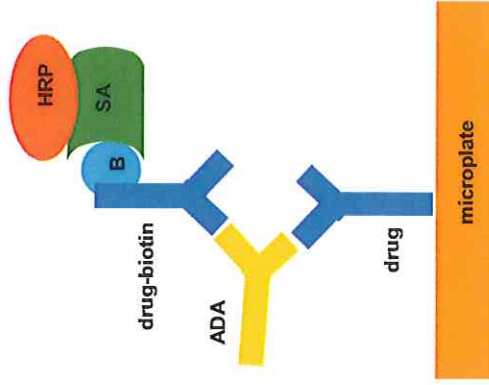


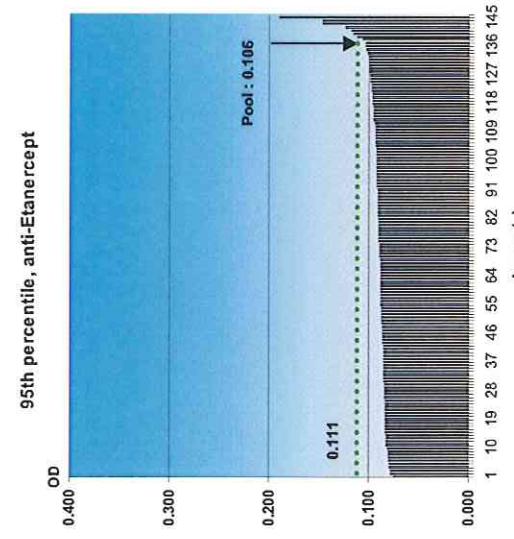
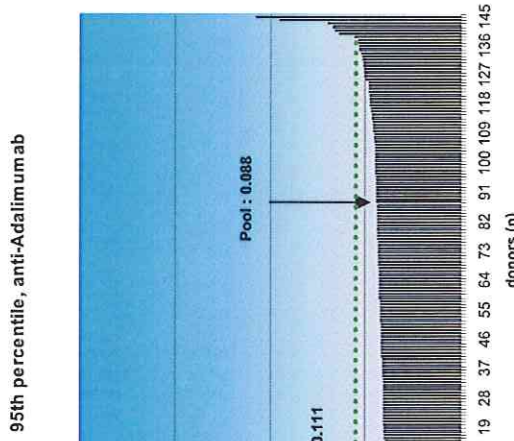
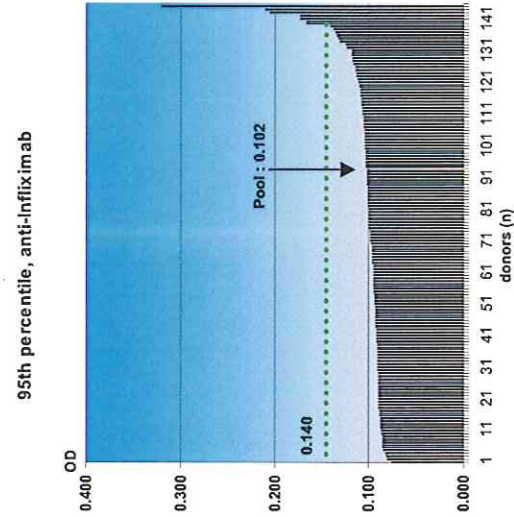
Introduction : Therapeutic agents like REMICADE®, HUMIRA® and ENBREL® are widely used to cure inflammatory diseases such as rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, etc. These drugs are able to block TNF α action which is responsible for the inflammatory state. But, during the treatment, patients may develop antibodies directed against the drug (ADA, anti-drug antibodies : anti-Infliximab, anti-Adalimumab, anti-Etanercept). Consequently, the treatment becomes less efficient. Bmd has developed immunoassays, using ELISA technique, in order to detect these antibodies.

