# Achieve the most unbiased, target-specific preamplification from limited samples using Prelude PreAmp Master Mix

Enjoy the benefits of preamplification—without bias.

- Preamplification from as little as 10 pg of starting material
- Least-biased preamplification of any commercially available preamplification mixes (obtain at least 2–3X fewer outliers)
- Superior preamplification of 162 breast cancer-related genes from FFPE tissue

## Introduction

Real-time PCR (qPCR), SNP genotyping, and target enrichment are powerful molecular techniques for a wide range of research and clinical applications. However, the usefulness of these techniques can be reduced by limited or precious starting materials, such as nucleic acids extracted from FFPE tissue or samples obtained in extremely small quantities or at dilute concentrations in a biofluid.

These challenges can be overcome using preamplification (originally referred to as "booster" PCR), which enables unbiased, target-specific amplification of cDNA and gDNA. The key consideration when performing preamplification is ensuring that your preamplification mix is sensitive enough to work with a low-input amount of template DNA, yet does not introduce bias. Bias can occur when PCR efficiency is poor for some of the targets, which can result in under- or over-representation of targets in the preamplified sample. These changes can then influence the data generated by downstream assays.

We developed Prelude PreAmp Master Mix using an optimized polymerase and buffer to maximize PCR efficiency for all targets in a preamplification reaction. This gives Prelude PreAmp Master Mix the highest level of unbiased preamplification of over 100 targets, while starting with as little as 10 pg of cDNA or gDNA.

## Results

### Get the benefits of preamplification while maintaining uniform representation

Limited samples that are undetectable by conventional qPCR can be successfully analyzed using preamplification (Figure 1). In this experiment, Human Universal QUICK-Clone II cDNA (Cat. # 637260) was assayed across the PrimerArray Cell Cycle (Human) panel (Cat. # PH002), which contains 88 genes relevant to the cell cycle and eight housekeeping genes. The experiment was performed using either 100 pg of cDNA that had not been preamplified (purple) or 100 pg of cDNA that was subjected to 10 cycles of preamplification using Prelude PreAmp Master Mix (blue). Notably, only a small subset of the 96 genes assayed from 100 pg of nonpreamplified cDNA were detectable ($C_t$ value <32). However, the 10-cycle preamplification prior to qPCR drastically improved these data, with all 96 genes now demonstrating $C_t$ values between 17 and 30 (Figure 1, blue).

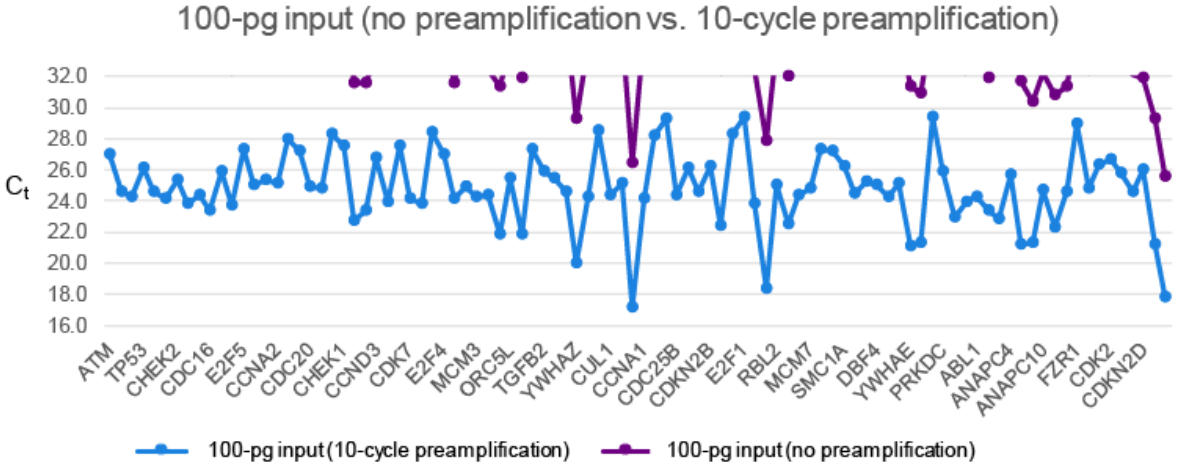


**Figure 1. The benefits of preamplification.** Please note that although the above graph displays data for all 96 target genes in the PrimerArray Cell Cycle (Human) panel, only some of the gene names are shown on the x-axis to ensure their readability. The complete list of target genes can be found here. Data points for nonpreamplified genes with $C_t$ >32 are not shown.

