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Validation of an immunoassay for anti-thymidine phosphorylase antibodies in patients with MNGIE treated with enzyme replacement therapy

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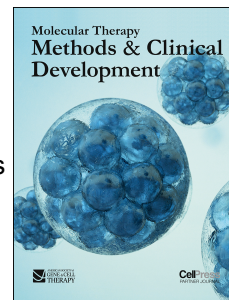
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1 **Title:** Validation of an immunoassay for anti-thymidine phosphorylase antibodies in patients  
2 with MNGIE treated with enzyme replacement therapy

3

4 **Running title:** Assay for anti-thymidine phosphorylase antibodies

5

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17

18 **Key words:** Assay validation; Bridging immunoassay; Enzyme replacement therapy;  
19 MNGIE; Thymidine phosphorylase

20

21

## 22 ABSTRACT

23 Erythrocyte encapsulated thymidine phosphorylase is recombinant *Escherichia coli*  
24 thymidine phosphorylase encapsulated within human autologous erythrocytes and is under  
25 development as an enzyme replacement therapy for the ultra-rare inherited metabolic  
26 disorder, mitochondrial neurogastrointestinal encephalomyopathy. This study describes the  
27 method validation of a two-step bridging electrochemiluminescence immunoassay for the  
28 detection of anti-thymidine phosphorylase antibodies in human serum according to current  
29 industry practice and regulatory guidelines. The analytical method was assessed for screening  
30 cut-point, specificity, selectivity, precision, prozone effect, drug tolerance and stability. Key  
31 findings were a correction factor of 129 relative light units for the cut-point determination; a  
32 specificity cut-point of 93% inhibition; confirmed intra-assay and inter-assay precision, assay  
33 sensitivity of 356 ng/mL; no matrix or prozone effects up to 25,900 ng/mL; a drug tolerance  
34 of 156 ng/mL; and stability at room temperature for 24 hours and up to 5 freeze-thaws.  
35 Immunogenicity evaluations of serum from three patients who received erythrocyte  
36 encapsulated thymidine phosphorylase under a compassionate treatment programme showed  
37 specific anti-thymidine phosphorylase antibodies in one patient. To conclude, a sensitive,  
38 specific and selective immunoassay has been validated for the measurement of anti-  
39 thymidine phosphorylase antibodies; this will be utilized in a phase II pivotal clinical trial of  
40 erythrocyte encapsulated thymidine phosphorylase.

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## 46 INTRODUCTION

47 Enzyme replacement therapies are typically applied to the treatment of individuals with  
48 inherited enzyme deficiency disorders, whereby the deficient enzyme is replaced by regular  
49 infusions of the normal counterpart, with the aim of decelerating the disease progression  
50 process. Current licenced preparations are either purified from natural human or animal  
51 sources, or produced by recombinant technologies, and thus have the potential to induce  
52 undesirable immune responses. Clinical experience has shown that the development of anti-  
53 enzyme antibodies is a common occurrence, with many of the approved enzyme replacement  
54 therapies exhibiting immunogenicity rates of 51 - 100%.<sup>1,2</sup> Clinical complications of  
55 immunogenic reactions include the modification of therapeutic efficacy and acute infusion  
56 reactions, such as anaphylaxis. Appropriately, the appraisal of anti-enzyme antibody  
57 formation is a crucial component of the clinical development programme, and is specifically  
58 relevant during the evaluation of the enzyme's efficacy and safety profile. There is thus a  
59 regulatory expectation that a valid, sensitive, specific and selective immunoassay is  
60 developed for measuring enzyme-specific antibody responses .<sup>3,4</sup>

61 Erythrocyte encapsulated thymidine phosphorylase (EETP) is under development as an  
62 enzyme replacement therapy for the rare metabolic disorder, mitochondrial  
63 neurogastrointestinal encephalomyopathy, abbreviated to MNGIE .<sup>5-7</sup> The disease is caused  
64 by mutations in the nuclear *TYMP* gene encoding for the enzyme thymidine phosphorylase  
65 (TP), leading to elevated concentrations of thymidine and deoxyuridine in cellular and extra-  
66 cellular compartments, and ultimately mitochondrial failure due to progressive accumulation  
67 of mitochondrial DNA (mtDNA) defects and mtDNA depletion.<sup>8-12</sup> Clinically, MNGIE  
68 manifests as leukoencephalopathy, ptosis and ophthalmoplegia, peripheral polyneuropathy  
69 and enteric neuromyopathy causing severe gastrointestinal dysmotility with cachexia.<sup>13</sup> The  
70 disorder invariably leads to death at an average age of 37.6 years.

