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LI-920 Microbial Cultivation and Molecular Characterization

November 2021

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Idaho National Laboratory

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MICROBIAL CULTIVATION AND MOLECULAR CHARACTERIZATION	Identifier: LI-920 Revision: 7 Effective Date: 11/02/2021 Page: 1 of 27

EES&T	Laboratory Instruction	**USE TYPE 4**	DCR Number: 691640

PURPOSE/SCOPE/APPLICABILITY

Research Activity Description (Provide a description to include the following):	
1.	**ACTIVITY LOCATION BY AREA, BUILDING NUMBER, AND LAB ROOM NUMBER:** REC IF 603 Labs A-2, A-5, A-8, A-9, B-7, B-8, B-9, 100, 101, 103, 104, 108
2.	**ACTIVITY LAB MANAGER: Sandy Fox** Phone: 6-4985; e-mail: Sandra.Fox@inl.gov **PRINCIPAL RESEARCHER: David Reed** Phone: 6-7788; e-mail: David.Reed@inl.gov **LABORATORY SPACE COORDINATOR: David ReedLABS: A-2, A-8, 101, 108** **LABORATORY SPACE COORDINATOR: Caitlin McNamaraLABS: B-7, B-8, B-9, 104** **LABORATORY SPACE COORDINATOR: Rebecca Brown** **LABS: A-9, 100** **LABORATORY SPACE COORDINATOR: Chris Orme** **LABS: 103, A-5**
3.	**PROGRAM OBJECTIVES:** The purpose of this work is to conduct microbiological, molecular biological and biochemical research using Risk Group (RG-1) organisms (e.g., bacteria, archaea, yeast, fungi, and algae), nucleic acids (DNA/RNA), and amino acids (proteins/enzymes). This research monitors microbes or microbial communities under a variety of growth conditions. This activity supports multiple programs. The work will involve cultivation, characterization of microbes, nucleic acids, and amino acids using standard microbiological, biochemical and molecular methods that include the following overlapping tasks: • Cultivation, including enrichment and isolation of microorganisms • Growth and activity measurements • Growth substrate preparation • Bioleaching elements with microbial processing • Preservation and disposal of microorganisms • Microscopic observation and photography

- Use of chemical laboratory techniques
- Processing of cell mass, DNA/RNA, proteins/enzymes and metabolites
- Working with recombinant or synthetic nucleic acid molecules and microorganisms
- Processing of off-site field samples (collection of environmental samples)

Experiments will be performed using good microbiological practices which are outlined in Appendix C of LWP-14621 "Laboratory Biological Experimentation Safety" and are summarized in Appendix A.

LI scope bounding conditions for activities:

- Will not include the cultivation of microorganisms from Risk Group 2 or higher
- Will not include radiological activities
- Will not include the use of vectors or genetic systems that induce the production of toxins or lead to pathogens
- Will not include plant recombinant or plant synthetic nucleic acids work

4. # PROJECT/ACTIVITY DESCRIPTION:

This LI describes standard microbiological and molecular laboratory methods (Methods for General and Molecular Bacteriology, Gerhardt et. al.)[1] used for the cultivation and characterization of microorganisms and their internal parts. Techniques include routine procedures related to general/basic microbiology, molecular biology, chemistry and biochemistry plus procedures related to the processing of metabolites, nucleic acids, and amino acids. Additionally, the LI will include cultivation of recombinant **or synthetic nucleic acid molecules from microorganisms.**

The environmental samples that will be used for these research activities are not expected to be taken from Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) sites or known to be contaminated with listed Resource Conservation and Recovery Act (RCRA) hazardous waste. However, work that may involve materials that are considered RCRA waste (e.g., electronic scrap) will be addressed with Waste Generation Services (WGS) or the Program Environmental Lead to ensure proper handling and disposal as well as determining if the work should be classified as a treatability study or not.

- If samples/materials are determined to be radioactive the work cannot be performed under this LI.
    - Ore (and tailings), phosphate, or coal (and coal ash) samples believed to contain rare earth elements and other metals with a potential for near background levels of radioactivity will be received by Rad Con in IRC-603 for a radiological survey and further direction depending upon the results of that survey. Natural derived samples (including those with rare earths) with surveys that indicate levels near or below background will be handled without any additional radiological controls.
    - In the event that the sample material is highly suspected to contain radiation and/or contamination or that the radiological control technician, supervisor, or engineer determines that there is a radiological concern in the material this work will not be performed under this LI.

Growth of Organisms:

Microbial research activities described in this LI involve the use of Risk Group-1 (RG-1) whole organisms, (e.g., bacteria, yeast and other fungi, viruses and algae) that are obtained from a culture collections (e.g., American Type Culture Collection, German Collection of Microorganisms), or other research institutions or environmental samples. Handling specific organisms will be evaluated on a case-by-case basis. Work with these organisms is done using Biosafety Level 1 (BSL-1) controls and practices.

Cultivation of microorganisms including recombinant microbes is performed using a variety of general defined and undefined medium (e.g., yeast extract, peptone) preparations. These media contain the organic and inorganic compounds necessary for cell growth with a pH range that varies between 0.5 and 11. Growth media is sterilized prior to use via autoclaving or filtration. It is then inoculated and incubated at the appropriate temperature, which, depending on the organism can vary between 4ºC and 100ºC. The cultures may be incubated using a shaker incubator, dry bead bath, and water bath or heat blocks. Cultivation occurs in a variety of vessels such as flasks, bottles, Chemostats, Petri dishes and culture tubes under aerobic or anaerobic conditions.

Cultivation of microbes may be include agriculture residue (e.g., corn stover, potato processing water, date palm), spent food from grocers or industry (e.g., cellulosic, fruit, vegetable, nut, legume), municipal disposal material (e.g., landfill or sanitation) or soil (e.g., ore, tailings, spring water). Materials will be preprocessed or pretreated by fractionating components, microwaving, heating, grinding, sieving, blending, filtering, chemical treating (e.g., acid, base) and/or enzymatic hydrolyzing. Cultivation substrates and nutrients may be sterilized (e.g., 0.22 µm filtration, autoclaving). Cultivation will be conducted as describe in Table 2.01 and media will be autoclaved prior to disposal.