It is also necessary to ensure that the preamplification is not introducing bias. For example, after preamplification, some of the genes in Figure 1 displayed $C_t$ values <20. The following experiment was performed to determine if this was due to biased amplification preferentially selecting these primers, or if these genes were merely expressed at higher levels in the starting sample: 100 pg of cDNA preamplified for 10 cycles with Prelude PreAmp Master Mix (blue) was compared to 20 ng of cDNA (purple) that had not been preamplified, using the same cell cycle panel (Figure 2). Critically, the $C_t$ values for all 96 genes were highly similar, demonstrating uniform representation. Thus, 10 cycles of preamplification using Prelude PreAmp Master Mix can make 100 pg of starting cDNA detectable using qPCR without introducing bias and maintaining uniform representation.

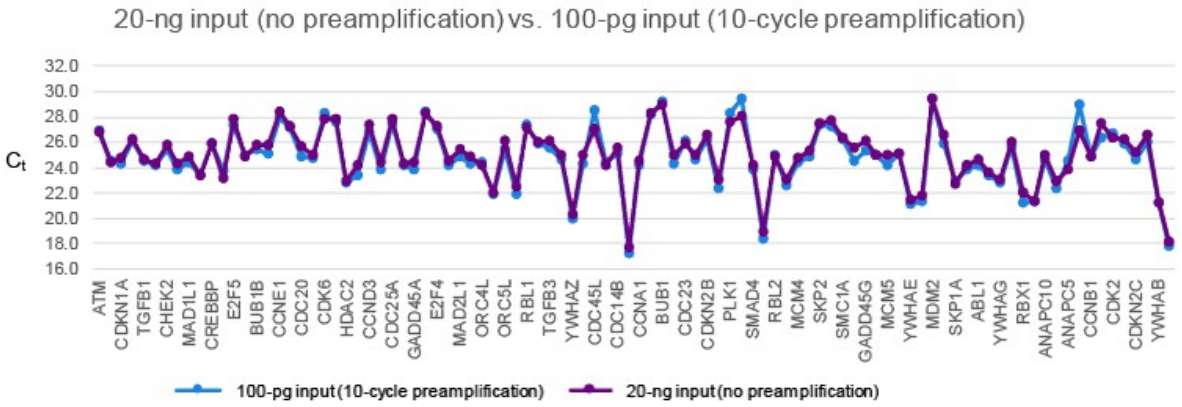



**Figure 2. Uniform representation following preamplification.** Please note that although the above graph displays data for all 96 target genes in the PrimerArray Cell Cycle (Human) panel, only some of the gene names are shown on the x-axis to ensure their readability. The complete list of target genes can be found here.

In extreme cases of either low sample input or genes with relatively low copy number, it may be necessary to perform 14 cycles of preamplification instead of 10. As before, more cycles introduce the potential for more bias. To address this, 100 pg of cDNA was preamplified for either 10 cycles (blue) or 14 cycles (purple) using Prelude PreAmp Master Mix and assayed across the same cell cycle panel as before. Notably, increasing the number of preamplification cycles from 10 to 14 maintained uniform representation (Figure 3).

