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भारतीय मानक मसौदा

बाह्यकोशिकीय पुशिकाएं – लक्षण वर्णन

Draft Indian Standard

Extracellular Vesicles – Characterization

ICS:07.080

Medical Biotechnology and Medical Nanotechnology Sectional Committee, MHD 20

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FOREWORD

(Formal Clause will be added later)

Extracellular vesicles (EVs) are membrane-bound vesicles released by cells that play a key role in intercellular communication by transporting proteins, lipids, and nucleic acids. EVs include exosomes, microvesicles, and apoptotic bodies, each varying in size, biogenesis, and function.

Characterizing EVs is essential for understanding their functions, and molecular cargo, and ensuring their quality for clinical and industrial applications. This process involves analysing their physical, molecular, and functional properties, which provide insights into their identity, biological activity, and therapeutic potential.

Given the diverse applications of EVs, MHD20 has developed this standard to guide characterization for researchers, clinicians, and industry professionals working in EV research, diagnostics, and therapeutics.

The list of Abbreviations are given in Annex A

In the preparation of this standard considerable assistance has been derived from the publications given in Annex B.

For the purpose of deciding whether a particular requirement of this standard is complied with the final value, observed or calculated, expressing the result of a test or analysis shall be rounded off in accordance with IS 2: 2022 ‘Rules for rounding off numerical values (*second revision*). The number of significant places retained in the rounded-off value should be the same as that of the specified value in this standard.

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Indian Standard

EXTRACELLULAR VESICLES – CHARACTERIZATION

1. SCOPE

This standard provides general considerations for the characterization of Extracellular Vesicles (EVs). It serves as a guidance document, outlining various test methods applicable to different use cases. Only those tests that have gained broad consensus for characterization are designated as mandatory, while other recommended methods are left to the user's discretion based on their Intended application.

2. NORMATIVE REFERENCES

Reference No	Title
IS 18637 (Part 1): 2024	Cleanrooms and associated controlled environments Part 1: Classification of air cleanliness by particle concentration (ISO 14644-1:2015, MOD)
IS/ISO 29701: 2010	Nanotechnologies - Endotoxin test on nanomaterial samples for in vitro systems - Limulus amebocyte lysate (LAL) test
IS 18514: 2024/ISO 22412: 2017	Particle Size Analysis — Dynamic Light Scattering (DLS)
IS 17932 (Part 1): 2023	Biological evaluation of medical devices Part 1: Evaluation and testing within a risk management process (ISO 10993-1 2018, MOD)

3 TERMS AND DEFINITIONS

3.1 Extracellular Vesicles (EVs): Membrane-bound vesicles released from cells, varying in size (30 nm - 10 µm), content, and function, which facilitate intercellular communication.

3.2 Exosomes: Small EVs (30 - 200 nm) of endosomal origin, released through the fusion of multivesicular bodies (MVBs) with the plasma membrane.

3.3 Microvesicles (MVs): Larger EVs (100 - 1000 nm) formed by the outward budding of the plasma membrane.

3.4 Apoptotic Bodies: Large vesicles (1 - 5 µm) released by cells undergoing apoptosis, containing cellular debris.

3.5 EV Cargo: The molecular content within EVs, including proteins, lipids, RNA (mRNA, microRNA), and DNA.

3.6 Cellular Debris: Refers to the fragmented remains of cells that result from cellular damage, death, or disintegration during the culture process.

4 GENERAL CONSIDERATIONS

4.1. Key aspects of aspects of EVs for consideration

As per the *Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches* published by the International Society for Extracellular Vesicles, the characterization techniques/ methods should determine the following aspects of EVs.

i.particle size

ii.particle number/ concentration

iii.particle morphology

iv.total protein content

v.total lipids content

vi.total RNA content

vii. protein markers

viii. non-protein markers

ix. EV-associated componen

5 PHYSICAL CHARACTERIZATION OF EVS

5.1. Physical characterization is essential for confirming the identity of EVs and distinguishing them from other biological components. EVs are highly heterogeneous, with exosomes typically ranging from 30 – 200 nm in diameter, while MVs and apoptotic bodies fall into different size ranges. By determining their size, one can differentiate between these subtypes and eliminate contaminants such as protein aggregates, lipoproteins, or cellular debris that may mimic EVs in size or appearance. Comprehensive characterization of EVs is necessary to confirm their identity, purity, and functionality.
5.2. Following parameters for physical characterization should be considered and done as per the indicated test methods.

