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REVIEW ARTICLE

Microbiological reference material (bacterial and fungal domains): Definition, production rules, use and need for establishment in Brazil

Material de referência microbiológico (domínios bacterias e fungos): definição, regras de produção, uso e necessidade de desenvolvimento no Brasil

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Abstract

Reference materials are crucial for laboratory quality assurance. They are widely used for the internal quality control of analytical tests, in the validation of methodologies and as test items in proficiency tests. Microbiological reference materials are represented mainly by reference strains, whose use in laboratory internal quality control is unquestionable and recommended by several microbiological manuals. In Brazil, the practice of producing microbiological reference materials has advanced in recent years in the development of specific materials for proficiency testing. However, the same did not occur with the establishment of reference strains due to a lack of incentive policies, maintaining the country dependant on the use of international reference strains. This article aims to emphasize the importance of the use of microbiological reference materials in laboratory quality control and discuss the need for the development of such materials in Brazil. The paper presents a brief explanation of microbiological reference materials and points out questions concerning the country's dependence on the acquisition of international biological materials. It also describes quality standards related to the production of these materials and the situation of culture collections and Brazilian reference laboratories that supply reference strains. The study also mentions practical recommendations on the subcultures of reference strains.

Keywords: Development; Reference strains; Internal quality control; Laboratory quality assurance; Proficiency test; Culture collections.

Resumo

Materiais de referência são ferramentas indispensáveis para a garantia da qualidade laboratorial. São amplamente utilizados no controle interno da qualidade de ensaios analíticos e na validação de metodologias, e como itens de ensaio em ensaios de proficiência. Materiais de referência microbiológicos são representados principalmente por cepas de referência, cuja utilização como controle interno de ensaios é inquestionável e recomendada por vários manuais microbiológicos. No Brasil, a prática de produção de materiais de referência microbiológicos avançou, nos últimos anos, no desenvolvimento de materiais específicos para ensaios de proficiência. O mesmo não ocorreu com estudos relacionados ao estabelecimento de cepas de referência, por falta de políticas de incentivo, mantendo a dependência do país no uso de cepas de referência internacionais. Este artigo tem como objetivos ressaltar a importância do uso de materiais de referência microbiológicos no controle da qualidade laboratorial e discutir a necessidade do desenvolvimento desses materiais no Brasil. O presente trabalho apresenta uma breve explicação sobre materiais de referência microbiológicos e aponta questões sobre a dependência do país na aquisição de materiais biológicos internacionais. Descreve os padrões de qualidade relacionados à produção desses materiais e a situação das coleções de cultura e dos laboratórios de referência brasileiros que fornecem cepas de referência. Este estudo menciona também recomendações práticas sobre subcultivos de cepas de referência.

Palavras-chave: Desenvolvimento; Cepas de referência; Controle da qualidade interno; Garantia da qualidade laboratorial; Ensaio de proficiência; Coleção de culturas.

1 Introduction

The use of reference material (RM) in analytical assays is widely employed by the scientific community and described in most official analysis manuals.

According to the ISO/GUIDE 30:2015 (International Organization for Standardization, 2015a), RM is a material homogenous and stable enough with respect to one or more specified properties, established for its intended use in a measuring process. Reference materials may be classified as certified or not, each of these categories being indicated for a specific use. A RM is considered a Certified Reference Material (CRM), when characterized by a metrologically valid procedure for one or more properties, accompanied by a certificate that offers the value of the specified property, as well as its associated uncertainty described in a metrological traceability statement. The application of a reference material without certified qualification is restricted to the control of the analytical performance in the repeatability and reproducibility of the assay (Camarinha et al., 2011). In the microbiology field, RMs may be used in several activities such as: taxonomy studies, identification of pathogens, antimicrobial susceptibility tests, scientific research and the quality control of laboratorial assays.

Certified Reference Material has applicability in more complex analytical processes. It should be used in analytical procedures with the objective of obtaining data with exact measurements, such as: validation, development of methods and collaborative study (International Organization for Standardization, 2017a).

Microbiological RMs are represented mainly by reference strains but may also be presented as replicable parts of microorganisms, such as genomic and plasmid DNA (De Baets et al., 2009a) and by toxins produced by these organisms (Zeleny et al., 2015; Weisemann et al., 2015).

