

Guidelines for the optimization of assays using HawkZ05 Fast Polymerase

Application Note

July 2018
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Introdution

Z05 Fast DNA polymerase is a mutant enzyme derived from the Z05 polymerase of the thermophilic eubacterium *Thermus* species Z05 and is designed for enhanced speed and efficiency. With both RNA- and DNA-dependent polymerase activity, Z05 Fast DNA polymerase can be used to assemble one-enzyme, one-step, one-tube RT-PCR reactions. All products of the HawkZ05 Fast portfolio use this thermostable and thermoactive Z05 Fast enzyme.

HawkZ05 Fast Polymerase exhibits excellent performance at high temperatures and is highly tolerant of inhibitors. As a result, the enzyme affords high flexibility in assay design that can be leveraged to, for example, create assays for complex targets from various samples while keeping assay architecture and protocol simple.

The complexity of a PCR assay makes optimizing reaction parameters challenging. Thus, this application note provides data and guidance to successfully incorporate HawkZ05 Fast Polymerase into your assay and adjust reaction conditions and components for optimal performance. The first section demonstrates the technical strengths of the HawkZ05 Fast Polymerase and the second outlines a strategy to optimize its performance in your hands.

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Technical strengths of HawkZ05 Fast

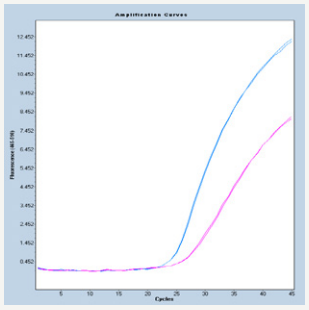
Products of the HawkZ05 Fast Polymerase portfolio are versatile because of the enzyme's hot start capability, dual polymerase activity (RNA as well as DNA-dependent activity), robustness against inhibitors, and compatibility with both multiplexing and lyophilization. The following is a profile of these technical strengths and their contribution to enzyme and assay performance.

Hot start feature

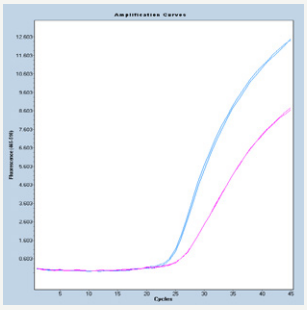
Analytical specificity is an essential parameter in the development and validation of a molecular assay.[1]
High reaction temperatures for reverse transcription and hot start mechanisms are important features to prevent nonspecific amplification and enhance assay specificity. Thermostable Z05 Fast Polymerase performs reverse transcription efficiently at high temperatures and is blended with an aptamer that regulates its activity with a reversible, temperature-dependent hot start mechanism. At low temperatures, the aptamer binds to the enzyme and inhibits its activity. At temperatures above
55 °C, the aptamer starts losing its affinity for the polymerase, and by 60 °C the enzyme is fully active. As the temperature drops below 55 °C at the end of the reaction, the aptamer binds to the DNA polymerase again and renders it inactive.

Exemplifies the performance of HawkZ05 Fast Polymerase at different reverse transcription temperatures. Performance was optimal with full enzyme activation at 60 °C. At 50 °C and 55 °C, a portion of enzyme molecules is still bound by the aptamer and thus, inactive. Nevertheless, when optimizing multiplex reactions, two or three reverse transcription holds at 55 °C, 60 °C and then 65 °C can achieve better specificity by regulating enzyme availability at the beginning of the reaction. In addition to following standard primer design guidelines,[2,3] make sure to design primers that can accommodate high-temperature reverse transcription reactions, as well as PCR anneal and extension steps at 60 °C or higher (Fig. 01).

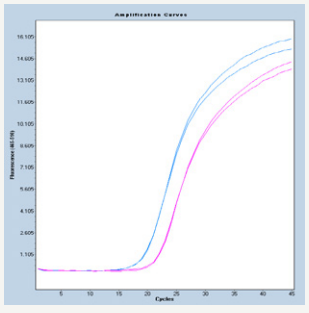
RT @50 °C



RT @55 °C



RT @60 °C



RNA conc.
100 ng
10 ng

01
Optimal temperature for reverse transcription is critical to maximize assay performance.

HawkZ05 Fast Polymerase was used to amplify GAPDH (FAM) at high and low RNA concentrations (100 ng and 10 ng) in 20 µl reactions with Tricine buffer. Reverse transcription was carried out at 50 °C, 55 °C or 60 °C followed by PCR. Performance is maximized at 60 °C, when the enzyme is fully active.

	RT Temperature		
RNA conc.	**50 °C**	**55 °C**	**60 °C**
100 ng	24.61	24.28	19.63
10 ng	26.89	26.15	21.44

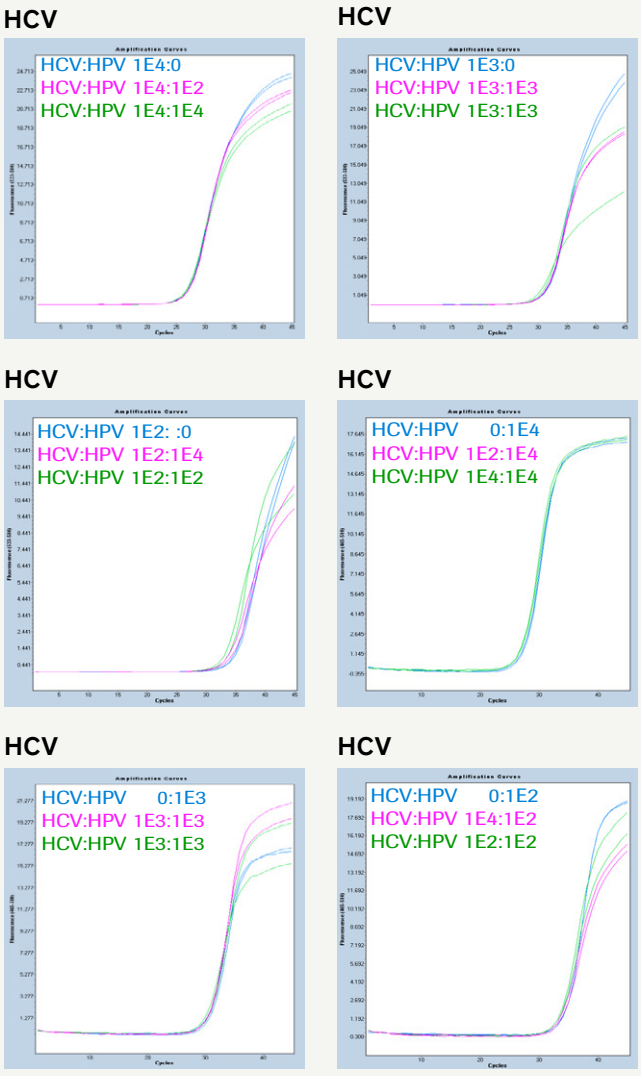
Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	50 °C or 55 °C or 60 °C	5 min
PCR	1	95 °C	30 sec
	45	95 °C	10 sec
		60 °C	30 sec

