Purpose built reverse transcriptases and Taq DNA Polymerases deliver high yield in the presence of inhibitors

Eric van der Walt, Phillip Venter, Bjarne Faurholm, Ross Wadsworth, Craig Marshall, Jen Pavlica, Leo Karamanof, Abre de Beer, Julie Walker – Watchmaker Genomics

WATCHMAKER GENOMICS

Introduction

Many advances in molecular diagnostic (MDx) applications are built upon the engineering of novel enzymes which permit access to information that was previously impossible to capture. To address the ever-evolving need for highly functional and purpose-built enzymatic tools, we utilized a combination of enzyme engineering platforms including a proprietary platform that allows us to select enzyme variants with features that are highly desirable in genomics and molecular diagnostic applications. Our platform enables engineering of a wide selection of DNA- and RNA-modifying enzymes that utilize large-scale, hypothesis-free libraries as selection inputs to both enhance enzyme activity and/or introduce novel activities. We present data on our engineering efforts of reverse transcriptases (RT) and Taq DNA Polymerases (Taq DNAP) — two enzymes that are commonly used to detect RNA and/or DNA viruses in patient samples for point of care (POC) diagnostics. Our screens identified RT variants with increased thermostability and tolerance to inhibitors and Taq DNAP variants with increased speed and tolerance to inhibitors.

Novel Enzymes Enable Omics' and MDx Applications

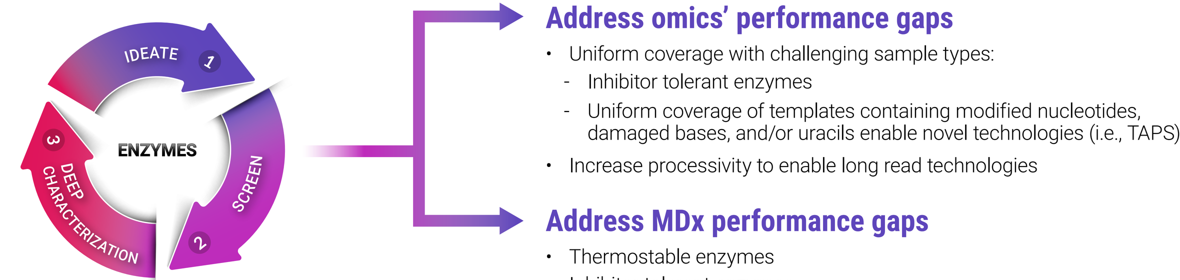

Figure 1. Overcoming performance gaps. Our enzyme engineering platforms are well suited to identify novel enzyme variants with properties desirable for genomics and molecular diagnostic (MDx) applications.

Engineering Core Competencies

Figure 2. Engineering capabilities. We take a three-pronged approach to enzyme engineering. Rationale design refers to the intentional modification of specific domains. In silico, or computational, design leverages algorithms to identify and introduce advantageous mutations. Directed evolution applies a screen to identify mutations of value within massive variant libraries.