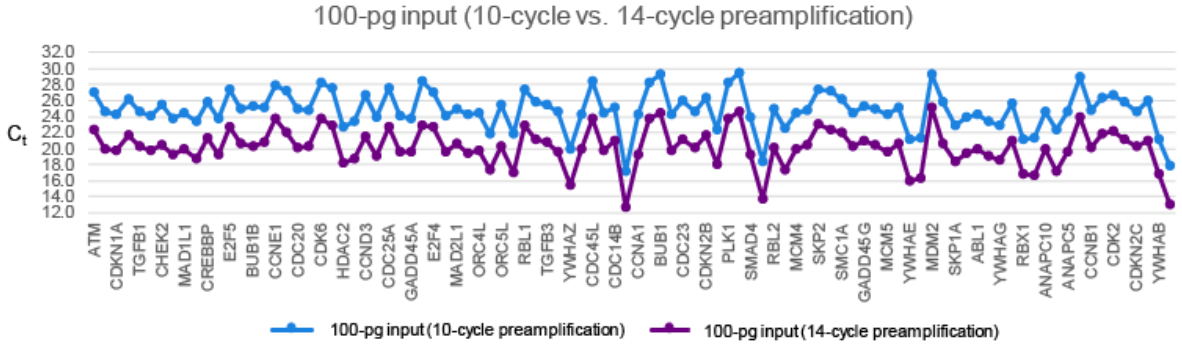



**Figure 3. Uniformity following either 10 or 14 cycles of preamplification.** Please note that although the above graph displays data for all 96 target genes in the PrimerArray Cell Cycle (Human) panel, only some of the gene names are shown on the x-axis to ensure their readability. The complete list of target genes can be found here.

To further demonstrate uniformity, we performed a delta-delta $C_t$ ($\Delta\Delta C_t$) analysis between the $C_t$ values generated by the 10- and 14-cycle preamplifications. A difference of less than ±0.75 is one of the most stringent methods of ensuring unbiased amplification. Critically, all 96 genes assayed met this requirement (Figure 4), demonstrating that Prelude PreAmp Master Mix provides excellent preamplification of 100 pg of cDNA with no introduction of bias—even when performing as many as 14 cycles of preamplification.

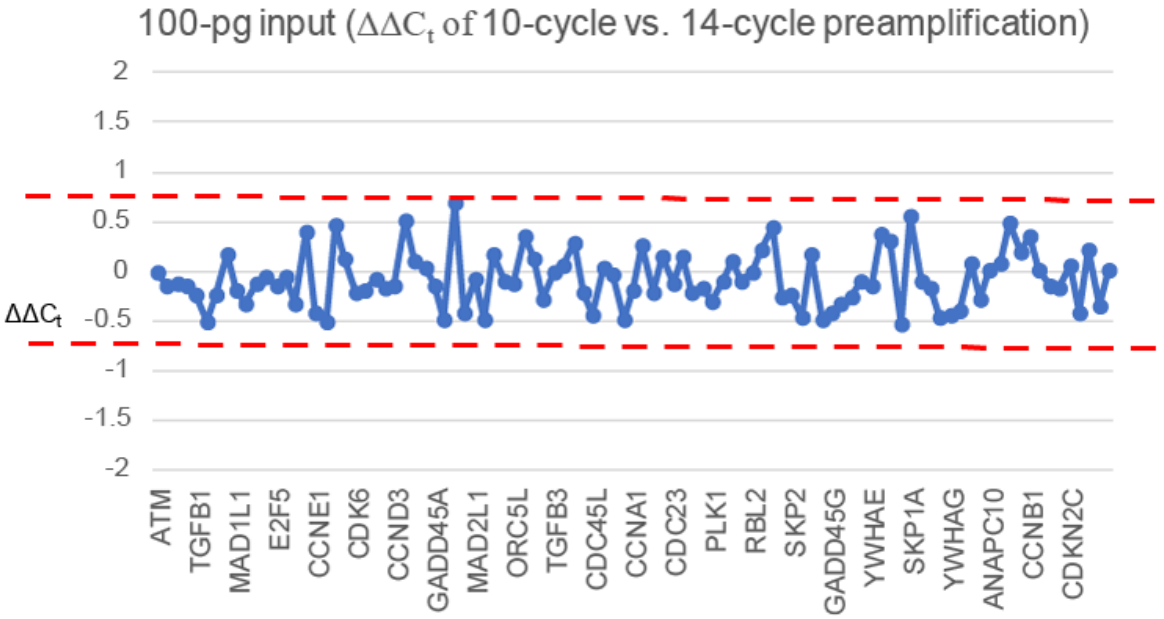



**Figure 4. $\Delta\Delta C_t$ analysis demonstrates unbiased preamplification between 10 and 14 cycles.** Please note that although the above graph displays data for all 96 target genes in the PrimerArray Cell Cycle (Human) panel, only some of the gene names are shown on the x-axis to ensure their readability. The complete list of target genes can be found here.

