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Original article

Collaborative study to evaluate the inter laboratory reproducibility for Gel Electrophoresis by SDS PAGE on human Erythropoietin drug samples

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ABSTRACT

Introduction: A collaborative study was carried out to determine the inter laboratory variability in purity test method on erythropoietin samples by Gel electrophoresis employing standard SDS PAGE in addition to as a pre-requisite for assuring the quality of test results reported by the laboratories. Eight different laboratories across the country participated in this study, which play an important role in Public Health Sector for availability of standard quality drugs and biologicals. All receiving identical material of Reference standard and the coded samples from the same batch. **Material and method:** A standard protocol was prepared complying with the requirements of Erythropoietin injection which is a published monograph in Indian Pharmacopoeia–(IP) 2014. Three independent experiments were carried out within a month of receipt of the samples. In this report we present the results of an Inter-Laboratory Comparison (ILC) study coordinated by Quality Management Unit (QMU) of NIB during January-September 2014 for assuring the quality of results of Identification of Recombinant Human Erythropoietin (rh-EPO injection) by Gel electrophoresis as a step forward for the continuous improvement towards accreditation and compliance to the standard ISO17025:2005. **Result:** All the eight laboratories provided satisfactory results and these results are further evaluated, ranked under Good (50%), Excellent (37.5%) and Fair (12.5%) none of the laboratories obtained "poor" rank. **Conclusion:** Participations in such studies reveal indicators for the assessment of quality risk management, could contribute to the refinement of the assay for reliable identification and purity test parameter assessment.

KEYWORDS: Assuring quality, Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis, Inter-laboratory comparison, quality control.

INTRODUCTION

This paper has presented several factors to consider while selecting the analytical methods to assess the identity, purity of the product erythropoietin injection by inter-laboratory comparison. Since no single method can provide data on all key product parameters, orthogonal analytical methods are always preferred.

Methods used under Good Laboratory Practice (GLP) or CGMP quality practices must be validated for their intended use. The strategies for qualifying and/or validating analytical methods should be based on the type of method, the nature of the product and the parameter to be evaluated with the data. Laboratories that adopt validated methods (e.g., compendial methods) must experimentally verify the suitable performance of these methods in the user environment and determine their acceptable quality, bulk and final product must be analyzed for identity, purity, concentration[1].

Pharmaceutical laboratories play an important role in drug regulation by providing test results of investigation on active pharmaceuticals ingredients, pharmaceutical products and excipients. Therefore, it is important that the results generated by these laboratories are accurate, precise and reproducible. Many approaches are in use for assuring the quality of test results produced by testing laboratories [1].

Participation in ILC provides laboratories with an objective of accessing and demonstrating the reliability of results they produce. ILC participation also provides independent verification of the competence of a laboratory and show commitment to the maintenance and improvement of performance [1]. ILC covers the entire process in a laboratory including receipt and storage of test samples, the experimental procedures carried out in the laboratory and transcription of the data, preparation of reports and the conclusion drawn from the reports. ILC studies are conducted to assess the quality of test results produced by Laboratories [2].

Erythropoietin (EPO) is the main regulator of human erythropoiesis. This sialoglycoprotein hormone consists of 165 amino acids that form a single polypeptide chain containing two intra-chain disulfide bonds and four potential glycosylation sites. The molecular mass of EPO is 30-34 kDa [3]. Epoetin alfa is the genetically engineered form of endogenous erythropoietin that has become the standard of care for erythropoietic support in renal anemia and cancer-associated anemias. Replacement therapy with epoetin has been shown to increase RBC mass, to decrease the need for blood transfusions, and to reduce the symptoms of anemia.

National Institute of Biologicals (NIB) is an autonomous institute under Ministry of Health & Family Welfare, Government of India having one of the mandates for Quality Control (QC) testing of Biologicals [4]. Quality control testing of biologicals includes a series of test for identification, potency, purity & safety [5]. The Institute is actively involved in QC testing of various Biologicals indigenously manufactured and imported to Indian market and thus playing prime responsibility in providing standard quality drugs in the interest of Public Health. The Institute is also developing written standards in collaboration with the industry and Indian Pharmacopoeia Commission for publication of Monographs in Indian Pharmacopoeia (IP) for strengthening indigenous industry by providing well validated methods for testing Identity, Safety, purity and Potency of various biological products.

