



iLite™ antialpha
For the Detection of Neutralizing Antibodies to human Interferon Alpha (IFN α)

Registered Office:
 Biomonitor Limited
 Unit 1 Business Innovation Centre
 NUI Galway, Ireland.
 Tel: +353 91 862664
 Fax: +353 91 862665
 Email: info@biomon.ie
 Web: www.biomon.dk

INTENDED USE:

The *iLite™ antialpha* assay is intended for the semi quantitative determination of neutralizing antibodies (NAbs) to human Interferon Alpha (IFN α) in serum, using luciferase generated bioluminescence. This kit is for Research Use Only.

BACKGROUND:

Interferon α has been widely used to treat chronic viral hepatitis and a wide variety of malignant diseases, including hairy cell leukaemia, basal cell carcinoma, chronic myeloid leukaemia and cutaneous T-cell lymphoma. Several different recombinant preparations of IFN α are available commercially, the most commonly used formulations include IFN α 2a and IFN α 2b. A number of studies have shown that development of anti-IFN α antibodies is correlated with a loss of IFN α treatment efficacy. Existing assays utilise either the anti-viral effect of IFN α (the cytopathic effect assay) or measure the IFN α induced gene products (Myxovirus resistance protein or mRNA). These assays are time consuming, expensive and show variability between laboratories.

The *iLite™ antialpha* assay is a simple and easy test format which provides researchers with a semi-quantitative estimate of NAbs to IFN α in serum.

ASSAY PRINCIPLE:

The test procedure involves the use of division-arrested interferon-sensitive cells in a bioassay capable of measuring IFN α bioactivity. NAbs interfere with the binding of exogenous IFN α and thereby reduce its bioactivity. A semi quantitative estimate of the amount of NAbs present is determined by titering the sample to a dilution that provides a reduction in activity from 50IU IFN α /ml to 5IU IFN α /ml ie 10 Laboratory Units (LU) to 1LU (in this assay 1LU is equivalent to 5IU IFN α /ml).

Kit Components	Quantity
<i>iLite™</i> Cells	1 vial
<i>iLite™</i> IFN Stock (950 IU IFN α 2b /ml)	1 vial
<i>iLite™</i> Diluent	1 vial
Bright-Glo™ Luciferase Assay Buffer	1 vial
Bright-Glo™ Luciferase Assay Substrate Contains dithiothreitol (DTT) [HARMFUL]	1 Vial
White-Walled Micro well Plate	1
Product Insert	1

SAMPLE STORAGE: Serum samples may be stored at 4°C for 1 week, or frozen if assay is not initiated within 1 week of sample collection. Repeated freeze thawing of samples should be avoided.

ASSAY PROCEDURE: It is recommended to initially screen the serum samples for presence of NAbs (example layout Table 3A), then progress with the titre of positive samples i.e. samples with ≤ 1 LU IFN α remaining, following a 10LU spike (example plate layout Table 3B).

The recommended method for screening involves testing each sample in parallel; one non spiked and the other spiked with the 'IFN Spike'. All samples should be tested in duplicate at a 1:2 dilution (final dilution in assay, 1:15 incorporating the 7.5 assay dilution).

1. Thawing Kit Reagents

Remove diluent and thaw at room temperature or 37°C. If the micro well plate has been stored at -80°C, it should be allowed to equilibrate at room temperature at this time. When the diluent has equilibrated, the interferon standard is thawed at room temperature. Allow approximately 25 min. for interferon standard to thaw.

2. Sample Preparation

Prior to the assay set up, the serum samples should be thawed at room temperature, mixed and heat inactivated by heating at 56°C for 30min.

Following heat inactivation, samples should be mixed and diluted appropriately immediately prior to the assay.

Samples for screening should be diluted 1:2. Samples for titering should be diluted according to the desired range -see Table 3C below for an example of possible dilutions . All dilutions should be carried out using the *iLite™* diluent.