Phototrophic microbes may be grown in vessels that are incubated under timer controlled fluorescent lighting in incubators equipped with lights or, if needed, custom built fluorescent and LED light systems. There are several classes of bacteria (e.g., sulfate reducers, methanogens, methanotrophs, sulfur oxidizers, and denitrifying bacteria) that may be cultivated and characterized under this LI that are capable of producing hazardous end-products. Potentially hazardous by-products that require special mitigation are listed in Table 2.01 “Risk and Controls”.

Growth and activity measurements, preservation and disposal of microorganisms:

Growth may be monitored by measuring the optical density using a spectrophotometer, microscopic enumeration, colony counts, most probable number, metabolite or gas production, or wet/dry biomass measurements. Inoculum may be transferred between vessels using pipettes, syringes, or inoculating loops using standard microbiological aseptic technique. Cells are collected from growth media by centrifugation or filtration and washed then stored in physiological saline, nutrient salts solutions or various buffers pending further use. Fungal agar plugs, obtained using a cork borer, may be homogenized in a blender and then used to prepare cultures. Cultures may be preserved for long term storage by freeze-drying or cold storage (-80ºC to 4°C) in buffer solutions or culture media containing glycerol or dimethyl sulfoxide (DMSO). Cryogenic materials, including dry ice and liquid nitrogen, may also be used in the preservation and storage of cultures and biological materials.

Following use, spent-liquid media and biological contaminated materials will be sterilized using an autoclave or bleach solution and neutralized prior to disposal. Refer to Table 2.04 for disposal of acidic and basic media and agar plates. These types of materials may be temporarily stored in

a fume hood prior to sterilization. When stored the items will be appropriately labeled to indicate pH and associated hazards (e.g., metal content or presence of antibiotic).

Chemical laboratory techniques:

Many of the methods used to characterize microbial activities involve common chemical laboratory techniques such as: weighing, measuring and dispensing of chemicals and reagents; use of compressed gases; filtration; use of low pressure and <25 in Hg vacuum equipment; measurement of pH, redox potential, temperature; extraction or crystallization of chemicals or metabolic products from aqueous or organic phases; use of ground-joint glassware; heating, cooling, freezing and freeze-drying of materials. Glassware may be soaked in <6N hydrochloric or <10% nitric acid to remove contaminating metals. Working in a fume hood, bottles and flasks will be filled with the acid and stored overnight in a large 10-20L polypropylene container.

Bioleaching is conducted as outlined in Table 2.01 with lixiviants (e.g., microbial spent medium, organic acids, mineral acids) on materials that contain metals (e.g., rare earth elements, copper, gold, tellurium, vanadium, nickel, cobalt, manganese) from natural and industrial materials (e.g., ore, ore tailings, phosphate clay, phosphogypsum, magnets, battery components, fluid catalytic cracking catalyst, phosphors, coal, ash, municipal solid waste) in conical plastic tubes or glass bottles/flasks shaking or stationary in incubators at 20-80°C. Leachate is filtered prior to analysis and spent materials are disposed as outlined in Table 2.04.

Microscopic observation and photography:

Microorganisms are attached to solid surfaces such as slides, membranes or minerals by natural adherence, filtration, heat or chemical treatment. Cells are prepared using various fixatives (such as 2% glutaraldehyde or 2% formamide), stains (such as Acridine orange or 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI)) and reagents including nucleic acid probes and antibodies, with and without fluorescent tags. Microorganisms or microbial cell products are visualized using a variety of microscopes including: light, epi-fluorescent and confocal. Images are captured and processed using standard photographic and digital imaging devices and associated software.

Biological cell mass, nucleic acid, proteins, and metabolite isolation:

Cell biomass, nucleic acid, proteins and metabolites can be isolated from growth medium and/or microbial cells. Growth medium can be filtered or centrifuged to remove cells for direct analysis. In contrast, cells can be isolated from growth medium using centrifugation and then lysed using a variety of physical and chemical methods for cellular analysis.

Physical methods of cell lysis include use of a French pressure cell, microfluidizer, sonicator, bead beater, blender and mortar and pestle. Protocols for using these techniques are derived from instrument manuals which are located near the instruments. These instruments are used infrequently and for short durations.

Chemical cell lysis may involve the addition of detergents or enzymes (e.g., lysozyme, protease K) and reducing agents (e.g., DTT) to inhibit protein degrading enzymes. Commercial cell lysis kits using similar reagents are also used similar to manufacturer directions.

Following cell lysis, membrane, soluble, and insoluble fractions can be separated via (ultra)centrifugation, suspended in various buffers and/or gradient material (e.g., sucrose, cesium chloride, dextran, PEG) and stored in a freezer or refrigerator pending further processing.

Metabolite analysis: Cultures may be grown in sealed bottles for head space gas analysis by Gas Chromatography (GC). Head space samples will be taken using a gas tight syringe and injected directly into the GC.

Protein and metabolite processing:

Protein may be processed with the addition of detergents (e.g., SDS, Tween, CHAPS), reducing agents (e.g., TCEP), and alkylating compounds (e.g., iodoacetamide) in addition to enzymatic digestion (e.g., trypsin). Proteins and peptides are further processed via electrophoresis (e.g., PAGE, capillary), chromatography (e.g., thin layer, various liquid processes such as HPLC) and mass spectrometry (various) often with addition of biological buffers and organic compounds (e.g., acetonitrile). Proteins are concentrated with membranes containing low molecular weight cut off masses by centrifugation or ultrafiltration often under air pressure.