Mixed polymerase activity

The dual polymerase activity of HawkZ05 Fast Polymerase enables carrying out RT-PCR and PCR using a single enzyme and provides the flexibility to detect RNA and DNA targets simultaneously in a single reaction, significantly reducing workflow complexity. To assess the performance of HawkZ05 Fast Polymerase in the simultaneous detection of RNA and DNA targets in a single reaction, we performed a duplex PCR (RT-PCR and PCR) containing different ratios of an RNA (Hepatitis C Virus (HCV); low, medium, high) versus a DNA (Human Papillomavirus (HPV); low, medium, high) target (Fig. 02).

Amplification of both analytes was robust at all DNA versus RNA concentration ratios, even when differences spanned several orders of magnitude. Neither the Cp nor the fluorescence values of the targets were impacted in the combined reaction. When designing mixed reaction assays, assess the amplification efficiency for each target in the presence of various concentrations of the other target or targets to characterize limits in amplification efficiency and sensitivity that arise from the multiplexed assay architecture.



02
One-enzyme, one-step and one-tube amplification of RNA and DNA targets.

Different ratios of RNA (HCV-Vic) and DNA (HPV-FAM) targets were detected simultaneously using HawkZ05 Fast Polymerase in a 20 µl reaction volume using Tricine buffer and the thermal cycling protocol shown below. The top panel shows amplification of high, medium and low copy numbers of the RNA target in the presence of high to low concentrations of the DNA target. Conversely, the bottom panel shows robust amplification of high, medium and low copy numbers of the DNA target in the presence of various RNA target concentrations. Amplification of both targets was robust in all combined reactions.

Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	60 °C	5 min
PCR	1	92 °C	30 sec
	45	92 °C	10 sec
		60 °C	30 sec

	DNA High	DNA Medium	DNA Low
RNA High	✔	✔	✔
RNA Medium	✔	✔	✔
RNA Low	✔	✔	✔

Inhibitor tolerance

Sensitivity and specificity of a PCR assay is influenced by several factors, including sample quality (e.g., FFPE samples, blood samples) and template purity (crude versus purified). Characterizing assay performance in the presence of "interfering components" is required for assay validation as an essential parameter for robustness and accuracy.[1] Mixed DNA polymerases are known to be resistant to many inhibitors from specimens,[4] and HawkZ05 Fast Polymerase is no exception. Without additives or a special formulation, the enzyme exhibits robust performance in the presence of common inhibitors found in samples or that result from sample preparation methods. Fig. 03 summarizes the inhibitor tolerance levels of HawkZ05 Fast Polymerase.

In a similar way, test any inhibitors that the intended sample type of your assay might carry. Assay performance improvements can be achieved by optimizing enzyme concentrations or using reaction additives.



03
Inhibitor tolerance levels of HawkZ05 Fast Polymerase.

HCV RNA template (1,000 copies) was amplified using HawkZ05 Fast Polymerase in Tricine buffer with the thermal cycling protocol shown below. The 20 µl RT-PCR reaction was spiked with various concentrations of inhibitors. Tolerance limit is defined as **≤3 cycles** change in Cp value and **≤45 %** change in fluorescence.

Thermal cycling protocol

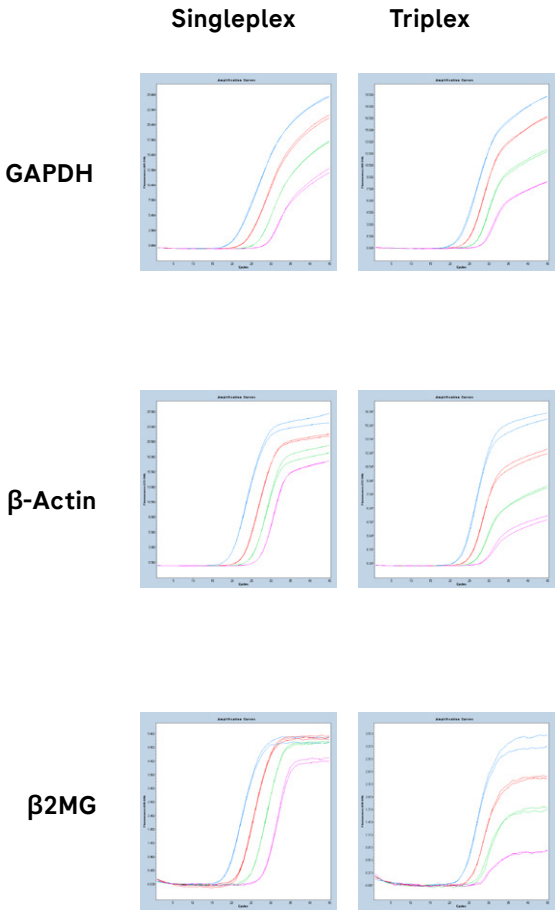
Step	Cycles	Temp	Time
RT	1	55 °C	5 min
	1	60 °C	5 min
	1	65 °C	5 min
PCR	1	95 °C	30 sec
	45	95 °C	15 sec
		60 °C	30 sec

Color indicates probable source of inhibitor

- **Sample prep**
- **Patient sample or sample collection tubes**

Multiplexing capacity

HawkZ05 Fast Polymerase enables simultaneous amplification of multiple targets in a single reaction, significantly reducing the number of reactions and amount of precious sample material needed. Fig. 04 is a comparison of triplex reactions to amplify GAPDH, β-actin and β2MG versus the singleplex reactions for each target. Total RNA used as template varied from 100 ng to 0.01 ng, and the reactions were carried out in Tricine buffer without employing additives, optimizing primer-probe combination, or increasing enzyme concentration. HawkZ05 Fast Polymerase robustly amplified the three targets across the dynamic range tested. Enhance the performance of your multiplex assay by adjusting enzyme amount, optimizing primer design, and testing different PCR cycling conditions and fluorophores for each target.