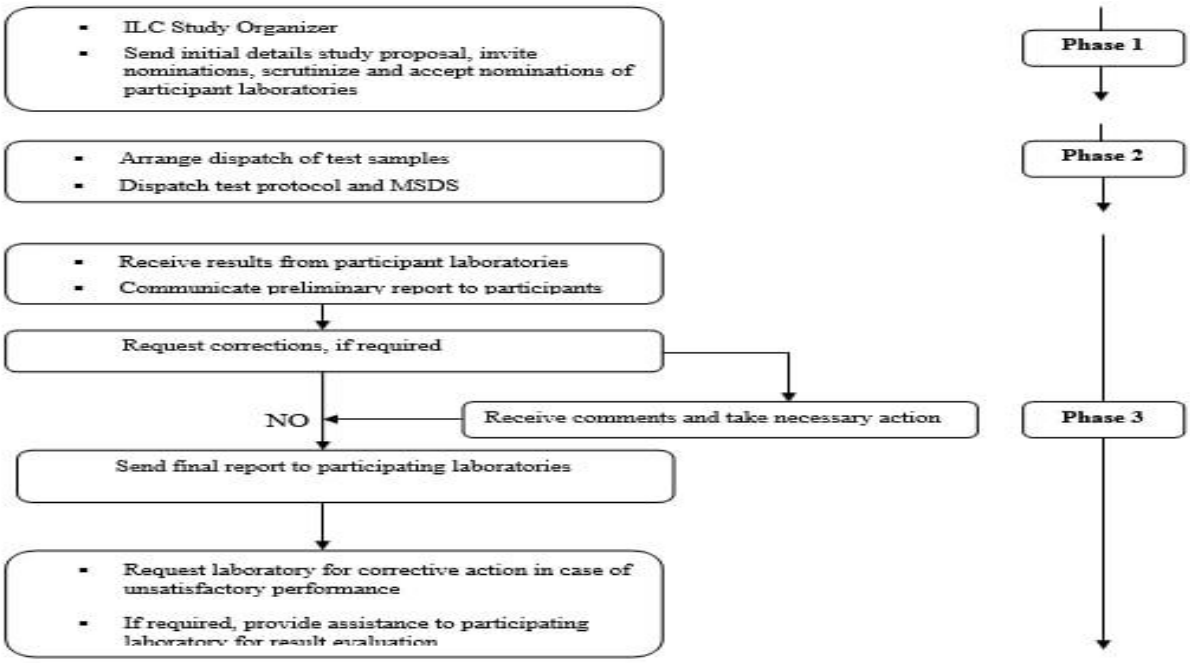
NIB is notified as Central Drugs Laboratory (CDL) by Government of India for testing of various Bio-therapeutics, Vaccines and Diagnostics. The Institute's testing laboratories are accredited for ISO/IEC 17025:2005 standard compliance with a vast and unique scope in Biological & Chemical testing areas. To strengthen the accredited scope and a way forward to continuous improvement for the best possible compliance with the standard (ISO/IEC 17025:2005), Quality Management Unit (QMU) of the institute had initiated organizing ILC studies for various qualitative tests of Identification, Purity and safety used for testing of biological products.

The gel electrophoresis is an important identification test that is adopted in the pharmacopeia for the identification and impurity of proteins [6]. With the objective of this study is to determine the inter laboratory variability in purity test method on erythropoietin samples by Gel electrophoresis employing SDS PAGE standard, Quality Management Unit (QMU) of NIB undertook an initiative for an ILC study on qualitative parameter of gel electrophoresis to assess the performance of participants laboratories and to analyze the deficiencies, if any, observed in the test.

