2025

iGEM Safety Standard

for
Engineered Live
Biotherapeutic Products

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AUTHORS & EDITORS

Peking University (Peking)

Zhejiang University (IZJU-China)

ZJE 浙江大学爱丁堡大学联合学院
ZJU-UoE INSTITUTE

FOREWORD

It is with immense pride that we introduce the iGEM Safety Standard for Engineered Live Biotherapeutic Products (LBPs). This document is the culmination of a dedicated, collaborative effort to address a critical and evolving challenge within the synthetic biology community: the safe and responsible development of engineered organisms intended for therapeutic use.

The inspiration for this standard was born from a shared sense of responsibility. As the Peking iGEM team prepared to host the iGEM Live Biotherapeutics Exchange Meeting on August 5, 2025, we connected with our peers from iZJU-China and discovered a mutual, deep-seated concern for the safety implications of our work. This dialogue was further enriched during the meeting, where iZJU-China’s insightful presentation on safety catalyzed our decision to transform our conversations into a tangible, lasting resource. From August to September 2025, our two teams embarked on a joint mission to create a comprehensive safety framework designed by iGEMers, for iGEMers.

This standard is intended to be more than a checklist; it is a guide to instill a proactive "regulatory mindset" from a project's inception. We believe that as iGEM projects grow in sophistication, our approach to safety must mature in parallel. This document translates complex professional regulatory principles into a rigorous yet feasible framework adapted for the iGEM context. Its structure is designed to guide teams through the entire LBP development lifecycle, beginning with foundational principles and adapted CMC concepts for rational chassis selection and payload design. It then covers the critical need for active biocontainment systems to ensure environmental safety, provides a practical framework for non-clinical safety evaluation through in vitro testing, and concludes by emphasizing the importance of thorough documentation and responsible conduct, including dual-use considerations.

This comprehensive standard would not have been possible without the synergistic collaboration between our two teams. The Peking University team took the lead on drafting the core content of the standard and engaged with industry experts to refine its principles. The iZJU-China team provided the

crucial context by meticulously researching and integrating relevant case studies from past iGEM projects, and masterfully handled the document's design and layout to ensure it was both informative and accessible.

Looking ahead, we hope this document serves as a foundational resource that empowers future iGEM teams to pursue ambitious therapeutic projects with confidence and a heightened sense of responsibility. Our ultimate goal is to contribute to a community-wide culture where safety is not an afterthought, but the bedrock upon which we build the future of synthetic biology.

Zhilan Mu

Human Practices Lead

On behalf of the 2025 Peking iGEM Team

The development of the *iGEM Safety Standard for Engineered Live Biotherapeutic Products (LBPs)* reflects our shared commitment to building a safer and more responsible foundation for synthetic biology.
When the iZJU-China and Peking iGEM teams first connected in mid-2025, our discussions quickly revealed a common vision — that as synthetic biology begins to enter therapeutic frontiers, *biosafety must evolve from reactive regulation to proactive design*.

From this shared belief grew a genuine collaboration. Our team focused on providing the practical dimension of the standard — systematically reviewing past iGEM projects, identifying representative biosafety strategies, and transforming these insights into case-based frameworks embedded throughout the document. We sought to ensure that each principle in this standard is not only scientifically sound but also achievable in real iGEM practice.

Working alongside the Peking team allowed us to experience how dialogue between different perspectives — academic, engineering, and ethical — can converge into a unified standard. This collaboration has been a vivid demonstration of the iGEM spirit: openness, co-creation, and learning through exchange.

Looking forward, we hope this document serves as both a benchmark and a catalyst — helping future teams design safer, more transparent, and more ethical living therapeutics. For us, this handbook is not the end of a project, but the beginning of a culture in which safety becomes the most creative and enduring form of innovation.

Canrong Long

Human Practices Lead

On behalf of the 2025 iZJU- iGEM Team

PART I: FOUNDATIONAL PRINCIPLES AND DEFINITIONS

1.1 Introduction to Engineered LBPs in the iGEM Context

The International Genetically Engineered Machine (iGEM) competition stands at the forefront of synthetic biology innovation. As projects grow in sophistication, many teams are now developing engineered microorganisms intended to diagnose, treat, or prevent human diseases. These projects fall under the regulatory category of Live Biotherapeutic Products (LBPs), necessitating a dedicated and rigorous safety framework. This document proposes a safety framework for any iGEM team developing an engineered LBP.

1.1.1 Defining Live Biotherapeutic Products (LBPs)

A Live Biotherapeutic Product is formally defined as a biological product that: 1) contains live organisms, such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is not a vaccine. Within the iGEM competition, this standard applies to any project where a live, genetically modified organism (GMO) is designed to exert a therapeutic, preventative, or diagnostic effect

in vivo (e.g., within the gastrointestinal tract) or on the human body (e.g., topically on the skin). The intended application as a therapeutic agent legally classifies the organism as a drug, irrespective of its origin.

The Zurich (2022) team developed an "inflammation-responsive therapeutic bacterium" using the probiotic Escherichia coli Nissle 1917, a Risk Group 1 strain. Through genetic engineering, they equipped this chassis with a dual "inflammation-sensing and therapeutic-secreting" circuit. When exposed to inflammation signals in the gastrointestinal tract of IBD patients, such as elevated reactive oxygen species, the engineered strain automatically secreted anti-inflammatory molecules (e.g., IL-10 analogs) to alleviate intestinal inflammation. This design represents a typical "in vivo therapeutic LBP," as it involves a live genetically modified probiotic that delivers targeted treatment within the human body (https://2022.igem.wiki/uzurich/).