04
Triplex versus singleplex RT-PCR using HawkZ05 Fast Polymerase.

GAPDH, β-actin and β2MG were amplified in triplex reactions across a range of template amounts (total RNA) and compared to the singleplex detection of the individual targets. Each 20 µl reaction was carried out in Tricine buffer without optimization of primer-probe combination, additives or increased amounts of enzyme. HawkZ05 Fast Polymerase amplified all three targets in the triplex reactions with comparable efficiency and across the dynamic range tested.

Total RNA	GAPDH	Triplex/GAPDH
100 ng	20,74	22,79
10 ng	24,215	24,96
1 ng	26,8	26,70
0,1 ng	28,86	28,10

Total RNA	β-Actin	Triplex/β-Actin
100 ng	20,48	23,22
10 ng	23,15	25,15
1 ng	25,32	26,42
0,1 ng	27,26	28,09

Total RNA	β2MG	Triplex/ β2MG
100 ng	18,79	23,57
10 ng	21,98	25,48
1 ng	25,23	26,75
0,1 ng	28,27	27,56

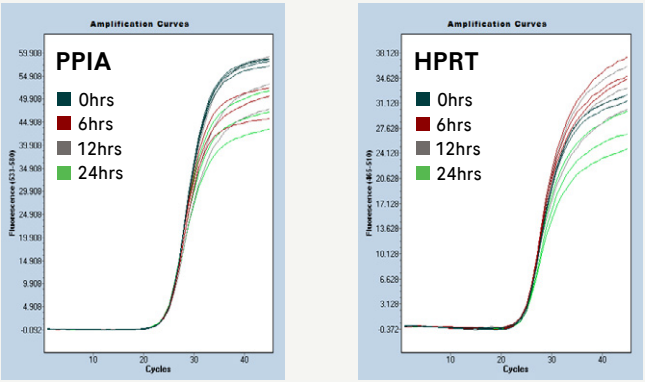
Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	60 °C	5 min
PCR	1	92 °C	30 sec
	45	92 °C	10 sec
		60 °C	30 sec

Room temperature stability

HawkZ05 Fast Polymerase and HawkZ05 Fast One-Step RT-PCR Master are stable at 2 – 8 °C for 4 and 12 weeks, respectively, see corresponding instructions for use. For high-throughput scenarios, where reaction plates are pipetted by automated dispensers and stacked up in holders before going into PCR instruments, stability at room temperature can greatly simplify workflows. To test the stability of HawkZ05 Fast Polymerase at room temperature, we set up RT-PCR reactions with the HawkZ05 Fast Master mix, which is designed without glycerol to be compatible with high-throughput automation. Complete reactions were set up for duplex RNA amplification and then tested after storage for 0, 6, 12 and 24 h at room temperature. No deterioration in performance based on Cp values or fluorescence was observed at any tested time point (Fig. 05).

Key aspects to consider for holding complete reactions at room temperature is the susceptibility of reactions to inhibitors from samples themselves or from sample preparation methods, and the impact of elevated temperatures for extended periods of time on the reaction components. Assessing these factors in a systematic approach, as in Fig. 05, allows adjusting assay parameters or protocol steps to ensure optimal reaction setup and performance.



05
Stability of HawkZ05 Fast One-Step RT-PCR Master at room temperature (25 °C).

Reactions were assembled using HawkZ05 Fast Lyo Mastermix with 50 ng of total RNA as template (reaction volume 10 µl) and incubated at room temperature (25 °C) for 0, 6, 12, 24 hrs. Subsequently, RT-PCR was performed using the thermal cycling protocol shown below. Storage of the complete reaction at room temperature did not impact the performance of HawkZ05 Fast Polymerase in the subsequent RT-PCR.

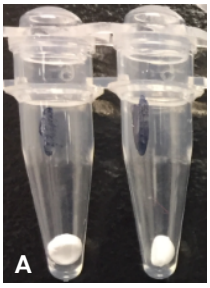
Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	55 °C	5 min
	1	60 °C	5 min
	1	65 °C	5 min
PCR	1	95 °C	30 sec
	45	95 °C	15 sec
		60 °C	30 sec

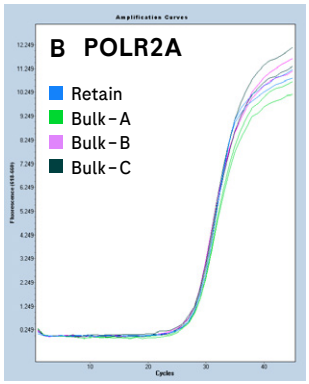
Designed for lyophilization

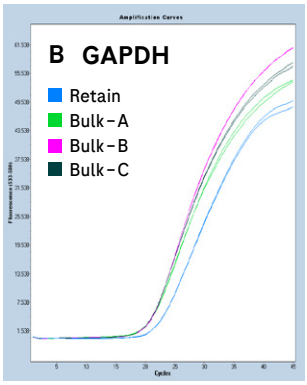
Lyophilization compatibility is a desired attribute for assay components because it simplifies shipping, storage and setup. HawkZ05 Fast Polymerase enzyme (≤0.1 % glycerol) and the HawkZ05 Fast One-Step RT-PCR Master (5× conc. with 0.5 % glycerol) are available with very low glycerol and are compatible with lyophilization and high-throughput automation. We examined the suitability of HawkZ05 Fast Polymerase for lyophilization. An external party lyophilized HawkZ05 Fast Master mix with three different stabilizers/bulking agents (A, B, C).The resulting pellets were reconstituted with water, used in the amplification of RNA targets, and the amplification performance was compared with retain material before bulking agents were added and lyophilized. Excellent correlation of Cp values and fluorescence intensities were observed before and after lyophilization (Fig. 6B). We also observed that the stabilizers used for lyophilization can, in some cases, slightly improve performance (Cps), as was the case for GAPDH (Fig. 6B). We also assessed the stability of beads when stored at room temperature for up to 4 weeks. As shown in Fig. 6C, lyophilized pellets remained stable during storage and displayed similar performance as the control pellets.

