DeQuanto® *E. Coli* HCP ELISA Kit

QT4003-ST

USER MANUAL

Immunoassay for quantitative determination of *E. Coli* HCP's in Bioprocess sample

96- Well (12X8 strips) Microtiter plate

Research Use Only (RUO)

Please read this user's manual carefully before using the kit

DENOVO BIOLABS PVT LTD
Version 1.1

NO. A-112, Ground Floor KSSIDC, Block-3, Electronic City Phase I, Bengaluru, Karnataka 560100, INDIA
Phone: +91-80-29575711; E-mail: info@denovobiolabs.com ; Web: www.denovobiolabs.com

TABLE OF CONTENTS

INTRODUCTION

Host cell proteins (HCP's) are proteins produced or encoded by the host organisms used to produce recombinant therapeutic proteins (1). Recombinant therapeutic proteins are usually produced by genetically-modified host cells using cell culture or fermentation technology (2). During the recombinant protein production, host cells also coproduce proteins related to the normal cell functions such as cell growth, proliferation, survival, gene transcription, protein synthesis, and etc. Other non-essential proteins may also be released to the cell culture or fermentation as a result of cell apoptosis/death/lysis (2). HCP's constitute a most important part of process-related impurities during recombinant therapeutic proteins production. The amount of residual HCP's in drug product is generally considered a critical quality attribute (CQA), due to their potential to affect product safety and efficacy. Therefore, it is a regulatory requirement to monitor the removal of HCP's in drug product during bioprocess development.

Industry-recommended practices for ligand-binding assays [3, 4, and 5] were used for validation of this kit. This DeQuanto® *E Coli* HCP ELISA kit has been developed for specific quantification of *E. Coli* HCP's in bioprocess sample with high sensitivity and reproducibility.

ASSAY PRINCIPLE

The DeQuanto® *E. Coli* HCP ELISA kit is an ELISA based immunoassay. The HCP's present in the sample or standard binds to pre coated Anti *E. Coli* HCP's antibodies on polystyrene microtiter plate. Non bound or the excess substances are removed by washing. Horseradish Peroxidase (HRP) labeled Anti *E. Coli* HCP's antibodies is added to the well which binds to the HCP's/Anti *E. Coli* antibodies complex in the wells. Following a wash to remove any unbound antibody-enzyme reagent, substrate solution is added to the wells. A color product is formed in proportion to the amount of HCP's present in the sample or standards. The color development is stopped by addition of a stop solution. The absorbance is measured at 450 nm in a microtiter plate reader. The concentration of HCP's in a sample can be interpolated from the standard curve.

MATERIALS PROVIDED AND STORAGE

The DeQuanto® *E. Coli* HCP ELISA kit is recommended to be stored as mentioned in table below.

Sl. No.	Description	Quantity	Volume	Strength/Conc.	Storage
1	Pre Coated 96-well microtiter plate (12X8 strips)	1 plate	NA	NA	-20°C
2	*E. Coli* HCP's Standard	1 Vial	50 µl	1.06 mg/ml	-20°C
3	Detection Antibody	1 Vial	20 µl	1000X	-20°C
4	Assay Diluent	1 Bottle	100 ml	NA	2-8°C
5	TMB Substrate	1 Vial	1.0 ml	20X	2-8°C
6	Wash Buffer-A	1 Bottle	70 ml	20X	2-8°C
7	Plate sealer	1 Nos.	NA	NA	RT

MATERIALS REQUIRED BY END-USER

All the following materials are required but not available with the kit.