Nucleic acid and protein separation:

Electrophoresis, the migration of ions in an electric field, separates nucleic acids and proteins. Agarose gels for electrophoresis are prepared by heating agarose in an electrolyte solution until the agarose is dissolved, and then pouring the resulting solution into a tray for solidification. At times, RNA or DNA may be electrophoresed through agarose gels that contain denaturants (e.g., formaldehyde) and proteins may be electrophoresed through non-denaturing or denaturing polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE). Following preparation, the gels are placed into an electrophoresis chamber, covered with an electrolyte buffer, loaded with sample, and exposed to an electric field for protein separation.

Nucleic acid and protein visualization:

Following electrophoresis, nucleic acids and proteins present in the gels are visualized using a variety of stains (< 100 µg/mL). Nucleic acids are generally stained using DNA intercalating dyes (e.g., ethidium bromide, SYBR). Proteins are visualized using prepared or commercial stains (e.g., Coomassie Brilliant Blue, silver nitrate) with reagents (e.g., methanol, acetic acid). After staining, the gels are viewed using either an ultraviolet transilluminator box or a UV-Vis viewing-imaging system (Alpha Imager). Proteins concentrations are determined with colorimetric assays (e.g., Lowry, Bradford with spectroscopy following) and enzyme activities are assayed using chromogenic, fluorogenic, and colorimetric reactions in gels or followed with spectroscopy measurements.

The Dinitrosalicylic acid (DNS) assay is a common colorimetric technique for the determination of reducing-sugars. DNS contains phenol; at room temperature phenol is in the crystalline form which will be used in a fume hood, heated to a liquid state in a secondary container in a 50°C water bath. Working on the bench top <3 mL per tube of the DNS reagent will be pipetted into several test tubes, covered with aluminum foil and placed in a test tube rack which is then placed in boiling water bath in the hood. The tubes will be cooled in the hood and then transferred to the bench top for pipetting into cuvettes or multiwall plates and analyzed via absorption spectroscopy.

Nucleic acid and protein hybridization:

Microbial derived nucleic acids may be hybridized to characterize specific gene sequences for their expression. Nucleic acid may be bound to membrane filters such as nitrocellulose and labeled with a fluorescent labeled nucleic acid probe. In situ hybridization is also performed where fluorescent nucleic acid probes are hybridized with whole microbial cells. The resulting preserved cells are then applied to microscope slides, hybridized with fluorescent probes and viewed using an epifluorescent microscope.

Following separation by gel electrophoresis, proteins are transferred onto a membrane, incubated with a blocking solution, and allowed to interact with an enzyme such as alkaline phosphatase or horseradish peroxidase conjugated to antibody. Binding of the antibody to the protein is detected by the addition of substrate, which the converts enzyme into a colored product that can be visually observed and photographed.

DNA amplification:

The polymerase chain reaction (PCR) is used as a method to amplify nucleic acid sequences and potentially add restriction enzyme sites for cloning. During PCR, template DNA or RNA is mixed with enzymes, buffers, and oligonucleotide primers in reactions < 100 microliters and heated up to 100°C in a thermocycler for amplification. The primer may include a fluorescent dye at extremely low concentrations and PCR additives may contain DMSO, formamide, glycerol, and/or non-ionic detergents. All stock reagents are typically 20 µL to 2 mL. Samples may be prepared on the bench top or in a PCR workstation. The workstation is a ductless, laminar flow enclosure equipped with a UV germicidal light and is used to decrease cross contamination with the environment and other DNA samples.

Recombinant or synthetic nucleic acid molecules manipulation:

These activities include nucleic acid manipulation, mutation, cloning, transfer and expression. Because this work involves the use of recombinant or synthetic nucleic acid molecules, form 420.11 “Notification of Intent to Work with Recombinant DNA Molecules, Large Scale Culture, or Biohazardous Agents” is associated with this document. DNA is manipulated for cloning or analysis using standard molecular methods (Molecular Cloning, Sambrook et. al.)[2]. Methods may include restriction digestion, ligation, (de)phosphorylation, etc., at small volumes <100 µL and temperatures <60°C. Introduction of nucleic acid into host cells is done using plasmids that carry genes of interest including reporter, antisense and drug-resistance genes.

Transformation, conjugation and electroporation are used to insert the plasmids into host cells. Transformation and conjugation are standard cell manipulation methods; whereas, electroporation is a physical technique. Electroporation is the application of high voltage electric field pulses of short duration in order to create temporary pores or holes in the membrane of cells to allow nucleic acid to enter the cell.

Plasmid DNA replicates and expresses a drug resistant gene inside the host (e.g., bacteria or yeast cell). This allows transformed microbes to grow in the presence of an antibiotic and serves as a means to identify and recover cells that potentially carry the gene inserts of interest. Antibiotic stock solutions (<500 mg/mL) are prepared and then added to growth medium at a final concentration of <500 µg/mL.

Processing of Off-Site Field Samples:

	Periodically environmental samples will be collected in the field for enrichments and isolation. Liquid and solid samples will be collected and brought back to the lab for processing. Liquid such as water and solids such as soil, mud or other debris will be used for microbial enrichments. 1. **Off-site field work:** Prior to the sampling effort the Principal Researcher will discuss the particulars with the Laboratory Manager to evaluate the field sampling situation and fill out form 420.15 "Off-Site Work Request" Additionally, the field sampling will be done in accordance with off-site work permits if required and abide by the safety procedures of the sampling location (e.g., Yellowstone National Park Thermal Area Safety Procedures)**.** **Biological Testing**: Water samples and solid samples collected for microbial cell counts will be amended in the field with a fixative for cell preservation. Samples will be disposed of per source composition and INL guidelines.
5.	**MAJOR EQUIPMENT USED IN ACTIVITY:** No major equipment will be used. All minor equipment is operated per manufacturer's instructions and manuals are available. For use of equipment that poses specific hazards, Performer Controlled Activity (PCA) work documents are followed (see Table 2.02).

1. RISK AND CONTROLS

Table 2.01 Risks and Controls

Gloves for handling chemical materials will be nitrile (>4 mil) unless chemical handling requirements dictate otherwise (see 420.07 for specific glove recommendations). Gloves and lab-coats are recommended but not required for handling biological samples. When hearing protection is required use ear plugs or ear muffs with a minimum NRR of 20.

