# Development of a Novel Exosome-based, Multiplexed RT-qPCR Technology for Rapid and Accurate Detection of Circulating Tumor Acquired Resistance Variants in *ESR1* at ≤ 0.1% Frequency


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## Summary

- Breast cancer is a complex disease often requiring ongoing monitoring and management to ensure the best possible patient outcomes. Monitoring for genetic mutations, including those in the Estrogen Receptor 1 (*ESR1*) gene, provides valuable information about tumor progression and response to treatment for select cases.
- We describe a comprehensive methodology for targeted clinical RT-qPCR monitoring of *ESR1* mutations in plasma that utilizes both exosomal RNA (exoRNA) and circulating cell-free DNA (cfDNA), interrogates 11 mutations, provides a streamlined workflow, and accommodates a range of minor allele fractions (MAF).
- We demonstrate consistent and specific RT-qPCR down to 3 *ESR1* mutants (in a background of 5000 wild-type (WT) copies, or 0.06% MAF), congruent with ultra-sensitive mutation detection from plasma.
- Preliminary testing supports the feasibility of the entire *ESR1* assay workflow with plasma samples from subjects with stage IV metastatic breast cancer (mBC) (HR+/HER2-) on active aromatase inhibitor therapy, +/- CDK 4/6 inhibitor for at least one year.


## Introduction

Hormone receptor-positive/human epidermal growth factor receptor 2-negative (HR+/HER2-) breast cancer is the most common type of breast cancer. Patients with HR+/HER2- mBC often become resistant to aromatase inhibitors commonly used in endocrine therapy (ET). *ESR1* ligand binding domain mutations are frequently detected in HR+ mBC and have been reported to be associated with ET resistance (1). It is estimated that 20-40% of mBC patients will develop resistance to treatment via mutations in *ESR1* (1). Recent studies have shown that monitoring of *ESR1* mutations in plasma may serve as a predictive biomarker of acquired resistance to ET, showcasing a strong need for sensitive nucleic acid-based assays (1). The 2023 FDA approval (3) of elacestrant for individuals with HR+/HER2-, *ESR1*-mutated breast cancer and the updated breast cancer NCCN guidelines (4), highlights the immediate need for timely and sensitive *ESR1* mutation detection.

## Materials and Methods

Due to the scarcity of samples containing *ESR1* mutations, plasmids containing the 11 clinically relevant mutations shown in Figure 1 and outlined in Table 1, were designed. These constructs contained a T7 promoter, allowing for in vitro transcription (IVT) of mutant *ESR1* transcripts. The resulting *ESR1* transcripts also included an additional 84-nucleotide exogenous sequence for precise quantification using droplet digital PCR (ddPCR) (BioRad QX200). IVT product was created by linearizing plasmids with BamHI (NEB), followed by MEGAshortscript T7 Transcription Kit (Thermo Fisher) and MEGAclear Transcription Clean-Up Kit (Thermo Fisher).

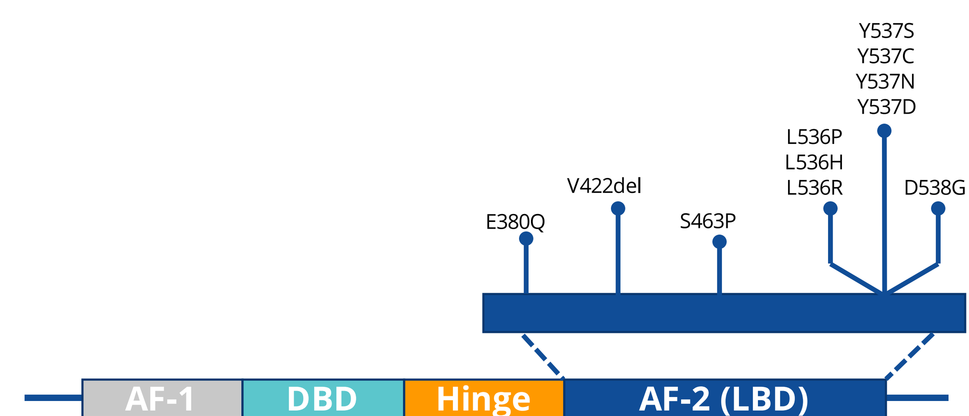



**Figure 1. *ESR1* acquired resistance mutations.** In patients with HR+/HER2- mBC, *ESR1* mutations are a common cause of acquired resistance to aromatase inhibitors. A key mechanism of endocrine resistance is mutation of the ligand-binding domain (LBD); mutations investigated in our preliminary studies within the LBD are shown.

