**Proposed Protocol for the Taq Polymerase Extension Assay April 2013**

**Materials:**

* **DNA Template and Primer**: The template for the polymerase assay is an 80-mer single stranded DNA, TAT2, custom synthesized by Sigma Custom Oligos. A 17-mer primer TAP1, complementary to the 17 bases at the 3’ end of TAT2 was also custom synthesized by Sigma Custom Oligos. (see table 1 for GC content and calculated Tm).
* **Reaction Components:** Native Taq Polymerase (with its buffer and MgCl2) is obtained from Life Technologies, dNTP mix from New England Biolabs, Quant-iT PicoGreen ds DNA Reagent from Molecular Probes.
* **Instruments:** The incubation for the activity assays are carried out in the BioRad CFX96 PCR machine (software: CFX Manager ver 1.6.541.1028). PicoGreen fluorescence is measured in the Lab Systems Fluoroskan Ascent Fluorescence 96/384 Well Plate Reader using a PCR plate adapter (Excitation 485nm; Emission 520nm).
* **Software:** Calculation of Tms of synthetic oligomers was done using Sigma DNA Calculator, <http://www.sigma-genosys.com/calc/DNACalc.asp>. Analysis of secondary structures and of primer-dimer formation was done according to Oligo Analyzer from IDT: <http://biotools.idtdna.com/analyzer/applications/oligoanalyzer/> . GraphPad Prism was used for all curve fittings.

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| **Table 1 : Oligos used in the study** | | | | |
| **Oligo Name (length)** | **Sequence** | **% GC** | **Tm** | **Description** |
| TAT2 (80 bases) | 5’- ACA CGT TAG GAA GAT GGA ATT GAT TGG ATC GAA GGA AAT AAA AGA AAT TAA GGC AAT GGT CTC CCG TCG GCG GCG CGA GC-3’ | 47.5 | 92oC | Template for Taq polymerase extension |
| TAT2comp (80 bases) | 5’- GCTCGCGCCG CCGACGGGAG ACCATTGCCT TAATTTCTTT TATTTCCTTC GATCCAATCA ATTCCATCTT CCTAACGTGT -3’ | 47.5 | 92oC | Complementary strand for TAT2 (used in PicoGreen Calibration) |
| TAT1 (17 bases) | 5’-GCT CGC GCC GCC GAC GG-3’ | 88 | 80oC | Primer for TAT2 |

**Method:**

* **Preparation of Template-Primer Mix (see Table 2 for details) :**

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| **Table 2: Composition of the Template-Primer mix** | | |
| Initial Conc | Reaction Component | Final Conc |
|  | Template (TAT2) | 20nM |
|  | Primer (TAP1) | 140nM |
| 10X | Taq Polymerase Buffer | 1X |
| 50mM | MgCl2 | 2mM |

1. The single stranded 80-mer template, TAT2 and it 17-mer primer TAP1 are mixed such that the primer is present in a 7 molar excess (this is the molar ratio used in the previous study) in 1X *Taq* reaction buffer (20 mM Tris-HCl, 50 mM KCl) containing 2mM MgCl2.
2. The holding and cooling steps for annealing are as follows (as used in the previous study): hold at 95oC for 1.5 minute, cool to 75oC over 30 minutes, hold at 75oC for 5 minutes, cool to 70oC over 9 minutes, hold at 70oC for 5 minutes, cool to 45oC over 45 minutes and then to room temperature on the bench. (The annealed primer-template complex mix is stored at room temperature for the duration of the assay for up to 24hrs. It is neither chilled nor frozen).

* **Set up of the Reaction (see Table 3 for details)**:

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| **Table 3: Assay Variables** | |
| Temperature | 70oC (in the initial trial) |
| Time points | 10secs, 20secs, 30secs, 40secs, 50secs, 1min, 1.5mins, 2mins, 5mins, 10mins |
| Enzyme Concentration | 0.02nM vs 0.36nM |
| dNTP Concentration | 200uM (for the initial trial) |
| Replicates |  |
| Reaction Volume | 20ul |

1. In the initial trial of the new protocol, one complete time course at 70oC will be performed for two enzyme concentrations (0.36nM used previously and 0.02nM which achieves the template: enzyme ration of 1000). Each time point will have 3 replicates. This implies 33 reactions for each enzyme concentration, a total of 66 reactions. The template-primer mix will be made/ annealed as a single batch for this entire set and will be split into two parts, one for each enzyme concentration. Each part will then be processed as follows:
2. The template-primer mix is split into 11parts, one for each assay time point (see table) and one for a no-Taq control. Taq Polymerase (appropriately diluted in the dilution buffer to achieve the final concentration stated in the table in each 20ul reaction) is added to the test fractions and water is added to the no-Taq control fraction.
3. The reaction is incubated at 70oC in the CFX96 (PCR machine) for 5mins (for equilibration of the Taq polymerase with the primer-template complex.
4. After equilibration, the polymerase reaction is initiated by addition of dNTPs (see table for concentration).
5. At the end of the incubation the reaction is immediately chilled to 0oC and EDTA is added to a final conc of 20mM.

* **Quantitation:**

1. 80ul of Picogreen reagent freshly diluted 178-fold in 1X TE is added to each reaction. The reaction is incubated in the dark for 5min at room temperature and fluorescence is measured in the Fluoroskan with excitation at 485nm and emission at 520nm.

* **Plots:**

1. The measured RFU is plotted against time. The curves are fitted using the equation for one-phase association kinetics. The initial reaction rate (del RFU/sec) is calculated using the corrected RFU values from the fitted curve. These calculated initial reaction rates are used to plot the Michaelis Menten curves (del RFU/sec vs [S]) for each assay temperature .