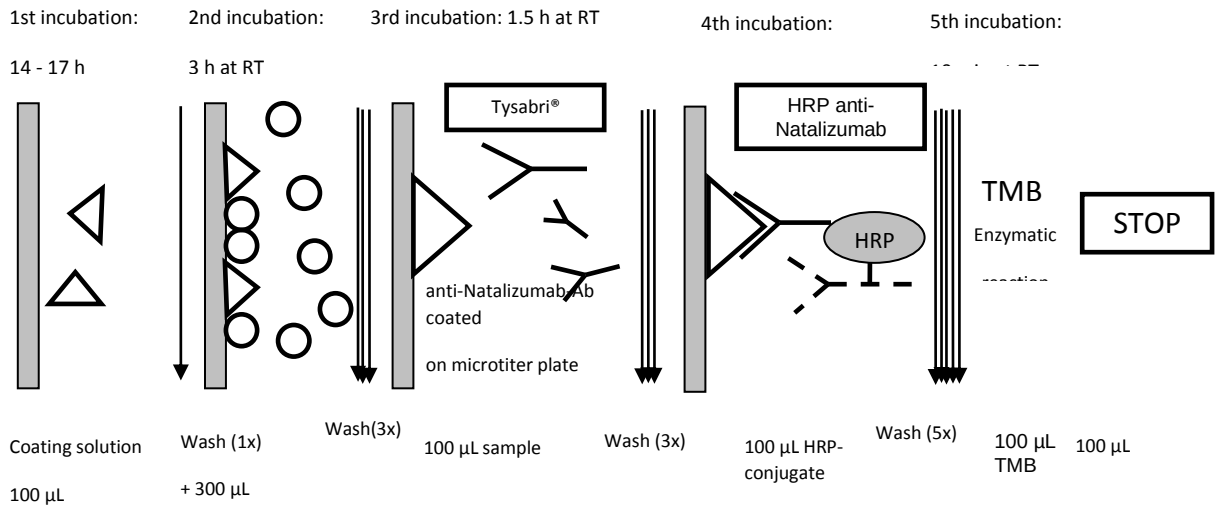
SUPPLEMENTARY DATA: Section 1

1 Validation of an ELISA-assay for the quantitative determination of natalizumab in serum samples.

Subject of this validation was the quantitative determination of Tysabri® (Natalizumab) in serum samples. This assay employed the quantitative antibody sandwich enzyme immunoassay technique (ELISA). A microtiter plate was coated with a specific anti-Natalizumab antibody. After blocking, calibration samples, quality control samples (QCs) or study samples were pipetted into the wells. After washing, the HRP-conjugated secondary anti-Natalizumab antibody was added to the wells. After washing away the mixture of secondary antibody and unbound substance, HRP bound in the complex was visualized by 3,3',5,5' tetramethylbenzidine (TMB) substrate solution. After stopping the enzymatic reaction with sulphuric acid, the intensity of the resulting colour was determined colourimetrically at 450 nm (reference wavelength at 620 nm). The colour intensity was proportional to the concentration of Tysabri® (Natalizumab) in the sample. For details on the method process see the following Figure 1:

Figure 1 Scheme of analytical method optimisation for natalizumab determination in plasma of cynomolgus monkeys.



1.1 Chemicals & equipment

Coating solution: 1 × PBS (Fisher Scientific, 14-190-169), coating antibody: Anti-Natalizumab,