Microbiological reference strains can be found in different presentations, such as: lenticular discs (Boyd et al., 2006), calcium carbonate pellets (Abdelmassih et al., 2014), freeze-dried cultures (Wessman et al., 2011; Brandão et al., 2013) and freeze-dried BioBall (De Baets et al. 2008; De Baets et al., 2009b). The different forms of production aim to increase the properties of the RMs, such as reducing matrix interference, variation amongst batch units (Phillipp et al., 2007), easy handling and dissolution of materials, as well as increased stability regarding the transport temperature and shelf life (Abdelmassih et al., 2014). The challenge for establishing and maintaining biological RM is the cells stability and viability. However, new challenges may be directed to the development of more robust, accurate and possibly commutable material (Phillipp et al., 2007).

In the quality assurance context, RMs are considered essential tools in analytical performance monitoring. The international reference standard ISO/IEC 17025:2017 (International Organization for Standardization,

2017b) that establishes "General Requirements for the Competence of Testing and Calibration Laboratories", presents the technical and management principles to be followed by a laboratory interested in assuring the quality of the services provided and demonstrating its technical competence. The use of RM is mentioned in ISO/IEC 17025:2017 as a technical requirement for the quality assurance of analytical results, through regular use as the internal control of analysis. The standard also highlights the importance of participation in proficiency tests or interlaboratory comparisons, carried out by the use of RM as a test item.

In Brazil the reference strains used for the internal control of assays are of international origin. The high costs and bureaucratic and sanitary requirements for importation hinder the acquisition of biological materials by Brazilian laboratories (Instituto Nacional de Controle de Qualidade em Saúde, 2008). In addition, there is currently a trend towards the emergence of technical and commercial barriers associated with biosafety and biosecurity on the international market that may hamper or even restrict the transit of biological products between countries (Canhos et al., 2007).

Thus, according to the Quality Standards, the development of reference strains in Brazil can offer greater autonomy and security in the availability of these materials and consequently greater support for laboratory activities.

This article presents a discussion about the importance of using microbiological reference materials in laboratory quality control, the need to produce these materials in Brazil and other relevant information about the subject in question. The review was elaborated from an exploratory study, carried out by bibliographical research based on important scientific publications, as well as technical reports, documents and laws using textual databases and electronic sites available on the Internet. The PubMed, CAPES Periodic and ScienceDirect databases were used in the search for scientific articles, focusing on the following descriptors: microbiological reference materials, reference strains, internal microbiological quality control, culture collections and microbiological resource centrers. The searches made in the databases were carried out between January and April 2017 and approximately 90 publications were obtained. From these, articles with relevant information on the production of microbiological reference materials and about culture collections were selected. Articles published before 2006 and incomplete articles, without titles or abstracts were excluded. Queries to the International Standards (ISO), pertinent to the subject, were made concerning documents available at the Oswaldo Cruz Foundation.

2 The use of reference material in food microbiology laboratories

Food microbiology laboratories play an important role in public health by assessing the quality requirements of the analyzed samples, based on criteria described in the legislation and by verifying foods involved in outbreaks of food poisoning. These laboratories must implement a quality assurance system to ensure high quality analytical procedures as well as accurate and reliable results (Rosas, 2009).

The use of validated official methods controlled by internal control (reference strains) and participation in quality external evaluation programs (proficiency testing) are important tools to be applied in routine procedures (Camaró-Sala et al., 2015).

Official international guidelines for the analysis of food and water microbiology, such as the Bacteriological Analytical Manual of American Food and Drug Administration (Food and Drug Administration, 2018), Microbiological methods of the International Organization for Standardization (ISO), Compendium of Methods for the Microbiological Examination of Foods (Salfinger & Tortorello, 2015), Microbiology Laboratory Guide book of the United States Department of Agriculture (United States Department of Agriculture, 2018) and Standard Methods for the Examination of Water and Wastewater (Baird et al., 2017), recommend the use of reference strains without the certification category in the quality control of the analytical methodologies.