Purpose Built Polymereases

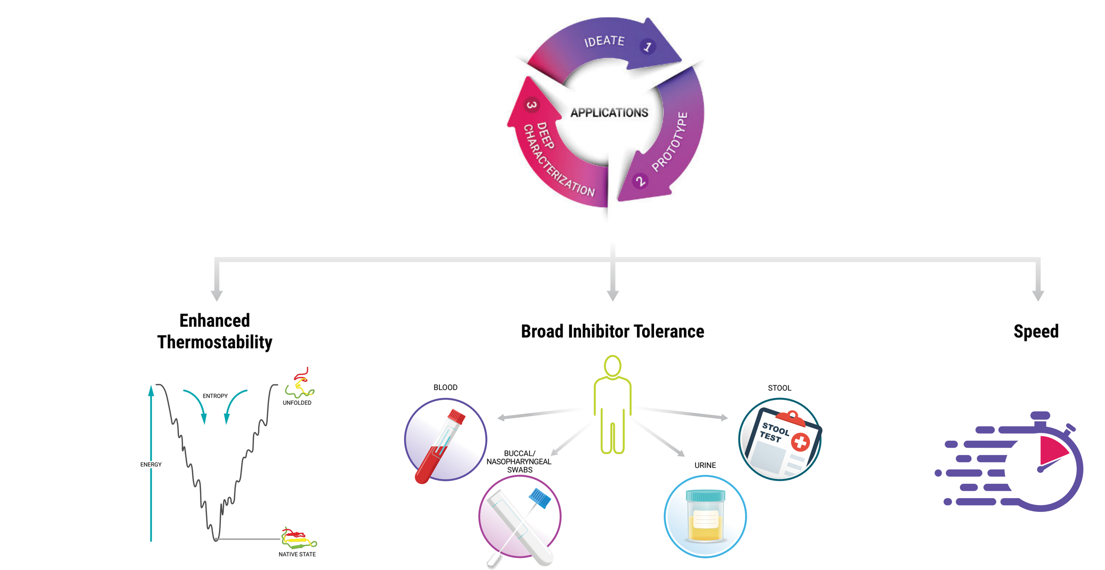

Figure 3. Purpose built Reverse Transcriptases and Taq DNA Polymerases. To address current pain points in the molecular diagnostic field, we aimed to identify mutations in reverse transcriptases and Taq DNAP that would confer enhanced thermostability, inhibitor tolerance and/or speed.

Reverse Transcriptases

	Optimal Temp.	Thermal Stability	Inhibitor Tolerance	Template Switching	RT-qPCR	RT-PCR	RNA-seq	scRNA-seq	5' RACE	3' RACE
StellarScript	42°C	+	+	+	+	+	+	+	+	+
StellarScript HT	42 – 50°C	++	++	-	++	++	+	-	-	+
StellarScript HT+	42 – 65°C	+++	+++	+	+++	+++	+	+	+	+

Table 1. Overview of Reverse Transcriptase for each enzyme. StellarScript portfolio enzymes properties and relevant applications. "+" signs indicate strength of a property or fit of an application an ideal choice for molecular diagnostic applications.

Taq DNA Polymerase Prototypes

Taq DNA Polymerase	Inhibitor Tolerance*	Polymerization Rate*	Fast PCR	Crude Sample Input
Hot Start aCat77	+	+	++	+
Hot Start aCat173	+++	++++	+++	+++
Hot Start aCat241	++++	+++	++	++++

*Relative to wild-type Taq DNAP

Table 2. Overview of Taq DNA Polymerases. We screened numerous Taq variants and identified three strong candidates. Hot Start aCat173 (HS-aCat173) Taq DNAP exhibits fast polymerization rates and robust inhibitor tolerance enabling fast PCR and amplification on challenging sample types. In addition, HS-aCat173 enables a broad buffer design space as it behaves optimally in a wide range of salt/ionic strength buffers. This makes HS-aCat173 an ideal candidate in a multitude of different applications and/or assays where various sample inputs are required.

Superior Thermostability

Increased thermostability enables reverse transcription at elevated temperatures to overcome RNA template secondary structure and generate cDNAs from difficult targets, such as viral RNA. It further improves specificity by minimizing nonspecific primer binding.