MATERIAL AND METHOD

The proposal of the ILC study on Gel Electrophoresis and consent form for participation was circulated by email in January 2014 to sixteen laboratories in India who have been the potential stakeholders of the bio therapeutic manufacturers, Pharmacopeia laboratories, National Control laboratories and academia laboratories. Study was planned in three phases and process flow steps of ILC study was shown in Figure 1. Phase 1 of the study included confirmation by participating laboratories for the ILC study. In phase 2, study samples were dispatched by NIB and participating laboratories reported their results after performing the gel electrophoresis. During phase 3 results were compiled and report was prepared and communicated to all participants.

Figure 1: Process flow showing the phases of inter laboratory collaborative study



Participants

Eight laboratories agreed to participate in the collaborative study for ILC. Participants are listed in Table 1 and the participants are referred to by a code number which is unrelated to their order of listing in the table. The participants include one from pharmacopoeia laboratory, one from National Testing Laboratory, six laboratories of biotech manufacturers in India. QMU of NIB served as the coordinating laboratory for this study.

Table 1: Participants of the ILC study from India

S. No.	Participant Name and Organization
1	Sriram Akundi and Harshad Joshi, Biocon Limited, Bangalore
2	Krithika Balasubramanian and Sridevi Khambhampati, Dr. Reddy's Laboratories, RR District, Hyderabad
3	Susobhan Das and Kinnary Vyas,Intas Pharmaceuticals Limited, Ahmedabad
4	Sunil Gairola,Serum Institute of India Limited, Pune
5	Alka Beotra, Shila Jain, National Dope Testing Laboratory, New Delhi
6	Mahesh Bhalgat and S. Muruganandham, Shantha Biotechnics Limited**,** Hyderabad
7	Ranjan Chakrabarti and G. Pradeep, USP India Private Limited, Hyderabad
8	P. S. Maruthi Sai and Nimesh Thaker, Zydus Biologics, Ahmedabad

Test Samples

Three preparations of test samples were provided to the participants with detail given in Table 2. Test samples selected for the study were finished recombinant biologicals products received for quality control testing at NIB and identity of these samples is not disclosed in this report. Test sample were coded and dispatched under cold chain conditions in March 2014. The consignment was accompanied with test protocol and material safety data sheet.

Table 2: **Details of study samples along with their Potency**

Sample No.	Sample Code	Potency/Concentration
Reference standard	NIB TS-81	1mg/ml
Sample 1	NIB/ILC/SDS-01	20,000 IU/ml
Sample 2	NIB/ILC/SDS-02	10,000 IU/ml
Sample 3	NIB/ILC/SDS-03	20,000 IU/ml

Testing of samples by SDS-PAGE

Test protocol provided to participants for gel electrophoresis by SDS PAGE method is based on Pharmacopoeia monograph published in Indian Pharmacopoeia-2014 [6, 7]. Participants were requested to follow the method detailed in the protocol and carry out required testing and to submit the results on prescribed data recording form. Instructions were given on critical requirements on sample receipt, storage and handling, essential equipment and images of electropherogram.

It was recommended that on receipt the material to be stored at 5 ± 3°C until use and the contents of each test sample & reference standard to be reconstituted as per procedure described. Information had to be filled by the Participating laboratories in the following sections of Data Recording Forms:

a. Gel Preparation& Equipment used
b. Chemicals/ Reagents/ Reference standards used
c. Preparation of the Reference & Test sample / solution
d. Gel Image – reducing/ non reducing conditions
e. Validity criteria
f. Data Analysis

SDS PAGE was performed as described by Laemmli Using 12% polyacrylamide gel. The proteins were prepared in non-reduced (intact) and reduced forms. For preparation of non-reduced protein samples, sample buffer (10% of the total volume of the sample) was added to the protein sample and then vortex for 2 minutes. For the preparation of reduced protein samples, reduced or reducing sample buffer (5% β-mercaptoethanol in sample buffer) was added to the protein sample (10% of the total volume of the sample). The mixture was vortex and boiled at 100^0C for 5 minutes. Electrophoresis was performed using vertical electrophoresis slab gel apparatus (Bio Rad Mini gel protean) at a constant voltage of 100 volts when samples were in the stacking gel. When the dye front reached the resolving gel, voltage was increased to 200 volts. The run was stopped when the dye front was 2 to 3 mm away from bottom edge of the gel. The gel was then silver stained using the commercial silver staining kit.

