

DHR-ICMR SPONSORED NATIONAL WORKSHOP ON

Biomedical Innovations in One Health
Research for Combating MDR/XDR
Pathogens through *In-Silico*, *In-Vitro* and
In-Vivo Methodologies

SPONSORED BY

Department of Health Research (DHR)
Indian Council of Medical Research (ICMR)
Ministry of Health & Family Welfare Government of India

2026
September
22-26
Tuesday - Saturday
8.00 - 17.00

Venue:
**Conference
Hall, PRC**



ABOUT THE WORKSHOP

This DHR-ICMR sponsored national workshop brings together clinicians, microbiologists, biomedical scientists, computational biologists and translational researchers to explore

integrated One Health approaches for combating antimicrobial resistance using in-silico, in-vitro and in-vivo methodologies

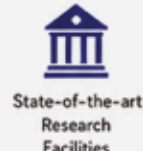
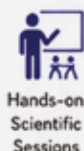


ABOUT PUSHPAGIRI RESEARCH CENTRE

Established in 2010, Pushpagiri Research Centre (PRC) is the Central Research Facility of Pushpagiri Medical Society. It houses advanced research

infrastructure, instrumentation & simulation facilities. It's an approved Ph.D. Centre, has a DHR- registered IRB and a CCSEA-approved Animal House with clinical and translational research facilities.

WORKSHOP HIGHLIGHTS



PROGRAMME AT A GLANCE



ORGANISED BY



Pushpagiri Research Centre (PRC)
Tiruvalla, Kerala - 689101

DHR-ICMR Sponsored National Workshop

Biomedical Innovations in One Health Research for Combating MDR/XDR Pathogens Through *In-Silico*, *In-Vitro* and *In-Vivo* Methodologies

Organised by: **Pushpagiri Institute of Medical Sciences and Research Centre (PIMSRC),
Tiruvalla, Kerala - 689101**

Date: **22nd - 26th September 2026**

INAUGURATION PROGRAMME (22nd September 2026)

Time: 09.00 AM – 10:00 AM

Venue: Lecture Hall - 1

<i>Prayer Song - PCAHS</i>		
Welcome Speech	:	Dr. Gaddam Vijaylakshmi (Vice Principal – UG Academics)
Presidential Address	:	Dr. Vikram Gowda N R (Vice-Principal – Administration & Co-PI)
Inaugural Address	:	Rev. Dr. Mathew Mazhavancheril (Director - PIMSRC)
<i>Lighting of Lamp</i>		
Felicitations Address	:	Dr. S. R. Madhan Shankar (Chief Technical and Operations Officer, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu)
Vote of Thanks	:	Dr. Aniket Naha (Scientist & PI)
<i>National Anthem</i>		

VALEDICTORY FUNCTION (26th September 2026)

Time: 10.00 AM – 12:00 PM

Venue: Lecture Hall - 1

<i>Prayer Song - PCAHS</i>		
Welcome Speech	:	Dr. Vikram Gowda N R (Vice-Principal – Administration & Co-PI)
Presidential Address	:	Rev. Dr. Mathew Mazhavancheril (Director - PIMSRC)
Guest Lecture	:	Dr. Geetanjali Sageena (Scientist-C, Division of HRD, DHR)
<i>00Felicitations of the Guest</i>		
AMR Updates in PMCH	:	Dr. Aneeta Mary Jacob (Associate Professor & HICC-in-charge)
Felicitations Address	:	Dr. Reena Thomas (Principal - PIMSRC)
Workshop Summary	:	Dr. Aniket Naha (Scientist & PI)
<i>Feedback from Participants</i>		
Bioradiance - 2027	:	Dr. Soumya RS (Scientist & Co-PI)
Vote of Thanks	:	Dr. Ampadi AN (Coordinator, PRC)
<i>Certificate Distribution</i>		
<i>National Anthem</i>		

DHR-ICMR Sponsored National Workshop
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Organised by: **Pushpagiri Institute of Medical Sciences and Research Centre (PIMSRC), Tiruvalla, Kerala - 689101**

Date: 22nd - 26th September 2026

WORKSHOP SCHEDULE

Time	Programme	
Day - 1 (22nd September, 2026)		
08.00 AM	Registration & Kit Distribution	
08.30 AM	Ice - Breaking Session and Pre Assessment	
09.00 AM	Inaugural Function	
09.30 AM	Introduction to PRC and Overview of 5-Days Workshop Program	
	Dr. Aniket Naha (Scientist and Student Coordinator, PRC)	
Tea Break		
10.00 AM	Lecture - 1	Speaker: Dr. Aravind R (Professor and Head, Department of Infectious Diseases, Government Medical College, Thiruvananthapuram, Kerala) Lecture Topic: Antimicrobial Resistance in the Clinical Era: Bridging Evidence, Stewardship, and Innovation for Better Patient Outcomes
11.00 PM	Lecture - 2	Speaker: Dr. Dhanya Sasidharan Palappallil (Professor, Department of Pharmacology, Government Medical College Kottayam, Kerala) Lecture Topic: The Role of Clinical Pharmacology in Addressing Antimicrobial Resistance: Challenges and Opportunities
Lunch Break		
01.00 PM	Lecture - 3	Speaker: Dr. S. R. Madhan Shankar (Chief Technical and Operations Officer, Kongunadu Arts and Science College, Founder and CEO, Ashwinis Herbs and Foods, Coimbatore, Tamil Nadu) Lecture Topic: The Invention Journey: Discovering Novel Beta-Lactam Inhibitors and Beyond
02.00 PM	Hands-on Session - I	Topic: Structural Bioinformatics - Protein Structure Modelling and Introduction to small compound databases (In-silico) Expert: Dr. Dhanya Sasidharan (Professor) and Dr. Aniket Naha (Scientist)
Tea Break		
3.30 PM	Hands-on Session - II	Topic: Structural Bioinformatics - Molecular Docking and Intermolecular Interaction Profiling (In-silico) Expert: Dr. Dhanya Sasidharan (Professor) and Dr. Aniket Naha (Scientist)
05.00 PM	Closing Remarks	

Day – 2 (23rd September, 2026)		
08.00 AM	Hands-on Session - III	Topic: Introduction to Data Repository – Database Search & Sequence Analysis (In-silico) Expert: Dr. Kumar KM (Associate Professor) and Dr. Aniket Naha (Scientist)
Tea Break		
10.00 AM	Lecture – 4	Speaker: Dr. Kumar KM (Associate Professor, Department of Bioinformatics, School of Life Sciences, Pondicherry Central University, Pondicherry) Lecture Topic: From Genome to Drug Target: Computational Strategies for AMR Drug Discovery
11.00 AM	Hands-on Session - IV	Topic: Structural Bioinformatics – Protein energy minimization, relative RMSD and Mutant generation and thermodynamics analysis Expert: Dr. Kumar KM (Associate Professor) and Dr. Aniket Naha (Scientist)
Lunch Break		
01.00 PM	Hands-on Session - V	Topic: Clinical Microbiology – Sterilization, Media Preparation & Handling Clinical Pathogens, Demonstration and GLPs to be practiced in BSL-2 facility (In-vitro) Expert: Dr. Janani Remyaprabha (Associate Professor) and Dr. Aniket Naha (Scientist)
Tea Break		
3.30 PM	Hands-on Session - VI	Topic: Clinical Microbiology – Morphological and Biochemical Characterization (In-vitro) Expert: Dr. Janani Remyaprabha (Associate Professor), Dr. Betsy A Jose (Associate Professor) and Dr. Aniket Naha (Scientist)
05.00 PM	Closing Remarks	
Day – 3 (24th September, 2026)		
08.00 AM	Hands-on Session - VII	Topic: Clinical Microbiology – Phenotypic Characterization (AST, MIC), mechanisms of antibiotic resistance and antibacterial assays (In-vitro) Expert: Dr. Keshavamurthy M. (Assistant Professor) and Dr. Aniket Naha (Scientist)
Tea Break		
10.00 AM	Lecture – 5	Speaker: Dr. Keshavamurthy M. (Assistant Professor, Department of Biotechnology, School of Applied Sciences, REVA University, Bengaluru) Lecture Topic: Exploring Medicinal Plant Endophytes for Novel Antimicrobials Against Multidrug-Resistant Pathogens
11.00 AM	Lecture – 6	Speaker: Dr. Sanjeev Nair (Professor (CAP), Government Medical College, Thiruvananthapuram, Kerala) Lecture Topic: Drug-Resistant Tuberculosis: Current Challenges, Advances, and Future Directions in Control and Management
Lunch Break		

01.00 PM	Lecture – 7	Speaker: Dr. Janani Remyaprabha Jnana Thapaswini (Associate Professor, Department of Rasa Sasthra Bhaishajya Kalpana, Santhigiri Ashram, Santhigiri Ayurveda Medical College, Olassery, Kerala) Lecture Topic: Bridging Ayurveda and Modern Science: Phytochemical Approaches to Infectious Disease Management
02.00 PM	Hands-on Session - VIII	Topic: Clinical Microbiology – Sterilization, Media Preparation & Handling Clinical Pathogens, Demonstration and GLPs to be practiced in BSL-2 facility (In-vitro) Expert: Dr. Keshavamurthy M. (Assistant Professor) and Dr. Aniket Naha (Scientist)
Tea Break		
03.30 PM	Hands-on Session - IX	Topic: Basic techniques in cell culture – Media Preparation, Cell Line Maintenance, Cell Seeding (In-vitro) Expert: Dr. Arun KB (Assistant Professor), Dr. Ajay Krishnan U and Dr. Soumya RS (Scientist)
05.00 PM	Closing Remarks	
Day – 4 (25th September, 2026)		
08.00 AM	Hands-on Session - X	Topic: Seeding cells and assay setup for fluorescence and cell proliferation assay: DCFDA and MTT assay – Part I Expert: Dr. Arun KB (Assistant Professor), Dr. Ajay Krishnan U and Dr. Soumya RS (Scientist)
Tea Break		
10.00 AM	Lecture – 8	Speaker: Dr. Arun KB (Assistant Professor, Department of Life Sciences, CHRIST (Deemed to be University), Bangalore, Karnataka) Lecture Topic: Reprogramming Host Defense: Advanced Cell Culture Approaches to Combat Drug-Resistant Tuberculosis
11.00 AM	Lecture – 9	Speaker: Dr. Rojin Abraham (Professor and HOD, Department of Neurosurgery, Believers Church Medical College Hospital, Tiruvalla, Kerala) Lecture Topic: Antimicrobial Resistance in Traumatic Brain Injury: Prevention and Management of Infectious Complications
Lunch Break		
01.00 PM	Hands-on Session - XI	Topic: Seeding cells and assay setup for fluorescence and cell proliferation assay: DCFDA and MTT assay - Part II Expert: Dr. Arun KB (Assistant Professor), Dr. Ajay Krishnan U and Dr. Soumya RS (Scientist)
Tea Break		
3.30 PM	Hands-on Session - XII	Topic: Data analysis: Fluorescence microscopy (DCFDA) and ELISA reader (MTT assay), HPLC Demonstration (In-vitro) Expert: Dr. Arun KB (Assistant Professor), Dr. Ajay Krishnan U and Dr. Soumya RS (Scientist)
05.00 PM	Closing Remarks	

<i>Day - 5 (26th September, 2026)</i>		
08.00 AM	Post Assessment	
09.00 AM	Lecture – 10	Speaker: Dr. Nebu George Thomas (Professor, Department of Periodontology, Pushpagiri College of Dental Sciences, Scientist, Stem Cell and Tissue Engineering Lab, PRC) Lecture Topic: Advances in Animal Studies in Biomedical Research
<i>Tea Break</i>		
<i>Valedictory Function</i>		
<i>Lunch Break</i>		
01.00 PM	Lecture – 11	Speaker: Dr. Sachin J. Shenoy (Scientist, Medical Devices Engineering, Sree Chitra Thirunal Institute of Medical Sciences and Technology, Trivandrum, Kerala) Lecture Topic: Research guidelines and regulations for biomedical research in small animals
02:00 PM	Hands-on Session - XIII	Topic: Animal House Visit and Observation of Experiments on Small Animals as per CCSEA guidelines Expert: Dr. Sachin Shenoy (Scientist) and Dr. Ampadi AN (Veterinary Officer)
<i>Certificate Distribution and Feedback</i>		
<i>Closing Remarks & Tea Break</i>		

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BASIC TECHNIQUES IN BIOINFORMATICS

Biological Databases & Data Retrieval

Theory

A biological database is a computerized archive used to store and organize biological data in such a way that information can be retrieved easily via a variety of search criteria. In general, it is a collection of structured, searchable biological contents. It should be updated periodically and cross-referenced with source information.

Importance of Biological Databases

- Exponential growth in biological data.
- Data (genomic sequences, 3D structures, 2D gel analysis, MS analysis, Microarrays....) are no longer published in a conventional manner, but directly submitted to databases.
- Importantly, it is one of the essential tools for biological research.

Databases and its types

One of the first biological sequence databases was probably the book, "**Atlas of Protein Sequences and Structures**" by Margaret Dayhoff and colleagues, first published in 1965. It contained the protein sequences determined at the time, and new editions of the book were published well into the 1970s.

Based on their contents, biological databases can be roughly divided into three main categories:

Primary databases: contain original biological data. They are archives of raw sequence or structural data submitted by the scientific community. Example: Protein Data Bank (PDB; coordinates of three-dimensional macromolecular structures)

Secondary databases: contain computationally processed or manually curated information, based on original information from primary databases. Example: UniProt, SCOP and CATH.

Composite Databases: Collection of multiple databases under single umbrella. Example: NCBI.

In essence, the scientific community frequently develop or involved in the development of databases that cater to a particular research interest. These are often called as **Specialized databases**.

Pitfalls of Biological Databases:

- One of the major problems associated with biological databases is overreliance on sequence information and related annotations, without understanding the reliability of the information.
- High levels of redundancy in the primary sequence databases.
- Annotations of genes can also occasionally be false or incomplete. It may lead to error propagation.
- Errors may be due to sequencing. Sometimes, gene sequences are contaminated with sequences from cloning vectors. There are also some errors that are simply caused by omissions or mistakes in typing.

Overall, the most fundamental skill in bioinformatics is the ability to retrieve the biological data with high precision together with an efficient and comprehensive search of the biological data scientific literature to find out what is known about a specific subject. All of you are familiar with web search engines and while they can be useful, they also turn up many items that have never undergone the test of scientific peer review. Thus, this exercise is NOT a search of the World Wide Web, but will introduce you to searching the published scientific literature using a database such as MEDLINE, Biological Abstracts or Chemical Abstracts. In addition, this exercise will also guide you through an effective way of biological data (say DNA and protein) retrieval from the existing databases.