1.1.2 Distinguishing from Probiotics and Foods

Many iGEM teams may select chassis organisms that are commonly found in fermented foods or commercial probiotic supplements. It is crucial to understand that once such an organism is genetically engineered to perform a therapeutic function, it is no longer considered a food or a supplement. Under regulatory frameworks established by agencies like the U.S. Food and Drug Administration (FDA), any substance intended for use in the diagnosis, cure, mitigation,

treatment, or prevention of disease meets the definition of a drug. Therefore, even if a project begins with a strain that has a presumption of safety from a food perspective, its development as an LBP requires adherence to the higher safety and quality standards applicable to medicinal products. This standard clarifies that the use of a "probiotic" or a Generally Recognized as Safe (GRAS) strain does not exempt a project from these rigorous safety requirements.

1.1.3 The iGEM Context and Regulatory Mindset

This standard acknowledges that iGEM projects are conducted in an academic, pre-clinical research setting, not in facilities compliant with Current Good Manufacturing Practices (CGMP). Consequently, the requirements outlined herein are adapted from professional guidelines issued by international regulatory bodies—including the FDA, the European Medicines Agency (EMA), and China's National Medical Products Administration (NMPA)—to be both rigorous and feasible within the scope of a typical iGEM project .

The primary purpose of this standard extends beyond a simple checklist of rules. It is designed to instill a "regulatory mindset" in iGEM participants. This involves a fundamental shift in perspective: from viewing a project as a simple proof-of-concept experiment to approaching it as the first step in a potential therapeutic development pipeline. By introducing foundational concepts like Chemistry, Manufacturing, and Controls (CMC), genetic stability, and biocontainment, this framework encourages a culture of responsibility, meticulous documentation, and proactive safety engineering from the project's inception. Adherence to this standard not only ensures the safety of the project but also elevates its scientific rigor, making the research more robust, reproducible, and valuable for future academic or commercial development. This approach better prepares participants for professional careers in biotechnology, where safety and quality are paramount.

1.2 Core Principles of LBP Safety

To provide a clear and memorable framework, this standard is built upon four fundamental pillars of safety. Every aspect of an LBP project, from initial design to final validation, should be assessed through the lens of these core principles.

1. Intrinsic Chassis Safety: The foundation of a safe LBP is the host microorganism itself. The chosen chassis should have a well-established safety profile, be free of intrinsic hazardous characteristics, and be thoroughly characterized before any genetic modification is introduced.
2. Genetic Payload Safety: All engineered genetic components—including genes, regulatory elements, and vectors—and the products they express should be demonstrably safe.

The design should proactively exclude elements that could be toxic, immunogenic, or otherwise harmful to the host or the human body.

3. Containment and Control: An engineered LBP should be designed to be controllable. This principle suggests the inclusion of robust biocontainment systems that prevent the organism's survival, proliferation, or genetic transfer outside of its intended operational environment. The organism's function should be confined to the target site and duration.
4. Final Product Verification: The complete, final engineered LBP should be empirically tested to verify its safety profile. This involves direct, in vitro testing to confirm that the final product does not exhibit unintended toxicity or other hazardous properties.

1.3 Glossary of Key Terms

To ensure clarity and consistent interpretation, the following terms are defined for the purposes of this standard.

1. Live Biotherapeutic Product (LBP): A biological product containing live microorganisms, such as bacteria or yeast, that is intended for the prevention, treatment, or cure of a disease or condition in humans.
2. Chassis: The host microorganism (e.g., a specific strain of Escherichia coli or Lactiplantibacillus plantarum) into which engineered genetic systems are introduced.
3. Genetic Payload: The entirety of the synthetic or modified genetic material introduced into the chassis. This includes all genes, promoters, terminators, ribosome binding sites, vectors, and other regulatory elements.
4. Biocontainment: The use of physical or biological measures to prevent the release, survival, and proliferation of genetically modified organisms in unintended environments.
5. Kill Switch: A genetically encoded circuit designed to induce cell death in response to a specific environmental signal (or the absence thereof), serving as a form of active biocontainment.
6. Auxotrophy: A state in which an organism is unable to synthesize a particular organic compound required for its growth. Engineering auxotrophy for a compound absent in the external environment can serve as a biocontainment mechanism.
7. Genetic Stability: The propensity of an engineered genetic construct to remain unchanged over many generations of cell division. Instability can lead to loss of function and failure of safety mechanisms.

8. Cytotoxicity: The quality of being toxic to cells. In this context, it refers to the potential of an LBP or its products to damage or kill human cells.
9. Chemistry, Manufacturing, and Controls (CMC): A comprehensive set of information and practices related to the drug substance and drug product that ensures product identity, quality, purity, and strength. This standard adapts key CMC principles for the iGEM context.
10. Generally Recognized as Safe (GRAS): A designation by the U.S. FDA that a substance added to food is considered safe by experts, and so is exempted from the usual Federal Food, Drug, and Cosmetic Act (FFDCA) food additive tolerance requirements. Strains with GRAS notification have a strong, publicly reviewed safety dossier.

PART II: LBP CHARACTERIZATION AND CONTROL (ADAPTED CMC)

This section translates the professional regulatory principles of Chemistry, Manufacturing, and Controls (CMC) into a "research-grade" framework suitable for the iGEM laboratory environment. The goal is to provide actionable steps and documentation suggestions that ensure the identity, purity, consistency, and stability of the engineered LBP. Implementing these principles is not merely a safety exercise; it is fundamental to conducting reproducible and high-quality science. By ensuring that experiments begin with consistent, well-characterized starting material, teams can mitigate a common source of experimental variability and failure.

