

## Measuring neutralizing antibodies to IFN $\alpha$ / $\beta$ using *iLite*<sup>™</sup> Type I IFN Assay Ready Cells

*This application note contains a suggested protocol and performance data. Each individual laboratory must set up their own method and perform relevant validations.  
For research and professional use only.*

### Background

Interferon alpha (IFN $\alpha$ ) has been widely used to treat chronic viral hepatitis and a wide variety of malignant diseases, including hairy cell leukemia, basal cell carcinoma, chronic myeloid leukemia and cutaneous T-cell lymphoma. Several different recombinant preparations of IFN $\alpha$  are available commercially; the most commonly used formulations include IFN $\alpha$ 2a and IFN $\alpha$ 2b. A number of studies have shown that development of anti IFN $\alpha$  neutralizing antibodies (NAbs) is correlated with a loss of IFN $\alpha$  treatment efficacy.

Interferon beta (IFN $\beta$ ) is well established as first line therapy in relapsing/remitting multiple sclerosis. The occurrence of neutralizing antibodies (NAbs) and binding antibodies (BAbs) to IFN $\beta$  has been widely reported. Subjects with NAbs have shown reduced response to treatment with IFN $\beta$ , having higher relapse rates, increased MRI activity and higher risk of disease progression. The frequencies and titers of NAbs vary depending on the preparation used, dose and frequency of administration and also the assay used to quantify them.

The *iLite*<sup>™</sup> Type I IFN Assay Ready Cells can be used for measuring presence of neutralizing antibodies to IFN $\alpha$  or  $\beta$ .

### Principle of the assay

The *iLite*<sup>™</sup> Type I IFN Assay Ready Cells are engineered cells optimized to express Firefly luciferase under the control of an IFN $\alpha$ / $\beta$  responsive promoter. IFN $\alpha$ / $\beta$  binds to the IFN $\alpha$ / $\beta$  receptor on the cell surface and activates the IFN $\alpha$ / $\beta$  regulated Firefly luciferase reporter gene construct. The Firefly luciferase signal can be measured in a luminometer following addition and incubation of luciferase substrate. The Firefly luciferase signal is proportional to the concentration of IFN $\alpha$ / $\beta$  in the sample (Fig.1).

In presence of IFN $\alpha$ / $\beta$  NAbs in test samples the amount of free IFN $\alpha$ / $\beta$  is reduced, resulting in a decreased stimulation of Firefly luciferase production. The Firefly luciferase signal is inversely proportional to the amount of IFN $\alpha$ / $\beta$  NAb activity in the samples. The amount of neutralizing IFN $\alpha$ / $\beta$  antibodies in a sample is given as its ability to neutralize a known amount of interferon.

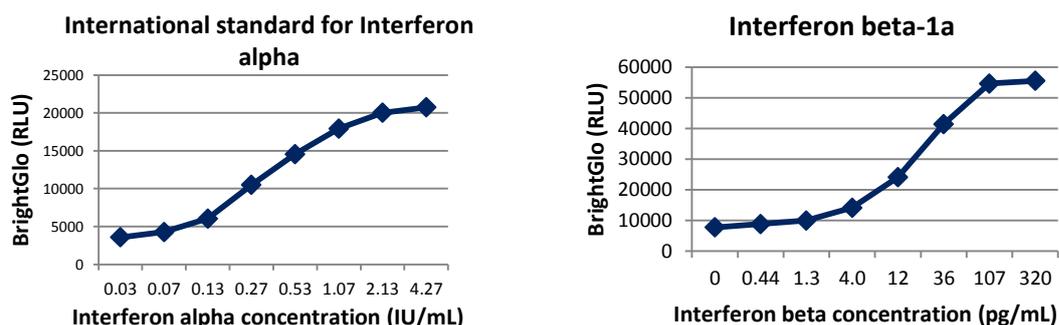


Fig 1. Concentration curves of interferon alpha (left graph) and interferon beta (right graph)

### Specimen collection

The *iLite™* Type I IFN Assay Ready Cells can be used for measuring concentration of IFN $\alpha/\beta$  and presence of neutralizing antibodies to IFN $\alpha/\beta$  in test samples including human serum.