Data Analysis and Preparation of Report

All the eight participant laboratories submitted their results in May 2014 on prescribed data recording forms. Information on material usage for SDS PAGE was compiled and results were analysed. A preliminary report prepared along with the deficiencies observed and communicated to the participating laboratories with a request to comment upon. Clarifications received from the participants were considered to resolve the deficiencies and final report was prepared and communicated to participants in September 2014.

RESULTS

The quality of SDS gels was graded by assigning ranks from "excellent" to "poor" to each parameter mainly Band quality, Lanes, Gel background and Smearing (Table 3).

Table 3: Quality grading of Gel Images

S. No	Parameter	Excellent	Good	Fair	Poor
1.	Band quality	All test sample and reference standard bands must be well established and sharp across the entire gel. Protein mol. weight markers should be resolved into discrete bands and distributed along 80 % of the gel	Slight band distortion in one lane but this does not interfere with analysis. A few bands are of low intensity (difficult to see clearly)	Some bands 2-3 are too thick but still analysable.	Bands too light or too thick to distinguish
2.	Lanes	Straight across length and width of the gel	Slight curving, still analysable	Significant curving, still analysable	Curving that interferes with analysis
3.	Gel background	Clear	Mostly clear background Minor unclarity, but does not affect analysis	Too many bands present, that may or may not make analysis difficult	Due to background analysis is difficult
4.	Smearing in Lanes	Not present	Smearing in a few lanes but bands are clear	Significant smearing in one or two lanes that may or may not make analysis difficult	Significant smearing in more than two lanes that may make analysis difficult

Quality grading has been done on the basis of the validity criteria and data analysis which was described in the protocol communicated to the participants. The Band Quality achieved by the participant laboratories was taken into account for overall scoring of ranks.

Details of gel preparation, run conditions and image acquisition information on gel percent used, gel thickness, molecular weight standard ladder received from the participant laboratories is described in Table 4.

Table 4: Performance evaluation of Participant Laboratories

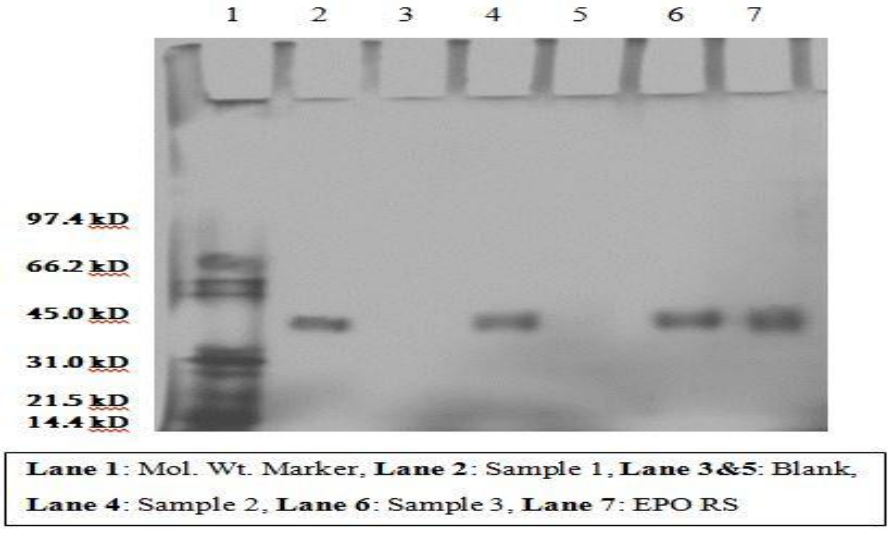
Laboratories Code	Band Quality	Lanes	Gel Background	Smearing in lanes	Remarks: Position of Test sample and reference standard
NIB/ ILC – Lab 01	**Good** Band distortion in molecular weight standard lane. NIB Reference Standard band is difficult to see clearly	**Good** Slight curving in molecular weight marker lane	**Excellent** Clear	**Good** Minor smearing in Molecular weight marker lane	• Test samples and NIB reference standard band lying near 42 kD marker • Test only performed for Reducing condition
NIB/ ILC – Lab 02	**Excellent** All bands well established and sharp, protein mol. wt markers are resolved into discrete bands & distributed along 80% of the gel	**Excellent** Straight across length and width of gel	**Excellent** Clear	**Excellent** No smearing	• Test samples and NIB reference standard band lying near 43 kD marker • Test performed for both reducing and non-reducing condition • In house working standard not run
NIB/ ILC – Lab 03	**Excellent** All bands well established and sharp, protein mol. wt markers are resolved into discrete bands and distributed along 80 % of the gel	**Excellent** Straight across length and width of gel	**Excellent** Clear	**Excellent** No smearing	• Test samples and NIB reference standard band lying near ~35 kD marker • Test performed for both reducing and non-reducing condition

