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Instructions For Use

iLite™ Infliximab NAb – Research Use Only (RUO) Kit

INTENDED USE:
The *iLite*™ Infliximab NAb assay is intended for *in vitro* diagnostic use for the semi-quantitative determination of neutralizing antibodies (NAbs) to Infliximab in serum, using luciferase generated bioluminescence. It should not be used for diagnostic purposes.

BACKGROUND:
Infliximab is a mouse-human chimeric antibody construct. The drug has proven to be immunogenic, and long-term use can therefore induce anti-Infliximab antibodies in some patients. This may cause secondary response failure and side-effects (acute and delayed infusion reactions, vasculitis, etc.). The iLite™ Infliximab NAb assay is the only commercially available bioassay for detecting neutralizing antibodies against Infliximab in serum. It is a simple and easy to use as it uses growth arrested cells hence culture of live cells is not required.

ASSAY PRINCIPLE:
The test procedure involves the use of division-arrested TNFα-sensitive cells in a bioassay capable of measuring TNFα bioactivity. The TNFα induced activation of the luciferase reporter gene construct is inversely proportional to the amount of Infliximab present. The amount of Infliximab present is inversely proportional to the amount of neutralizing antibodies to Infliximab. A semi-quantitative estimate of the amount of NAbs present in a serum sample is determined by titering the sample to a dilution where the neutralizing effect of the antibodies is no longer distinguishable from an antibody-negative control.

Materials Provided

iLite™ TNF alpha Cells	1.5 ml	Dual-Glo® Luciferase Buffer	10 ml
Control +	75 µl	Dual-Glo® Luciferase Substrate	1 Vial (Contains dithiothreitol (DTT), HARMFUL)
Control -	100 µl	Dual-Glo® Stop & Glo® Buffer	10 ml
REAG Infliximab	4 ml	Dual-Glo® Stop & Glo® Substrate	100 µl
TNF alpha	7 ml	Clear Microwell Plate	1
DIL A	7.5 ml	White Microwell Plate	1
DIL B	20 ml	Product Insert	1
REAG Blank	200 µl		

Additional Material Required

Micropipette covering ranges 20µl - 1000µl	Incubator 37°C, 5% CO_2
Multichannel Pipette covering ranges 30µl - 150µl	Sterile Reservoir
Polypropylene tubes (Sterile) 2ml & 14ml	Microplate Luminometer and appropriate software, ensure software is correctly installed and that operators are fully trained in its use.

ASSAY DESCRIPTION:

QUICK GUIDE

Thawing Kit Reagents and Samples – **do not thaw cells and substrate reagents at this stage**	Clear microwell plate in monoplicate
Dilute samples (titering)	
Add standards, controls and samples to clear Microwell Plate in monoplicate	
Add 50 µl of REAG Infliximab to wells **- except the wells indicated "Blank"**	
Add 50 µl of REAG Blank to the wells indicated "Blank"	
Cover the wells and mix the contents	
Incubate at 37°C in 5% CO_2 for 30 min	
Add 100 µl of TNF alpha to wells	
Cover the wells and mix the contents	
Incubate at 37°C in 5% CO_2 for 30 min **Thaw and dilute the cells** **Commence thawing substrate reagents**	
Transfer 50 µl serum/infliximab/TNFα solution to white-walled microwell plate in duplicate	White microwell plate in duplicate
Add 50 µl of diluted cells to wells	
Cover the wells and mix the contents	
Incubate at 37°C in 5% CO_2 for 3 h	
Equilibrate the white-walled microwell plate to room temperature	
Add 80 µl Dual-Glo® Luciferase reagent per wells, read at luminometer 10 min after addition of Dual-Glo substrate	
Add 80 µl Dual-Glo® Stop & Glo® reagent per wells, read at luminometer 10 min after addition of Stop-Glo substrate	

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 for determination of antibody titre of the positive samples (containing Nabs).

Screening of Samples: Tests for the presence/absence of NAbs in the serum sample (example layout Table 3)

Titering of Samples: Samples which tested positive for NAbs in the screening assay are then further tested to determine the antibody titre (example layout Table 4).

1. Sample Preparation

Prior to the assay setup, the serum samples should be equilibrated to room temperature and mixed.

2. Thawing Kit Reagents

Equilibrate the microwell plates, DIL A, DIL B, REAG Blank, REAG Infliximab, TNF alpha, Control+, and Control- to room temperature. DO NOT THAW THE CELLS OR THE SUBSTRATE REAGENTS AT THIS STAGE.