### Material and equipment needed

| Material and equipment  | Suggested supplier   | Reference                                  |
|---|--|--|
| <i>iLite™</i> Interferon $\alpha/\beta$ Assay Ready Cells                           | Euro Diagnostica   | BM3049                                     |
| Diluent B, RPMI 1640  | Euro Diagnostica   | BM3134, <i>iLite™</i> Diluent B            |
| Firefly luciferase substrate only   | Promega  | E2610, Bright-Glo™ Luciferase Assay System |
| Interferon $\alpha$   | NA   | NA   |
| Interferon $\beta$  | NA   | NA   |
| White walled micro well plate suitable for luminescence                             | PerkinElmer  | 6005680                                    |
| Microplate Luminometer with appropriate reading software – no filter on luminometer | Contact Euro Diagnostica for list of recommended suppliers | NA   |
| Incubator, 37 °C with 5% CO <sub>2</sub>  | NA   | NA   |
| Water bath, 37 °C   | NA   | NA   |
| Single-channel and multi-channel pipettes with polypropylene disposable tips        | NA   | NA   |
| Polypropylene tubes or plate for dilution of samples and cells                      | NA   | NA   |
| Single-use polypropylene reservoir  | NA   | NA   |
| Plate shaker  | NA   | NA   |
| Timer   | NA   | NA   |

### Preparation of standard samples and controls

Construct three standard samples (S1, S2, S3) by diluting interferon in, for example, diluent RPMI 1640 with 10% heat inactivated fetal calf serum, to appropriate concentrations. Suggested concentrations and volumes can be found in Table 1.

Table 1. Preparation of interferon standards. For dilutions RPMI 1640 with 10% heat inactivated fetal calf serum (HFCS) can be used.

| Standards/controls | Interferon standards |                                   |
|--------------------|----------------------|-----------------------------------|
|                    | Total volume         | Interferon concentration. (IU/mL) |
| S1                 | 5.5 mL               | 18.75                             |
| S2                 | 500 $\mu$ L          | 1.875                             |
| S3                 | 5.5 mL               | 0                                 |

The concentrations of S1 and S2 need to be determined for each type of interferon and for each cell lot. It is important to ensure that the measurements are performed in the linear range of the interferon dilution curve. For an optimal performance the RLU signal of S2 should fall within 20% of ED50 (Fig.2).

Example:

S1: 20000 RLU

S2: 10000 RLU

S3: 4000 RLU

ED50:  $20000 + 4000 / 2 = 12000$

S2/ED50:  $10000 / 12000 = 0.83$

(Acceptance range: 0.80-1.20 )

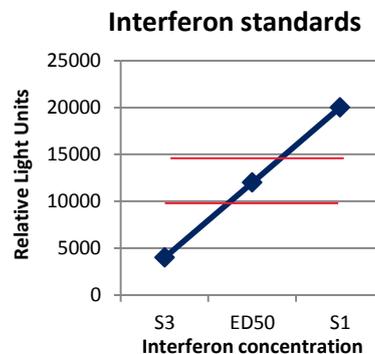


Fig 2. Standard S2 should fall within 20% of ED50 (red lines)

If the S2/ED50 ratio is lower than 0.80, the interferon concentration of S1 and S2 should be increased and if the ratio is above 1.2, the interferon concentration of S1 and S2 should be decreased. Ensure that S1 always contains 10 times the interferon concentration of S2.

Positive controls may be prepared from pools of NAb positive patient samples or commercially available anti-interferon antibodies. Diluent may be used as a negative control.

It is important to ensure a consistent matrix effect (serum concentration) between standards, control and samples throughout the assay.