Safety glasses with side shields are required for all laboratory activities (exception includes microscopy). Substantial footwear (e.g., leather shoes), sleeved shirts, and long pants required for all activities.

Activity/Task	Hazard	Engineering Control	Administrative Control	Personal Protective Equipment (PPE)
Growth and handling of microbes (including recombinant or synthetic nucleic acid molecules)	Biological hazards		Decontaminate work surfaces (when used for microbial procedures) daily when in use and after spills using either a 10% bleach solution (1% sodium hypochlorite), ethanol (70-85% by weight) or commercial decontamination solution When practical wash hands after handling live microorganisms, after removing gloves, and prior to leaving the laboratory area If gloves are worn and become contaminated, take appropriate measures to avoid spreading the contamination to surfaces such as instruments, bench tops, notebooks and doors	

			Autoclave gloves and other contaminated items prior to disposal Do not pipette by mouth; use mechanical pipetting devices.	
Cultivation and handling of microbes (including recombinant or synthetic nucleic acid molecules) **when aerosols could be generated potentially releasing the microbe**	Generation of aerosols when transferring culture, filtering, or centrifuging Antibiotic sensitivity	Use aerosol barriers with centrifuge, filtration or filtration devices and aerosol-filter pipette tips If desired perform transfers in a biological safety cabinet (BSC) to contain aerosols	Use disposable inoculation loops and autoclavable toothpicks or allow metal loops to dry prior to flame sterilization, avoid splashing or spilling of viable materials Use sealed capped bottles at volumes per manufacturer recommendation Visually inspect sealing O-rings and grease periodically Briefing of hazards and sensitivities Staff will be informed of hazards and sensitivities associated with antibiotics and disinfectants	
Filtering recombinant or synthetic nucleic acid molecules	Generation of aerosols or a spilled sample could be released		Use RG-1 microorganisms, non-pathogenic vectors, in-line filters, secondary containment, and quality seals	
Handling and cultivation of spore-forming fungi	Release of spores	Perform transfers in BSC to reduce release of spores	Decontaminate work surfaces with 10% bleach solution (1% sodium hypochlorite); avoid splashing or spilling of viable materials	

Cultivation of phototrophic microbes (i.e., cyanobacteria, algae)	Fire hazard due to the heat generation of the lighting systems		Consult electrical authority having jurisdiction (AHJ) to assess electrical and lighting set-up prior to use	
Use of carbon dioxide and air to enhance photosynthetic growth	Asphyxiation, over pressurization		The air flow rates into the vessels will be less than 200 ml/min and the CO_2 flow will be less than 2 mL/min (i.e., ~1% of the total gas stream will consist of CO_2) Vent vessels to prevent over pressurization Off gases will be vented to the room air and will include un-utilized CO_2 and O_2; Note: Use of CO_2 in confined work areas (e.g., environmental chambers, closed incubators) will be conducted under a separate LI	
Performing general instrument maintenance	Exposure to electrical components/electric shock		Turn off and unplug instrument. Exclusive cord and plug control within eyesight and hands reach of individual performing activity.	
Handling cultures and materials (e.g., DNS tube assay, enzyme vial assays) when incubated at temperatures >50ºC	Contact skin burn, splashing of hot liquids	Use of chemical fume hood if chemical is an inhalation risk per Form 420.07. Heat blocks are enclosed, and the sample trays act as barriers between the heat block and the operator		Wear heat/steam resistant gloves when handling glassware, tubes, vials or metals
Cultivation of cultures or bioleaching lixiviant (e.g., spent media) in	Samples may contain heavy metals	Transfer , cultivate, or bioleach in chemical fume hood if	If gloves are worn and become soiled, take appropriate measures to avoid spreading the material to	

contact with potentially hazardous metals (e.g., ore, ore tailing, magnet swarf, catalyst, rare earth metals, phosphors, coal, coal ash, phosphates, municipal solid waste, bottom ash, magnets, battery cathode, and other recyclable electronic materials)	Waste samples may be considered RCRA, industrial or hazardous wastes	materials pose an inhalation risk at a specific processing step	surfaces such as instruments, bench tops, notebooks and doors If using waste material, then consult the R&D Program Environmental Lead to determine whether the activity might be considered a RCRA treatability study and have additional requirements	
Drying materials at temperatures above the vaporization temperature for those that contain hazardous volatiles or organics	Potential generation of inhalation and flammability hazards	Put oven or muffle furnace in a chemical fume hood if chemical is an inhalation risk from metal or organics	Contact IH for monitoring Make sure that oven components are compatible with the oven temperature	
Analyzing headspace with Gas Chromatography (GC)	Chemical exposure, inhalation hazard	Use standard gas mixes for GC calibration (H_2S, CO) in the hood; Vent GC with trunk line		
Biological hydrogen sulfide production (<0.2mg/bottle/hr maximum) or H_2S generation from solid sulfur reduction at pH 2-12.5	Potential generation of inhalation and flammability hazards Asphyxiant	Transfer in chemical fume hood; grow cultures in sealed vials, or anaerobic chamber with charcoal absorbent	Contact IH to determine if monitoring is required or to discuss if additional controls are needed Keep away from flames or other ignition sources	
Biological methane and hydrogen production	Potential generation of flammability hazards	Transfer in chemical fume hood; grow cultures in sealed vials, or anaerobic chamber	Contact IH to determine if monitoring is required	

