

# PRIMAGAM<sup>®</sup> - The primate IFN- $\gamma$ test

**B. Schroeder**

{Prionics AG, Schlieren, Switzerland}

Correspondence to: Bjoern.Schroeder@prionics.com

## 1. Introduction

On December 2<sup>nd</sup> 2010, Prionics gave a presentation about PRIMAGAM<sup>®</sup> - the primate interferon- $\gamma$  test, at the German Primate Center (DPZ) in Göttingen, Germany. This paper summarizes the most relevant aspects of the presentation.

### 1.1. History of Tuberculosis

It is thought that the origin of *Mycobacterium (M.)* causing tuberculosis (TB) was initially found in soil. During the domestication of cattle, which occurred between 10,000 and 25,000 before Christ (B.C.), the passage of mycobacteria to humans and its adaptation occurred shortly thereafter. Typical TB causing deformities on bones are apparent in Egypt mummies, and successful TB isolation (part of *M. tuberculosis*) in Neolithic skeleton has been performed.

In recorded history, Assyrian clay tablets described patients coughing blood (700 B.C.). Two hundred years later, Hippocrates wrote of patients with consumption, i. e. wasting away associated with chest pain (Greek term: phthisis) and blood in the sputum. The start of urban development causing epidemics in Europe occurred in the 16th and 17th century and achieved a peak in middle of 19th century. In 2010, still 2 million people are dying yearly worldwide on TB, and yearly 8 million new infections occur.

The human TB causing agent, *Mycobacterium tuberculosis*, could be identified in 1882 by Robert Koch, and “his” tuberculins are still used in TB diagnostics since 1907. Antibiotic treatment is available since the late 1940s. Currently, there is no effective vaccine for TB prevention in the market. The Bacille Calmette–Guérin (BCG) vaccines have a questionable efficacy profile.

## 1.2. Tuberculosis - Diagnostic Test Approaches

TB is a disease, for which no diagnostic gold standard is available. There are a couple of principle diagnostic tests available: Culture, PCR, Tuberculin testing (Caudal Fold test (CFT), Single Intradermal Cervical Test (SICT), Comparative Intradermal Tuberculin Test (SICCT)). In non-human primates, eyelid injections or abdominal skin injections are the principle diagnostic tests for TB diagnosis.

All the above mentioned tests for non-human primates are stressful for the animals due to the need of anaesthesia to perform the test and a second anaesthesia for reading the results. Thus, a blood based in-vitro assay like the PRIMAGAM<sup>®</sup> test for optimal TB control is needed. PRIMAGAM<sup>®</sup> is a rapid in vitro blood-based assay of cell mediated immune response to *M. bovis* tuberculin purified protein derivatives (PPD) for the diagnosis of tuberculosis infection in non-human primates. PPD antigens or synthetic antigen cocktails are presented to lymphocytes in whole blood culture. The resulting production of interferon  $\gamma$  (IFN-  $\gamma$ ) by the TB-exposed cells is then detected using a monoclonal antibody-based sandwich enzyme immunoassay (EIA).

## 2. PRIMAGAM<sup>®</sup>

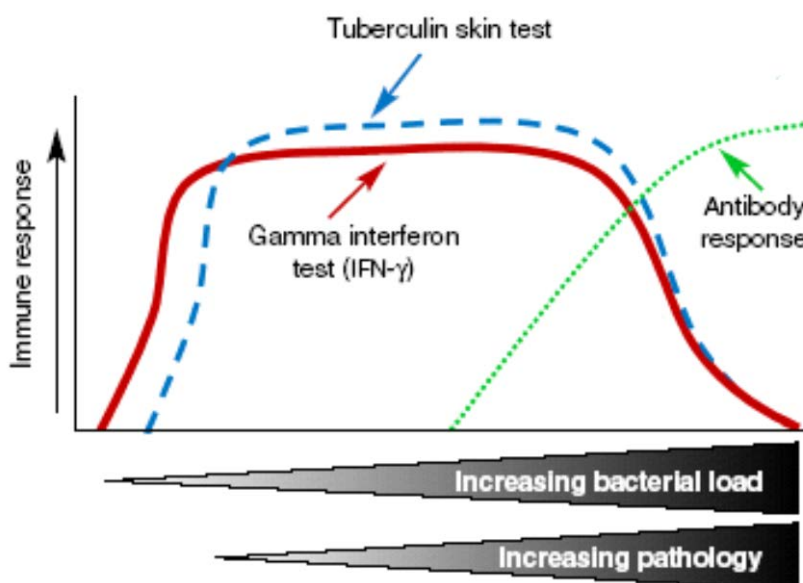
### 2.1. Principles of PRIMAGAM<sup>®</sup>