human, clone AbD21375 (HCA248, abdserotec), batch no. 1703, washing buffer: 1 × PBS (Fisher Scientific, 14-190-169), 0.05% (v/v) Tween 20 (Merck 822184), blocking solution: 3% BSA Fraction V (Merck 112018) in 1 x PBS, assay buffer (for sample dilution): PBS (Fisher Scientific, 14-190-169) + 1% BSA Fraction V (Merck 112018) + 10% Cynomolgus monkey blank serum (pool of 10 Cynomolgus monkeys provided by LPT), calibration standard: Tysabri® (Natalizumab), 20 mg/mL. Tysabri® (20 mM Sodium Phosphate Buffer (Na-Pi), pH 6.5, 15 mM NaCl; βProt = 23.97 mg/mL; Lot 1420793) was stored at +2°C to +8°C. 30 µL aliquots were made and stored at -80°C. The calibration standards were prepared fresh from the stock solutions and used directly after preparation. On the day of experiment, aliquots were diluted in dilution buffer to 25, 12.5, 6.25, 3.13, 1.56 and 0.78 ng Tysabri®/mL freshly. Secondary antibody: HRP conjugated anti-Natalizumab, human, clone AbD21375_hlgG1 (HCA249P, abdserotec), batch no. NN0915, substrate solution: TMB-Fertiglösung (Abcam, ab171522), stop solution: H2SO4 (0.5 M) (Honeywell, 10315797; Lot G1600), pooled Cynomolgus blank serum of 5 male and 5 female individuals. Equipment used: Flat bottomed MTPs, PP, Maxisorb 442404 type (Nunc), with Magellan software V6.4/7.2 on Tecan host computer, Microtiterplate reader / ELISA-Reader, Sunrise (Tecan Deutschland GmbH, 74564 Crailsheim, Germany), incubator Titramax 1000 + Inkubator 1000 (Heidolph).

1.2 Samples preparation and method validation

For the validation of the analytical method, replicate analyses of samples containing known amounts of analyte (QC samples) were conducted. QC samples are matrix (in this study pooled Cynomolgus monkey blank serum) spiked with analyte. For validation of serum sample analysis, pooled Cynomolgus monkey serum was spiked to concentrations of approx. 85.7 and 857 µg/mL, respectively. The spiked matrices were diluted into the range of the standard curve. Five concentrations representing the entire range of the standard curve were studied (QC samples): 0.78 ng/mL, 2.3 ng/mL, 4.5 ng/mL, 10 ng/mL and 25 ng/mL (corresponding to the anticipated LLOQ (LLOQ-QC), one within approx. 3x of the LLOQ (low QC sample), one near the center (middle QC), one near the upper boundary of the standard curve (high QC) and one corresponding to the anticipated ULOQ (ULOQ-QC)). The LLOQ is the lowest standard of the assay (see also Section 3.8.6). The ULOQ is the highest standard of the assay. These stock solutions were generated by spiking Cynomolgus monkey blank serum with appropriate amounts

of Tysabri® (Natalizumab) and further dilution with an appropriate diluent. To achieve the concentration of the high QC (10 ng/mL) a dilution factor of 1:85700 was appropriate, mimicking the dilution factor for the theoretically highest concentrated study samples. Validation criteria are described in Table 1

Table 1**.** Validation criteria ELISA method for determination of natalizumab concentration in plasma samples of cynomolgus monkeys.

Parameter	**Method for the Determination of Tysabri® (Natalizumab) in Cynomolgus Monkey Serum**
Calibration /Standard curve (Range: 0.78 ng/mL to 25 ng/mL)	Inaccuracy below ± 20% (± 25% at LLOQ/ULOQ) Accuracy: 84.6% to 114.1% Imprecision below 20%CV (25% at LLOQ/ULOQ) Imprecision: ≤ 10.55%CV Coefficient of correlation ≥ 0.990 (nominal conc. vs. actual conc.) Coefficient of correlation (r): 0.999
Accuracy	Inaccuracy below ± 20% (± 25% at LLOQ/ULOQ) Accuracy: 83.6% to 115.8% Inaccuracy (mean): 7.79%
Precision	Imprecision below 20%CV (25% at LLOQ/ULOQ) Intra-assay Imprecision: ≤ 7.87%CV Inter-assay Imprecision (overall): 8.75%CV Total error below 30% (40% at LLOQ/ULOQ) Total error: 2.9% to 16.7%
Stability	No apparent degradation >±20% of nominal value (>±25% at LLOQ/ULOQ) was detected after 1 and 3 freeze / thaw cycles (with a freezing period of at least 24 hours per cycle). The determination of long-term stability after 2 and 3 months at ≤-15°C is still in progress.