## Protocol

### Sample dilution and pre-incubation

If human serum is analysed for neutralizing antibodies against IFN $\alpha/\beta$ , serum samples should be heat in-activated at 56°C for 30 minutes prior to analysis, to inactivate potential residual Interferon  $\alpha/\beta$ . The three standard samples should be analysed as quadruplicates. Control sample and unknown samples should be analysed in duplicates. Control sample and unknown samples should be analysed with and without a known amount of interferon (one duplicate with and one duplicate without interferon) as the amount of neutralizing IFN $\alpha/\beta$  antibodies in a sample is given as its ability to neutralize a known amount of interferon. Twenty unknowns can be analysed per 96 well plate.

### Pre incubation with interferon

1. Design a plate layout with 4 wells assigned to each standard, control and sample. Use a white-walled plate suitable for luminescence determinations.
2. Pre-dilute samples: 60  $\mu$ L sample with 100  $\mu$ L Diluent B.
3. Add to standard wells in white plate: 80  $\mu$ L of S1, S2 and S3 to 4 wells each + 20  $\mu$ L RPMI containing 37.5% heat inactivated human serum to all 12 wells.
4. Sample wells and control wells:
  - Add to two wells 20  $\mu$ L sample + 80  $\mu$ L **S1**
  - Add to two wells 20  $\mu$ L sample + 80  $\mu$ L **S3**

5. Cover the plate with a lid, mix by gently swirling and incubate for 30 minutes at 37°C with 5% CO<sub>2</sub>.

#### **Cell dilution**

6. Thaw the vial of cells in a 37°C water bath 15 minutes prior to completion of the incubation. Invert the vial a minimum of 10 times to ensure a uniform cell suspension.
7. Dilute 2.5mL cells with 5.5mL *iLite™* Diluent B

#### **Cell incubation**

8. Add 50 µL diluted cells to each well.
9. Place the lid on the plate, mix and incubate for 18 hours at 37 °C with 5% CO<sub>2</sub>.

#### **Adding substrate solutions**

10. Thaw the vial of Bright-Glo™ Luciferase Assay System 30 minutes prior to completion of the incubation. Prepare the substrate according to the supplier's instructions and add 50 µL per well. Mix and protect the plate from light. Read in a luminometer after 2 minutes incubation at room temperature.

#### **Determination of neutralizing antibodies to Interferon α or β**

Calculate the mean Relative Light Units (RLU) for each data point. A sample is considered positive for neutralising interferon antibodies if the RLU signal is reduced by tenfold, i.e. below the signal of S2. Calculate the ratio between every unknown sample and S2.

If the ratio is > 1.0 the sample is considered negative for neutralizing antibodies to interferon.  
If the ratio is ≤ 1.0 the sample is considered positive for neutralizing antibodies to interferon.

#### **Quality Control**

If the following criteria are met, the standards are considered valid:

Ratio between S2 and ED50: 0.8 – 1.2

If the following criteria are met, the test run is considered valid:

Positive control: Positive for NAb

Negative control: Negative for NAb

If the following criteria are met, a sample result may be considered valid:

% CV of duplicates ≤ 20%

The ratio between individual samples and S3 is between 0.7 – 1.3.

Ratios < 0.7 indicate a toxic effect on the cells by the sample and rejection of the result should be considered.

Ratios > 1.3 indicates intrinsic interferon-like activity in the sample, which may affect detection of NAb. Rejection of the result should be considered.

### **Precautions**

-This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.

-Use and handle the material and instruments referenced according to the supplier's/manufacturer's instructions or product specifications accompanying the individual material and instruments.

-Dispose of all sample specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed as though potentially infectious.