The customer's wish is to achieve 100 % specificity and 100 % sensitivity of a diagnostic test. Based on fundamental characteristics of T helper cell 1 stimulation, this is impossible to achieve. The immune system is individual and may vary between primates (cellular subsets e. g. B-cells, T-cells). Additionally, the environmental impact on each animal (genetic background, stress, age, infectious status, herd environment) might be different. Although the T-cell stimulation pathway is in general similar between primates, small variances of environmental or external factors directly influence the range of sensitivity and specificity of the test system. As a consequence , relative variances in the assay outcome are one of the characteristics of all used stimulation approaches (skin test, IFN-  $\gamma$  test). As a gold standard is not available, the customer's need might be either a very high specificity or acceptance of lower sensitivity or *vice versa*.

The advantage of the IFN- $\gamma$  test is the early detection of TB compromised animals in a colony in comparison to standard humoral immune response test systems. In the subclinical phase, mainly the cellular immune response is dominant, which normally

correlates with a low bacterial load. Within the progressive course the bacterial load increases and the IFN- $\gamma$  response decreases. On the other hand, the humoral immune response increases.

Figure 1: Comparison of IFN- $\gamma$  diagnostic test in comparison with test based on the humoral immune response.



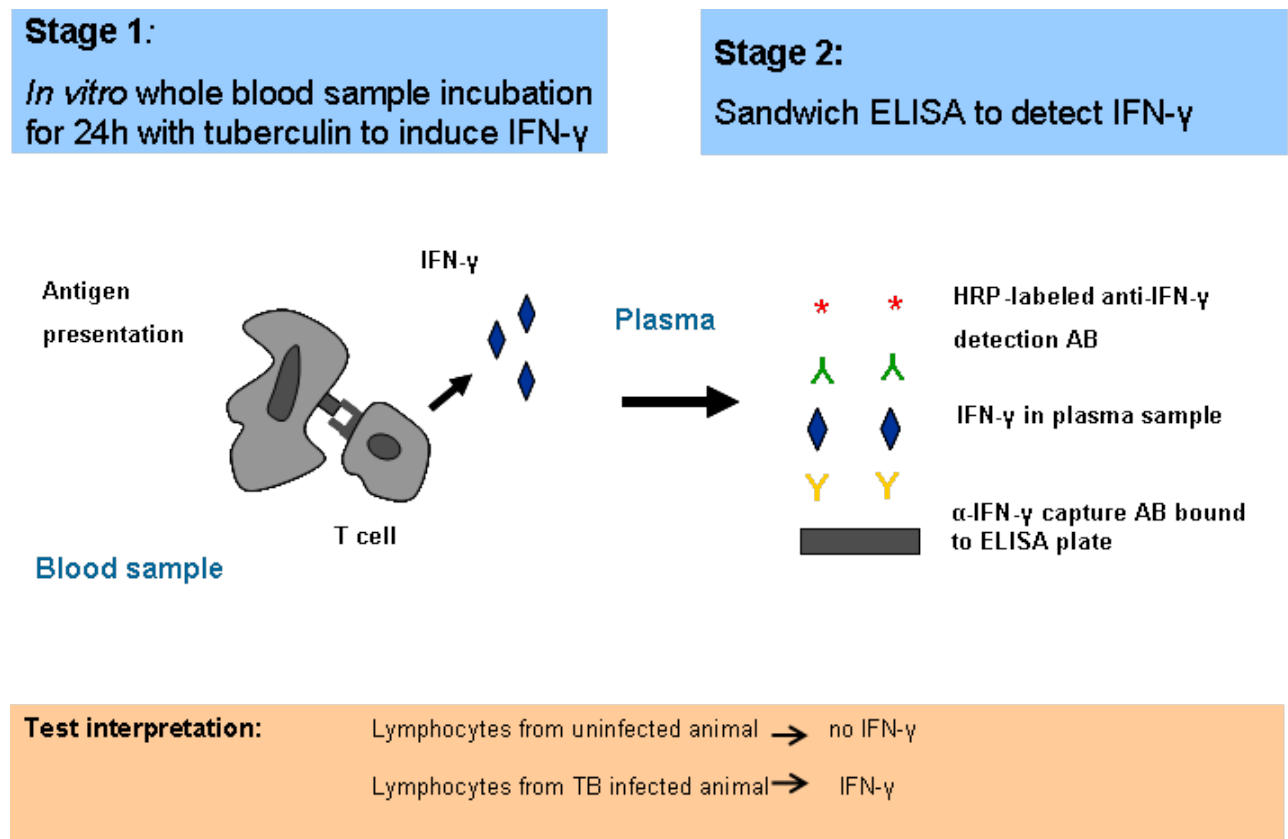
Vordermeier *et al.*, The Veterinary Record, July 10, 2004