Sr. No.	Parameter	Description	Test methods	Additional Information
1	Particle size and distribution *	Size range (30–200 nm for exosomes) and uniformity of particles	NTA (Nanoparticle Tracking Analysis), DLS (Dynamic Light Scattering) or other validated method	Identifies EV subtypes, checks purity, and helps understand biological function
2	Particle number/ concentration*	Number of particles per volume	NTA, RPS (Resistive Pulse Sensing) or other validated method	Ensures proper dosing and standardization
3	Morphology *	Structure and integrity (spherical or cup-shaped vesicles with lipid bilayer)	TEM, Cryo-EM, AFM or other validated method	Confirms vesicle structure and rules out contaminants
4	Surface charge **	Particle stability and tendency to aggregate	Zeta Potential Analyzer or other validated method	Indicates stability and interaction potential
5	Density **	EV density for separation and purity assessment	Density Gradient Ultracentrifugation or other validated method	Helps isolate EVs from contaminants
6	Refractive index **	Optical property relevant to imaging and sizing	Refractive Index Analysis or other validated method	Ensures accuracy in optical measurement methods (e.g., NTA)
7	Aggregation state **	Presence of clumped particles	DLS, TEM or other validated method	Detects aggregation that may affect function or data

				accuracy
8	Purity indicators **	Co-isolated impurities (lipoproteins, proteins, debris)	TEM, Marker Analysis, Flow Cytometry or other validated method	Ensures high-purity EVs for reliable research and therapeutic use
* Mandatory tests ** Additional informative / Optional tests				

Note 1: The techniques listed are not exhaustive; other suitable methods may also be employed to meet the minimum criteria for establishing EV identity, purity, and structural integrity.

Note 2: For the aforementioned tests, equipment, and analyses, relevant BIS/ISO standards should be cross-referenced and followed wherever applicable.

6 MOLECULAR CHARACTERIZATION OF EVS

6.1. Molecular characterization of EVs is a critical step in understanding the biochemical composition of EVs, specifically their proteins, lipids, and nucleic acids. This deeper understanding is vital for confirming EV identity, revealing their functional properties, and ensuring their utility in clinical and research settings. Molecular characterization is essential for distinguishing EVs from contaminants, confirming sample purity, and ensuring safety and efficacy in functional and therapeutic applications.
6.2. By detecting molecular markers such as tetraspanins (e.g., CD63, CD81, CD9) and cytosolic proteins (e.g., TSG101, Alix), one can validate the presence of EVs and exclude impurities like free proteins or lipoproteins. This profiling provides insights into EVs' roles in intercellular communication, disease processes, and biomarker discovery, revealing disease-specific biomolecules for non-invasive diagnostics and personalized medicine. Additionally, it ensures therapeutic EVs carry functional bioactive molecules and remain contaminant-free, supporting clinical translation. Standardized molecular markers enhance reproducibility, while detailed profiling elucidates EV biogenesis, release, and uptake, optimizing research and therapeutic strategies. Following parameters for Molecular characterization should be considered and done as per the recommended test methods.

Sr. No.	Parameter	Description	Test methods	Additional Information
1	Total protein content *	Quantify total protein in an EV preparation	Colorimetric, fluorometric or other validated method	Useful for normalization and comparing EV preparations.
2	Total lipid content *	Quantify total lipid content in an EV preparation	Colorimetric, fluorometric, chromatography or other validated method	Useful for normalization and comparing EV preparations.
3	Total RNA content *	Quantify total RNA content in an EV preparation	Capillary electrophoresis, Quant- iT RiboGreen RNA kit or other validated method	Useful for normalization and comparing EV preparations.
4	Protein markers[#] *	Detect surface protein markers like CD63, CD81, CD9	Western Blot, Flow Cytometry or other validated method	Confirm EV identity and helps classify EV subtypes
		Detect cytosolic protein markers like TSG101, Alix, HSP70	Western Blot, Flow Cytometry or other validated method	Confirm EVs have typical internal cargo, ensuring vesicle integrity
		Detect purity markers like albumin, ApoB, Calnexin.	Western Blot, Flow Cytometry or other validated method	Assess sample purity for reliable experiments and therapeutic use
5	Non-protein markers **	Detect lipids (e.g., phosphatidylserine), glycans, or nucleic acids	Mass Spectrometry, Raman Spectroscopy or other validated	Reflects EV bi-layer integrity and cytosolic features.