Experiment 1

Experiment: Retrieval of nucleotide sequence from primary nucleotide database (NCBI)

PROTOCOL

1. Open Browser (Chrome or Firefox) from your system
2. Visit the NCBI homepage [<https://www.ncbi.nlm.nih.gov/>]
3. Select 'Nucleotide database' and paste the query accession number 'FR695881' in the search bar and press 'Search'
4. The nucleotide sequence information will be displayed in GenBank File Format
5. Observe the sequence information
6. Click on 'FASTA' for the sequence
7. The nucleotide sequence of FR695881 is displayed
8. Click on 'Send to' and choose destination as 'File'
9. Click on 'Create File'
10. The sequence will get downloaded as 'sequence.fasta'

11. Open the downloaded file in 'Notepad or WordPad'

QUERIES AND RESULTS

Accession no: FR695881.1

- a. Retrieve the FASTA sequence
- b. Mention number of base pairs
- c. Provide the gene information
- d. Write the organism's name

Experiment 2

Experiment: Retrieval of Protein sequence from protein database (UNIPROT)

PROTOCOL

1. Open Browser (Chrome or Firefox) from your system
2. Visit the UNIPROT homepage [<https://www.uniprot.org/>]
3. Select 'UniProtKB' and paste the query protein ID 'P06280' in the search bar and press 'Search'
4. The protein information will be displayed in UniProt File Format
5. Click on 'Sequences' for the sequence retrieval
6. The amino acid sequence of P06280 is displayed
7. Observe the protein sequence information
8. Click on 'FASTA' and select the sequence in FASTA file format
9. Copy and paste the sequence in 'Notepad or WordPad'

QUERIES AND RESULTS

Retrieve the following information for the above given Uniprot ID P06280

- a. Protein name
- b. Gene name
- c. Organism name
- d. Write the length and Molecular weight of the protein
- e. Retrieve the FASTA sequence of the corresponding protein

Experiment 3

Experiment: Retrieval of Protein structure from structure database (PDB)

PROTOCOL

1. Open Browser (Chrome or Firefox) from your system
2. Visit the PDB database [<https://www.rcsb.org/>]
3. Search for the query protein '5FAS' in the search bar and press 'Search'
4. The entire protein information will be displayed in PDB File Format
5. View the protein structure by clicking '3D View'
6. Observe the protein structure information
7. Click on 'Download Files' and select 'PDB format' to download the protein structure in *.pdb* file format
8. The protein structure will get downloaded as '5fas.pdb'
9. User can use pymol or Rasmol for protein visualization and further analysis

QUERIES AND RESULTS

- a. Protein Name
- b. Organism
- c. Experiment Method
- d. Resolution
- e. Molecule
- f. Sequence Length
- g. Chains
- h. Stoichiometry

Experiment 4

Experiment: Access of secondary biological data from various biological databases (SOPMA)

PROTOCOL

1. Open Browser (Chrome or Firefox) from your system
2. Visit SOPMA with the given link
[https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html]
3. Paste the given query sequence in the space provided
4. In Number of Conformational States, Select 'Helix, Sheet, Turn, Coil'
5. Press 'Submit' with the other default parameters
6. The details of secondary biological data of the query protein gets displayed
7. The protein structure will get downloaded as '5fas.pdb'

QUERIES AND RESULTS

Predict the secondary structure of the above given sequence and paste the result.

MRSLLILVLCFLPLAALGKVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAK
FESNFNTQATNRNTDGSTDYGILQINSRWWCNDGRTPGSRNLCNIPCSALLSS
DITASVNCAKKIVSDGNGMNAWVAWRNRCKGTDVQAWIRGCRL

- a. Sequence Length
- b. Percentage of α -helix
- c. Percentage of β -turn
- d. Percentage of extended strand
- e. Percentage of Random Coil

PAIRWISE SEQUENCE ALIGNMENT

Theory:

When a new sequence is found, the structure and function can be easily predicted by doing sequence alignment. Since it is believed that, a sequence sharing a common ancestor would exhibit similar structure or function. Greater the sequence similarity, greater is the chance that they share similar structure or function.

Sequence alignment can be of two types i.e., comparing two (pair-wise) or more sequences (multiple) for a series of characters or patterns. Of note, pair wise alignment can be executed by two different algorithm such as Needleman-Wunsch and Smith-Waterman algorithm. For instance, Needleman-Wunsch algorithm is to generate Global alignment. On the other hand, Smith-Waterman algorithm is to generate the local alignment between pair of sequences. Alignment of three or more biological nucleotides or protein sequences, simply defines multiple sequence alignment. The genes which are similar may be conserved among different species.

In global alignment, Alignment is carried out from beginning to end of both sequences to find the best possible alignment across the entire length between the two sequences.

This method is more applicable for aligning two closely related sequences of roughly the same length. For divergent sequences and sequences of variable lengths, this method may not be able to generate optimal results

In Local alignment, Local alignment on the other hand, does not assume that the two sequences in question have similarity over the entire length. It only finds local regions with the highest level of similarity between the two sequences and aligns these regions without regard for the alignment of the rest of the sequence regions.

This approach is more appropriate for aligning divergent biological sequences containing only modules that are similar, which are referred to as domains or motifs.

Experiment 5

Experiment: Pairwise alignment using dot plot [EMBOSS: dotmatcher]

PROTOCOL

➤ Sequence Retrieval

1. Open Browser (Chrome or Firefox) from your system
2. Visit the UNIPROT homepage [<https://www.uniprot.org/>]
3. Select 'UniProtKB' and paste the query protein with Uniprot IDs 'P02768'; 'P02770' and 'S9WI87' in the search bar and press 'Search'
4. The protein information will be displayed in UniProt File Format
5. Click on 'Sequences' for the sequence retrieval and then click on 'FASTA' to retrieve the sequences
6. Copy and paste all the sequences in 'Notepad or WordPad'

➤ EMBOSS: dotmatcher

7. Visit the EMBOSS: dotmatcher homepage [<http://emboss.bioinformatics.nl/cgi-bin/emboss/dotmatcher>]
8. Insert the FASTA sequences of Target and Query sequences
 - a. Uniprot ID: 'P02768' with Uniprot ID: 'P02770'
 - b. Uniprot ID: 'P02768' with Uniprot ID: 'S9WI87'
9. Press 'Run dotmatcher' after providing suitable titles and with other default parameters
10. Take a snapshot of the output file using 'Snipping Tool or print-screen option'

QUERIES AND RESULTS

- a. Find out which two sequences are closely related
- b. Provide the alignments (Screenshot)

Experiment 6

Experiment: Pairwise alignment using dynamic programming

- ✓ Local Alignment
- ✓ Global Alignment

PROTOCOL FOR LOCAL ALIGNMENT

➤ Sequence Retrieval

➤ For Local Alignment [EMBOSS Water]

(EMBOSS Water uses the Smith-Waterman algorithm (modified for speed enhancements) to calculate the local alignment of two sequences)

1. Visit the EMBOSS Water homepage
[\[https://www.ebi.ac.uk/jdispatcher/psa/lalign\]](https://www.ebi.ac.uk/jdispatcher/psa/lalign)
[\[https://www.biochemintelli.com/localalign/\]](https://www.biochemintelli.com/localalign/)
2. Select 'PROTEIN' for protein query sequence
3. Insert the FASTA sequences of Target and Query sequences
 - a. Uniprot ID: 'P02768' with Uniprot ID: 'P02770'
 - b. Uniprot ID: 'P02768' with Uniprot ID: 'S9WI87'
4. Press 'Submit' with the other default parameters
5. The result will get displayed in the subsequent page

QUERIES AND RESULTS

- a. Mention the percentage of identity for both the combinations
- b. Mention the percentage of similarity for both the combinations
- c. Mention the percentage of gaps for both the combinations
- d. Provide the alignments for both the combinations (Screenshot)

PROTOCOL FOR GLOAL ALIGNMENT

➤ **Sequence Retrieval**

➤ **For global Alignment [EMBOSS Needle]**

(EMBOSS Needle reads two input sequences and writes their optimal global sequence alignment to file)

1. Visit the EMBOSS Needle homepage
[\[https://www.ebi.ac.uk/jdispatcher/psa/emboss_needle\]](https://www.ebi.ac.uk/jdispatcher/psa/emboss_needle)
2. Select 'PROTEIN' for protein query sequence
3. Insert the FASTA sequences of Target and Query sequences
 - a. Uniprot ID: 'P02768' with Uniprot ID: 'P02770'
 - b. Uniprot ID: 'P02768' with Uniprot ID: 'S9WI87'
4. Press 'Submit' with the other default parameters
5. The result will get displayed in the subsequent page

QUERIES AND RESULTS

- a. Mention the percentage of identity for both the combinations
- b. Mention the percentage of similarity for both the combinations
- c. Mention the percentage of gaps for both the combinations
- d. Provide the alignments for both the combinations (Screenshot)

BLAST - BASIC LOCAL ALIGNMENT SEARCH TOOL

Theory:

The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches.

BLAST is a heuristic method of local alignment. It is based on the concepts that homologous sequences are likely to contain a short high scoring similarity region a hit. Each hit gives a seed that BLAST tries to extend on both sides. The algorithm has 3 major steps. i) Seeding – identify where to start alignment ii) Extension – extending alignment from seeds and iii) Evaluation – Determine which alignments are statistically significant. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

Traditional BLAST programs:

Program	Database	Query
BLASTN	Nucleotide	Nucleotide
BLASTP	Protein	Protein
BLASTX	Protein	Nucleotide translated into protein
TBLASTN	Nucleotide translated into protein	Protein
TBLASTX	Nucleotide translated into protein	Nucleotide translated into protein

Experiment 7

Experiment: Heuristic Sequence Alignment using BLAST/ FASTA

➤ Sequence alignment using BLAST tool

11. Open Web Browser (Chrome or Firefox) from your system
12. Retrieve the query sequence in FASTA file format using either NCBI (Nucleotide sequence: <https://www.ncbi.nlm.nih.gov/>) or Uniprot (for Protein sequence: <https://www.uniprot.org/>)
13. Copy and paste all the sequences in 'Notepad or WordPad'
14. Visit the BLAST homepage: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>
15. Select the type of BLAST (Nucleotide BLAST or Protein BLAST) based on your requirement
16. Paste the FASTA sequences of the Query protein/nucleotide
17. Select the database to compare

18. Enter the BLAST button at the bottom.
19. Observe the results for highly similar sequences.

QUERIES AND RESULTS

1. Retrieve sequence for Uniprot ID: P02768
 - a. Provide basic information such as protein name, organism name and sequence length.
2. BLAST the query sequence against PDB Database.
 - a. List top 5 PDB IDs with 100% identical structure available
 - b. Which is best similar structure for your query sequence?
 - c. Please fill the following details from your results.

Sl. No.	Description	Query coverage	E-value	Identity (%)	Accession

Sequence alignment using FASTA tool

1. Open web Browser (Chrome or Firefox) from your system
2. Retrieve the query sequence in FASTA file format using either NCBI (Nucleotide sequence: <https://www.ncbi.nlm.nih.gov/>) or Uniprot (for Protein sequence: <https://www.uniprot.org/>)
3. Copy and paste all the sequences in 'Notepad or WordPad'
4. Visit the FASTA homepage: <https://www.ebi.ac.uk/Tools/sss/fasta/>
5. Select the Database from the list of databases
6. Paste the FASTA sequences of the Query protein/nucleotide sequence
7. Enter the Submit button at the bottom.
8. Observe the results for highly similar sequences.

QUERIES AND RESULTS

1. Retrieve sequence for Uniprot ID: P02768
2. Search the query sequence Uniprot-KB Database and provide the below information for the top 5 protein sequences.

Sl. No.	Description	Query coverage	E-value	Identity (%)	Accession

MULTIPLE SEQUENCE ALIGNMENT AND PHYLOGENETIC TREE CONSTRUCTION

Theory:

A natural extension of pairwise alignment is multiple sequence alignment, which is to align multiple related sequences to achieve optimal matching of the sequences. As the process generates multiple matching sequence pairs, it is often necessary to convert the numerous pairwise alignments into a single alignment, which arranges sequences in such a way that evolutionarily equivalent positions across all sequences are matched.

There is a unique advantage of multiple sequence alignment because it reveals more biological information than many pairwise alignments can. For example, it allows the identification of conserved sequence patterns and motifs in the whole sequence family, which are not obvious to detect by comparing only two sequences. Many conserved and functionally critical amino acid residues can be identified in a protein multiple alignment. Multiple sequence alignment is also an essential prerequisite to carrying out phylogenetic analysis of sequence families and prediction of protein secondary and tertiary structures. Multiple sequence alignment also has applications in designing degenerate polymerase chain reaction (PCR) primers based on multiple related sequences.

We can find many tools for multiple sequence alignment like MSA DIALIGN, CLUSTAL series, MAFT, MUSCLE, T-Coffee, BlastAlign, etc.

Key terms

Conserved regions: In biology, during the evolutionary time there may be some regions called group of bases or a sequence of nucleotides preserved as such in DNA, those sequences or a region, if seen in next generations called as Conserved regions. **Consensus Sequence:** In a Nucleotide or an amino acid sequence, each base pair (an amino acid or a nucleotide) may occur more frequently at a particular region in different sequences of nature.

Experiment 8

Experiment: Multiple sequence alignment and the Construction of Phylogenetic trees using Clustal- Omega tool

PROTOCOL

1. Open Browser (Chrome or Firefox) from your system
2. Retrieve the query sequence in FASTA file format using NCBI or Uniprot
3. Copy and paste all the sequences in 'Notepad or WordPad'

4. Visit the Clustal-Omega tool homepage:
<https://www.ebi.ac.uk/Tools/msa/clustalo/>
5. Select the necessary parameters
6. Paste the FASTA sequences of the Query DNA/Protein sequences
7. Enter the submit button at the bottom
8. Observe the results

QUERIES AND RESULTS

Retrieve the nucleotide sequences using NCBI accession numbers:

- a) NM_001291897
 - b) XM_012496623
 - c) XM_012041171
 - d) XM_003777677.2
 - e) XM_006750095
 - f) XM_004278069
1. Write the gene names, source organism and sequence lengths of each sequence
 2. Run multiple sequence alignment analysis
 3. Find out the longest highly conserved regions (Paste the alignment).
 4. Give the screenshot of phylogenetic tree.

HOMOLOGY MODELLING OF PROTEIN STRUCTURES

Theory:

Homology modelling has matured into an important technique in structural biology, significantly contributing to narrowing the gap between known protein sequences and experimentally determined structures. Fully automated workflows and servers simplify and streamline the homology modelling process, also allowing users without a specific computational expertise to generate reliable protein models and have easy access to modelling results, their visualization and interpretation. The SWISS-MODEL server, which pioneered the field of automated modelling 25 years ago and been continuously further developed.

In SWISS-MODEL, the default modelling workflow consists of the following main steps: Input data, Template search, Template selection, Model building and Model quality estimation.

The principle behind it is that if two proteins share a high enough sequence similarity, they are likely to have very similar three-dimensional structures. If one of the protein sequences has a known structure, then the structure can be copied

to the unknown protein with a high degree of confidence. Thus, in Homology modelling, a 3D protein model of a target sequence is generated by extrapolating experimental information from an evolutionary related protein structure that serves as a template.

The modelled structure can be validated by means of Ramachandran plot before further analysis. For instance, when ϕ and ψ angles of amino acids of a particular protein are plotted against each other, the resulting diagram is called a Ramachandran plot. This plot maps the entire conformational space of a peptide and shows sterically allowed and disallowed regions.

