

***iLite*TManti-Human IFN beta 1b**

For the Detection of Neutralizing Antibodies to Interferon beta 1b (IFNβ 1b)

INTENDED USE:

The *iLite*TM anti-human IFN beta 1b assay is intended for the semi-quantitative determination of neutralizing antibodies (NABs) to human Interferon beta 1b (IFNβ 1b), using luciferase generated bioluminescence.

BACKGROUND:

IFNβ, is well established as first line therapy in relapsing/remitting multiple sclerosis. The occurrence of NABs and binding antibodies (BAbs) to IFNβ 1b has been widely reported. Subjects with NABs have shown reduced response to treatment with IFNβ 1b, having higher relapse rates, increased MRI activity and a higher risk of disease progression. The frequencies and titres of NABs vary depending on the preparation used, dose and frequency of administration and also the assay used to quantify them. Existing assays utilise either the anti-viral effect of IFNβ (the cytopathic effect assay) or measure the IFNβ-induced gene products (myxovirus resistance protein A). These assays are time consuming, expensive and show variability between laboratories.

The *iLite*TM anti-human IFN beta 1b assay is a Reporter Gene Bioassay which is a simple and easy to use for detecting neutralization of IFN-beta 1b activity.

ASSAY PRINCIPLE:

The test procedure involves the use of division-arrested IFNβ-sensitive cells in a bioassay capable of measuring IFNβ bioactivity. NABs interfere with the binding of exogenous IFNβ to cell receptors and thereby reducing its activity. A semi-quantitative estimate of the amount of NABs present in serum is determined by titrating the sample to a dilution that provides a Tenfold Reduction Unit (TRU/ml¹) in activity (i.e. from 10 Laboratory Units/ml to 1 LU/ml).

ASSAY PROCEDURE:

It is recommended to initially screen the serum samples for presence/absence of NABs using the *iLite* kit and then using a second *iLite* kit, determine the antibody titre of the samples containing NABs.

Screening of Samples: Tests for residual IFN present in the serum sample and serum-induced toxicity (samples spiked with Diluent) as well as the presence/absence of NABs in the serum sample (samples spiked with IFN) (example layout Table 4)

Titering of Samples: Samples which tested positive for NABs in the screening assay are titered to determine the antibody titre of serum samples (example layout Table 5).

1. Sample Preparation

Prior to the assay setup, the serum samples should be equilibrated to Room Temperature (RT), mixed and heat-inactivated by heating at 56°C for 30 min.

2. Thawing Kit Reagents and Samples

Remove the micro well plate, *iLite*TM diluent, *iLite*TM IFN Stock and *iLite*TM Positive Control, Negative Control, and Serum Samples and allow to equilibrate to RT. Allow approx 20 min for all components except for diluent (approx 1hr unless thawed at 37°C). **DO NOT THAW THE CELLS AT THIS STAGE.**

3. Preparation of Standard Curve & 'IFN Spike'

Standard Curve: Construct a standard curve with the following activities; 0, 0.33, 0.82, 2.05, 5.12, 12.8, 32, and 80 IU IFN-beta 1b/ml, using *iLite*TM IFN Stock and *iLite*TM diluent. Mix the thawed *iLite*TM IFN Stock with a pipette prior to use. Serially dilute the IFN Stock with diluent in sterile polypropylene tubes, not supplied). (Table 1)

IFN Spike: Prepare the 'IFN Spike' using *iLite*TM IFN Stock and *iLite*TM diluent (Example in Table 2)

Table 1: IFN stock dilution recommended for construction of standard curve using IFNβ 1b Standard. Quantities are sufficient for 2 reps of each activity

| Sample | Volume of Diluent (µl) | Volume of IFN (µl) | Dilution Factor | Activity of standard (IU/ml) | Final Activity of standard in assay (IU/ml) |
|--------|------------------------|--------------------|-----------------|------------------------------|---|
| A | 450 | 65 Stock | 7.9167 | 120 | 80.00 |
| B | 300 | 200 A | 2.5 | 48 | 32.00 |
| C | 300 | 200 B | 2.5 | 19 | 12.80 |
| D | 300 | 200 C | 2.5 | 8 | 5.12 |
| E | 300 | 200 D | 2.5 | 3.1 | 2.05 |
| F | 300 | 200 E | 2.5 | 1.2 | 0.82 |
| H | 300 | 200 F | 2.5 | 0.5 | 0.33 |
| I | 300 | N/A N/A | N/A | 0.00 | 0.00 |