1.3 Results

Calibration curve

Sets of test item standards of Tysabri® were analysed in the range of 0.78 to 25 ng/mL. The accuracy of the back-calculated concentrations ranged from 84.6% to 114.1% (Tysabri®) for all concentrations and was within the admissible limits of 80% and 120% (75% and 125% at LLOQ/ULOQ). The mean inaccuracy per concentration for all standards was ≤±9.3%. The

coefficient of variation (%CV; %RSD) of all analysed samples per concentration was below the admissible limit of 20% (25% at LLOQ/ULOQ). The coefficient of variation (%CV; %RSD) was ≤10.55% for each concentration of the test item standards. The coefficient of correlation (r) was 0.999 (Tysabri®) for back-calculated concentrations and therefore above the admissible limit of ≥0.990. All calibration standards met the admissible limits. The re-calculated concentrations corresponded to the nominal concentrations, indicating the absence of a systematical calibration error. The detector response (OD) of the lowest calibration standard (0.78 ng/mL) was equal or higher than the mean OD of 10 blank samples added to the standard deviation of the OD of these blank samples multiplied with 10, so that the lowest calibration standard (0.78 ng/mL) was accepted as the lower limit of quantification (LLOQ).

Accuracy and precision

The accuracy and the intra-assay and inter-assay precision of the method were determined by calculation of test item samples at concentrations of 0.78, 2.3, 4.5, 10 and 25 ng/mL. The accuracy of all results of Tysabri® samples was within the admissible limits of 80% to 120% (75% to 125% at LLOQ/ULOQ). The recovery ranged from 83.6% to 115.8% (Tysabri®) for all concentrations. The mean inaccuracy per concentration ranged from 7.0% to 8.9% for Tysabri® determined for samples at concentrations of 0.78, 2.3, 4.5, 10 and 25 ng/mL. The overall mean of inaccuracy of the method was 7.79% (Tysabri®). The intra-assay and inter-assay imprecision of all Tysabri® samples were below the admissible limit of ≤ 20%CV (≤ 25% at LLOQ/ULOQ). The coefficient of variation (%CV, %RSD) determined by intra-assay analyses ranged from 0.85% to 7.87% for Tysabri® for all concentrations. The inter-assay imprecision (%CV, %RSD) per concentration of the analytical method ranged from 6.88% to 9.72% for Tysabri®. The overall inter-assay imprecision for Tysabri® was 8.75%. All samples met the admissible limit for the total error (%TE) of ≤ 30% (≤ 40% at LLOQ/ULOQ). The total error (%TE) ranged from 2.9% to 16.7% (Tysabri®) for all concentrations in all assays.

Stability

The freeze / thaw stability of the analyte (Tysabri®) in matrix (Cynomolgus monkey serum) was tested after 1 and 3 freeze / thaw cycles (with a freezing period of at least 24 hours per cycle). Two concentrations (85.7 and 857 µg/mL) were tested directly after preparation (no freeze / thaw cycle) and after 1 and 3 freeze / thaw cycles. Before measurement the samples were diluted to concentrations of 0.78, 2.3, 4.5, 10 and 25 ng/mL, respectively, and the freeze / thaw stability

was evaluated. The mean recovery of all Tysabri® samples after 1 or 3 freeze / thaw cycles was within the admissible limits of 80% to 120% (75% to 125% at LLOQ/ULOQ). The mean recovery of the samples ranged from 87.5% to 109.4% (Tysabri®) for all concentrations.