## Preamplification with less than 100 pg of starting material

As demonstrated above, Prelude PreAmp Master Mix can perform 14 cycles of preamplification on 100 pg of cDNA without introducing bias. However, no commercially available preamplification mixes have demonstrated lower inputs, with many requiring as much as 1 ng of input. Based on the performance of Prelude PreAmp Master Mix, it can even be utilized for situations where less than 100 pg of starting material is present, which can occur with limited or precious samples, such as FFPE tissue and liquid biopsies, or rare targets such as circulating or cell-free DNA. To demonstrate this, 12.5 pg, 50 pg, or 100 pg of cDNA were subjected to 14 cycles of preamplification using Prelude PreAmp Master Mix. These samples were then assayed using the same 96-target cell cycle panel as above. Regardless of the starting input amount, all genes maintained uniform representation with no introduction of bias (Figure 5). Since Prelude PreAmp Master Mix can work with as little as 12.5 pg of starting material, it provides an unrivaled level of sensitivity.

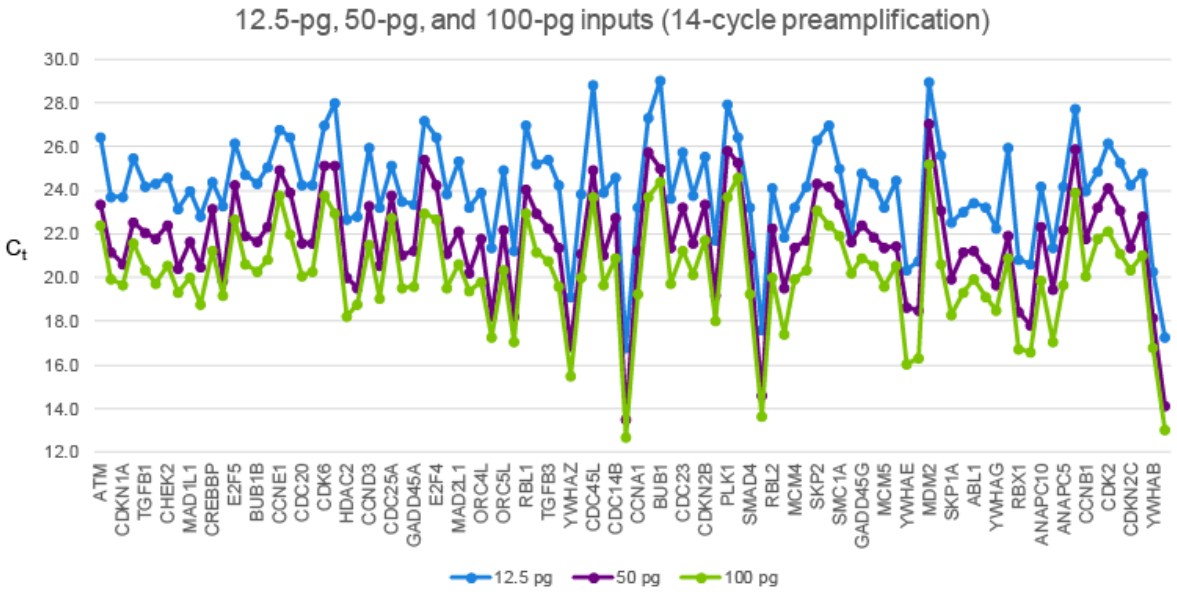



**Figure 5. Sensitive, unbiased preamplification using as little as 12.5 pg of starting material.** Please note that although the above graph displays data for all 96 target genes in the PrimerArray Cell Cycle (Human) panel, only some of the gene names are shown on the x-axis to ensure their readability. The complete list of target genes can be found here.