			Keep away from open flames, sparks or other ignition sources (e.g., Bunsen burners)	
Sterilization of low pH (pH<5) biological liquids	Potential generation of chlorine gas if treated with household bleach	Autoclave only (in secondary container)		
Sterilization or decontamination of biological liquids containing flammable concentrations of organic compounds	Potential generation of inhalation and flammability hazards if autoclaved	Use of chemical fume hood to control vapors	Use appropriate chemical disinfecting agent, consult with IH and WGS as needed	
Decontamination of recombinant or synthetic nucleic acid molecules and/or biological materials, including antibiotics	Biological contamination		Autoclave or otherwise sterilize (e.g., 10% bleach) all viable materials prior to disposal	
General Chemical Handling	Inhalation, skin/eye exposure	Use of chemical fume hood if chemical is an inhalation risk per Form 420.07	Review product safety data sheet (SDS)	Gloves contaminated with hazardous chemicals may need to be disposed of as hazardous waste; use of a dust mask is optional for chemicals that are powdery upon weighing
Handling Particularly Hazardous Substances (PHS) (e.g., carcinogens,	Inhalation, ingestion,	Use of chemical fume hood if chemical is an inhalation risk per Form 420.07	Post sign in lab as a designated PHS area; Limit use to as small as feasible	

reproductive toxins and/or highly toxic agents)	skin/eye exposure		Consult with IH to determine monitoring issues and requirements	
Cell extractions	Liberation of chlorine and hydrogen gas	Perform task in chemical fume hood Use containers (Hungate tubes, orange cap bottles) that handle increased pressure or keep cap cracked open	Contact IH for monitoring chlorine gas	
Handling corrosive chemicals (when containers can be easily handled in fume hood).	Inhalation, skin/eye exposure	Handle in chemical fume hood (sash used as a barrier; adjust height to protect face and eyes in the event of a splash)		Safety glasses, lab coat and nitrile gloves or chemical specific gloves per Form 420.07 (e.g., butyl rubber) For acids >1 M wear an apron when transferring contents of large primary containers into smaller containers or using >500mL
Handling corrosive chemicals (when containers cannot be easily handled in a fume hood)	Inhalation, skin/eye exposure			Chemical splash goggles, face shield, lab coat, apron and double nitrile gloves or chemical specific gloves per Form 420.07 (e.g., butyl rubber)
Handling and use of acids (e.g., acetic, hydrochloric, nitric and sulfuric acid)	Inhalation, skin/eye exposure	Handle in fume hood .		Chemical splash goggles, face shield, lab coat, double nitrile gloves or chemical

				specific gloves per Form 420.07 (e.g., butyl rubber) and an apron when transferring large primary containers into smaller containers or using >500mL.
Handling of acids and bases for pretreatment of biomass (e.g., corn stover, date palm, municipal solid waste)	Inhalation, skin/eye exposure	Conduct lytic steps (e.g., uncovered slurry preparation, transfers and filtration) in fume hood. Use fume hood sash as secondary barrier. Lower hood sash to a position that protects the face and neck against splash.		Lab coat and gloves. For steps that cannot be conducted in hood and splash or spillage is a concern, use face shield, lab coat, double nitrile gloves or chemical specific gloves per Form 420.07 (e.g., butyl rubber) and an apron.
Use of acid bath when immersion or prolonged exposure may occur	Inhalation, skin/eye exposure, splashing	Handle in chemical fume hood		Goggles, face shield, lab coat, apron, and gloves
Handling chemicals with significant inhalation risks including materials that have fine powders (phosphors, coal ash, etc.)	Inhalation, ingestion, skin/eye exposure	Transfer in chemical fume hood	If transfers cannot be made in a hood, contact IH to determine what additional controls, including monitoring for airborne exposure levels	
Handling and use of flammable liquids and combustible materials	Fire or explosion	Transfer in a fume hood	Keep away from flames or other ignitions sources; Store flammable solvents in approved cabinet	
Handling heavy metals (Pb, Cd, Hg, etc.,) samples	Inhalation; Ingestion; Skin/eye exposure;	Samples will be handled in a laboratory hood if dust may be created	Contact IH if levels do or may exceed 1000 ppm	Lab coat and gloves

	acute/chronic toxicity			
Preparation of DMSO stock solution from DMSO reagent bottle	Skin, eye and mucous membrane exposure	Handle in fume hood		Lab coat and gloves
Handling solutions that contain diluted DMSO or solvents (e.g., acetonitrile, hexane, methanol, tetrahydrofuran)	Skin, eye and mucous membrane exposure	Handle in fume hood	Handle with care	Lab coat and and nitrile gloves or chemical specific gloves per Form 420.07 (e.g., butyl rubber)
Discarding unused hazardous chemicals, empty vendor containers, contaminated PPE or spill residues	Potential mismanagement of hazardous waste		Contact WGS for guidance	
Handling cryogenic liquids (Transferring and use of ≤ 1 liter)	Cryogenic burn			Use of tongs if appropriate
Handling cryogenic liquids (Transferring and use of > 1 liter)	Cryogenic burn			Face shield, safety glasses w/ side shields, cryogenic gloves, substantial foot wear and lab coat or apron
Handling cryogenic liquids (Filling a Dewar from the filling station)	Cryogenic burn, noise			Face shield, safety glasses w/ side shields, cryogenic gloves, substantial footwear and lab coat or apron and hearing protection
Handling and use of compressed gas cylinders with	Over pressurization event, pinch	Use of cylinder cart and cylinder racks with chains; Use of purchased pressure release	Transport cylinders with valve cap secured hand tight. Secure cylinder	Substantial footwear such as leather shoes or composite toe caps for

equipment (i.e., glove box or anaerobic chambers)	point, asphyxiation	valves and gas regulators; ultrafiltration device and membranes will be located downstream of pressure relief valve and operated per manufacturer's instruction	at user location and empty storage area Limit number of cylinders in use; contact IH to monitor asphyxiation hazard if two or more cylinders are in use on a manifold at once	handling and transporting cylinders
Pressurized system set-up (e.g., ultrafiltration cell, anaerobic chamber, gassing station manifold)	Over pressurization event	Use pressure relief valves; minimal allowable operating pressure set to less than pressure relief valve	Pressure system assembler to assemble; qualified independent peer to review setup. Document the following in a laboratory notebook: Configurational changes Reaction conditions (temperature, pressure, materials, time, maintenance performed)	
Use of electrical equipment (electrophoretic unit and electroporator)	Contact or equipment failure can result in electric shock, arc-flash burn, fire, thermal burn, or blast	The equipment is: **a.** listed by a Nationally Recognized Testing Laboratory (NRTL) (e.g., UL, CSA, FM, etc.) or, **b.** approved by INL National Electrical Code (NEC) Authority Having Jurisdiction (AHJ) if there is no NRTL listing or, **c.** approved by INL NEC AHJ if designed, fabricated, and assembled at INL	Verify that NRTL (e.g., UL, CSA, FM, etc.) marking(s) or, INL NEC AHJ approval sticker(s) are present prior to use	
Use of blender	Noise		Contact IH to monitor noise levels posting the "High Noise Area" if	