PRIMAGAM<sup>®</sup> is a modification of the original BOVIGAM<sup>®</sup> test, a blood-based assay of cell mediated immunity for the diagnosis of bovine tuberculosis in cattle. Animals infected with mycobacteria can be identified by measuring the cytokine IFN- $\gamma$  against tuberculin, an antigen used to aid in the diagnosis of TB infection. PPD antigens are presented to lymphocytes in whole blood cultures, and the production of IFN- $\gamma$  from the stimulated T cells is detected using a monoclonal antibody-based sandwich enzyme immunoassay (EIA). Lymphocytes from uninfected animals do not produce IFN- $\gamma$  to tuberculin PPD antigens and, hence, IFN- $\gamma$  detection correlates with infection.

The PRIMAGAM<sup>®</sup> test involves two stages. In the first stage, blood samples are incubated overnight with antigen (e. g. tuberculin PPD) to stimulate the lymphocytes to produce IFN- $\gamma$ . In the second stage, IFN- $\gamma$  in the plasma supernatants of each blood aliquot is determined using a sandwich EIA. IFN- $\gamma$  in the sample binds to antibodies to primate IFN- $\gamma$

bound to a solid support and is visualized with a second anti-primate IFN- $\gamma$  antibody labelled with an enzyme that generates a colour signal. Colour development is proportional to the amount of bound IFN- $\gamma$ .

Figure 2: Illustration of the PRIMAGAM<sup>®</sup> assay.



## 2.2. Controls

All acute viral and bacterial infections may cause a basal level of INF- $\gamma$  production in individual species, which is measured as NIL value in the test system. Beside CD4+T cells, several other cell types are able to produce IFN- $\gamma$ .

Viability of cells should be evaluated in all non-human primate samples in order to ensure that cells are not compromised by any reasons. The viability of whole blood cells can be measured by use of different stimulation agents e. g. superantigens (SEB) or mitogens (PWM). Samples with low optical density (OD) values indicate that the cells in the stimulation approach do not demonstrate sufficient viability (bovine samples > 0.5 OD). The described approach is only a measurement of the general viability of cells. It is not a

stimulation control for the IFN- $\gamma$  assay as, for at least for PWM, we do not need an interaction between antigen presenting cells (APCs) and T-cells. Figure 3 summarizes the different non-human primate species, for which mitogens have been tested.

Figure 3: Mitogens which have been tested in different primate species with PRIMAGAM<sup>®</sup>.