**Table 1. *ESR1* mutations with chromosomal location specifics**

| Common Name | Cosmic ID | Coordinate GRCh38 | RefBase | AltBase |
|---|---|---|---|---|
| E380Q | COSV52782264 | Chr6:152011697 | G | C |
| V422del | COSV52789441 | Chr6:152061020..152061022 | GTGG | G |
| S463P | COSV52784970 | Chr6:152094402 | T | C |
| L536P | COSV52782930 | Chr6:152098785 | T | C |
| L536H | COSV52795259 | Chr6:152098785 | T | A |
| L536R | COSV52787207 | Chr6:152098785 | T | G |
| Y537S | COSV52783938 | Chr6:152098788 | A | C |
| Y537C | COSV52782924 | Chr6:152098788 | A | G |
| Y537N | COSV52784978 | Chr6:152098787 | T | A |
| Y537D | COSV52804811 | Chr6:152098787 | T | G |
| D538G | COSV52781024 | Chr6:152098791 | A | G |

Contrived samples were created by adding 50, 5, or 3 copies of mutant (MUT) *ESR1* plasmid DNA into a background of 5000 copies of ultrasonicated (Covaris) WT cell line DNA for 1%, 0.1%, and 0.06% MAF, respectively. Assay improvements have streamlined the Prototype *ESR1* qPCR Assay* into 3 multiplex qPCR reactions using modified QuantideX® reagents (Asuragen) targeting 11 *ESR1* mutations and an internal PCR control for analysis on the QuantStudio™ 5 Dx Real-time PCR System (Thermo Fisher) (Table 2).

RT-qPCR target enrichment was performed with 50, or 10 copies of MUT *ESR1* IVT product into a background of 10,000 copies of ultrasonicated WT cell line DNA using SuperScript VILO cDNA Synthesis Kit (Thermo Fisher) for 0.5% and 0.1% MAF, respectively. The reverse transcription (RT) product underwent a pre-amplification (Pre-Amp) PCR for sample enrichment and diluted Pre-Amp product was added to 3 multiplex *ESR1* qPCR reactions for analysis on the QuantStudio (Table 3).

Plasma samples collected from subjects with stage IV mBC (HR+/HER2-) on active aromatase inhibitor therapy, +/- CDK 4/6 inhibitor for a minimum of 1 year, underwent exoRNA and cfDNA co-isolation from PAXgene® Blood ccfDNA Tubes (CE-IVD, Qiagen) processed through a 0.8 µm filter followed by the ExoLution Plus Isolation Kit* (Exosome Diagnostics). RT was completed on the entire exoRNA/cfDNA eluate using SuperScript VILO cDNA Synthesis Kit. The RT product was passed through a Pre-Amp PCR, diluted, and added to 3 multiplex *ESR1* targeted qPCR reactions that included an internal PCR control for analysis on the QuantStudio (Figure 2, Table 4). Singleplex ddPCR assays (BioRad) of the 11 *ESR1* mutations were used for mutation status confirmation.

## Results

**Table 2. Prototype multiplex *ESR1* qPCR assays can detect MAFs at 0.06% or 3 MUT copies for all 11 mutations without WT signal, showing large improvement in specificity from previously singleplex assays.** 4-8 replicates of ultrasonicated WT cell line DNA and 2-4 replicates of varying proportioned amounts of mixed MUT and ultrasonicated WT cell line DNA were run in qPCR for 60 cycles to investigate the sensitivity of each multiplex qPCR assay. As seen in the overview table in **2A.)** the optimized multiplex qPCR prototype assay sensitivity was maintained down to 3 MUT copies in the background of 5000 WT copies (0.06% MAF) for all 11 *ERS1* mutations with greater than 16 delta Cts from averaged WT Ct values. The internal PCR control consistently amplified when in the presence of WT cell line DNA for the multiplex assays, data not shown. ND = signal not detected at 60 cycles. **2B.)** Prototype singleplex qPCR *ESR1* assays for improvement comparison and streamlined workflow. S463P was not assessed in singleplex.