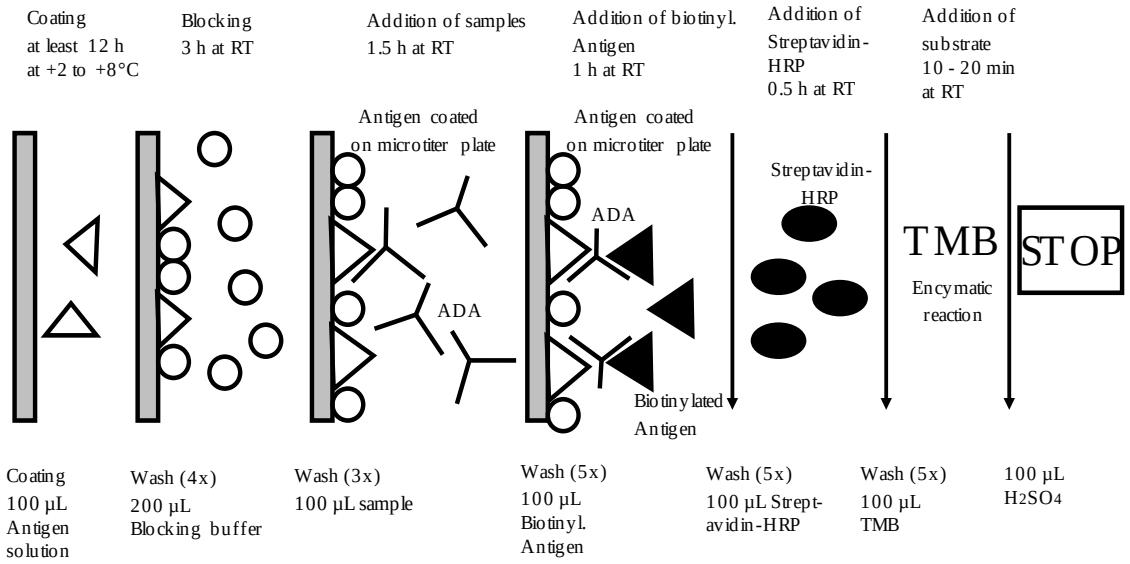
2 Validation of an ELISA-assay for the determination of the anti-drug antibody (ADA) response induced in cynomolgus monkeys against natalizumab.

The immunogenicity of Tysabri® was assessed in screening assay and specificity confirmation assay for positive samples. The analytical method was validated by determination of the normalization factor for in-study screening cut-point calculation, the sensitivity, specificity and linearity of the screening assay, the spike concentration for specificity cut-point determination, the specificity cut-point, the concentration of the low quality control sample (QC) and the acceptance criteria for QC samples. Validation was performed with regard to precision (repeatability and intermediate precision) and assay interference by matrix components or Tysabri®. ADA assays require establishment of two critical decision parameters: a 'screening cut-point' for the screening assay and a 'specificity cut-point' for the specificity confirmation assay. The screening cut-point enables the classification of ADA results as either antibody negative or reactive samples (potential positive samples). The screening cut-point was determined by means of statistical analysis of negative samples. The reactive samples undergo further characterization to categorize them as positive versus negative (non-specific reactive) samples by means of a specificity confirmation assay (signal inhibition by competition with drug). The magnitude of signal inhibition required for a sample to be deemed as containing drug-specific ADA is termed specificity cut-point and was experimentally established.

In this study, the bridging ELISA format was used for the detection of ADA. A microtiter plate was coated with Tysabri®. After blocking, the positive control (a polyclonal monkey anti-Tysabri®), the negative control (monkey pool serum), calibration samples or the quality controls (QCs) were added to the wells. After having added the samples, the biotinylated drug was added. Unbound antibody was removed by washing, followed by addition and incubation of peroxidase-conjugated streptavidine. After removing the unbound conjugate by washing, the chromogenic substrate 3,3´,5,5´tetramethylbenzidine (TMB) was added and incubated. After stopping the enzymatic reaction with sulphuric acid, the intensity of the resulting colour was determined

colourimetrically at 450 nm (reference wavelength at 620 nm). The colour intensity was proportional to the concentration of ADA in the sample (Figure 2).

Figure 2 Scheme of analytical method optimisation for ADA determination in plasma of cynomolgus monkeys.



Coating buffer, 1 × PBS 006 (Fisher Scientific, 14-190-169), washing buffer, 1 × PBS (Fisher Scientific, 14-190-169), 0.05% (v/v) Tween 20 (Merck 822184), blocking solution 2% BSA (Sigma Aldrich A7906) in 1 × PBS, sample dilution buffer, 1% BSA (Sigma Aldrich A7906) in 1 × PBS, Positive Control / Calibration standard monkey polyclonal antibody to Tysabri® at a concentration of 0.7 mg/mL (provided by LPT and BioGenes), for calibration standards diluted in 1 % BSA/PBS + 10 % pooled monkey blank serum to 50, 25, 12.5, 6.25, 3.13 1.57 and 0.79 ng/mL. Negative control 1% BSA/PBS + 10% monkey blank serum (pooled from 30 different drug naïve animals).Negative control for specificity testing: Human IgG (Sigma, product no. I4506; Lot. 121M7009V); diluted in 1% BSA/PBS + 10% pooled Cynomolgus monkey serum to 50, 25, 12.5, 6.25, 3.13 1.57 and 0.79 ng/mL. Biotinylated antigen: Biotinylated Tysabri® (Tysabri®; biotinylated by Biogenes); diluted 1:50000 in sample dilution buffer. Peroxidase-conjugated streptavidin: Peroxidase-conjugated streptavidin (Jackson ImmunoResearch, 016-