2.1 Chassis Strain Selection and Justification

The selection of a safe chassis is one of the most important decisions in an LBP project. The safety of the final product is fundamentally dependent on the intrinsic properties of the host organism.

2.1.1 Prioritizing Strains with a History of Safe Use

It is recommended that teams select a chassis organism from Risk Group 1 and provide a robust justification for its safety. The strongest justification comes from selecting strains with a documented history of safe use in humans.

1. Recommendation: It is strongly recommended that teams use chassis strains that have received a "no questions" letter in response to a Generally Recognized as Safe (GRAS) notification submitted to the U.S. FDA. The GRAS notification inventory is a public database containing extensive safety data and provides a strong foundation for a safety argument. Examples include various strains of Lactiplantibacillus plantarum, Bacillus coagulans, and Bacillus subtilis.

The Zurich (2022) team used Escherichia coli Nissle 1917, a Risk Group 1 probiotic with a long record of safe human use, as the chassis for an “inflammation-responsive therapeutic bacterium” targeting IBD. They avoided introducing antibiotic resistance genes and relied on inflammation-inducible promoters to control therapeutic molecule secretion, reducing risks of horizontal gene transfer. This combination of historical safe use and careful design highlights why E. coli Nissle 1917 is a robust LBP chassis (https://2022.igem.wiki/uzurich/).

The Utrecht (2017) team employed cells from Polypedilum vanderplanki, a non-pathogenic lower eukaryote classified as Risk Group 1, for their OUTCASST diagnostic system. These desiccation-tolerant cells could be stored dry for months and reactivated without risk of uncontrolled proliferation. Whole-genome sequencing confirmed the absence of pathogenic genes, and the desiccated cells successfully carried a CRISPR-Cas detection module that remained active after six months, demonstrating both safety and stability (https://2017.igem.org/Team:Utrecht/).

2. Guidance: The use of any Risk Group 2 organism as an LBP chassis is strongly discouraged and should be approached with caution, requiring an exceptional justification for its necessity and a detailed plan for managing the associated risks, subject to explicit review and approval by the iGEM Safety and Security Committee.

Pseudomonas aeruginosa is a Risk Group 2 organism that can cause pulmonary infections and requires BSL-2 practices. If considered as a "lung-targeted LBP" chassis (e.g., for cystic fibrosis therapy), its use would demand strict risk control. Following the logic of the 2017 Utrecht team, this could involve CRISPR knockout of lasR and rhlR to attenuate virulence, an oxygen- and temperature-sensitive dual kill switch to restrict survival to lung-like conditions, and submission of a detailed safety package—including modification maps, risk matrices, and emergency plans—for review by the iGEM Safety and Security Committee (https://2017.igem.org/Team:Utrecht/).

2.1.2 Comprehensive Baseline Characterization

Before any genetic modification, it is crucial to unambiguously identify and characterize the wild-type chassis strain. This baseline is essential for evaluating the effects of subsequent engineering.

1. Identity Verification: The identity of the chassis strain should be confirmed using a definitive method, with 16S rRNA gene sequencing being a highly recommended minimum. The sequence should be compared to a reference database to confirm species and, if possible, strain identity.

LINKS_China (2021) team isolated a Komagataeibacter strain from commercially available kombucha and confirmed its identity using colony PCR amplification of the 16S rRNA gene followed by Sanger sequencing. They constructed a phylogenetic tree to verify its taxonomic position and its relation to closely related strains. Their workflow—

"isolation → colony-PCR (16S) → Sanger sequencing → BLAST/phylogenetic analysis" — provides a complete and reliable method for chassis strain identity verification (https://2021.igem.org/Team:LINKS_China).

2. Intrinsic Property Documentation: Teams are encouraged to document the intrinsic properties of their chosen chassis. This could include:
    1. Antibiotic Resistance Profile: The susceptibility of the wild-type strain to a standard panel of clinically relevant antibiotics should be determined. This baseline is critical to ensure that the engineering process does not inadvertently confer new antibiotic resistances.
    2. Screening for Virulence Factors: A literature review and, if available, a genomic analysis of the chassis strain should be conducted to screen for the presence of known virulence factors, toxins, or other hazardous genes (e.g., hemolysins, enterotoxins). This aligns with regulatory expectations to fully describe and characterize the product strain.

Marburg (2023) tested wild-type Agrobacterium strains for their natural sensitivity to several commonly used antibiotics, including ampicillin, chloramphenicol, and tetracycline. The strains were susceptible to these antibiotics, with K599 showing higher sensitivity. Some strains exhibited natural resistance or tolerance to streptomycin or spectinomycin, which the team considered when selecting appropriate antibiotic resistance markers (https://2023.igem.wiki/marburg/).

2.2 Genetic Modification Design and Construction

The genetic payload should be designed with safety as a primary consideration, a principle often referred to as "Safe-by-Design."

2.2.1 Safe-by-Design Principles for Genetic Payloads

The design of all engineered parts, circuits, and vectors should proactively minimize potential risks.

1. Component Screening: All genetic sequences (e.g., for protein expression) should be screened against public databases to identify any homology to known allergens, toxins, or virulence factors.

Manchester (2019) performed allergenicity prediction for their kill-switch protein by comparing its amino acid sequence against public allergen databases like AllergenOnline. Using a sliding 80-amino-acid window, they identified subsequences with ⩾ 35% identity to known allergens. In all cases, similarity was below the threshold, concluding low allergenic risk. The team documented the tool, database, thresholds, and results,

redesigning or excluding any segments with high homology to known allergens (https://2019.igem.org/Team:Manchester).