**2A.**

| MUT Copies | 50 | 50 | 5 | 3 | 0 |
|---|---|---|---|---|---|
| WT Copies | 0 | 5000 | 5000 | 5000 | 5000 |

| Tube # | Target | 100% MAF Avg. Ct | 1% MAF Avg. Ct | 0.1% MAF Avg. Ct | 0.06% MAF Avg. Ct | 100% WT Avg. Ct | WT - MUT 0.1% Avg. Ct | WT - MUT 0.06% Avg. Ct |
|---|---|---|---|---|---|---|---|---|
| 1 | D538G | 31.73 | 31.56 | 35.01 | 36.19 | 52.83 | 17.82 | 16.64 |
| | S463P | 31.04 | 31.24 | 34.92 | 35.49 | 57.25 | 22.33 | 21.76 |
| 2 | Y537S | 31.02 | 30.93 | 34.31 | 36.53 | ND | 25.69 | 23.47 |
| | Y537C | 33.94 | 33.28 | 35.92 | 37.22 | | 24.08 | 22.78 |
| | Y537N | 31.15 | 31.23 | 34.75 | 36.81 | | 25.25 | 23.19 |
| | Y537D | 31.85 | 31.63 | 36.21 | 35.61 | | 23.79 | 24.39 |
| | E380Q | 31.70 | 31.04 | 34.21 | 35.03 | ND | 25.79 | 24.97 |
| 3 | L536R | 30.32 | 30.21 | 35.97 | 34.30 | ND | 24.03 | 25.70 |
| | L536H | 30.32 | 30.40 | 33.58 | 35.37 | | 26.42 | 24.63 |
| | L536P | 31.83 | 32.42 | 34.73 | 35.65 | | 25.27 | 24.35 |
| | V422del | 33.43 | 34.08 | 37.30 | 38.44 | ND | 22.70 | 21.56 |

**2B.**

| MUT Copies | 50 | 50 | 5 | 3 | 0 |
|---|---|---|---|---|---|
| WT Copies | 0 | 5000 | 5000 | 5000 | 5000 |

| Tube # | Target | 100% MAF Avg. Ct | 1% MAF Avg. Ct | 0.1% MAF Avg. Ct | 0.06% MAF Avg. Ct | 100% WT Avg. Ct | WT - MUT 0.1% Avg. Ct | WT - MUT 0.06% Avg. Ct |
|---|---|---|---|---|---|---|---|---|
| 1 | D538G | 32.98 | 33.16 | 37.20 | 38.07 | 47.08 | 9.88 | 9.01 |
| 2 | Y537S | 30.58 | 30.52 | 36.56 | 35.70 | 42.85 | 6.29 | 7.15 |
| 3 | Y537C | 32.47 | 32.42 | 37.89 | 37.67 | 44.70 | 6.81 | 7.03 |
| 4 | Y537N | 30.92 | 30.35 | 35.36 | 33.80 | 40.25 | 4.89 | 6.46 |
| 5 | Y537D | 30.98 | 30.52 | 34.20 | 36.22 | 44.43 | 10.23 | 8.21 |
| 6 | E380Q | 30.09 | 30.02 | 35.96 | 35.04 | 42.05 | 6.10 | 7.02 |
| 7 | L536R | 30.84 | 30.78 | 35.49 | 35.11 | 39.80 | 4.31 | 4.69 |
| 8 | L536H | 30.64 | 31.04 | 34.15 | 34.73 | 42.38 | 8.23 | 7.65 |
| 9 | L536P | 32.52 | 33.05 | 36.04 | 36.22 | 38.10 | 2.06 | 1.89 |
| 10 | V422del | 31.75 | 31.84 | 35.96 | 36.26 | 43.40 | 7.45 | 7.14 |