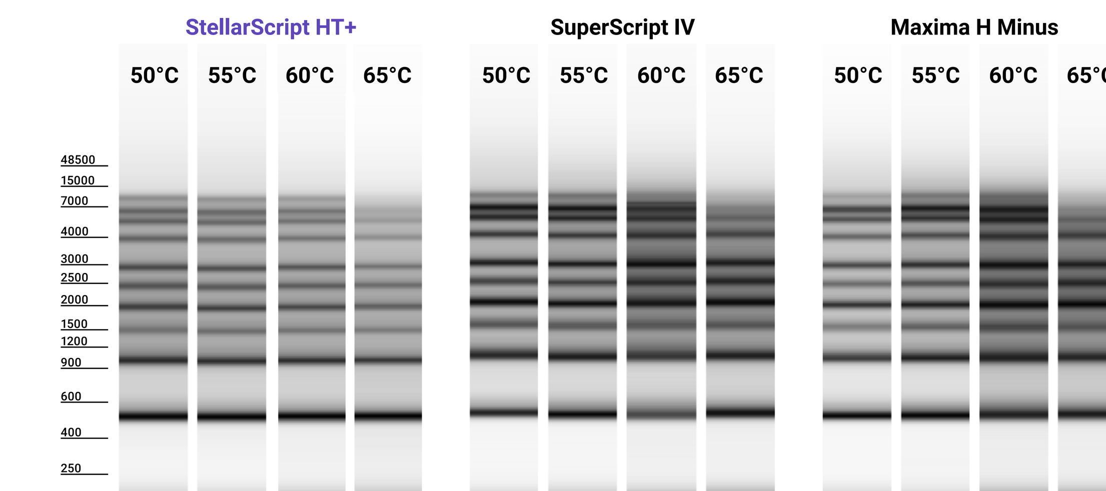

Figure 4. StellarScript HT+ provides equivalent processivity at elevated temperatures. Watchmaker's StellarScript HT+ and ThermoFisher Scientific's SuperScript IV and Maxima H Minus were run in an oligo-dT-primed first strand synthesis at 50°C, 55°C, 60°C, or 65°C for 30 min using a 0.5 to 9 kb RNA ladder as template. All enzymes have robust processivity up to 60°C.

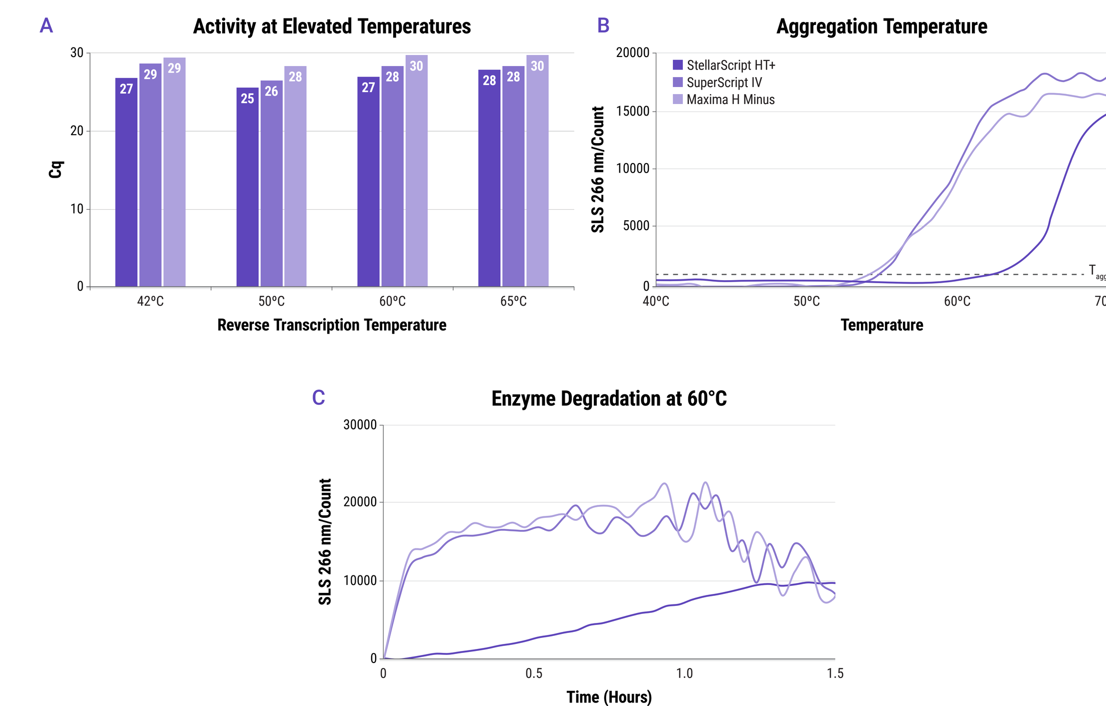

Figure 5. StellarScript HT+ delivers superior thermostability. (A) Watchmaker's StellarScript HT+ and ThermoFisher Scientific's SuperScript IV and Maxima H Minus were run in an oligo-dT-primed first strand synthesis was employed for 25 min using 10 ng total liver RNA, as indicated. Resulting cDNA mass was assessed via qPCR using primers targeting the 5' end of β-actin gene to generate a 90 bp amplicon. StellarScript HT+ produced higher yields (indicated by lower Cq values) than SuperScript IV and Maxima H Minus at any evaluated temperature, indicating improved efficiency at elevated temperatures. **(B)** Enzymes were further assessed via static light scattering under increasing temperature to determine their respective aggregation temperatures, at which point protein unfolding begins and **(C)** during a time course study at 60°C to measure their stability in reverse transcription reaction conditions. The enhanced thermostability of StellarScript HT+ is demonstrated by its increased aggregation temperature and improved stability over time at 60°C.

