A quantitative fluorescence-based steady-state assay of DNA polymerase
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Introduction
Fluorescent dyes that bind DNA have been demonstrated as a useful alternative to radionucleotides for the quantification of DNA and the in vitro measurement of the activity of DNA polymerases and nucleases. However, this approach is generally used in a semi-quantitative way to determine relative rates of reaction. In this report, we demonstrate a method for the simultaneous quantification of DNA in both its single-strand and double-strand forms using the dye PicoGreen. This approach is used in a steady-state assay of DNA polymerase Klenow fragment exo⁻, where we determine $k_{cat}$ and $K_m$ values for the DNA polymerase that are in excellent agreement with literature values.

Abbreviations
KF, Klenow fragment of DNA polymerase I; PG, PicoGreen.
many years to quantify DNA in such techniques as real-time PCR and quantitative PCR [30–33]. Fluorescent dyes have been shown to bind to DNA in a variety of different ways [34], PG has been shown both to intercalate into the double helix of DNA with a frequency of approximately one dye molecule per four base pairs of dsDNA and also to bind along the phosphate backbone surface using electrostatic interactions along with groove binding [35]. PG displays high affinity to DNA, which lends itself to a large linear dynamic range. The fluorescence enhancement of PG upon association with dsDNA has been reported to be ~2000-fold and detection of DNA concentrations ranging from low picomolar to micromolar by PG has been demonstrated as the Kd for PG/DNA is in the nanomolar range (dependent upon buffer conditions) [34–36]. The fluorescence enhancement of PG when bound to ssDNA is significantly less than that when bound to dsDNA and the fluorescence sensitivity to dsDNA is not greatly affected by the presence of ssDNA [36]. As a result, PG lends itself to studying the production of dsDNA from a single-stand template, and therefore also to the study of DNA polymerase activity. Indeed, a PG-based DNA polymerase assay has been demonstrated, but this approach has not yet progressed beyond the qualitative or comparative analysis of dsDNA production [14,15]. Similarly, PG has also been employed to semi-quantitatively monitor the hydrolysis of dsDNA catalysed by DNA nuclease [37].

In this report, we present a fluorescence-based assay for the study of DNA polymerase kinetics based on the selective detection of dsDNA by PG. We focus on the steady-state turnover of Klenow fragment (KF) exonuclease, which is a well-characterized model polymerase [2,3,38–44]. We have designed the assay to simplify the study of polymerases, with the removal of nucleotide analogues and the need to visualize results, indirectly, using gel electrophoresis. This assay can be used to obtain steady-state parameters such as the apparent Michaelis–Menten constant (Km) and, as it is quantitative, the velocities Vmax and kcat. The assay consumes relatively small quantities of DNA, nucleotides and enzyme and can be performed using a conventional fluorometer, simply first requiring the creation of a standard curve.

**Results and discussion**

In order to monitor dsDNA production during a polymerase assay using a fluorophore such as PG, the fluorescence from PG bound to dsDNA (fds) must be deconvoluted from the total observed fluorescence from the sample, fobs:

\[ f_{\text{obs}} = f_{\text{PG}}[\text{PG}] + f_{\text{dNTP}}[\text{PG} \cdot \text{dNTPs}] + f_{\text{ds}}[\text{PG} \cdot \text{dsDNA}] + f_{\text{ss}}[\text{PG} \cdot \text{ssDNA}] \]

(1)

Under the instrumental and buffer conditions used throughout this study (given in Materials and methods), we measured fPG = 0.85 ± 0.23 counts per second (cps) per nM PG. By titrating dNTP into a solution of 3 μM PG (the PG concentration typically used throughout this study), a value of f_{\text{dNTP}} = 0.050 ± 0.002 cps (nm dNTP)^{-1} was determined (data not shown). Next, 50mers of dsDNA and ssDNA were titrated into 3 μM PG to determine fds = 580 ± 18 cps (nM dsDNA)^{-1} and fss = 36 ± 2 and 50 ± 3 cps (nM ssDNA)^{-1} (Fig. 1). The dsDNA was made by annealing a 1 : 1 ratio of T7pR30 : T7pR30rc (Table 1) and the different values of fss are for the T7pR30 and T7pR30rc oligonucleotides, respectively. The variability in fss suggests that the magnitude of the fluorescence enhancement upon DNA binding to PG may have some sequence dependence.