Experiment 9

Experiment: Protein structure homology modelling using automated protein structure homology-modelling server

SWISS- MODEL PROTOCOL

1. Open Browser (Chrome or Firefox) from your system
2. Retrieve the query sequence in FASTA file format from Uniprot database
3. Copy and paste all the sequences in 'Notepad or WordPad'
4. Visit the SWISS-Model tool homepage: <https://swissmodel.expasy.org/>
5. Click on Start Modelling
6. Paste the **Query protein** sequence in FASTA format
7. Select "Search for the templates"
8. Observe the results
9. Select the best models based on the query coverage and the Percentage of Identity
10. Click on Build Models
11. Click on Structure Assessment option to check the quality of the model structure
12. Download the modelled structure in PDB file format

QUERIES AND RESULTS

Model the protein structure for the following sequence using homology modelling approach and report the observations

>Query Sequence | Test Example

```
MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPF
EDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP
ERNECFQLQHKDDNPPLRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLF
FAKRYKAAFTTECCLKCASLQKFGERAFAKAWAVARLSQRFPKAEFAEVSKLVTDLTQVHTE
CCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDAKDVFLGM
FLYFYARRHPDYSVVLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIK
QNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCKKHPEAKRMPCA
EDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTF
```

HADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCCKADDKETCFA
EEGKKLVAASQAALGL

Experiment 10

Experiment: Operations in Swiss-pdbviewer 4.1.0 (SPDBV)

PROTOCOL

1. Energy Minimization from SPDBV

- File → Open .pdb file [open the file to be energy minimized]
- Select → All
- Tools → Compute energy (forcefield) → OK
- *Note down E_i*
- Tools → Energy Minimization → OK
- *Note down E_f*
- File → Save Current Layer

2. Relative RMSD Calculation from SPDBV

- File → Open parent structure
- File → Open refined structure
- Select → All
- Fit → Magic Fit
- Fit → Calculate RMS
- *Note down the relative RMSD score*

3. Generation of Mutants in SPDBV

- File → Open .pdb file → Load parent structure
- Wind → Control Panel → Click Label
- Rotate → Shift → Zoom (*Based on the requirement*)
- Click → Mutate (*Change to desired residue*)
- Induce All mutations & accept the change
- File → Save Current Layer

Experiment 11

Experiment: Perform molecular docking using Auto Dock

PROTOCOL

1. Create a folder with the following files

- protein.pdb
- ligand.pdb
- autodock4.exe
- autogrid4.exe

NOTE: names within the directory should be given without spaces

2. Open Auto Dock and Set working directory
 - File → Preferences → Set → Paste the link of the working directory → Set → Dismiss
3. Next is the Protein Preparation,
 - File → Read molecule → select protein.pdb → ok
 - Edit → hydrogens → add → polar only → ok
 - Edit → hydrogens → merge nonpolar
 - Edit → charges → add Kollman charges → ok
 - File → save → write PDB → protein_1.pdb → save
4. Select Ligand
 - Ligand → input → open → ligand.pdb → ok
 - Ligand → output → save as pdbqt → save → [ligand.pdbqt gets generated]
5. Click on Grid
 - Grid → macromolecule → open → select protein_1.pdb → ok → save as protein_1.pdbqt → [protein.pdbqt gets generated]
 - Grid → set MAP types → choose ligand → select ligand
 - Grid → grid box → maximize x, y, z dimensions
 - File → close saving current
 - Grid → output → save gpf → name as grid.gpf
 - Run → run autogrid → choose *autogrid4.exe* as program path name
 - Parameter file name → grid.gpf → grid.dlg → LAUNCH
6. Now select Dock
 - Docking → macromolecules → set rigid filename → select protein_1.pdbqt
 - Docking → ligand → choose → select ligand → accept
 - Docking → search parameter → genetic algorithm → accept
 - Docking → docking parameter → accept
 - Docking → output → Lamarckian → dock.dpf → save
 - Run → run autodock → program pathname *autodock4.exe* → parameter file name dock.dpf → LAUNCH
7. Analyse
 - Analyse → docking → open → dock.dlg → open → OK → OK
 - Analyse → macromolecule → choose → select
 - Analyse → conformations → play ranked by energy
 - Click "&" → show info

Note down: - Binding energy (kcal/mol) and inhibition constant (μ M or nM)
 - Build current → write complex → protein_ligand_complex.pdb → save
8. Visualization: Visualize the docked protein-ligand complex using Pymol/ Discovery studio/ Chimera

SAFETY CONCERNS AND GOOD LABORATORY PRACTICES

Safety in a microbiology laboratory is important in the prevention of infection that might be caused by the microorganisms being studied. In addition, many of the reagents, equipment, and procedures used are potentially hazardous. Attention to proper procedures and prudent laboratory practices are required for your safety and protection. So there are some general laboratory practices which needs to be performed to ensure safety among ourselves and also to others.

General Good Laboratory Practices

- Prepare for each laboratory period by reading each exercise and by becoming familiar with the principles and methods involved. Familiarity with the exercise decreases chances of an accident. Also, advance preparation allows you to use your time efficiently to complete the experiment.
- Wear your lab coat at all times in the laboratory. This helps ensure that no solution or hazardous chemical is accidentally deposited on your clothes or skin, protecting you and your clothes from contamination and stains.
- Bring only those materials pertinent to your lab work. All other items such as cell phones, books and bags should be stored away from your work area.
- Most of the equipment in the lab are very expensive and sensitive; please handle them properly and clean them after every use. Refrain from using any equipment unless you are familiar with it or under supervision and maintain cleanliness of the lab at all times.
- Label all solutions and chemicals properly with your name, date, and the reagent's or solution's name. Labelling is critical to avoid improper use or disposal of material.
- After the laboratory session, maintain good hygiene by washing your hands before leaving the laboratory.
- In the event of any accident or injury, report immediately to the laboratory instructor so that prompt and proper action can be taken.

In addition, here are some **microbial laboratory safety practices** and procedures which needs to be taken care of

- Treat all microorganisms as potential pathogens. A student who has a compromised immune system or has had a recent extended illness should talk with the instructor before working in the microbiology laboratory.
- Sterilize equipment and materials. All materials, media, tubes, plates, loops, needles, pipettes, and other items used for culturing microorganisms should be sterilized by autoclaving. Otherwise, use commercially sterilized products.
- Disinfect work areas before and after use. Use a disinfectant, such as a 10% bleach or 70% ethanol solution, to wipe down benches and work areas both before and

after working with cultures. Also be aware of the possible dangers of the disinfectants.

- Never pipette by mouth. Use pipette bulbs or pipetting devices for the aspiration and dispensing of liquid cultures.
- Do not eat or drink in the lab, nor store food in areas where microorganisms are stored. Keep your fingers out of your mouth. Cover any cuts on your hands with a bandage. Gloves may be worn as extra protection.
- Autoclave or disinfect all waste materials. All items to be discarded after a class should be placed in a biohazard autoclave bag and autoclaved 30 to 40 minutes at 121° C at 20 pounds of pressure.
- Clean up spills with care. Cover any spills or broken culture tubes with disinfectant; then cover with paper towels. After allowing the spill to sit with the disinfectant for a short time, carefully clean up and place the materials in a biohazard autoclave bag to be autoclaved. Wash the area again with disinfectant.

BASIC TECHNIQUES IN MICROBIOLOGY

STERILISATION

It is the process by which an article, surface or media is completely made free from all microorganisms, either vegetative or spores forms. In a microbiology laboratory, sterilisation is essential because culture media, glassware, instruments, solutions and other materials must not contain viable microorganisms that could:

- Contaminate experimental cultures;
- Interfere with experimental results;
- Compromise antimicrobial susceptibility or microbiological assays;
- Cause laboratory-acquired infections;
- Or result in the release of viable biological material into the environment.

Disinfection

It is the process of destruction or removal of all pathogens or organisms capable of producing infections in the living cells but not necessarily spores. All organisms may not be killed but the number is reduced to a level that is no longer harmful to health.

Sterilisation vs. Disinfection

Features	Sterilisation	Disinfection
Primary purpose	Eliminate viable microorganisms	Reduce/eliminate many or most pathogenic microorganisms

Bacterial spores	Designed to destroy/inactivate spores when the process is appropriate and validated	May not destroy spores
Application	Media, instruments, laboratory materials, certain wastes	Work surfaces, equipment surfaces and other environmental areas
Requirements	Requires an appropriate validated process	Effectiveness depends on the disinfectant, concentration, contact time and organism
Examples	Requires an appropriate validated process	70% alcohol for appropriate surface disinfection

Principle of Sterilisation

Different sterilisation methods destroy microorganisms through different physical or chemical mechanisms. The major approaches can be broadly classified as:

A. Physical methods

1. Heat
2. Radiation
3. Filtration

B. Chemical methods

1. Chemical sterilants
2. Gas sterilisation

The choice of method depends on the material being sterilised, its heat/moisture sensitivity, its physical characteristics and the intended use.

A. Physical methods of sterilisation

1. Heat sterilisation

Can be mainly classified into

Moist heat sterilisation

Moist heat may be employed in the following ways:

- Temperature below 100°C

(a) Pasteurization

Process of sterilisation of packaged and liquid foods to a mild temperature below 100°C.

Common methods include

- **LTLT** (Low temperature long time): heating to 63°C for 30 minutes
- **HTST** (High temperature short time): heating to 72°C for 15 seconds
- **UHT** (Ultra high temperature): heating to 138-150°C for 1-2 seconds

(b) Inspissation

Sterilisation technique utilising 80-85°C temperature for 30 minutes for 3 consecutive days.

Mechanism involves the heating during the first day to kill all the vegetative bacteria. The resting periods at room temperature allow any surviving spores to germinate into vegetative cells, which are then killed during the subsequent daily heating cycles.

- Temperature at 100°C

(a) Boiling at 100°C

This method of sterilisation kills almost all vegetative cells but doesn't ensure complete sterilisation. Recommended for sterilisation of pipette, rubber stoppers, scalpels, scissors.

(b) Tyndallisation

It is an **intermittent** or **fractional** sterilisation method in which the articles to be sterilized are exposed to steam at 100°C for 30-45 minutes on 3 successive days. In this way, it ensures the removal of spores.

- Temperature above 100°C

Principle

Moist heat sterilisation uses **steam under pressure** to achieve temperatures higher than those possible with boiling water at atmospheric pressure.

The major mechanism is **heat-induced denaturation and coagulation of cellular proteins**, resulting in loss of microbial viability.

The most important laboratory equipment using this principle is the autoclave.

Autoclave

It is an effective and reliable method for sterilising laboratory materials and decontaminating biological waste when it is used correctly. Steam under pressure allows temperatures such as **121°C** to be achieved, facilitating inactivation of heat-resistant microorganisms including spores.

Dry heat sterilisation

Dry heat uses **hot air** rather than steam.

Principle

Microbial destruction occurs primarily through **oxidative damage and dehydration of cellular components**.

Mainly consist of

(a) Red heat

Materials are held on the flame of the Bunsen burner till it becomes red hot. This type of sterilisation is used in case of inoculating loops, needles, forceps.

(b) Flaming

Flaming technique is used for the sterilisation of mouth of culture tubes, slides, scalpels were they are not made red hot.

(c) Incineration

It is a dry heat waste treatment process that burns organic and infectious materials at extreme temperature 800°C to 1200°C to completely destroy microorganism and reduce them to ash.

(d) Hot-air oven

It is an instrument which utilises dry heat for the sterilisation of

- certain glassware;
- metal instruments; and
- selected dry, heat-stable materials.

The temperature and time setting for the hot air oven is 160°C for 30 minutes or 180°C for 20 minutes.

2. Sterilisation by radiation

They are considered as cold sterilisation method which utilises radiations to destroy the DNA of the microorganisms.

Types of radiations used mainly include ionizing radiation which are high energy rays like gamma rays (γ), X- rays which has got high penetration power and non-ionising radiations involving low energy rays like, infra-red, UV light which have low penetration power and are mainly used for surface sterilisation.

3. Sterilisation by filtration

Filtration is the preferred method of sterilizing heat sensitive liquid and gases without exposure to denaturing heat. Rather than destroying contaminating microorganisms, it simply removes them. Used for sterilizing antibiotic solutions, toxic chemicals, vaccines, and carbohydrates, which are all heat sensitive.

The liquid or gas is passed through a filter, a device with pores too small for the passage of microorganisms, but large enough to allow the passage of the liquid or gas. Most commonly used filter is composed of **nitrocellulose and has a pore size of 0.22µm**. The size of the bacteria ranges from 0.3 to 0.5 µm Thus a filter of 0.22µm retains all bacteria and **spores** but not all viruses.

Sterilizing-grade filtration achieves microbial removal by physically retaining microorganisms within a validated filter system; it does not depend on killing the microorganisms.

Filtration of liquids: The solution is passed through a **cellulose acetate or cellulose nitrate membrane** with a vacuum (i.e, by applying negative pressure in the filter paper) or by forcing the solution through filter paper by imposing positive pressure above the fluid.

Filtration of air: High-Efficiency Particulate Air (HEPA) filters designed to remove organisms larger than 0.3 µm from isolation rooms, operating rooms, and Biosafety cabinet (LAF).

Types of filters	Material used	Application
Depth filter (HEPA)	Borosilicate glass fibers	Air sterilization
Membrane filter	cellulose acetate, cellulose nitrate or polysulfone.	Liquid sterilization
Candle filter	Carbon steel, polypropylene (PP)	Purification of water
Asbestos filter (Seitz filter)	Cellulose & diatomaceous earth	Thermolabile solution sterilisation
Sintered glass filter	Mesh layers of metals, polymers	

B. Chemical methods of sterilization

Chemical sterilization is the process of removal of microorganisms using chemical bactericidal agents. Appropriate to use for heat-sensitive materials like plastics, fiber optics, and biological specimens.

1. Chemical sterilants

The equipment which needs to be sterilized are submerged into liquid sterilants to kill all the viable microorganisms and spores. Examples include:

70% alcohol

Why is 70% alcohol a better disinfectant than 100% alcohol?

- (i) takes more time in evaporation from the surface, so contact time is increased
- (ii) penetrates in the cell wall at a slower rate when compared to that of 100% which would penetrate instantly creating a protein covering, protecting the cell wall

Sodium Hypochlorite

Aka Liquid bleach is another liquid chemical that can be used as a disinfectant. Submerging devices for 20-24 hours in liquid bleach might kill pathogenic organisms

2. Gaseous sterilization

Gaseous sterilization involves the process of exposing equipment or devices to different gases in a closed heated or pressurized chamber. More effective technique as gases can pass through a tiny orifice and provide more effective results. Examples include:

Ethylene oxide

Ethylene oxide (EO) gas is a common gas used for chemical treatment applied to sterilize or disinfect different types of equipment. Ethylene oxide (EO) gas is a common gas used for chemical treatment applied to sterilize, pasteurize, or disinfect different types of equipment.

Formaldehyde

Another important highly reactive gas which is used for sterilization. It possesses broad-spectrum biocidal activity and has found application in the sterilization of reusable surgical instruments, specific medical, diagnostic and electrical equipment, and the surface sterilization of powders.

LABORATORY INSTRUMENTS

Class II Biosafety Cabinet

A Class II Biological Safety Cabinet (BSC) is a primary containment device designed to provide protection to the laboratory worker, the surrounding environment, and the biological material being handled. It is particularly important when laboratory procedures may generate infectious aerosols or splashes.

Working principle

The protection provided by a Class II BSC is based on controlled airflow combined with HEPA filtration. A simplified airflow pattern is:

Room air → Front opening → Inward airflow → Work zone



HEPA-filtered downward airflow



Work surface → HEPA-filtered exhaust/recirculation.

The three levels of protection are provided

Protection	How it is achieved
Personnel protection	Inward airflow at the front opening helps contain aerosols
Product/sample protection	HEPA-filtered airflow across the work area reduces contamination
Environmental protection	Exhaust air is HEPA filtered

Applications

Class II BSCs are used when work requires primary containment of potentially infectious biological material, particularly when procedures can generate aerosols or splashes. Applications include:

- Handling of clinical or potentially infectious specimens
- Bacterial culture-related procedures with aerosol risk
- Preparation or manipulation of potentially infectious samples
- Procedures involving pipetting or other activities that may generate aerosols
- Molecular diagnostic procedures involving potentially infectious clinical material

Safety measures

Before starting work

- Only trained and authorised personnel should operate the BSC.
- Confirm that the cabinet is within its required certification/validation period.
- Ensure the front intake grill and exhaust pathway are unobstructed.

- Disinfect the working surface using the laboratory-approved disinfectant.
- Wear appropriate PPE according to the risk assessment and laboratory SOP.
- Arrange materials so that unnecessary obstruction of airflow is avoided.

During work

- Work with deliberate, controlled movements.
- Avoid blocking the front air-intake grille.
- Do not overcrowd the cabinet.
- Keep clean and potentially contaminated materials appropriately separated.
- Minimise activities that could generate aerosols.

After work

- Decontaminate the work surface according to the laboratory SOP.
- Remove waste using the appropriate biomedical-waste procedure.
- Decontaminate potentially contaminated materials before removal when required by the SOP.
- Report unusual airflow, alarms, damage or other cabinet malfunction immediately.
- Do not independently open or service HEPA filters or other critical cabinet components.