Robust Inhibitor Tolerance

Inhibitors can dampen reverse transcription efficiency and impact sensitivity in applications such as RT-qPCR. Improved robustness in the presence of common inhibitors safeguards performance and ensures compatibility across a wide range of sample types and sources. StellarScript HT+ delivers the highest level of inhibitor tolerance across the StellarScript portfolio.

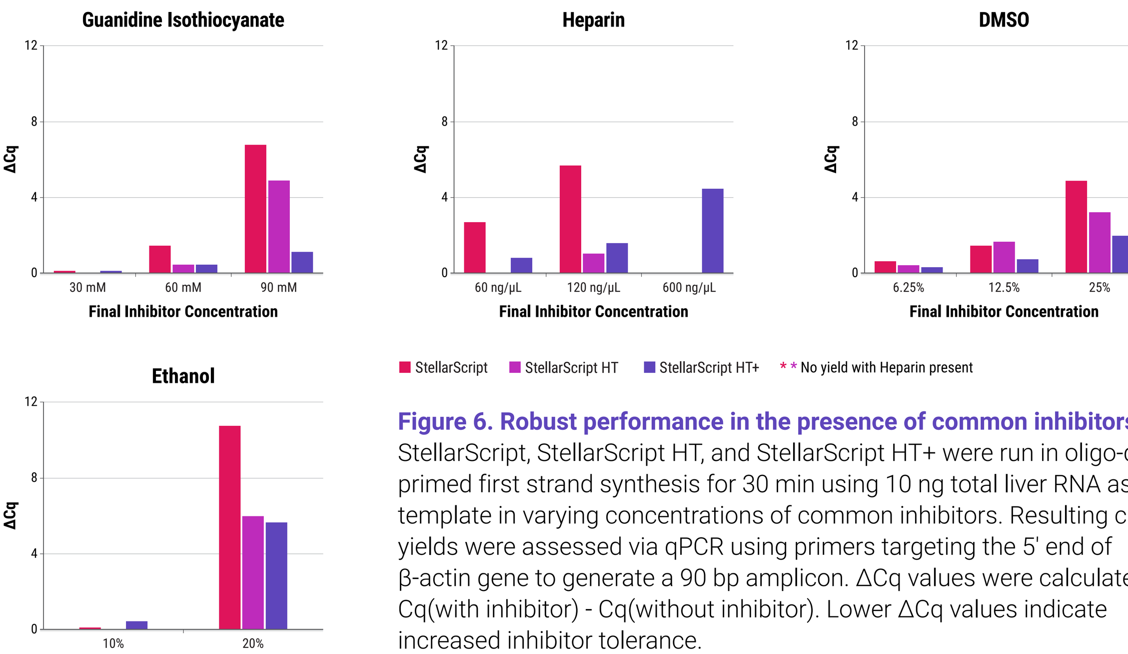

Figure 6. Robust performance in the presence of common inhibitors. StellarScript, StellarScript HT, and StellarScript HT+ were run in oligo-dT-primed first strand synthesis for 30 min using 10 ng total liver RNA as template in varying concentrations of common inhibitors. Resulting cDNA yields were assessed via qPCR using primers targeting the 5' end of β-actin gene to generate a 90 bp amplicon. ΔCq values were calculated: Cq(with inhibitor) - Cq(without inhibitor). Lower ΔCq values indicate increased inhibitor tolerance.

High Throughput Taq DNAP Variant Performance Mapping

Directed evolution of Taq DNAP was performed in the presence of inhibitory molecules. The screening effort generated a wealth of data, revealing high resolution sequence-function relationship of modifications that increase or decrease enzyme activity and/or enzyme tolerance to inhibitory molecules (Figure 7).