2. Minimizing Repetitive DNA: The reuse of genetic parts (e.g., the same promoter or terminator) introduces repetitive DNA sequences. Such sequences are substrates for homologous recombination, a major cause of genetic instability that can lead to the deletion of the engineered construct. Teams should, where feasible, use collections of characterized, non-repetitive genetic parts to enhance the long-term stability of their system.

Aalto-Helsinki (2023) optimized their expression constructs to reduce genetic instability by removing internal repetitive DNA sequences, enhancing fragment synthesis success, and limiting homologous recombination sites. Intrinsic terminators within coding regions were eliminated, and Shine-Dalgarno-like sequences, promoter-like motifs (e.g., -35 boxes and Pribnow Box), were removed to prevent unintended transcription initiation. Silent mutations were used to preserve protein coding while reducing sequence redundancy, ensuring compatibility with assembly and codon optimization standards (https://2023.igem.wiki/aalto-helsinki/).

3. Vector Choice: The choice of vector is critical. Self-transmissible or mobilizable plasmids should be avoided. The selection marker used should not confer resistance to a clinically important antibiotic.

Imperial College London (2022) designed a sense-and-response system, engineering Bacillus subtilis spores to display chitinases that break down fungal cell walls. The spores, modified to display a mutant germinant receptor that binds chitin monomers, trigger a cascade enabling germination and production of antifungal peptides upon detection. These modifications were introduced using a self-digesting plasmid with CRISPR technology to ensure the system is free of foreign DNA, ensuring biosafety and high durability (https://2022.igem.wiki/imperial-college-london/).

2.2.2 Thorough Documentation of Genetic Modifications

Meticulous documentation is essential for reproducibility and safety assessment. For every engineered strain, teams are encouraged to provide:

1. Complete Sequence Information: The full sequence of all engineered plasmids and/or the sequence of the integrated genetic construct and its flanking genomic regions.
2. Annotated Parts Table: A detailed list of all genetic parts used, including their source (e.g., another organism, a parts registry number), function, and any modifications made.

3. Construction Methodology: A clear description of the methods used to construct the plasmids and/or create the final engineered strain. For genomic integrations, the exact site of integration should be specified and verified.

2.2.3 Assessment of Metabolic Burden

Introducing a synthetic genetic circuit invariably imposes a metabolic burden on the host cell by consuming resources (e.g., ATP, amino acids, ribosomes). High metabolic burden reduces the host's fitness, creating strong evolutionary pressure to inactivate or delete the foreign DNA. This is a direct threat to the project's function and safety, as it can lead to the loss of essential control elements like kill switches. Teams should consider the metabolic burden of their design and are encouraged to measure its impact on the host's growth rate compared to the wild-type chassis.

2.3 Principles of Manufacturing and Control for iGEM Labs

To ensure that the LBP used for all safety testing is consistent and well-defined, teams may find it beneficial to adopt principles adapted from industrial CMC practices.

2.3.1 Two-Tiered Cell Banking System

Serial re-culturing of strains from plates can lead to the accumulation of mutations and genetic drift. To ensure consistency throughout the project, teams are encouraged to establish a simple cell banking system.

1. Master Cell Bank (MCB): After a final engineered strain is constructed and sequence-verified, a large batch of culture could be prepared from a single colony, aliquoted, and cryopreserved. This is the MCB. It can remain untouched for the remainder of the project.
2. Working Cell Bank (WCB): A single vial from the MCB can be used to create a second, smaller set of cryopreserved aliquots. This is the WCB. All experiments for the duration of the project can then be initiated from a WCB vial. This practice ensures that every experiment starts from the same genetic source, greatly enhancing reproducibility.

2.3.2 Standardized Production Protocols

To ensure that different batches of the LBP are comparable, teams are encouraged to develop and document a Standard Operating Procedure (SOP) for its production. This SOP could specify, at a minimum:

1. Media composition and preparation.

2. Inoculation procedure (e.g., volume from WCB).
3. Growth conditions (temperature, aeration, vessel type).
4. Growth duration or harvest point (e.g., based on optical density).
5. Harvesting and preparation procedure (e.g., centrifugation, washing, resuspension buffer).

TUDelft (2020) received the Safety Commendation honor for establishing comprehensive standard operating procedures (SOPs) to ensure project safety. These SOPs included norms for operating the biosafety cabinet, standardizing the sample collection, transportation, and preservation process at 4° C, to maintain sample purity and prevent leakage (https://2020.igem.org/Team:TUDelft).

2.3.3 Final Product Release Specifications

Before proceeding with the safety evaluation experiments described in Part IV, it is advisable for teams to define a set of quality control specifications for their LBP "drug product." Every batch produced for testing should aim to meet these criteria.

1. Identity: Confirmation that the harvested cells are the correct engineered strain. This can be done via a method like colony PCR using primers specific to the genetic construct.

The UM_Macau (2020) team developed BREAC, a biofilm-removal system, and placed among the top five in the Environment track. To ensure product identity and safe application, they engineered E. coli with synthetic sensing and degradation modules, and added ferritin expression to enable magnetic recovery. This allowed the engineered strain to be effectively retrieved after use, preventing environmental leakage and confirming the identity of the intended engineered chassis (https://2020.igem.org/Team:UM_Macau).

2. Purity: An assessment of the absence of contaminating microorganisms. This is typically achieved by streaking a sample on a non-selective agar plate and observing for uniform colony morphology.