Participation in proficiency tests allows for a comparison of laboratory performance with other laboratories when analyzing homogeneous and stable materials from the same batch in a round test (Thompson et al., 2006). This is an important tool when used in the evaluation of laboratory performance, able to verify the accuracy and reliability of the results (Stang & Anderson, 2013). Proficiency tests enable the identification

of tendencies not pointed out by other methods of evaluation, suggesting the need for corrective actions in the analytical system (São José et al., 2011). In Brazil, up to the first decade of century 2000, food microbiology laboratories that wanted to participate in proficiency tests had to look for international suppliers, since there were no national providers. After a period of encouragement for the development of test items for proficiency testing by national providers, the scenario changed, with the emergence of Brazilian providers, mainly in the area of food and water microbiology. Table 1 shows the Brazilian food and water microbiology proficiency test providers according to information obtained in the international database EPTIS (Bundesanstalt für Materialforschung und -prüfung, 2018).

Table 1. Brazilian Food and Water Proficiency Test providers according to the EPtis database.

Provider	Property tested	Product groups	Accreditation
Ezequiel Dias Foundation	**Qualitative**: coliforms, *E. coli*	Water	Data not available on website
Metrological Network – RS	**Qualitative:** total coliforms, *E. coli* **Quantitative**: total and thermotolerant coliforms, *E. coli,* heterotrophic bacterial count	Water	Data not available on website
CONTROLLAB	**Qualitative**: *P. aeruginosa*, sulphite-reducing clostridia, *E. coli, Enterococcus* sp., total and thermophilic coliforms **Quantitative**: heterotrophic bacteria, *S. aureus,* total coliforms, mesophilic microorganisms	Water	Accredited on the basis of ABNT NBR ISO/IEC 17043
	Qualitative: *L. monocytogenes*, *Salmonella* spp. **Quantitative**: total and thermotolerant coliforms, *E. coli*, moulds and yeasts, viable aerobic mesophilic microorganisms, *B. cereus*, coagulase positive staphylococci, sulphite-reducing clostridia	Food, drinking water	
PEP-SENAI SC Lanal	**Qualitative**: *Salmonella* spp., *L. monocytogenes, Campylobacter* **Quantitative**: yeasts and moulds, lactic bacteria, coliforms MPN, *B. cereus*, *C. perfringens*, *S. aureus*, coagulase positive staphylococci, Enterobacteriaceae, thermotolerant coliforms, sulphite-reducing clostridia, psychotropic bacteria	Food, drinking water	Accredited on the basis of ABNT NBR ISO/IEC 17043
	Qualitative: *E. coli,* total and thermotolerant coliforms **Quantitative**: heterotrophic bacteria, *C. perfringens*, total and thermotolerant coliforms, *E. coli*, *P. aeruginosa*, *Enterococcus* sp	Water	
SAAS Group	**Qualitative**: total and faecal coliforms **Quantitative**: total and faecal coliforms, heterotrophic bacteria	Food, drinking water	Data not available on website
National Institute of Health Quality Control INCQS/FIOCRUZ	**Qualitative**: *Salmonella* spp., *L. monocytogenes* **Quantitative**: coliforms, *B. cereus*, coagulase positive staphylococci, thermotolerant coliforms, total aerobic mesophilic count; mycotoxins	Food, drinking water	Accredited on the basis of ABNT NBR ISO/IEC 17043 for mycotoxins and pesticides
Metrological Network - MG	**Quantitative**: total and thermotolerant coliforms, *E. coli*, heterotrophic bacteria, faecal streptococci	Water	Data not available on website
Prowater Interlaboratorial Program	**Quantitative**: heterotrophic bacterial count	Water	Data not available on website
Basic Sanitation Company of São Paulo State	**Qualitative**: *E. coli* **Quantitative**: heterotrophic bacterial count	Water	Data not available on website

3 Production guidelines for biological materials

Due to their great diversity, microorganisms are essential to life and to the advancement of science. Microbial strains can present diverse applications in teaching activities, as well as in the areas of agronomy, biotechnology, pharmacology and public health (Overmann, 2015).

Culture collections are fundamental in preserving biodiversity and in providing authentic microbiological resources (Sharma & Shouche, 2014).

Considering the need for access to quality biological materials in the development of the bio-economy, the Organization for Economic Co-operation and Development (OECD) has been working to define a Biological Resource Centre (BRC) as a culture collection that provides high quality services and plays a key role in the development of a sustainable bio-economy (Organization for Economic Co-Operation and Development, 2001). Thus, culture collections with BRC status exhibit a high standard of quality and expertise, being recognized by the international scientific community and industry (Janssens et al., 2010).

