not authorise another.
- Strain custody with unique identifiers recording construct version, integration locus and responsible custodian, with inventory reconciliation against stored material.
- Calibration data, raw measurements and analysis versions retained with change history. Because the realistic harm pathway runs through a wrong reading rather than a dangerous organism, data integrity carries the same weight as strain custody.
- No release outside contained use and no use in public demonstration.
- Any change of host, product, scale, configuration, sample matrix, intended user or decision context invalidates the assessment and requires a new one.

10. Residual position and review triggers

We set out our own assessment so that this document is not read as advocacy.

ICHNOS has not been tested in any production process, at any scale, in any configuration. The circuit operates in laboratory yeast and nowhere else. The application with the clearest commercial value, which is fragile and expensive mammalian cell culture, sits in a host the circuit has never been built for and the host it does work in is one the experts described as robust enough that it often does not need this. The sentinel configuration, which is the most elegant answer to the contamination problem, rests on a representativeness assumption we were asked about directly and could not demonstrate.

We have also not yet chosen the area of production this application is for and we now treat that as the open item rather than as a detail to settle later. Until it is chosen the stressors of interest and the sampling points cannot be fixed, because both follow from the product and the process. The one step that does not wait on that choice is the responsiveness check described in 3.1, which asks only whether the circuit reacts to bioreactor-type conditions at all.

What we do have is a well-defined question worth answering, namely whether a specific process failure damaged a specific batch, identified for us by people who run bioreactors. We also have a first experiment that can be done with the cells and equipment we already have and a staged route that starts entirely outside the production line. That is the honest state of this application. This protocol is written to make the first step of it safe rather than to make the whole of it sound finished.

Review triggers. Any change of host, construct, configuration, product, scale or decision context. An unexpected result that cannot be explained by the controls. Any spill, exposure, failed inactivation or containment deviation. Any proposal to use an output outside the advisory role defined in Section 8.

Approval record. Institutional biosafety review, site quality acceptance and residual-risk acceptance are not recorded, because they have not occurred. This protocol is a proposal for how such work could be approached and must not be cited as evidence that it has been approved.