XNA Increases Assay Sensitivity in Sanger Sequencing, qPCR, NGS and CRISPR Mutant Screening

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INTRODUCTION

XNA[1] is a small DNA oligo analog that binds to wildtype sequence with 100% sequence match Due to the high-affinity binding nature, the XNA binding to the wildtype sequence blocks its amplification in PCR. In contrast, the mutant sequence with one or more mismatch to the XNA sequence is able to be amplified and therefore gets enriched. The enrichment of mutant sequence allows mutation detection with much better sensitivity for Sanger sequencing, qPCR, NGS, and CRISPR screening qPCR assay.

Here we present examples of assay sensitivity improvement by XNA in different technique platform or applications. In BRAF Sanger sequencing[2], the assay sensitivity is increased from traditional 15 to 20% to 0.04% and JAK2 qPCR assay, the sensitivity from traditional qPCR 1% sensitivity to 0.05% sensitivity. For NGS panel, we have shown that the mutation can be detected much earlier and sequencing depth can be significantly decreased when XNA is present than without XNA.

METHODS

Sanger Sequencing

BRAF wildtype or V600E fragment was amplified by PCR in the presence of BRAF XNA, and Sanger sequencing was performed by Sequetech (Mountain View, CA).

qPCR Assay

JAK2 qPCR assay that detects the JAK2 V617F in the absence or presence of XNA were performed at different concentrations of V617F variant (variant allele frequency, VAF) from 0.039% to 5%. The qPCR reactions were performed on either Bio-Rad CFX 384, Thermo Fisher QuantStudio 5, or Roche 480 II. The qPCR products were sent for Sanger sequencing by Sequetech.

NGS Assay

Important somatic mutation targets (OptiSeq™ Dual Cancer Mini-MRD kit) designed in the NGS panel were tested in the presence or the absence of the target XNAs. The targeted NGS was performed according to the kit instruction manual.

CRISPR Mutant Screening

The customized CRISPR mutant screening kit was used to detect Trim62 variants. The kit is based on a qPCR assay that detects single point mutations or small indels located between the primers. SYBR Green qPCR assays was performed on a Bio-Rad CFX384 instrument.

RESULTS

XNA increases Sanger sequencing sensitivity from 15-20% to 0.04%VAF

Sanger sequencing sensitivity is low - thus preventing its use in companion diagnostics. XNA-based Sanger sequencing improves sensitivity >100-fold[2].

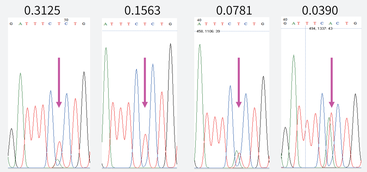


Figure 1A. BRAF V600E mutation can be detected at 0.039% VAF in a Sanger sequencing reaction. The arrows point to the mutation peak (red, T) or wild-type (green, A). The sequence reads the anti-sense strand.

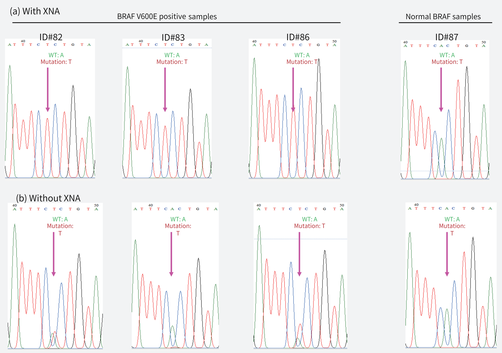
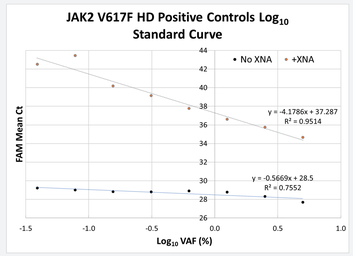


Figure 1B. BRAF V600E mutation is detected in samples #82 and #86, but is missed in #83 in regular Sanger sequencing. XNA identifies the #83 BRAF V600E mutation.

XNA increases JAK2 qPCR sensitivity

XNA added to JAK2 qPCR reaction allows the mutation to be detected at 0.039% (1.2 copies), while regular qPCR has the assay sensitivity at 1%.



	FAM Mean Ct	
VAF (%)	No XNA	+XNA
5	27.69	34.67
2.5	28.33	35.76
1.25	28.77	36.60
0.625	28.92	37.79
0.313	28.81	39.16
0.156	28.84	40.19
0.078	29.02	43.47
0.039	29.23	42.53
0	29.11	48.21

Figure 2. JAK2 XNA inhibits the wildtype sequence amplification, but not the mutant. It allows 1.2 copy (0.039% VAF) of mutant to be detected (confirmed by Sanger sequencing) in a linear range from 0.039 to 5% of JAK2 V617F allele.

XNA allows mutations detected earlier in NGS, reducing sequencing depth

Low-frequency somatic mutations are harder to be detected in targeted NGS panels with 50 to 100x depth. Deep sequencing is necessary to find these mutations. Addition of XNA into NGS panels allows discovery of the mutations with less sequencing depth compared to NGS without XNA.

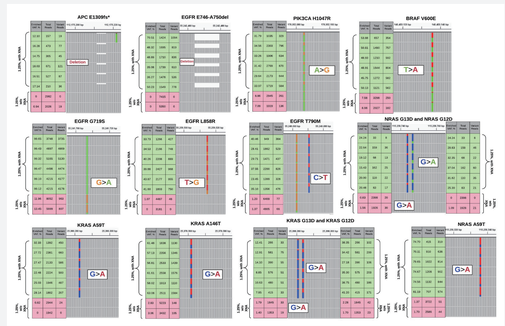
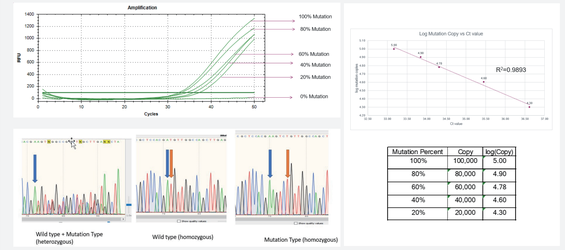


Figure 3. XNA decreases sequencing depth for finding mutations.

CRISPR Mutant Screening by XNA-based qPCR

XNA-based qPCR allows quick screening of gRNAs, editing efficiency in pools, and identification of individual mutant clones.



Mutation Percent	Copy	log(Copy)
100%	100,000	5.00
80%	80,000	4.90
60%	60,000	4.78
40%	40,000	4.60
20%	20,000	4.30

Figure 4. Top left: amplification curve of standard. Top right: standard curve on calculation of editing efficiency. Bottom left: individual cones confirmed by Sanger sequencing.

CONCLUSION

- XNA can increase genomic assay sensitivities including Sanger sequencing, qPCR and NGS. Low detection limit as low as one copy allows XNA to be a potential tool for MRD assay development.
- XNA-based Sanger sequencing can detect low-frequency mutation detection otherwise missed by regular Sanger sequencing.
- XNA-based NGS panel allows low-frequency mutation detection without deep sequencing, saving cost for CDx MRD detection.

REFERENCE

1. Powell et al. (2015) High Sensitivity Detection of Tumor Gene Mutations. BAOJ Cancer 001.
2. https://www.biorxiv.org/content/10.1101/2021.08.14.456349v1

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