3. Dilution of Samples

Samples should be mixed and diluted using DIL B (screening and 1st tittering dilution) and DIL A (subsequent tittering dilutions).

Samples for Screening: Dilute directly in clear plate: 20 µl sample (neat serum) + 30 µl DIL B. Final dilution in assay is 1 in 20.

Samples for Titering: Dilute in polypropylene tubes: Recommended dilutions of samples are from 1 in 20 to 1 in 2560 (Table 1). Samples with a titer > 2560 could be re-tittered starting at a higher dilution of sample, but always perform a 2 in 5 dilution of serum in DIL B before subsequent dilution in DIL A – this will ensure constant serum concentration.

Table 1: Dilution of samples for determination of titer (2-fold)

Dilution	Volume of Diluent B (µl)	Volume of Sample (µl)	Volume of Diluent A (µl)	Dilution Factor	Final Sample Dilution
A	150	100 neat serum		2.5	20
B		100 A	100	5	40
C		100 B	100	10	80
D		100 C	100	20	160
E		100 D	100	40	320
F		100 E	100	80	640
G		100 F	100	160	1280
H		100 G	100	320	2560

4. Addition of Standards, Controls and Samples to clear Plate (Table 2)

A plan of the intended location of each sample, control and standard on the clear microwell plate should be generated in advance to assist with this step (see example in Tables 3 & 4). All samples, controls and standards are pipetted in monoplicate on the **clear** microwell plate; these will later be transferred in duplicate to the **white** microwell plate.

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

Add the various standards, controls and samples to the wells of the **clear** plate as follows (example plate layout shown in Table 3 or Table 4 as applicable)

- 50 µl of DIL A to the wells indicated either "St 40" or "Blank".
- 50 µl of Control+ to the well indicated "Control+".
- 50 µl of Control- to the well indicated "Control-".
- **Screening**: 20 µl of neat serum sample and 30 µl of DIL B to the wells indicated "Sp" as per plate layout in Table 3. Mix the contents in the wells by gently swirling the plate a few times
- **Titering**: 50 µl of pre-diluted serum sample (Table 1) to the wells indicated "Sp" as per plate layout in Table 4.
- 50 µl of REAG Infliximab to all the wells except the wells indicated "Blank".
- 50 µl of REAG Blank to the wells indicated "Blank".
- Place the lid on the microwell plate and mix the contents in the wells by gently swirling the plate a few times.
- Incubate at 37°C in 5% CO_2 for 30 min.
- Add 100 µl of TNF alpha to all the wells.
- Place the lid on the microwell plate and mix the contents in the wells by gently swirling the plate a few times.
- Incubate at 37°C in 5% CO_2 for 30 min.

5. Addition of *iLite*™ Cells and serum/infliximab/TNFα solutions to white-walled microwell plate (Table 2A)

Ensure tips are changed between each duplicate addition to avoid cross-contamination.

- Thaw the vial of cells quickly by agitation in a 37°C water bath 5 min prior to completion of the incubation. Invert the vial a minimum of 10 times to ensure a uniform cell suspension.
  (One can also remove the Substrate reagents from the freezer at this stage (Dual-Glo® Luciferase Buffer, Dual-Glo® Luciferase Substrate, Dual-Glo® Stop & Glo® Buffer, and Dual-Glo® Stop & Glo® Substrate). Store the substrate reagents until use at room temperature.)
- Dilute the cells 1 in 5. Add the entire contents of the *iLite*™ Cells vial to 6 ml DIL B in a sterile tube.
- Transfer 50 µl of serum/infliximab/TNFα solution from the **clear** Plate (Plate 1) in duplicate to the **white** microwell plate (Plate 2); eg. transfer 50 µl from column $1_{Plate\ 1}$ to column $1_{Plate\ 2}$ + 50 µl from column $1_{Plate\ 1}$ to column $2_{Plate\ 2}$, column 3 to column 3+4, column 5 to column 5+6, column 7 to column 7+8, column 9 to column 9+10, and column 11 to column 11+12.
- Invert diluted cells approx. 10 times to ensure an uniform cell suspension then transfer into a sterile multichannel reservoir.
- Add 50 µl of diluted cells to each well.
- Replace the lid on the white microwell plate, mix the contents in the wells by gently swirling the plate a few times.
- Incubate at 37°C in 5% CO_2 for 3 hrs.

6. Addition of Substrate reagents to white-walled microwell plate (Table 2A)

Ensure tips are changed between each duplicate addition to avoid cross-contamination.