Cooling Centrifuge

A cooling centrifuge (refrigerated centrifuge) is a laboratory instrument used to separate components of a sample by rapid rotation while maintaining the sample at a controlled temperature. It is useful when samples are sensitive to heat generated during centrifugation.

Working principle

The centrifuge separates sample components based on differences in their size and density. During rotation, centrifugal force causes denser particles to move towards the bottom of the tube, forming a pellet, while less dense components remain in the liquid supernatant. The refrigeration system helps maintain the required temperature during centrifugation.

Applications

- Separation of serum or plasma from blood.
- Pelletting and concentration of cells or microorganisms from suspensions.
- Processing biological samples for molecular and biochemical analyses.

- Separation of temperature-sensitive biological materials under controlled conditions.

Safety measures

- Always balance the tubes properly before centrifugation.
- Check tubes and rotor for cracks or damage before use.
- Close the lid securely before starting the centrifuge.
- Never open the lid while the rotor is moving.
- When handling potentially infectious samples, use appropriate sealed rotors or safety cups as required by the laboratory SOP and risk assessment.
- In case of tube breakage or spillage, follow the laboratory's spill and decontamination procedure.

Autoclave

An autoclave is a laboratory sterilization equipment that uses saturated steam under pressure to sterilize suitable heat- and moisture-resistant materials and to decontaminate appropriate biological waste. It is commonly used for culture media, glassware and other autoclavable laboratory materials.

Working principle

An autoclave works by exposing materials to saturated steam at high temperature and pressure for a specified time.

Vapour pressure is the pressure exerted by a vapour when it is in equilibrium with its liquid phase at a given temperature. In a closed autoclave, heating water produces steam. As steam pressure increases, its corresponding saturation temperature also increases, allowing temperatures above the normal boiling point of water (121°C at 15 psi) to be achieved.

When saturated steam comes into contact with a cooler material, it condenses and releases latent heat. This moist heat penetrates the material and causes the destruction or inactivation of microorganisms, including bacterial spores, when an appropriate validated cycle is used.

Applications

- Sterilization of culture media and laboratory solutions
- Sterilization of suitable glassware and heat-resistant laboratory materials
- Sterilization of autoclavable laboratory instruments and supplies

- Decontamination of appropriate biological waste before disposal
- Preparation of sterile materials for microbiological and other laboratory procedures.

Safety measures

- Only trained personnel should operate the autoclave
- Do not overload the chamber; allow adequate space for steam penetration and circulation
- Use only containers, materials and packaging that are suitable for autoclaving
- Do not autoclave flammable, volatile, toxic or corrosive chemicals unless specifically approved for the equipment and validated procedure
- Ensure the door is properly closed and locked before starting the cycle
- Never open the autoclave while it is pressurized
- Wait until pressure and temperature have reached the appropriate safe level before opening
- Wear appropriate heat-resistant gloves and other required PPE when unloading
- Use appropriate chemical and/or biological indicators as required to monitor sterilization effectiveness

Soxhlet Apparatus

Soxhlet apparatus is the laboratory equipment used for the continuous extraction of desired compounds from solid materials using a liquid solvent. Soxhlet extraction is a continuous solid-liquid extraction technique used to extract soluble phytochemicals from dried plant material. A suitable solvent is repeatedly boiled, condensed, and passed through the plant material. When the extraction chamber fills, the solvent containing the extracted compounds siphons back into the boiling flask. This cycle is repeated, allowing efficient extraction using a relatively small volume of solvent.

Procedure

Prepare the plant material: Wash, dry and finely powder the plant material. Excess moisture should be removed where appropriate.

Load the sample: Place a measured quantity of the powdered plant material into a Soxhlet thimble and position it in the extraction chamber.

Add solvent: Add an appropriate solvent (e.g., ethanol, methanol, hexane, or another validated solvent) to the round-bottom flask.

Assemble the apparatus: Connect the boiling flask, Soxhlet extractor and condenser securely. Ensure all joints are properly fitted.

Heat: Heat the solvent gently using a heating mantle or water/oil bath, depending on the solvent and apparatus. The solvent vaporizes and condenses in the condenser.

Extraction cycles: The condensed solvent repeatedly fills the chamber and contacts the plant material. Once the solvent reaches the siphon level, it flows back into the boiling flask carrying dissolved phytochemicals.

Continue extraction: Allow repeated cycles until the extraction is considered complete according to the selected method.

Recover the extract: After cooling, separate the solvent from the extract using an appropriate evaporation method, such as a rotary evaporator, if required.

Store: Store the concentrated extract in a properly labelled, suitable container under conditions appropriate for the compounds being studied.

Safety Measures

- Use the correct solvent according to the target phytochemicals and laboratory protocol.
- Many extraction solvents are flammable and/or toxic; perform the procedure in a functional fume hood.
- Never use an open flame for heating organic solvents.
- Use a heating mantle or suitable controlled heating system and avoid overheating the solvent.
- Ensure the condenser has adequate cooling-water flow and that all glassware connections are secure.
- Do not overfill the boiling flask or extraction thimble.
- Allow the apparatus to cool before dismantling.
- Wear appropriate PPE: lab coat, safety goggles and solvent-compatible gloves.
- Handle and dispose of used solvents as chemical waste according to institutional procedures.
- Inspect glassware for cracks or damage before assembly

Rotary evaporator

A rotary evaporator is commonly used after plant extraction to remove the extraction solvent and concentrate the extract. It is particularly useful when the solvent needs to

be removed under relatively mild conditions to reduce unnecessary exposure of heat-sensitive phytochemicals.

Principle

Rotary evaporation is based on evaporation of a solvent under reduced pressure. The main principles are:

- Vacuum lowers the boiling point of the solvent
- The extraction flask is rotated, producing a thin film of liquid over the flask wall
- Rotation increases the surface area available for evaporation and promotes more uniform heating
- A controlled water bath supplies heat
- The solvent vapour passes into a condenser, where it is cooled and converted back into liquid
- The condensed solvent is collected in a receiving flask
- The plant extract remains increasingly concentrated in the evaporation flask

Key concept

Reduced pressure + controlled heating + rotation → efficient solvent evaporation at a lower temperature.

Procedure

Prepare the extract

- Obtain the plant extract using the selected extraction method and solvent.
- Remove large particulate plant material by appropriate filtration if required.

Transfer the extract

- Transfer the filtrate/extract into a suitable round-bottom evaporation flask.
- Do not overfill the flask, as this increases the risk of bumping and carryover.

Set up the rotary evaporator

- Ensure the evaporation flask, condenser, receiving flask and other connections are properly secured.
- Check that the condenser cooling system is operating.
- Ensure the vacuum system is connected appropriately.

Set the water bath

- Select a temperature appropriate for the solvent and the stability of the compounds being concentrated.
- The temperature should not be unnecessarily high, particularly when working with potentially heat-sensitive phytochemicals.

Start rotation

- Start rotation at a suitable speed.
- Rotation spreads the extract as a thin film and facilitates evaporation.

Apply vacuum gradually

- Reduce the pressure gradually rather than suddenly.
- Observe the extract for excessive foaming or bumping.
- Adjust the vacuum, rotation and bath conditions if vigorous boiling occurs.

Evaporate the solvent

- Continue evaporation while monitoring the process.
- The solvent vapour is condensed and collected in the receiving flask.

Concentrate the extract

- Continue until the desired degree of solvent removal/concentration is achieved.
- Avoid unnecessary prolonged heating or drying if the objective is only concentration.

Finish the operation

- Release the vacuum before removing the evaporation flask, following the instrument's operating procedure.
- Stop rotation and remove the flask carefully.
- Recover the concentrated plant extract using an appropriate container.

Clean the apparatus

- Clean the glassware and other components according to the laboratory SOP and solvent-specific waste-disposal requirements.

pH Meter

A pH meter is an electronic instrument used to measure the acidity or alkalinity of an aqueous solution by determining its hydrogen-ion activity. It is commonly used in microbiology and biomedical laboratories to check and adjust the pH of culture media, buffers and other laboratory solutions.

Working principle

A pH meter uses a pH-sensitive glass electrode and a reference electrode to detect an electrical potential that varies with the hydrogen-ion activity of the solution. The instrument converts this electrical signal into a pH value and displays the result.

Accurate measurement requires the electrode to be properly calibrated using standard buffer solutions. Temperature can affect pH measurement, so temperature compensation or measurement at the appropriate temperature should be considered.

Applications

- Checking and adjusting the pH of microbiological culture media.
- Preparation and verification of buffer solutions.
- Measuring the pH of laboratory reagents and aqueous samples.
- Monitoring pH during microbiological and biochemical experiments.
- Quality control of solutions where pH is a critical parameter.

Safety measures and precautions

- Calibrate the pH meter using appropriate standard buffers before measurement, as specified by the instrument SOP.
- Handle the glass electrode carefully; the sensing bulb is fragile.
- Rinse the electrode with appropriate distilled/deionized water between measurements and avoid cross-contamination.
- Do not wipe the glass bulb vigorously; gently blot it where appropriate.
- Keep the electrode properly stored in the recommended storage solution when not in use; do not allow it to dry.
- Ensure that the electrode is sufficiently immersed in the sample according to the manufacturer's instructions.
- Allow the reading to stabilize before recording the pH.

Microscope

A microscope is an optical instrument used to observe objects or structures that are too small to be clearly seen with the naked eye. A compound light microscope uses a system of lenses and visible light to produce a magnified image of a specimen.

Working principle

A compound light microscope works by magnifying a specimen using two lens systems – the objective lens and the eyepiece. Light passes through the specimen and is focused by the objective lens to form a magnified image. The eyepiece further magnifies this image for observation.

Different objective lenses provide different magnifications. The total magnification is calculated by multiplying the magnification of the objective lens by that of the eyepiece.

Applications

- Observation of bacterial, fungal and other microscopic structures.
- Examination of stained microbial smears.
- Study of cell morphology and cellular structures.
- Examination of biological specimens in clinical and research laboratories.
- Assessment of culture characteristics and microscopic morphology.

Safety measures and precautions

- Carry the microscope with both hands, supporting the base and arm.
- Handle slides carefully to avoid breakage and contamination.
- Start focusing with the low-power objective and progress to higher magnification as required.
- Use the fine adjustment for precise focusing at higher magnification.
- When using an oil-immersion objective, use only the appropriate immersion oil and clean the objective after use.
- Clean lenses only with proper lens-cleaning material; do not use ordinary tissue or abrasive materials.
- Switch off the illumination and cover the microscope after use.

Colorimeter

A colorimeter is an analytical instrument used to determine the concentration of a substance in a solution by measuring the intensity of colour produced by the substance or by a suitable chemical reaction. It is commonly used for quantitative analysis of coloured biological and biochemical samples.

Working principle

A colorimeter works on the principle of colorimetry, in which the amount of light absorbed by a coloured solution is related to the concentration of the absorbing substance. A selected wavelength of light is passed through the sample, and the instrument measures the amount of light transmitted through it.

The measured absorbance is generally related to concentration according to the Beer–Lambert law, within the applicable concentration range. A blank is used to account for background absorbance, and standards of known concentration may be used to establish a calibration relationship.

Applications

- Quantitative estimation of biochemical substances in laboratory samples.
- Measurement of products formed in enzyme and biochemical assays.
- Determination of microbial growth by measuring optical density/turbidity, where appropriate.
- Quantification of coloured reaction products in microbiological and research assays.
- Routine analysis of solutions in teaching, research and laboratory quality-control procedures.

Safety measures and precautions

- Select the appropriate wavelength/filter for the assay.
- Use clean, suitable cuvettes and handle them by the opaque/frosted sides where applicable.
- Ensure that the cuvette is free from fingerprints, droplets and air bubbles before measurement.
- Use an appropriate blank and standards according to the assay protocol.
- Keep the sample volume and cuvette orientation consistent between measurements.
- Do not spill liquids into the instrument or place a wet cuvette inside it.
- Clean the sample compartment after use if any spill occurs.

Orbital Shaking Incubator

An orbital shaking incubator is a laboratory instrument that combines controlled-temperature incubation with continuous orbital shaking. It is used to grow microorganisms or maintain biological cultures while providing uniform mixing and aeration.

Working principle

The instrument uses an electrically driven platform that moves in a circular orbital motion at a controlled speed. The shaking continuously mixes the culture, improving contact between the cells and the surrounding medium and promoting uniform distribution of nutrients and oxygen.

At the same time, a temperature-control system maintains the chamber at the selected temperature, providing controlled conditions for microbial growth.

Applications

- Cultivation of microorganisms in liquid media.
- Growth of bacterial cultures requiring continuous agitation and aeration.
- Preparation of microbial cultures for downstream microbiological and molecular studies.
- Studies involving microbial growth and metabolite production under controlled conditions.
- Mixing and incubation of biological cultures where both temperature control and agitation are required.

Safety measures

- Ensure culture flasks or containers are securely positioned on the shaking platform.
- Do not overload the platform or exceed the recommended speed and load.
- Keep containers properly closed to prevent spillage and contamination.
- Check that the platform is stable before starting the shaker.
- Follow the laboratory SOP for handling cultures and decontaminate the instrument after use.

CULTURE MEDIA

Culture media are prepared nutrient formulations used to support the growth, isolation, maintenance, identification, and/or enumeration of microorganisms under laboratory conditions.

Basic requirements for a culture media

- Carbon source
- Energy source
- Nitrogen source
- Minerals and salts
- Growth factors

Classification of Culture Media

Culture media can be classified according to several criteria:

I . Based on physical state

- Liquid media (broth)**
In broth media, nutrients are dissolved in water, and bacterial growth is indicated by a change in broth's appearance from clear to turbid. Examples: nutrient broth, glucose broth, brain-heart infusion (BHI) broth, alkaline peptone water.
- Semi-solid media**
It is used to check if bacteria is motile or not. Prepared by addition of agar in less concentrations so that they won't get solidified (0.2-0.5%). Examples: Stuart's and Amies transport media
- Solid media**
Any liquid medium can be made solid by the addition of certain solidifying agents. Agar agar (simply called agar) is the most commonly used solidifying agent which is obtained from some species of red algae seaweed such as the genera *Gelidium* or *Gracilaria* Examples: MacConkey agar, chocolate agar, nutrient agar, blood agar,

II . Based on composition

- Basal/simple media**
Simple media are those which contain only basic nutrients required for the growth of ordinary organisms (non-fastidious microbes) and used as a general-purpose media. Example: peptone water, nutrient broth and nutrient agar.
- Complex/undefined media**

It contains a variety of ingredients such as meat juices and digested proteins. The exact chemical composition of these ingredients can be highly variable. Example: Nutrient broth is used for the preparation of meat infusion broth, meat extract broth, digest broth

iii. Defined/synthetic media

A defined medium (also known as chemically defined or synthetic medium) is a medium which is made only from pure, known chemical compounds, no yeast, animal, or plant tissue is present.

It contains known concentration of ingredients, like sugar (glucose or glycerol) and nitrogen source (such as ammonium salt or nitrate as inorganic nitrogen). Examples: Mineral glucose medium

iv. Special-purpose media

These are customised media used for the isolation, identification and preservation of microorganisms which cannot be grown in simple media. Examples: enriched media, enrichment media, selective media

III. Based on function or purpose

i. Enriched media

Enriched media are basal media supplemented with additional nutrients or growth-promoting substances to support the growth of fastidious microorganisms.

Common enrichment substances include:

- blood
- serum
- egg
- specific growth factors

Examples:

1. Blood agar

Blood agar consists of a suitable basal agar supplemented with 5% blood. It supports the growth of many clinically important bacteria and allows observation of haemolysis.



Figure 1: Blood agar showing α β and γ haemolysis

2. Chocolate agar

Chocolate agar is prepared by heating blood-containing medium at a temperature 75°C so that red blood cells are lysed.

It provides additional growth factors and is used for organisms with demanding nutritional requirements.