The XJTLU-China (2023) team, working on biosorbents for heavy metal and silver removal from industrial wastewater, emphasized product purity by avoiding live cells altogether. They immobilized functional proteins directly onto carriers, thereby eliminating risks of microbial contamination, horizontal gene transfer, and unintended environmental release. This approach elevated the purity standard from “no microbial contamination” to “no viable organism release” (https://2023.igem.wiki/xjtlu-china/).

3. Viability and Concentration: A quantitative measure of the number of live cells per unit of product, typically reported as Colony Forming Units per milliliter (CFU·mL−1).

This is a critical parameter for dosing in subsequent experiments and serves as an analogue for potency in an LBP.

The SCU-China (2023) team engineered E. coli strains to reduce hydrogen sulfide levels in wastewater by targeting sulfate-reducing bacteria. To validate performance in simulated aquatic environments, they employed CFU counting to quantify viable cells, tightly controlled the dosage of bacterial inoculum, and established standardized procedures. This ensured both reproducibility of results and compliance with safety requirements regarding viability and concentration (https://2023.igem.wiki/scu-china/).

2.4 Genetic Stability Assessment

Engineered microorganisms are subject to evolutionary pressures that can lead to the mutation or loss of the synthetic genetic construct, especially if it is burdensome. An unstable safety feature is an ineffective one. For example, a kill switch that is rapidly inactivated by mutation provides a false sense of security. Therefore, demonstrating the stability of the engineered construct is a key safety consideration.

2.4.1 Recommended Stability Study

It is highly recommended that teams perform an experiment to assess the genetic stability of their engineered LBP, with a particular focus on the stability of any biocontainment mechanisms.

1. Suggested Method: A serial passaging experiment is a good approach.

1. Initiate a culture from the WCB.
2. Grow the culture under standard conditions, without selective pressure if the system is designed to function without it (e.g., chromosomally integrated constructs).
3. Serially dilute and re-inoculate the culture into fresh media every 24 hours for a period sufficient to accumulate a significant number of generations (a minimum of 50 generations is recommended).
4. At the end of the experiment, isolate a representative sample of the population (e.g., 10-20 individual colonies).
5. Analyze each isolate to confirm the structural integrity of the genetic construct (e.g., via PCR, restriction digest of a rescued plasmid, or sequencing) and its function (e.g., reporter expression, kill switch activity).

2.4.2 Reporting and Strategies for Enhancing Stability

The results of the stability study should be documented, reporting the percentage of the population that retained the intact and functional construct. If instability is observed, teams should discuss the potential causes and propose strategies for improvement. A robust strategy for enhancing stability is to integrate the genetic payload directly into the host chromosome, as this removes the burden of plasmid replication and ensures the construct is passed to daughter cells more reliably than a plasmid.

PART III: BIOCONTAINMENT AND ENVIRONMENTAL SAFETY

An engineered LBP intended for use in the human body is, by definition, designed for release into a complex, uncontrolled ecosystem (e.g., the gut). This creates a unique safety challenge: the organism must survive and function in its target environment but should be prevented from persisting if it exits that environment (e.g., through excretion) and from transferring its genetic material to other microbes. This section outlines recommendations for active biocontainment systems to mitigate these risks.

3.1 The Case for an Active Biocontainment System

The potential for an engineered organism to proliferate in the environment or transfer its genetic payload to indigenous microbiota represents a significant ecological and safety risk. Therefore, this standard strongly advocates for the inclusion of active biocontainment measures.

1. Recommendation: Any engineered LBP designed to function within the human body or any other non-contained environment should ideally include at least one robust, genetically encoded, active biocontainment mechanism. Standard laboratory practices (passive containment) are insufficient for LBP projects. This is a critical component for project safety.
2. Definition of an Active System: An active biocontainment system is one that is encoded in the organism's DNA and is designed to cause growth arrest or cell death upon leaving the designated operational environment. This reframes biocontainment not as a simple safety add-on, but as a central engineering challenge of the LBP project itself. The design, construction, and validation of the biocontainment system should be approached with the same scientific rigor as the therapeutic function.

3.2 Evaluation of Biocontainment Mechanisms

Teams are encouraged to select, implement, and experimentally validate a suitable biocontainment mechanism. The choice of mechanism and its trigger should be directly relevant to the LBP's intended application.

3.2.1 Examples of Biocontainment Mechanisms

The following are examples of well-documented biocontainment strategies. Teams may use these or other scientifically sound approaches from the literature.

1. Kill Switches: These are genetic circuits that induce the expression of a lethal protein (toxin) in "non-permissive" conditions. The design of a robust kill switch often involves balancing the expression of a toxin and a corresponding antitoxin. Examples include:
    1. Environment-Sensing Switches: Circuits that respond to a specific molecule that is present in the target environment but absent elsewhere. The "Deadman" switch, for example, requires the presence of a specific small molecule to repress toxin expression; its removal leads to cell death.

The CAU-China team (2024) integrated an oxygen–nitrogen dual-responsive suicide switch in their "Nodule Factory" project. Low oxygen in nodules activated nifH to sustain antitoxin VapB, while low nitrogen activated glnK to produce sgRNA repressing VapC. Once bacteria escaped into high-oxygen, high-nitrogen environments, both signals disappeared, VapB was lost, and VapC triggered self-destruction. This dual sensing confined survival to the root nodule microenvironment. (https://2024.igem.wiki/cau-china/)

    2. Temperature-Sensitive Switches: For gut-based LBPs, a "Cryodeath" circuit can be designed to activate toxin expression when the organism is exposed to temperatures below human body temperature (e.g., room temperature), simulating excretion into the environment.