## Unrivaled performance when compared to other preamplification mixes

When it comes to performing preamplification, there are multiple master mixes to choose from. Prelude PreAmp Master Mix was designed to offer the best preamplification with the least amount of bias. To compare the performance of Prelude PreAmp Master Mix against other leading preamplification mixes (Figure 6), 10 pg of cDNA was subjected to either 10 or 14 cycles of preamplification with Prelude PreAmp Master Mix [TBU] using a 96-target cell cycle panel. Three other master mixes (TaqMan PreAmp Master Mix [ABI], SsoAdvanced PreAmp Supermix [BioRad], and PerfeCTa PreAmp SuperMix [Quanta]) were also tested using the manufacturer's recommended protocols. $\Delta\Delta C_t$ was calculated using GAPDH as a normalizer. A threshold of ±0.75 was used to determine whether amplification was unbiased (Table I). Notably, Prelude PreAmp Master Mix performed the best, with 92/96 assays within ±0.75 (96%). Other master mixes could not match up, with some only amplifying 75% of the assays without bias. Thus, Prelude PreAmp Master Mix offers the ultimate confidence in unbiased preamplification from limited samples.

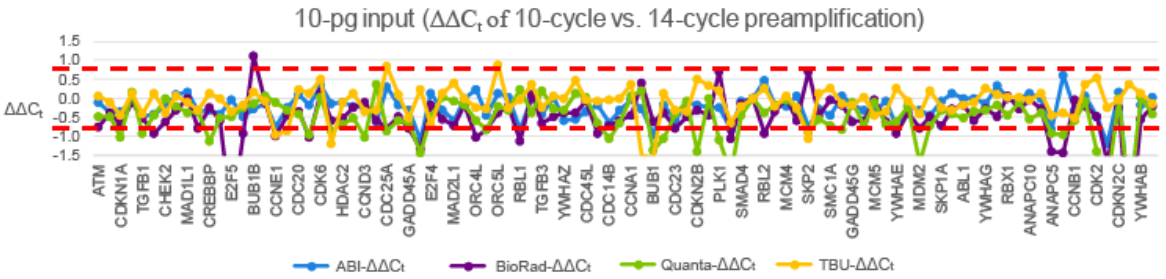


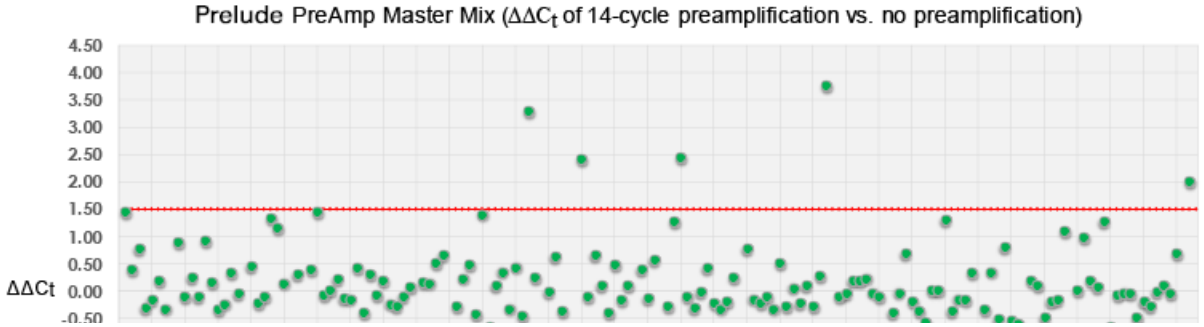

**Figure 6. Prelude PreAmp Master Mix provides less biased preamplification than other master mixes.** Please note that although the above graph displays data for all 96 target genes in the PrimerArray Cell Cycle (Human) panel, only some of the gene names are shown on the x-axis to ensure their readability. The complete list of target genes can be found here.

| Product | No. assays within ±0.75 | Percent bias |
|---|---|---|
| Prelude PreAmp Master Mix | 92/96 | 4% |
| TaqMan PreAmp Master Mix | 87/96 | 9% |
| SsoAdvanced PreAmp Supermix | 72/96 | 25% |
| PerfeCTa PreAmp SuperMix | 73/96 | 24% |