71 EETP is produced by encapsulating recombinant *Escherichia coli* (*E.coli*) TP within  
72 autologous erythrocytes *ex vitro*; the loaded cells are then infused into the patient. The  
73 rationale for this approach is based on thymidine and deoxyuridine diffusing across the  
74 erythrocyte membrane via nucleoside transporters into the cell where the encapsulated  
75 enzyme catalyses their metabolism to the normal products. The administration of EETP under  
76 a compassionate treatment programme has shown a sustained reduction or elimination of  
77 plasma thymidine and deoxyuridine concentrations, translating into clinical  
78 improvement.<sup>5,6,14,15</sup> EETP therapy has the advantage of prolonging the circulatory half-life  
79 of the enzyme and potentially minimising the immunogenic reactions, which are frequently  
80 observed in enzyme replacement therapies administered by the conventional route.

81 We describe here the validation of a two-step immunoassay method for the detection of anti-  
82 TP antibodies in human serum for supporting a phase II pivotal clinical trial of EETP. The  
83 analytical method was assessed for screening cut-point, specificity, intra- and inter-assay  
84 precision, sensitivity, selectivity, drug tolerance, prozone effect and stability.

85

## 86 RESULTS

87 The key results from this validation study are presented in Table 1.

88

### 89 **Disease state matrix**

90 Of the seven disease matrix samples from untreated patients that were screened, five were  
91 negative for anti-TP antibodies. The difference in the mean instrument responses between  
92 the patient and normal matrix samples was 10.1%; this was not considered to be significant  
93 (see Supplemental Table 1), indicating that the same cut point can be applied (see below).

94

95 **Screening and specificity cut-point**

96 The signal distribution for the 51 NC samples was normally distributed ( $p > 0.05$ ) with no  
97 outliers. The validation cut-point was calculated to be 898.5 relative light units (RLU), see  
98 Table 2, first iteration.

99 Statistically significant differences were evident between the means for analyst, day, plate,  
100 analyst by plate, analyst by day and analyst by day by plate interactions ( $p < 0.001$ ) and also  
101 the variances ( $p < 0.001$ ) indicating a dynamic screening cut-point (see online Supplemental  
102 Figure 1). Each analyst was analysed separately to determine the source of these differences.  
103 For Analyst 1 there were significant differences between the means for day, plate and their  
104 interaction, but not the variances, indicating a floating screening cut-point. For Analyst 2,  
105 there were significant differences between the means for day, plate and their interaction and  
106 the variances, indicating a dynamic cut-point. Due to practical limitations of using a dynamic  
107 cut-point, the validation study continued using Analyst 1, thereby applying a floating cut-  
108 point which was calculated as 1066.6 RLU, see Table 2, second iteration. The correction  
109 factor for the screening cut-point for Analyst 1 was estimated to be 128.6 RLU and this was  
110 applied to subsequent assays.

111 An analysis of the specificity cut-point data revealed a normal distribution and one outlier  
112 which was excluded. The fixed specificity cut-point was calculated to be 93% inhibition  
113 (Figure 1). Statistically significant differences were evident between the means for analyst,  
114 day, plate, analyst by day, day by plate and analyst by day by plate interactions ( $p < 0.001$ ).

115

116 **Sensitivity**

117 Assay sensitivity analysis was determined from data generated by Analyst 1 only and was  
118 calculated as 356 ng/mL, see Supplemental Table 2.

119

## 120 **Controls**

121 The NC samples were below the cut-point, the LPC samples above the cut-point and the HPC  
122 samples at the high end of the dynamic range for both intra and inter assay analyses, and are  
123 therefore considered suitable. Controls pre-incubated in the presence of 12,500 ng/mL TP  
124 demonstrated RLUs below the cut-point in both intra and inter-assay analyses, with  
125 inhibitions ranging between 80.6 to 98.8%, Table 3.

126 Assay drift was not observed, as indicated by a mean difference in response readings of the  
127 control samples at the beginning and end of the assay plate being within  $\pm 30\%$ , when  
128 compared to each other (data not shown).

129

## 130 **Drug tolerance**

131 The drug tolerance of the analytical method was determined at 156 ng/mL (Figure 2).

132

## 133 **Selectivity**

134 All low and high spiked samples were above the cut-point without TP and below the cut-  
135 point with TP. Inhibitions at the confirmatory drug concentration (12,500 ng/mL) were  
136 observed for the high spiked patient and control samples (Figure 3). Matrix effects with  
137 regard to the therapeutic enzyme and disease state matrix are therefore not considered  
138 significant.

139

**140 Prozone**

141 The instrument response readings remained above the assay cut-point, therefore prozone  
142 effects were not observed up to a serum anti-TP antibody concentration of 25,900 ng/mL,  
143 2.59-fold higher than the HPC (data not shown).