			method	
6	Localization of EV Components **	Determine whether EV-associated components such as proteins, RNAs, glycans are luminal, membrane-bound or external.	Mild protease/ nuclease digestion, permeabilization assays, antibody accessibility assays or other validated method	Helps understand EV function, cargo delivery mechanisms, and interaction with recipient cells
7	Protein profiling **	Comprehensive profiling of proteins in EV preparations	Mass Spectrometry or other validated method	Identifies EV-specific proteins; critical for functional and therapeutic studies
8	Lipid profiling **	Comprehensive profiling of lipis in EV preparations	Mass Spectrometry, Lipidomics, Thin-Layer Chromatography (TLC) or other validated method	Helps understand membrane structure and vesicle stability
9	RNA Profiling **	Comprehensive profiling of RNAs in EV preparations	Q-PCR, RNA-Seq or other validated method	Reveals functional EV cargo; useful for biomarker discovery
10	DNA content and/or profiling **	Quantify total DNA content and its profiling in EV preparation	qPCR, PicoGreen assay or other validated method	Useful for studying gene transfer and identifying DNA-based biomarkers
11	Glycans profiling **	Comprehensive profiling of glycans in EV preparations	Lectin blotting, Mass Spectrometry, Chromatography or other validated method	Reveals glycosylation patterns that influence EV targeting,

				uptake, and immune interactions
12	Functional markers**	Specific EV components linked to physiological or pathological state	Q-PCR, Western blotting or other validated method	Key for diagnostics, prognosis, and personalized medicine
***** Mandatory tests ** Additional informative / Optional tests				

#Follow the five-component framework as per MISEV2023 for reporting claims about EV protein content.

Note 1: The techniques listed are not exhaustive; other suitable methods may also be employed to meet the minimum criteria for establishing EV identity, purity, and structural integrity.

Note 2: For the aforementioned tests, equipment, and analyses, relevant BIS/ISO standards should be cross-referenced and followed wherever applicable.

7. FUNCTIONAL CHARACTERIZATION OF EVS

7.1. Functional characterization of EVs is essential for understanding their biological roles and therapeutic potential. While physical and molecular characterization focus on their size, structure, and composition, functional characterization evaluates how EVs interact with biological systems, including their effects on cells, tissues, and organs. This process is vital for identifying EVs' roles in health, disease, and their potential therapeutic applications.

7.2. EVs facilitate intercellular communication by carrying proteins, lipids, and RNAs that influence cellular processes. Studying these interactions reveals EV involvement in immune regulation, tissue repair, and disease mechanisms such as cancer and viral infections. Functional characterization helps explore how EVs mediate these processes, guiding the development of EV-based therapies.

7.3. Functional characterization is key for optimizing EV isolation techniques and improving therapeutic strategies. By assessing the biological activity of isolated EVs, researchers can identify the most effective methods for obtaining high-quality EVs with specific properties. Engineering EVs to target particular cells or deliver therapeutic cargo enhances their therapeutic potential, making them promising candidates for drug delivery systems, especially for diseases like cancer, neurodegenerative disorders, and autoimmune conditions.

7.4. The following parameters for functional characterization should be considered and assessed using

the indicated test methods, depending on the designated purpose of the study. These tests are not mandatory, and additional evaluations may be performed based on the specific end-use applications of the EVs.

Sr. No.	Parameter	Description	Test Methods	Additional Information
1	Cellular uptake	Evaluation of how EVs are internalized by target cells	Flow cytometry, confocal microscopy or other validated method	Indicates delivery efficiency; essential for therapeutic applications
2	Targeting efficiency	Assessment of EVs' ability to bind specifically to target cells	Flow cytometry, confocal microscopy or other validated method	Shows precision of EV delivery; important for targeted therapies
3	Cargo delivery	Ability of EVs to deliver proteins, RNA, or other molecules to cells	Flow cytometry, confocal microscopy or other validated method	Confirms functional delivery of therapeutic cargo
4	Immune modulation	Evaluation of EVs' effects on immune cells	ELISA, Flow Cytometry, Cytokine Assays or other validated method	Helps design EVs for immunotherapy and reduce immune-related side effects
5	Tumorigenesis & metastasis	Studying EVs' role in cancer progression and tumor environment	In vivo Tumor Models, Migration & Invasion Assays or other validated method	Supports cancer diagnostics and development of anti-cancer strategies
6	Gene expression modulation	Analysis of EV-induced changes in gene expression	RT-qPCR, RNA-Seq or other validated method	Key for evaluating EVs in gene regulation and therapeutic modulation