**Table I. Prelude PreAmp Master Mix displays less bias than other preamplification master mixes.**

## Superior preamplification of 162 breast cancer-related genes in FFPE tissue

Prelude PreAmp Master Mix was also tested using a 162-target breast cancer panel on FFPE tissue. RNA was extracted from FFPE tissue samples using NucleoSpin totalRNA FFPE (Cat. # 740982.10) and cDNA was generated using PrimeScript RT Master Mix (Perfect Real Time) (Cat. # RR036A). The cDNA was then subjected to 14 cycles of preamplification using Prelude PreAmp Master Mix. The preamplified product was then assayed using a custom 162-target breast cancer panel and detected using SmartChip TB Green Gene Expression Master Mix (Cat. # 640211) on the SmartChip Real-Time PCR System (Cat. # 640022). $\Delta\Delta C_t$ values were calculated using the median of six internal housekeeping genes as a normalizer and plotted for all assays in the panel (Figure 7). A threshold of ±1.5 $C_t$ was used to determine whether amplification was unbiased, due to the high variability in assay performance and sample quality. Notably, the preamplification of this large number of targets in a challenging sample type, FFPE tissue, resulted in very little preamplification bias, with 97% of the assays (157 out of 162) falling within the acceptable threshold.



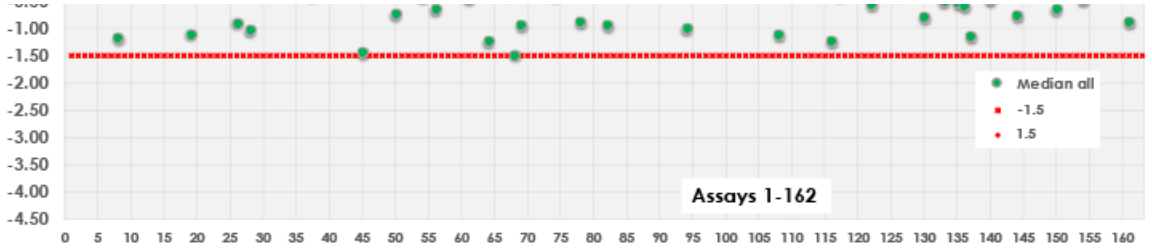



**Figure 7. Prelude PreAmp Master Mix provides highly unbiased preamplification when assaying 162 breast cancer targets in FFPE tissue.**

A similar experiment was performed using TaqMan PreAmp Master Mix, according to the manufacturer's recommendations. $\Delta\Delta C_t$ values were once again calculated using the mean of six internal housekeeping genes as a normalizer and plotted for all assays in the panel (Figure 8). A threshold of ±1.5 $C_t$ was used to determine whether amplification was unbiased, due to the high variability in assay performance and sample quality. Subsequent analysis revealed that preamplification using this mix was more biased, with only 91% of the targets falling within a threshold of ±1.5—triple the amount of bias observed when using Prelude PreAmp Master Mix. Thus, when compared with TaqMan PreAmp Master Mix, Prelude PreAmp Master Mix performs better due to less biased preamplification (Table II).