The HNU-China team (2022) used a λ phage CI857-based temperature switch for intestinal LBPs. At 30 °C, CI857 repressed the R promoter, but at 37 °C it dissociated, activating expression. Integrated into a suicide system, this ensured survival in the gut and elimination in ambient environments. (https://2022.igem.wiki/hnu-china/)

2. Engineered Auxotrophy: This strategy involves deleting a gene essential for the synthesis of a vital nutrient (e.g., an amino acid or nucleotide precursor). The LBP can then only survive if this nutrient is supplied externally. For this to be an effective biocontainment strategy, the chosen nutrient should be readily available in the target environment (e.g., the gut) but scarce in the external environment (e.g., soil or water).

The Wageningen_UR team (2021) engineered E. coli and P. putida to form a cross-feeding auxotrophic community, surviving only in cattle barn biofilters. Added quorum sensing and methane triggers caused cell death outside this niche, creating a multilayered containment system. (https://2021.igem.org/Team:Wageningen_UR)

3.2.2 Biologically Relevant Triggers

A critical aspect of biocontainment design is the selection of a trigger that reliably distinguishes between the "permissive" (target) and "non-permissive" (external) environments. A kill switch dependent on a synthetic, lab-specific chemical inducer (e.g., anhydrotetracycline, ATc) is not a valid biocontainment strategy for an LBP intended for use in the gut, as the inducer will not be present upon excretion. Teams should justify that their chosen trigger is biologically relevant. For a gut LBP, relevant triggers could include:

1. Temperature: The drop from ~37° C in the gut to ambient temperature.

The HNU-China team (2022) applied a λ phage CI857 temperature switch, keeping E. coli viable at 37 ° C in the gut but eliminating it at lower environmental temperatures. (https://2022.igem.wiki/hnu-china/)

2. Oxygen: The shift from the anaerobic environment of the colon to an aerobic external environment.

The PTSH-Taiwan team (2023) integrated an oxygen-sensitive promoter into their "SarcoTreat" LBP. In hypoxic intestines, toxin expression was suppressed, but in oxygen-rich environments it was induced, killing the bacteria. (https://2023.igem.wiki/ptsh-taiwan/)

3. Nutrient Availability: The absence of a specific gut-associated nutrient.

The NCKU_Tainan team (2019) engineered E. coli Nissle by deleting can and dapA, linking survival to gut-specific CO_2 and nutrients. This ensured the strain died in external environments, and the project won the Grand Prize. (https://2019.igem.org/Team:NCKU_Tainan)

3.2.3 Key Performance Indicators and Experimental Validation

Teams are encouraged to experimentally validate their biocontainment system and report on two key performance indicators.

1. Killing Efficiency / Escape Frequency: This measures the effectiveness of the system in eliminating the population upon activation.
    1. Procedure: An experiment can be designed to compare the number of viable cells (CFU) in permissive conditions versus non-permissive conditions. For a temperature-sensitive kill switch, this would involve plating cultures incubated at 37° C (permissive) and at room temperature (non-permissive).

2. Calculation: The escape frequency is calculated as the ratio of CFU·mL−1 in non-permissive conditions to CFU·mL−1 in permissive conditions.
3. Benchmark: The system should aim to demonstrate a high killing efficiency. Referencing guidelines from the National Institutes of Health (NIH), the target escape frequency should be less than 10−8. However, acknowledging the constraints of an iGEM project, a demonstrated escape frequency of less than 10−6 is a valuable benchmark for this standard.

2. Evolutionary Stability: As established in Part II, the genetic circuit encoding the biocontainment mechanism is subject to inactivating mutations.

1. Procedure: The serial passaging experiment described in Section 2.4 should be performed. The functional integrity of the biocontainment system can then be tested in the isolates from the end of the experiment.
2. Benchmark: A high percentage of the population (>99%) should retain the fully functional biocontainment system after prolonged growth without selection. An unstable kill switch is not a valid biocontainment system.

PART IV: NON-CLINICAL SAFETY EVALUATION

After characterizing the LBP and validating its control mechanisms, we advocate for direct experimental safety testing on the final, engineered product. This section outlines a suggested in vitro safety assessment to build a preliminary safety profile. The methods are selected to be feasible in a standard university cell culture facility while providing meaningful data on the LBP's potential to cause harm to human cells. The following table provides a summary of the key safety indicators and the recommended methods for their assessment, serving as a practical checklist for iGEM teams.

Table 1: Key Safety Indicators and Recommended Assessment Methods for iGEM LBP Projects

Safety Indicator	iGEM-Feasible Assessment Method	Acceptance Criteria / Goal	Rationale / Link to Principle
Chassis Identity	16S rRNA Gene Sequencing	>99% sequence identity to the intended reference strain.	Confirms the foundational organism is correct. (Principle 1)
Intrinsic Safety	Antibiotic Susceptibility Profiling; Literature/Genomic screen for virulence factors.	No acquisition of new antibiotic resistances; Absence of known virulence factors.	Ensures the starting organism is not inherently hazardous. (Principle 1)
Genetic Construct Integrity	Diagnostic PCR and/or DNA Sequencing of the final construct.	Correct amplicon size(s); Sequence matches the intended design.	Verifies that the engineered payload was constructed correctly. (Principle 2)
Genetic Stability	Serial Passaging (>50 generations) followed by PCR/functional analysis of isolates.	>99% of the population retains the intact, functional construct.	Ensures the LBP and its safety features are not easily lost through mutation. (Principles 2 & 3)
Biocontainment Efficacy	Kill Switch Induction Assay (CFU plating under permissive vs. non-permissive conditions).	Escape frequency $<10^{-6}$.	Confirms the active containment system effectively eliminates the LBP outside the target environment. (Principle 3)