			levels are determined to exceed 85 dBA.	
Use of sonicator, microfluidizer or similar equipment	Noise	Operate sonicator in sound enclosure when available	Contact IH to monitor noise levels;	Hearing protection
Use of glassware under vacuum (1/3 HP; <25 in Hg vacuum)	Broken glass, flying glass shards		Inspect glassware for defects; use properly designed glassware; Use low vacuum pump with a limit of 25 in Hg	Wear ANSI level 4 protective gloves to handle broken glass
Use of razor blades, box cutters or scalpels	Laceration		Dispose of blades in sharp item container; work awareness	Wear ANSI level 4 cut resistant gloves When fine cutting control is required as is the case for cutting DNA from agarose gels, then wear double layers of nitrile gloves. Scalpel is recommended (vs razor blade)
Use of needles	Puncture		Dispose of needles in sharp item container; work awareness	
Use of syringes on filters	Disengagement of filter from syringe and exposure to chemical,	Shielding, luer-lock syringe	If luer-lock is not available do not apply too much pressure (that could separate filter from syringe)	Eye protection, face shield if luer-lock is not available
Ergonomic hazards	Prolonged standing, repetitive pipetting, extensive		Identify appropriate tools and work techniques to minimize musculoskeletal stress and repetitive body motions. Assess and optimize workstation and equipment ergonomics	

	microscope work		Take a 5 min break after a maximum of 30 minutes of repetitive tasks Contact IH with questions or concerns Floor mat or ergonomic shoes to relieve stress from prolonged standing; ergonomic chairs	
UV transilluminator	Eye and skin damage	Illuminator only functions with door closed Plexiglas covering attached to illuminator	Situational awareness	Wear safety glasses and face shield that have protection against UV wavelengths

Table 2.02 Performer Controlled Activity Lists

The following work tasks are performed using TEM-21221, Performer Controlled Work (PCA) lists.

Task	PCA List Number and Title
Harvesting cultures, separating nucleic acid, proteins, metabolites	LST-450; Performer Controlled Activity for Operation of the Ultracentrifuge
Harvesting cultures, separating nucleic acid, proteins, metabolites	LST-452; Performer Controlled Activity for Operating Centrifuges
Sterilization of media, glassware, and supplies	LST-453; Performer Controlled Activity for Operating the Autoclaves
Decontaminating glassware for RNA work, processing biological cell mass	LST-457; Performer Controlled Activity for Using Drying Ovens and Muffle Furnaces
Cellular rupture for enzymes	LST-521; Performer Controlled Activity for Operating the Microfluidizer
Microbial cultivation, recombinant or synthetic nucleic acid molecules cultivation	LST-812; Performer Controlled Activity for Using Bunsen Burners

Table 2.03 Risk and Controls.

Hazard scenarios that require mitigation.

See Table 2.01

Table 2.04 Waste Generation.

Type of Waste	Generation Location	Anticipated Volume	Container Type	Disposal Method
Biological debris-including recombinant or synthetic nucleic acid molecules (Agar plates-neutral pH, used pipette tips, wipes and towels used to clean up a biological spill)	A-2, A-5, A-8, A-9, B-7, B-8, B-9, 103, 104	10 cu. Ft/Yr	Polypropylene autoclave bag or autoclavable containers	Autoclave the bags and dispose in general trash receptacles
Elementary neutralization of solutions with pH <5 or ≥9; Non-RCRA solutions	A-2, A-5, A-8, A-9, B-7, B-8, B-9, 103, 104	20-50 L/Yr	NA	Dispose of in municipal sewer drain after neutralization
Empty reagent bottles or gas cylinders	A-2, A-5, A-8, A-9, B-7, B-8, B-9, 103, 104	NA	NA	ICMS empty container box in laboratory; gas cylinder “empty” cage
Glass (broken glassware)	A-2, A-5, A-8, A-9, B-7, B-8, B-9, 103, 104	3 cu. Ft./Yr	Cardboard containers	General trash
Flammable hazardous waste (generated from chromatography, assays, culturing, including kits) (e.g., acetone, acetonitrile, ethanol, hexane, isoamyl alcohol, tetrahydrofuran, methanol, isoprene)	A-8, A-9, B-7, B-8, B-9,	< 5 L/Yr	Chemically compatible bottles (250 mL-4 L)	WGS
Industrial waste from enzyme assays, protein purification, molecular biology, including kits (e.g., dyes (DNS), >3M imidazole, and >0.1% DMSO, formaldehyde, glutaraldehyde, certain antibiotics)	A-2, A-8, A-9, B-7, B-8, B-9	< 5 L/Yr	Chemically compatible bottles (250 mL-4 L)	WGS
Hazardous or sewer regulated wastes (e.g., heavy metals; Ag≥5ppm; As≥5ppm; Ba≥100ppm, Cd≥1ppm; Cr≥5ppm; Hg≥0.2ppm; Pb≥5ppm; Se≥1ppm) such as those generated by bioleaching	A-8, A-9, B-7, B-8, B-9	10-50 L/Yr	Labeled leak proof containers	WGS