030-084); diluted 1:5000 in PBS. Substrate solution: TMB: TMB-Fertiglösung (Sigma, T4444), diluted 1:2 in distilled water. Stop solution: H2SO4: H2SO4 (1 N) (AppliChem, 181059.1211). Equipment see section 1.1.

2.2 Samples preparation and method validation

The microtiter plates were coated with 50 ng Tysabri® per mL coating buffer. 100 µL of the Tysabri® solution were dispensed into each well. The solution was dispersed evenly over the bottom of the well by carefully tapping on the side of the microtiter plate (visual control). The plate was covered with an adhesive film and incubated overnight on a horizontal shaker (approx. 120 rpm) in the refrigerator.The contents were briskly shaken out of the wells. The wells were rinsed 4 times with washing buffer (250 µL per well). The plate was stricken sharply on absorbent paper to remove residual droplets. 200 µL blocking solution were dispensed into each well and incubated for 3 hours on a horizontal shaker (approx. 300 rpm) at room temperature. After blocking, the contents were briskly shaken out of the wells. The wells were rinsed 3 times with washing buffer (250 µL per well). Then the wells were stricken sharply on absorbent paper to remove residual droplets. Then 100 µL blank, calibration standard and samples were added into the wells. The plate was covered and incubated on a horizontal shaker (approx. 300 rpm) at room temperature for 1.5 hour. After the sample incubation, the contents were briskly shaken out of the wells. The wells were rinsed 5 times with washing buffer (250 µL per well). The wells were stricken sharply on absorbent paper to remove residual droplets. Then 100 µL biotinylated Tysabri® (diluted 1:50000) were added into each well. The plate was covered and incubated on a horizontal shaker (approx. 300 rpm) at room temperature for 1 hour. The contents of the wells were briskly shaken out. The wells were rinsed 5 times with washing buffer as described above. Then 100 µL peroxidase-conjugated streptavidin (0.2 µg/mL) was added per well. The plate was covered and incubated on a horizontal shaker (approx. 300 rpm) for 0.5 hour at room temperature. The contents of the wells were briskly shaken out. The wells were rinsed 5 times with washing buffer as described above. Then 100 µL substrate solution (diluted 1:2) were quickly added per well. The plate was incubated in the dark at room temperature. The enzymatic reaction was stopped after 10-20 minutes by adding 100 µL of Stop Solution to each well. Immediately after stopping the enzymatic reaction, the intensity of the resulting colour was

determined colourimetrically at 450 nm (reference wavelength at 620 nm) with a microtiter plate reader. The colour intensity was proportional to the concentration of ADA in the sample.

The standard curve was composed of six concentration points: 50, 25, 12.5, 6.25, 3.13, 1.57 and 0.79 ng/mL in negative control buffer. Coefficient of variation (%CV) of concentration values from at least five measurements per concentration point had to be less than or equal to 20% and the %CV for the lowest value (0.79 ng/mL) had to be less or equal to 25%. The concentration of the high quality control sample was chosen from the linear range of the calibration curve, just below the upper plateau of the curve. In this validation study 35 ng/mL (concentration on the plate) of the positive control served as high QC. A control sample including 5 ng/mL of the positive control served as intermediate quality control (twice; at the start and the end of the plate). In this validation study, 0.7 ng/mL of the positive control served as low QC. Coefficient of variation (%CV) from at least five measurements per concentration of the low, intermediate and high positive control had to be less than or equal to 15%. Coefficient of variation (%CV) of the individual values of each concentration of the low, intermediate and high positive control of all evaluated microtiter plates had to be less than or equal to 25%. 80% - 120% (75% - 125% at LLOQ/ULOQ) acceptance criteria for the in-study phase were determined in this validation study.