Common Name	Genus & Species	No.	Mitogen*				
			PHA	ConA	PWM	SEA	SEB
Chimpanzee	<i>Pan troglodytes</i>	51	+++	+	+	+	+
Gibbon	Not specified	2	++++	++	ND	ND	ND
Gibbon – white cheeked	<i>Hylobates leucogenys</i>	3	++++	ND	++++	+++	++++
Gibbon – siamang	<i>Hylobates syndactylus</i>	3	++++	++++	++++	ND	++++
Gorilla	<i>Gorilla gorilla</i>	28	++	+	++	++	++
Green monkey – vervet	<i>Chlorocebus aethiops</i>	5	-	+	+++	ND	++++
Guenon/De Brezza's	<i>Cercopithecus neglectus</i>	8	-	++	++	+	+
Guereza	<i>Colobus guereza</i>	8	-	++	++	++++	+++
Langur – ebony leaf	<i>Trachypithecus auratus auratus</i>	10	++	++++	+++	++++	++++
Langur – dusky	<i>Trachypithecus obscurus</i>	2	ND	ND	++++	+++	+++
Lemur – ring-tailed	<i>Lemur catta</i>	7	-	-	-	-	-
Lemur – ruffed	<i>Varecia variegata</i>	2	-	+	-	-	-
Lemur – white fronted	<i>Eulemur fulvus albifrons</i>	1	ND	ND	-	ND	ND
Macaque – crab eating	<i>Macaca fascicularis</i>	158	-	++	-	ND	+
Macaque – lion tail	<i>Macaca silenus</i>	7	+	-	-	-	-
Macaque – pig tail	<i>Macaca nemestrina</i>	70	-	+	+	+	+
Macaque – rhesus	<i>Macaca mulatta</i>	203	-	+	ND	ND	+
Mandrill	<i>Mandrillus sphinx</i>	8	+++	+	+++	ND	++
Marmoset	<i>Callithrix jacchus</i>	2	ND	ND	+++	ND	+++
Orangutan	<i>Pongo pygmaeus</i>	13	++++	++	+++	++++	++++
Owl monkey	<i>Aotus lemuinus griseimembra</i>	12	ND	ND	ND	ND	+++
Squirrel monkey	<i>Siamiri sciureus</i>	17	+	ND	++++	ND	++++
Spider monkey	<i>Ateles geoffroyi</i>	5	-	-	+	+	+
Tamarin – cotton top	<i>Saguinus oedipus</i>	6	ND	ND	ND	ND	+
Tamarin – emperor	<i>Saguinus imperator</i>	1	ND	ND	+++	ND	ND
Tamarin – golden lion	<i>Leontideus rosalia</i>	4	-	-	+	+	++
<b>Total</b>		<b>656</b>					

- Relative amounts of IFN- $\gamma$  stimulated by each mitogen; ND = not done.
- Key:
- |                               |                                 |
|-------------------------------|---------------------------------|
| + Absorbance readings > 0.15  | +++ Absorbance readings > 1.00  |
| ++ Absorbance readings > 0.50 | ++++ Absorbance readings > 2.00 |
| - Absorbance readings < 0.15  | ND= Not done                    |

### 2.3.1. Specificity

Specificity and sensitivity of PRIMAGAM<sup>®</sup> have been validated for *Macaca fascicularis* and *Macaca mulatta*. In a first trial, specificity was validated on 69 *Macaca fascicularis*. This

work was undertaken by Dr. JoAnn Yee, University of California, USA, where 69 juvenile and adult captive-bred cynomolgus macaques (*Macaca fascicularis*) have been investigated. Gender distribution was 21 females and 33 males (gender not recorded for 15 animals). Average age was 4.5 years (+/- 1 year). All animals were skin test negative at the time of investigation. Three animals had a history of false positive skin test reactions. 69 animals were tested, 0 found to react positively in the PRIMAGAM<sup>®</sup> test. Conclusively, the specificity for cynomolgus macaques was found to be 100 % (Literature: Species testable by PRIMAGAM<sup>®</sup> test, USDA Approval Dossier, 2005, not published).

In addition, specificity of PRIMAGAM<sup>®</sup> has been validated for rhesus macaques. This work was also undertaken by Dr. JoAnn Yee, University of California, USA. She investigated 41 juvenile and adult captive-bred rhesus macaques, of which 23 were females and 18 were males. Average age was 2.6 years (+/- 2.7 month), and all animals were skin test negative. 41 animals have been tested, 0 found to react positively in the PRIMAGAM<sup>®</sup> test. Conclusively, the specificity for rhesus macaques was found to be 100 % (Literature: Species testable by PRIMAGAM<sup>®</sup> test, USDA Approval Dossier, 2005, not published).

### 2.3.2. Sensitivity

The sensitivity of PRIMAGAM<sup>®</sup> has been tested for *Macaca fascicularis* and *Macaca mulatta*. All work was done by Dr. JoAnn Yee, University of California, USA. A total of 54 captive-bred cynomolgus macaques and 22 rhesus macaques were investigated. Age and gender were not recorded. Skin test positive animals were necropsied. TB status was confirmed on basis of gross lesions, histopathology and positive culture results consistent with *M. tuberculosis* complex infection (Literature: Species testable by PRIMAGAM<sup>®</sup> USDA Approval Dossier, 2005, not published). Table 1a compares the outcome of the mutual comparison of skin test versus (vs.) PRIMAGAM<sup>®</sup> tested animals.

PRIMAGAM<sup>®</sup> sensitivity was 88.2 %, which was identical ( $p = 0.68$ , McNemar's Chisquare test) with those of the skin test (84.4 %). All 37 cynomolgus macaques without tuberculosis were PRIMAGAM<sup>®</sup> test negative, and 36/37 animals were also negative in the skin test (97.3 %).