Relative Variant Activity of All Mutations from an Inhibitor Screen

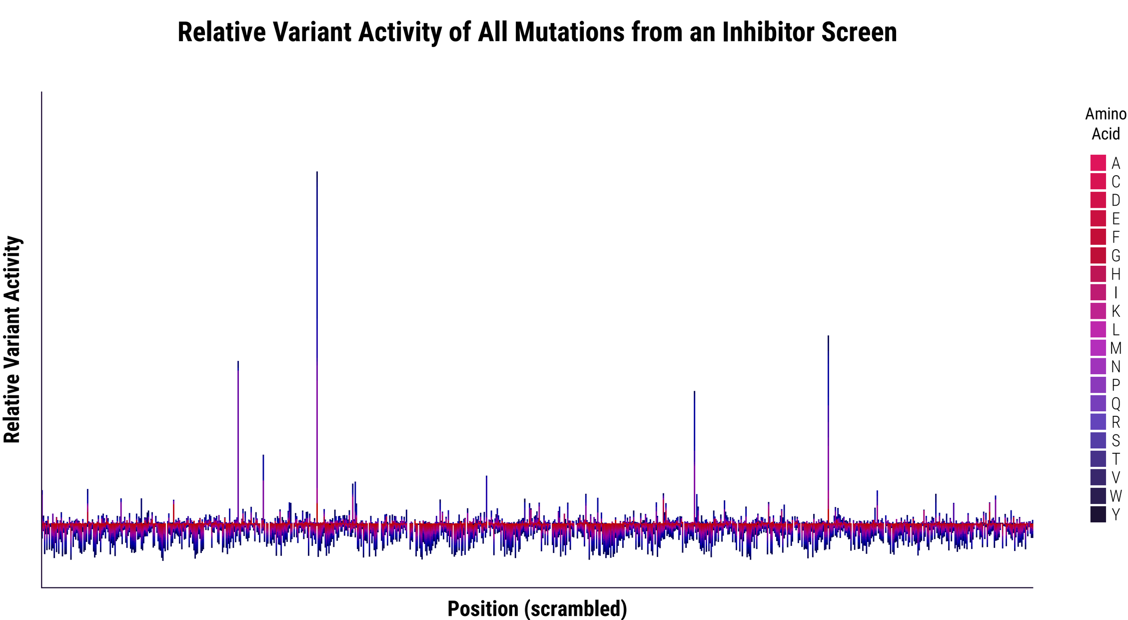

Figure 7. Inhibitor tolerance screen shows domain "hotspots" that confer enhanced inhibitor tolerance. A comprehensive single site variant library of Taq DNAP was screened for increased PCR activity in the presence of inhibitors. Performance of all amino acid substitutions at all positions is shown. To generate a measure of "relative activity" mutants activity is compared to the activity of WT enzyme under the same condition.

Taq DNAP Inhibitor Tolerance

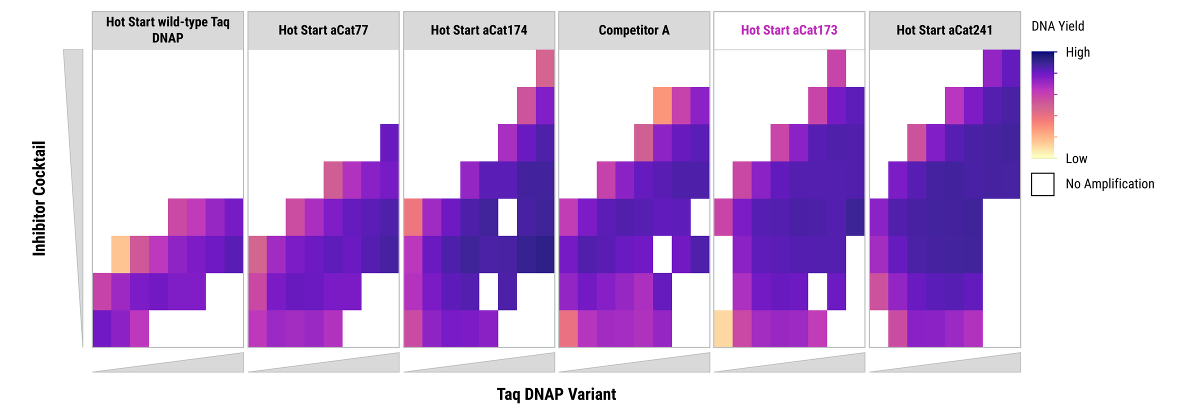

Figure 8. Enzyme vs inhibitor cocktail matrix of Taq DNAP prototypes and competitors. A 66 base pair target was amplified off of 100 copies of human genomic DNA with each Taq DNAP variant in a probe based qPCR assay. To characterize improved inhibitor tolerance, a matrix of increasing enzyme and inhibitor cocktail concentrations were tested. Cq values are represented as DNA yield and shown as a heatmap. The Taq DNAP variants are ranked from left to right from least inhibitor tolerant to most inhibitor tolerant: HS WT Taq DNAP < HS-aCat77 < HS-aCat174 < Competitor A < HS-aCat173 < HS-aCat241.

