Engineered reverse transcriptases and Taq DNA polymerases deliver robust yield in the presence of clinically relevant inhibitors

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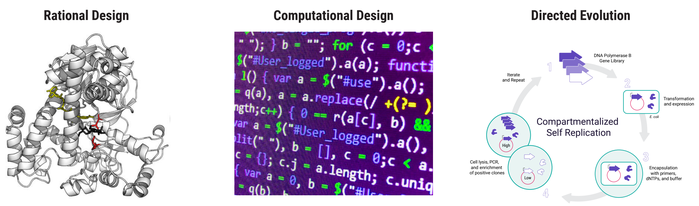
Introduction

All biological information is read, written, and edited by enzymes. If you improve the enzymes, you improve data quality. Reverse transcriptase (RT) and Taq DNA Polymerase (Taq DNAP) are commonly used to detect RNA and/or DNA viruses in patient samples for point-of-care (POC) diagnostics. Crude patient samples bring over inhibitors that inhibit enzymes leading to a decrease in enzymatic activity potentially leading to an increase in false-negative results. In addition, there is a need for faster turnaround time for patient diagnosis which can be enabled by developing faster and more sensitive Taq DNAPs. We set out to develop engineered reverse transcriptases and Taq DNA Polymerases that exhibit enhanced thermostability, inhibitor tolerance, sensitivity, specificity, and/or speed.

Enzyme Engineering

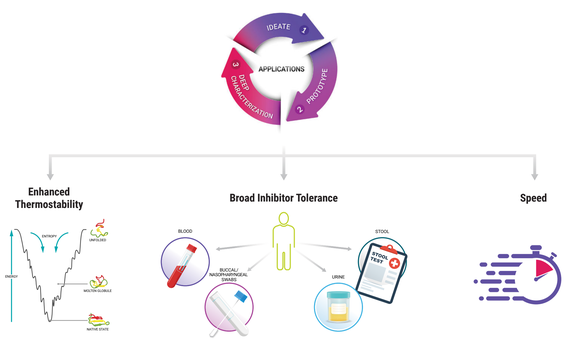
Rational design, *in silico*-based approaches, and a high-purity prototype screen were used to rapidly purify and characterize over 20 RT and Taq DNAP prototypes. Enzymes were screened iteratively for improved thermostability, inhibitor tolerance, and speed. RTs were characterized in RT-qPCR, RNA-seq, a thermal shift assay, and an RNA ladder assay to identify highly thermostable and inhibitor-tolerant variants. We characterized engineered Taq DNAPs in a polymerization assay and qPCR to identify fast and inhibitor-tolerant polymerases.

From our reverse transcriptase development efforts, we commercialized StellarScript HT+. StellarScript HT+ exhibits best-in-class thermostability and inhibitor tolerance. From our Taq DNAP development efforts, we characterized variants that exhibit enhanced speeds, inhibitor tolerance, and high sensitivity and specificity. Based on these key attributes, StellarScript HT+ and WMG Taq DNAP variants are well suited to give robust yields from various clinically relevant inputs. We were successful in engineering RT and Taq DNAP variants that are highly thermostable, inhibitor tolerant, and thus will enable breakthroughs in molecular diagnostics.



Experimental Approach

Reverse transcriptase (RT) and Taq DNA Polymerase (DNAP) engineering. We combined rational design and *in silico* based approaches and used a high-purity prototype screen to rapidly purify and characterize >20 RT and Taq DNA Polymerases prototypes. We took an iterative approach to screen engineered RTs and Taq DNAPs for improved thermostability, inhibitor tolerance, and speed. We characterized engineered RTs in RT-qPCR, RNA-seq, a thermal shift assay, and an RNA ladder assay to identify highly thermostable and inhibitor tolerant variants. We characterized engineered Taq DNAPs in a polymerization assay and qPCR to identify fast and inhibitor-tolerant polymerases.



Engineered Polymereases

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. StellarScript portfolio enzymes properties and relevant applications. "+" signs indicate strength of a property or fit of an application for each enzyme. StellarScript HT+ has best-in-class thermostability and inhibitor tolerance making it an ideal choice for molecular diagnostic applications.

Taq DNA Polymerase Prototypes

Taq DNA Polymerase	Inhibitor Tolerance*	Polymerization Rate*	Broad Buffer and Sample Compatibility*
aCat77	+	+	++
aCat174	+++	+++	+++
aCat241	+++	+++	+

Figure 1. Overview of Taq DNA Polymerases. We screened numerous Taq variants and identified three strong candidates. aCat174 Taq DNA Polymerase exhibits fast polymerization rates and robust inhibitor tolerance enabling fast PCR and amplification on challenging sample types. In addition, buffer additives have little to no effect on overall yield making aCat174 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.