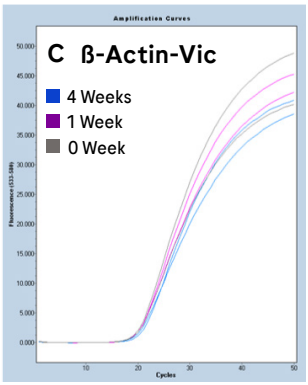
Assay architecture (ie., using an enzyme alone or adding further reaction components), storage conditions for master mixes or other assay components after lyophilization (ie., refrigerated or at room temperature), and the use of bulking or stabilizing agents are all important considerations for optimizing an assay. While lyophilization can build convenience into shipping and handling of assay components, make sure to assess the robustness of your assay before and after lyophilization, and under conditions that can arise during storage to make appropriate handling recommendations if necessary.



(A) HawkZ05 Fast One-Step RT-PCR Master mixes were lyophylized by a third party with bulking agents A, B or C. (B) The master mix pellets were reconsitituted in water and reactions were set up with total RNA as template and primer-probes for PolR2A (Cy5) and GAPDH targets (Vic) in a 20 µl reaction. Retain material (prior to addition of bulking agents) was used for comparison. RT-PCR was carried out using the cycling protocol shown below to compare amplification performance of the master mixes before and after lyophilization.
(C) Lyophilized master mix pellets were then stored at room temperature for 1 and 4 weeks before reconstituting with water and setting up a RT-PCR to detect β-actin (Vic) using total RNA as template in a 20 µl volume. Storage at room temperature did not impact the performance of the tested master mixes.







06
Performance of HawkZ05 Fast One-Step RT-PCR Master (with Mn cofactor) after lyophilization.

	POLR2A	GAPDH
Retain	28.4	22.13
Bulk-A	28.14	20.84
Bulk-B	27.71	21.00
Bulk-C	28.01	20.75

Room temperature storage of the lyophilized MMx

	4-Wks	1-Wks	0-Wks
Bulk-A	20.61	20.28	20.26
Bulk-B	21.39	22.26	22.30
Bulk-C	22.09	21.46	21.47

Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	55 °C	5 min
	1	60 °C	5 min
	1	65 °C	5 min
PCR	1	95 °C	30 sec
	45	95 °C	15 sec
		60 °C	30 sec

Working with HawkZ05 Fast

Integrating HawkZ05 Fast Polymerase into your assay opens novel opportunities to streamline workflows and shorten turnaround times. The enzyme is robust to a range of reaction conditions, which is advantageous for assay optimization. The following is a summary of studies done at Roche CustomBiotech that can serve as a starting point for the optimization of your assays.

Optimization guidelines

HawkZ05 Fast Polymerase provides the flexibility to design assays for complex targets from various samples, as it performs well at high temperatures and is also tolerant of inhibitors. The enzyme's performance can be enhanced by optimizing reaction components, conditions and additives, bearing in mind that sample and target requirements or limitations should be accounted for as early in assay development as possible.
The complexity of a PCR assay makes it difficult to serially optimize one factor after another. However, using Design of Experiments (DOE) approaches significantly reduces assay development time and results in a streamlined final assay with optimal performance.

Determine the optimal thermal cycling protocol for your assay

HawkZ05 Fast Polymerase is engineered for fast reverse transcription rates, which you can leverage to minimize overall assay time-to-result without compromising quality. We amplified various copy numbers of an HCV target under standard and fast cycling conditions (Table 01). As shown in Fig. 07 A, HawkZ05 Fast Polymerase demonstrates good performance and sensitivity under fast cycling conditions and across the dynamic range tested. Cps at lower target concentrations were delayed under fast cycling conditions, however, a balance between turnaround time (TAT) and Cp values can be achieved by coupling fast reverse transcription with standard PCR cycling conditions (Table 01).

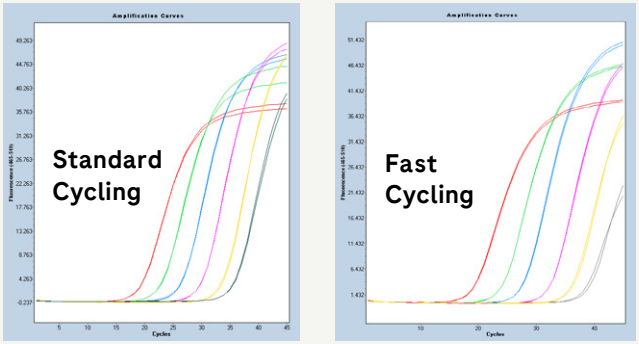
A single-temperature hold step at 60 °C for 2 – 5 minutes is sufficient for effective reverse transcription of most targets for real-time PCR (<200 nucleotides). When working with long targets (>300 nucleotides) or with complex secondary structures, assay sensitivity can be improved either with a longer (~10 minutes) reverse transcription hold time, or by using a three-step reverse transcription:

- **First hold at 55 °C:** This step provides stability for primers with low melting temperatures and can be useful in cases where RNA target and reverse transcription primers are slightly mismatched (e.g., viral RNA targets).
- **Second hold at 60 °C:** At this temperature, the enzyme is fully active and is dissociated from the aptamer.
- **Third hold at 65 °C:** A final high-temperature step relaxes complex secondary structures in RNA targets, allowing more efficient cDNA synthesis.

Step	Cycles	Standard Cycling		Fast Cycling	
		Temp	Time	Temp	Time
RT	1	55 °C	5 min		
	1	60 °C	5 min	60 °C	5 min
	1	65 °C	5 min		
PCR	1	92 °C	30 sec	92 °C	30 sec
	45	92 °C	15 sec	92 °C	1 sec
		60 °C	30 sec	60 °C	10 sec
Time to completion		74 min		44 min	

T01
Fast and standard thermal cycling protocols.