144

**145 Stability**

146 Anti-TP antibodies were stable up to 24 hours at room temperature and for up to five cycles  
147 of freeze-thaw at -70°C (Figure 4). The precision (% CV) of the instrument responses was ≤  
148 20%.

149

**150 Evaluation of serum samples from treated patients**

151 Serum samples from three patients were analysed before treatment and at different time  
152 points during treatment, Table 4. The mean instrument responses for all pre-treatment  
153 samples were below the assay cut-point. For patient 1, one sample after 9 months of  
154 treatment was above the cut-point. For patient 2, all samples during the treatment phase were  
155 above the cut-point. For patient 3, one sample after 5 months of treatment was above the cut-  
156 point. All positive samples from patient 2 were found to be specific in the confirmatory  
157 assay. Positive samples for patients 1 and 3 were confirmed as non-specific antibodies as the  
158 inhibition was below the specificity point of 93%.

159

**160 DISCUSSION**



161 Autologous erythrocyte-mediated enzyme replacement is employed as a strategy for  
162 preventing or minimising the development of immune reactions against therapeutic enzymes.  
163 Our experience includes the treatment of a patient with adenosine deaminase deficiency with  
164 erythrocyte encapsulated adenosine deaminase and the administration of EETP to 5 patients  
165 with MNGIE under a compassionate use programme.<sup>5, 6, 14-17</sup> A recombinant *E.coli* source of  
166 GMP TP has been developed to support a clinical trial of EETP. Although erythrocyte  
167 encapsulation would be predicted to reduce the immunogenicity of the enzyme, an  
168 intravascular release of TP from damaged erythrocytes is likely to evoke an immunogenic  
169 reaction against a protein of bacterial origin. The evaluation of the immunogenicity of  
170 therapeutic enzymes is an important aspect of clinical development as the formation of anti-  
171 enzyme antibodies can negatively influence the efficacy and safety of the proposed treatment.

172

173 In this study, we validated a method for the detection of anti-TP antibodies in the serum of  
174 patients treated with EETP according to published recommendations for the design and  
175 optimisation of immunoassays for the detection of host antibodies against therapeutic  
176 proteins.<sup>3, 4, 18-21</sup> To minimise the false positive rate and to increase specificity, a two-step  
177 analysis was adopted; a screening assay for the identification of anti-TP positive patient  
178 samples, followed by an assay for confirming the presence of anti-TP antibodies. Due to  
179 having the potential to detect all antibody isotypes and classes produced in an immune  
180 response, an electrochemiluminescent bridging immunoassay platform was selected. Fifty-  
181 one individual control serum samples were used to determine the 95% confidence interval  
182 used as the cut point factor. The cut-point factor was added to the mean signal for the pooled  
183 NC serum on each plate to establish the cut point. In the second analysis step, a confirmation  
184 assay was developed to confirm the specificity of putatively positive samples identified in the  
185 screening assay. In this approach, PC samples were pre-incubated with and without a high

186 concentration of TP to inhibit the assay signal beyond the cut point value; inhibition above  
187 the cut-point confirmed the presence of anti-TP antibodies. Ideally, cut-point assessments  
188 should be conducted using disease state serum samples, however for rare diseases, obtaining  
189 a sufficient number of patient samples is challenging. To address possible differences  
190 between control and diseases matrices, assay selectivity testing was assessed in patient and  
191 NC matrix samples. The bioanalytical guidelines of the EMA and FDA recommend the  
192 testing of at least ten individual sources of sample matrix, however because of the rarity of  
193 MNGIE, only seven patient matrix samples were available for testing.<sup>22,23</sup> The mean  
194 instrument responses between the patient and NC matrix samples nevertheless were not  
195 significantly different, therefore demonstrating the absence of disease matrix effects. Testing  
196 a larger number of samples will be contemplated during the clinical trial when more patients  
197 will be available.

198

199 The assay provided an adequate sensitivity of 356 ng/mL of polyclonal antibodies in serum,  
200 this is in the accepted range of 250 – 500 ng/mL in serum for antibody assays in clinical  
201 trials.<sup>24</sup> Drug tolerance was 156 ng/mL; in patient compassionate use studies, plasma levels  
202 of free TP are undetectable and therefore assay interference by free TP is considered  
203 negligible.