Table 2: IFN dilution recommended for preparation of the 'Spike IFN' Quantities are sufficient for 80 wells.

| Sample | Volume of Diluent (µl) | Volume of IFN(µl) | Dilution Factor | Activity of 'Spike' (IU/ml) | Final Activity of 'Spike' in assay (IU/ml) |
|--------|------------------------|-------------------|-----------------|-----------------------------|--|
| A | 3908 | 400 | Stock | 88.21 | 50 |
| B | 3500 | 3500 | A | 44.10 | 25 |

Note: Calculations are for preparation of a 'Spike' final activity of 25 IU IFN/ml. Refer to Certificate of Analysis for the activity of the 'IFN spike' to be prepared as the activity may vary depending on the kit lot and adjust the calculations accordingly.

4. Dilution of Samples

Samples should be mixed and diluted appropriately using the *iLite*TM diluent. All samples should be tested in duplicate

Samples for Screening: Diluted 1 in 2 (e.g. 45 µl sample + 45 µl *iLite*TM diluent

Final dilution in assay is 1 in 20, incorporating the 1 in 10 assay dilution.

Samples for Titering: Recommended Dilutions of samples are from 1 in 20 to 1 in 540 (Table 3). Samples can be diluted further as required or samples with a TRU > 540 be re-titered starting at a higher dilution of sample eq 400-10,800

Table 3: Dilution of Sample for Determination of TRU/ml

| Sample | Volume of Diluent (µl) | Volume of Sample (µl) | Dilution Factor | Final Sample Dilution |
|--------|------------------------|-----------------------|-----------------|-----------------------|
| A | 45 | 45 neat | 2 | 20 |
| B | 60 | 30 A | 3 | 60 |
| C | 60 | 30 B | 3 | 180 |
| D | 60 | 30 C | 3 | 540 |

5. Addition of Standards and Samples 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 4 & 5).

Add the various standards and samples to the wells as follows:

Ensure tips are changed between each dilution/addition to avoid cross-contamination. Ensure adequate mixing of each dilution.

- Add 100 µl of IFNβ1b standard (0-80 IU /ml) in duplicate to generate a standard curve.
- Add 85 µl of the 'IFN Spike' to the wells as per plate layout.
- Add 85 µl of diluent to the wells as per plate layout – if testing for cell toxicity or residual IFN.
- Add 15 µl of diluted serum samples to the wells containing 'IFN Spike' or diluent as appropriate.
- Replace the lid on the microwell plate, and incubate at 37°C in 5% CO₂ for 30 min. mix the contents in the wells by gently swirling the plate a few times.
- Thaw the vial of cells using a 37°C water bath 15 min prior to completion of the incubation (3-4 min with agitation) then leave the thawed cells sit in the 37°C waterbath for a further 11-12 min; i.e. cells should be in 37°C water bath for 15 min). Invert the vial a minimum of 10 times to ensure a uniform cell suspension. Dilute the cells 1 in 4 : Add 2 ml of cells to 6 ml of diluent in a sterile tube. Invert approx. 10 times to ensure a uniform cell suspension then transfer into a sterile multichannel reservoir.
- Add 50 µl of diluted cells to each well.
- Replace the lid on the microwell plate, mix the contents in the wells by gently swirling the plate a few times and incubate at 37°C in 5% CO₂ for 18 hrs.
- Approx. 30 min prior to completion of incubation, prepare the Bright GloTM substrate by thawing the Bright-GloTM 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-GloTM Luciferase Assay Buffer to the Substrate, replace cap and mix gently by inversion.
- Add 50 µl Bright-GloTM substrate system per well using a multichannel micropipette, ensure contents of wells are mixed by pulling liquid up and down the pipette.
- 2 min after addition of substrate determine the luminescence using a microplate luminometer (e.g. VictorTM Light luminometer, PerkinElmer LAS, Seer Green, Bucks, UK)