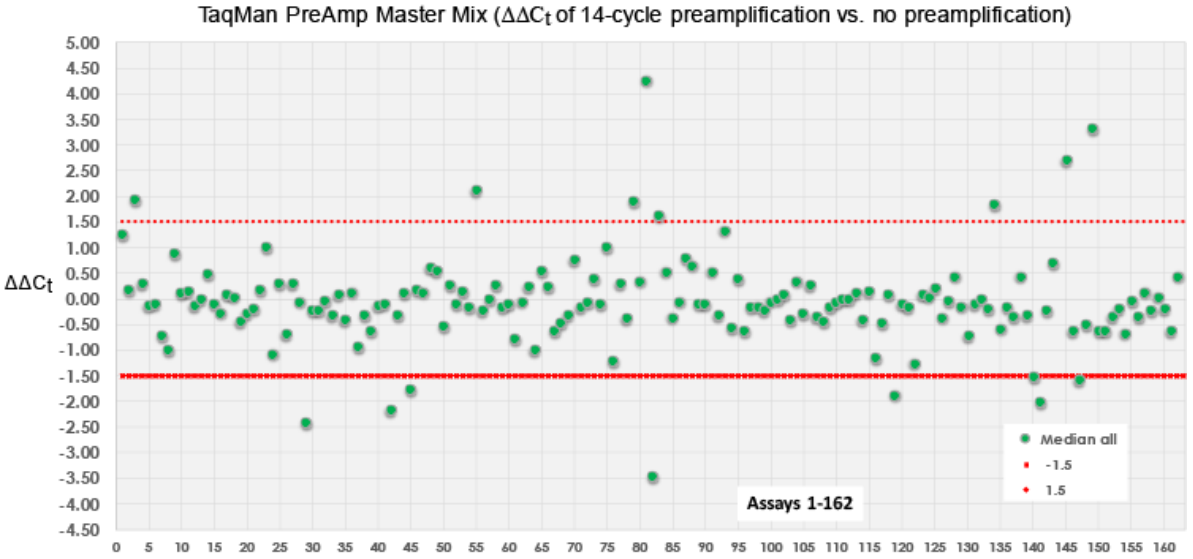



**Figure 8. TaqMan PreAmp Master Mix displays more bias than Prelude PreAmp Master Mix when assaying 162 breast cancer targets in FFPE tissue.**

| Product | No. assays within ±1.5 | Percent bias |
|---|---|---|
| Prelude PreAmp Master Mix | 157/162 | 3% |
| TaqMan PreAmp Master Mix | 142/162 | 9% |

**Table II. Prelude PreAmp Master Mix provides more unbiased preamplification than TaqMan PreAmp Master Mix.**

## Conclusions

As researchers grapple with increasingly challenging sample types—which are limited and precious—preamplification has emerged as a powerful technique to sensitively and consistently obtain enough material for qPCR, SNP genotyping, and target enrichment analyses. However, to ensure the best results, preamplification must be sensitive enough to work with the lowest starting inputs (<100 pg) while introducing the lowest possible amount of bias. Prelude PreAmp Master Mix is a powerful master mix that works with inputs as low as 10 pg of cDNA/gDNA and provides the most unbiased preamplification when compared to other available preamplification master mixes.

## Methods

### Preamplification using cDNA

Multiple starting concentrations of Human Universal QUICK-Clone II cDNA (Cat. # 637260) were preamplified for the specified number of cycles using either Prelude PreAmp Master Mix, TaqMan PreAmp Master Mix, SsoAdvanced PreAmp Supermix, or PerfeCTa PreAmp SuperMix, according to the manufacturers' recommendations. Preamplified cDNA was assayed across the PrimerArray Cell Cycle (Human) panel (Cat. # PH002), which contains 88 genes relevant to the cell cycle and 8 housekeeping genes, using SmartChip TB Green Gene Expression Master Mix (Cat. # 640211) on the SmartChip Real-Time PCR System (Cat. # 640022). $\Delta\Delta C_t$ was calculated using GAPDH as a normalizer and plotted for all assays in the panel according to the methods described in Livak and Schmittgen 2001. A threshold of ±0.75 $C_t$ was used to determine whether amplification was unbiased or not.

### Preamplification using RNA from FFPE tissue

RNA was extracted from FFPE tissue samples using NucleoSpin totalRNA FFPE (Cat. # 740982.10) and cDNA was generated using PrimeScript RT Master Mix (Perfect Real Time) (Cat. # RR036A). The cDNA was the subjected to 14 cycles of preamplification using Prelude PreAmp Master Mix or TaqMan PreAmp Master Mix according to the manufacturer's recommendations. The preamplified product was then assayed using a custom 162-target breast cancer panel and detected using SmartChip TB Green Gene Expression Master Mix (Cat. # 640211) on the SmartChip Real-Time PCR System (Cat. # 640022). $\Delta\Delta C_t$ was calculated using GAPDH as a normalizer and plotted for all assays in the panel according to the methods described in Livak and Schmittgen 2001. A threshold of ±0.75 $C_t$ was used to determine whether amplification was unbiased.

## References

Livak, K. J. & Schmittgen, T. D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods* **25**, 402–408 (2001).

## Related Products

| Cat. # | Product | Size | Price | License | Quantity | Details |
|---|---|---|---|---|---|---|
| 638541 | Prelude™ PreAmp Master Mix | 40 Rxns | $480.00 | | | |
| | Prelude PreAmp Master Mix uses an optimized 2X PCR mix for unbiased preamplification of over 100 targets, starting from a limited input of cDNA or gDNA (10 pg–100 ng). Amplified products can be used for downstream real-time PCR (qPCR), genotyping, or target enrichment analysis. | | | | | |
| | Documents / Components / Image Data | | | | | |
| 638542 | Prelude™ PreAmp Master Mix | 10 Rxns | $140.00 | | | |

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