204

205 No specific anti-TP antibodies were detected in patients 1 and 3, determined using the  
206 confirmatory assay. However, in patient 2 positive anti-TP antibodies were detected after 8  
207 months of treatment (after nine administrations of EETP) onwards. The development of anti-  
208 TP antibodies does not necessarily predict the development of adverse events in patients, but  
209 could potentially impact on the efficacy of TP by inhibiting the pharmacological activity of

210 the enzyme through the formation of immune complexes. Another clinical consequence of  
211 anti-body formation is cross-reactivity with an endogenous protein, which performs a key  
212 physiological function. The development of specific anti-TP antibodies in Patient 2 did not  
213 raise any specific concerns with regard to the efficacy of encapsulated TP, as depletion of the  
214 plasma metabolites improved over the 5.5 years of administration and clinical improvements  
215 were also recorded.<sup>15</sup> Nevertheless, heterogeneity in patient antibody responses are often  
216 observed and thus sufficient data should be compiled during clinical development to  
217 characterise antibody response variability. Guidelines of the FDA and EMA recommend that  
218 specific antibody responses are further analysed for neutralizing capacity.<sup>3, 4, 25</sup> Neutralising  
219 antibody assay validation was not included in this study, and although we anticipate that it is  
220 unlikely that neutralising antibodies will be formed due to the encapsulation of TP in the  
221 erythrocyte, a relevant assay will be validated during clinical development and prior to  
222 marketing authorisation applications. Pre-clinical studies with EETP demonstrated specific  
223 anti-TP antibodies in 2/18 treated dogs and 19/60 treated BALB/c mice.<sup>7</sup> The development  
224 of specific antibodies against TP is not a surprising observation since senescent erythrocytes  
225 are naturally sequestered from the vascular compartment by macrophages of the monocyte-  
226 macrophage system, which is able to present antigens to T lymphocytes. We have previously  
227 shown that humoral responses can be elicited by the administration of erythrocyte  
228 encapsulated antigens to BALB/c mice.<sup>26</sup> One of the advantages of employing the autologous  
229 erythrocyte is that the development of antibodies against the carrier is unlikely, and indeed  
230 this has not been encountered in 25 years of clinical experience.

231

232 To conclude, this assay has appropriate performance characteristics and is considered suitable  
233 for the detection of anti-TP antibodies in human serum. Further assay refinement will be

234 implemented during clinical development to include the validation of a neutralising antibody  
235 assay and detection of IgE antibodies.

236

## 237 MATERIALS AND METHODS

238 This validation study was designed to adhere to recommendations for the validation of  
239 immunoassays used for detection of host antibodies against biotechnology products  
240 according to FDA and EMA immunogenicity guidelines and in compliance with Good  
241 Laboratory Practice (GLP) standards.<sup>3, 4, 18-21, 24</sup>

### 242 **Reagents**

243 All reagents were supplied by Meso Scale Discovery, UK unless otherwise stated. The wash  
244 buffer was phosphate-buffered saline (PBS) with 0.05% Tween 20 (Sigma Chemical  
245 Company, UK). Blocker A solution consisted of 5% (w/v) Blocker A in phosphate buffer; the  
246 assay buffer was 1 volume of 5% Blocker A solution and 4 volumes of wash buffer; and the  
247 Read buffer (4×) was diluted 1 in 2 with ultra-high purity grade water.

248 Recombinant *E. coli* (TP, 13 mg/mL) produced by the methodology employed for the  
249 manufacture of clinical GMP material was employed for the development and validation of  
250 this immunoassay (Diatheva, Italy). A 12,500 ng/mL working solution of TP was prepared by  
251 dilution in assay buffer. Biotinylated and sulfo-TAG TP conjugates were prepared as the  
252 capture and detection antigens, respectively, as described previously and were used to  
253 formulate a conjugate mastermix complex working solution containing 300 ng/mL biotin and  
254 300 ng/mL sulfo-TAG in assay buffer.<sup>27</sup>

255

### 256 **Negative and positive human serum controls**

257 A negative control (NC) human serum pool was prepared from 15 individual human samples  
258 which had been screened against a positive control calibration curve for the presence of anti-  
259 TP antibodies and stored at  $-20^{\circ}\text{C}$  until required. Positive human serum controls (PC) were  
260 prepared from affinity-purified rabbit anti-TP antibody (0.518 mg/mL, custom produced by  
261 Open Biosystems, Huntsville, USA) diluted with NC sera to produce the low PC just above  
262 the cut-point (LPC, 400 ng/mL) and a high PC giving approximately 75% of the maximum  
263 signal (HPC, 10,000 ng/mL). Prior to analysis, the NC and PC samples were diluted 1 in 10  
264 with assay buffer.

265

#### 266 **Samples from patients with MNGIE**

267 To ascertain that the normal matrix was representative of the disease state matrix, seven  
268 individual treatment-naïve disease state human serum samples were screened and analysed  
269 alongside ten individual NC samples.