Laboratories Code	Band Quality	Lanes	Gel Background	Smearing in lanes	Remarks: Position of Test sample and reference standard
NIB/ ILC – Lab 04	**Good** Reference standard bands are of low intensity.	**Good** Slight curving in Molecular weight marker lane	**Excellent** Clear	**Excellent** No smearing	• Test samples and NIB reference standard band lying near 45 kD • Test performed in both reducing and non-reducing condition
NIB/ ILC – Lab 05	**Excellent** All bands well established and sharp, protein mol. wt markers are resolved into discrete bands and distributed along 80 % of the gel	**Good** Slight curving in Molecular weight marker lane	**Excellent** Clear	**Excellent** No smearing	• Test samples and NIB • reference standard band lying near 45 kD marker band • Test performed in both reducing and non-reducing condition
NIB/ ILC – Lab 06	**Fair** Test sample bands too thick. Sample 1 band intensity very low in both reducing and non-reducing condition	**Good** Slight curving in molecular weight marker and test sample bands, still analysable	**Fair** Too many bands in few lanes	**Fair** Significant smearing in few lanes (Gel Image in Reducing condition)	• Test samples 2 and 3 and NIB reference standard band lying at 37kD marker band but sample 1 difficult to analyze • Test performed in both reducing and non-reducing condition
NIB/ ILC – Lab 08	**Fair** Test sample band in non-reducing condition image is of low intensity. EPO reference standard band is too thick but still analyzable	**Good** Slight curving in Molecular weight band	**Excellent** Clear	**Good** Minor smearing in molecular weight marker bands	• Test samples and NIB reference standard band lying near 37kD marker band • Test performed in both reducing and non-reducing condition
NIB/ ILC – Lab 10	**Fair** Test sample and reference standard band are analyzable although too thick	**Good** Slight curving in reference standard lane	**Excellent** Mostly clear	**Good** No smearing	• Band in test solution matches in position with reference band. • Test performed only for reducing condition.

The study was carried out by participation of 8 different laboratories across the country. Figure 2 depicts the standard SDS-PAGE pattern of Erythropoietin sample on 12.5% gel. The SDS electrophoretic profile of the erythropoietin contains only one major band identified at approximately 34 kDa in respect of a solution of molecular weight markers suitable for calibrating SDS Polyacrylamide gels in range of 14.4 -97.4 kDa. The electropherogram obtained with test solution is expected to show a single diffuse band corresponding in position and intensity to the single band seen in the electropherogram obtained with reference solution. Quality grading of gel images were documented by considering 4 parameters viz., Band quality, Lanes, Gel background, Smearing in Lanes mentioned**.**