- Tween-20
- 2N H_2SO_4
- Deionized or ultra pure water
- Analytical grade sodium chloride (NaCl)
- Microtiter plate reader capable of measuring absorbance at 450 nm (Reference wave length 600 nm is optional)
- Software capable of 4PL or 5PL parameter logistic curve fitting for data analysis

ASSAY DURATION

The approximate time taken for each step of the assay are shown in the table below

Sl. No.	Step	Duration
1	Reagent thawing and preparation	30 minutes
2	Analyte addition and incubation	90 minutes
3	Detection Antibody Addition and Incubation	50 minutes
4	Substrate Addition and Measurement	30 minutes
5	Calculation of Results	15 minutes

PRECAUTIONS

1. User should be trained with ELISA based assays and test procedure
2. All reagents have to be at room temperature before use, except the detection antibody (should be stored at -20°C to ensure the stability)
3. Avoid repeated freeze/thaw cycles for all reagents
4. Handle all reagents wearing gloves and other protective gears
5. Do not pipette any reagents by mouth
6. Use calibrated pipettes and devices only
7. Sodium azide inactivates HRP, do not use sodium azide-containing solutions
8. Any modification in the standard assay procedure may influence the kit performance
9. The described pipetting volume, incubation time, temperature steps should be performed according to the user manual
10. TMB/H_2O_2 Substrate and detection antibody are light sensitive, hence should not be exposed to light
11. Avoid physical contact with Stop solution
12. Centrifuge all vials briefly before use
13. Use disposable pipette tips for each transfer to avoid cross contamination
14. Any samples which are out of assay range should be repeated using different dilutions
15. Reagents from different batch/lot are not interchangeable
16. Do not mix reagents from different kit batches/lots
17. The waste disposal should be performed according to your laboratory regulations

REAGENT PREPARATION

WASH BUFFER-A

1. Thaw the wash buffer at RT until it is a clear solution
2. Prepare 1000 ml of 1X wash buffer-A, using 50 ml of 20X Wash buffer-A and make up the volume to 1000 ml with ultra pure water
3. Add 29.22 NaCl to 1X wash Buffer-A and dissolve it with the help of magnetic stirrer for 10 minutes.

WASH BUFFER-B

1. Aliquot 500 ml of wash buffer-A
2. Add 0.05% (250 µl) of Tween-20 solution and mix it for 10 minutes on magnetic stirrer at RT

ASSAY DILUENT

1. Allow it to mix on rocker for 10 minutes or by gentle manual mixing at room temperature
2. Assay diluent should appear as a clear solution after mixing
3. Assay diluent is ready to use solution

TMB SUBSTRATE

1. Dilute the 20X TMB substrate to 1X solution in ultra pure water (600 µl of TMB substrate to 11.4 ml of ultra pure water)
2. Mix the 1X solution thoroughly on vortex mixer or manually

STANDARDS

1. Prepare a sub stock of 21.2 µg/ml (21200 ng/ml) as given in table below:

Required sub-stock Conc. (µg/ml)	Volume of Main-stock to be taken (µl)	Volume of Assay Diluent (µl) to be taken	Final volume (µl)	Dilution factor
21.2	20	980	1000	1:50

2. Prepare standards 1 to 10 and zero standard (blank) in assay diluent as given in the table below

Sub stock of *E. Coli* HCP's protein (ng/ml)	Volume from sub stock (µl)	Assay Diluent Volume (µl)	Final concentration (ng/ml)	Standard No
21200.00	359.91	640.09	7630.00	1
7630.00	400.00	600.00	3052.00	2
3052.00	400.00	600.00	1220.80	3
1220.80	400.00	600.00	488.32	4
488.32	400.00	600.00	195.33	5
195.33	400.00	600.00	78.13	6
78.13	400.00	600.00	31.25	7
31.25	400.00	600.00	12.50	8
12.50	400.00	600.00	5.00	9
5.00	400.00	600.00	2.00	10
-	-	300.00	0.00	Blank

CAUTION

- ***Vortex gently to mix during each step***
- ***Use appropriate pipette range and do not change pipette in between the assay procedure***

QUALITY CONTROL

1. Prepare minimum of three QC samples in assay matrix
2. Keep the diluent buffer control in two replicates

DETECTION ANTIBODY

1. Dilute the 1000X Detection Antibody in assay diluent at 1:1000 (take 15 µl of 1000X detection antibody and add to 15 ml of assay diluent)
2. Gently mix the detection antibody before use