Examples of organisms that may be recovered on chocolate agar include:

- *Haemophilus influenzae*
- *Neisseria gonorrhoeae*
- *Neisseria meningitidis*



Figure 2: Chocolate agar

ii. Enrichment media

Enrichment media are a form of extremely selective media that only permits a specific species of microbe to grow in it while preventing undesirable, commensal, or contaminating microbes from growing.

As a result, enrichment media can be used to concentrate on a certain type of microorganism while recovering it from the rest of the microorganisms present in the media.

Enrichment media are typically liquid in their consistency.

1. Selenite F broth

2. Alkaline peptone water (APW) is used as an enrichment medium for *Vibrio*, particularly when *Vibrio cholerae* is suspected.

It is a type of media used for the selective isolation of a specific type of bacteria while inhibiting the growth of others. It's an agar-based medium that is used to isolate microorganisms in laboratory.

These media are used to isolate particular bacteria from specimens where mixed bacterial flora is expected.

1. MacConkey agar

Figure 3: MacConkey agar showing LF and NLF

2. Mannitol Salt Agar (MSA)

MSA contains a high concentration of sodium chloride (7.5%), which inhibits many bacteria while allowing salt-tolerant organisms such as *Staphylococcus* species to grow.

MSA is actually both selective and differential, because mannitol fermentation can be detected using a pH indicator.

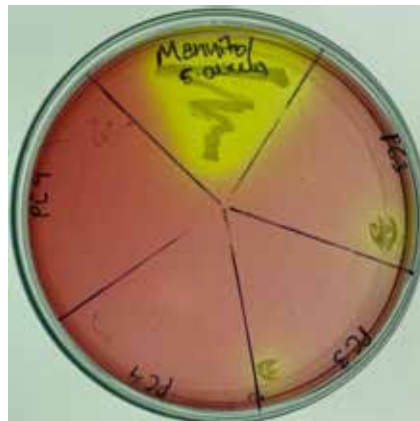


Figure 4: MSA agar with positive results indicating mannitol fermentation

3. TCBS agar

Thiosulfate-Citrate-Bile Salts-Sucrose (TCBS) agar is a selective and differential medium used for the isolation of *Vibrio* species.

4. EMB agar

EMB agar is selective for gram-negative bacteria. The dye methylene blue in the medium inhibits the growth of gram-positive bacteria; small amounts of this dye effectively inhibit the growth of most gram-positive bacteria and bile salt inhibits non-intestinal bacteria.



Figure 5: EMB agar

5. CLED agar

Cystine-Lactose-Electrolyte-Deficient (CLED) medium is generally used for diagnostic routine urinary bacteriology as a non-selective medium capable of supporting the growth of most urinary pathogens.

It is also a differential medium and supports the growth of urinary pathogens and contaminants and gives good colonial differentiation without the spread of *Proteus* species due to its lack of electrolytes.

Components:

L-cystine supports the growth of the cysteine-dependent dwarf coliform colony. Lactose is a fermentable carbohydrate that provides carbon and energy. Organisms capable of fermenting lactose will lower the pH and change the color of the medium from green to yellow. The color change is indicated by the presence of the pH indicator Bromothymol Blue.

Yellow Colonies: Indicate lactose-fermenting bacteria (such as *E. coli*).

Blue or Green Colonies: Indicate non-lactose-fermenting bacteria (such as *Pseudomonas*).



Figure 6: CLED agar

iv. Differential media

It contains indicators or substrates that allow microorganisms growing on the medium to be distinguished from one another based on a visible biochemical reaction.

The medium does not necessarily prevent the growth of other organisms.

Example:

1. MacConkey agar contains lactose and a pH indicator.
Lactose-fermenting bacteria → acid production → coloured/pink colonies
Non-lactose-fermenting bacteria → pale/colourless colonies
2. Blood agar differentiates organisms according to their haemolytic reaction:
 β -haemolysis → complete haemolysis
 α -haemolysis → partial haemolysis/greenish discoloration
 γ -haemolysis → no haemolysis
3. DNase agar contains polymerised DNA which is mainly used to check whether the bacteria produce the enzyme deoxyribonuclease (DNase).

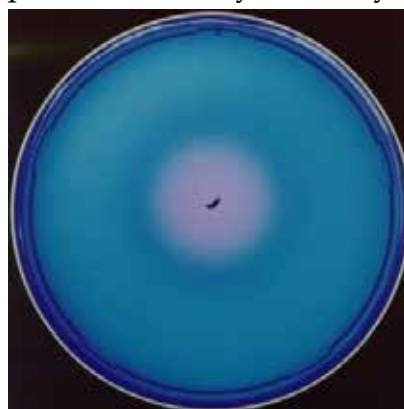


Figure 7: DNase agar with halo zone

v. Indicator media

They are specialised microbiology culture media that change color or appearance to identify and differentiate microorganisms based on their biochemical activity.

Indicator media contains specific sugars, nutrients, or chemical dyes (such as phenol red, neutral red, or methylene blue)

Examples: MacConkey agar indicates lactose fermenting property. Lactose fermenters (LF) produce pink coloured colonies while non-lactose fermenters (NLF) are colourless which is indicated with the help of neutral red indicator.

Wilson Blair medium contains sulfite which acts as an indicator. *Salmonella. typhi* reduces the sulfite into sulfide in the presence of glucose and the colonies appear as black metallic sheen

vi. Transport media

Transport media are designed to maintain the viability of microorganisms in a clinical specimen during transportation to the laboratory without allowing significant multiplication and to prevent the overgrowth of contaminating organisms. It does not contain any growth factors, and they can be semi solid or liquid in consistency.

Examples

Stuart's transport medium

Used for transporting various clinical specimens, particularly swab specimens. It is mainly used for the transport of gonococci

Amies transport medium

A modified transport medium used for maintaining viability of organisms during transport. Used for the transportation of gonococci.

Cary-Blair transport medium

Widely used for transportation of enteric bacterial pathogens in stool specimens. Used for the transportation of *Campylobacter* and *Vibrio cholerae*

IV. Based on oxygen requirement

i. Aerobic media

Used for the culturing of microorganisms which can grow in the presence of atmospheric oxygen.

Examples:

Nutrient agar

Blood agar

MacConkey agar

ii. Anaerobic media

They are specialized growth formulations designed to cultivate bacteria that cannot survive or multiply in the presence of oxygen. They are also called reducing media. **Reducing media** contain substances like L-cysteine, sodium thioglycolate are added that reduce the oxidation-reduction potential of the medium and thereby create conditions more favourable for anaerobic organisms.

Examples: **Thioglycollate broth**

Thioglycollate broth is particularly useful for demonstrating the **oxygen requirements** of microorganisms.

A typical pattern can be interpreted as:

Growth pattern	Interpretation
Throughout the tube	Facultative/oxygen-tolerant growth pattern
Concentrated at top	Obligate aerobe
Concentrated at bottom	Obligate anaerobe
Thin band just below surface	Microaerophile
Uniform throughout	Aerotolerant anaerobe

Robertson's cooked meat medium

This media contains nutrient broth with meat infusion. Liquid paraffin is layered over the broth to prevent the entry of air. The unsaturated fatty acid in the meat acts as reducing agent and absorb oxygen while it also contains nutrients. It is used for culturing *Clostridium* spp.

STAINING TECHNIQUES

Staining, in microbiology, can be defined as a technique which is used to enhance and contrast a biological specimen at the microscopic level.

Types of Stains

Acidic stain

Contains negatively charged acid radicals and binds to positively charged cell structures. Example: Acid fuchsin, Eosin, Rose Bengal.

Basic stain

Contains positive charged basic radicals and binds to negatively charged particles.

Example: crystal violet, methylene blue, basic fuchsin.

Neutral stain

Both positively and negatively charge imparts different colour to different components.

Example: Geimsa's stain, Leishman's stain

Types of Staining Techniques

(i) Simple staining

It is a technique that stains the bacterial cells in a single colour by using a single dye and they cannot be used to differentiate between different types of organisms. Simple stains are used to stain whole cells or to stain specific cellular components.

(ii) Differential staining

Differential staining is **a staining process which uses more than one stain.**

Using multiple stains can be better to differentiate between different microorganisms or structures/cellular components of a single organism.

- Gram staining
- Acid fast staining
- Capsule staining

Bacterial smear preparation:

Smearing is the distribution of bacterial cells on the slide for the purpose of viewing under the microscope.

Method:

Aseptically a small sample of the culture is spread over a slide surface. Then it is allowed to dry. Heat fixation is done to help the cell adhere to the surface of the slide.

Air dried smears are passed through flame two-three times. Do not overheat. Allow slide to cool before staining. This slide can be used for staining.

Gram staining

Gram staining differentiates the bacteria into two types

- Gram positive

The cell walls of gram-positive bacteria have a thick layer of peptidoglycan and lipid content is low.

- Gram negative

Cell wall made of thin peptidoglycan layer and contains high lipid content.

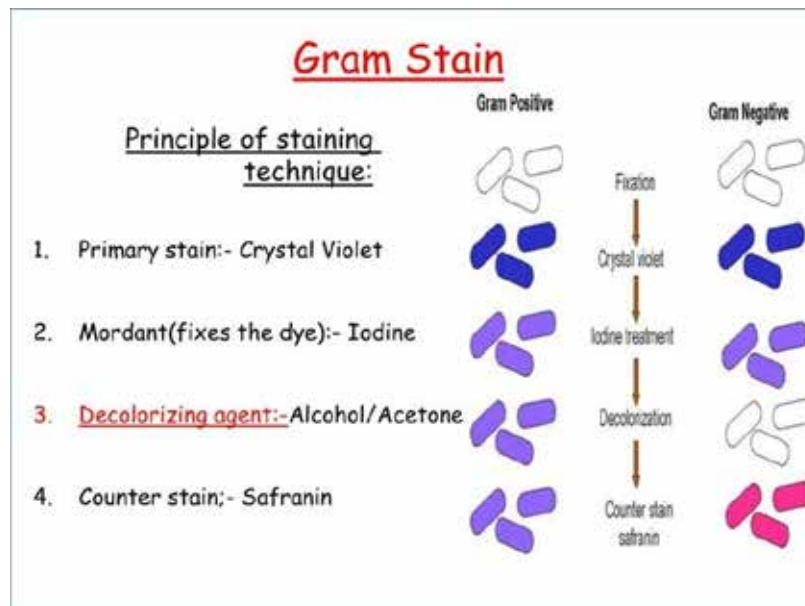


Figure 8: Gram's staining

For gram positive bacteria,

- When decolorizing solution (ethanol or a mixture of ethanol and acetone) is added it interacts with lipids in the cell wall.
- Decolorizer dehydrate and shrink cell wall which closes the pores and prevents the stain from exiting the cell.
- Ethanol cannot remove the Crystal Violet-Iodine complex that is bound to the thick layer of peptidoglycan of gram positive bacteria and appears purple in color.

For gram negative bacteria

- cell wall also takes up the CV-Iodine complex but due to the thin layer of peptidoglycan and outer layer which is formed of lipids, CV-Iodine complex gets washed off.
- When counterstain, positively charged safranin, is added, it interacts with the free negatively charged components in Gram-Negative cell wall and membrane and bacteria becomes pink/red.

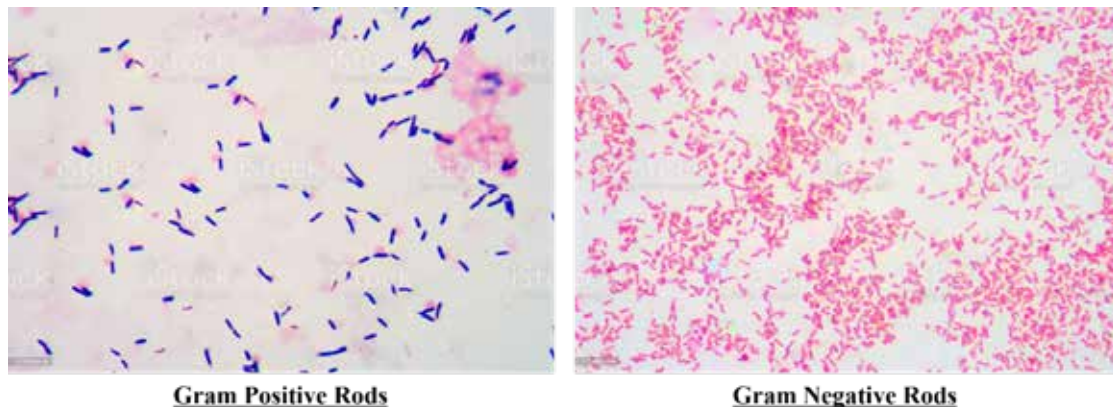


Figure 9: Gram positive and Gram negative rods

Acid fast staining (Ziehl-Neelsen staining)

- It is the differential staining techniques where bacteria are differentiated into acid fast and non-acid fast strains.
- **Organisms which are not stained by simple or Gram staining method, particularly the member of genus *Mycobacterium*, are resistant and can only be visualized by acid-fast staining.**

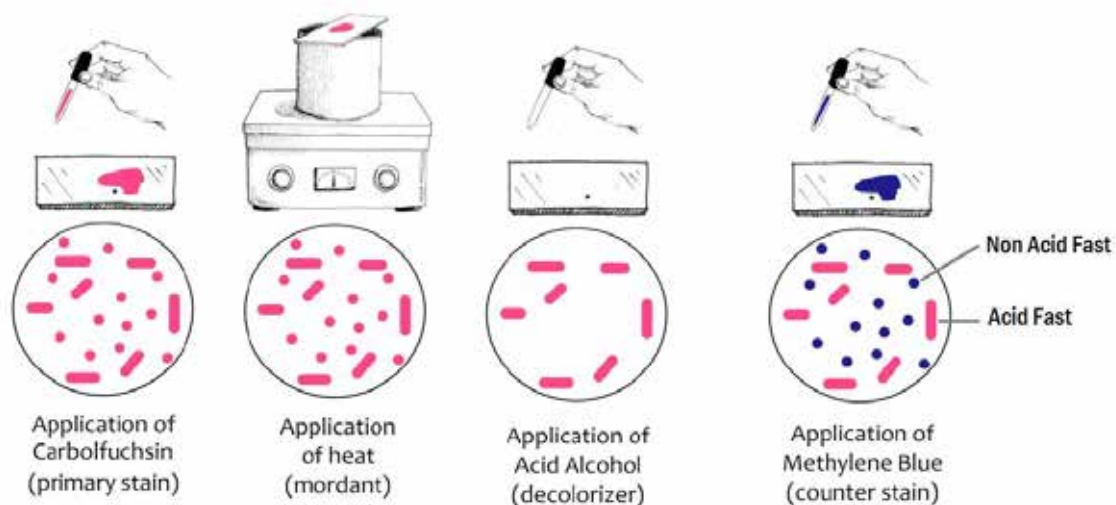


Figure 10: Acid fast staining procedure

Principle

Primary stain interacts with cell wall mycolic acid. The acid alcohol does not decolorise the stain from the acid fast bacteria. So, the bacteria are observed as red/pink while the secondary stain give contrasting colour to the background.

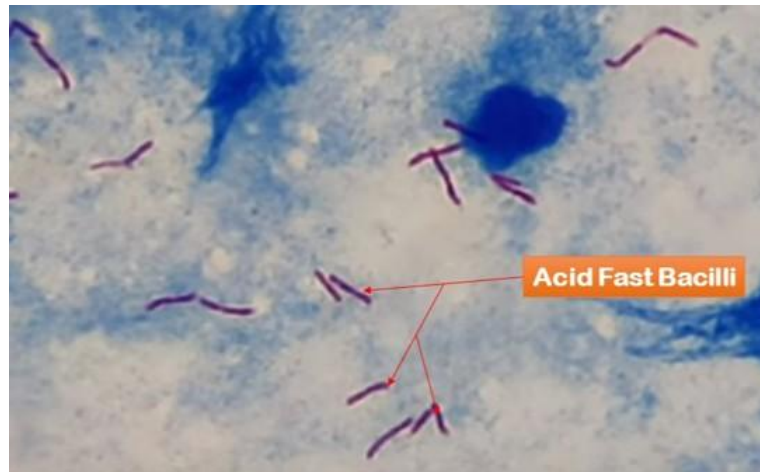


Figure 11: acid fast bacteria observed as reddish colour

Capsule staining (Negative staining)

Capsules are outermost layer which provides pathogenicity to most of the bacteria. They are composed of polysaccharide and are non-ionic so no acidic nor basic stains can be added for the visualization of the capsule. So instead of staining the cells the background is stained so that it provides a contrast. That is why they are called as negative staining. Indian ink or nigrosine is used for negative staining.