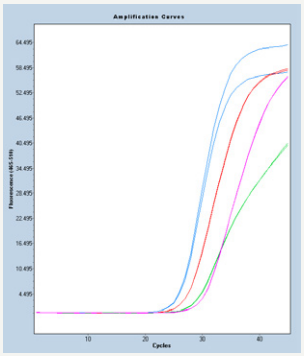
Fig. 07 B shows the amplification of a target with complex secondary structure. Different sized amplicons were generated from the highly conserved GC-rich 5' UTR region of HCV by coupling one forward primer with different reverse primers.[5] The ability of HawkZ05 tFast polymerase to amplify complex regions affords flexibility in selecting target location and length. You can also improve assay performance by adjusting the amplification cycles. For example, short anneal and extension steps can increase the specificity and sensitivity of the reaction. If working with a GC-rich amplicon, you can improve amplification efficiency by extending the denaturation step of each cycle to up to 15 seconds and increasing the denaturation temperature from 92 °C to 94 °C.



07 A
Performance of HawkZ05 Fast Polymerase under standard and fast cycling conditions.

Copy Number	Std RT Std Cycling	Fast RT Std Cycling	Fast RT Fast Cycling
1 E6	19.35	19.26	19.24
1 E5	23.04	23.29	24.12
1 E4	26.64	27.09	27.90
1 E3	30.20	32.05	32.71
1 E2	33.93	35.22	36.33
1 E1	35.92	36.43	38.73

PCR reactions were setup with HawkZ05 Fast Polymerase in Tricine buffer to amplify HCV RNA (Vic) using different thermal cycling protocols (Table 1). The enzyme shows good performance and sensitivity under fast cycling conditions.



Amplicon Size	CP's
62 bp	26.23
157 bp	27.72
222 bp	28.96
304 bp	30.55

07 B
Amplification of different sized amplicons from GC-rich and complex 5' non-coding regions of HCV.

Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	55 °C	5 min
	1	60 °C	5 min
	1	65 °C	5 min
PCR	1	95 °C	30 sec
	45	95 °C	15 sec
		60 °C	30 sec

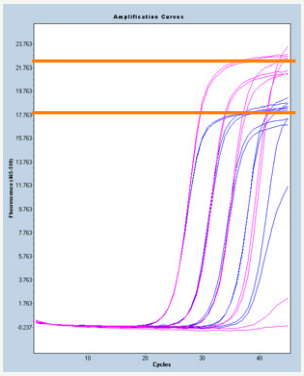
A 20 µl reaction with primers and probe (FAM) designed according to Chen et al., 2016.[5] resulted in different sized amplicons of GC-rich and complex non-coding regions of HCV. HawkZ05 Fast Polymerase efficiently amplified all amplicons, regardless of size and complexity.

Assess the need for cofactors and determine their optimal concentration

Depending on cofactors used, HawkZ05 Fast Polymerase exhibits both RNA-dependent and DNA-dependent DNA polymerase activity or mostly DNA-dependent DNA polymerase activity. In the presence of manganese (Mn) as a cofactor, HawkZ05 Fast Polymerase has both activities, enabling RT-PCR amplification in a one-step, one-tube setup. In the presence of magnesium (Mg) as a cofactor, HawkZ05 Fast Polymerase exhibits mostly DNA-dependent DNA polymerase activity. Thus, if your assay requires only DNA amplification, you can use either manganese or magnesium as a cofactor, as demonstrated in Fig. 08. Manganese resulted in lower Cp values when amplifying DNA, especially with lower copy numbers, while magnesium cofactor gave consistently higher fluorescence (15 – 18 %) at each of the template concentrations.

Manganese promotes the dual polymerase activity of HawkZ05 Fast Polymerase (Fig. 09). Adding magnesium as a cofactor can slightly boost fluorescence signals but also increase Cp values.

For most assays, the recommended 1.5 mM final concentration of manganese acetate delivers highly sensitive and specific RT-PCR but can be optimized within the range of 1.5 and 3.0 mM. In general, lower manganese or magnesium ion concentrations may result in poor yield and too high concentrations may produce nonspecific products. Combining manganese and magnesium cofactors can improve fluorescence intensity as well as the shape of amplification curves. Furthermore, optimizing cofactors and their concentration can help overcome inhibition from sample components, such as EDTA in blood samples.



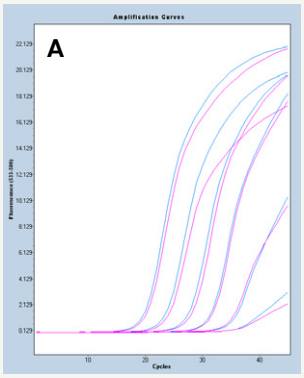
08
Influence of cofactors on HawkZ05 Fast Polymerase performance in amplifying DNA.

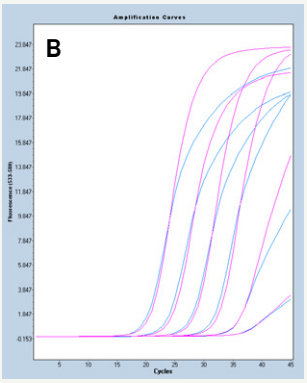
Starting from various copy numbers, HPV DNA was amplified in a 20 µl reaction supplemented with either Mn or Mg cofactors in Tricine buffer. Cp values were slightly lower when Mn was used as a cofactor, while Mg generated higher fluorescence signals at all template concentrations.

