RayBio® Human NANOG IQELISA Kit

Catalog #: IQH-NANOG

User Manual
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Caution:
Extraordinarily useful information enclosed

RayBiotech
Empowering your proteomics

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RayBiotech, Inc.

RayBio® Human NANOG IQELISA Kit Protocol

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I. INTRODUCTION

The RayBio® **I**mmuno **Q**uantitative **E**nzyme **L**inked **I**mumuno**S**orbent **A**ssay (IQELISA) is an innovative new assay that combines the specificity and ease of use of an ELISA with the sensitivity of real-time PCR. This results in an assay that is simultaneously familiar and cutting edge and enables the use of lower sample volumes while also providing more sensitivity. The RayBio® Human NANOG IQELISA Kit is a modified ELISA assay with high sensitivity qPCR readout for the quantitative measurement of Human NANOG in serum, plasma, and cell culture supernatants. This assay employs an antibody specific for Human NANOG coated on a 96-well PCR plate. Standards and samples are pipetted into the wells and NANOG present in a sample is bound to the wells by the immobilized antibody. The wells are washed and a detection affinity molecule is added to the plates. After washing away unbound detection affinity molecule, primers and a PCR master mix are added to the wells and data is collected using qPCR. C_t values obtained from the qPCR are then used to calculate the amount of antigen contained in each sample, where lower C_t values indicate a higher concentration of antigen.

II. REAGENTS

1. NANOG Microplate (Item A)**: 96 well PCR plate coated with anti-Human NANOG.
2. Wash Buffer I Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials of recombinant Human NANOG.
4. Assay Diluent B (Item E): 15 ml of 5x concentrated buffer.
5. Detection Affinity Reagent for NANOG (Item F): 2 vials of a 4x concentrated solution of anti-Human NANOG affinity reagent.
6. IQELISA Detection Reagent (Item G): 1 mL of a 10x concentrated stock.
7. Primer Solution (Item I): 1.5 mL vial.
8. PCR Master Mix (Item J): 1.4 mL vial.
9. PCR Preparation buffer (Item K): 1mL vial of 10x concentrated buffer.
10. Final Wash Buffer (Item L): 10 mL vial of 10x concentrated buffer.

***The PCR plate used is a 0.2 mL, non-skirted 96-well plate (ThermoFisher, cat. no.: AB0600). Please ensure compatibility with your PCR machine prior to purchase. For additional information contact technical support (techsupport@raybiotech.com).*

III. STORAGE

May be stored for up to 6 months at 2°to 8°C from the date of shipment. Standard (recombinant protein) should be stored at -20°C or -80°C (recommended at -80°C) after reconstitution. Opened PCR plate or reagents may be stored for up to 1 month at 2° to 8°C. Note: the kit can be used within one year if the whole kit is stored at -20°C. Avoid repeated freeze-thaw cycles.

IV. ADDITIONAL MATERIALS REQUIRED

1. Real-time PCR instrument, Bio-Rad recommended
2. Precision pipettes to deliver 2 µl to 1 mL volumes.
3. Adjustable 1-25 mL pipettes for reagent preparation.
4. 100 mL and 1 L graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. Log-log graph paper or computer and software for data analysis.
8. Tubes to prepare standard or sample dilutions.
9. Heating block or water bath capable of 80°C

V. REAGENT PREPARATION

1. Bring wash buffer, samples, assay diluents, and PCR plate to room temperature (18 - 25°C) before use. PCR master mix and Primer solution should be kept at 4°C at all times.

2. Sample dilution: If your samples need to be diluted, 1x Assay Diluent B should be used for dilution of serum/plasma samples.

   Suggested dilution for normal serum/plasma: 2 fold*.