The inhibitor tolerant prototypes in our initial screens were tested for tolerance to common inhibitors such as urine and bile salts. The buffer salt concentration has a strong effect on inhibitor tolerance thus a matrix of inhibitor vs salt was performed. Our Taq DNAP prototypes show superior inhibitor tolerance in comparison to three main competitors and wild-type Taq DNAP (Figure 9).

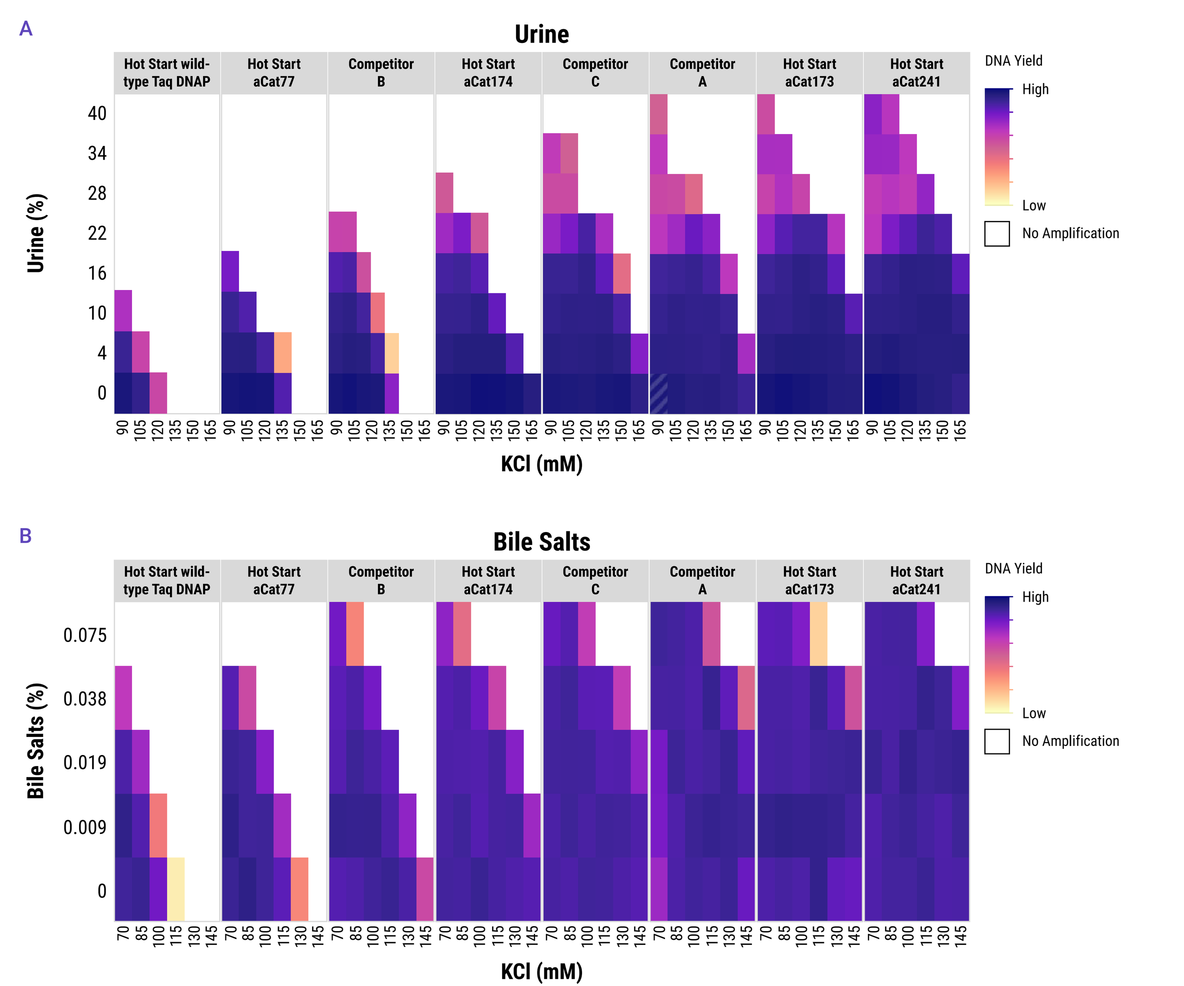

Figure 9. Inhibitor vs salt matrix of Taq DNA polymerase prototypes and competitors. PCR targets of ~100 base pairs were amplified off of ~500 copies of template DNA using equimolar concentrations of each Taq DNAP variant in probe based qPCR assays. A matrix of increasing inhibitor and salt was performed. **(A)** Urine. **(B)** Bile salts. Cq values are shown as a heatmap. The Taq DNAP variants are ranked from left to right from Least inhibitor tolerant to most inhibitor tolerant: HS WT Taq DNAP < HS-aCat77 < Competitor B < HS-aCat174 < Competitor C < Competitor A < HS-aCat173 < HS-aCat241.

Taq DNAP Polymerization Speed

The polymerization speed of each Taq DNAP variant was investigated to determine if the variants could be used in a fast PCR setting. The assay was performed at various salt concentrations as each polymerase has a unique optimal salt concentration. HS-aCat173 show polymerization speeds that are significantly faster than those of wild-type Taq DNAP and our competitors (Figure 10).