Direct Cytotoxicity	Co-culture with a relevant human cell line (e.g., Caco-2 for gut LBPs); Measurement of cell viability via MTT or LDH assay.	>90% cell viability relative to an untreated control. The engineered LBP should not be significantly more cytotoxic than the wild-type chassis.	Directly tests whether the final LBP product is toxic to human cells. (Principle 4)

4.1 Framework for In Vitro Safety Assessment

The safety assessment follows a logical progression. Having established the identity and stability of the LBP (Principles 1, 2, 3), the final step is to evaluate its direct interaction with human cells (Principle 4). This provides the most direct evidence of its potential for toxicity.

4.2 Cytotoxicity Testing

Cytotoxicity testing is a standard and essential method for the biological evaluation of medical products and devices. It provides a direct measure of a substance's potential to damage living cells.

4.2.1 Recommendation and Rationale

It is highly recommended that teams assess the cytotoxicity of their final, engineered LBP on a relevant human cell line. This experiment aims to answer a critical safety question: "Does the engineered organism, as a whole, cause harm to human cells upon direct contact?"

4.2.2 Recommended Methodology

1. Cell Line Selection: The choice of cell line should be relevant to the LBP's intended site of action. For an LBP designed to function in the gastrointestinal tract, a human intestinal epithelial cell line such as Caco-2 or HT-29 is highly recommended.
2. Co-culture Experiment:
    1. Human cells are cultured to form a confluent monolayer in a multi-well plate.
    2. The LBP, prepared according to the standardized protocol (Section 2.3.2), is added to the wells at one or more defined concentrations (e.g., at a multiplicity of infection (MOI) of 10:1 or 100:1 bacteria to human cells).
    3. The co-culture is incubated for a defined period (e.g., 4-24 hours).
4. Essential Controls: The experiment should include the following controls for valid interpretation:
    5. Untreated Control: Human cells with media only (represents 100% viability).

6. Chassis Control: Human cells co-cultured with the unmodified, wild-type chassis strain at the same concentration as the LBP. This is crucial for determining if the genetic modification itself introduced cytotoxicity.
7. Positive Control: Human cells treated with a substance known to be cytotoxic (e.g., Triton X-100 or ethanol) to ensure the assay is working correctly.
8. Viability Measurement: After incubation, the viability of the human cells can be measured using a standardized assay:
9. MTT Assay: This colorimetric assay measures the metabolic activity of the cells. A reduction in activity is indicative of cell stress or death .
10. Lactate Dehydrogenase (LDH) Assay: This assay measures the release of the LDH enzyme from damaged cells into the culture supernatant. An increase in LDH activity indicates a loss of cell membrane integrity, a hallmark of cytotoxicity.

UM-Macau (2023) conducted adhesion and co-culture experiments using the human intestinal epithelial cell line Caco-2. Under both pH 7 and pH 5 conditions, engineered bacteria were co-incubated with confluent Caco-2 monolayers for 2 hours, after which non-adherent bacteria were washed off and adherent cells were quantified microscopically. The results demonstrated that strains expressing cp19k exhibited significantly higher adhesion to the Caco-2 surface compared to control strains. Prior to performing the experiment, the team consulted with the iGEM Safety Committee and restricted the scope to exclude any DNA/RNA extraction, thereby ensuring regulatory compliance and boundary control. To further improve methodological consistency, it is recommended that Caco-2 co-culture assays include pre-defined multiplicities of infection (MOI, e.g., 10:1 or 100:1), defined incubation windows (4 – 24 hours), and three control groups (untreated, chassis-only, and positive control) to assess whether genetic engineering introduces additional cytotoxicity (set MOI & time window; include negative/parental/positive controls) (https://2023.igem.wiki/um-macau/).

UNILausanne (2020) employed the MTT assay on Caco-2 human cells to evaluate the impact of engineered bacteria on cell viability. After 24 hours of incubation, metabolic activity was quantified colorimetrically, with untreated controls, chassis-only controls, and positive controls included to ensure data robustness. The results showed that engineered bacteria did not significantly reduce Caco-2 viability, indicating that the modifications did not introduce additional cytotoxic effects. This practice aligns well with the recommended control design and viability measurement outlined in this standard. Where feasible, the MTT (metabolic activity) assay may be combined with the LDH

(membrane integrity) assay for dual-parameter interpretation, enhancing robustness and maintaining consistency with the "viability measurement" requirements in Section 4.2.2 (https://2020.igem.org/Team:UNILausanne).

4.2.3 Interpretation of Results

The results should be reported as the percentage of cell viability relative to the untreated control. The primary comparison is between the engineered LBP and the wild-type chassis control. A safe LBP should not exhibit significantly greater cytotoxicity than its parent strain. An acceptable result would be cell viability remaining above 90% and no statistically significant difference compared to the chassis control.

4.3 Characterization of Secreted Products

If the LBP's therapeutic mechanism involves the secretion of a protein or small molecule, it is important to assess the safety of that specific payload component.