Non-hazardous, non-sewer regulated, non-recycled, Non-biological debris (PPE, lab ware, towels and wipes used to sterilize work areas)	A-2, A-5, A-8, A-9, B-7, B-8, B-9, 103, 104	20-50 L/Yr	NA	Dispose in the municipal sewer drain or in general trash receptacles
Recycled plastics (non–chemical lab ware, pipette tip, plastic ware)	A-2, A-5, A-8, A-9, B-7, B-8, B-9, 103, 104	4 cu. Ft/Yr	Blue plastic recycle bin	Recycled plastics
Recycled liquid waste (e.g., machine oil)	A-8, A-9, B-7, B-8, B-9	< 5 L/Yr	Chemically compatible bottles	WGS
Sewer restricted waste from assays (e.g., phenol)	A-8, A-9, B-7, B-8, B-9	2-10 L/Yr	Chemically compatible bottles	WGS
Sharps (Razor blades, sharps, needles)	A-2, A-5, A-8, A-9, B-7, B-8, B-9, 103, 104	<5 L/Yr	Hard sided	Autoclave hard sided container, place in an opaque bag and place in general trash receptacles
Oxidizers or oxidizing solutions (e.g., sodium chlorite, ammonium nitrate, ammonium persulfate)	A-8, A-9, B-7, B-8, B-9	< 5 L/Yr	Chemically compatible bottles	WGS
Precious Metals (e.g., gold)	??	??	??	Precious metal recycle through Property Management

List any special needs/requirements for storage and handling wastes.

None.

Method to clean up spills:

- Microbial work areas and spills will be wiped up using a 10% household bleach solution (<1% sodium hypochlorite) if medium pH is 5-8, alcohol (ethanol, 70-85% by weight), or a commercial disinfectant.
- DNA recombinant or synthetic nucleic acid molecules spill to be reported to the BSO and IBC chair.
- Chemical spills will be cleaned up in accordance with manufacturer and facility guidelines.
- The Principal Researcher and Lab Manager will be contacted if a spill of over 100 mL occurs.
- WGS and the IRC Building Manager maybe contacted in the event of hazardous chemical spills.

Special instructions for phenol spill:

- Place a phenol emergency first aid kit near the phenol handling area (plastic bag containing small bottle of PEG-300/PEG-400/or glycerin, packaged gauze pads, butyl rubber gloves)

- If eye contact occurs, immediately flush eyes with WATER for 15 minutes, then seek prompt medical attention
- If skin contact occurs
  - remove contaminated clothing
  - put on butyl rubber gloves (if hand has not been exposed)
  - remove contents from plastic bag
  - open a few packages of gauze pads
  - pour PEG or glycerin on to a gauze pad
  - gently wipe over exposed area to remove phenol, then place gauze pad into the plastic bag
  - repeat process until all visible traces of phenol have been removed from the skin
  - seek prompt medical attention while continuing to gently wipe skin with PEG/glycerin-soaked gauze pads (changing pads frequently)
  - NOTE: if any contaminated clothing has been removed, place it into a plastic bag, and contact WGS for disposal.

Drips from acid bath:

- Personal protective equipment (PPE) will be worn, care will be taken to minimize drips, and spills will be wiped up using a baking soda water solution.

INL Spill Notification Team can be contacted at (208) 241-6400 for spills of any size.

Describe anything else that may be relevant for waste disposal purposes:

- Biosafety Level-1 wastes can be collected in covered or uncovered, labeled, leak-proof containers or in the hood until decontaminated.
- The bulk of the waste generated by these research activities are non-regulated, circumneutral, spent culture media and disposable bottles, tubes, and pipettes. These materials are typically sterilized by autoclaving and then poured down the drain or placed in general trash receptacles.
- Ethidium bromide containing gels are allowed to dry and then disposed of as solid industrial waste through Waste Generator Services (WGS). Ethidium bromide present in liquids is sequestered and concentrated using commercially available “EtBr Green Bags.” After use the bags are disposed of as solid industrial waste and water is poured down the drain.
- Aqueous waste containing inorganic acids or bases and non-regulated components are autoclaved and then neutralized and discarded in the drain as non-regulated sewer waste.
- Liquid wastes containing viable microbes and hazardous components (inducing antibiotics) are decontaminated prior to placement in the SAA.

2.1 Training Required

Activity	Training Required
All laboratory activities	00INL670-Laboratory Awareness Training 00INL722-Laboratory Personnel Protective Equipment (PPE) 000INL13 Chemical Hygiene Training

Use of hazardous chemicals and materials	SMJS992B - Flammable and Combustible Materials SMJS992E - Irritants, Corrosives and Sensitizers SMJS992P - Chemicals with Specific Target Organ Effects SMJS992Q - Particularly Hazardous Substances SMJS992R - Reactive and Unstable Chemicals SMJS992S - Agents which present Significant Exposure via Inhalation or Skin Absorption
Working with liquid nitrogen	SMJS992N - Cryogenic Materials
Use of compressed gases	0INL1041-Compressed Gas Safety
Recombinant or synthetic nucleic acid molecules/microbiological materials	LWP-14621 Laboratory Biological Experimentation Safety SMJS992M Biohazardous Agents & Recombinant or synthetic nucleic acid molecules
Waste generation and disposal	QN000SAA-Satellite Accumulation Area Training
Repetitive work (prolonged standing, repetitive pipetting, extensive microscope, and BSC work)	0INL1491-Laboratory Ergonomics Training
Set-up of Pressurized Systems (e.g., anaerobic chamber, gassing station) by qualified staff	QN000PSA-INL Pressure System Assembler
PR Recombinant or synthetic nucleic acid molecules	RRNIH000 - NIH Guidelines for research involving recombinant or synthetic nucleic acid molecules.