Capsule Staining Protocol

1. Place 1 drop of Indian ink near one edge of a clean slide.
2. Aseptically get a loop of culture given to you and mix it with the ink drop on slide.
3. Use a second slide as spreader and spread the mixture first by touching the short edge of spreader to the mixture.
Let the mixture run across the edge and then spread the liquid across the length of the slide by holding the spreader tilted.
4. Let it air dry completely, no heat fixation and no washing, Heat deteriorates capsule
5. Cover the smear with Safranin and wait for 1 min.
6. Rinse with dH₂O.
7. Perform blotting and air dry if necessary.
8. Observe with 100x objective

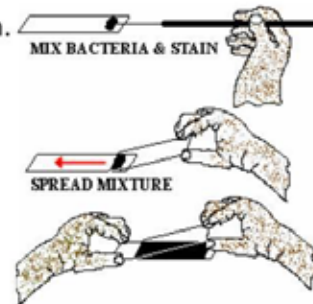


Figure 12: Capsule staining procedure

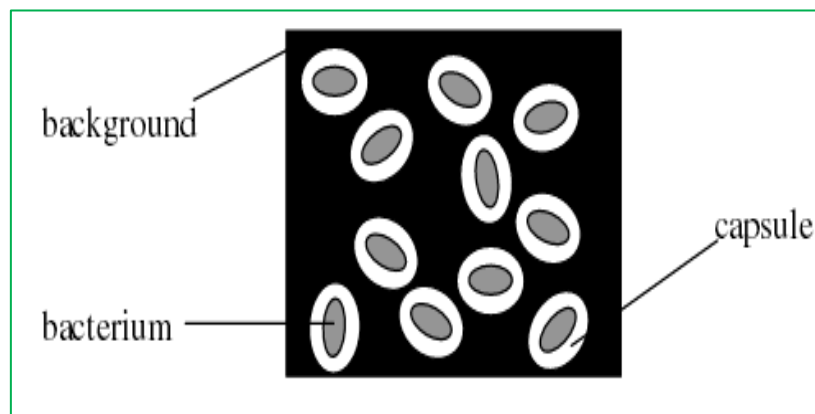


Figure 13: Negative staining

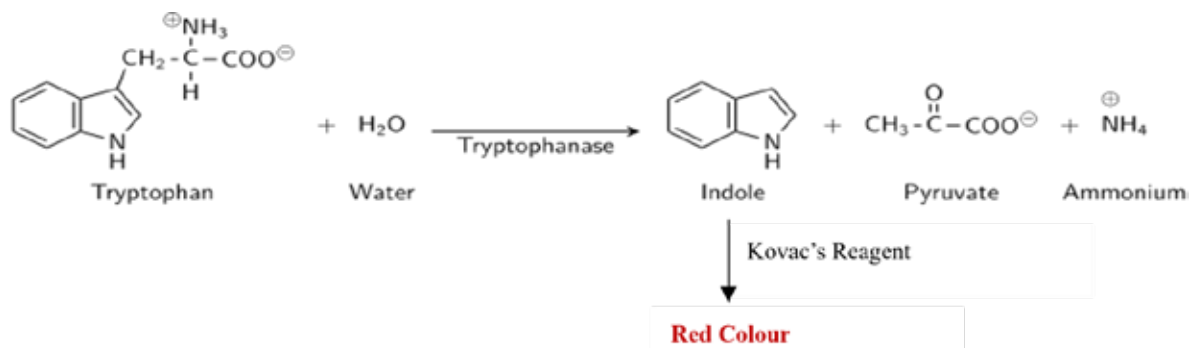
BIOCHEMICAL CHARACTERISATION

Principle

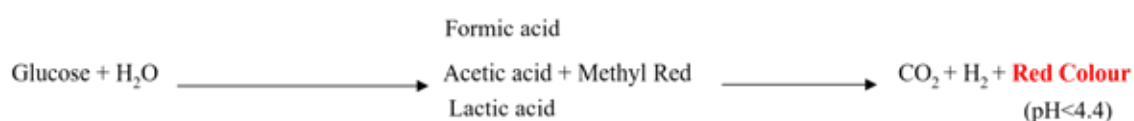
IMViC reactions are a set of four useful reactions that are commonly employed in the identification of members of family Enterobacteriaceae. The four reactions are: Indole test, Methyl Red (MR) test, Voges Proskauer (VP) test and Citrate utilization test.

1. **Indole test:** Some bacteria can produce indole from amino acid tryptophan using the enzyme typtophanase. Production of indole is detected using Ehrlich's reagent

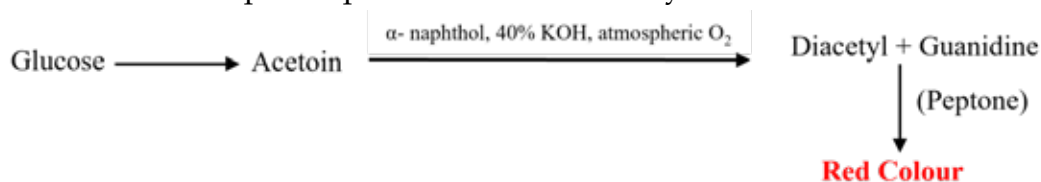
or Kovac's reagent. Kovac's reagent consists of para-dimethyl aminobenzaldehyde, isoamyl alcohol and conc. HCl. Indole reacts with the aldehyde in the reagent to give a red colour. An alcoholic layer concentrates the cherry red colour as a ring at the top.



2. **Methyl red (MR) test:** This is to detect the ability of an organism to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce large amounts of acids from glucose fermentation that they overcome the buffering action of the system. Methyl Red is a pH indicator, which remains red in color at a pH of 4.4 or less.

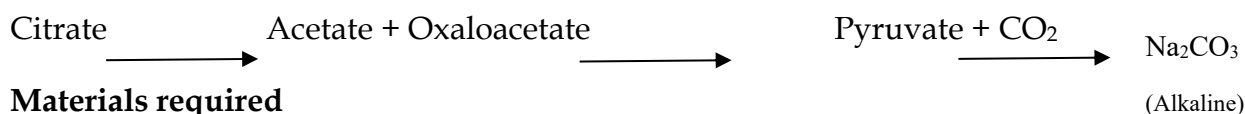


3. **Voges Proskauer (VP) test:** VP test detects butylene glycol producers. Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In this test two reagents, alpha-naphthol and 40% KOH are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone, in the presence of alpha-naphthol to produce red color. Role of alpha-naphthol is that of a catalyst and a color intensifier.



4. **Citrate utilization test:** This test detects the ability of an organism to utilize citrate as the sole source of carbon and energy. Bacteria are inoculated on a medium containing sodium citrate and a pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which is utilized as sole source of nitrogen. Utilization of citrate involves the enzyme citritase, which breaks down citrate to oxaloacetate and acetate. Oxaloacetate is further broken down to pyruvate and CO_2 . Production of Na_2CO_3 as well as NH_3 from utilization of sodium citrate and

ammonium salt respectively results in alkaline pH. This results in change of medium's color from green to blue.



Materials required

- Pure culture of bacteria
- Sterile test tubes
- Inoculating loop
- Bunsen Burner
- Marker pen

Reagents required

- Peptone water / SIM Media
- Kovac's Reagent
- MRVP Broth
- Methyl Red Reagent
- Barritt's Reagent A and Barritt's Reagent B
- Simmon's Citrate Agar

Procedure

1. **Indole test:** Bacterium to be tested is inoculated in peptone water, which contains amino acid tryptophan and incubated overnight at 37°C. Following incubation few drops of Kovac's reagent are added. Kovac's reagent is more sensitive in detecting indole production in anaerobes and non-fermenters. Formation of a red or pink coloured ring at the top is taken as positive.
2. **Methyl red (MR) test:** The bacterium to be tested is inoculated into glucose phosphate broth, which contains glucose and a phosphate buffer and incubated at 37°C for 24 hours. Over the 24 hours the mixed-acid producing organism must produce sufficient acid to overcome the phosphate buffer and remain acid. The pH of the medium is tested by the addition of 5 drops of MR reagent. Development of red color is taken as positive. MR negative organisms produce yellow color.
3. **Voges Proskauer (VP) test:** Bacterium to be tested is inoculated into glucose phosphate broth and incubated for at least 48 hours. 5 drops of alpha-naphthol is added to the test broth and shaken. 3 drops of 40% KOH is added to the broth and shaken. The tube is allowed to stand for 15 minutes. Appearance of red color is taken as a positive test. The negative tubes must be held for one hour, since maximum color development occurs within one hour after addition of reagents.
4. **Citrate utilization test:** Bacterial colonies are picked up from a straight wire and inoculated into slope of Simmon's citrate agar and incubated overnight at 37°C. If the organism has the ability to utilize citrate, the medium changes its color from green to blue.

Other test also includes

5. **Catalase test:** Demonstrates the presence of catalase, an enzyme that catalyzes the release of oxygen from hydrogen peroxide which is considered to be toxic for the bacteria.

1 drop of hydrogen peroxide solution is poured over a 24-hour nutrient agar slope culture of the test organism and the tube is held in a slanting position.

Alternatively, a small amount of the culture to be tested is picked from a nutrient agar slope with a clean sterile platinum loop or a clean, thin glass rod and dip it in a drop of 10 percent hydrogen peroxide on a clean glass slide.

- Positive test – Immediate bubbling indicating the formation of oxygen
- Negative test – No bubbling indicating absence of oxygen

6. **Oxidase test:** Used to detect the presence of cytochrome c oxidase enzyme which is mainly involved in ETC (Electron Transport Chain). The presence of the enzyme is detected by smearing the bacteria onto sterile oxidase disc which contains a dye p-phenylenediamine dihydrochloride which turns purple on interaction with bacteria that produces oxidase.

7. **Coagulase test:** Coagulase is an enzyme that converts fibrinogen into fibrin, leading to clot formation. This enzyme acts as a virulence factor, helping bacteria evade the immune system. There are two different forms of coagulase: free coagulase and bound coagulase. The bound coagulase is called the clumping factor and is detected rapidly by a slide test. The free coagulase, in turn, is detected in the test tube because of the formation of a clot. The bacteria which produce coagulase enzyme shows agglutination once the plasma is added.

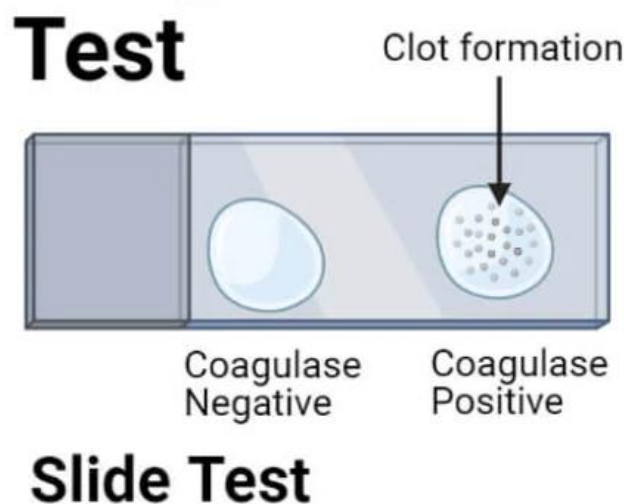


Figure 14: Positive results for coagulase test

Antibiotic Susceptibility Test

Kirby-Bauer Disc Diffusion Method

Principle

This method is also called the agar diffusion method or the disk diffusion method. The procedure followed is simply that a filter disk impregnated with an antibiotic is applied to the surface of an agar plate containing the organism to be tested and the plate is incubated at 37°C for 24-48 hours. As the antimicrobial agent diffuses from the filter paper into the agar, the concentration decreases as a function of the square of the distance of diffusion.

At some distance from each disk, the antibiotic is diluted to the point that it no longer inhibits microbial growth. The effectiveness of a particular antibiotic is shown by the presence of growth-inhibition zones. These zones of inhibition (ZOIs) appear as clear areas surrounding the disk from which the substances with antimicrobial activity diffused. The diameter of the ZOI can be measured with a ruler and the results of such an experiment constitutes an antibiogram.

Thus, a “zone of inhibition” can be observed and measured to determine the susceptibility to an antibiotic for that particular organism. The measurement is compared to the criteria set by the Clinical and Laboratory Standards Institute (CLSI) which are globally recognised standards developed to ensure quality, consistency and accuracy in medical laboratories. They provide standard methods and breakpoints to test how bacteria and fungi respond to drugs. Based on the criteria, the organism can be classified as being Resistant (R), Intermediate (I) or Susceptible (S).

The culture broth used for the test should have density of about 1.5×10^8 colony forming units (CFU/ml) by comparing its turbidity with 0.5 **McFarland standard**. They are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range to standardize microbial testing.

Materials required

1. Bacterial pure culture in broth
2. Mueller Hinton Agar (MHA)
3. Antibiotic Discs
4. Cotton Swab
5. Forceps

Procedure

A. Inoculation of Mueller Hinton Agar

1. Dip a sterile cotton swab into the bacterial suspension. To remove excess liquid, rotate the swab several times with a firm pressure on the inside wall of the tube above the fluid level
2. Using the swab, streak the Mueller-Hinton agar plate to form a bacterial lawn
3. To obtain uniform growth, streak the plate with the swab in one direction, rotate the plate 90° and streak the plate again in that direction
4. Repeat this rotation 3 times
5. Allow the plate to dry for approximately 5 minutes
6. Use an Antibiotic Disc Dispenser to dispense discs containing specific antibiotics onto the plate
7. Using sterile sticks or loops, gently press each disc to the agar to ensure that the disc is attached to the agar
8. Plates should be incubated overnight at an incubation temperature of 37°C in inverted position

B. Reading and interpreting zone sizes

1. After overnight incubation measure the zone sizes (area of no growth around the disk) in millimetres using a ruler or template
2. Interpret the results as Resistant or Susceptible for each antibiotic

Well Diffusion Method

The agar well diffusion method is a laboratory test used to measure the antimicrobial activity of plant extracts, antibiotics, or other chemical substances.

Principle

The technique is based on the diffusion of an antimicrobial substance through solid agar. When the test substance is placed in a well:

Antimicrobial in well → diffusion through agar → inhibition of bacterial growth → zone of inhibition

The diameter of the resulting zone can be measured in millimetres. A larger zone may indicate greater apparent antimicrobial activity under the specific experimental conditions, but zone size is affected by several factors, including:

- concentration and volume of the antimicrobial
- diffusion characteristics of the compound
- molecular size and physicochemical properties

- agar composition and depth
- inoculum density
- incubation conditions
- stability of the antimicrobial in the medium

Therefore, zone diameter alone is not a direct measurement of MIC or antimicrobial potency. Standardized AST methods establish relationships between zone diameters and MICs using validated methodology.

Materials Required

- MHA
- Pure culture of the test organism
- Sterile saline or another validated diluent for inoculum preparation
- Sterile Petri plates
- Sterile cotton swab
- Sterile cork borer/well punch or equivalent device
- Micropipette and sterile tips
- Test antimicrobial preparations
- Appropriate controls
- Incubator
- Ruler for measuring inhibition zones

For standardized AST of many common aerobic bacteria, Mueller-Hinton agar (MHA) is the standard medium used for disk diffusion. EUCAST specifies Mueller-Hinton media for its standardized disk-diffusion method, with supplemented versions for specified fastidious organisms.