1. Procedure: The LBP is cultured according to its SOP. The culture is then centrifuged, and the supernatant is passed through a 0.22 µm filter to remove all bacteria. This sterile-filtered supernatant, containing the secreted product, is then applied to the human cell line, and a cytotoxicity assay is performed as described above.
2. Rationale: This experiment helps to de-convolute the effects of the live bacteria from the effects of the secreted therapeutic molecule. It can reveal if the payload itself is cytotoxic, independent of the chassis.

MIPT-MSU (2021) engineered mammalian cells to produce vesicles carrying specific therapeutic payloads. These vesicles were separated from the producer cells, collected from the culture supernatant, and processed under sterile conditions. The vesicles were then applied directly to HT-29 human colorectal adenocarcinoma cells. During subsequent incubation, the researchers observed clear GFP fluorescence signals within the recipient cells, indicating that the nucleic acids and membrane proteins contained in the vesicles successfully entered the target cells and exerted their intended function. This result demonstrates that the secreted product itself can exert a measurable biological effect on human cells independently of the chassis. By adopting a workflow of "separating the secreted product → applying it directly to human cells," the team was able to distinguish non-specific effects of the host chassis from the independent activity of the payload. This approach is fully consistent with the principle emphasized in this manual, "differentiating live bacterial effects from those of secreted molecules in safety evaluation," and provides a valuable precedent for other teams. We recommend that

future teams conducting similar experiments adopt such standardized workflows, including the necessary control groups (untreated, chassis control, and positive control) to ensure that the safety assessment of secreted products is both reliable and reproducible (https://2021.igem.org/Team:MIPT_MSU).

4.4 Final LBP Safety Profile Documentation

To conclude their safety evaluation, teams are encouraged to synthesize all the data generated in accordance with Parts II, III, and IV of this standard into a single, comprehensive "LBP Safety Profile" document. This document serves as the capstone summary of the project's safety engineering and validation efforts. It should be a clear, well-organized report that presents the methods, results, and conclusions for each safety assessment, demonstrating a thorough and responsible approach to the development of the engineered LBP.

PART V: DOCUMENTATION AND RESPONSIBLE CONDUCT

A core part of responsible innovation is thorough documentation and reflection on the project's broader implications. This ensures that the work is transparent, reproducible, and thoughtfully considers its potential impact.

5.1 Recommended LBP Safety Dossier

For teams working on LBP projects, we suggest compiling a "Safety Dossier" as a best practice for documenting your safety-related work. This is not a formal submission requirement but rather a way to organize and present your safety considerations comprehensively. A well-structured dossier could include:

1. A Summary of Adherence: A checklist or summary indicating which of the safety considerations outlined in this document were addressed in your project.
2. Key Results: A concise summary of the key findings from your safety-related experiments (e.g., "The chassis was confirmed as L. plantarum by 16S sequencing," "The kill switch demonstrated an escape frequency of 1.5×10^{-7}," "Cytotoxicity assay showed 95% cell viability, with no significant difference from the wild-type chassis.").
3. Supporting Documentation: A collection of the detailed information that supports your safety evaluation. This could include:
    1. The chassis safety justification (Section 2.1).
    2. The standardized production protocol (SOP) (Section 2.3.2).
    3. The complete data and analysis from the genetic stability study (Section 2.4).
    4. The complete data and analysis from the biocontainment validation experiment (Section 3.2.3).
    5. The final, comprehensive LBP Safety Profile document (Section 4.4), which includes the cytotoxicity data.

5.2 Ethical Considerations and Dual-Use

Beyond empirical safety testing, responsible innovation requires reflection on the broader societal and ethical implications of the technology being developed. The creation of an organism designed to modulate human health carries a significant responsibility. As part of your project's development, we encourage teams to consider and discuss the following:

1. Intended Use and Patient Population: Who is the intended user of this LBP? What is the specific condition it aims to treat? Are there any vulnerable populations involved?
2. Potential for Misuse (Dual-Use): Could the technology or the engineered organism be intentionally misused to cause harm? For example, could a system for delivering a therapeutic protein be re-engineered to deliver a toxin? Teams should demonstrate that they have considered these possibilities and, if applicable, describe any design features that might mitigate such risks.
3. Long-Term and Societal Impact: What are the potential long-term consequences of widespread use of such an LBP? How might it affect the human microbiome or the environment? This section encourages teams to think beyond the immediate scope of their project and engage with the broader conversation about the responsible deployment of synthetic biology.

This reflective exercise is aligned with iGEM's core mission of fostering a community of synthetic biologists who are not only technically proficient but also ethically and socially conscious.

ZJU-China (2021) created a dedicated section entitled “Dual use and possible danger” in their project’s Safety page, where they reflected on the potential misuse of their technology in unintended contexts. The team explicitly highlighted the need to establish boundaries in both information disclosure and experimental practices, and combined these with biosecurity measures to prevent misuse. This demonstrates that their focus went beyond laboratory-level compliance, as they proactively identified potential dual-use risks and responded with concrete measures, aligning with the principle emphasized in this manual of “anticipating risks and proposing mitigation strategies.” (https://2021.igem.org/Team:ZJU-China)

CAU-China (2021), in their Implementation page, stated the need to “consider possible dual-use … means to prevent dual-use,” integrating dual-use risk assessment with end-user scenarios and usage safety. This reflects an approach where ethical reflection is embedded directly into the application pathway (https://2021.igem.org/Team:CAU_China).

ECUST-China (2020), in their Safety page, incorporated dual-use concerns into a broader biosecurity framework, emphasizing “safety, security, and responsible conduct” as a unified standard. This provided a model for establishing a consistent ethical and safety language at the early stages of project development (https://2020.igem.org/Team:ECUST_China).

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