The differential fluorescence enhancement of PG bound to dsDNA versus ssDNA (fds/fss) observed in Fig. 1B is 11–16, which is of the same order as previously reported [36]. To alternatively determine fds and fss under conditions similar to those used during a polymerase assay, one ssDNA 50-mer (T7pR50; Table 1) was titrated (with annealing) against its reverse complement (T7pR50rc). These data, shown in Fig. 2, were fitted to a quadratic function (Eqn 2) to simultaneously determine fds and fss.

\[
\frac{f_{\text{ss}}}{f_{\text{ds}}} \approx \frac{f_{\text{ds}}[\text{dsDNA}]}{f_{\text{ss}}[\text{ssDNA}]} \\
\text{[dsDNA]} = \frac{\text{length}/2}{(\text{O}1 + \text{O}2 + \text{K}_d) - \sqrt{(\text{O}1 + \text{O}2 + \text{K}_d)^2 - 4\text{O}1\text{O}2}} \\
\text{[ssDNA]} = \text{length}[\text{O}1 + \text{O}2] - \text{[dsDNA]}
\]

(2)

Here, length is the ssDNA (oligonucleotide) length (i.e. 50) and [O1] and [O2] are the concentrations of the T7pR50 and T7pR50rc oligonucleotides. f_{\text{dNTP}} and f_{\text{PG}} can also be omitted in the approximation of f_{\text{obs}}, as their contributions are negligible. Fitting the data in Fig. 2B yielded fds = 562 ± 9 cps·nm^{-1}, fss = 53 ± 2 cps·nm^{-1}, and Kd = 2.9 ± 0.7 nm. These values are
comparable to those determined in Fig. 1 and yield $f_{ds}/f_{ss} = 10.6 \pm 0.1$. The observed $K_d$ is in good agreement with previous reports [34–36]. Consequently PG can be used to discriminate between dsDNA and ssDNA. The difference in fluorescence emission between PG bound to dsDNA and ssDNA (~11-fold) is consistent between experiments. This is important to consider, as it has been shown that this difference in fluorescence enhancement is dependent upon buffer conditions [36].

The data in Figs 1 and 2 show that it is possible to accurately determine the concentration of dsDNA or ssDNA in reaction mixtures containing dsDNA, ssDNA and PG if the concentration of the other species is known. However, if we consider an idealized polymerase-catalysed reaction (Eqn 3), ssDNA and dNTPs are stoichiometrically converted to dsDNA.

$$\text{dsDNA}_n \cdot \text{ssDNA}_m + \text{dNTP} \xrightarrow{\text{polymerase}} \text{dsDNA}_{n+1} \cdot \text{ssDNA}_{m-1} + \text{PPi}$$  (3)

The change in $f_{obs}$ as the reaction progresses ($\Delta f_{obs}$) then allows the determination of the amount of dsDNA produced ($\Delta[\text{dsDNA}]$) without knowledge of the concentration of any of the species:

$$\Delta f_{obs} \approx (f_{ds} - f_{ss})\Delta[\text{dsDNA}] = f_A \Delta[\text{dsDNA}]$$  (4)

We will use a value of $f_A = 520 \pm 30 \text{ cps-nm}^{-1}$, which was determined using $f_{ds}$ and $f_{ss}$ values averaged
between those determined in Figs 1 and 2. While this value will vary depending on the fluorometer and instrument parameters employed, it can be readily determined using the method described in Fig. 1 and/or Fig. 2. Note that if absolute values of $V_{\text{max}}$ and $k_{\text{cat}}$ are not required, then relative velocities can be readily determined by comparison with a reference reaction. This removes the necessity of the creation of a standard curve to determine $f_A$ in Eqn (4).