- Add the entire contents of the Dual-Glo® Luciferase Buffer to the Dual-Glo® Luciferase Substrate, replace cap and mix gently by inversion.
- Add 80 µl Dual-Glo® Luciferase reagent per well using a multichannel micropipette, ensure contents of wells are mixed by pulling liquid up and down the pipette.
- 10 min after addition of substrate (protect the plate against light) determine the luminescence using a microplate luminometer (e.g. Victor ™ Light luminometer, PerkinElmer LAS, Seer Green, Bucks, UK) – Firefly luciferase reading. Read without lid.
- Add the entire contents of the Dual-Glo® Stop & Glo® Substrate to the Dual-Glo® Stop & Glo® Buffer, replace cap and mix gently by inversion.

- Add 80 µl Dual-Glo® Stop & Glo® reagent per well using a multichannel micropipette, ensure contents of wells are mixed by pulling liquid up and down the pipette.
- 10 min after addition of substrate (protect the plate against light) determine the luminescence using a microplate luminometer – Renilla reading. Read without lid.

Table 2: Overview of addition of reagents to clear plate (per well[Δ])

Reagent	Standard 40 (St40)	Blank	Sample (Sp)	Control-	Control+
Diluted Sample*	-	-	50 µl*	-	-
Control -	-	-	-	50 µl	-
Control+	-	-	-	-	50 µl
DIL A	50 µl	50 µl	-	-	-
REAG Blank	-	50 µl	-	-	-
REAG Infliximab	50 µl	-	50 µl	50 µl	50 µl
TNF alpha	100 µl	100 µl	100 µl	100 µl	100 µl

[Δ] Only use column 1, 3, 5, 7, 9, and 11 (monoplicate) – as per plate layout example shown in Table 3 or Table 4
* Samples for Screening: Dilute directly in plate; 20 µl of serum sample and 30 µl of DIL B.
Samples for Titering: Dilute as shown in Table 1

Table 2A: Overview of addition of reagents to white microwell plate (per well)

Reagent	Volume
Serum/infliximab/TNFα solution	50 µl
iLite ™ TNF alpha Cells (diluted 1 in 5)	50 µl
Dual-Glo® Luciferase reagent	80 µl
Dual-Glo® Stop & Glo® reagent	80 µl

Table 3: Example plate layout for screening of samples

	Column 1	Column 3	Column 5	Column 7	Column 9	Column 11
A	St 40	Sp 1	Sp 9	Sp 27	Sp 25	Sp 33
B	St 40	Sp 2	Sp 20	Sp 28	Sp 26	Sp 34
C	St 40	Sp 3	Sp 21	Sp 29	Sp 27	Sp 35
D	St 40	Sp 4	Sp 22	Sp 20	Sp 28	Sp 36
E	Blank	Sp 5	Sp 23	Sp 21	Sp 29	Sp 37
F	Blank	Sp 6	Sp 24	Sp 22	Sp 30	Sp 38
G	Control+	Sp 7	Sp 25	Sp 23	Sp 31	Sp 39
H	Control-	Sp 8	Sp 26	Sp 24	Sp 32	Sp 40

Legend: St = Standard, Sp = Sample

Table 4: Example plate layout for determination of titer

	Column 1	Column 3	Column 5	Column 7	Column 9	Column 11
A	St 40	Sp 1-A	Sp 2-A	Sp 3-A	Sp 4-A	Sp 5-A
B	St 40	Sp 1-B	Sp 2-B	Sp 3-B	Sp 4-B	Sp 5-B
C	St 40	Sp 1-C	Sp 2-C	Sp 3-C	Sp 4-C	Sp 5-C
D	St 40	Sp 1-D	Sp 2-D	Sp 3-D	Sp 4-D	Sp 5-D
E	Blank	Sp 1-E	Sp 2-E	Sp 3-E	Sp 4-E	Sp 5-E
F	Blank	Sp 1-F	Sp 2-F	Sp 3-F	Sp 4-F	Sp 5-F
G	Control+	Sp 1-G	Sp 2-G	Sp 3-G	Sp 4-G	Sp 5-G
H	Control-	Sp 1-H	Sp 2-H	Sp 3-H	Sp 4-H	Sp 5-H

Legend: St = Standard, Sp = Sample.: Sp 1-A = Sample 1 – Dilutiion A (20 fold final dilution)
Dilutions as shown in Table 1

ANALYSIS OF RESULTS:

Calculation of threshold for antibody-negative control sera

1. The Renilla normalized TNFα activity ie Firefly luciferase/Renilla luciferase (FL/RL) ratio of "St 40" is equivalent to FL/RL ratio of an antibody-negative sera. The threshold for an antibody-negative serum is set to 1.4 × FL/RL of "St 40". If the FL/RL ratio of a sample is above this threshold, the serum is considered to contain neutralizing antibodies to Infliximab.

