Reverse Transcription Cycling Achieves Greater Than 100% cDNA Conversion With Implications For Ultra-High Sensitivity of Clinically-Actionable, Oncogenic RNA Mutations

Melissa Church, Rebecca Rinehart, Shobha Gokul, Liangjing Chen, Stela Filipovic-Sadic, and Gary J Latham
Asuragen, a Bio-Techne Brand, Austin, TX

Poster # TT043

Summary

- Oncogenic RNAs are biomarkers with diagnostic, prognostic and predictive clinical implications in many cancers and specimen types.
- Methods for detecting oncogenic RNAs typically require reverse transcription to cDNA, which is vulnerable to RNA drop-outs at low copy numbers.
- To improve the sensitivity and robustness of RNA detection, we developed "RT cycling," a linear amplification method using a thermostable reverse transcriptase, an RNA-protective buffer and high-temperature cycling.
- Here, we demonstrate that cancer-associated RNAs can be amplified by at least 10-fold through cDNA synthesis, leading to accurate quantification of variants that are missed using conventional reverse transcription.

Introduction

RNA biomarkers are present at low copy numbers in many specimens, especially liquid biopsies and formalin-fixed, paraffin-embedded (FFPE) samples. Although RT-PCR is the method of choice for RNA detection, particularly for clinical applications, reverse transcription (RT) is a stoichiometric process. As a result, low-copy RNA templates have "one shot" to be commuted into cDNA. If this conversion is incomplete, then information about the RNA molecule is irrevocably lost. To address this limitation, we developed a novel technology that combines single-enzyme RT-PCR with high-temperature RT that preserves RNA intactness and cycles cDNA synthesis. The ability to generate multiple copies of cDNA from a single copy of RNA offers "multiple shots on target" to both capture RNA-level information and amplify it. Here, we describe this RT cycling method, which relies on optimized buffer and cycling conditions, and thermostable RNA-dependent DNA polymerases.

Materials and Methods

We evaluated multiple thermostable polymerases, including HawkZ05 Fast Polymerase (Roche), for their ability to amplify RNA targets derived from cell lines, synthetic materials, and non-small cell lung cancer FFPE biopsies. RT cycling reactions were performed using mixtures of mutant RNA in wild-type sample RNA with a single gene-specific primer. Digital PCR (QX200, Bio-Rad) was used to calculate the number of cDNA copies across cycle numbers.

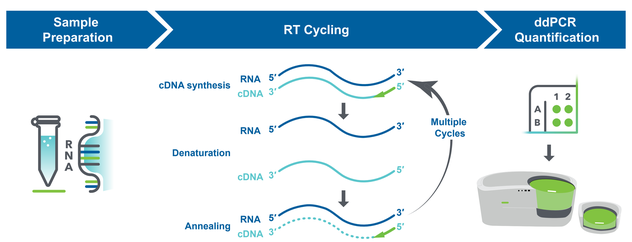


Figure 1. Workflow. RNA was either in vitro-transcribed from synthetic DNA or was isolated from cell lines or FFPE. RNA samples then underwent amplification by thermocycling at multiple cycles using a thermostable RT-PCR enzyme. The resultant cDNA was quantified via droplet digital PCR to obtain absolute copy numbers.

Proof-of-concept data only. Future availability and performance cannot be ensured.
Presented at AMP 2022

Results

2A

Polymerase	Background	Target	Copies Expected	Copies Detected: 1 cycle	5 cycles	10 cycles	20 cycles	30 cycles	Fold Changes: 5 cycles/ 1 cycle	10 cycles/ 1 cycle	20 cycles/ 1 cycle	30 cycles/ 1 cycle
HawkZ05	None	Mutant	100	144	236	416	2580	12780	1.6	2.9	17.9	88.8
HawkZ05	Cell line RNA	Mutant	100	106	382	582	882	Saturated	3.6	5.5	8.3	Saturated
		Wild-type	2000	2140	8120	14040	Saturated	Saturated	3.8	6.6	Saturated	Saturated