HPV Copy number	Mn -1.5 mM	Mg -1.5 mM
1.00E + 05	24.09	24.53
1.00E + 04	28.01	28.51
1.00E + 03	31.36	31.95
1.00E + 02	34.73	36.5
1.00E + 01	37.8	–

Thermal cycling protocol

Cycles	Temp	Time
1	92 °C	30 sec
45	92 °C	10 sec
	60 °C	30 sec





	Manganese concentration	
HCV Copy number	1.5 mM	2.1 mM
1.00E + 05	23.95	23.02
1.00E + 04	27.16	27.24
1.00E + 03	31.65	31.71
1.00E + 02	35.02	35.29
1.00E + 01	35.66	35.9

	Cofactor concentration	
HCV Copy number	Mn -1.5 mM	Mn -1.5 mM Mg -1.5 mM
1.00E + 05	19.92	20.81
1.00E + 04	23.41	24.42
1.00E + 03	28.16	28.99
1.00E + 02	31.52	32.70
1.00E + 01	34.59	36.09

09
Influence of cofactors on HawkZ05 Fast Polymerase performance in amplifying RNA.

Various copy numbers of HCV RNA were amplified in a 20 µl reaction in Tricine buffer using Mn as cofactor at 1.5 mM and 2.1 mM concentration (A) and Mn or a combination of Mn and Mg as cofactors (B). HawkZ05 Fast Polymerase showed robust amplification of the RNA target with Mn as a cofactor.

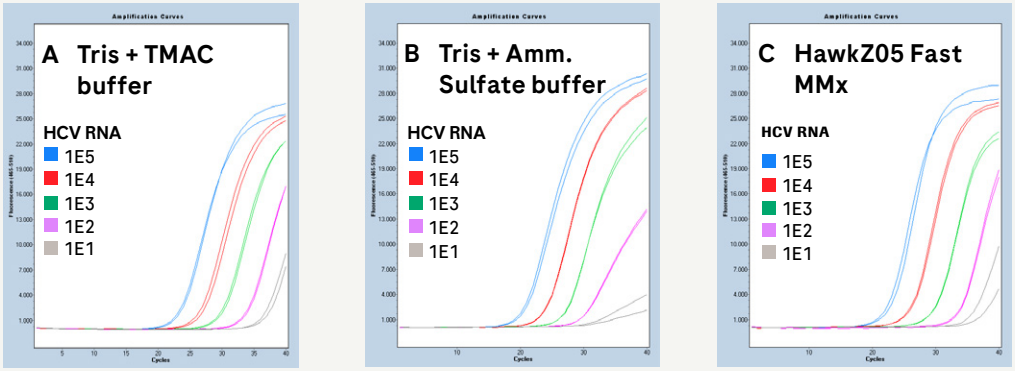
Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	60 °C	5 min
PCR	1	92 °C	30 sec
	45	92 °C	10 sec
		60 °C	30 sec

Choose or formulate an appropriate buffer

In selecting or formulating a buffer for your assay, HawkZ05 Fast Polymerase allows a flexible choice between Tricine-based or Tris-based buffers. The enzyme performs well in both buffers, however, Tricine/bicine buffers tolerate a wider cofactor concentration range for optimization than Tris buffers.[6]

HawkZ05 Fast Polymerase showed good performance and sensitivity with both Tricine and Tris-based buffers. In addition to the choice of buffer, buffer pH and concentration also impact the performance of an enzyme. HawksZ05 Fast Polymerase delivers good performance in a pH range of 8.0 – 8.3 for Tricine buffers and 8.3 – 8.6 for Tris-based buffers. Potassium and ammonium salts can play an important role in denaturing template strands and enhancing the performance of a polymerase. HawkZ05 Fast can tolerate high amounts of salts (e.g., up to 150 mM potassium) and this feature should be leveraged as an optimization strategy in addition to pH and buffer concentration.



10
HawkZ05 Fast Polymerase performance in Tris and Tricine buffers.

Various copy numbers of an HCV RNA target were amplified in a 20 µl reaction volume using different buffers:
- (A) Tris buffer with TMAC (75 mM) and a combination of Mg and Mn cofactors (1.5 mM each)
- (B) Tris buffer with ammonium sulfate (20 mM) and a combination of Mg and Mn cofactors (1.5 mM each)
- (C) Tricine buffer with potassium acetate (110 mM) and Mn as cofactor (1.5 mM). Amplification by HawkZ05 Fast Polymerase was robust in all three buffers.

Copy	Tris + TMAC buffer	Tris + Amm. Sulfate buffer	HawkZ05 Fast Mx
1 E5	23.53	20.75	22.82
1 E4	26.87	24.09	26.38
1 E3	30.06	27.20	30.00
1 E2	34.21	29.85	34.08
1 E1	35.00	30.81	35.00

Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	60 °C	5 min
PCR	1	92 °C	30 sec
	45	92 °C	10 sec
		60 °C	30 sec

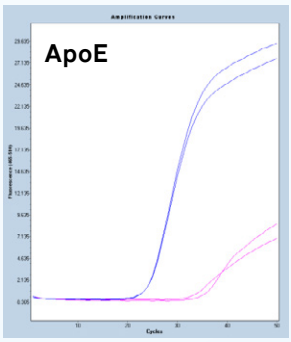
Consider additives to optimize the amplification of complex or GC-rich templates

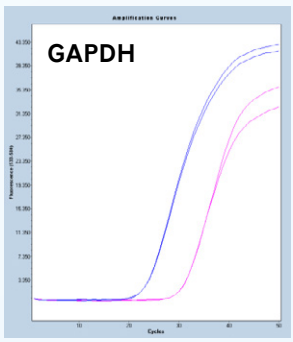
The high temperatures at which HawkZ05 Fast Polymerase can perform reverse transcription accommodates robust amplification of complex GC-rich targets. Additives like dimethyl sulfoxide (DMSO), betaine or glycerol can further improve assay sensitivity for targets with high GC content or with extensive secondary structures.

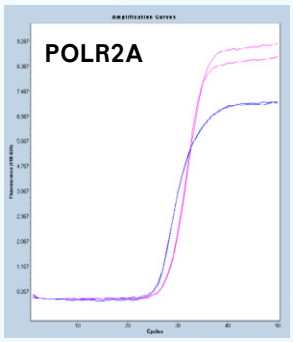
Incorporating DMSO into the RT-PCR has a positive influence on the performance of HawkZ05 Fast One-Step RT-PCR Master and improves overall detection limits of targets. Fig. 11 depicts the impact of DMSO on the detection of three targets in a triplex assay. At all template concentrations tested, amplification curves of all targets showed lower Cp values.