Drug	Active principle	Type
REMICADE®	Infliximab	Chimeric IgG ₁
HUMIRA®	Adalimumab	Fully humanized IgG ₁
ENBREL®	Etanercept	Fusion protein consisting of 2 extracellular domains of the human p75 TNF receptor, linked to the Fc portion of human IgG ₁

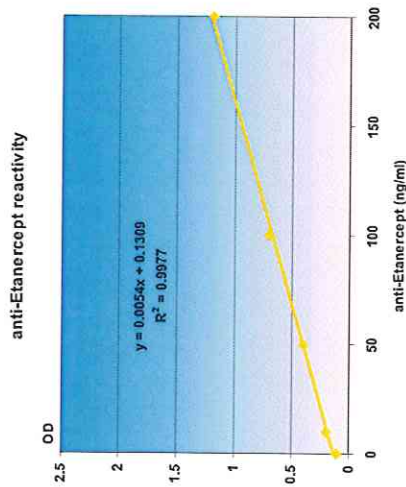
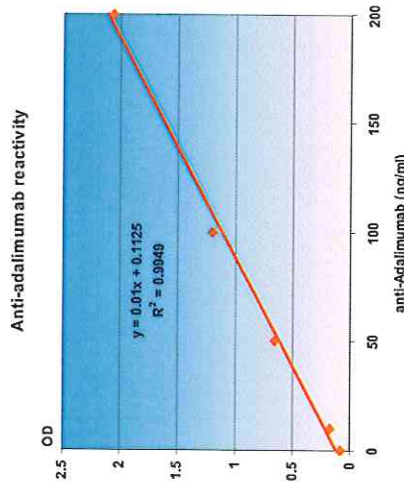
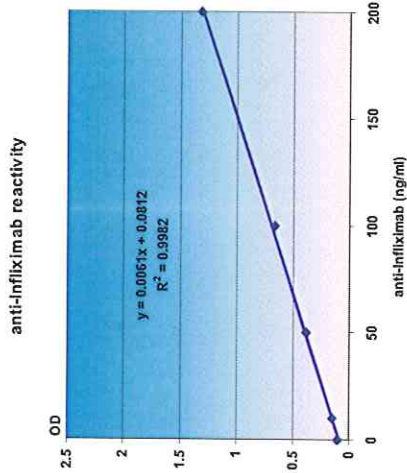
Test principle : we use a « bridging » immunoassay. First of all, the wells of a 96 polystyren microplate (microplate) are coated with the drug. Then, a blocking solution is added to block all the uncoated surface. Serum samples, diluted into a buffer solution are added to the wells : if ADA are present, they can bind to the coated drug. After a wash step, biotinylated-drug (drug-biotin) is added : biotinylated drug and the coated drug are linked in a « bridging » assay format with the aid of ADA. After a new wash step, streptavidin (SA) conjugated to peroxidase (HRP) is added. A new wash step is conducted, then, peroxidase « substrate solution » is added to obtain a colored solution. Once the « stop solution » is added, measurement of optical density (OD) can be done on a spectrophotometer. The color intensity (reactivity) is proportional to the amount of ADA detected.



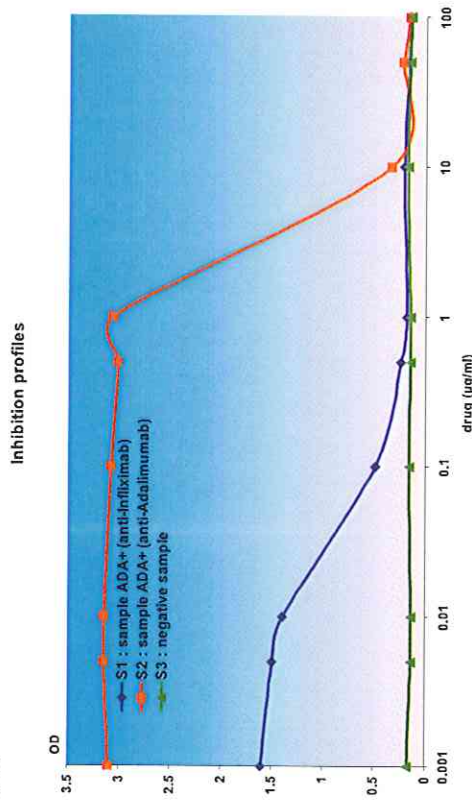
Cut-point determination : a sample is considered « positive » if the OD is above the cut-point. To determine the assay cut-point, we tested 144 serum-samples from healthy individual donors. We also tested a « Pool » sample composed of 10 healthy individual donors. We chose the 95th percentile (7 samples giving an OD above the assay cut-point) to determine the cut-point. OD can drift according to the day of the test, so we calculated a normalization factor (NF) which is defined as : OD from « Pool » x NF = OD cut-point. The NF are 1,37 ; 1,26 and 1,05 for anti-Infliximab, anti-Adalimumab and anti-Etanercept respectively. The figures below show the value of the 95th percentile (dotted green line), and the « Pool » value, for each immunoassay.