2B

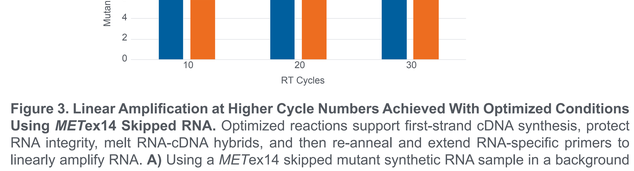
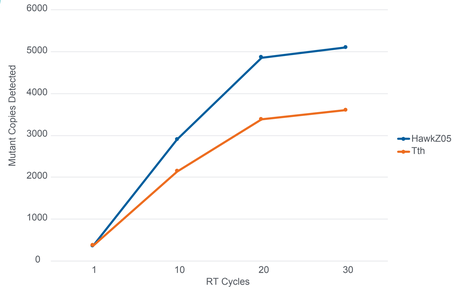


Figure 2. Non-linear Amplification Using 95°C Denaturation Temperature is Associated With RNA Degradation. A) A representative RT-PCR polymerase, HawkZ05, was used to test synthetic *MET* exon 14 skipped mutant RNA both by itself and in a background of cell line RNA. Linear amplification was detected up to 10 or 20 cycles of RT, after which signals became saturated and exponential amplification was observed. This is likely due to RNA hydrolysis and degradation at 95°C during the thermocycling protocol, resulting in fragmented RNAs that can serve as forward sense primers. **B)** Bioanalyzer results of synthetic RNA after exposure to 80°C in an RNA-protective buffer compared to 95°C. Complete degradation of RNA was observed at 95°C after 30 minutes, whereas RNA at 80°C remained considerably more intact. Preservation of RNA intactness at elevated temperatures is critical to enable cycling and linear amplification during cDNA synthesis.

3A



3B

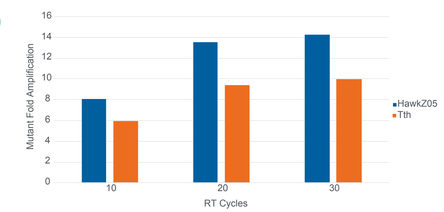


Figure 3. Linear Amplification at Higher Cycle Numbers Achieved With Optimized Conditions Using *MET*ex14 Skipped RNA. Optimized reactions support first-strand cDNA synthesis, protect RNA integrity, melt RNA-cDNA hybrids, and then re-anneal and extend RNA-specific primers to linearly amplify RNA. **A)** Using a *MET*ex14 skipped mutant synthetic RNA sample in a background of wild-type cell line RNA, HawkZ05 and Tth enzymes showed linear amplification up to 20 cycles, after which additional amplification was observed but linearity was lost. **B)** Fold changes from 1 cycle demonstrate that up to 13-fold amplification of mutant copies was achieved at 20 cycles.

4A

Number of RT Cycles	*ESR1* D538G Mutant Copies Detected	*ESR1* Exon 8 Wild-type Copies Detected	% Variant Detected
1	225	652	26%
5	973	2960	25%
10	1699	5140	25%
20	2556	7990	24%

4B

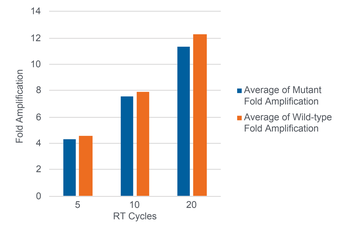


Figure 4. Linear RT Cycling Demonstrated With a Second Oncogenic RNA Target, *ESR1*. A mixture of synthetic *ESR1* D538G mutant RNA in synthetic *ESR1* exon 8 wild-type RNA was tested in the RT cycling workflow using HawkZ05 polymerase. Slight modifications from the optimized *MET* protocol, such as an increased denaturing temperature of 85°C, were made based on GC content of the new target. **A)** Both mutant and wild-type copies successfully achieved >100% cDNA synthesis, while preserving the spike-in variant allele frequency. **B)** Mutant and wild-type RNAs showed similar fold amplification, yielding 11-fold and 12-fold amplification of mutant and wild-type templates, respectively, after 20 RT cycles.