To optimize the incorporation of additives into your assay, follow these general guidelines, as listed in the corresponding instructions for use:

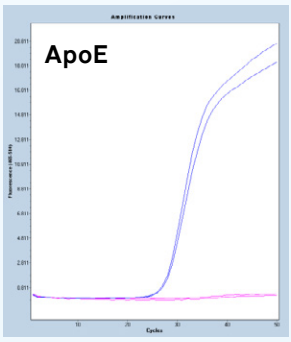
- DMSO at a final concentration of 1 % to 5 %
- Betaine at a final concentration of 0.5 % to 3 %
- Glycerol at a final concentration of 2 % to 15 %
- A combination of DMSO and Glycerol may also be optimized, with final concentrations ranging between 5 and 15 %

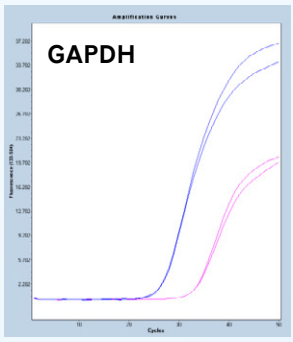


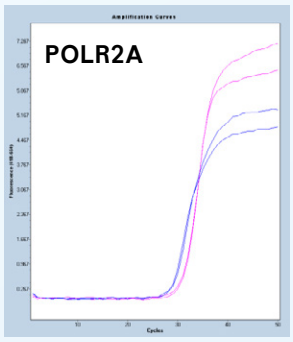




10 ng RNA	Control	DMSO
ApoE	33.93	24.63
GAPDH	31.10	28.84
POLR2A	28.21	25.74

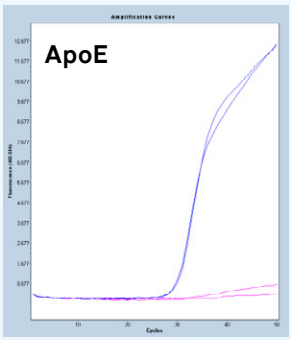


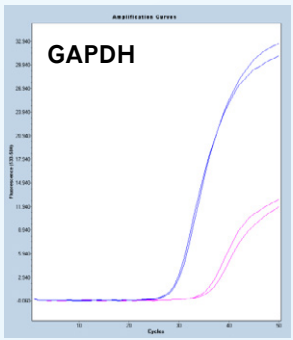


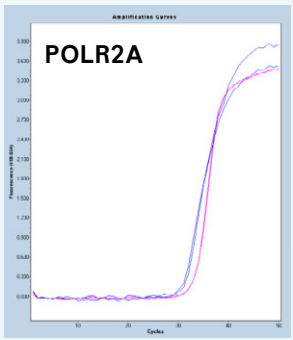


1 ng RNA	Control	DMSO
ApoE	ND	27.59
GAPDH	33.49	27.03
POLR2A	30.78	28.64

*ND Not detected







0.1 ng RNA	Control	DMSO
ApoE	ND	30.06
GAPDH	35.55	29.58
POLR2A	32.79	31.07

*ND Not detected

11
Influence of DMSO on the detection of GC-rich target ApoE with HawkZ05 Fast Polymerase.

HawkZ05 Fast One-Step RT-PCR Master was used to amplify the GC-rich target ApoE in a triplex assay in the presence or absence of DMSO (5 %). Three concentrations of total RNA were tested in a 20 µl reaction that additionally amplified GAPDH and POLR2A. DMSO improved detection limits of ApoE and reduced Cp values for all three targets.

Thermal cycling protocol

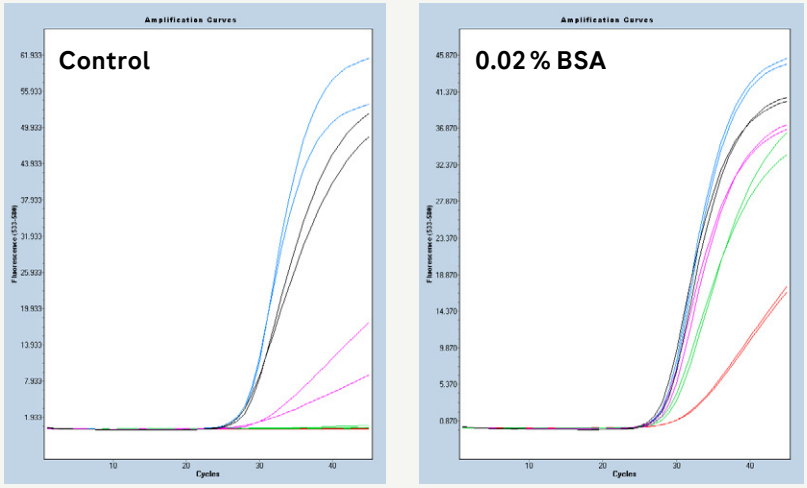
Step	Cycles	Temp	Time
RT	1	55 °C	5 min
	1	60 °C	5 min
	1	65 °C	5 min
PCR	1	92 °C	30 sec
	45	92 °C	10 sec
		60 °C	30 sec

Examine the impact of carryover inhibitors from samples

HawkZ05 Fast Polymerase tolerates certain inhibitors present in nasal swabs, stool, sputum or crude samples, as well as serum and plasma from blood mixed with EDTA or citrate (Fig. 03). Further improvements to tolerance levels can be achieved by using more enzyme or additives like BSA, gelatin, and T4 Gene 32 protein. The choice of additive and the improvement in performance depends on the type of inhibition.[7] For example, hematin inhibits PCR assay performance by binding to polymerases. Such inhibition can be mitigated with additives like BSA, which provide alternative binding sites for hematin.[8] As shown in Fig. 12, the addition of BSA at a final concentration of 0.02 % improved assay tolerance of hematin from 6.25 µM to 25 µM.