ASSAY PROCEDURE

PRE-PROCESSING OF 96-WELL MICROTITER PLATE

1. Thaw the pre-coated 96-well microtiter (12X8 strips) at room temperature for 15 minutes
2. Wash the plate with 1X wash buffer-A, allowing 2 minutes for soaking
3. Decant off the contents of the plate

CAUTION

- ***Thaw only required number of pre coated strips***
- ***Use multichannel pipette***
- ***Keep the programs for plate washer ready (if automated washer is used)***

ANALYTE ADDITION

1. Prepare standards, QC samples and test samples
2. Add 100 µl of standards, QC and test or specimen samples to the wells of the microtiter plate as per the plate template
3. Seal plates with adhesive plate sealer
4. Incubate the microtiter plate at RT (~25°C) for 1 hour on shaking mode at 300 rpm.

CAUTION

- ***Refer the sample plate layout given at the end of this user manual, for standards and control***
- ***Before addition of samples to the microtiter plate, keep the Plate template ready.***

DETECTION ANTIBODY

1. Discard the contents of each well and wash 3 times with 1X wash buffer-B and followed by 3 washes with 1X wash buffer-A, allowing 2 minutes for soaking between each wash step
2. Blot the microtiter plate on absorbent paper to remove any residual reagent from the wells
3. Add 100 µl of Detection Antibody solution to each well of the microtiter plate
4. Seal the microtiter plate with adhesive plate sealer
5. Incubate the microtiter plate at RT (~25 °C) for 45 minutes

SUBSTRATE ADDITION AND MEASUREMENT

1. Discard the contents of each well and wash 3 times with 1X wash buffer-B and followed by 3 washes with 1X wash buffer-A, allowing 2 minutes for soaking between each wash step
2. Blot the microtiter plate on absorbent paper to remove any residual reagent from the wells
3. Add 100 µl/well of 1X TMB substrate solution and incubate the plate in dark at RT (~25°C) for 15 minutes
4. Add 50µl/well of Stop solution to stop the reaction
5. Read the absorbance in micro plate reader set to 450 nm, set the reference wavelength to 600 nm

CAUTION

- ***Add stop solution in the same order of addition of TMB substrate to the plate***

ASSAY SUMMARY

1. Prepare all reagents as instructed in the user manual

2. Add 100 µl of standard, quality control and test/specimen samples to each well of microtiter plate

3. Seal the microtiter plate with adhesive plate sealer and incubate at RT (~25°C) for 1 hour on shaking at 300 rpm

4. Decant the contents of the microtiter plate and wash the microtiter plate 3 times with wash buffer-B, followed by 3 times with wash Buffer-A, allowing 2 minutes of soaking between each wash step

5. Blot the plate on absorbent paper to remove any residual reagent from the wells

6. Add 100 µl of detection antibody to each well of the microtiter plate and incubate for 45 minutes at RT (~25°C)

7. Repeat the steps 4 and 5

8. Add 100 µl of 1X TMB solution to each well of the microtiter plate and incubate for 15 minutes in dark at RT (~25°C)

9. Add 50 µl of stop solution (2N H_2SO_4) to each well

10. Read the absorbance at 450 nm and 600 nm as reference wave length

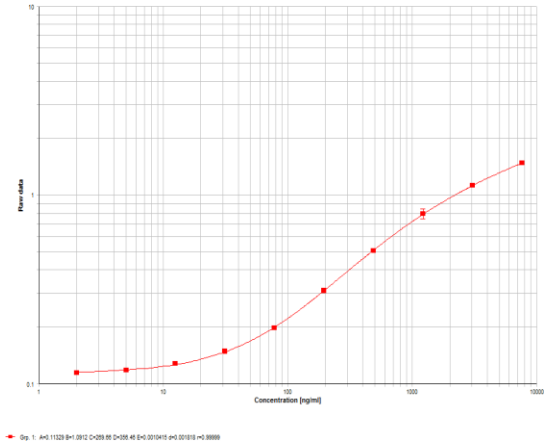
CALCULATION OF RESULTS

After the absorbance is read at 450 nm and 600 nm as reference wave length, construct a standard curve of difference data using software capable of generating four or five parameter logistic (4PL or 5PL) curve fit. Absorbance of the test/specimen and the QC samples are interpolated from the standard curve. Report the values of test/specimen samples within the assay range.