3. IFN Standards Preparation for Standard Curve

a. Construct a standard curve at 0, 1.25, 2.5, 5, 10, 20, 40 & 80 IU IFN/ml, using the IFN stock and diluent.

b. Mix the thawed IFN stock with a pipette prior to use. Label sterile polypropylene tubes (not supplied) and serially dilute the IFN stock with diluent as directed in Table 1 in these tubes.

Ensure adequate mixing of each dilution. Pipette tips should be changed between each dilution.

4. IFN Spike Preparation

c. Prepare the 'IFN Spike' from the IFN stock (Table 2). This 'spike' (80 μ l) will be used to spike the serum samples.

5. Addition of Reagents to Plate

A plan of the intended location of each sample/standard on the microwell plate should be generated in advance to assist with this step (see example in Table 3a). Add the various standards and samples to the wells as follows;

- i. 100 μ l of interferon standards (0-80 IU IFN/ml) in duplicate to generate a standard curve, taking care not to cross contaminate wells (a multi-channel micro-pipette may be used).
- ii. Add 80 μ l of the 'IFN Spike' to the wells which will contain spiked samples.
- iii. Add 80 μ l of diluent to the wells which will contain un-spiked samples.
- iv. Add & gently mix 20 μ l of diluted (minimum 1:2) unknown samples in duplicate to the appropriate wells, taking care not to cross contaminate wells.
- v. Replace the lid on the micro well plate, mix the contents in the wells by gently swirling the plate a few times and incubate at 37°C in 5% CO₂ for 30 minutes.
- vi. 3-4 minutes prior to completion of the incubation rapidly thaw the vial of cells using at 37°C water bath. Thawing should be accomplished within 3-4 minutes.
- vii. Invert the vial a minimum of 10 times to ensure a uniform cell suspension.
- viii. Transfer 3ml of cells into a sterile tube and add 3ml of diluent to the cells. Invert the diluted cells a minimum of 10 times to ensure a uniform cell suspension and transfer the diluted cells to a sterile multichannel reservoir.
- ix. Gently add 50 μ l of cells to each well. Ensure tips are changed between each addition to avoid cross contamination.
- x. Replace the lid on the micro well plate, mix the contents in the wells by gently swirling the plate a few times and incubate at 37°C in 5% CO₂ for 6 hrs.
- xi. Approx. 30 min prior to completion of incubation, prepare the Bright Glo™ substrate (10ml) by thawing the Bright-Glo™ Luciferase Assay Buffer and Substrate at room temperature (RT) e.g. placing vial in RT water (Do NOT use a 37°C water bath). Then add the entire contents of the Bright-Glo™ Luciferase Assay Buffer to the Substrate, replace cap and mix gently by inversion.
- xii. Add 50 μ l Bright-Glo™ substrate system per well using a multichannel micro-pipette, taking care not to cross contaminate wells.

6. Reading Plate Luminescence

Determine the luminescence emitted by the samples using a micro-plate luminometer (e.g. Victor™ Light luminometer, PerkinElmer LAS, Seer Green, Bucks, UK) 2 minutes after addition of substrate.

ANALYSIS OF RESULTS:

1. Best Fit Curve Generation

- A. Generate the best fit curve using at least 3 points on the graph with an R² value of ≥ 0.95 (the following example uses Microsoft Excel however any other appropriate software will suffice)
- B. Open a new worksheet and construct a data table.