BRC status is given to institutions able to safely maintain and distribute their microbial sources, genetic material (genomic DNA) and toxins, together with associated information. A BRC introduces specialized knowledge into their microbial cultures, providing systemization and protection to their intellectual property and applying procedures of quality assurance, biosafety and bio-protection, as well as database services with respect to the available collection (Overmann, 2015).

Suppliers of biological reference materials should follow the production rules listed in the international standards, and preferably be accredited for these procedures as a means to demonstrate their quality and competence. Among the standard document available for this practice are the Good Laboratory Practices and the International Organization for Standardization (ISO) documents. The quality standards most used by suppliers of reference materials are: ISO 9001:2015 (International Organization for Standardization, 2015b), including previous editions, which establishes “Quality Management Systems for Institutions that Perform Production or Services”; ISO/IEC17025:2017, which describes the guidelines for the implementation of the Quality System and the fundamental criteria that allow laboratories to improve their ability to produce reliable results in their technical operations, and ISO 17034:2016 (International Organization for Standardization, 2016), including previous editions (ISO GUIDE 34), which presents the “General Requirements for the Competence of Producers of Reference Material” (International Organization for Standardization, 2009). The production and quality control of RM must follow the quality criteria of the standard used and amongst the controls described in the ISO 17034:2016 standard are the homogeneity and stability tests.

In the case of biological reference material, the controls applied should guarantee the purity of the culture, and the authenticity and stability of the phenotypic and genotypic characteristics of these materials over time (Day & Stacey, 2008).

In this context, the implementation of the “Best Practice Guidelines for Biological Resource Centres” of the OECD is indicated. This document is supplemented by the guidelines of Good Practices in Bio-protection for BRC, which consist of institutional and personal safety procedures that institutions must use in order to avoid loss, theft, misuse or intentional release of pathogens or their parts. The guide discusses the importance of the implementation of good practices to acquire, characterize, maintain and supply biological material. According to this document, different controls must be applied to assure the

characteristics of a preserved microorganism, including: (a) culture purity; (b) viability; (c) authenticity (preservation of the phenotypic and genotypic features of a specie); (d) genetic integrity throughout time of storage and use (Organization for Economic Co-Operation and Development, 2007).

4 Production and supply of reference strains in Brazil

There are no official reference strains producers in Brazil at present, and thus the biological reference strains used for the internal control of the assays are from international providers. The acquisition of these materials is hampered by high importation costs as well as by sanitary and commercial barriers imposed on the importation of biological materials.

The main international microbiological RM providers include: the American Type Culture Collection (ATCC), the German Collection of Microorganisms and Cell Cultures (DSMZ); the National Collection of Type Cultures (NCTC) in England and the Institute Pasteur (CIP) in France. The description of the worldwide providers of biological reference material can be found in Table 2.

With regard to certified reference material, the offer is even more restricted, and the material must be acquired directly from the international providers. A search of the International Database for Certified Reference Materials "COMAR" allows one to obtain information on the CRM available on the world market (Bundesanstalt für Materialforschung und -prüfung, 2017). The important suppliers of certified reference strains are cited in Table 2.

These facts show the dependence of Brazil on obtaining reference micro-organisms, certified or not, from international suppliers. This finding points to the need for financial support for the development and establishment of national reference strains. A national reference can be understood as a microorganism isolated from Brazilian territory, characterized phenotypically and genotypically, and produced following the relevant standard guidelines.

Thus, it is necessary to adapt the laboratorial infrastructure of the Brazilian culture collections and CBRs following the requirements specified in the quality assurance systems, so that they can establish and provide high quality biological reference materials. At the same time, it is essential to train specialized human resources to ensure the reliability of the services provided, based on the biosafety and biosecurity guidelines.

In 2014, with the prospective of encouraging the production of reference materials in the country, the National Council for Scientific and Technological Development together with the National Health Surveillance Agency, presented the National Council for Scientific and Technological Development/National Health Surveillance Agency edict Nº 05, search line: Studies of Methodologies, Development of Reference Materials for the Conformity Assessment of Products and Processes to Support the Laboratorial Component of Health Surveillance Actions. A scientific project was developed (Rosas, 2018), culminating in the establishment of two certified microbiological reference materials of Brazilian origin, which in the future will be available free of charge to the Brazilian public laboratories.