   Threshold = 1.4 × Mean FL/RL of St 40 ie Mean TNFα activity* / Mean Renilla activity* of St 40)

   * TNFα activity = Firefly luciferase RLU reading, Renilla activity = Renilla RLU reading

Calculation of serum interference in a sample

2. The Renilla activity of a sample is expected to be the same as the Renilla activity in the assay standards if the serum does not contain interfering factors and the number of cells is constant throughout the plate. If the Renilla activity of a sample is > 30 % different from the Renilla activity of the assay standards ("St 40" and "Blank"), the serum is considered to contain interfering factors (or the cells numbers are too variable) and the antibody determination is therefore invalid.

   Interference ratio = Mean Renilla activity of sample / Mean Renilla activity of Blank and St 40

   Serum interference if Interference ratio < 0.7 or Interference ratio > 1.3

Calculation of normalized TNF activity in a sample

3. The normalized TNF activity is found by dividing the TNFα activity (Firefly luciferase reading) with the Renilla activity (Renilla reading). The normalized TNF activity is calculated for each respective replicate.

   Normalized TNF activity in sample = TNFα activity / Renilla activity

Determination of neutralizing antibodies (NAbs) to Infliximab in a sample

4. Screening: If the normalized TNF activity of a sample is > threshold the sample is regarded as positive for Nabs to Infliximab. If the normalized TNF activity of a sample is ≤ threshold the sample is regarded as negative for NAbs to Infliximab.

   NAb positive: Normalized TNF activity in sample > threshold
   NAb negative: Normalized TNF activity in sample ≤ threshold

5. Titering: Create an antibody titre curve as an XY scatter plot of the data with sample dilution on the X axis and normalized TNF activity on Y axis [both linear scale]. Draw a line through the data points. The titre of the samples is defined as the dilution where the line intersects the threshold. If the line through the data points does not intersect the threshold, the titre cannot be determined. If the line through the data points is below the threshold, the sample is negative for NAbs to Infliximab (eg. titre < 20). If the line through the data points is above the threshold, the sample titre is > the highest sample dilution and the sample in question requires further dilution to obtain an accurate titre.

Example

Example of raw data (FL RLU's and RL RLU's) processing from titering samples performed as shown in Table 4

Table 5: Example of Firefly luciferase RLU (FL-RLU) reading of titering plate (TNFα activity)

	1	2	3	4	5	6	7	8	9	10	11	12
A	463	395	1580	1608	897	834	1556	1593	1628	1554	960	956
B	375	365	1303	#1301	725	669	1677	1659	1655	1775	1019	1133
C	392	423	976	937	531	469	1428	1511	1603	1787	1233	1186
D	412	392	547	519	389	425	985	943	1291	1268	1367	1405
E	1851	1859	526	465	414	355	669	672	803	744	1275	1326
F	1865	1892	444	428	369	349	490	475	539	532	867	805
G	856	906	403	386	385	326	445	432	424	453	583	609
H	201	231	416	445	423	395	359	401	445	373	502	457

Table 6: Example of Renilla RLU (RL-RLU) reading of titering plate (Renilla activity)

	1	2	3	4	5	6	7	8	9	10	11	12
A	#145598	#149547	142614	144841	143460	144502	145017	143932	146057	143494	144878	144449
B	#149227	#149604	142307	#139165	146803	145009	148675	146204	149272	146546	152956	142564
C	#155168	#151099	142971	147586	153033	146732	147858	151247	150347	148589	153584	141591
D	#146576	#153510	152893	151474	152177	154184	153626	147923	154958	154544	156110	146703
E	150432	158380	152541	153527	146803	141271	149209	147674	149305	148748	147871	146899
F	154607	156077	156176	154481	156131	156686	152491	154662	156158	154782	151013	143649
G	152315	161211	156589	155070	159255	165049	161434	159129	156141	153441	151154	144133
H	154282	157424	151246	150896	162052	162286	164424	163359	164110	163354	178826	178667