EXAMPLE DATA

This standard curve results are provided for demonstration purpose only.

The standard curve should be run for each assay.



Standard	Conc (ng/ml)	Mean Abs
S1	7630.00	1.474
S2	3052.00	1.117
S3	1220.80	0.793
S4	488.32	0.507
S5	195.32	0.311
S6	78.13	0.197
S7	31.25	0.148
S8	12.50	0.127
S9	5.00	0.117
S10	2.00	0.114
Blank	0.00	0.113

ASSAY CHARACTERISTICS

VALIDATION SUMMARY

Denovo Biolabs have validated this assay in assay diluent according to industry-recommended practices for ligand-binding assays [3, 4, and 5]. However, such validation is generic in nature and it is intended to only supplement but not substitute specific validation as required by regulations or otherwise in each case.

STANDARD CURVE MODEL

The calibration standards were generated by spiking *E. Coli* HCP's protein in assay diluent. The standard curve consisted of ten non-zero standards (from 7630 ng/ml to 2.0 ng/ml). A 5PL model was used to fit the standard curve. The regression model was accepted as the %RE of the back-calculated value for at least 75% calibrators was within 20% of nominal concentration, except at the ULOQ and LLOQ where it was within 25%.

ACCURACY AND PRECISION

Accuracy (%RE) of all QC samples was within 20% across all batches. Inter-assay and pooled (cumulative) intra-assay precision (%CV) of each QC sample was ≤ 20%. The total error was within 20%.

SELECTIVITY

ELISA and western blot analysis against DH5 and BL21 strains of *E. Coli* indicates the conserved proteins. Thus this kit should be useful for detecting HCP's from *E. Coli* strains. The end user must evaluate the kit and validate the reactivity for specific samples.

SENSITIVITY

In all batch runs LLOQ-QC at 12.5 ng/ml showed %RE within the 20% range confirming the sensitivity of the assay to be 12.5 ng/ml.

DRIFT

The drift parameter was evaluated by placing QC samples at different positions of the 96-well microtiter plate in an intra-assay batch. All the three QCs samples passed the drift criteria and the %CV of all the QC samples were within $\leq$ 20%, except LLOQ-QC where it was within $\leq$ 25%.

DILUTION LINEARITY

From the sub-stock a 1000X, ULOQ sample was made which was further diluted into other QC samples. Each of these QC samples was tested for their %RE values. All near QC samples passed the acceptance criteria. 1000X-ULOQ did not pass the criteria due to Hooks Effect.

ABBREVIATIONS

RT	Room Temperature
Abs	Absorbance
Conc.	Concentration
HRP	Horse Radish Peroxidase
HCP	Host cell protein
HCP's	Host cell proteins
TMB	3, 3', 5, 5'-Tetramethylbiphenyl-4, 4'-Diamine
ELISA	Enzyme Linked Immunosorbent Assay
%CV	Coefficient of variations
%RE	Relative Error
PL	Parameter Logistic
QC	Quality Control
CTRL	Control
ULOQ	Upper Limit of Quantification
HQC	High Quality Control
LQC	Lower Quality Control
LLOQ	Lower Limit of Quantification
°C	Degree Celsius
µg	Microgram
mg	Milligram
µl	Microliter
Eg.	Example
ml	Milliliter
IgG	Immunoglobulin
ng	Nanogram
nm	Nanometer
2N H_2SO_4	2 Normal Sulphuric Acid
RPM	Revolutions Per Minute