It is worth emphasizing the need to implement state policies in Brazil that can subsidize long-term investments for culture collections and CBRs in the production of microbiological reference strains, in order to lead the country to self-sufficiency in this area.

Table 2. International reference microorganism providers, their products, quality certification/accreditations and BRC Status.

Provider	Reference Microorganisms (bacterial and fungal domains)		Quality Certification/ Accreditation referring to the production of reference materials	BRC Status
	Reference material	Certified reference material		
American Type Culture Collection (ATCC)	Bacterial and fungal species	Bacterial and fungal species	ISO 9001:2015 ISO GUIDE 34:2009 ISO/IEC 17025:2005	Yes
German Collection of Microorganisms and Cell Cultures (DSMZ)	Bacterial and fungal species	Bacterial and fungal species	ISO 9001:2015	Yes
National Collection of Type Cultures (NCTC)	Bacterial and fungal species	Bacterial and fungal species	ISO 9001:2015 ISO/IEC 17025:2005	Yes
Institut Pasteur (CIP)	Bacterial and fungal species	Data not available on provider's website	ISO 9001:2000 NF 96 900:2009	Yes
Institute for Reference Material and Measurements - European Commission's Science" (IRMM)	------	Bacterial and fungal species	ISO GUIDE 34:2009 ISO/IEC 17025:2005 ISO/IEC 17043:2010	Data not available on provider's website

ISO 9001-Quality management; ISO GUIDE 34-General requirements for the competence of reference material producers; ISO/IEC 17025-General requirements for the competence of testing and calibration laboratories; NF 96 900-Quality of Biological Resource Centres (BRC); ISO/IEC 17043- Conformity assessment -- General requirements for proficiency testing. Data from September 2018.

5 The development of Brazilian culture collections and reference laboratories as BRC

Culture collections are differentiated in service, reference and work collections, depending on their activities. The culture of services plays a fundamental role in the characterization, maintenance and distribution of microbial resources, aiming at the supply of products and services with assured quality (Sette et al, 2007).

Since the beginning of the century, Brazilian researchers have joined efforts to assist the development of the country's culture collections. According to Vazoller & Canhos (2005), the international culture collections of industrialized countries had their activities consolidated as infrastructure to provide services, at the end of the last century. However, such development did not occur with the collections of services in Brazil, due to the lack of definitions of continuing state policies that would guarantee long-term support.

Many advances have been noted over the years, as described in the text below but without continuing guidelines for the development of Brazilian culture collections. Thus, it is essential to define long-term strategies for the strengthening and modernization of these collections. Amongst the many challenges to be overcome is the development of institutional capacity, related to infrastructure and human resources.

In order to modernize and harmonize the culture collections, the OECD established a Global Network of BRC. In 2001 it published the document "Biological Resource Centres: underpinning the future of life

sciences and biotechnology" (Organization for Economic Co-Operation and Development, 2001). Brazil has been following the commitment of the OECD in its attempt to implement the Global BRC Network. In 2002, the document "System for Conformity Assessment of Biological Material" was published by the Brazilian Ministry of Science and Technology (MST), aiming at providing a set of support actions focused on the reorganization, development and harmonization of microbiological collections of institutional services. Resources were applied to support actions for the improvement of infrastructure and in the implementation of quality management procedures. These actions promoted the development of the Information System for Collections of Biotechnological Interest - SICol. They also triggered an agreement between the National Institute of Industrial Property and the National Institute of Metrology, Quality and Technology, which resulted in a project to build the Brazilian Centre of Biological Material for the deposit of patented materials in the Inmetro facilities National Institute of Metrology, Quality and Technology in Rio de Janeiro, as an integral part of the Brazilian BRC network (Canhos et al., 2009).

In 2007, the Biotechnology Development Policy was established through Decree 6041 of August 2nd, 2007 of the Brazilian MST, with emphasis on improving the infrastructure of microbiological collections of services, aiming at structuring the Brazilian BRC network (Brasil, 2007). As a result of the national strategy for the creation of the Brazilian BRC network, institutional modernization is occurring, with the implementation of quality management procedures in collection systems which hold reference collections and relevant collections such as the Oswaldo Cruz Foundation collections in the area of health, the Brazilian Agricultural Research Corporation in the area of agriculture, and the Campinas State University in the area of environment and industry (Canhos et al., 2009).