1. Threshold = 1.4 × (402 / #150041) = 0.00375
2. Interference ratio = 143728 / 151652 = 0.948; no serum interference
3. Normalized TNF activity in sample = 1303/ 142307 = 0.00916, #1301/ #139165 = #0.00935,
   Mean of Normalized TNF activity in sample = (0.00916+#0.00935)/2 = 0.00926
4. NAb positive since 0.00926 > 0.00375
5. See Figure 1; Sample 1: titre = 150, Sample 2: titre = 60, Sample 3: titre = 480, Sample 4: titre = 580, Sample 5: titre = 1500

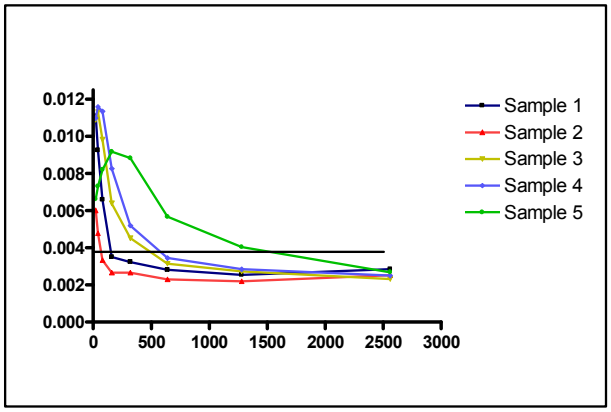

Figure 1: Determination of sample titre (data from Table 5 and 6, setup as Table 4). Sample dilution on the X axis and FL/RL ratio on Y axis. Sample titre is found where lines cross threshold = 0.00375. Sample 1: titre = 150, Sample 2: titre = 60, Sample 3: titre = 480, Sample 4: titre = 580, Sample 5: titre = 1500

QC CRITERIA: For a valid assay the following criteria should be met: **Control-** = Negative for NAb, **Control+** = Positive for NAb

For a valid sample the following criteria should be met: % CV of normalized TNFα activity ≤ 20 % and 0.7 ≥ Interference ratio ≤ 1.3

LIMITATIONS OF USE

- The test can only be used for the semi quantification of neutralizing antibodies to Infliximab.
- The test can only be used on sera with < 0.65 µg/ml endogenous Infliximab.
- Repeated freeze thawing of samples should be avoided. Serum samples may be stored at 4 °C for 1 month, or frozen for extended storage.

PERFORMANCE CHARACTERISTICS

Specificity: *iLite*™ cells in the *iLite*™ Infliximab NAb Bioassay are specific for TNFα. No cross reactivity was observed with IL-2 or rhTGF β1. No cross reactivity was observed with NAbs against Adalimumab.

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

Limit of detection: The minimum limit of detection is a titre of 20

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 ≥ 0.65 µg/ml Infliximab 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 7: Inter and Intra Assay Variation for *iLite*™ Infliximab NAb bioassay

Precision Tested	% CV
Inter Lot & Day (3 Lots x 3 Days)	23
Intra Assay (3 Locations on Plate)	12
Inter Operator (3 Operators)	4

Performance Evaluation

Table 8: Results of the *iLite*™ Infliximab NAb assay when compared to RIA assay

iLite™ Infliximab Nab Bioassay	RIA Ab				Total
	High pos	Medium pos	Low pos	Negative	
Positive	15	6	0	0	21
Negative	1	11	6	59	77
Total	16	17	6	59	98

Analytical Sensitivity = 54 %
Analytical Sensitivity$_{\text{High + Medium only}}$ = 64 %
Analytical Sensitivity$_{\text{High only}}$ = 94 %

Analytical Specificity > 99 %

Overall Accuracy = 82 %

Correlation of *iLite*™ Infliximab NAb assay v's RIA by Spearmans correlation is r = 0.81, p <0.0001

PRECAUTIONS:

SAFETY

- *iLite*™ Infliximab NAb assay 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*™ TNF alpha 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 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 80ºC** storage.
- All kit reagents are stored at -80ºC and are stable as supplied until the expiry date shown.

- Cells 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

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

REFERENCES

1 Lallemand C, Kavrochorianou A, Steenholdt C, Bendtzen K, Ainsworth MA, Meritet J-F, Blanchard B, Lebon P, Taylor P, Charles P, Alzabin S, Tovey MG. Reporter gene assay for the quantification of the activity and neutralizing antibody response to TNFalpha antagonists. J Immunol Meth. 373, 229-239 (2011)

Refer to Web site for other relevant references: www.biomonitor.dk

INTERPRETATION OF SYMBOLS

Expiry date	
Storage Temperature	
Name and address of manufacturer	
Catalogue Number	REF
Batch Number	LOT
Research Use Only	RUO