REFERENCES

1. X. Wang, A.K. Hunter, and N.M. Mozier, *Biotechnol. Bioeng.* 103 (3), pp. 446-58, 2009.

2. M. Jin, et al., *Biotechnol. Bioeng.* 105 (2), pp. 306-16, 2010.

3. DeSilva *et al* (2003), *Recommendations for the Bioanalytical Method Validation of Ligand-binding Assays to Support Pharmacokinetic Assessments of Macromolecules*, Pharmaceutical Research, **20 (11)***:* 1885-1900*.*

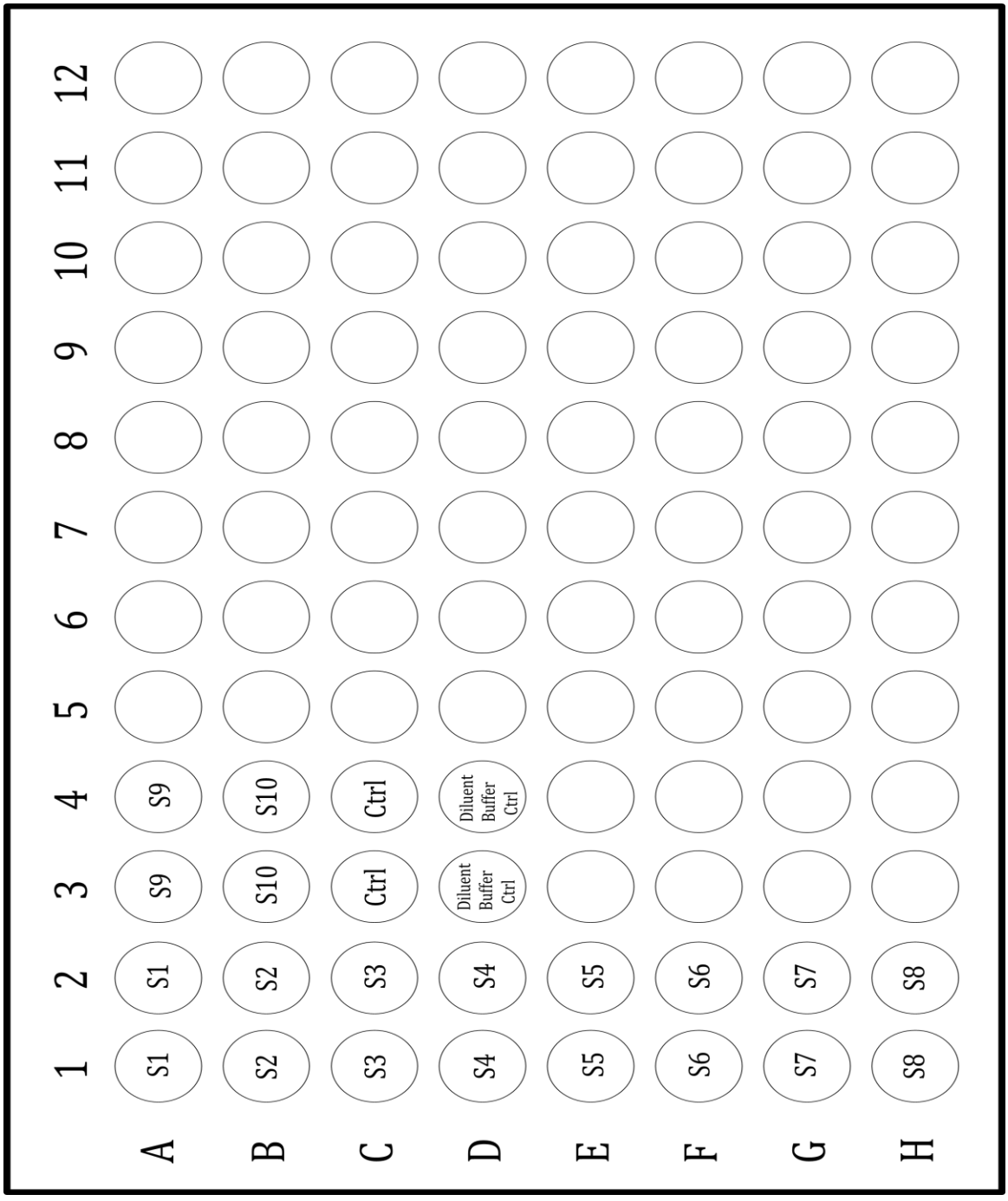
4. Findlay *et al*. (2000) *Validation of Immunoassays for bioanalysis: A pharmaceutical industry perspective*. J. Pharm. Biomed. Anal. **21**, 1249-73.