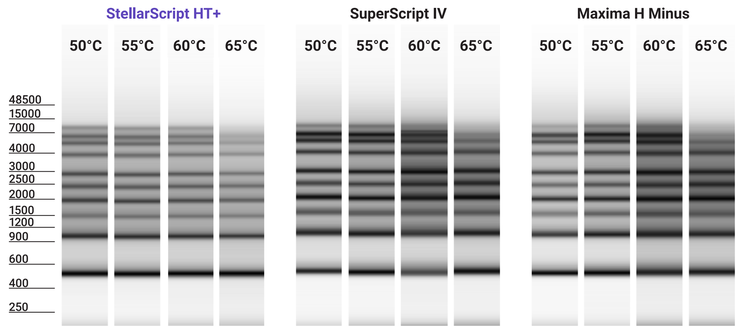


Figure 2. StellarScript HT+ provides equivalent processivity at elevated temperatures. Watchmaker's StellarScript HT+ and ThermoFisher Scientific's SuperScript IV and Maxima H Minus were run in an oligo-dT-primed first strand synthesis at 50°C, 55°C, 60°C, or 65°C for 30 min using a 0.5 to 9 kb RNA ladder as template. All enzymes have robust processivity up to 60°C.

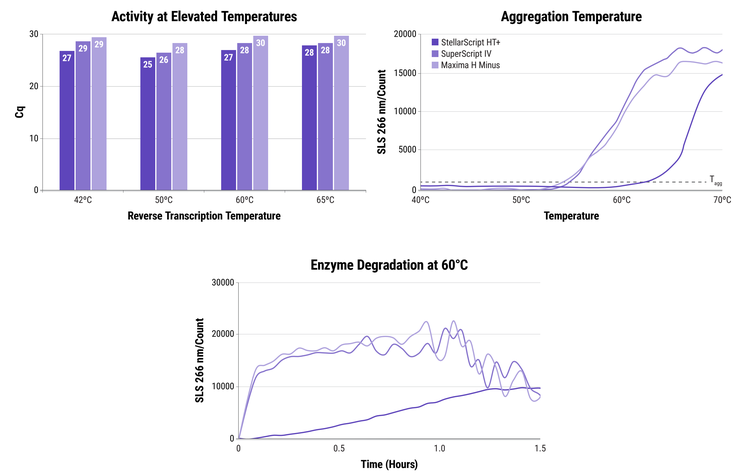


Figure 3. StellarScript HT+ delivers superior thermostability. (A) Watchmaker's StellarScript HT+ and ThermoFisher Scientific's SuperScript IV and Maxima H Minus were run in an oligo-dT-primed first strand synthesis was employed for 25 min using 10 ng total liver RNA, as indicated. Resulting cDNA mass 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.

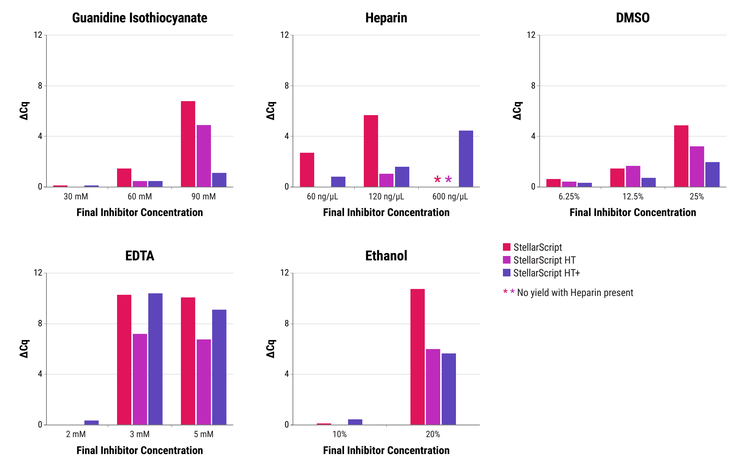


Figure 4. Robust performance in the presence of common inhibitors. StellarScript, StellarScript HT, and StellarScript HT+ were run in oligo-dT-primed first strand synthesis for 30 min using 10 ng total liver RNA as template in varying concentrations of common inhibitors. Resulting cDNA yields were assessed 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.