270 Serum samples from three patients with a confirmed diagnosis of MNGIE who had received  
271 2 to 4 weekly infusions of EETP (3.9 to 108 U/Kg body weight) were collected pre-treatment  
272 and at a number of time points after therapy initiation. Samples were stored at  $-80^{\circ}\text{C}$  in a  
273 temperature-monitored freezer until sample analysis, by a two-tiered process, a screening  
274 assay to identify samples positive for anti-TP antibodies, followed by a confirmatory assay to  
275 establish if the antibodies were specific to TP. NC, LPC and HPC samples were included in  
276 each assay run. Approval for the study was obtained from the National Research Ethics  
277 Service Committee. Patient informed consent was obtained prior to the start of treatment.

278

#### 279 **Assay procedure**

280 Assays were performed using an electrochemiluminescent bridging immunoassay. Briefly,  
281 NC, PCs and test samples were diluted in assay buffer with and without TP for 1 hour at  
282 room temperature, after which 75 $\mu$ L were added to wells of a polypropylene 96-well plate  
283 (Fisher Scientific, UK) followed by 75  $\mu$ L conjugate mastermix. The plates were covered  
284 and incubated at room temperature for 2 hours with shaking at 800 rpm. Following this, 350  
285  $\mu$ L Blocker A solution were added to the appropriate wells of a streptavidin gold plate, which  
286 was then covered and incubated at room temperature for 2 hours with shaking at 800 rpm.  
287 The streptavidin plate was then washed three times with 350  $\mu$ L wash buffer per well using a  
288 plate washer; the last wash was aspirated and the plate blotted dry by inversion over  
289 absorbent paper. Two 50  $\mu$ L aliquots from each well of the polypropylene 96-well plate were  
290 transferred to corresponding duplicate wells in the streptavidin plate, which was then covered  
291 and incubated at room temperature for 1 hour, with shaking at 800 rpm. This was followed  
292 by three washes with 350  $\mu$ L wash buffer per well using a plate washer; the last wash was  
293 aspirated and the plate blotted dry by inversion over absorbent paper. Finally 150  $\mu$ L Read  
294 buffer (2x) were added to each well the plate read on a MSD Sector Imager 6000 within 10  
295 minutes.

296

## 297 **Method validation parameters**

### 298 *Reagent optimisation*

299 Design Expert was used to optimise the concentrations of biotinylated TP and Sulfo-TAG TP.

300

### 301 *PC standard curve for assay sensitivity determination*

302 PC calibration curves were prepared from working standards (n=2, in duplicate) and  
303 processed using a 4-parameter logistical algorithm; this fitting routine was applied throughout  
304 the determination of screening and specificity cut-point, and assay sensitivity.

305

#### 306 *Screening and specificity cut-point*

307 The screening cut-point was assessed to determine the threshold for identifying samples as  
308 negative or potentially positive (equal or above the cut-point) for the presence of anti-TP  
309 antibodies. The methodology applied was that of Shankar *et al.*<sup>18</sup> where the purpose was to  
310 determine the type of cut-point required (floating, fixed or dynamic), calculate the cut-point  
311 value and determine the specificity (confirmation) cut-point. Fifty-one individual human  
312 serum samples were measured in duplicate, by two analysts, over three plates, on three days.

313

314 The specificity cut-point assay is employed to determine whether samples identified as  
315 potentially reactive in the screening assay are positive or negative for anti-TP antibodies. The  
316 same source of serum samples that were employed in the screening cut-point assay were pre-  
317 incubated with TP at a concentration of 12,500 ng/mL, this being ten times the lowest  
318 concentration that was observed to fall below the screening cut-point during assay  
319 development. Each assay run included a PC standard curve, NC, LPC and HPC samples, with  
320 and without TP.

321

#### 322 *Assay sensitivity*

323 The sensitivity of the assay is defined by the lowest concentration at which a PC antibody  
324 preparation consistently provides a positive signal in the assay. This was calculated as the

325 mean concentration obtained by interpolation of the plate-specific cut-point value against the  
326 PC curve on each of the 18 assay runs described above, and then determining the lowest  
327 concentration that is measured as positive 95% of the time. Instrument responses, RLUs for  
328 the PC samples were assessed according to their relation to the cut-point.

329

#### 330 *PC and NC sample suitability*

331 Intra-assay precision was determined by the replicate analysis of NC (4 independent  
332 preparations of NC, 3 independent preparations of LPC and 3 independent preparations of  
333 the HPC samples in one assay run. An additional set of control samples pre-incubated with  
334 12,500 ng/mL TP was also analysed. Inter-assay precision was determined from the replicate  
335 analysis of 4 independent preparations of NC, 2 independent preparations of LPC and 2  
336 independent preparations of HPC samples, with and without TP on 15 occasions spanning 4  
337 different days, by two analysts. Assay drift was assessed by analysing control samples (+ pre-  
338 incubation with TP) in the first and last columns of the assay plate.

