Original Research Paper

Volume - 16 | Issue - 09 | September - 2026 | PRINT ISSN No. 2249 - 555X | DOI : 10.36106/ijar

Biotechnology

PLATE-BASED ASSAYS FOR PROCESS-RELATED IMPURITY QUANTIFICATION: A FOCUSED REVIEW ON ELISA AND MICROPLATE METHODS FOR HCP, HCD AND RPA

Ms. Prajyot Kulkarni* Student, Department of Biotechnology, KIT's College of Engineering (Empowered Autonomous), Kolhapur, Maharashtra*Corresponding Author

Dr. Medha Petkar Associate Professor, Department of Biotechnology, KIT's College of Engineering (Empowered Autonomous), Kolhapur, Maharashtra

ABSTRACT Process-related impurities such as host cell protein (HCP), host cell DNA (HCD) and residual Protein A (rPA) are mandatory quality attributes for monoclonal antibodies [1]. While orthogonal characterization by LC-MS/MS and NGS is gaining importance, ELISA, Threshold total DNA assay and plate-based qPCR in 96-well format remain regulatory workhorses for GMP lot release [2,3]. Unlike safety-centric reviews, this review focuses exclusively on analytical principles of plate-based assays: reagent generation, coverage analysis, Threshold principle, qPCR for CHO DNA and IgY ELISA for Protein A. Key challenges such as antibody coverage gap, matrix interference, hook effect, dilutional linearity and drug tolerance are discussed with mitigation approaches [5,7]. Emerging platforms such as Gyrolab, AlphaLISA and Single Molecule Counting (SMC) ELISA that retain microplate workflow while improving sensitivity are also reviewed [8].

KEYWORDS : Plate-based assay, ELISA, HCP, HCD, Residual Protein A, Threshold assay, Assay validation

1. INTRODUCTION

India's biopharmaceutical sector is rapidly transitioning from generics to biosimilars. Monoclonal antibodies produced in Chinese Hamster Ovary (CHO) cells dominate this pipeline. Purification leaves trace levels of host-derived and process-added impurities. Regulatory agencies including USFDA, EMA and CDSCO classify HCP, HCD and rPA as process-related impurities that must be monitored per ICH Q6B [1]. Although characterization now uses advanced techniques, routine QC still depends on 96-well microplate formats due to low cost, high throughput and established validation history [3]. All three major PRIs have compendial plate-based methods but their assay design is fundamentally different. This review consolidates practical knowledge on how these assays are built, why conventional assays under-report, and how validation should be performed as per ICH Q2(R2) [2]. This focus fills a gap where very few dedicated reviews exist on plate-based methods.

2. Principle Of Plate-based Assays

Microplate assays rely on capture reagent immobilization, specific analyte binding and enzymatic signal amplification in a polystyrene well. The most common format is sandwich ELISA, where capture antibody is coated overnight, sample is incubated, and HRP-conjugated detection antibody generates color with TMB substrate [3]. Advantages include testing 80 samples per plate in duplicate, low volume (<100 µL), sensitivity in ng/mL to pg/mL range and regulatory acceptance under USP <1132> [3]. For PRIs, the challenge is detecting 1-100 ppm of heterogeneous analytes in presence of 10-150 mg/mL therapeutic antibody. This high product-to-impurity ratio creates significant matrix interference and requires determination of Minimum Required Dilution (MRD) to reduce interference while maintaining sensitivity [2].

3. Host Cell Protein Elisa

3.1 Reagent Generation

Process-specific HCP ELISA uses polyclonal antibodies raised against HCPs from a null cell line, i.e., host cells transfected with empty vector and cultured under identical conditions as production cells but without product gene expression [6]. The null harvest containing 2000-3000 HCPs is concentrated, adjuvanted and used to immunize goats or rabbits over 3-4 boosts. The IgG fraction is affinity purified and used as both capture and detection antibody in sandwich format. This approach is superior to generic commercial CHO HCP kits which use broad CHO proteome and may miss process-specific upregulated HCPs [6].

3.2 Coverage Analysis

Regulators now expect demonstration of antibody coverage for the specific process as per USP <1132> [3]. Coverage is assessed by two-dimensional electrophoresis (2D-PAGE) followed by Western blotting, where total HCPs are separated by isoelectric point and molecular weight and stained by silver stain versus blot probed with anti-HCP antibody [5]. Percent coverage is calculated as number of spots detected by antibody divided by total silver stained spots. Acceptable coverage is typically >65%, but low molecular weight (<15 kDa) and hydrophobic membrane proteins are often missed [5]. High-risk HCPs such as Phospholipase B-like 2 (PLBL2), Lipoprotein Lipase (LPL), Lysosomal Phospholipase A2 (LPLA2) and Cathepsin D are weakly immunogenic and under-represented [5,8]. These hitchhiker HCPs bind to the antibody product itself and escape capture, causing polysorbate degradation even when total HCP ELISA shows <100 ppm [8]. Hence orthogonal LC-MS/MS is required for characterization.