Table 1a: Direct comparison of the assay outcome of the skin test vs. PRIMAGAM<sup>®</sup> test in macaques.

		Skin test		
		Positive	Negative	Total
PRIMAGAM	Positive	12	3	15
	Negative	3	36	39
	Total	15	39	54

Table 1: From: Species testable by PRIMAGAM<sup>®</sup> USDA Approval Dossier, 2005, not published.

In rhesus macaques, 22 animals have been tested (table 1b). All 8 animals (36 %) with confirmed tuberculosis were detected as positive reactants by skin test and PRIMAGAM<sup>®</sup> test. Sensitivity and apparent specificity were both 100 %.

Table 1b: Direct comparison of the assay outcome of the skin test vs. PRIMAGAM<sup>®</sup> - rhesusmacaques.

		Skin test		
		Positive	Negative	Total
PRIMAGAM	Positive	8	0	8
	Negative	0	14	14
	Total	8	14	22

Conclusively, the sensitivity of PRIMAGAM<sup>®</sup> test for tuberculosis in macaques was found to be 92 % (100 % for rhesus and 88.2 % for cynomolgus macaques).

In an independent trial published by Garcia et al. (2004), 58 feral cynomolgus macaques of Mauritius origin and 22 female and male rhesus macaques were investigated at Stanford University School of Medicine, California, USA. The age was not recorded. All skin test positive animals were necropsied, and TB status was confirmed on basis of gross lesions, histopathology and positive culture results consistent with *M. tuberculosis* complex infection. Tables 2 and 3 summarize the results of this trial.

Table 2: Two different cut-offs with PRIMAGAM<sup>®</sup> were compared with necropsy results.

Cutoff	PRIMAGAM	Necropsy		PV (%)
		Positive	Negative	
0.05 OD	Positive	17	1	94
	Negative	8	38	83
0.01 OD	Positive	22	3	88
	Negative	3	36	92

Table 3: Skin test results compared with necropsy results.

Cutoff	TST	Necropsy		PV (%)
		Positive	Negative	
Grade 3	Positive	23	6	79
	Negative	4	36	90

The PRIMAGAM<sup>®</sup> demonstrated a good sensitivity (68%) and excellent specificity (97%).

Decreasing the threshold cutoff ends up in an increased sensitivity of 92%

The overall conclusion is consequently that PRIMAGAM<sup>®</sup> can be used for the TB testing for a number of different primate species. So far as known, baboons are the only primate species in which contradictory results of the PRIMAGAM<sup>®</sup> use have been reported.

#### 2.4. Test interpretation

A lot of different factors (which are not completely under control by the investigator, but have been taken into consideration) potentially have an impact on PRIMAGAM<sup>®</sup> test result interpretation. Among other things, these are the immune system and the immunological status of each individual animal (e.g. cellular subset of APCs and T-cells, immunosuppression), the infection status with other environmental mycobacteria, the viability of cells (e.g. measured by stimulation with mitogens), the investigated species, the breeding situation ((in)-breed, captive or feral animals), origin of primate species (country), stress, treatment with non-steroidal anti-inflammatory drugs (NSAIDs) and/or corticosteroids, handling of blood samples with correct consideration of the stimulation



time, the time from bleeding until stimulation, use of different tuberculins (e. g PPD vs. peptides), age (e. g. cytotoxic T-cells in juvenile animals can cause false positive results). According to the package insert, the test validity has to be verified by the following specifications:

- Nil value: < 0.2 OD units
- Viability control: individual minimum OD value for each mitogen must be fulfilled
- NC: < 0.15 OD units
- PC: > 1.0

If one of the criteria above could not be met the test interpretation should not be initiated and the test has to be repeated. One should be aware that animals with an acute infection may demonstrate higher Nil OD values than 0.2. The impact of immune suppression on the test result should also be taken into account (e.g. SIV infected primates and NSAIDs).

The test interpretation is:

- If OD of PPD-B – PPD-A > 0.050 OD units → presence of *M. tuberculosis* infection likely
- If OD values are outside the linear range of the ELISA (OD units 0.1 – 2.0) → samples should be diluted with non-stimulated plasma (instability of IFN- $\gamma$ )

A single versus a duplicate well approach can lead to a different interpretation of the test result. As the test variation is around 10 %, small differences can cause contradictory results as outlined in table 4.