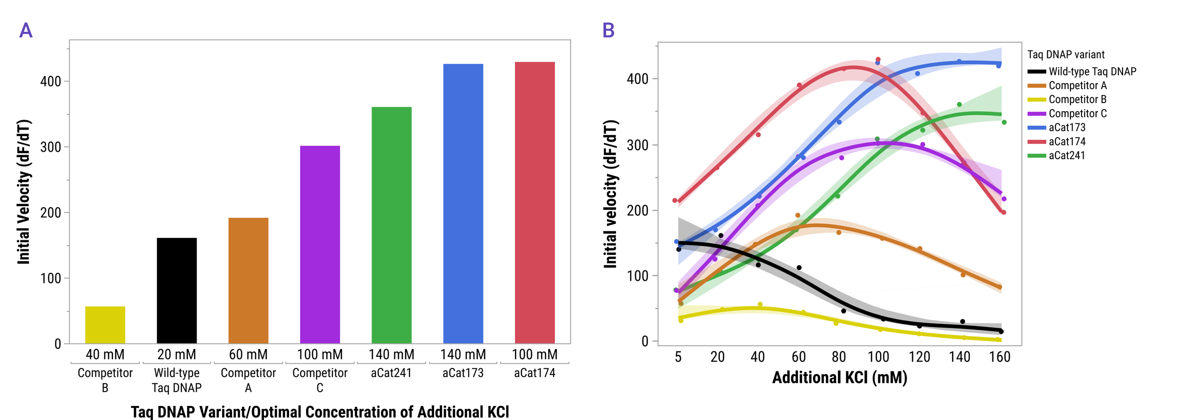

Figure 10. Polymerization speed of the Taq DNAP prototypes and competitors. A pre-primed defined ssDNA template is extended by the Taq DNAP variants, at equimolar concentration, at 72°C. The EvaGreen fluorescence increase over time was measured. The initial velocity of the kinetic readout was taken as a representation of the polymerization speed. The assay was performed at increasing buffer KCl concentrations to assess the optimal polymerization speed. **(A)** The maximum initial velocity of each polymerase at their optimal buffer salt concentration. **(B)** Initial velocity of each polymerase at increasing concentrations of KCl.

Conclusions

- Watchmaker Genomics' enzyme engineering platforms identify polymerase variants with **enhanced activities** for molecular diagnostic applications.
- **StellarScript HT+** has enhanced thermostability and inhibitor tolerance.
- We identified several Hot Start Taq DNAP variants that have enhanced inhibitor tolerance and speed. **HS-aCat173** is currently available for beta testing.

For Research Use Only. Not for use in diagnostic procedures.
© 2024 Watchmaker Genomics. All trademarks are property of their respective owners.

M292