- C. Calculate the mean RLU (in this example n=2) for each of the data points to be used in the generation of the standard curve (e.g. 1.25, 2.5, 5, 10, 20, 40 & 80 IU/ml for the standard IFN curve).
- D. Create a XY scatter plot of the data, briefly:
 - a. Highlight the data and click on the chart wizard icon or the menu bar, use "Insert" and then "Chart". When the chart wizard dialog box appears, chose XY (Scatter), and the subtype with data points connected with smooth points. Then select "Next >" twice, enter the table information; in this case *iLite™ antialpha* standard curve for the title, IFN α IU/ml on X axis and RLU on Y axis. Click "Finish".
 - b. Bring cursor over the X-axis values and double click, which will open the "Format Axis" box.
 - i. Click on the "Scale" Tab and make the following adjustments to the X-axis settings;
 - ii. Enter 1 into the "minimum" box,
 - iii. Enter 1 into the "Value (y) axis crosses at" box,
 - iv. Click on the "Logarithmic scale" box. Then click "OK".
- E. Now right-click on one of the points of the plot. Choose in the menu bar "Chart" then "Add Trendline". When the 'Add Trendline' dialog box appears, choose logarithmic on the Type tab. Click the 'Options' tab, be sure to check/click the "display equation on chart", and the "R-squared Value". Click "OK" and Excel should now show a graph of the best-fit straight line through the data points (the regression line) and the logarithmic regression equation and R² value should be displayed.
- F. Select the best fit three points for the curve; in this example (standard curve-graph 1 below) four points (2.5-20 IU/ml) is a sufficient fit. When R² is close to 1, the regression curve is a good fit for the data.

Figure1: Example of Standard Curve for *iLite™ antialpha*

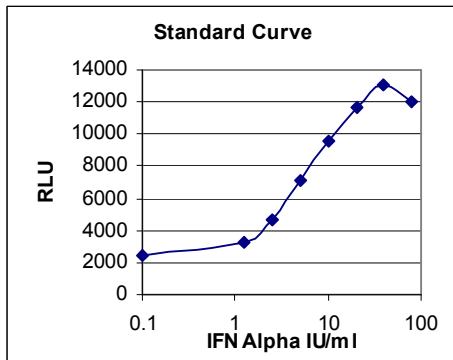
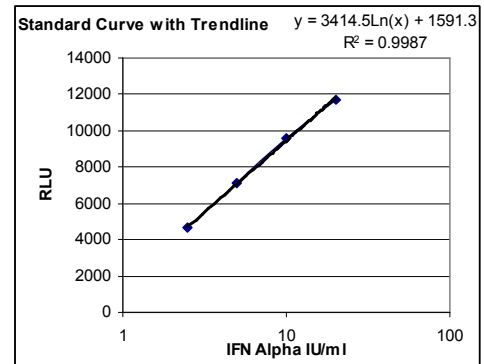


Figure 2: Example of Standard Curve for *iLite™ antialpha* with Trendline



2. Calculation of IFN Activity of the unknown samples
 - a. The logarithmic regression equation can now be used to equate the mean RLU values of "unknown samples" see example in Figure 2 - IFN activity (IU/ml) or in Figure 4 - Antibody titres.
 - b. The regression equation for the standard curve in Figure 2 is $y = 3414.5(x) + 1591.3$
 - c. Where $y = \text{RLU}$ for unknown sample, x can be calculated using the following formula; $x = \text{EXP}((y - c)/m)$.
Using this equation an unknown sample that yields a mean RLU of 9606 is equated to having an IFN activity of 10.5 IU/ml i.e. $\text{EXP}((9606 - 1591.3)/ 3414.5) = 10.5$
3. Determine the Laboratory Units Remaining of unknown sample.
 - a. Divide the IFN activity for unknown sample by the spike concentration at 1LU ie 5IU/ml.
In example above $10.5/5 = 2.1 \text{ LU}$
4. Determination of results of Samples which were Screened
If the RLU of the spiked sample $\leq 5\text{IU/ml}$ (1LU) = NAb's detected, this sample may be further processed in another assay to determine the antibody titre.
If the RLU of the spiked sample $> 5\text{IU/ml}$ (1LU) = NAb's not detected.
5. Determine the Antibody Titre of a sample
Construct the antibody titre curve (Figures 3&4) & construct a trendline to the antibody curve following procedures as above.
 - a. Calculate the RLU that corresponds to 5IU/ml (1LU) using the following equation from the standard curve
 $y = m * \ln(x) + c$
 $y = (3414.5 * \ln(5)) + 1591.3 = 7087$
 - b. Calculate the antibody titres of sample corresponding to the RLU at 5IU/ml (1LU) from the antibody dose response trendline (Fig. 4) using the equation as above
 $x = \text{EXP}((y - c)/m)$
In example $x = \text{EXP}((7087 - (-12358))/6133.7) = 24$
Therefore the antibody titre in this example = 24