2.3 Results

Calibration curve

A set of calibration standards in the range of 35, 17.5, 8.75, 4.38, 2.19, 1.10, and 0.55 ng/mL was analysed. The calculated sensitivity (including a 5% rejection rate) in 1:10 diluted matrix was determined as 0.576 ng ADA/mL corresponding to 5.76 ng ADA/mL in undiluted serum. Sets of test standards (anti-Tysabri®) were analysed in the range of 0.79 to 50 ng/mL. The coefficient of variation (%CV; %RSD) was ≤6.16% for each concentration of the test standard and thus below the admissible limit of 20% (25% at LLOQ). The re-calculated concentrations corresponded to the nominal concentrations, indicating the absence of a systematical calibration error. The lowest standard on the calibration curve (0.79 ng/mL) was accepted as the lower limit of quantification (LLOQ) (internal pre-validation experiments). Samples at a level of 35 ng/mL of the positive control served as high control sample (QC), 5 ng/mL as intermediate QC and 0.7 ng/mL as low QC. During sensitivity assessment, the calculated concentration of the low QC (including a 1%

rejection rate) was determined as 0.693 ng of the positive control per mL. As a result, samples with concentrations of 0.7 ng ADA/mL were used as low quality control (QC) samples. The concentration of the low QC (0.7 ng/mL) was below the lowest calibration standard (LLOQ) and thus no valid quantification could be performed.

Accuracy and precision

The intra-assay and inter-assay precision of the method were determined by calculation of the %CV of QC samples at concentrations of 0.7, 5 and 35 ng/mL analysed in six independent runs on 3 different days. The determination of screening assay precision revealed that the results of the samples met the acceptance criteria. The coefficient of variation (%CV, %RSD) determined by intra-assay analyses ranged from 2.99% to 8.47% for the high QC and from 2.12% to 6.61% for the intermediate QC. The inter-assay imprecision (%CV, %RSD) of the analytical method ranged from 6.75% (high QC) to 4.80% (intermediate QC). The accuracy (recovery) for the high QC ranged from 88.2% to 117.9% and 91.8% to 111.2% for the intermediate QC. The mean of inaccuracy was 5.30% for the high QC and 4.10% for the intermediate QC. The concentration of the low QC (0.7 ng/mL) was below the lowest calibration standard (LLOQ) and thus no valid quantification could be performed. The inter-assay precision of the specificity confirmation assay was determined by calculation of the %CV of the signal inhibition of QC samples at concentrations of 0.7, 5 and 35 ng/mL analysed in six independent runs on 3 different days. The coefficient of variation (%CV, %RSD) determined by inter-assay analyses was 0.69% for the high QC and 2.34% for the intermediate QC. These values were below the admissible limit of 25%.

Stability

SUPPLEMENTARY DATA: Section 2

Table 2. Tissues collected for preservation & histopathology

Adrenal gland	Muscle (skeletal, leg)
Aorta abdominalis	Nerve (sciatic)
Bone (os femoris with joint)	Oesophagus
Bone marrow (os femoris)	Ovary
Brain (cerebrum, cerebellum, brain stem,	Pancreas
hippocampus, paraventricular parts)	Pituitary
Caecum	Prostate
Epididymis	Salivary glands (mandibular,
Eye with optic nerve	parotid, sublingual)
Gall bladder	Seminal vesicle
Gross lesions	Skin (left flank)
Heart (left/right ventricle, septum)	Spinal cord (3 sections)
Infusion site	Spleen
Intestine, large (colon, rectum)	Sternum
Intestine, small incl. Peyer's patches	Stomach
(duodenum, jejunum, ileum)	Testicle
Kidney and ureter	Thymus
Lacrimal gland	Thyroid (incl. parathyroids)
Liver (3 sections: left (medial), right (medial)	Tissue masses or tumours
and caudal lobe)	(incl. regional lymph nodes)
Lungs (with mainstem bronchi and	Tongue (incl. base)
Bronchioles; 3 sections: one through	Trachea (incl. larynx)
each of three different lobes)	Urinary bladder
Lymph node (1, cervical)	Uterus (incl. cervix and oviducts)
Lymph node (1, mesenteric)	Vagina
Mammary gland	