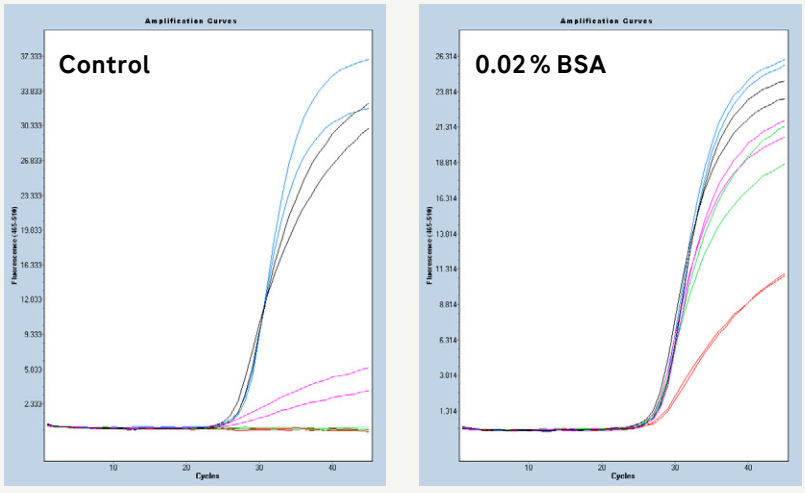
Knowledge of potential inhibitors in specific sample types and their mode of inhibition is helpful in choosing an appropriate additive to overcome PCR inhibition. Table 02 is a compilation of PCR enhancers and how they exert their effect.

Target: HCV



A duplex RT-PCR reaction using HawkZ05 Fast One-Step RT-PCR Master was set up with different concentrations of the inhibitor hematin in the presence or absence of 0.02 % BSA. 5 ng of total RNA was spiked with 1,000 copies of HCV. BSA enabled amplification of the HCV target even at the highest inhibitor concentration tested (25 µM).

Target: HPRT



12
Reducing hematin inhibition by addition of BSA.

Thermal cycling protocol

Step	Cycles	Temp	Time
RT	1	55 °C	5 min
	1	60 °C	5 min
	1	65 °C	5 min
PCR	1	95 °C	30 sec
	45	95 °C	15 sec
		60 °C	30 sec

Additive	Recommended Concentration in Reaction [a]	Effect of Additive [a]
Ammonium sulfate [$(NH_4)_2SO_4$]	5 – 30 mM	Facilitates DNA strand separation
Bovine serum albumin *	50 – 500 ng per 50 µl reaction	Binds many PCR inhibitors found in tissue samples
Dimethylsulfoxide (DMSO)	2 – 10 % v/v	Lowers T_m of target DNA to enhance annealing
Dimethylformamide (DMF)	<10 % v/v [b]	Lowers T_m of target DNA to enhance annealing
Betain		Lowers T_m of target DNA to enhance annealing
Formamide	1.25 – 10 % v/v [b]	• Changes Tm of primer-template hybridization to increase specificity and yield • Stabilizes Taq DNA Polymerase
Gelatin	0.01 – 0.10 % w/v	Stabilizes Taq DNA Polymerase
Glycerol	5 – 15 % v/v	Stabilizes Taq DNA Polymerase
PEG 6000	5 – 15 % v/v	Stabilizes Taq DNA Polymerase
SDS	less than 0.01 % w/v [b]	Prevents aggregation of polymerase
Spermidine		Reduces nonspecific binding of polymerase to template DNA
T4 Gene 32 protein *	0.05 – 0.1 nmol per 50 µl reaction	Changes T_m of primer-template hybridization to increase specificity and yield
Triton X-100	0.01 % v/v	Prevents aggregation of polymerase
Urea	1 – 1.5 M [b]	Lowers T_m of target DNA to enhance annealing

T02
Effect of additives on a standard PCR.

References

[a] Part of the information in this table from Aoyagi (2001). The effects of the additives were determined only for reactions with Taq DNA Polymerase.

[b] Higher concentrations are inhibitory.

*Available from Roche CustomBiotech

Conclusion

Ultimately, the exact procedure and array of tests needed to optimize an assay is dictated by the relevant sample types and the chosen targets. Several references offer additional information for choosing the correct PCR equipment and reagents.[9,10] Nevertheless, with its reversible hot start and dual polymerase activity, HawkZ05 Fast Polymerase is a highly flexible and robust enzyme that delivers sensitive, consistent and highly specific performance, which can greatly facilitate the assay optimization process. HawkZ05 Fast Polymerase opens new possibilities in assay design for more streamlined assay workflows through compatibility with various buffers, tolerance of lyophilization, and enablement of high-throughput scenarios.

Appendix

Materials

A) Template: HCV, HPV from ATCC®; Total RNA, Total cDNA from Clontech®.

B) Primer-Probes: HCV (FAM & Vic), HPV (FAM), β-Actin (FAM and Vic), GAPDH (FAM and Vic), HPRT1 (FAM), PP1A (Vic), ApoE (FAM) from Life Technologies®; β2MG (Cy5), POLR2A (Cy5) HCV different amplicon size from IDT®.

C) HawkZ05 Fast DNA Polymerase,
200 U/µl (≤0.1 % Glycerol); Cat. No. 07731329103
HawkZ05 Fast DNA Polymerase,
40 U/µl; US Cat. No. 07731264103

D) HawkZ05 Fast One-Step RT-PCR kit,
5 ml plus manganese acetate; 05987687190
HawkZ05 Fast One-Step RT-PCR kit,
50 ml plus manganese acetate; 05987695190

E) HawkZ05 Fast reaction buffer (5×):

Buffer composition used
250 mM Tricine
550 mM potassium acetate
0.05% (v/v) Tween 20
25% (w/v) Glycerol
pH 8.0 +/- 0.1
Adjust the pH of Tricine using potassium hydroxide

Unless otherwise specified, the final reaction includes 200 µM of each dNTP, an enzyme concentration of 0.5 U/µl of reaction volume (10 U/20 µl reaction volume) and 1.5 mM manganese acetate.

F) Tris reaction buffers (5×)

Tris TMAC buffer	Tris ammonium sulfate buffer
250 mM Tris-HCl, pH 8.5 (25 °C)	375 mM Tris-HCl, pH 8.5 (25 °C)
375 mM TMAC	100 mM $(NH_4)_2SO_4$
0.05% TWEEN®20	
0.05% gelatin 7.5	
mM $MgCl_2$	

Unless otherwise specified, the final reaction includes 200 µM of each dNTP, an enzyme concentration of 0.5 U/µl of reaction volume (10 U/20 µl reaction volume) and 1.5 mM manganese acetate.

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