Figure 3: Example of Antibody Dose Response Curve

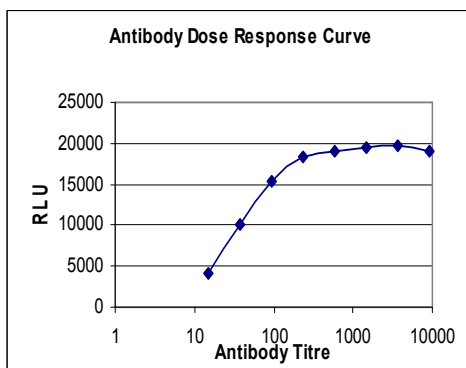


Figure 4: Example of Antibody Dose Response Curve with Trendline

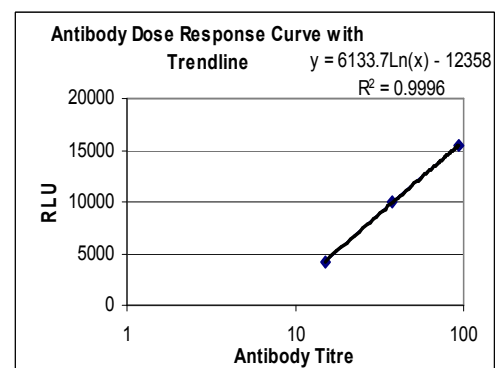


Table 1: IFN stock dilution recommended for construction of standard curve using IFN α Standard.

Quantities are sufficient for 2 replicates of each activity

ID	Volume of Diluent (μ l)	Volume of IFN (μ l)	Dilution Factor	Activity/ml of Standard	Final Activity/ml of Standard in assay
A	519	75 Stock	7.92	120	80
B	300	300 A	2	60	40
C	300	300 B	2	30	20
D	300	300 C	2	15	10
E	300	300 D	2	7.5	5
F	300	300 E	2	3.7	2.5
G	300	300 F	2	1.9	1.25
H	300	N/A	N/A	0	0

Table 2: IFN dilution recommended for preparation of the 'Spike IFN' (50IU/ml) for quantities sufficient for 80 wells.

Volume of Diluent (μ l)	Volume of IFN Stock (μ l)	Dilution Factor	Activity/ml of 'Spike'	Final Activity/ml of 'Spike' in assay
6393	700	10.133	93.75	50.00

Table 3A: Example plate layout for Screening of Samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	80	80	NS-S1	S-S1	NS-S5	S-S5	NS-S9	S-S9	NS-S13	S-S13	NS-S17	S-S17
B	40	40	NS-S1	S-S1	NS-S5	S-S5	NS-S9	S-S9	NS-S13	S-S13	NS-S17	S-S17
C	20	20	NS-S2	S-S2	NS-S6	S-S6	NS-S10	S-S10	NS-S14	S-S14	NS-S18	S-S18
D	10	10	NS-S2	S-S2	NS-S6	S-S6	NS-S10	S-S10	NS-S14	S-S14	NS-S18	S-S18
E	5	5	NS-S3	S-S3	NS-S7	S-S7	NS-S11	S-S11	NS-S15	S-S15	NS-S19	S-S19
F	2.5	2.5	NS-S3	S-S3	NS-S7	S-S7	NS-S11	S-S11	NS-S15	S-S15	NS-S19	S-S19
G	1.25	1.25	NS-S4	S-S4	NS-S8	S-S8	NS-S12	S-S12	NS-S16	S-S16	NS-S20	S-S20
H	0	0	NS-S4	S-S4	NS-S8	S-S8	NS-S12	S-S12	NS-S16	S-S16	NS-S20	S-S20