   *Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

3. Assay Diluent B should be diluted 5-fold with deionized water.

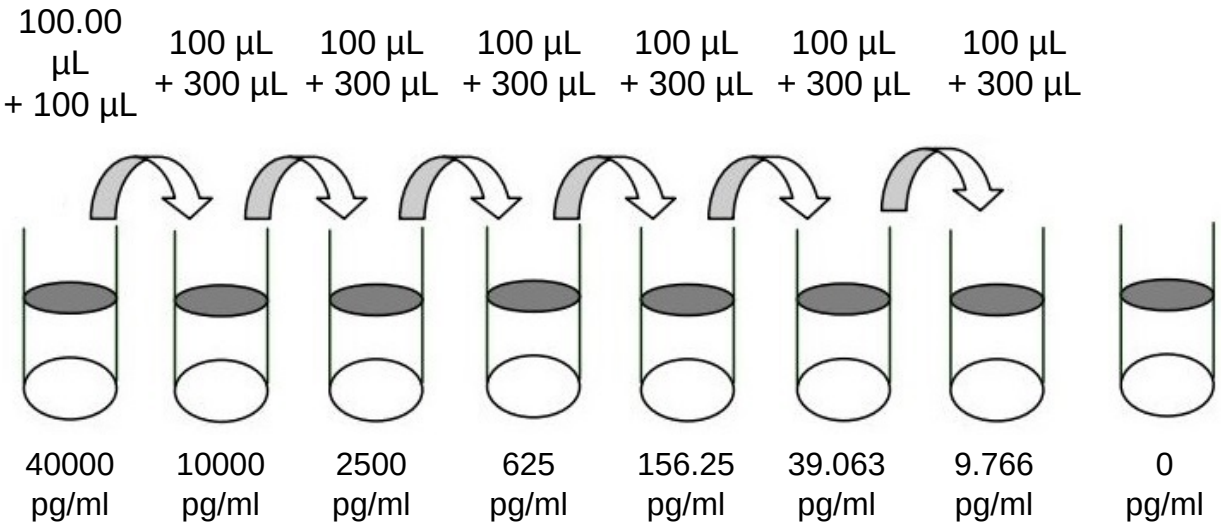
4. Briefly spin the Detection Antibody vial before use. Add 25 µL of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). This concentrate should be diluted 80-fold with 1x Assay Diluent B and used in step 4 of the Assay Procedure.

5. PCR preparation buffer should be transferred to a 15 mL tube and diluted with 9 mL of deionized or distilled water before use.

6. Final Wash Buffer should be transferred to a 15 mL tube and diluted with 9 mL of deionized or distilled water for every 1 mL of 10x concentrate used before use.

7. Preparation of standard: Preparation of standard: Briefly spin a vial of Standards. Add 400 µl 1X Assay Diluent B into Standards vial to prepare a 80 ng/ml standard solution. Dissolve the powder thoroughly by a gentle mix. Transfer 100 µl of the 100ng/ml stock solution into a new tube and mix with 100 µl 1x Assay Diluent B to generate a 40,000

pg/ml standard. Pipette 300 µl 1X Assay Diluent B into each tube. Use the 40,000 pg/ml standard solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1X Assay Diluent B serves as the zero standard (0 ng/ml).



8. If the Wash Buffer Concentrate (20x) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1x Wash Buffer.

9. Prepare the IQELISA detection reagent by calculating how much will be needed. This may be accomplished by multiplying the number of wells to be assayed by the volume you plan to use per well. Once the volume of IQELISA detection reagent is known, prepare the reagent by diluting it 1:10 with deionized water and mixing thoroughly.

VI. ASSAY PROCEDURE

Optional Visual Aid: IQELISA [Good Laboratory Practice Guide]

1. Bring all reagents and samples to room temperature (18 - 25°C) before use. It is recommended that all standards and samples be run in triplicate. Partial plate runs may be accomplished by cutting the PCR plate into the desired number of strips using a pair of sturdy scissors, wire cutters, or shears. The remainder may be saved and used for a later date. If this is done, the PCR Plate Film should also be cut to a suitable size.

2. Add 10-25 µL of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Volumes should be consistent between all wells, samples, and

standards. As little as 10 µL can be used if sample volume is limited, however this increases the chance of technical error. Ensure there are no bubbles present at the bottom of the wells. Dislodge any bubbles with gentle tapping or with a pipette tip being careful not to contact the sides or bottom of the well. Cover well and incubate for 1.5 - 2.5 hours at room temperature.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (100 µL) using a multi-channel Pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

4. Add 25 µL of prepared Detection Antibody (Reagent Preparation step 4) to each well. Incubate for 1 hour at room temperature with gentle shaking.

5. Discard the solution. Repeat the wash as in step 3.

6. Add 50 µL of prepared IQELISA detection reagent and incubate 1 hour with rocking (Reagent Preparation step 9)

7. Discard the solution. Repeat the wash as in step 3, for a total of 6 washes.

8. Add 75 µL of Final wash buffer to each well and incubate for 4 minutes with rocking. Remove the solution from each well and blot against paper towels.

