DeQuanto® Human TNF alpha ELISA Kit

QT4002

USER MANUAL

Immunoassay for quantitative determination of Human TNF alpha in human serum or plasma

Research Use Only (RUO)

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

DENOVO BIOLABS PVT LTD

Version 1.0

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INTRODUCTION

Tumor Necrosis Factor-alpha (TNF-α) is a multifunctional inflammatory cytokine/adipokine belonging to TNF super-family[1], secreted by macrophage/monocytes, which is involved in systemic inflammation and stimulates acute phase reaction[2,3].

ASSAY PRINCIPLE

The DeQuanto® Human TNF alpha ELISA kit is an ELISA based immunoassay. The TNF alpha present in the sample binds to pre coated Anti TNF-α monoclonal antibody on polystyrene microtiter plate. Non bound or the excess substances are removed by washing. Biotinylated polyclonal Anti TNF alpha antibody is added to each well. After washing, Streptavidin Horseradish Peroxidase (HRP) is added to the wells which bind to the biotinylated antibody in the wells. Following a wash to remove any unbound antibody-enzyme reagent, substrate solution is added to the wells. A colored product is formed in proportion to the amount of TNF alpha 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 TNF alpha in a sample can be interpolated from the standard curve.

MATERIALS PROVIDED AND STORAGE

The DeQuanto® Human TNF alpha ELISA kit is recommended to be stored as mentioned in table below.

Sl. No.	Description	Quantity	Volume	Strength/Conc.	Storage
1	Capture Antibody	1 Vial	60 µl	230X	-20°C
2	Recombinant Human TNF alpha Standard	1 Vial	40 µl	0.6 mg/ml	-20°C
3	Biotin Conjugated Antibody*	1 Vial	40 µl	1000X	-20°C
4	Streptavidin-HRP	1 Vial	20 µl	2500X	-20°C
5	Assay Diluent	1 Bottle	100 ml	1X	2-8°C
6	TMB Substrate	1 Vial	1.0 ml	20X	2-8°C
7	Wash Buffer-A	1 Bottle	70 ml	20X	2-8°C
8	Plate sealer	1 Nos	NA	NA	RT

*Detection Antibody

MATERIALS REQUIRED BY END-USER

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

- Human Serum or Plasma
- Tween-20
- 2N H_2SO_4
- Maxisorp or equivalent quality polystyrene microtiter plate
- 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	Coating capture antibody	Overnight
2	Blocking of plates	120 minutes
3	Reagent thawing and preparation	30 minutes
4	Analyte addition and incubation	90 minutes
5	Detection Antibody Addition and Incubation	30 minutes
6	Streptavidin-HRP Addition and Incubation	30 minutes
7	Substrate Addition and Measurement	30 minutes
8	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

SPECIMEN COLLECTION AND STORAGE

SERUM

Use serum clot tube and allow the blood sample to coagulate at room temperature (RT) for 30 minutes. Centrifuge at 5000 RPM for 10 minutes at RT. Aliquot the clear serum and store at -20°C. Avoid repeated freeze/ thaw cycles.

PLASMA

Use K_2 EDTA as anticoagulant for blood collection and allow at RT for 30 minutes. Centrifuge the sample at 5000 RPM for 10 minutes at RT. Aliquot the clear plasma and store at -20°C. Avoid repeated freeze/ thaw cycles.

NOTE: Grossly haemolyzed, lipemic or contaminated sample (serum or Plasma) may lead to inaccurate results and is not recommended to use with this procedure.

It is recommended that the end user must validate assay procedure using either serum or plasma.

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

WASH BUFFER-B

1. Aliquot 500 ml of wash buffer-A
2. Add 0.1% (500 µl) of Tween-20 solution and mix it for 10 minutes on magnetic stirrer at RT

COATING BUFFER

1. Use 1X wash buffer-A as coating buffer.
2. Dilute the capture antibody using this buffer.

ASSAY DILUENT

1. Thaw the Assay diluent at RT until it is a clear solution
2. Use this assay diluent buffer for assay matrix and test sample dilution

ASSAY MATRIX

1. Dilute human serum or plasma at MRD (1:10) in assay diluent
2. Use this assay matrix for standards and quality control preparation

CAUTION

- *Human serum or plasma used for assay matrix must be from healthy volunteers*

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 6000 ng/ml by diluting the TNF alpha Standard (0.6 mg/ml) in neat human serum or plasma (5 µl of TNF alpha standard in 495 µl of human serum or plasma)
2. Prepare a working stock of 600 ng/ml from 6000 ng/ml sub stock as given in table below

Required Working stock Conc. (pg/ml)	Volume of Sub-stock to be taken (µl)	Volume of Assay Matrix (µl) to be taken	Final volume (µl)	Dilution factor
600000	50	450	500	1:10

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

Sub stock of Standard (pg/ml)	Volume from sub stock (µl)	Assay Matrix Volume (µl)	Final concentration (pg/ml)	Standard No
600000.00	40	1460	16000.00	1
16000.00	400	600	6400.00	2
6400.00	400	600	2560.00	3
2560.00	400	600	1024.00	4
1024.00	400	600	409.60	5
409.60	400	600	163.84	6
163.84	400	600	65.54	7
65.54	400	600	26.21	8
26.21	400	600	10.49	9
10.49	400	600	4.19	10
-	-	300	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