Limit of detection : The limit of detection is defined as the amount of ADA required to obtain an OD equal to the cut-point. Calibration curves were made with monoclonal antibodies directed against Infliximab, Adalimumab and Etanercept. Back-calculating the limit of detection from the curves resulted in a concentration of 9,6 ng/ml, 1,5 ng/ml and 2,8 ng/ml for anti-Infliximab, anti-Adalimumab, and anti-Etanercept respectively.



Test conducted on a population treated with anti-TNF α : 18 samples gave « positive » results (14 « positive » anti-Infliximab and 4 « positive » anti-Adalimumab) out of 115 serum-samples from patients treated with anti-TNF (REMICADE[®] or HUMIRA[®]). The 14 « positive » anti-Infliximab samples were confirmed « positive » with a kit already on the market. To confirm our results, we tested the 18 « positive » samples in a second test : inhibition assay. It consists in adding an increasing amount of drug into serum-samples to block ADA reactivity : if ADA are present, the reactivity is inhibited. But if ADA are not present, the level of reactivity is about the same as in the first test. The 18 samples tested showed a drop of reactivity below the drug was added. So, these samples were confirmed « positive » (ADA+). The figure below shows inhibition profiles for one « positive » anti-Infliximab sample (E1), for one « positive » anti-Adalimumab sample (E2), and for a « negative » ADA sample (E3), tested in the first test.



Other performances. Specificity : samples known as interferences agents were tested : rheumatoid factors, cryoglobulins, hypergammaglobulinemia, C1q protein, bilirubin, lipemic aspect, etc. All samples were « negative ». Serum samples from patients suffering from an auto-immune disease (SLE, Sjögren syndrome, CREST, systemic sclerosis, polydermatomyositis, mixt connective, etc.) were also tested. All samples were « negative ».

Precision : 4 samples (anti-Infliximab and anti-Adalimumab) and standards from the calibration curves were tested. Each sample and standard were tested four times in one assay. We performed four assays on four different days. The coefficient of variation (CV) was below 10% intra-assay and below 15% inter-assays. **Rugdeness** : Time (+/-15min) and temperature (from 15°C to 30°C) of incubation steps do not affect the results. **Stability** : reagents stability was not affected by temperature conditions which means the performances should remain stable in time.

Conclusion : Bmd has developed 3 immunoassays to detect antibodies directed against Infliximab, Adalimumab, and Etanercept. These semi-quantitative assays should help clinicians to monitor the state of their patients. These fast (<4h) and easy-to-use immunoassays are sensible (ng/ml), specific (interferences not detected) and precise (CV<15%). « Positive » samples (ADA+) from patients treated with REMICADE[®] were confirmed « positive » with a kit already on the market. « Positive » samples from patients treated with REMICADE[®] or HUMIRA[®] were confirmed « positive » when tested in a inhibition assay from Bmd. No « positive » anti-Etanercept sample was found.

- References** :
- Ram Raj Singh, et al., TNF α blockade in human diseases : mechanisms and future. Clin.Immunol., 2008 February ; 126(2):121-136
 - Anthony R. Mire-Sluis, et al., Recommendation for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. Journal of Immunological Methods, 333 (2008) 1-9
 - Eugen Koren, et al., Recommendation on risk-based strategies for detection and characterization of antibodies against biotechnology products. Journal of Immunological Methods, 289 (2004) 1-16.
 - Isabel Bittel, Feedback from European Regulators on real life submissions. Immunogenicity for Biologics, 8 september 2009, Prague.
 - Ana T. Menendez., Detection and characterisation of immunogenicity of therapeutic biologics. Immunogenicity for Biologics, 8 september 2009, Prague.