9. Add 75 µL of 1x PCR preparation buffer to each well and incubate for 10 seconds before removing the buffer. Blot the plate after the buffer is removed to ensure complete removal of the buffer.

10. Add 10 µL of the Primer solution to each well of the plate. At this stage the plate can be covered and stored at -20°C for use the next day if needed.

11. Add 10 µL of PCR Master Mix to each well and pipette thoroughly to mix the well (at least 3x up and down).

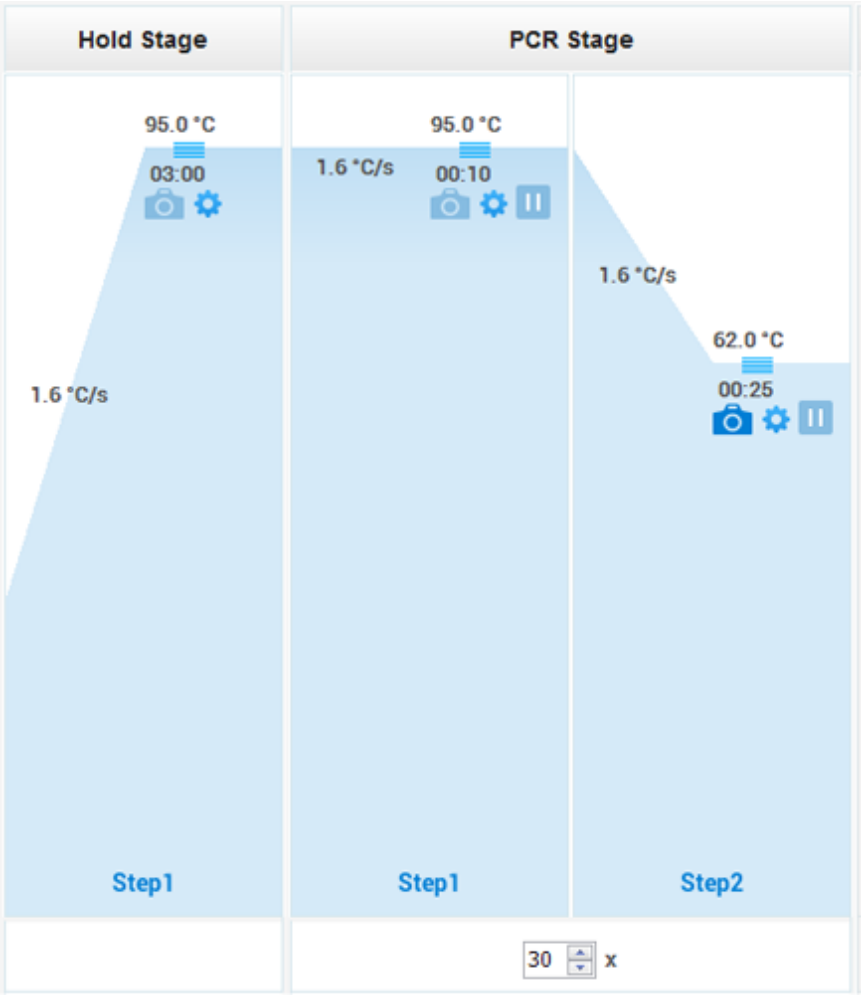
12. Cover the plate with the supplied PCR Plate Film, taking care to insure the film is completely and even pressed onto the plate, creating an air tight seal around each well of the plate.

    Optional Visual Aid: Sealing the plate [qPCR]

13. Place the plate into a real-time PCR instrument using a FITC compatible wave length for detection with the following settings for cycling

    1. 2 minute activation at 95°C
    2. 15 seconds 95°C denaturation
    3. 25 seconds 60°C annealing/extension
    4. Repeat steps 2 and 3 34x*

    *Optional: Include a melt curve to view potential plate contamination that can cause high background and lower the sensitivity. This can be seen in the visual aid on YouTube.



VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.
2. Add 25 µL standard or sample to each well. Incubate 1.5 - 2.5 hours at room temperature.
3. Add 25 µL Detection Antibody to each well. Incubate 1 hour at room temperature.
4. Add 50 µL of IQELISA Detection Reagent to each well. Incubate 1 hour
5. Add 10 µL Primer solution and 10 µL of PCR master mix to each well
6. Run real-time PCR

VIII. CALCULATION OF RESULTS

The primary data output of the IQELISA kit is C_t values. These values represent the number of cycles required for a sample to pass a fluorescence threshold. As the DNA is amplified additional fluorescent signal is produced, with each cycle resulting in an approximate doubling of the DNA. Therefore, higher levels of DNA (directly related to the amount of antigen in the sample) result in lower C_t values.