339

#### 340 *Drug tolerance*

341 The tolerance of the assay to free TP was assessed by pre-incubation of the LPC for one hour  
342 in TP over the final concentration range of 39.1 to 40,000 ng/mL. A sample without TP was  
343 also analysed.

344

#### 345 *Selectivity*

346 Assay selectivity was assessed to determine whether the assay was affected by the disease  
347 state matrix or by the potential existence of therapeutic TP in serum samples. Individual



348 control (n=10) and disease (n=7) serum samples were unspiked and spiked with anti-TP  
349 antibodies at low (400 ng/mL) and high (10,000 ng/mL) concentrations. Two aliquots of  
350 sample were prepared and incubated for 1 hour, one aliquot with buffer and the other aliquot  
351 with assay buffer containing free TP (12,500 ng/mL). The samples were distributed over four  
352 assay runs. The percentage inhibition of signal in the presence of free TP was calculated  
353 using the formula described in Data handling and statistics.

354

### 355 *Prozone*

356 Assay prozone caused by high anti-TP antibody levels was investigated by serial dilution of a  
357 high spiked sample (containing anti-TP antibodies at a concentration of 25,900 ng/mL) with  
358 assay buffer.

359

### 360 *Stability*

361 The effect of anticipated sample handling conditions on assay performance were evaluated,  
362 specifically bench top storage at room temperature (nominally 22°C) and repeated freeze-  
363 thaw cycles. Room temperature effects were assessed by thawing one set of PC samples for  
364 approximately 24 hours (expected maximum duration that samples would be left thawed) and  
365 an addition set for baseline assessment, just prior to analysis (n=3, in duplicate). The effect  
366 of repeated freeze-thaw cycles on the stability of anti-TP antibodies was assessed by  
367 subjecting PC samples to three and five freeze-thaw cycles, with each cycle consisting of a  
368 minimum of two hours at room temperature, followed by storage at -70°C for at least 12  
369 hours (n = 3, in duplicate). An additional set of PC samples for baseline assessment was  
370 thawed prior to analysis. Stability was verified if the mean precision (% CV) and mean  
371 percent difference from the baseline responses were  $\leq 20\%$ .

372

373 **Data handling and statistics**

374 Instrument responses are reported as mean values of RLU. All data acquisition, processing  
375 and evaluations were performed using the Watson Laboratory Information Management  
376 System version 7.2, Microsoft Excel and Meso Scale Discovery Workbench version 3.0.185.  
377 Data for cut-point calculation was analysed using SAS Version 9.1.3.

378

379 Assay cut-point evaluation was performed using the statistical methodology described by  
380 Shankar *et al.*<sup>18</sup> Measurements for each of the 51 human serum samples (n=18, in duplicate)  
381 were averaged and tested for normality using the Shapiro Wilk's test<sup>28</sup>; logarithmic (base 10)  
382 or square root transformation was applied to non-normally distributed data. An assessment  
383 for outliers was made using the Studentized Deleted Residuals whereby residues  $<-3$  or  $>3$   
384 standard deviations (SD) were excluded. Once outliers were removed, data was reassessed for  
385 normality; the validation cut-point was defined as the 95% Quantile for non-normally  
386 distributed data, or the mean + 1.645\*SD for normally distributed data.

387

388 To assess the type of screening cut-point to apply, an analysis of variance method was  
389 applied to assess for any analyst, plate and day differences on either the untransformed or  
390 transformed data, depending on the outcome of the Shapiro Wilk's test above. Analyst, plate,  
391 day and their interactions were set as fixed factors, whereas subject was included as a random  
392 effect. Levene's test for homogeneity of variance was performed.<sup>29</sup> A fixed screening cut-  
393 point was indicated if there were no differences or variances, whereas a floating cut-point  
394 was reported if there were differences between means only, otherwise a dynamic screening  
395 cut-point was required.<sup>18</sup> The Correction Factor was calculated as the validation cut-point

396 minus the mean of the NC values from the validation runs. The screening cut-point was  
397 defined as either the validation cut-point or the mean of NC values from the in study run +  
398 Correction Factor, depending on whether the means and variances between runs were similar.

399

400 The fixed specificity cut-point was calculated using the method of Shankar *et al.*<sup>18</sup> For each  
401 sample the percentage inhibition of signal in the presence of free TP was calculated as  
402 follows:

$$\text{Signal inhibition (\%)} = 100 \times \left[ 1 - \left( \frac{\text{drug inhibited sample}}{\text{uninhibited sample}} \right) \right]$$

403 Data was assessed for outliers and normal distribution and treated accordingly, as described  
404 above. For normally distributed data, the fixed specificity cut-point was calculated as mean %  
405 inhibition + 3.09 x SD. For data not normalised by transformations, the specificity cut-point  
406 was calculated as median + 99% quantile. Analysis of variance techniques were applied to  
407 assess for analyst, plate and day differences.