Table 4: Example plate layout for Screening of Samples

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|------|------|------|------|------|------|-------|-------|-------|-------|-------|-------|
| A | 80 | 80 | PC | PC | NC | NC | S8-S | S8-S | S8-D | S8-D | S16-S | S16-S |
| B | 32 | 32 | S1-S | S1-S | S1-D | S1-D | S9-S | S9-S | S9-D | S9-D | S17-S | S17-S |
| C | 12.8 | 12.8 | S2-S | S2-S | S2-D | S2-D | S10-S | S10-S | S10-D | S10-D | S18-S | S18-S |
| D | 5.12 | 5.12 | S3-S | S3-S | S3-D | S3-D | S11-S | S11-S | S11-D | S11-D | S19-S | S19-S |
| E | 2.05 | 2.05 | S4-S | S4-S | S4-D | S4-D | S12-S | S12-S | S12-D | S12-D | S16-D | S16-D |
| F | 0.82 | 0.82 | S5-S | S5-S | S5-D | S5-D | S13-S | S13-S | S13-D | S13-D | S17-D | S17-D |
| G | 0.33 | 0.33 | S6-S | S6-S | S6-D | S6-D | S14-S | S14-S | S14-D | S14-D | S18-D | S18-D |
| H | 0 | 0 | S7-S | S7-S | S7-D | S7-D | S15-S | S15-S | S15-D | S15-D | S19-D | S19-D |

Table 5: Example plate layout for Determination of TRU/ml

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|------|------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| A | 80 | 80 | NC | NC | S2-20 | S2-20 | S4-20 | S4-20 | S6-20 | S6-20 | S8-20 | S8-20 |
| B | 32 | 32 | PC | PC | S2-60 | S2-60 | S4-60 | S4-60 | S6-60 | S6-60 | S8-60 | S8-60 |
| C | 12.8 | 12.8 | S1-20 | S1-20 | S2-180 | S2-180 | S4-180 | S4-180 | S6-180 | S6-180 | S8-180 | S8-180 |
| D | 5.12 | 5.12 | S1-60 | S1-60 | S2-540 | S2-540 | S4-540 | S4-540 | S6-540 | S6-540 | S8-540 | S8-540 |
| E | 2.05 | 2.05 | S1-180 | S1-180 | S3-20 | S3-20 | S5-20 | S5-20 | S7-20 | S7-20 | S9-20 | S9-20 |
| F | 0.82 | 0.82 | S1-540 | S1-540 | S3-60 | S3-60 | S5-60 | S5-60 | S7-60 | S7-60 | S9-60 | S9-60 |
| G | 0.33 | 0.33 | | | S3-180 | S3-180 | S5-180 | S5-180 | S7-180 | S7-180 | S9-180 | S9-180 |
| H | 0 | 0 | | | S3-540 | S3-540 | S5-540 | S5-540 | S7-540 | S7-540 | S9-540 | S9-540 |

Legend: PC = Positive Control, NC = Negative Control, S1-S = Sample #1 + Spike, S1-D = Sample #1 + Diluent, S1-20 = Sample #1 at a 1 in 20 final dilution + Spike
 Shading indicates well containing 'IFN Spike'

ANALYSIS OF RESULTS:

- Calculate the mean Relative Light Units (RLU) for each of the data point used in the generation of the standard curve and the antibody titre curve. Create an XY scatter plot of the data with IFN activity/antibody dilution on X axis [log scale] and RLU on y axis [linear scale] - the following example uses Microsoft Excel Add a trendline [Logarithmic] on the linear portion of the graphs and get the regression equations.