3.3 Failure Modes

Two major failure modes are observed in HCP ELISA [5]. First, poor dilutional linearity due to matrix effect of the drug product. If sample diluted 1:2, 1:4, 1:8 does not show linear recovery, matrix interference is suspected and MRD must be increased [2]. Second, under-recovery due to HCP-product association. Pre-treatment with chaotropic agents such as urea or acid dissociation is sometimes required to release bound HCPs. Validation must include spike recovery of 70-130% across at least three dilutions to prove parallelism as per ICH Q2(R2) [2].

4. Host Cell Dna - Threshold And Qpcr

Host Cell DNA is considered oncogenic and infectious risk factor as per WHO guidance [9]. Two plate-based approaches are accepted for its quantification. The Threshold assay is a microplate assay based on binding of single-stranded DNA to a nitrocellulose membrane via single-stranded DNA binding protein [4]. Total DNA from sample is first extracted using proteinase K digestion to remove protein, then heat denatured to single strand, hybridized with biotinylated ssDNA binding protein, captured on streptavidin coated membrane in 96-well plate and detected by anti-ssDNA antibody conjugated to urease. It is rapid (<4 hours) but non-specific to host species and measures total residual DNA [4]. In contrast, plate-based qPCR is species-specific and performed in 96/384 well plates using primers for conserved repetitive sequences such as SINE or LINE elements in CHO genome [4,9]. DNA is extracted by silica column, then amplified using TaqMan probe. qPCR offers sensitivity up to 1 pg/mL and is required to demonstrate size distribution <200 bp after purification. However, qPCR is prone to inhibition by high protein concentration and requires careful extraction validation and internal control [4]. Regulatory limit is <10 ng per dose as per WHO and FDA [9].

5. Residual Protein A - Elisa And Drug Tolerance

Protein A is a bacterial protein from Staphylococcus aureus used as affinity ligand for capture chromatography. Leaching occurs due to proteolytic clipping, low pH elution at pH 3.5 and resin aging over 200 cycles [7]. Residual Protein A is highly immunogenic and can cause anaphylaxis. It is measured by sandwich ELISA using mammalian anti-Protein A antibodies or preferably non-immune chicken IgY which binds Protein A via Fc-independent mechanism, thus avoiding

interference from human IgG [7]. The unique challenge in this assay is drug tolerance. Therapeutic mAb itself contains Fc region that can compete with assay antibodies for residual Protein A, leading to false negative results [7]. To mitigate, samples are pre-treated with acid dissociation at pH 3.5 for 30 minutes followed by neutralization and heating at 50°C to disrupt mAb-Protein A complexes before plating [7]. Modern alkali-stabilized resins such as MabSelect SuRe and PrismA have reduced baseline leaching from historical 15-20 ppm to <2 ppm, but assay remains mandatory per USP <1130> [4]. Acceptance limit is generally <10 ppm in drug substance [7].

6. Validation Challenges As Per Ich Q2(r2)

All PRI plate assays must be validated for specificity, accuracy, precision and robustness as per ICH Q2(R2) [2]. Common issues include Hook Effect - At very high impurity concentration, signal decreases due to saturation of both capture and detection antibodies separately, leading to false low result. This is checked by testing up to 1000 ng/mL [5]. Dilutional Linearity and Parallelism - Required to prove matrix does not interfere. ICH Q2(R2) requires spike recovery 70-130% across minimum three dilutions with CV <20% [2]. Drug Tolerance - Maximum product concentration where 100 pg/mL impurity can still be detected with acceptable recovery [7]. Reagent Criticality - Polyclonal antibody lot-to-lot variation is the biggest source of variability and requires bridging study with 2D-Western blot and titration curve comparison when new critical reagent lot is introduced [3,5]. LOQ is defined as lowest concentration with CV <20% and recovery 80-120% [2].