The rate of dsDNA produced during steady-state turnover of KF exo$^-$ was initially investigated using a continuous assay with a saturating concentration (1 mM) of dNTPs and 100 nM of the T7pR30 template with a two-fold excess of T7 primer annealed. With annealed T7 primer, the T7pR30 template has a 5’ overhang of 30 nucleotides that can be filled in during the reaction. The assay was performed in the presence of PG and the change in [dsDNA] was quantified by monitoring the fluorescence emission from PG at 525 nm. PG was found to strongly inhibit the reaction with an apparent $K_I = 30 \pm 8$ nM (Fig. 3). It is notable that this value is of the same order as the $K_d$ reported for DNA binding to PG [34,36] and of that determined in Fig. 2. Similar inhibition has been reported previously [14] and can be rationalized if polymerase binding to the template DNA is inhibited when template DNA has bound PG present; essentially, PG appears to be acting like a competitive inhibitor. While it may be possible to perform continuous assays in the presence of low concentrations (sub-μM) of PG, this is problematic. In order for PG to linearly report on [dsDNA], it must be saturating relative to the final amount of dsDNA produced. With 100 nM of a 50-mer template (as in Fig. 3), a minimum PG concentration of 1.25 μM is required to allow PG to bind, on average, to every fourth base pair of dsDNA [35]. If a longer template is used (see later),
then more PG is required and/or the concentration of template must be reduced, which reduces the sensitivity of the assay. This is also assuming that intercalation is the largest contributor to fluorescence enhancement/emission, given that PG can also associate with DNA via electrostatics and groove binding [35].

As an alternative to optimizing the conditions of the continuous assay, a stopped assay was explored. The reaction conditions were similar to those used in Fig. 3 and typically contained 10 or 100 nM primed template and 0–500 μM dNTPs. The reaction was initiated by the addition of 5 or 50 nM KF exo− and aliquots were quenched by the addition of 3 μM PG at 10 s intervals. We used PG to quench the reaction as EDTA (a common polymerase quencher) was found to reduce the fluorescence of PG/DNA solutions. This is most probably due to EDTA sequestering metal ions (i.e. Mg2+), which have been shown to enhance PG fluorescence [45,46]. No activity was measured with 3 μM PG in Fig. 3, and care was taken during the stopped assay to minimize the time between quenching and fluorescence measurement. The stopped assay with T7pR30 template is shown in Fig. 4. The reaction progress curves show the reaction going to completion with higher dNTP concentrations. In these cases, the template is limiting and the reaction stops when the single-strand 5′ overhang of the template is filled in. This was confirmed by performing assays with progressively longer templates with 50, 80 and 100 nucleotides of 5′ overhang (Table 1; data not shown). These data showed the reaction saturating after increasingly longer intervals, with correspondingly longer linear regions and higher final fluorescence levels. One additional advantage of a stopped assay is that the concentration of the reaction can be diluted upon quenching in order to ensure that PG is saturating. Thus, it is possible to use longer templates without compromising the sensitivity of the assay.

As the progress curves measured are non-linear, these data were fitted to a quadratic function to extract the initial velocities (Eqn 5) with example fits shown in Fig. 4B.

\[
\Delta f = \left( \frac{V_{\text{initial}}}{2} \right) \left\{ (c + t_{\text{sat}} + t) - \sqrt{(c + t_{\text{sat}} + t)^2 - 4t_{\text{sat}}t} \right\}
\]

Equation (5) provides a model-free method of fitting non-linear time course data in order to determine the slope of the initial linear region, i.e. \( V_{\text{initial}} \). The \( t_{\text{sat}} \) and \( c \) parameters are phenomenological and describe the time when the reaction has saturated (~ 60 s in Fig. 4B) and the curvature around this point, respectively.

The initial velocity data show a hyperbolic dependence of the observed velocity on dNTP concentration (Fig. 5). By fitting these data to a Michaelis–Menten expression (Eqn 6), apparent \( V_{\text{max}} \) and \( K_m \) values were determined for each of the four templates (Fig. 5; Table 2).
Fig. 5. Michaelis–Menten steady-state data. The dNTP dependence of the observed initial reaction velocity data extracted from stopped assays of KF exo− (50 nM) with 100 nM T7pR50 (circles) and T7pR100 (squares) templates. The inset shows the same data on a log concentration axis and the data are fitted to Eqn (6) with fitted values given in Table 2.