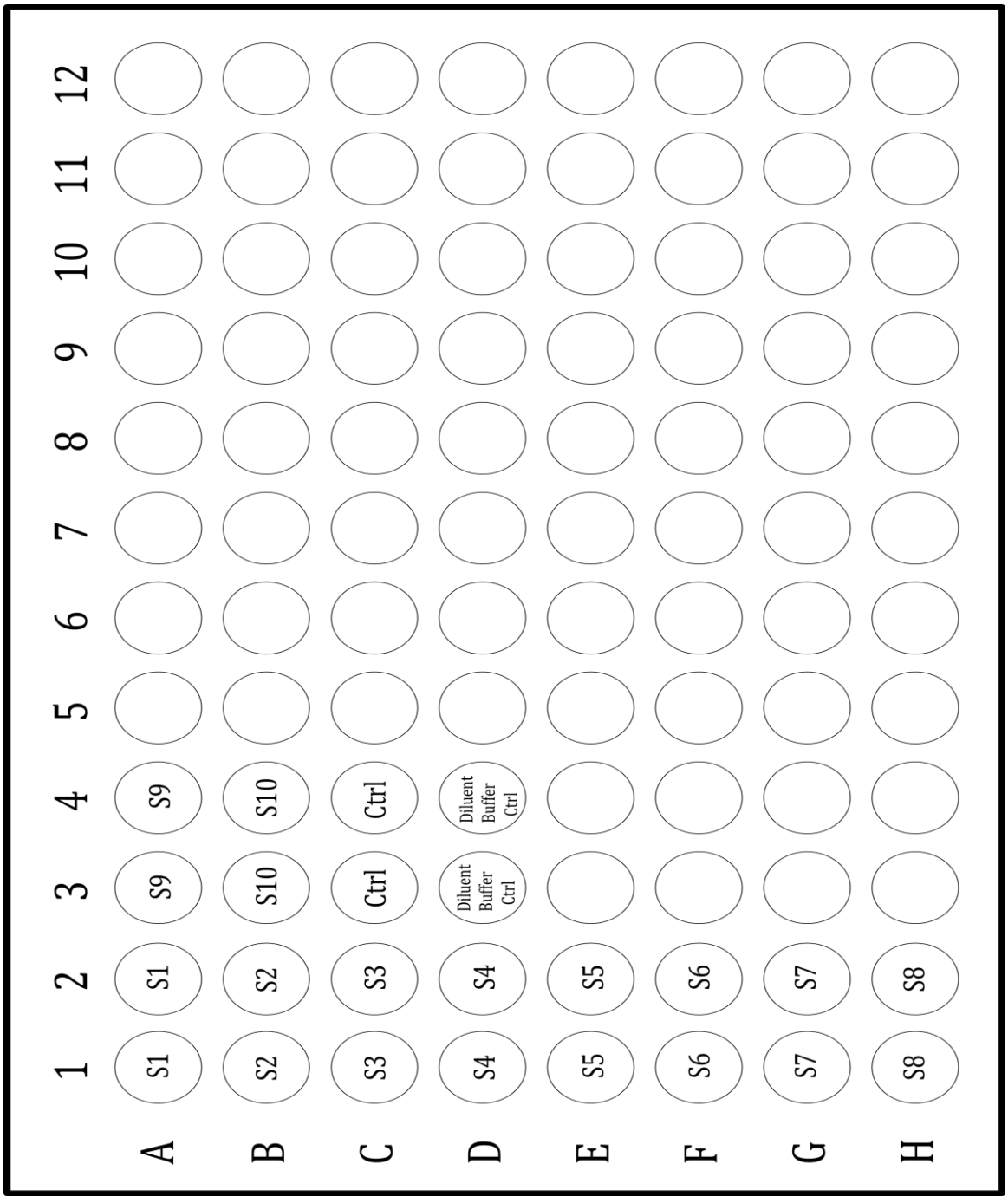
5. Kelley and DeSilva (2007)*; Key Elements of Bioanalytical Method Validation for Macromolecules*, AAPS Journal, **9 (2)**, E156-E163.