5A

FFPE RT input (ng)	Mutant Copies Expected after 1 Cycle	RT Cycles	Mutant Copies Detected	Wild-type Copies Detected	Mutant Fold Amplification	% Variant Detected
5.6	54	1	44	124	N/A	26
		5	174	424	4.0	29
		10	316	638	7.2	33
		20	406	738	9.2	35
0.56	5.4	1	5.8	24	N/A	19
		5	15	32	2.6	32
		10	22	56	3.8	28
		20	26	88	4.5	23
0.28	2.7	1	4	9.4	N/A	30
		5	8.6	22	2.2	28
		10	16	18	4.0	47
		20	13.4	46	3.4	23

5B

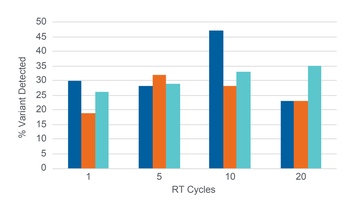


Figure 5. FFPE RNA Fraction Variant Preserved up to 20 Cycles. A) FFPE sample containing ~30% *MET*ex14 skipped mutant (previously characterized by NGS) was tested using HawkZ05 polymerase at three different RT inputs: 0.28, 0.56, and 5.6 ng. Linear amplification was observed to at least 5-10 RT cycles in all reactions, though reaction efficiency dropped off at 20 cycles. B) The variant allele fraction of ~30% was well preserved throughout 20 cycles of amplification.

6A

Sample	Expected Mutant Copies	Mutant Copies Detected: 1 cycle	5 cycles	10 cycles	20 cycles	Average % Variant Detected: 1 cycle	5 cycles	10 cycles	20 cycles
0.1% Positive	1	0	1.8	3.8	4.6	0.4%	0.1%	0.1%	0.1%
0.1% Positive	1	1.6	0	3	7.2				
0.5% Positive	5	4.4	11	7.6	22	1.3%	0.6%	0.5%	0.6%
0.5% Positive	5	6	6.2	16	22				
1% Positive	10	6	10.8	20	42	1.5%	0.9%	0.8%	1.0%
1% Positive	10	7.4	16	18	38				
5% Positive	50	18	82	116	148	6.8%	5.6%	4.6%	4.5%
5% Positive	50	42	78	102	210				
10% Positive	100	52	154	260	394	11.5%	9.2%	10.1%	10.1%
10% Positive	100	46	126	270	540				
RT-NTC	0	0	0	0	0	N/A	N/A	N/A	N/A
RT-NTC	0	0	0	0	0				

6B

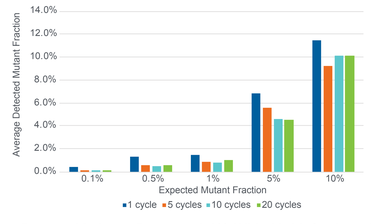


Figure 6. RT Cycling Detects Variants Down to 0.1% With 10-20 Cycles of RT Which Can Be Missed Using Single-step RT. A) Titrations of *MET*ex14 skipped mutant cell line RNA with wild-type cell line RNA at fractions ranging from 0.1% to 10% were assessed in duplicate reactions using HawkZ05 polymerase. Linear amplification of both mutant and wild-type copies was observed up to 20 cycles. In extremely low-variant samples where only 1 mutant copy was expected in a background of ~1000 wild-type copies, some replicates were not detected at lower RT cycles. At 10 or 20 cycles of RT, mutant copies were consistently detected for all samples while retaining the expected fraction variant. **B)** Average detected variant allele fraction was most variable at 1 cycle of RT. At both 10 and 20 cycles, fraction variant was consistent with the known mixing ratio and well maintained across samples down to 0.1%.

Conclusions

- We demonstrated that optimized reagent and RT cycling conditions both maintain RNA intactness and enable linear amplification over multiple rounds of cDNA synthesis, resulting in 5 to 10-fold amplification in low-quality samples such as FFPE.
- This innovative approach is well suited for RNA targets with low copy number or poor RT conversion efficiency.
- The method has potential to improve the analytical sensitivity of RNA detection for many applications, including the quantification of clinically-actionable mutations and monitoring of resistance in oncology using samples such as FFPE tumor biopsies or liquid biopsies.

References

1. Latham, G. (2022). Methods of RNA Amplification (U.S. Patent No. 11,236,384). US. Patent and Trademark Office

Asuragen
a bio-techne brand