DETECTION ANTIBODY(BIOTIN CONJUGATED ANTIBODY)

1. Dilute the 1000X Detection Antibody in assay diluent at 1:1000 (take 10 µl of 1000X detection antibody and add to 10 ml of assay diluent)
2. Gently mix the detection antibody before use

STREPTAVIDIN-HRP

1. Dilute the 2500X Streptavidin-HRP in assay diluent at 1:2500 (take 4 µl of 2500X Streptavidin-HRP and add to 10 ml of assay diluent)
2. Gently mix the solution before use

ASSAY PROCEDURE

PRE-PROCESSING OF 96-WELL MICROTITER PLATE

1. Take required number of microtitre plate strips.
2. Prepare the 1X capture antibody in coating buffer.
3. Add 100 µl per well of the microtitre plate.
4. Incubate the microtitre plate at 4°C for overnight.

BLOCKER ADDITION

1. Following day, keep the microtiter plate at room temperature for 5 minutes
2. Decant off the contents of the plate
3. Add 200 µl of assay diluent per well of the plate
4. Incubate the microtitre plate at RT for 2 hour
5. Decant off the contents of the plate

CAUTION

- ***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

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 (BIOTIN CONJUGATED 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 1 hour

STREPTAVIDIN-HRP

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 Streptavidin-HRP 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 30 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. Coat the capture antibody and incubate for overnight at 4°C.

3. Block the microtitre plate and incubate for 2 hours at RT

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

5. Seal the microtiter plate with adhesive plate sealer and incubate at RT (~25°C) for 1 hour

6. 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

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

8. Add 100 µl of detection antibody to each well of the microtiter plate and incubate for 1 hour at RT (~25°C)

9. Repeat the steps 6 and 7

10. Add 100 µl of Streptavidin-HRP to each well of the microtiter plate and incubate for 30 minutes at RT (~25°C)

11. Repeat the steps 6 and 7

12. 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)

⇩

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

⇩

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

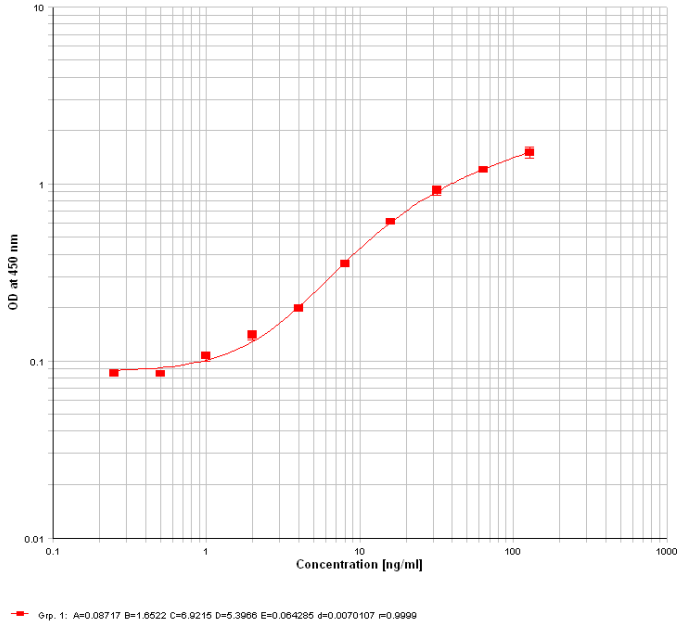
CALCULATION OF RESULTS

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 (pg/ml)	Mean Abs
S1	16000	2.242
S2	6400	1.894
S3	2560	1.304
S4	1024	0.844
S5	409.6	0.459
S6	163.84	0.271
S7	65.54	0.186
S8	26.21	0.145
S9	10.49	0.123
S10	4.19	0.113
Blank	0.00	0.1084

ASSAY CHARACTERISTICS

VALIDATION SUMMARY

Denovo Biolabs have validated this assay in human serum matrix according to EMA Bio-analytical Method Validation Guidelines [4] and industry-recommended practices for ligand-binding assays [5, 6 and 7]. 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 TNF alpha in human serum/plasma at MRD 1:10. The standard curve consisted of ten non-zero standards (from 16000 pg/ml to 4.19 pg/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

Ten different human plasma matrices were tested for selectivity in a single batch experiment by recovery studies at two QC points. 80% of 10 different individual plasma samples passed the acceptance criteria of %RE within 20% except at LLOQ where it was within 25%.