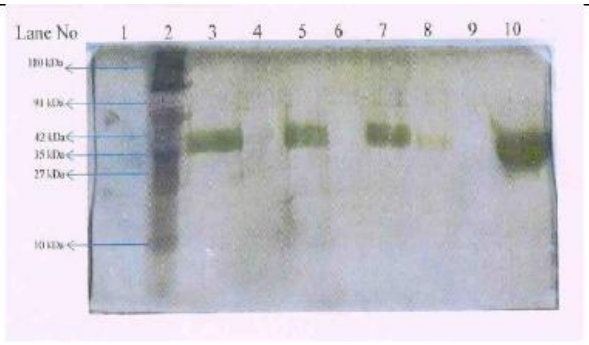
The laboratory wise performance was evaluated in Table 4 to rank laboratories into excellent, good, fair or poor. The ranking of gel images for the above four parameters was done based on the visual observations of the images provided by the laboratories as shown in the Figure 3 below.

Figure 2: Standard chromatogram of rh- erythropoietin (EPO)

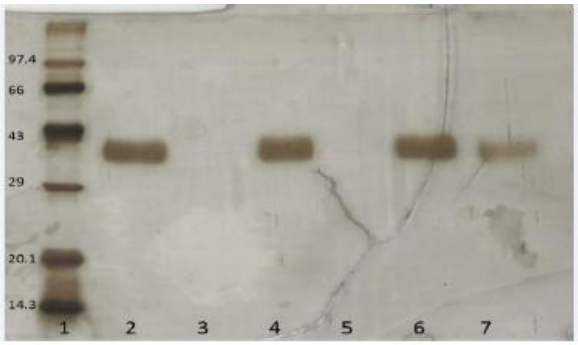


Lane 1: Mol. Wt. Marker, **Lane 2**: Sample 1, **Lane 3&5**: Blank, **Lane 4**: Sample 2, **Lane 6**: Sample 3, **Lane** 7: EPO RS

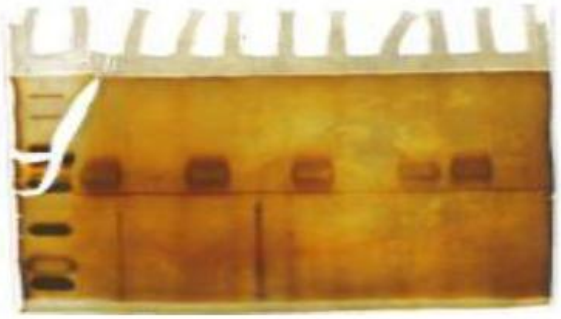
Figure 3: Gel images reducing conditions received from Participating Laboratories