TROUBLESHOOTING

Problem	Probable Causes	Solution
High or Low OD value of test samples	Test samples contain analyte concentrations greater than assay range	Repeat the assay with multiple dilutions of the test samples.
	Test Samples contain no or below detectable levels of analyte	Test sample contains the drug level below the LLOQ
High OD value of blank/ controls (-ve)	Matrix interference	Perform higher dilution than MRD (minimum required dilution). Ensure the diluent buffer blank/ controls (-ve) OD are acceptable.
High % CV (High variability in duplicate OD values)	Unequal volumes	Ensure the calibrated pipettes
		Ensure pipette tips are tightly secured while adding the solution to each well
	Inadequate washing	Ensure the wash system is working properly if the automated wash station is used. Also ensure the manual washes
	Non-homogenous solution	Ensure that all solution prepared are adequately mixed.
	Edge effect	Use plate sealer and maintain the temperature as recommended
	Cross-well contamination	Ensure the good pipetting practice
	Inadequate mixing of TMB substrate solution	Ensure the TMB substrate solution is mixed thoroughly before use
Low signal	Standard was incompletely reconstituted or the storage was inappropriate	Reconstitute standard according to the user's manual Ensure the kit reagents are not expired
	Reagents added to wells with incorrect concentrations	Cross check for calculation and pipetting errors
	Incorrect incubation time or temperature	Adhere to the recommended assay procedure
Standard curve with R^2 value <0.95	Pipetting error	Ensure the pipetting and the analyst training

PLATE LAYOUT




1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
S1 S2 S3 S4 S5 S6 S7 S8
S1 S2 S3 S4 S5 S6 S7 S8
S9 S10 Ctrl Diluent Buffer Ctrl
S9 S10 Ctrl Diluent Buffer Ctrl

NOTES

NOTES

NOTES

CUSTOMIZED SERVICES

PRECLINICAL/CLINICAL SAMPLE ANALYSIS
ELISA based validation and clinical or pre-clinical sample analysis.

POLYCLONAL ANTIBODY DEVELOPMENT
PAb development against peptide or protein, peptide designing, high titer antibody purification, characterization.

MONOCLONAL ANTIBODY DEVELOPMENT
MAb development against peptide or protein, peptide designing, best reacting/stable hybridoma development, sub cloning, small scale/large scale hybridoma culture and antibody purification, characterization.

CLONING, PROTEIN EXPRESSION PURIFICATION
Gene synthesis, site directed mutagenesis, cloning and expression in bacterial systems and other customized molecular biology services.

ANTIBODY CONJUGATION
With HRP, FITC, Biotin, ALP and many more molecules.

CELL BASED ASSAYS
Cell based assays, neutralization assays and potency ratio estimation of drugs with reference drugs, drug efficacy assessment and customized in-vitro, in-vivo assay development.

CUSTOMIZED ELISA BASED ASSAY/METHOD DEVELOPMENT
Different formats of ELISA based assay development.

For any queries/enquiries related to our products or services, please contact us by mailing us at info@denovobiolabs.com or call us at +91-80-29575711