408

409 In the event of differences between analysts for either inhibited or uninhibited samples, the  
410 sensitivity analysis was performed separately for each analyst. Each dilution curve was  
411 analysed using a four-parameter model. For a floating cut-point, separate curves were  
412 analysed for each plate, whereas for a fixed cut-point the data were combined from all plates.  
413 The screening assay cut-point determined to be appropriate for the method was back-  
414 calculated onto the standard curve for each plate to obtain the log concentrations of the  
415 screening cut-points. These were averaged across all plates and a 95% confidence interval  
416 obtained for the overall mean on the log scale. The back transformed upper 95% confidence  
417 interval was calculated, which was defined as the sensitivity of the assay.

418

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422

## 423 AUTHOR CONTRIBUTIONS

424 M.L, D.P, CG, J.H., M.S. and B.B. contributed to the design and implementation of the

425 research, to the analysis of the results and to the writing of the manuscript.

426

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508 **Figure 1. Specificity cut-point.** To establish the specificity cut-point, 51 individual control  
509 serum samples were pre-incubated with TP at a concentration of 12,500 ng/mL, and analysed  
510 in duplicate by two analysts over three plates on three days to assess % signal inhibition.  
511 Significant differences were observed between the means for analyst, day, plate, analyst by  
512 day, day\*plate and analyst\*day\*plate interactions ( $p < 0.001$ ). Data is expressed as mean %  
513 signal inhibition  $\pm$  SD.

514

515 **Figure 2. Assay tolerance to free TP.** Instrument response as function of TP concentration.  
516 The LPC was spiked with TP over a concentration range of 39.1 to 40,000 ng/mL and  
517 incubated for 1 hour before analysis. The blue arrow indicates the assay drug tolerance.

518

519 **Figure 3. Assay selectivity.** Individual control (n=10) and disease (n=7) serum samples,  
520 were unspiked or spiked with anti-TP antibodies at low (400 ng/mL) and high (10,000  
521 ng/mL) concentrations. Two aliquots of each sample were prepared and incubated for 1 hour,  
522 one aliquot with buffer and the other aliquot with free TP (12,500 ng/mL). Dotted line  
523 represents assay cut-point. Data is expressed as log mean RLU  $\pm$  SD.

524

525 **Figure 4. Stability of anti-TP antibody after 24 hours at room temperature and after**  
526 **repeated freeze-thaw cycles.** The dashed lines represent the assay cut-point range. Data is  
527 expressed as log mean RLU  $\pm$  SD.

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530 **Table 1. Summary of key validation parameters for the assessment of anti-TP**  
 531 **antibodies in human serum.**

<b>Validation Parameter</b>	<b>Results</b>
Positive control standard range	2.44 ng/mL to 10,000 ng/mL
Correction factor for cut-point calculation	129 RLU <sup>a</sup>
Screening cut-point	Floating cut-point
Specificity cut-point	93.0%
Assay sensitivity	356 ng/mL
<i>Intra-assay performance</i>	<i>Precision, CV (%)</i>
Negative control	14.5
Low positive control	11.1
High positive control	1.0
<i>Inter-assay performance</i>	<i>Mean precision, CV (%)</i>
Negative control	43.3
Low positive control	40.6
High positive control	30.5
Assay drift	Not present
Minimum required dilution (MRD)	1 in 10
Selectivity (matrix effects)	Not present
Prozone	Not present up to 25,900 ng/mL
Drug tolerance	Tolerant up to 156 ng/mL
Confirmatory drug concentration	12,500 ng/mL
<i>Stability</i>	
Room temperature	Up to 24 hours
Freeze-thaw	Up to five freeze-thaw cycles

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533 <sup>a</sup> RLU; relative light unit

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541 **Table 2. Screening cut-point determination.** The cut point was determined using 51  
 542 individual lots of serum, analysed in duplicate, by two analysts, over 3 plates, on 3 days. The  
 543 cut-point was calculated as the  $RLU + 1.645 * SD$ . The first iteration represents data analysed  
 544 from Analysts 1 and 2. Data from Analyst 2 were removed for the second iteration.