Robust Inhibitor Tolerance

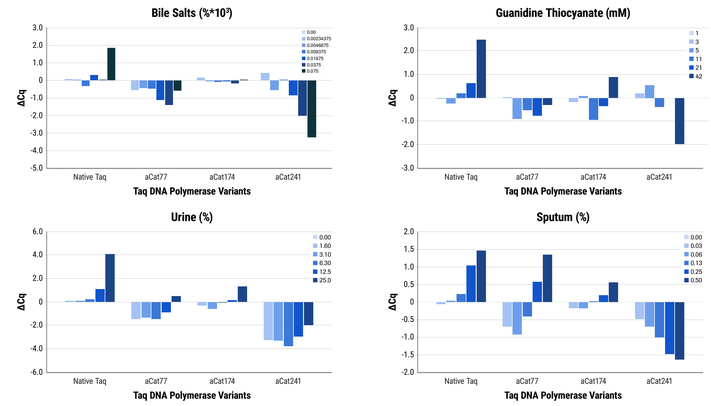


Figure 5. Robust performance in the presence of common inhibitors. Each Taq Polymerase variant was run in a qPCR assay with increasing concentrations of common inhibitors to assess inhibitor tolerance. Impact to DNA yield was assessed by calculating the ΔCq value: Cq (with inhibitor) - Cq (without inhibitor). Lower or negative delta Cq indicates more efficient amplification in the presence of inhibitors. aCat174 Taq DNA Polymerase delivers the broadest level of inhibitor tolerance across a wide variety of sample inputs. aCat241 Taq DNA Polymerase offers robust inhibitor tolerance but requires additional buffer optimization to find optimal performance.

Taq Polymerase Variants Enable Fast PCR

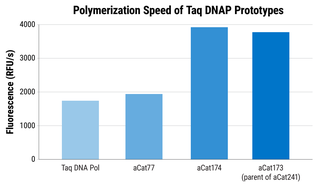


Figure 6. Elongation assay. A reaction containing 250 ng M13mp18 ssDNA, primer, standard reaction buffer, SYBR green and Taq variants at equimolar concentrations was incubated at 50°C and fluorescence per second was measured to determine the speed of each variant. aCat174 and aCat173 (parent of aCat241) have twice the polymerization rate of native Taq DNA Polymerase making them excellent candidates for fast PCR.

RT and Taq Polymerase Compatibility

RT: aCat72 (Units)	Taq Pol: aCat174 (Units) 0.24	0.6	1.5
0	14.1	13.9	14.0
2			20.2
6			
20			

RT: StellarScript HT+ (Units)	Taq Pol: aCat174 (Units) 0.24	0.6	1.5
0	14.0	13.8	14.0
2	18.2	17.8	15.7
6	20.8	19.5	17.9
20			

RT: aCat72 (Units)	Taq Pol: aCat241 (Units) 0.24	0.6	1.5
0	13.8	14.1	14.4
2		20.9	20.2
6			
20			

RT: StellarScript HT+ (Units)	Taq Pol: aCat241 (Units) 0.24	0.6	1.5
0	13.7	14.0	16.1
2	19.7	18.6	18.9
6	19.9		
20	20.6		

Low Cq — High Cq

Figure 7. RT & Taq Polymerase variant compatibility assay. A qPCR reaction containing a 440bp DNA standard, primers, a Taq DNA Polymerase variant and varying amounts of RT was run under standard qPCR conditions. DNA yield was assessed via Cq values of reactions where specific amplification occurred. Darker purple indicates higher DNA yields while lighter colors indicate lower or no DNA yield. StellarScript HT+ is the least inhibitory in qPCR and has high compatibility with aCat174.

Key Takeaways

- **Enzyme engineering** is a key technology pillar at Watchmaker Genomics. We utilize several approaches to enable; 1) high-purity prototype characterization, or 2) high-throughput selection screens. Both approaches output novel enzymes with desirable phenotypes (i.e., inhibitor tolerance, speed, thermostability, etc.).
- Watchmaker Reverse Transcriptase, **StellarScript HT+**, has enhanced thermostability and inhibitor tolerance making StellarScript HT+ an ideal candidate for cDNA generation on clinical samples.
- Watchmaker **Taq DNA Polymerase Prototypes** exhibit fast polymerization rates and enhanced inhibitor tolerance making them ideal enzymes for fast PCR and clinical samples.
- Watchmaker **RNase Inhibitor** improves cDNA yield when added to first strand synthesis cDNA reactions by inhibiting pancreatic ribonuclease (RNase A, B, and C).