7. Next Generation Plate-based Platforms

To overcome limitations of conventional ELISA while keeping plate workflow familiar to QC, new microfluidic plate platforms have emerged [8]. Gyrolab (Gyros Protein Technologies) uses nanoliter centrifugal microfluidics in a compact disc format with 112 microstructures. It reduces matrix interference due to short contact time and sample consumption to only 5 µL, ideal for high concentration mAb [8]. AlphaLISA (PerkinElmer) is a bead-based luminescent proximity assay in 384-well plates requiring no wash steps; donor and acceptor beads come into proximity when analyte is present, generating chemiluminescence. It offers higher drug tolerance for rPA detection up to 100 mg/mL. SMC-ELISA or Single Molecule Counting ELISA uses single photon counting to detect fluorophore labeled detection antibody, achieving sensitivity down to 0.05 ng/mL, 20 times more sensitive than conventional ELISA [8]. For Indian laboratories, cost of these platforms is high, so conventional ELISA with improved sample pre-treatment using arginine and Tween 20 remains pragmatic, while qPCR is preferred over Threshold because qPCR instruments already exist for mycoplasma testing [4].

7.1 Practical Implementation In Indian Biosimilar Settings

In Indian CRO and biosimilar settings, generic CHO HCP Kit 3G is widely used due to cost, but may miss clone-specific impurities [6]. Generating in-house null cell line reagents is recommended for better coverage. Many QC labs prefer qPCR over Threshold because real-time PCR instruments are already available [4,9]. For rPA, chicken IgY ELISA is preferred because it avoids interference from human IgG [7]. Drug tolerance can be improved by acid dissociation at pH 3.5, addition of 0.5 M arginine or 0.1% Tween 20 in diluent, and use of high-capacity streptavidin plates [7]. Validation must demonstrate spike recovery at low (1 ng/mL), mid (10 ng/mL) and high (50 ng/mL) levels in presence of 50 mg/mL product [2]. Documenting Minimum Required Dilution and parallelism is essential for CDSCO and USFDA submissions and is a common query in audits [1,2].

8. CONCLUSION

Plate-based assays will remain frontline QC tools for HCP, HCD and rPA for next decade due to simplicity, cost-effectiveness and regulatory history [3,4]. Limitation is not sensitivity but specificity and coverage [5,8]. Indian biosimilar industry should invest in process-specific HCP reagent generation rather than relying solely on generic kits to meet global expectations [6]. Future QC will adopt tiered approach: conventional ELISA, Threshold and qPCR for routine release and in-process controls, and orthogonal high-sensitivity platforms like Gyrolab and SMC-ELISA plus LC-MS/MS for characterization of high-risk HCPs [8]. Focus should shift from merely achieving low LOD to demonstrating robust drug tolerance, dilutional linearity and comprehensive coverage as per ICH Q2(R2) and Q14 [2].

Table 1: Comparison of Plate-Based Methods for Process-Related Impurities

Impurity	Plate Method	Capture Reagent	Sensitivity	Key Limitation
HCP	Sandwich ELISA [3,5]	Polyclonal anti-HCP (Goat/Rabbit)	1-5 ng/mL	Coverage gap, hitchhiker HCPs [8]
HCD	Threshold Assay [4]	ssDNA binding protein	2 pg/mL	No species specificity, total DNA only
HCD	qPCR (96-well plate) [4,9]	SINE/LINE primers	1 pg/mL	Extraction dependent, PCR inhibition
rPA	Chicken IgY Sandwich ELISA [7]	Chicken IgY anti-Protein A	0.1 ng/mL	Drug interference, needs acid dissociation [7]

REFERENCES

1. ICH Q6B - Specifications: Test Procedures and Acceptance Criteria for Biotechnological Products, 1999.
2. ICH Q2(R2) Validation of Analytical Procedures, 2023 and ICH Q14 Analytical Procedure Development.
3. USP<1132> Residual Host Cell Protein Measurement - General Chapter, USP-NF 2024.
4. USP<1130> Nucleic Acid-Based Techniques and Protein A Detection - General Chapter.
5. Zhu-Shimoni M. et al., Host cell protein testing by ELISA and the use of orthogonal methods, Biotechnol Bioeng. 2014;111(8):1513-1524.
6. Wang X. et al., Development of process-specific HCP ELISA using null cell line, BioProcess Int. 2022.
7. Eaton et al., Assay development for residual Protein A clearance and detection, J Immunol Methods. 2020.
8. Bracewell D.G. et al., Towards HCP quantification by LC-MS/MS as orthogonal method, Biotechnol Bioeng. 2015.
9. WHO Guidelines on evaluation of similar biotherapeutic products (SBPs), 2009 - HCD limit <10 ng/dose.