Calculate the mean C_t for each set of triplicate standards, controls and samples. Subtract the C_t value of each sample from the control to obtain the difference between the control and sample (Delta C_t). Plot the values of the standards on a graph using a log scale for concentration on the x axis. This graph is the quickest way to visualize results, although not the most accurate. If this method is used the concentration of unknown samples can be estimated using a logarithmic line of best fit.

The line of best fit will have an equation $y = m\ln(x)+b$, where y is the Delta C_t value and x is the concentration. It may be helpful to use 5 significant figures for m and b to minimize rounding errors. To calculate the concentration of unknown sample this can be entered into Excel in the following format

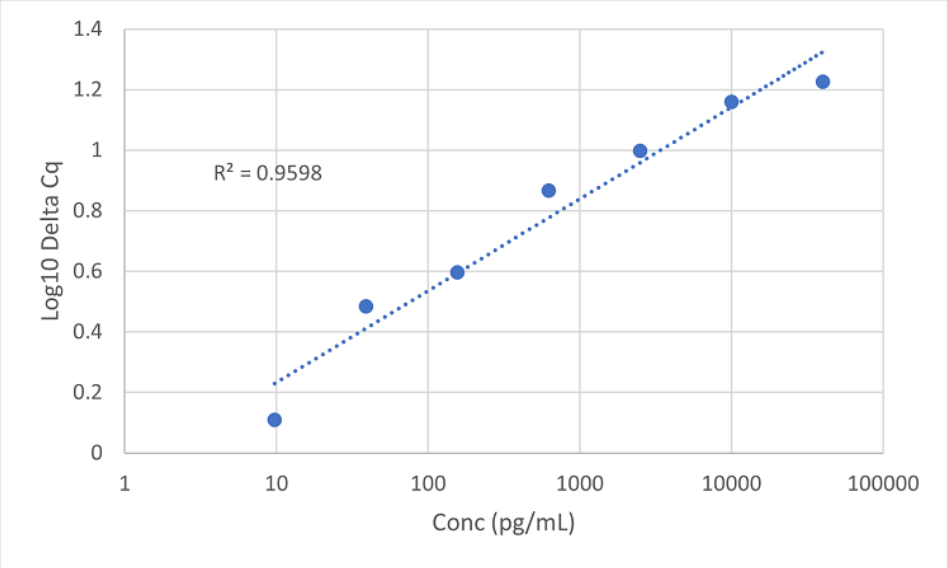
=EXP((y-b)/m))

Where y is the Delta C_t obtained during the assay, and b and m are obtained from the line of best fit.

Alternatively, for a more accurate representation linear regression may be used. Both the Delta C_t and Concentration can be transformed using a log base of 10, plotted on a graph as described above, along with a line of best fit (using a linear model). The equation of this line may be used to calculate the antigen concentration of unknown samples. This is the method used for the analysis spreadsheet for IQELISA available online.

A. TYPICAL DATA

These data are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY and RECOVERY

The minimum quantifiable dose of NANOG is typically 9.76 pg/ml, however levels as lower than 9.76 pg/ml may be detected outside of the quantification range.

Serum spike tests show recovery is 109% with a range from 113% to 104%.

Intraplate CV is below 10% for all samples and Interplate CV is below 15%.

X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Poor standard curve	• Inaccurate pipetting • Improper standard dilution	• Check pipettes • Briefly centrifuge standards and dissolve the powder thoroughly by gently mixing
Low signal	• Too brief incubation times • Inadequate reagent volumes or improper dilution	• Ensure sufficient incubation time. Assay procedure step 2 may be done overnight • Check pipettes and ensure correct preparation
Large CV	• Uneven pipetting • Bubbles present in wells	• Check pipettes • Lightly tap or use pipette tip to dislodge from bottom of well
High background	• Plate is insufficiently washed • Contaminated wash buffer • Improper Tm	• Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer • Check run parameters and calibrate instrument
Low sensitivity	• Improper storage of the IQELISA kit • Improper Tm	• Store your standard at <-20°C after reconstitution, others at 4°C. • Check run parameters and calibrate instrument

This product is for research use only.