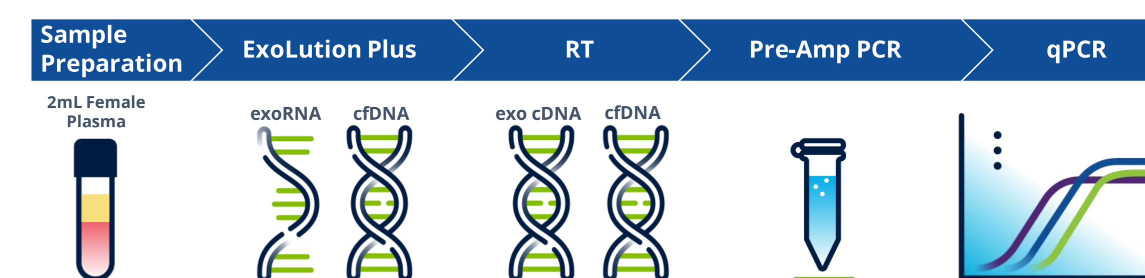


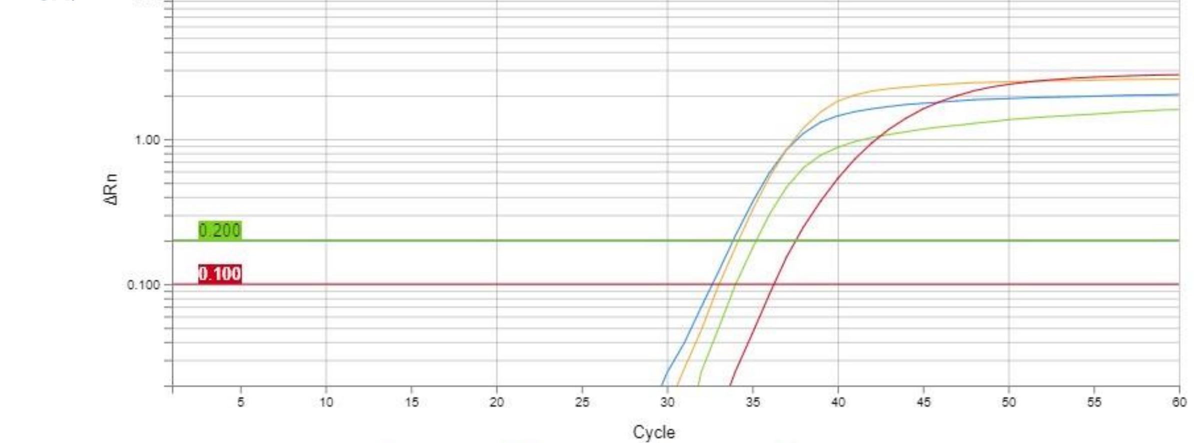

**Figure 2. Prototype *ESR1* assay workflow.** Complete assay workflow from 2mLs of plasma processed using the ExoLution Plus Isolation Kit. RT was completed on the co-isolated exoRNA and cfDNA, followed by Pre-Amp PCR for sample enrichment. Diluted Pre-Amp PCR product was transferred to 3 tubes for multiplex interrogation of the 11 *ESR1* mutations by qPCR. An internal PCR control was included in each multiplex reaction tube for sample QC purposes.

**Table 3. Incorporation of RT and Pre-Amp PCR maintained prototype *ESR1* qPCR assay sensitivity with no reduction in specificity.** 3 replicates of varying proportioned amounts of MUT IVT and ultrasonicated WT cell line DNA underwent RT and Pre-Amp PCR prior to 60 cycles of multiplex qPCR to investigate the sensitivity with the entire *ESR1* assay workflow (Fig. 2). The prototype assay successfully amplified 0.5% MAFs or 50 MUT copies in the background of 10,000 WT copies for all 11 *ESR1* mutations. The internal PCR control consistently amplified when in the presence of WT cell line DNA, data not shown. ND = signal not detected at 60 cycles. # = well failed internal control and *ESR1* qPCR, excluded for lack of template.

| MUT Copies | 50 | 10 | 0 |
|---|---|---|---|
| WT Copies | 10,000 | 10,000 | 10,000 |