Table 2. Apparent Michaelis–Menten parameters derived from fits of Eqn (6) (with fitting errors) to stopped steady-state assays of the dNTP dependence of KF exo− with 50 nM template.

| Template | \( k_{\text{cat}} \) (s \(^{-1} \)) | \( K_m \) (μM) | \( k_{\text{cat}}/K_m \) (μM \(^{-1} \) s \(^{-1} \)) |
|----------|--------------------------------|----------------|---------------------------------|
| T7pR30   | 1.43 ± 0.05                    | 4.2 ± 0.6      | 0.34 ± 0.06                     |
| T7pR50   | 1.55 ± 0.03                    | 5.3 ± 0.5      | 0.29 ± 0.03                     |
| T7pR80   | 0.93 ± 0.03                    | 2.8 ± 0.5      | 0.33 ± 0.07                     |
| T7pR100  | 1.22 ± 0.02                    | 8.0 ± 0.6      | 0.15 ± 0.02                     |

\[
V_{\text{obs}} = V_{\text{max}}[\text{dNTP}]/(K_m + [\text{dNTP}]) \quad (6)
\]

As template was in excess over enzyme in these experiments, apparent \( k_{\text{cat}} \) values were determined by dividing \( V_{\text{max}} \) by the concentration of polymerase. While there is some variability in the measured \( k_{\text{cat}} \) and \( K_m \) values, they are similar to literature values reported using steady-state methods [4,47]. Interestingly, \( k_{\text{cat}}/K_m \), which is a measure of productive dNTP capture, is significantly less variable. The variability in \( k_{\text{cat}} \) and \( K_m \) values may arise due to sequence variations between our templates. In our experiments, 30–100 nucleotides of dsDNA were synthesized (depending on the template) when the assay was allowed to go to completion.

The dependence of the T7pR80 template concentration on the stopped assay was next explored by performing the assay with saturating dNTPs (500 μM). KF exo− was used at 5 nM to allow sufficient time to acquire a reasonable reaction time course (data not shown). These data were again fitted to Eqn (5) to extract initial velocities that were then fitted to a ‘tight-binding’ expression (Eqn 7; \([E]_0\) is the initial enzyme concentration) to extract apparent \( V_{\text{max}} \) and \( K_m \) values for DNA binding to the polymerase (Fig. 6).

\[
V = \left( V_{\text{max}}/2[E]_0 \right) \left\{ (E) + [\text{dNTP}]_0 + K_m \right\} - \sqrt{(E) + [\text{dNTP}]_0 + K_m}^2 - 4[E]_0[\text{dNTP}]_0 \right\} \quad (7)
\]

Values of \( V_{\text{max}} = 1.6 ± 0.2 \) s \(^{-1} \) and \( K_m = 7 ± 3 \) nM were determined from the data in Fig. 6.

Finally, the dependence of the KF exo− concentration on the stopped assay was investigated with 50 nM T7pR100 template and saturating dNTPs (500 μM). It became progressively more difficult to extract reliable initial velocities from the time courses as the enzyme concentration was increased, as the reaction reached completion more quickly (Fig. 7, inset). The enzyme concentration dependence on the observed velocity appears to show saturation behaviour (Fig. 7) and these data were fitted to Eqn (7) to determine apparent \( V_{\text{max}} = 2.9 ± 0.3 \) s \(^{-1} \) and \( K_m = 22 ± 10 \) nM values. It is interesting to note that the apparent \( K_m \) values determined from Figs 6 and 7, respectively, are not significantly different and are similar to values determined for the association of KF with DNA using pre-steady-state methods [3]. It may be possible to study the processivity of the DNA polymerase using this assay, but a rapid-quench approach is likely to be required if significantly shorter templates are to be used.
In order for an enzyme reaction to be at steady-state, it has been argued that the following condition, \( \varepsilon \), must be met: \( \varepsilon = n_{\text{cat}}(K_m + S_0) \ll 1 \) [48]. While this criterion may not be strictly valid for non-Michaelis–Menten mechanisms such as that of DNA polymerase (which is multi-step and effectively has two substrates), it can still aid in the interpretation of enzyme turnover data. The dNTP dependences (Fig. 4; Table 2) were measured under conditions where \( \varepsilon < 0.01 \), while the template and enzyme dependences were, by necessity, measured with \( 0.08 < \varepsilon < 0.7 \) and \( 0.3 < \varepsilon < 3.2 \), respectively. Consequently, it would appear that our assay is capable of measuring steady-state turnover of DNA polymerase but only for nucleotide incorporation. This is likely to be a problem for other steady-state assays, and it is interesting to note that the value of \( k_{\text{cat}} \) determined in this paper is similar to those determined from the dNTP dependences (Table 2).