- Calculate the RLU at the Midpoint of the linear region of the standard curve which equals 1LU/ml

$$\text{Midpoint} = (Y1+Y2)/2$$

Y1 = RLU value of the lowest point on the trendline.
 Y2 = RLU value of the highest point on the trendline

- Calculate the IFN activity at the midpoint (1 LU/ml) from the Standard Curve

$$\text{IFN activity at Midpoint} = X = \text{EXP}((Y-C)/M) \quad Y = \text{RLU value}$$

- If samples have been screened calculate the LU remaining in the sample. Neutralizing antibodies in sample if LU remaining = ≤ 1 LU/ml.

$$\text{LU Remaining in Sample} = \text{IFN activity of sample} / \text{IFN activity of midpoint (1LU/ml)}$$

- Calculate the antibody titre corresponding to the RLU at 1 LU/ml from the Antibody Dose Response Curve

$$\text{Antibody Titre} = x = \text{EXP}((y -c)/m).$$

If the RLU of the midpoint is outside the range of the RLU's obtained in the antibody titre curve, the titre cannot be determined and the sample in question requires further dilution to obtain an accurate titre. In the example layout in Table 3B the maximum dilution of the sample tested is 1 in 540.

- Calculate the Laboratory Units used in the assay, the acceptable range is 5 - 15 LU/ml

$$\text{Laboratory Units used in assay} = \text{'Spike' activity} / \text{IFN activity at 1 LU/ml}.$$

- Calculate Tenfold Reduction Units (TRU/ml) using the following formula;

$$t = f(n-1/9) \quad f = \text{reciprocal of the Antibody dilution at 1LU,} \\ n = \text{LU used in the assay}$$

Example using data displayed in graphs Fig 2 and Fig. 4

- Midpoint** = (5150 + 28200)/2 = 16675
- Using Regression equation from Standard Curve (Fig. 2)
 $y = 6500.5x + 12173 = 16675$
IFN Activity at Midpoint (X) = EXP((16675-12173)/6500.5) = 2.0 IU/ml.
- Sample Activity = 1.9 IU/ml, Midpoint Activity = 2.0 IU/ml
 $1.9/2.0 = 0.95 \text{ LU/ml} = \text{NAb's in sample}$
- Using Regression equation from antibody dose response curve (Fig. 4)
 $y = 7282x + (-31481) = 16675$
Antibody Titre (X) = EXP((16675 + 31481)/7282) = 745
- Laboratory Units** used in assay = $25/2.0 = 12.51 \text{ LU/ml}$
- TRU/ml** = $745 \times (12.51 - 1)/9 = 952$

Figure 1: Example of Standard Curve

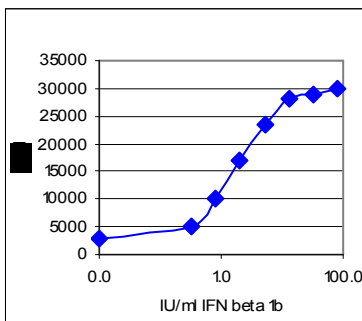


Fig. 2: Example of Standard Curve with Trendline

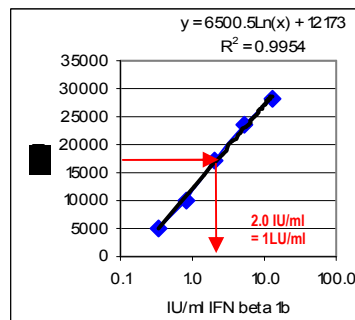


Fig. 3: Example of Neutralization Curve

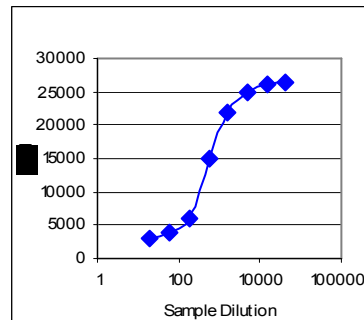
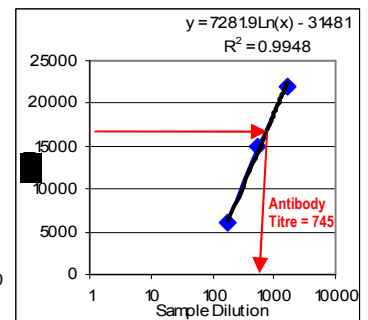


Fig. 4: Example of Neutralization Curve with Trendline



LIMITATIONS OF USE

- Samples with high endogenous IFN γ , IFN β , or IFN α (e.g. as a result of viral infection) should not be used.
- Samples with a high lipid, bilirubin or haemoglobin content (visible by eye) should be excluded as these can interfere with the bioluminescence determinations.
- The test can only be used for the semi quantification of neutralizing antibodies to IFN β 1b. This test cannot distinguish between neutralizing antibodies to the different IFN β isotypes i.e. IFN β 1a and IFN β 1b.
- Repeated freeze thawing of samples should be avoided. Serum samples may be stored at 4°C for 1 week, or frozen for extended storage.