| Tube # | Target | 0.5% MAF MUT Channel Rep 1 | Rep 2 | Rep 3 | 0.1% MAF MUT Channel Rep 1 | Rep 2 | Rep 3 | WT MUT Channel Rep 1 | Rep 2 | Rep 3 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | D538G | 33.93 | 34.34 | 34.75 | ND | 38.54 | 36.79 | ND | ND | ND |
| | S463P | 34.84 | 35.14 | 34.62 | 36.50 | ND | ND | ND | ND | ND |
| 2 | Y537S | 30.40 | 30.35 | 30.43 | 31.95 | 31.64 | 32.41 | ND | ND | ND |
| | Y537C | 34.54 | 34.72 | 33.82 | ND# | 35.62 | 35.53 | | | |
| | Y537N | 29.52 | 29.70 | 29.60 | 32.41 | 32.36 | 32.75 | | | |
| | Y537D | 31.00 | 30.56 | 30.37 | 32.46 | 32.77 | 32.56 | | | |
| | E380Q | 29.89 | 30.57 | 30.04 | 32.71 | 33.68 | 33.68 | ND | ND | ND |
| 3 | L536R | 30.32 | 30.26 | 30.37 | 34.82 | 35.08 | 37.71 | ND | ND | ND |
| | L536H | 30.60 | 30.96 | 30.74 | 33.79 | 35.07 | 35.27 | | | |
| | L536P | 32.05 | 32.00 | 29.74 | 34.09 | 33.56 | 35.35 | | | |
| | V422del | 32.88 | 33.19 | 33.49 | 37.17 | 36.13 | 35.02 | ND | ND | ND |

**3A.**




**3B.**

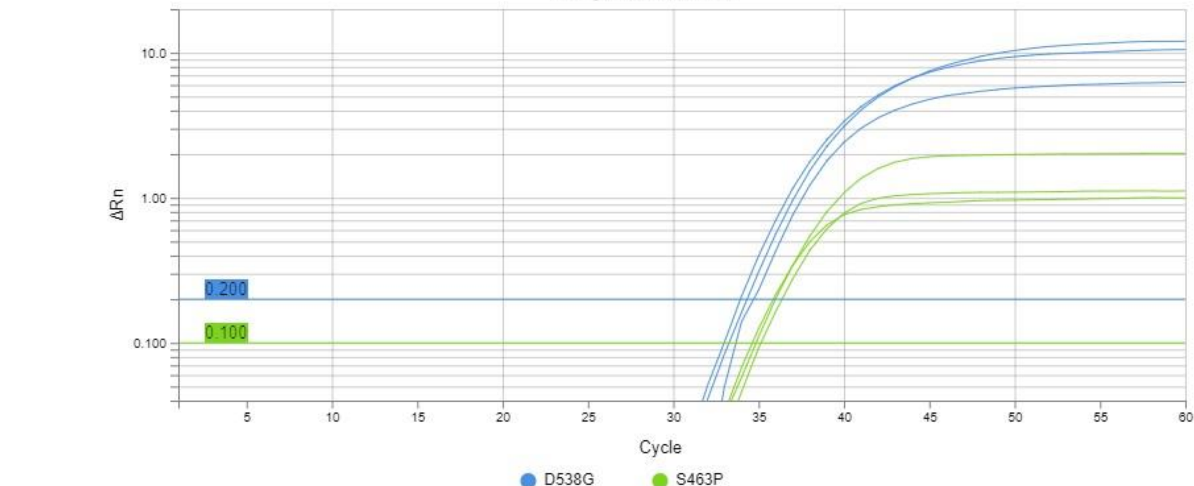



**Figure 3. Prototype multiplex *ESR1* assay qPCR amplification curves. 3A.)** qPCR amplification curve showcasing 10 copies of MUT IVT in a background of 10,000 copies WT cell line DNA for L536R/H/P and V422del. **3B.)** Triplicate qPCR amplification curves with 50 copies of MUT IVT in a background of 10,000 copies WT cell line DNA for D538G and S463P.

**Table 4. Evaluation of prototype *ESR1* assay with stage IV mBC plasma samples.** Plasma samples (2mL) collected from subjects with stage IV mBC (HR+/HER2-) on active aromatase inhibitor therapy, +/- CDK 4/6 inhibitor for a minimum of 1 year, underwent exoRNA and cfDNA co-isolation from PAXgene® Blood ccfDNA Tubes (CE-IVD, Qiagen) followed by the ExoLution Plus Isolation Kit (Exosome Diagnostics). Assay workflow (Fig. 2) followed RT, Pre-AMP PCR, and triplicate multiplex qPCR reactions targeting 11 *ESR1* mutations. The internal PCR control (Control Channel) amplified when in the presence of minimum required sample input, serving as a sample QC. Two subject plasma samples, out of the five tested, failed the internal PCR control, data not shown. ND = signal not detected at 60 cycles in the MUT Channel, indicating no *ESR1* mutations present in the 3 subject plasma samples. Singleplex ddPCR assays (BioRad) of the 11 *ESR1* mutations confirmed the 3 mBC subject plasma samples to be WT down to 5 positive droplets. ddPCR background noise was ≤5 positive droplets.