Table 4: Input of singlet and duplicate well approach with minor differences in the OD values. First interpretation: Each row is assessed as independent test values. Test result can be positive or negative. Second interpretation: The two values have to be assessed as a double well approach and the mean is calculated. As an outcome single well test evaluation can cause different interpretation than double approaches. Additionally, small variances can end up in a complete different assessment.

	Value 1	Value 2	Mean
PPDB	0.5	0.5	0.5
PPDA	0.45	0.5	0.475
$\Delta$ Value	0.05	0	0.025
Interpretation	Positive	Negative	Negative

	Value 1	Value 2	Mean
PPDB	0.5	0.5	0.5
PPDA	0.44	0.46	0.45
$\Delta$ Value	0.06	0.04	0.05
Interpretation	Positive	Negative	Positive

### 3. Conclusions

- IFN- $\gamma$  test result interpretation in non-human primates must be in context with all other available information.
- In non-human primates PRIMAGAM<sup>®</sup> can be used for all TB tested animals as a primary screening test or as an auxiliary test in combination with other tests.
- Due to the relative low numbers of TB tests in non-human primates, a data exchange between zoos and primate centers is important to increase the data basis for correct interpretation of **each** species.
- As the IFN- $\gamma$  test assay is not easy to handle, some centralized reference centers in Europe might be useful to optimize the TB diagnosis in non-human primates.

### 4. Prionics Tuberculosis Testing Service

Prionics offers a full service for IFN- $\gamma$  testing of primates. In an easy four step approach, Prionics is able to conduct the IFN- $\gamma$  testing and analysis of non-human primates for customer's convenience.

#### Step 1:

Prionics provides the PPD-tuberculins together with a convenient transportation system, which ensures stable 37° C temperature for at least 24 hours.

#### Step 2:

Customer collects whole blood and adds the respective amount of PPDs to the respective sample volume. The blood collection tube containing the stimulated whole blood sample will be placed into the transport device (figure 3).

Figure 4: Transport device – TempShell and TempFrames.



The 37° C TempShell (left) is designed for the incubation of blood during transportation. 37° C TempShells and TempFrames (right) are suitable to replace electric incubating boxes. A liquid crystal thermometer indicates the actual temperature. The TempShell must simply be pre-heated at 37 to 38° C until the fluid is completely liquid. During the solidification, the elements will keep the temperature stable between 37 and 35° C if transported into the TempFrame. The complete system is reusable.

Step 3:

The complete box has to be sent back to Prionics. Stimulation of the whole blood sample occurs during transport.

Step 4:

Tuberculosis testing on the sample is conducted with PRIMAGAM<sup>®</sup> at the diagnostic lab of Prionics under GLP conditions. Analyses and data interpretation will be conducted at Prionics.

## 5. ETHICAL ISSUES

Tuberculosis testing has several ethical implications, which should be considered before the initiation of the testing. The following list of questions should be considered for the individual tuberculosis testing in non-human primates:

- What would be the consequence of a TB true positive result for the individual primate, animal care taker, breeding situation, herd situation, pre-movement situation etc.?
- What is the level of false negative or false positive results I am willing to accept?

- What is the consequence of potentially false positive or false negative results for the individual primate, animal care taker, breeding situation, herd situation, pre-movement situation, etc.?
- What is really the best testing regimen in my situation (e.g. quarantine unite, age of primates, single housing vs. herd housing)?
- Is antibiotic treatment vs. sacrificing of positive reactors possible in my situation?
- Do I fairly balance the economic interests with a potential ethical impact of the test result?

The recommendations of the European Primate Veterinary Association Working Group on Tuberculosis may give guidance to some aspects of the addressed questions.

### **References**

Bushnitz, M., Lécu, A., Verreck, F., Preussing, E., Rensing, S., and Mätz-Rensing, K.: Guidelines for the prevention and control of tuberculosis in nonhuman primates: recommendations of the European Primate Veterinary Association Working Group on Tuberculosis. *J. Med. Primatol.*, 38, 59-69, 2009.

Garcia, M.A., Yee, J., Bouley, D.M., Moorhead, R. and Lerche, N.W.: Diagnosis of Tuberculosis in Macaques, using whole-blood in vitro interferon Gamma (PRIMAGAM®) testing, *Comp. Med.* 54, 86-92, 2004.