In this report, we have demonstrated a quantitative steady-state kinetic assay of the stoichiometric conversion of ssDNA to dsDNA catalysed by DNA polymerases, based on the differential fluorescence of PG when bound to dsDNA and ssDNA. PG was found to be a potent inhibitor of the KF exo\(^{-}\) reaction with an apparent inhibition constant of \( \sim 30 \text{ mM} \). However, by performing a stopped assay, Michaelis–Menten parameters (\( k_{\text{cat}}, K_m, k_{\text{cat}}/K_m \)) for DNA polymerase KF exo\(^{-}\) were determined and found to be in good agreement with previously reported values. The assay was found to be reliable over a range of DNA, enzyme and nucleotide concentrations and offers a viable alternative to conventional kinetic assays of DNA polymerase that employ radionucleotides. We also see no reason why this stopped assay cannot be applied to other DNA polymerase enzymes (and possibly reverse transcriptase enzymes), although the inhibition of the enzyme by PG should be confirmed first or an alternative quencher used. By employing a fluorescence plate reader, it is anticipated that a full steady-state assay of an enzyme could be performed in a few minutes. This could then lead to a screening assay, not only to examine enzyme kinetics, but potentially also to look at, for example, polymerase inhibitors.

**Materials and methods**

KF exo\(^{-}\) and NEB buffer 2 (10 mM Tris/HCl, 50 mM NaCl, 10 mM MgCl\(_2\), 1 mM dithiothreitol, pH 7.9) were obtained from New England Biolabs (Hitchin, Hertfordshire, UK). KF exo\(^{-}\) was monitored between batches by SDS/PAGE (single band at \( \sim 68 \text{ kDa} \)) and its concentration was determined spectrophotometrically \( (e_{290} = 55 \text{ 450 M}^{-1}\text{cm}^{-1}) \) [43,44]. dNTPs were obtained from Peqlab Ltd (Sarisbury Green, Hampshire, UK). PG was obtained from Invitrogen (Life Technologies, Paisley, UK) and its concentration was determined by \( (e_{390} = 70 \text{ 000 M}^{-1}\text{cm}^{-1}) \) [36]. PG was always used at a concentration below 4 \( \mu \text{M} \) for fluorescence experiments to avoid inner filter effects.

HPLC-purified DNA oligonucleotides were obtained from Eurofins MWG Operon (London, UK) and their sequences are given in Table 1. The sequences are designed to anneal to the T7 primer (from the T7 promoter sequence, explaining their nomenclature), leaving a 30-100 nucleotide 5’ overhang. This region of ssDNA is randomized and has approximately equal ratios of A:T:C:G. No secondary structure is predicted in any of the sequences. Oligonucleotides were annealed by heating the mixture to 95 °C for 10–15 min in buffer 2 (see below) before cooling to room temperature over at least 45 min. Unless stated otherwise, primed templates used for steady-state kinetics were made using a template oligo: T7 primer ratio of 1:2.

Fluorescence measurements were performed with an Edinburgh Instruments FLS920 spectrometer (Edinburgh, UK) with a 450 W xenon arc lamp light source, double excitation and emission monochromators and a red-sensitive cooled photomultiplier detector. Quartz fluorescence cells (Starna Scientific Ltd, Hainault, UK) with a 100 \( \mu \text{l} \) cell volume and 10 mm excitation pathlength were used. All measurements were performed at room temperature (\( \sim 23 \text{°C} \)). Care was taken to minimize photobleaching of PG by minimizing the excitation energy using a 0.5 nm excitation slit width. For all measurements, a 5 nm emission slit width and 0.1 s averaging time were used when acquiring spectra.

Steady-state reactions were performed in NEB buffer 2. The standard assay consisted of 100 \( \mu \text{M} \) DNA template, 5...
or 50 nM polymerase and equal quantities of each dNTP (combined concentrations are given in the text). Reactions were typically initiated by the addition of the polymerase. Discontinuous assays were stopped with 3 µM PG and measurements were made within 10 min of stopping. No significant change in fluorescence was observed upon waiting longer before measurement.

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