Table 3B: Example plate layout for Determination of Antibody Titre

	1	2	3	4	5	6	7	8	9	10	11	12
A	80	80	S1-15	S1-15	S2-15	S2-15	S3-15	S3-15	S4-15	S4-15	S5-15	S5-15
B	40	40	S1-38	S1-38	S2-38	S2-38	S3-38	S3-38	S4-38	S4-38	S5-38	S5-38
C	20	20	S1-94	S1-94	S2-94	S2-94	S3-94	S3-94	S4-94	S4-94	S5-94	S5-94
D	10	10	S1-234	S1-234	S2-234	S2-234	S3-234	S3-234	S4-234	S4-234	S5-234	S5-234
E	5	5	S1-586	S1-586	S2-586	S2-586	S3-586	S3-586	S4-586	S4-586	S5-586	S5-586
F	2.5	2.5	S1-1465	S1-1465	S2-1465	S2-1465	S3-1465	S3-1465	S4-1465	S4-1465	S5-1465	S5-1465
G	1.25	1.25	S1-3662	S1-3662	S2-3662	S2-3662	S3-3662	S3-3662	S4-3662	S4-3662	S5-3662	S5-3662
H	0	0	S1-9155	S1-9155	S2-9155	S2-9155	S3-9155	S3-9155	S4-9155	S4-9155	S5-9155	S5-9155

Table 3C: Dilution of Sample for Determination of Antibody Titre

Sample	Final Sample Dilution	Volume (μ l)	Diluent	Dilution Factor
A	15	42 neat	42	2
B	38	34 A	51	2.5
C	94	34 B	51	2.5
D	234	34 C	51	2.5
E	586	34 D	51	2.5
F	1465	34 E	51	2.5
G	3662	34 F	51	2.5
H	9155	34 G	51	2.5

Legend:

NS-S1 Non Spiked Sample 1
 S-S1 Spiked Sample 1
 S1-234: Sample 1 @ 1:234 final dilution
 Final dilution: Dilution in the well (inc. 7.5 assay dilution).

QC CRITERIA

If the criteria below are not met, the assay is considered invalid and must be repeated.

- The correlation coefficient for the trend line (R^2) of the standard curve must be ≥ 0.95 using at least 3 points on the standard curve.
- Replicate RLU values for individual standard curve points must have a CV of $\leq 15\%$

e.g.

Sample Activity	RLU	Mean RLU	%CV
5 IU/ml	8792	8520.5	3.19
	8249		

LIMITATIONS OF USE

- Samples with high endogenous IFN α , or IFN β (e.g. as a result of viral infection) should not be used.
- Samples with a high lipid, bilirubin or hemoglobin content (visible by eye) should be excluded as these can interfere with the bioluminescence determinations.
- Samples from patients with autoimmune disease eg. Lupus SLE may interfere with the assay due to the presence of a soluble form of IFN AR2 and should not be used.
- The *iLite™ antialpha* kit contains IFN α . 2b as the drug reference standard.

PERFORMANCE CHARACTERISTICS

Measuring Range: The measuring range of this assay starts with a sample titre of 15 and samples may be diluted to obtain the appropriate titre required for antibody titre determination, therefore, there is no maximum range of detection.

Limit of detection: The minimum limit of detection is a sample titre of 15 (see measuring range), and there is no maximum limit of detection.

Interference: Samples with a high lipid, bilirubin or hemoglobin content (visible by eye) and samples with high endogenous Beta or Alpha interferon can interfere with the bioluminescence determinations and must not be used.

Warranty: The performance data presented here was obtained using the procedure described. Any change or modification of the procedure, not recommended by Biomonitor Ltd, may affect the results, in which case Biomonitor Ltd disclaims all warranties, expressed, implied or statutory, including implied merchantability and fitness for use. In the case of such an event, Biomonitor Ltd shall not be liable for damages, direct or consequential.