Parameter	RLU <sup>a</sup>	
	First iteration	Second Iteration
Mean	797.9	914.3
n	36	18
SD	61.2	92.6
Cut point	898.5	1066.6
Mean of negative controls	814.2	938.0
Correction factor		128.6

545 <sup>a</sup> RLU; relative light unit

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560 **Table 3. Intra and inter-assay analysis of control samples with and without pre-**  
 561 **incubation with TP.**

Control sample	RLU <sup>a</sup> Mean ± SD	Intra assay		RLU Mean ± SD	Inter-assay	
		CV (%)	% inhibition		CV (%)	% inhibition
NC	865 ± 126	14.5		483 ± 209	43.3	
NC+TP <sup>b</sup>	ND	ND	ND	87 ± 10	11.0	80.6
LPC	1505 ± 167	11.1		919 ± 373	40.6	
LPC +TP	151 ± 8	5.3	90.0	103 ± 27	25.8	88.9
HPC	18111 ± 181	1.0		12680 ± 3873	30.5	
HPC + TP	265 ± 3	1.0	98.5	158 ± 59	37.4	98.8

562 <sup>a</sup>RLU; relative light unit

563 <sup>b</sup>TP; thymidine phosphorylase

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577 **Table 4. Screening analysis and confirmatory assay of positive patient samples**

Patient ID	Treatment (months)	Screening Assay (-TP <sup>a</sup> )	% inhibition	Confirmatory assay Specificity	
1	Pre-treatment	Negative			
	9	Positive	73.7	Non-specific	
	15	Negative			
	21	Negative			
	28	Negative			
2	Pre-treatment	Negative			
2	8	Positive	95.5	Specific	
	16	Positive	98.8	Specific	
	22	Positive	97.3	Specific	
	28	Positive	98.1	Specific	
	35	Positive	98.5	Specific	
	41	Positive	98.8	Specific	
	49	Positive	99.1	Specific	
	60	Positive	99.0	Specific	
	73	Positive	97.6	Specific	
	3	Pre-treatment	Negative		
		6	Positive	75.6	Non-specific

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579 <sup>a</sup>TP; thymidine phosphorylase

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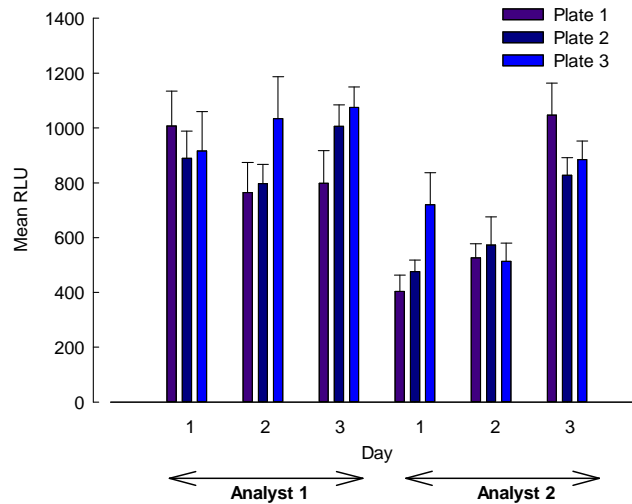
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**Supplemental Figure 1. Screening cut-point.** To establish the screening cut-point, 51 individual control serum samples were analysed in duplicate by two analysts over three plates on three days. Significant differences were observed between means for analyst, day, plate, analyst\*plate, analyst\*day and analyst\*day\*plate interactions ( $p < 0.001$ ) and variances ( $p < 0.001$ ). Data is expressed as mean RLU  $\pm$  SD.



**Supplemental Table 1. Comparison of instrument responses to negative control and disease state matrices.**

Serum sample	Instrument response (RLU <sup>a</sup> )	
	Mean $\pm$ SD	CV (%)
Disease (n=5)	1169 $\pm$ 388	33.2
Healthy control (n=10)	1301 $\pm$ 141	10.9

<sup>a</sup> RLU; relative light unit

**Supplemental Table 2. Sensitivity analysis for Analyst 1 for each of 9 plates.**

Plate	Mean RLU <sup>a</sup> Negative control	Screening cut point <sup>b</sup> (RLU)	Log sensitivity level
1	1037.0	1165.6	1.171
2	981.0	1109.6	2.233
3	905.0	1033.6	2.422
4	920.5	1049.1	3.114
5	818.0	946.6	2.472
6	810.5	939.1	2.243
7	987.5	1116.1	1.803
8	1107.0	1235.6	2.119
9	875.5	1004.1	2.431
<b>Log mean sensitivity level + SD</b>			2.223 $\pm$ 0.528
<b>Log mean sensitivity Upper 95% CI<sup>c</sup></b>			2.551
<b>Mean sensitivity Upper 95% CI</b>			355.53

<sup>a</sup> RLU; relative light unit

<sup>b</sup> Screening cut point = Negative control mean + Correction Factor (128.6)

<sup>c</sup> Upper log mean sensitivity = Mean + (SD/ $\sqrt{n}$ )\*t

ACCEPTED MANUSCRIPT