PERFORMANCE CHARACTERISTICS

Specificity: *iLite* is specific for NAbS for human IFN β 1b. No cross reactivity was observed with IFN α . Cross reactivity was observed with IFN β 1a.

Measuring Range: The measuring range of this assay starts with a sample of 20 TRU/ml and samples may be diluted to obtain the appropriate titre required for TRU/ml determination, therefore there is no maximum range of detection.

Limit of detection: The minimum limit of detection is a sample of 20 TRU/ml (see measuring range).

Interference: Samples with a high lipid, bilirubin or hemoglobin content (visible by eye) can interfere with the bioluminescence determinations and should not be used. Samples with high endogenous IFN β - or IFN α levels, should 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*TM anti-human IFN beta 1b assay

| | % CV |
|-----------------|------|
| Inter Lot & Day | 23.7 |
| Intra Assay | 16.1 |
| Inter Operator | 23.6 |

Clinical Data

Table 5: % Accuracy of the *iLite*TM anti-human IFN beta 1b when compared to CPE assay

| <i>iLite</i> TM anti-human IFN-beta 1b | CPE | | Total |
|---|----------|----------|-------|
| | Positive | Negative | |
| Positive | 51 | 0 | 51 |
| Negative | 0 | 50 | 50 |
| Total | 51 | 50 | 101 |

Diagnostic Sensitivity $\geq 99\%$
Diagnostic Specificity $\geq 99\%$
Diagnostic Accuracy $\geq 99\%$
The correlation of *iLite* anti-human IFN beta 1b v's CPE by Spearmans Correlation is $r = 0.99$, $p < 0.0001$

Table 6: *iLite*TM anti-human IFN beta 1b results of Normal Donor Sera (IFN naïve)

| | <i>iLite</i> + | <i>iLite</i> - |
|-------------------|----------------|----------------|
| Normal Donor Sera | 0 | 25 |

Table 7: QC Criteria

| QC Criteria | If the criteria below are not met, the assay is considered invalid: | |
|-------------|---|---------------------------------|
| % CV of RLU | $\leq 15\%$ | Negative Control > 1.5 LU/ml |
| LU spiked | 5 -15 LU/ml | Positive Control ≤ 1 LU/ml |
| r^2 | ≥ 0.95 | |

PRECAUTIONS:

SAFETY

- iLite*TM anti IFN β 1b 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 microorganism (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 microorganisms 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 setup. Do not use kit or individual reagents past their expiry dates.
- Do not mix or substitute reagents from different kit lot numbers.
- Deviation from the procedure eg. performing the assay outside the time, temperature and volume ranges provided, may produce erroneous results and should be repeated using the correct procedure.
- Care must be taken not to contaminate components, and always use fresh pipette tips 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 30 min of thawing.
- Standards should be used within 30 min of thawing.
- Diluent should be used on the day of thawing.
- Luciferase reagent should be used immediately after reconstitution (or within 2 weeks if frozen immediately after reconstitution, at -20°C)

FREQUENTLY ASKED QUESTIONS (FAQ'S): Refer to Web site for FAQ's www.biomonitor.dk

REFERENCES

- Grossberg SE, Kawade Y, Kohase M, Yokoyama H, Finter N. The neutralization of interferons by antibody. I. Quantitative and theoretical analyses of the neutralization reaction in different bioassay systems. J Interferon Cytokine Res 2001; 21: 729-742.
 - Grossberg SE, Kawade Y, Kohase M, Klein JP. The neutralization of interferons by antibody. II. Neutralizing antibody unitage and its relationship to bioassay sensitivity: the tenfold reduction unit. J Interferon Cytokine Res 2001; 21: 743-755.
- Refer to Web site for other relevant references: www.biomonitor.dk