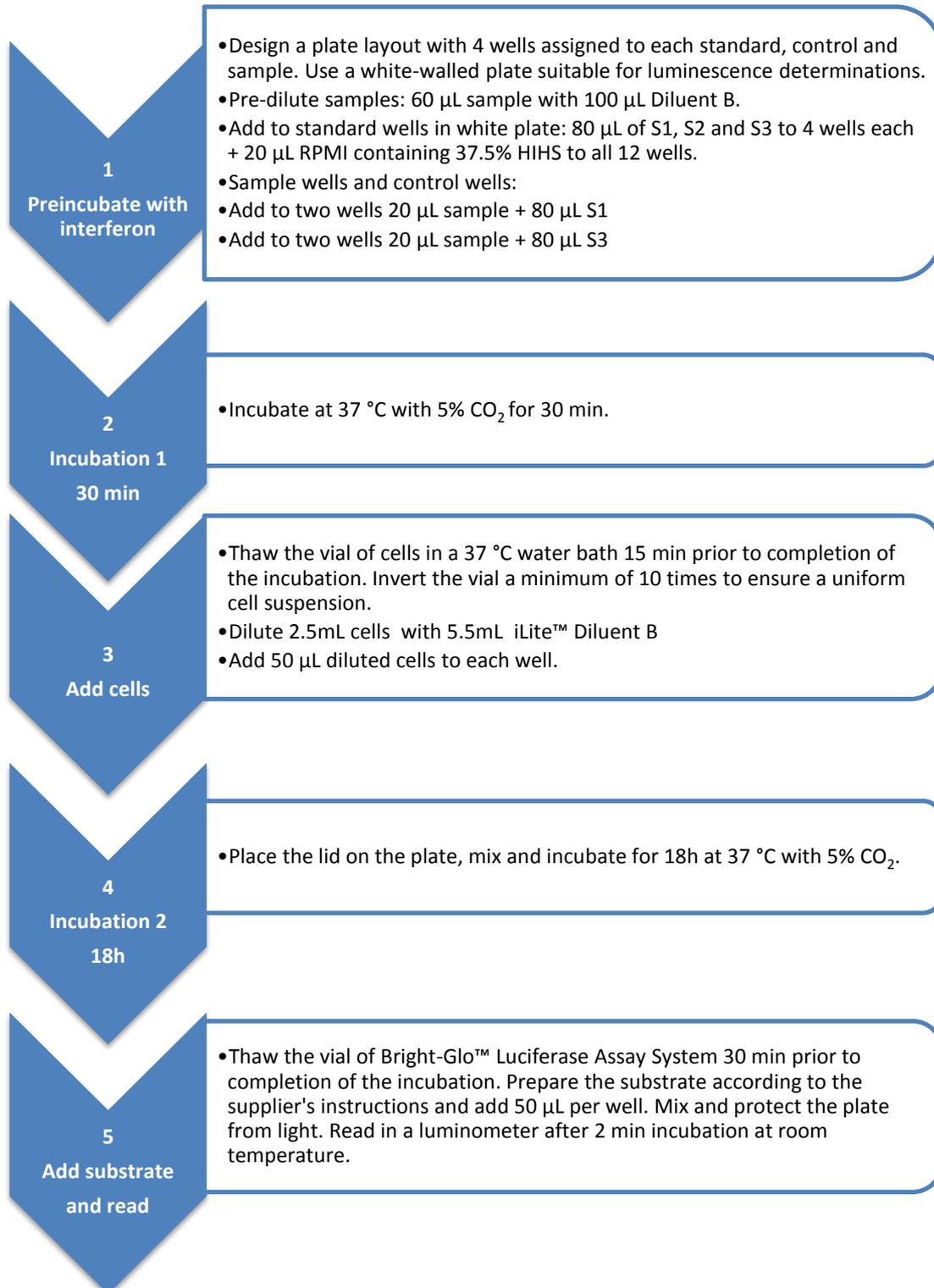
- Residues of chemicals and preparations are generally considered as biohazardous waste, and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

### **Propriety Information**

In accepting delivery of *iLite™* Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third party recipient, and only to use them directly in assays. Biomonitor *iLite™* cell-based products are covered by patents which are the property of Euro Diagnostica AB and any attempt to reproduce the delivered *iLite™* Assay Ready Cells is an infringement of these patents.



## Quick Guide – measuring neutralizing antibodies to IFN $\alpha$ / $\beta$ using iLite™ Type I IFN Assay Ready Cells



### Troubleshooting and FAQ

Please consult Euro Diagnostica's website [www.eurodiagnostica.com](http://www.eurodiagnostica.com)