In the perspective of the institution of Brazilian culture collections, the Ministry of Science Technology and Innovation established Ordinance Nº 409, of April 15th, 2014 aiming to create the Brazilian BRC Network and its structure (Brasil, 2014). In 2016, this law was revoked and replaced by Ordinance Nº 130 of the Ministry of Science Technology and Innovation, that regulates the current definitions and structural rules of the Brazilian BRCs, effective as from February 18th, 2016 (Brasil, 2016a).

Considering the need to define guidelines for Brazilian BRCs, the National Institute of Metrology, Quality and Technology established the standard NIT-DICLA-061: 2012 (Instituto Nacional de Metrologia, Qualidade e Tecnologia, 2012), which describes the requirements for the accreditation of reference material production activities carried out by the BRCs in the domain of microorganisms. The document indicates the use of the ISO Standards 17034:2016 and ISO/IEC 17025:2005, as well as the application of the guidelines described in the "Best Practices Guidelines for Biological Resource Centres" of the OECD.

In 2017, the Johanna Döbereiner Biological Resource Centre of the Brazilian Agricultural Research Corporation was inaugurated, as a shelter for microorganisms of importance in agriculture (Empresa Brasileira de Pesquisa Agropecuária, 2017).

Still in the national context, the need for future Brazilian microbiological RM producers who intend to use national genetic resources for research purposes, development and the commercialization of biological products should be noted, in order to comply with the rules of the Biodiversity Law Nº 13.123 regulated on May, 20th, 2015 by the Presidency of the Republic of Brazil (Brasil, 2015) by Decree Nº 8.772 and approved on May 11th, 2016 by the Presidency of the Republic of Brazil, which established the legal framework of the biodiversity and rules for access to Brazilian genetic property (Brasil, 2016b).

6 Recommendations for the practice of sub-culturing reference microorganisms

There is consensus that the number of subcultures applied to a reference strain must be reduced in order to minimize the chance of contamination, or of phenotypic and genotypic changes. Thus, international standard methods describe guidelines regarding the maximum number of cultures from a genuine reference

microorganism. According to the United States Pharmacopoeia (United States Pharmacopoeia, 2016) and the European Pharmacopoeia (European Directorate for the Quality of Medicine & Health-Care, 2013) guidelines on microbiological tests of non-sterile pharmaceutical products, microbial cultures derived from an original strain, should not undergo more than five passages. These recommendations have been accepted and followed by laboratories and industry worldwide, also with the accordance of the provider ATCC (American Type Culture Collection, 2003).

In a guiding technical bulletin, ATCC defines questions regarding the number of passages of a reference strain. It explains first that steps for the recovery of frozen strains or the suspension of lyophilized strains shall not be considered passages. A passage corresponds to the transfer of the microorganism to a fresh culture media, broth or agar. Thus, any other subculture indicates a new passage that should not happen more than five times (American Type Culture Collection, 2003).

In order to favor a better use of the reference strains used in routine, the USP recommends the preparation of a seeding batch, producing several replicates from an original reference strain. The batch must be preserved at the proper temperature for prolonged storage, the use of liquid nitrogen or a freezer below -50ºC being recommended, this step being considered the first passage. Working cultures can be prepared from the seeding batch, considered the second passage (United States Pharmacopoeia, 2016).

7 Conclusion

In light of the importance of the use of reference strains to assure the quality of laboratorial activities in microbiological analysis, and considering the dependence of the country on the acquisition of reference strains from international providers, it is necessary to encourage the production of national reference strains, certified or not, based on official production technical standards, aiming to meet the internal demand of Brazilian laboratories that seek increased reliability of their analytical results.

The current situation of national laboratories that need more accessible reference strains to control their assays, and the situation of national collections that provide derived international reference strains, motivates an urgent discussion with the country's health authorities. The Brazilian Ministry of Health, the Sanitary Surveillance Agency and the official accreditation organization, need to be in agreement to decide the future of Brazil in this area, facilitating the action of all the agencies, providers, legislators and controllers involved, which, in turn, could result in greater autonomy in the production of microbiological RM and, subsequently, more support to the quality of laboratorial activities, as well as the possibility of a reduction in the costs of analyses.

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