SENSITIVITY

In all batch runs LLOQ-QC at 65.5 pg/ml showed %RE within the 20% range confirming the sensitivity of the assay to be 65.5 pg/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
PK	Pharmaco Kinetics
TMB	3, 3', 5, 5'-Tetramethylbiphenyl-4, 4'-Diamine
TNF	Tumor Necrosis Factor
ELISA	Enzyme Linked Immunosorbent Assay
%CV	Coefficient of variations
%RE	Relative Error
MRD	Minimum Required Dilution
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
W/V	Weight by Volume
IgG	Immunoglobulin
ng	Nanogram
nm	Nanometer
2N H_2SO_4	2 Normal Sulphuric Acid
K_2 EDTA	Ethylene Di-amine Tetra Acetic acid (Di-Potassium salt)
RPM	Revolutions Per Minute

REFERENCES

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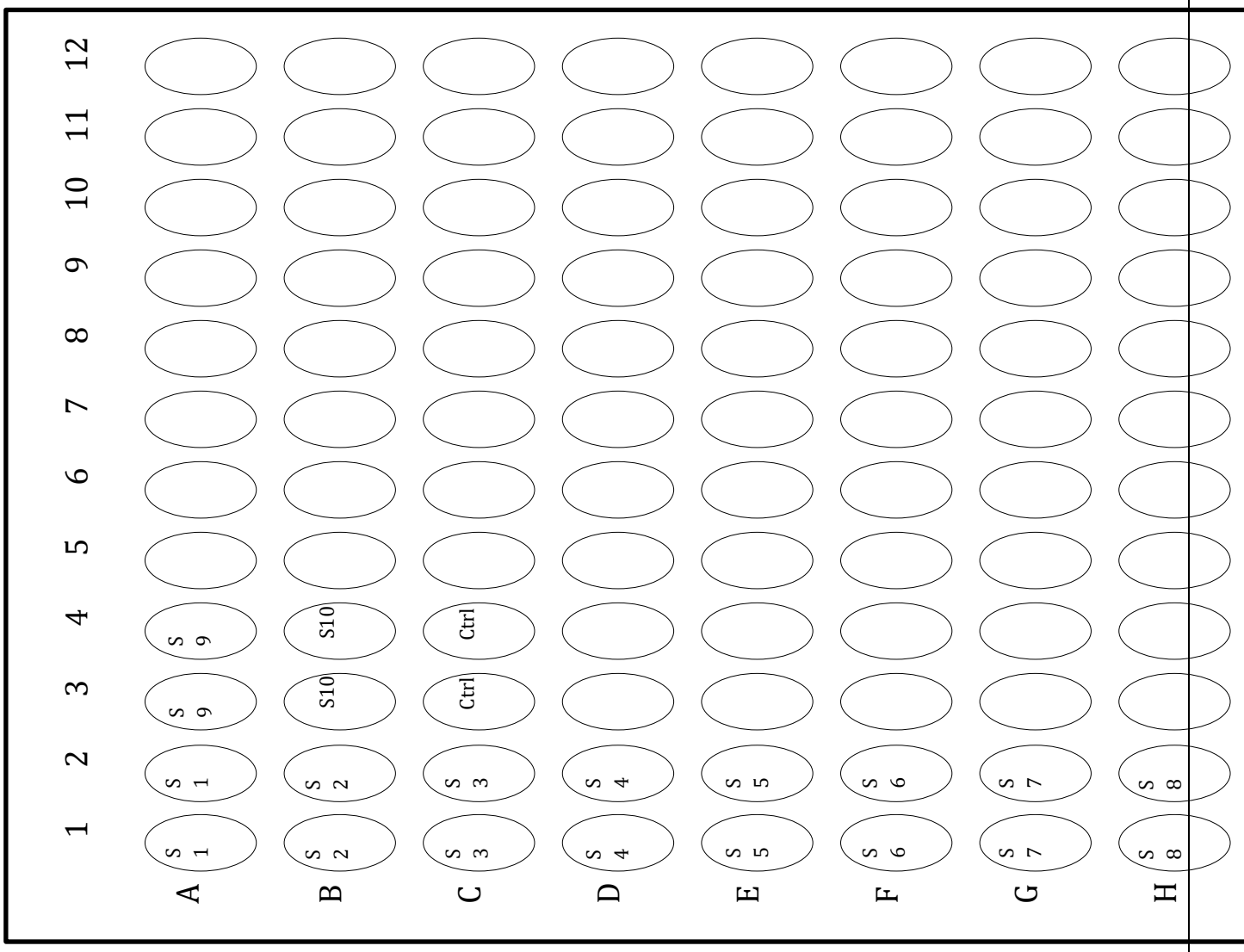
2. Hehlgans, T. and K. Pfeffer (2005) Immunology **115**:1
3. Wu, Y. and B.P. Zhou (2010) Br. J. Cancer **102**:639
4. *Guideline on bioanalytical method validation;* 21 July 2011 EMEA/CHMP/EWP/192217/2009 Committee for Medicinal Products for Human Use (CHMP)
5. DeSilva *et al* (2003), *Recommendations for the Bioanalytical Method Validation of Ligand-binding Assays to Support Pharmacokinetic Assessments of Macromolecules*, Pharmaceutical Research, **20 (11)***:* 1885-1900.
6. Findlay *et al*. (2000) *Validation of Immunoassays for bioanalysis: A pharmaceutical industry perspective*. J. Pharm. Biomed. Anal. **21**, 1249-73
7. 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 % 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



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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.

PHARMACOVIGILENCE
Clinical/post marketing drug safety reporting medical writing

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 28528900, 01, 02

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