Procedure

Preparation of the test organism

Prepare a fresh, pure culture of the test microorganism. Prepare and standardize the inoculum according to the laboratory's validated AST procedure. For standardized AST, inoculum density is a critical variable because differences in inoculum can alter the measured inhibition zone.

Inoculation of the agar plate

Using a sterile swab, inoculate the standardized bacterial suspension uniformly over the surface of the agar to obtain a confluent lawn of growth after incubation. Allow the inoculated surface to settle/dry sufficiently according to the laboratory SOP before making the wells.

Preparation of wells

Using a sterile well punch/cork borer, make appropriately sized wells in the agar. Remove the agar plugs carefully without damaging the surrounding agar. The wells should be clearly separated so that the resulting inhibition zones do not overlap.

Addition of test substances

Add a defined volume and concentration of each antimicrobial preparation into the respective wells. Include appropriate controls, such as:

- Negative/vehicle control – confirms whether the solvent or vehicle itself produces inhibition
- Positive control – an antimicrobial with known activity, where appropriate

The volume, concentration and identity of every test preparation should be recorded.

Incubation

Incubate the plates under the appropriate temperature, atmosphere and duration for the test organism and validated method. These conditions should not be arbitrarily changed because growth conditions influence the size of the inhibition zone.

Measurement

Following incubation, examine the plates for zones of growth inhibition. Measure the diameter of each zone in millimetres, preferably across two perpendicular axes when the zone is not perfectly circular and record the result.

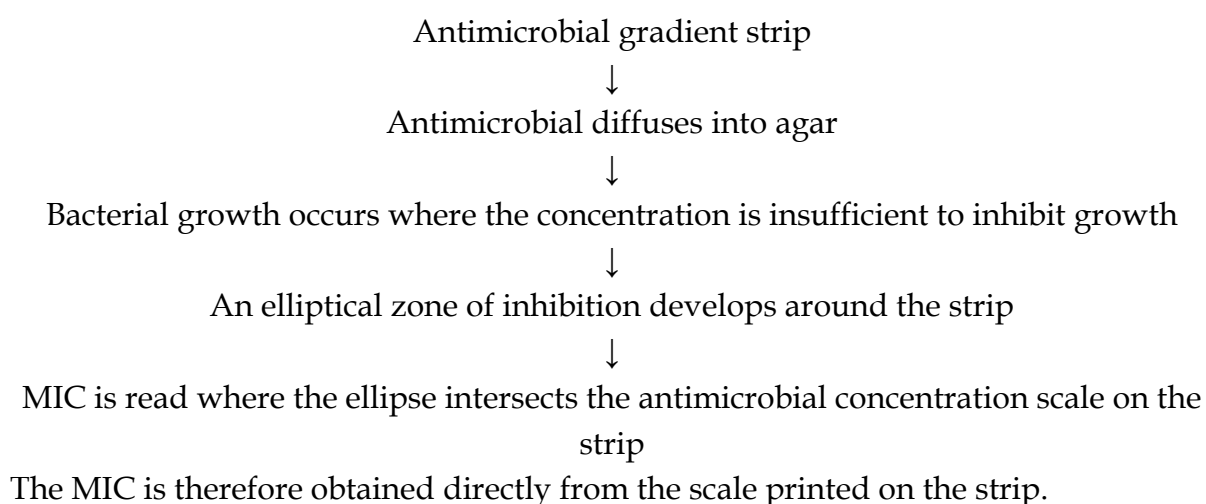
Minimum Inhibitory Concentration (MIC) – Gradient Diffusion Strip Method

The Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial agent that prevents visible growth of a microorganism under defined test conditions. MIC is an important quantitative parameter in antimicrobial susceptibility testing because, unlike a zone of inhibition, it provides an antimicrobial concentration associated with inhibition of visible bacterial growth.

One commonly used method for determining MIC is the antimicrobial gradient diffusion strip method, commercially available as systems such as Etest®. The strip contains a continuous, predefined gradient of antimicrobial concentrations along its length.

Principle

The test is based on diffusion of an antimicrobial concentration gradient from a strip into an inoculated agar surface. The basic process is:



The MIC strip

An MIC gradient strip is a thin, plastic strip containing a predefined concentration gradient of an antimicrobial agent. The concentration generally increases progressively from one end of the strip to the other. The strip has:

- an antimicrobial concentration scale
- the antimicrobial identity/code
- a concentration gradient
- a defined surface for placement onto inoculated agar

Commercial gradient strips are calibrated so that the concentration corresponding to the point where the inhibition ellipse intersects the strip can be read directly.

Procedure

Prepare the bacterial inoculum

Prepare a fresh, pure culture of the test organism. Prepare the inoculum to the density specified by the validated method being used. Accurate inoculum standardisation is essential because inoculum density can influence the MIC result.

Inoculate the agar

Using a sterile swab, inoculate the standardized bacterial suspension uniformly over the surface of the appropriate susceptibility-testing agar to produce a confluent bacterial lawn. Organisms for which standardised gradient diffusion testing is appropriate, the manufacturer's instructions and the relevant CLSI/EUCAST methodology should be followed for medium, inoculum, incubation atmosphere and other conditions.

Place the MIC strip

Using sterile forceps or the recommended applicator, place the strip antimicrobial-containing side in contact with the agar surface. Ensure that:

- the strip lies flat against the agar
- there are no air bubbles beneath it
- the strip is not moved after contact with the agar

Incubate

Incubate the plate under the specified conditions for the organism and antimicrobial being tested. The incubation temperature, atmosphere and duration should follow the manufacturer's instructions and the applicable standardised method.

Read the MIC

After incubation, an elliptical zone of inhibition should be visible around the strip. Locate the point where the edge of the inhibition ellipse intersects the concentration scale on the strip. The value at this intersection is recorded as the MIC. The intersection between the inhibition ellipse and the strip scale is the critical reading point.

BASIC TECHNIQUES IN CELL CULTURE

Introduction

Cell culture is a foundational biological technique that involves isolating cells from tissue sources and maintaining them within a strictly controlled artificial environment. Conceptualized in the early twentieth century for embryological studies, it has evolved into a standard methodology across cellular biology, pharmacology, cancer research, and regenerative medicine. Its primary goal is to provide isolated cells with the essential physicochemical conditions required to survive, proliferate, or differentiate outside a living organism.

At its core, cell culture simulates an *in vivo* environment using *in vitro* laboratory equipment. Cells are maintained in a liquid medium supplying essential nutrients, including amino acids, vitamins, glucose, and inorganic salts often supplemented with serum or growth factors to stimulate proliferation. To sustain these cells, incubators continuously regulate environmental parameters by maintaining a standard 37°C temperature, high humidity, and a 5% CO₂ atmosphere to stabilize the pH of the medium.

Cultures generally fall into two functional types: adherent and suspension. Adherent cells (e.g., fibroblasts, epithelial cells) require attachment to tissue-culture-treated plasticware to form a monolayer, while suspension cells (e.g., blood cells) float freely in liquid media. Regardless of type, cells progress through distinct growth phases: an initial lag phase, an exponential log phase of rapid division, and a plateau phase triggered by nutrient depletion or contact inhibition.

Sustaining long-term viability requires routine subculturing, or passaging, where cells are detached, diluted, and transferred into fresh vessels. Because isolated cells lack an immune system, strict aseptic techniques within biological safety cabinets are critical to prevent microbial contamination. Ultimately, by offering a homogeneous cell population free from systemic bodily variables, cell culture allows researchers to manipulate cellular pathways, screen drugs, model human diseases, and study metabolic processes with high reproducibility.

Media Preparation in Cell Culture

Media preparation is a critical process in animal cell culture that provides isolated cells with the essential chemical, physiological, and nutritional parameters required for *in vitro* survival and proliferation. Because cultured cells lack internal homeostatic regulatory systems, the growth medium serves as a synthetic replacement for extracellular fluid and blood plasma.

Core components of culture media

A balanced culture medium consists of defined basal nutrients supplemented with functional additives:

1. **Serum supplementation:** Fetal Bovine Serum (FBS) or Fetal Calf Serum (FCS) is added (usually 5-10% v/v) to provide hormones, attachment factors, binding proteins, and essential growth factors.
2. **Basal nutrient salt solutions:** Essential amino acids, vitamins, glucose (energy source), inorganic salts, and trace elements.
3. **Buffering systems:** Typically, sodium bicarbonate (NaHCO_3) combined with gaseous CO_2 (or organic buffers like HEPES) to strictly maintain physiological pH between 7.2 and 7.4.
4. **Phenol red indicator:** A pH-sensitive dye that visually signals medium acidification (yellow) or alkalinization (purple/pink).
5. **Antibiotics/antimycotics:** Reagents such as Penicillin-Streptomycin are used judiciously during preparation to prevent opportunistic bacterial and fungal contamination.

Culture media preparation

Dissolve the pre-formulated powdered medium (eg: DMEM/MEM) in ultrapure water at room temperature under sterile conditions with continuous stirring. Next, add sodium bicarbonate (NaHCO_3) to establish the core buffering system. The pH is then adjusted slightly below the final desired physiological level (around pH 7.1), as degassing during subsequent vacuum filtration causes the pH to rise to the target range of 7.2 to 7.4. Because culture media contain heat-labile vitamins, amino acids, and serum proteins, heat sterilization by autoclaving is unsuitable and leads to chemical inactivation.

Sterilization is instead accomplished by pressure or vacuum filtration through a sterile 0.22 μl cellulose acetate or polyethersulfone (PES) membrane filter inside a Class II Biological Safety Cabinet.

Following sterile filtration, heat-inactivated Fetal Bovine Serum (FBS) and required supplements or antibiotics are added aseptically. The complete medium is then aliquoted into sterile glass or tissue-culture-grade plastic bottles, sampled for quality-control sterility checks, and stored at 2°C to 8°C protected from light to prevent photo-oxidation of photosensitive nutrients such as riboflavin and tryptophan. The media should be syringe filtered before use to remove contaminants while storage or handling.

Cell Line Maintenance

Cell line maintenance involves the routine monitoring, feeding, and passaging required to keep cultured cells in a state of healthy, continuous proliferation. Cultures must be inspected daily using an inverted microscope to assess cell morphology, density, and signs of microbial contamination. As cells proliferate, they progress from the lag phase into the exponential log phase. To prevent them from reaching late confluence, where nutrient exhaustion, pH drop, and contact inhibition trigger cell arrest or apoptosis, cells must be subcultured before reaching 80-90% confluence. For adherent cell lines, subculture requires washing the monolayer with calcium- and magnesium-free balanced salt solution (such as DPBS) to remove trypsin-inhibiting serum, followed by enzymatic dissociation using a Trypsin-EDTA solution. Once detached, trypsin activity is neutralized by adding serum-containing fresh medium. The cells are then resuspended, counted to determine viability and seeding density, and subcultured into new culture vessels at an optimal split ratio. Consistent adherence to routine feeding schedules, precise split ratios, and detailed passage numbering ensures genetic stability, phenotypic reproducibility, and long-term culture health.

Cell Seeding (*In-Vitro*)

Cell seeding is the process of transferring a precisely quantified single-cell suspension into fresh culture vessels to initiate new cell growth. Following enzymatic detachment and centrifugation, the cell pellet is resuspended in a known volume of complete growth medium. Cell concentration and viability are determined using a hemocytometer or automated cell counter, typically via the trypan blue dye exclusion assay.

Calculating and seeding cells at an optimal density is critical. If seeded too low, cells fail to establish essential paracrine signaling and experience a prolonged lag phase or cell death. Conversely, over-seeding leads to rapid nutrient depletion and premature confluence. Once the correct volume of single-cell suspension is added to the culture vessel containing pre-warmed medium, the vessel must be gently rocked in a figure-eight pattern to ensure uniform cell distribution across the growth surface before incubation at 37°C in a 5% CO₂ atmosphere.

Seeding Cells and Assay Setup for Fluorescence and Cell Proliferation Assay

1. DCFDA Cellular ROS Assay

The 2',7'-dichlorofluorescein diacetate (DCFDA) assay relies on a two-step intracellular reaction mechanism involving enzymatic cleavage followed by chemical oxidation:

- a) **Cell Permeation and Enzymatic Cleavage:** DCFDA is a cell-permeable, lipophilic, non-fluorescent compound that readily diffuses across the cell membrane into the cytoplasm. Once inside the cell, intracellular esterases cleave the two acetate groups, converting DCFDA into 2',7'-dichlorofluorescein (DCFH).
- b) **Intracellular Trapping:** Unlike its parent compound, DCFH is hydrophilic and membrane-impermeable, trapping the probe inside the cytoplasm.
- c) **Oxidation to Fluorescent Product:** In the presence of cellular reactive oxygen species (ROS), including hydroxyl radicals ($\cdot\text{OH}$), peroxy radicals ($\text{ROO}\cdot$), and hydrogen peroxide (H_2O_2) in conjunction with cellular peroxidases, DCFH undergoes a two-electron oxidation to form 2',7'-dichlorofluorescein (DCF).
- d) **Signal Detection:** DCF is a highly fluorescent compound with excitation/emission maxima at **485nm/535nm**. The intensity of the green fluorescence signal is directly proportional to the concentration of intracellular ROS accumulated within the cells.

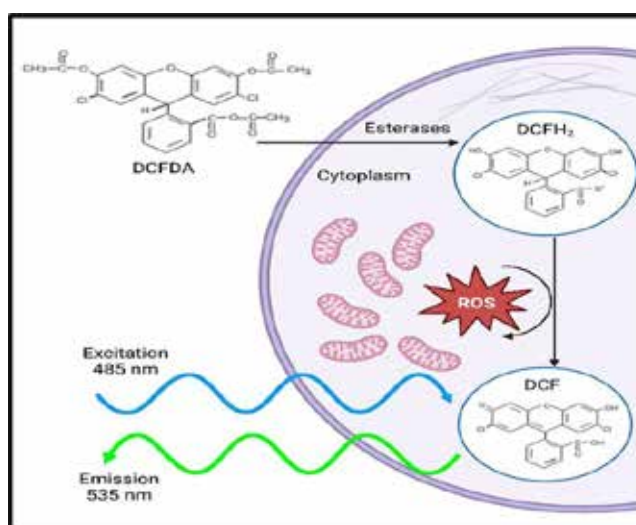


Figure 15: Mechanism of DCFDA assay

Protocol

The DCFDA assay workflow begins by seeding 2.0×10^4 cells per well in 100 μl of complete growth medium into a clear-bottom 96-well microplate. The plate is incubated at 37°C in 5% CO_2 for 18-24 hours until the monolayer reaches 70-80% confluence. Following cell attachment, the cells are immediately exposed to 100 μl of test compounds, negative controls, or positive ROS inducers (such as 100 μl H_2O_2) prepared in serum-free, phenol red-free media, followed by dark incubation at 37°C for the designated induction period. After incubation, the treatment medium is aspirated followed by the cells are loaded by adding 100 μl of a freshly prepared

DCFDA working solution. diluted in PBS or HBSS. The cells are incubated at 37°C in the dark for 30-45 minutes to permit probe uptake and intracellular cleavage by cellular esterases. Finally, intracellular oxidation is quantified by measuring top-read fluorescence on a microplate reader at an excitation wavelength of 485nm and an emission wavelength of 535nm.

Result

Under an inverted fluorescence microscope (excitation ~485 nm, emission ~535 nm), cells labeled with DCFDA display clear visual differences in intracellular fluorescence intensity based on their oxidative state. Untreated, healthy control cells exhibit minimal to weak baseline green fluorescence, appearing as faintly outlined cytoplasmic green signals against a dark background, reflecting normal physiological ROS levels. Upon induction of oxidative stress, such as treatment with a known pro-oxidant like hydrogen peroxide (H₂O₂) or a toxic compound, there is a dramatic, dose-dependent increase in bright green fluorescence localized entirely within the cytoplasm. The intensity of the green glow directly reflects the degree of intracellular DCF accumulation.