7	Angiogenesis	Assessment of EV influence on new blood vessel formation	Tube Formation Assay, In vivo Angiogenesis Models or other validated method	Important for regenerative medicine and cancer research
8	Cell proliferation & survival	Effects of EVs on cell growth and viability	MTT Assay, BrdU Assay, Live/Dead Assay or other validated method	Helps assess impact on cell health, relevant for therapy development
9	Regeneration	Studying EVs' ability to support tissue regeneration	In vitro or in vivo regenerative models	Critical for therapeutic applications in regenerative medicine
10	Endotoxin testing	Detection of bacterial endotoxins (LPS) in EV samples	LAL Assay or other validated method	Ensures safety and biocompatibility for clinical or therapeutic use
11	Toxicity & immunogenicity	Assessment of harmful effects or immune response caused by EVs	Cytotoxicity Assays, Flow Cytometry, ELISA or other validated method	Confirms safety of EVs for in vivo or clinical applications

Note 1: The techniques listed are not exhaustive; other suitable methods may also be employed to meet the minimum criteria for establishing EV identity, purity, and structural integrity.

Note 2: For the aforementioned tests, equipment, and analyses, relevant BIS/ISO standards should be cross-referenced and followed wherever applicable.

8. RECOMMENDED ENVIRONMENTAL CONTROLS FOR EV CHARACTERIZATION FACILITIES

8.1. GMP-integrated characterization for therapeutic purposes: When EVs are developed for clinical-grade or therapeutic use, environmental controls become critical—especially during in-line quality control and quality assurance (QC/QA) testing. In such cases, characterization steps like sterility testing, endotoxin assessment, and potency assays must be conducted in cleanroom environments compliant with IS 18637 (Part 1): 2024, typically ranging from *Class 5 to Class 8*, depending on the nature of the test.

8.2. Routine research and non-clinical characterization: For exploratory research, academic investigations, and routine EV testing that are not directly tied to GMP workflows, a *controlled laboratory environment* is generally sufficient. A *controlled lab* refers to a dedicated space where parameters like temperature, humidity, and access are regulated to support experimental consistency. While strict particle filtration and classification are not mandatory in such settings, adopting basic contamination control practices enhances reproducibility and data quality.

8.3. Where applicable, laboratories involved in EV characterization should aim to comply with *ISO/IEC 17025:2017* to ensure technical competence, method validation, and the reliable generation of consistent results.

9. DOCUMENTATION

9.1. Records of characterization helps in assessing the results and will help in assessing the unintended pitfall in QC/QA. The documentation of EV characterization serves as a vital tool for ensuring the safety, efficacy, and reproducibility of EV preparations. By documenting critical quality attributes and other essential data, stakeholders across various fields—ranging from basic research to clinical application—can ensure that EVs meet the required standards for their intended use.

9.2. The following shall be considered for documentation:

9.2.1. *Standard operating procedures:* detailed methodologies followed for characterizing various aspects of EVs, including size, morphology, and content.

9.2.2. *Instrument calibration and maintenance records:* ensure to keep calibration, maintenance and performance validation records of all characterization instruments used in the EVs characterization.

9.2.3. *Characterization data sheets:* Records particle concentration, size distribution, morphology, and molecular content.

9.2.4. *Quality control records:* Tracks internal quality assessments, deviations, and corrective actions.

9.3. All measurements should be conducted with appropriate controls, including reference EV standards where applicable. The experimental details, including instrument settings, calibration, and normalization methods, must be documented and reported. EV isolation and purification methods should be specified to contextualize the characterization data. All documents must be periodically reviewed, updated, and maintained as per regulatory and institutional requirements.

ANNEX A
(Foreword)
LIST OF ABBREVIATIONS

EV	Extracellular Vesicle
TEM	Transmission Electron Microscopy
Cryo-EM	Cryogenic Electron Microscopy
AFM	Atomic Force Microscopy
NTA	Nanoparticle Tracking Analysis
DLS	Dynamic Light Scattering
RPS	Resistive Pulse Sensing
QC/QA	Quality Control / Quality Assurance
RT-qPCR	Reverse Transcription quantitative Polymerase Chain Reaction
RNA-Seq	RNA Sequencing
Q-PCR	Quantitative Polymerase Chain Reaction
TLC	Thin-Layer Chromatography
GMP	Good Manufacturing Practice
ELISA	Enzyme-Linked Immunosorbent Assay

Annex B

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