Reproducibility

Table 4: Inter and Intra Assay Variation for *iLite™ antialpha*

	% CV
Inter Lot & Day	5.2
Intra Assay	7.4
Inter Operator	3.2

Clinical Data

Table 5: % Accuracy of the *iLite™ antialpha* when compared to CPE assay

	CPE-	CPE+
<i>iLite</i> -	19	0
<i>iLite</i> +	0	11
Accuracy = 100%		

Correlation between *iLite™ antialpha* and CPE (n=30): $r = 0.97$; $p < .00001$

PRECAUTIONS:

SAFETY

- *iLite™ antialpha* is for Research Use Only.
- *iLite™ antialpha* is intended for use by qualified laboratory staff only.
- The kit contains a stable transfected cell line of human origin and all materials should be treated as potentially infectious.
- In accordance with EU regulations (90/219/EEC), the transfected cell line (*iLite™* cells) is classified as a Class 1 Genetically Modified micro-organism (GMM), and should be handled and disposed of in a licensed contained-use facility in accordance with these regulations (biohazardous waste should be inactivated prior to disposal by autoclaving or using bleach). When used in accordance with the manufacturer's instructions the requirements of EC Directive 90/219/EEC of 23 April 1990 on the contained use of genetically modified micro organisms are deemed to have been met.
- Wear protective clothing, disposable latex gloves and eye protection when handling specimens and performing the assay. Wash hands thoroughly when finished. If contact occurs rinse off immediately with water and seek medical advice.
- Residues of chemicals, preparations and kit components are generally considered as biohazardous waste. All such materials should be disposed of in accordance with established safety procedures.
- The Luciferase Substrate contains dithiothreitol (DTT) and is therefore classified as HARMFUL (R22-36/37/38 HARMFUL IF SWALLOWED. IRRITATING TO EYES, RESPIRATORY SYSTEM AND SKIN). The reconstituted reagent is not known to present any hazard as the concentration of DTT is less than 1%. However, we recommend the use of laboratory protective clothing as described above when working with these reagents.
- Dispose of all clinical specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed as though potentially infectious.
- Do not pipette materials by mouth and never eat or drink at the laboratory workbench.

PROCEDURAL

- To ensure kit performance the protocol should be reviewed in its entirety prior to use.
- The Kit is for single use only. Kit components cannot be used if thawed and refrozen.
- Aseptic technique should be followed during assay set-up.
- Do not use kit or individual reagents past their expiry date.
- Do not mix or substitute reagents from different kit lot numbers.
- Deviation from the protocol provided may cause erroneous results.
- Performing the assay outside the time, temperature and volume ranges provided may produce invalid results. Assays not falling within the established time, temperature and volume ranges must be repeated.
- Care must be taken not to contaminate components and always use fresh pipette for each sample and component.
- All equipment should be calibrated prior to use.
- Frozen components should be thawed per reagent preparation instructions (see below), and mixed appropriately prior to use to ensure homogeneity.
- The packaging integrity of the kit should be confirmed prior to use to confirm absence of leaks.

RECEIPT, STORAGE AND STABILITY

- Upon receipt confirm that adequate dry-ice is present and the kit is frozen. Immediately transfer to **minus 80°C** storage.
- All kit reagents are stored at -80°C and are stable as supplied until the expiry date shown.
- Cells should be used within 15 minutes of thawing.
- Standards should be used within 30 minutes of thawing.
- Diluent should be used on the day of thawing.
- Luciferase substrate reagent should be used immediately after reconstitution (or within 4 weeks if frozen at -80°C immediately after reconstitution)

ADDITIONAL MATERIALS REQUIRED

- Micropipettes covering the following ranges (5µl-1000µl) and multichannel pipettes covering the following ranges (20µl-100µl).
- Micro-plate luminometer & appropriate software (e.g. Victor™ Light luminometer, PerkinElmer LAS, Seer Green, Bucks, UK). Ensure software is correctly installed in the luminometer and operators follow manufactures instructions and are fully trained in its use.
- Liquid Reservoir (sterile).
- Incubator 37°C, 5% CO₂.
- Polypropylene tubes (1.0ml sterile)