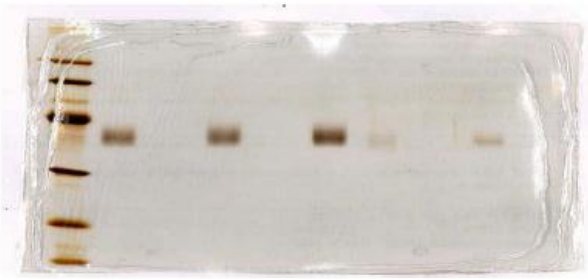
NIB/ ILC – Lab 01



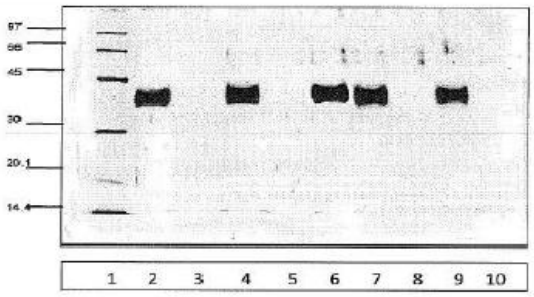
NIB/ ILC – Lab 02



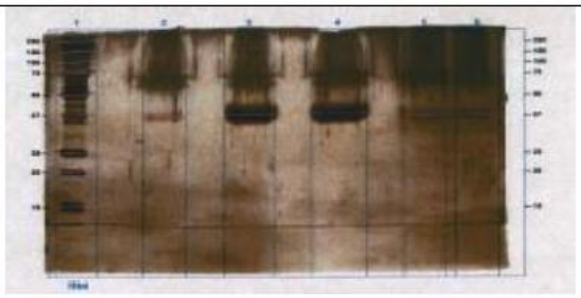
NIB/ ILC – Lab 03



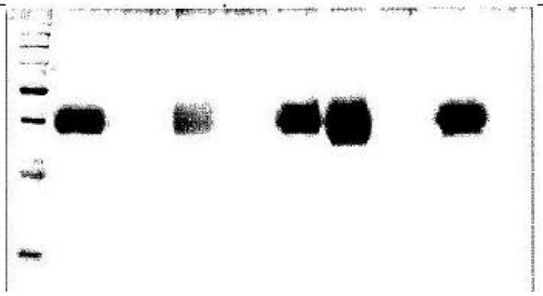
NIB/ ILC – Lab 04



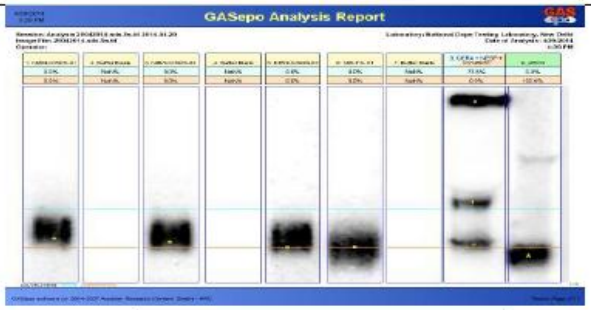
NIB/ ILC – Lab 05



NIB/ ILC – Lab 06



NIB/ ILC – Lab 08



NIB/ ILC – Lab 10

DISCUSSION

The participant laboratories were further assigned overall ranking based on the scores they obtained for the band quality. The overall performance of participant laboratories is depicted in terms of percentage . None of the labs obtained "poor" overall rank, one laboratory (12.50%) obtained "fair" rank, 4 laboratories (50%) "good" rank, and 3 laboratories (37.50%) obtained an "excellent" rank (Table 5).

Table 5: Overall rank assigned to Participant Laboratories

Evaluation	Rank assigned to the participant laboratory	Overall performance evaluation	Total no. of participated laboratories	Percentage Evaluation
Excellent	NIB/ILC-02 NIB/ILC-03 NIB/ILC-05	03 Labs	N = 8	37.5%
Good	NIB/ILC-01 NIB/ILC-04 NIB/ILC-08 NIB/ILC-10	04 Labs		50%
Poor	NIB/ILC-06	01 Labs		12.5%

The participating laboratories obtained the best results for the parameters "Gel Background" and "Smearing" for which, respectively, 6 (75%) and 5 (62.5%) laboratories obtained the rank "excellent". No Laboratory obtained the rank "poor" for any parameter. For the parameter "Lanes", 6 (75%) laboratories obtained "good" rank. However, minor weaknesses were identified in some technical areas, mainly "Band Quality", for which the "fair" rank was assigned.

The results of the study indicate that compendial monograph in which regulatory methods for these product attributes given are limited for qualitative assessments like Electrophoresis based methods discussed in this study. SDS-Gel Electrophoresis is a complex laboratory procedure, potentially affected by several sources of error, like,

1. Inappropriate handling of samples/ solution
2. Dilution errors
3. Pipetting errors
4. Inappropriate qualification/ Calibration of the equipment
5. Preparation and staining of gel
6. Reporting errors

CONCLUSION

Thus decisions regarding the control of the process and the quality of the product are based on data generated by analytical tests. If there are design flaws in the test methods, unrecognized sources of method variation, or a method is chosen that cannot support the specification requirements, the data will inevitably be inadequate, inaccurate or unreliable. So, while it is certainly critical to understand the process by which biotechnological product is produced, it is equally vital to understand the methods of analysis that are applied to the product.

This ILC study concludes for the risk based approach initiative as every product and every process has an associated risk and testing laboratories should have methodology for evaluating the risk and generating intervention for Risk Management Plan.

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