2. Cell Viability Assay (MTT Assay)

The MTT assay is a quantitative colorimetric assay used to measure cell viability, proliferation, and cytotoxicity. The assay relies on the reduction of the water-soluble, yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), by metabolically active cells. Intracellular dehydrogenase enzymes (such as lactate dehydrogenase) in viable cells reduce MTT into insoluble, dark purple formazan crystals. Because dead cells lose their metabolic activity, they cannot perform this conversion. The accumulated formazan crystals are subsequently dissolved using an organic solubilization solution (such as DMSO or SDS-based reagents), releasing the purple dye into solution. The absorbance of this dissolved solution, measured spectrophotometrically at 570 nm, is directly proportional to the number of metabolically active, viable cells present in the culture.

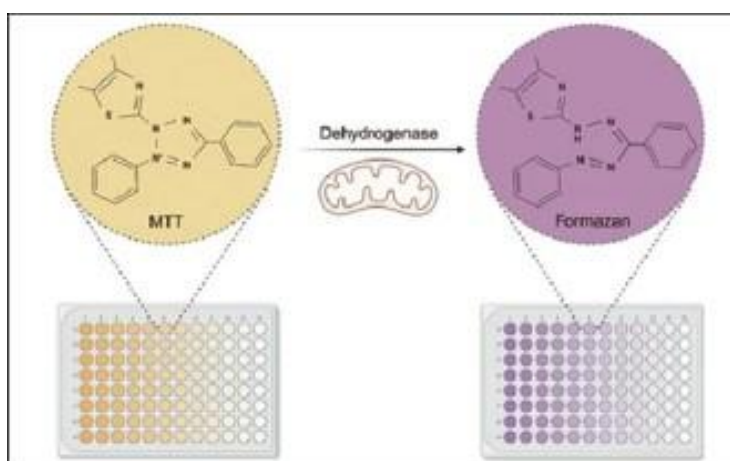


Figure 18: Mechanism of MTT assay

Protocol

To perform the assay in a 96-well format, cells are harvested, counted, and seeded at an optimized density (typically 1×10^4 to 2×10^4 cells per well) in 100 μ l of complete growth medium, then incubated for 18-24 hours at 37°C in a 5% CO₂ atmosphere to allow attachment. After treating the cells with the desired experimental compounds or media for the designated incubation period, 100 μ l of reconstituted MTT stock solution is added to each well (to reach a final concentration equal to 10% of the well volume). The microplate is wrapped in aluminum foil to protect the light-sensitive reagent and returned to the 37°C incubator for 2 to 4 hours. Following incubation, 100 μ l of solubilization solution is added to each well, and the plate is mixed gently on a gyratory shaker for 30 minutes or incubated overnight to ensure complete dissolution of the formazan precipitates. Finally, the absorbance is recorded on a microplate reader at 570nm (primary wavelength) using a reference wavelength above 600nm (such as 630nm) to subtract non-specific background signals.

$$\% \text{ of cell viability} = (\text{OD of cells} / \text{OD of negative control}) \times 100$$

Result

Microscopically, successful reduction of MTT is confirmed by observing dark purple, needle-shaped intracellular formazan crystals accumulating within the cytoplasm of viable cells under an inverted light microscope. Cells displaying robust metabolic activity will show high concentrations of these purple precipitates, whereas damaged or dying cells exhibit minimal to no crystal formation. Quantitatively, the net specific absorbance is calculated by subtracting the reference wavelength reading and the medium-only blank control values from the primary 570nm absorbance reading. Higher absorbance values directly correlate with enhanced cell viability and proliferation, whereas a dose-dependent decrease in absorbance indicates cytotoxic activity or reduced cellular growth induced by the test treatment.

HPLC

High-Performance Liquid Chromatography (HPLC) is an advanced analytical technique used to separate, identify, and quantify individual components within a complex mixture. The system operates by utilizing a high-pressure pump to propel a liquid solvent, known as the mobile phase, through a stainless-steel column packed with tiny solid adsorbent particles, referred to as the stationary phase. When a liquid

sample is injected into the stream, its constituent molecules travel through the column and interact with the stationary packing material. The degree of interaction depends on the physical and chemical properties of each analyte, such as its polarity, charge, or molecular size. Compounds that interact weakly with the stationary phase migrate quickly and elute first, whereas compounds that interact strongly are retained longer, resulting in distinct separation based on characteristic retention times.

As the separated components exit the column, they pass through a high-sensitivity detector, such as a UV-Vis spectrophotometer, photodiode array (PDA), fluorescence detector, or mass spectrometer, which measures the concentration of each emerging compound. The detector converts these chemical signals into electrical output, producing a chromatogram where each separated compound appears as an individual peak. The retention time of a peak identifies the specific compound, while the area under the peak is directly proportional to its concentration. Due to its exceptional precision, high resolution, and automated reproducibility, HPLC is widely utilized across pharmaceutical quality control, environmental monitoring, forensic testing, and chemical manufacturing.

BASIC TECHNIQUES IN ANIMAL HOUSE

Comprehensive Analysis of Animal Ethics, Regulatory Frameworks, and Operational Compliance Under the CCSEA in India

Introduction and Historical Context of Animal Experimentation

The ethical dimension of utilizing non-human creatures for scientific discovery, biomedical education, and safety testing has long been a subject of profound philosophical and societal debate. In India, the governance of animal welfare in laboratories is rooted in statutory law, specifically the **Prevention of Cruelty to Animals (PCA) Act of 1960**. Prior to the enactment of formal legislative oversight, animal usage across various institutions often lacked systematic uniformity, leading to significant variations in handling, housing and experimental procedures.

To address these shortcomings and ensure that animals are not subjected to unnecessary pain or suffering before, during, or after the performance of experiments, the Central Government established the **Committee for the Control and Supervision of Experiments on Animals (CCSEA)**. Operating under the administrative purview of the **Department of Animal Husbandry and Dairying (DAHD)** within the **Ministry of Fisheries, Animal Husbandry and Dairying (MoFAH&D)**, the CCSEA serves as the statutory watchdog ensuring that scientific integrity does not supersede moral accountability. The legal architecture was further strengthened through the notification of the **Breeding of and Experiments on Animals (Control and Supervision) Rules, 1998** and subsequent critical amendments in **2001** and **2006**.

Statutory Foundation and Evolution of Rules

The legal framework governing animal experimentation in India is structured to maintain a delicate balance between encouraging scientific progress and enforcing absolute ethical restraint.

The Prevention of Cruelty to Animals Act, 1960

Chapter IV of the PCA Act, 1960, explicitly addresses experimentation on animals. Section 14 protects the performance of experiments involving operations on animals provided they are conducted for the purpose of advancing new discoveries in physiological knowledge, saving or prolonging life, alleviating suffering, or combating diseases in human beings, animals, or plants. Section 15 empowers the Central Government to constitute the CCSEA, and Section 17 outlines the duties of the Committee to enact rules ensuring humane treatment.

Evolution through 1998, 2001, and 2006 Rules

- **The 1998 Rules:** Formally established the mandatory creation of Institutional Animal Ethics Committees (IAECs), instituted rules for the registration of breeders and establishments, and mandated basic standards for animal stocking, caging, and record-keeping.
- **The 2001 Amendments:** Broadened the definition of an "experiment" to encompass the acquisition of knowledge of a biological, psychological, ethological, physical, or chemical nature, including routine diagnostics, testing activities, and the establishment of transgenic stocks. It also refined qualification criteria for personnel supervising experiments and regulated the import of genetically defined rodents.
- **The 2006 Amendments:** Incorporated revolutionary ethical concepts into Indian law, including the formal recognition of the **4th R (Rehabilitation)**, explicit parameters for euthanasia, the preference for animals lower on the phylogenetic scale, and the mandatory inclusion of aftercare and rehabilitation costs into research budgets.

Core Ethical Principles and the Framework of the "Rs"

Modern animal experimentation under CCSEA guidelines is governed by universally acknowledged bioethical principles, tailored to Indian regulatory standards through recommendations by expert consultative groups.

1. The Phylogenetic Scale and Replacement

In accordance with Rule 9(bb) of the amended rules, experiments must be designed using the minimum number of animals necessary to yield statistically valid results at a 95% degree of confidence. Furthermore, investigators must prioritize species lowest on the phylogenetic scale—such as invertebrates, birds, and rodents—before considering higher-order mammals. Replacement alternatives that avoid animal use entirely must be given full consideration, and sound scientific justification must be provided if available alternatives are bypassed.

2. Avoidance and Minimization of Pain and Suffering

Research personnel must operate on the fundamental assumption that procedures causing pain or suffering in humans will induce similar distress in animals. All invasive procedures expected to cause more than momentary or slight pain must be conducted under adequate sedation, analgesia, or general anaesthesia. The use of paralytic agents like curare without concurrent deep anaesthesia causing loss of consciousness is strictly prohibited.

3. Aftercare, Cost Scaling, and Rehabilitation

It establishes that investigators are legally responsible for the post-experimental welfare, aftercare, and rehabilitation of animals. Crucially, the financial costs of rehabilitation must be integrated directly into the research budget, scaled in positive correlation with the sentience level of the animal. Rehabilitation must extend until the animal can resume a normal existence, handled either by the research establishment or by authorized animal welfare organizations.

4. Strict Parameters for Euthanasia

Euthanasia is treated as an exception rather than a routine procedure. Euthanasia is permissible only under three strict conditions:

- When an animal is permanently paralyzed, incapable of independent locomotion, or unable to perceive its environment in an intelligible manner.
- When an experimental procedure leaves the animal with recurring, long-term extreme pain and suffering.
- When non-termination of the animal's life would pose a direct biological threat to humans or other animals.

Institutional Mechanisms: The Role and Responsibilities of the IAEC

The operational linchpin of ethical compliance at the grassroots level is the **Institutional Animal Ethics Committee (IAEC)**.

Composition and Quorum

An IAEC must comprise specific personnel to ensure balanced oversight:

- Five internal members: one biological scientist, two scientists from different biological disciplines, a veterinarian involved in animal care, and the scientist-in-charge of the animal facility (with the Chairperson and Member Secretary appointed from among them).
- External members nominated by the CCSEA: a Main Nominee, a Link Nominee, an external scientist, and a socially aware non-scientific member.

A minimum quorum of six members is mandatory for conducting meetings, and decisions must be reached through consensus. Crucially, the presence of the main CCSEA nominee (or link nominee) and the socially aware nominee under specified guidelines is vital for the validity of proceedings.

Scope of Authority

The IAEC is fully empowered to review, evaluate, and approve project proposals involving small laboratory animals (rodents, rabbits, guinea pigs). However, for research protocols involving large animals higher on the phylogenetic scale (such as dogs, primates, equines, and farm animals), the IAEC acts solely as a recommending body; final statutory approval must be secured directly from the CCSEA in New Delhi.

Operational Standards for Animal House Facilities and Husbandry

To maintain optimal physiological and psychological health in laboratory animals, the CCSEA Guidelines for Laboratory Animal Facility (2015) mandate stringent controls over environmental and managerial parameters.

Environment and Macro-Climate

Animal houses must be physically isolated from human habitations, administrative offices, and sources of excessive noise, dust, and vibration. Environmental controls must maintain ambient temperatures between 18°C and 29°C and relative humidity between 30% and 70%. Ventilation systems must ensure 12 to 15 complete air cycles per hour, keeping animal rooms under proper negative or positive pressure depending on containment needs.

Housing, Caging, and Social Environment

Cages must be constructed from durable, seamless, and easily sanitizable materials like polypropylene, polycarbonate, or stainless steel. Space allocations must permit freedom of movement, normal postural adjustments, and species-specific social interactions. Because social isolation causes severe psychological trauma – particularly in gregarious species like dogs and non-human primates – group housing or compatible pairing should be prioritized unless explicitly contraindicated by experimental protocols.

Nutrition, Water, and Sanitation

Animals must be provided with fresh, nutritionally balanced, and contaminant-free diets stored in vermin-proof conditions. Continuous access to potable, clean drinking water must be maintained. Rigorous sanitation schedules, including automated cage washing at high temperatures (minimum 82.2°C) and safe waste disposal via incineration, are mandatory to prevent cross-contamination and the outbreak of infectious diseases.

Special Protocols: Care, Management, and Reuse of Large Animals

Certain scientific undertakings – such as the production of biologicals, hyperimmune plasma, and anti-toxins – rely heavily on large animals, particularly equines.

Equine Management in Biological Production

Equines selected for serum production must undergo rigorous pre-purchase soundness examinations, including screening for Glanders (Mallein test), Equine Infectious Anemia (Coggins test), haemoproteoan parasites, and comprehensive hematological and hepatic-renal function profiling. Bleeding schedules must strictly adhere to physiological safety limits: drawing no more than 5% of circulating blood volume every two weeks or 15% every four weeks, paired with mandatory plasma volume replacement using warm isotonic fluids. Furthermore, the duration of an equine's active participation in serum production is strictly regulated, followed by mandatory retirement and rehabilitation rather than commercial sale or auction.

Restrictions on the Reuse and Rehabilitation of Dogs

Dogs utilized in toxicological, pharmacokinetic, or telemetry research are subject to strict limitations. Reuse is heavily restricted and requires explicit, individual authorization from the CCSEA. Individual dogs may not be housed in laboratory facilities or subjected to reuse for a cumulative period exceeding three years. Upon completion of their experimental lifespan or breeding cycle (limited to five whelping cycles), dogs must be rehabilitated through structured adoption programs facilitated by trusted Animal Welfare Organizations (AWOs) and approved by the CCSEA.

Documentation, Record Keeping, and Digital Governance via the CCSEA Portal

Transparency and accountability are maintained through rigorous documentation across all registered establishments.

Mandatory Registers and Forms

Establishments and principal investigators must maintain continuous records, including:

- **Form A:** Application for initial registration and facility profiling.
- **Form B:** Detailed research protocols submitted for ethical evaluation and approval.
- **Form C:** Comprehensive stock registers tracking the acquisition, breeding, and transfer of animals.

- **Form D:** Individual project records tracking the exact number of animals utilized and procedures performed.
- **Form E:** Quarterly statements detailing the commercial trade or transfer of laboratory animals.

The CCSEA Online Web Portal

Launched to streamline regulatory workflows and promote transparency under the Digital India initiative, the CCSEA web portal enables establishments to submit online applications for new registrations, license renewals, IAEC reconstitutions, protocol approvals for large animals, and the digital uploading of meeting minutes and annual inspection reports by CCSEA nominees.

Conclusion

The regulatory ecosystem governing animal ethics in India, anchored by the PCA Act of 1960 and administered through the robust oversight of the CCSEA, establishes an exhaustive standard for humane scientific conduct. By integrating the principles of the Three Rs (Replacement, Reduction and Refinement), phylogenetic scaling, mandatory institutional ethics review, rigorous veterinary care, and structured post-experimental rehabilitation, India ensures that scientific innovation proceeds hand-in-hand with unyielding moral responsibility toward sentient animal life.

[illegible]

Approvals and Accreditations of PRC

- **PRC is a Scientific and Industrial Research Organization (SIRO) recognized by the Department of Scientific and Industrial Research (DSIR), Ministry of Science & Technology, Government of India**
- **PRC is an approved Ph.D. Centre under the Kerala University of Health Sciences (KUHS), Chinmaya University and Helsinki University, Finland**
- **PRC got an active Institution Review Board (IRB) that is registered to Director of Health Research (DHR)**
- **PRC has an Institutional Animal Ethics Committee and CCSEA approved Animal House Facility for Research and inhouse breeding of laboratory Animal's**
- **PRC hosts several ongoing national and international projects from eminent funding agencies**