2.2 Lessons Learned

Date submitted	Identifier	Site Area	Title
04/2009	NA	NA	The importance of knowing the differences between Laboratory (chemical) hoods, clean benches (laminar flow

			hoods) and biosafety cabinets. April 2009, http://safety.dri.edu/Hazards/LessonsLearned/laminar_flow.pdf
03/22/2008	2008-0208	DOE LL	Autoclave Testing Results for Sterilizing Biological Hazards in Environmental Samples. [Biological samples were shipped off site prior to verification of their sterility- Sterilization of biological samples should be confirmed prior to shipment if a biological hazard could be present.]
09/11/2008	2008-0279	DOE LL	First Degree Sunburn from Ultraviolet (UV) Trans-illuminator. [Employ was exposed to UV light while loading agarose gels to the box for excising DNA. The threshold limit of exposure for this light was 15 seconds. UV light should be turned on only after gel is loaded. There are no immediate warning symptoms to indicate UV overexposure. Reported symptoms, which typically appear hours after overexposure, include varying degrees of erythema (sunburn) or photokeratitis (welder's flash).]
07/09/2013	LBNL-LL-13-0019	DOE LB	Handling of an Extremely Heavy Gas Cylinder Results in Injury. [A 341 lbs gas cylinder that looked identical to the 133 lbs variety fell and rolled onto a graduate student's right foot, causing multiple broken bones in the foot. It is important to understand the hazards associated with handling compressed gas cylinders particularly weight.]
01/30/2014	HDI POC		Razor blades always pose hazards when handled. It is important to focus on the task at hand and use caution, appropriate tool, and cut resistant gloves when cutting with any blade. [A researcher preparing frozen tissue samples using a single-edge razor blade picking up and laying down the blade repeatedly accidentally picked it up and used it upside down and received a deep laceration on index finger.]

3. PREREQUISITES

Use of recombinant and synthetic molecules will be conducted per NIH guidelines for research involving recombinant or synthetic nucleic acid molecules, Section III-E and III-F. The researcher will evaluate form 420.11 prior to beginning recombinant work and if the 420.11 requires a modification the PR will notify the Laboratory Manager. Experimental work may begin simultaneously with initiation of IBC review for work that falls under Section III-E and is exempt from review for work that falls under Section III-F.

PR to provide briefing of NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules

Laboratory scales and balances are calibrated on an annual basis and should be verified to be within the calibration date prior to usage.

Phenol emergency kit will be prepared and located near work that requires dispensing phenol.

3.1 Additional LIs Supporting this LI

LI-714 Microbial Cultivation, Characterization and Metabolite Analyses, Bradley Wahlen, PR

3.2 Collocated Research Activities for this LI

Laboratory Instructions performed in the area of this research are located in the Laboratory Space Manual. Prior to performing any activities in this Laboratory Instruction, the Laboratory Space Coordinator and the Principal Researcher must evaluate and coordinate the work activities to ensure compatibility between area activities. Personnel, who have a reasonable risk of exposure to collocated hazards, must have a familiarity level knowledge of the collocated research activities and the associated hazards and controls that are authorized for this area. The LSC will convey this information to such personnel.

4. FACILITY CONDITIONS

This work requires the following facility conditions: certified biological safety cabinets, operating and tested fume hoods, safety showers and eyewash stations located in vicinity. For facility work or modifications, review the building Tenant Use Agreement (TUA) and notify the Building Specialist.

5. INSTRUCTIONS

Operate equipment per manuals located nearby (e.g., drawer), in laboratory filling cabinet or online.

6. POST-PERFORMANCE ACTIVITIES

Post-performance activities will include project clean-up, and proper disposition of wastes. Archived materials that are no longer part of an active project and could potentially be dispositioned as RCRA hazardous waste, if determined to no longer be of use/value, require written justification by the laboratory manager for storage.

7. ABNORMAL OPERATIONS

None.

8. RECORDS

Pertinent data will be recorded in registered laboratory notebooks per MCP-2875, “Proper Use and Maintenance of Laboratory Notebooks.”

9. REFERENCES

1) Methods for General and Molecular Bacteriology, P. Gerhardt (ed), 1994, American Society for Microbiology.

2) Molecular Cloning, A Laboratory Manual, Sambrook *et. al*., 1989, 2nd Edition, Cold Springs Harbor Laboratory Press.

3) LWP-14620-"Chemical Hygiene Plan"

4) LWP-14621-"Laboratory Biological Experimentation Safety"

5) MCP-2875-"Proper Use and Maintenance of Laboratory Notebooks"

10. APPENDIXES

1.1 Appendix A, "Laboratory Biological Experimentation Safety", LWP-14621, "Appendix C Good Microbiological Practices"

Appendix A

"Laboratory Biological Experimentation Safety," LWP-14621, Appendix C

Good Microbiological Practices

1. Work surfaces (when used for microbiological experiments or procedures involving live organisms) are decontaminated at least once a day, and after spills. For example, bench surfaces may be wiped down with ethanol (70–85% by weight), diluted household bleach (1% sodium hypochlorite), 5% hospital-type Lysol, or any approved hospital-grade disinfectant used according to the manufacturer's instructions. Ensure decontamination solutions are chemically compatible with media or other chemicals that may be contacted. Microbiologically contaminated solutions and cleaning materials must be disposed of as directed in the appropriate hazardous waste disposal procedures.
2. All microbiologically contaminated liquids and solids are decontaminated prior to disposal to ensure that cultured live microorganisms are not disposed of improperly.
3. Procedures will be documented by laboratory workers and PIs as follows:
    A. For microbial decontamination and verification of efficiency.
    B. For verification of data.
    C. To ensure maintenance of this information in laboratory notebooks, and to provide job-specific training on appropriate microbial decontamination procedures.
4. Eating, drinking, smoking, and the application of cosmetics are not permitted in the work area. Food is stored only in designated refrigerators.
5. Hands are washed after handling live microorganisms or biohazardous agents and prior to leaving the laboratory.
6. Aerosols containing live microorganisms or biohazardous agents are minimized.
7. Laboratory coats and shoe covers are recommended to prevent contamination or soiling of personal clothing.
8. If gloves are worn and become contaminated, take appropriate measures to avoid spreading the contamination to surfaces such as instruments, doors, or bench tops. Gloves and other contaminated items are decontaminated in an autoclave and disposed in biohazard bags.
9. Segregate sharp objects (needles, broken glass, etc.) into separate disposable containers.