| Tube # | Target | Sample 1 MUT Channel Rep 1 | Rep 2 | Rep 3 | Sample 1 Control Channel Rep 1 | Rep 2 | Rep 3 | Sample 2 MUT Channel Rep 1 | Rep 2 | Rep 3 | Sample 2 Control Channel Rep 1 | Rep 2 | Rep 3 | Sample 3 MUT Channel Rep 1 | Rep 2 | Rep 3 | Sample 3 Control Channel Rep 1 | Rep 2 | Rep 3 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | D538G | ND | ND | ND | 37.43 | 34.78 | 35.19 | ND | ND | ND | 35.65 | 36.19 | 35.36 | ND | ND | ND | 36.24 | 35.76 | 35.34 |
| | S463P | ND | ND | ND | | | | ND | ND | ND | | | | ND | ND | ND | | | |
| 2 | Y537S/C/N/D | ND | ND | ND | 35.08 | 35.42 | 35.69 | ND | ND | ND | 34.54 | 34.06 | 35.21 | ND | ND | ND | 35.55 | 34.84 | 34.12 |
| | E380Q | ND | ND | ND | | | | ND | ND | ND | | | | ND | ND | ND | | | |
| 3 | L536R/H/P | ND | ND | ND | 35.35 | ND | 36.12 | ND | ND | ND | 35.94 | 34.05 | 34.95 | ND | ND | ND | 35.01 | 35.30 | 34.47 |
| | V422del | ND | ND | ND | | | | ND | ND | ND | | | | ND | ND | ND | | | |

**4.**

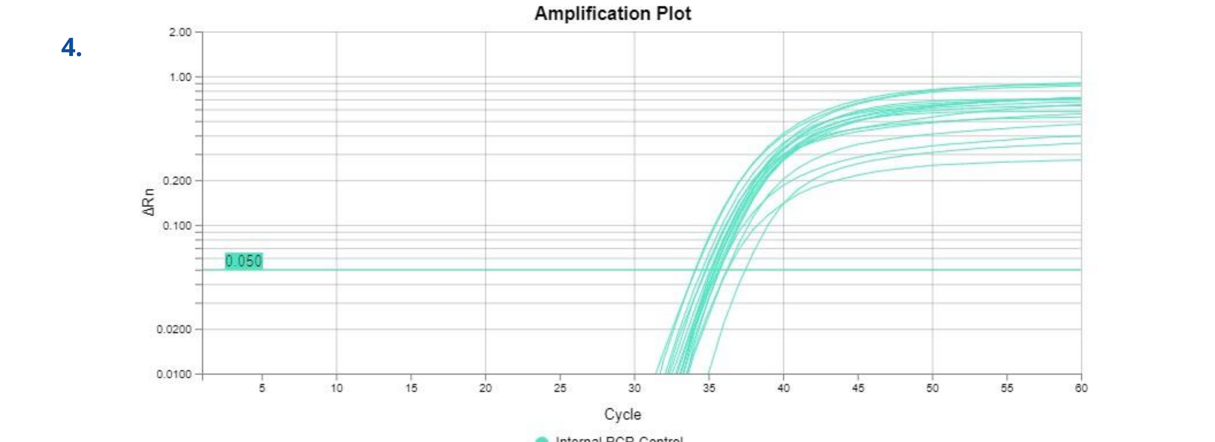



**Figure 4. Representative internal PCR control qPCR amplification curves from the subject plasma samples.**

## Conclusions

- A fast, efficient, and sensitive exosome-based *ESR1* RT-qPCR mutation assay panel was developed and evaluated, demonstrating the reliable and specific detection of rare variants in a model liquid biopsy system utilizing the ExoLution Plus Isolation Kit.
- Continual *ESR1* assay improvement has allowed for multiplex capabilities, incorporation of an internal PCR control, and decreased WT false positive amplification signal.
- This prototype technology has the potential to address several challenges associated with mutation monitoring in liquid biopsies by expanding the detection of mutant analytes to include exosomal RNA, improving analytical sensitivity through novel reagents and optimized multiplex reactions, and increasing accessibility by addressing the broad install base of qPCR instruments.


**References:**

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