Abstract #4351

Multiplex detection of G12/G13 KRAS mutations with an electrochemiluminescent hybridization assay

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1 Introduction

Oncogenic Kirsten rat sarcoma virus (KRAS) mutations are the most prevalent cancer mutations in all human tumors. Genotyping the KRAS gene has become increasingly important with the emergence of new evidence highlighting differences in downstream signaling pathways, tumor microenvironment composition, treatment responses, and prognoses linked to specific point mutations in the G12 or G13 codons. FDA approval of G12C-specific anti-KRAS drugs highlights the importance of the development of reliable assays to interrogate the mutation status of a tumor sample. Currently available genotyping assays are based on NGS, PCR, or ddPCR. These methods can be time-consuming, costly, suffer from amplification bias, or require follow-up testing or bioinformatics skills to analyze. Building on Meso Scale Discovery's (MSD) existing technology, we aimed to develop a method for the identification of eight KRAS mutations located on the G12-G13 codons in a single reaction.

2 MSD Platform

MSD Technology

MSD's electrochemiluminescence detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® and MULTI-SPOT® microplates.

Electrochemiluminescence Technology

- Minimal non-specific background and strong responses to analyte yield high signal-to-background ratios.
- The stimulation mechanism (electricity) is decoupled from the response (light signal), minimizing matrix interference.
- Only labels bound near the electrode surface are excited, enabling non-washed assays.
- Labels are stable, non-radioactive, and directly conjugated to biological molecules.
- Emission at ~620 nm eliminates problems with color quenching.
- Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- Carbon electrode surface has 10X greater binding capacity than polystyrene wells.
- Surface coatings can be customized.

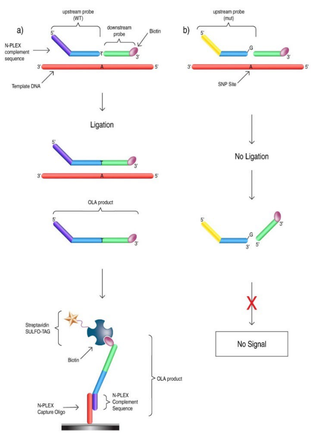
3 Assay Design

Basis of the N-PLEX® Platform

N-PLEX plates contain 10 unique capture oligonucleotides that are bound to their corresponding spot on the electrode surface. Detection of a nucleic acid sequence of interest is accomplished by hybridization of one or more probes with sequence complementary to these capture oligos and the nucleic acid of interest, followed by detection via electrochemiluminescence (i.e. biotin/streptavidin SULFO-TAG interactions). Blocking, hybridization, and detection are completed using proprietary MSD® buffers and diluents.

Probe Design

Upstream probes are designed to carry the N-PLEX complementary sequences on their 5' end followed by a KRAS-specific probe region with the mutation site on the 3' end of the probe. Downstream probes are universal for the codon being investigated and are biotin labeled on the 3' end. Probe length was selected to provide melting temperatures around 64°C for all upstream and downstream probes.



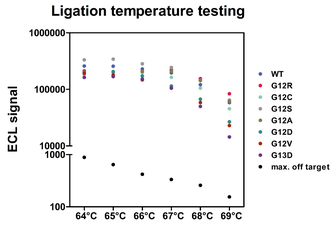
Oligonucleotide Ligation Assay (OLA) concept

- Genomic DNA extraction should be carried out using a protocol and reagents suitable for the sample type. An RNAse treatment step is recommended to be included in the protocol.
- Target regions are PCR-amplified using KRAS-specific primers to enrich for the region of interest and decrease assay backgrounds.
- Upstream and downstream probes are pre-mixed and added to the diluted PCR amplicons.
- A high fidelity thermostable DNA ligase is added to the samples and multiple cycles of denaturation and ligation are performed in a thermocycler for strict temperature control, creating mutation and N-PLEX spot-specific ligation products. Ligation temperatures close to the melting temperature of the probes provides a high differentiation rate between perfectly matched and mismatched target-probe hybrids.
- To increase the signal to noise ratio, the OLA products are denatured in the presence of excess blocking oligos.
- The OLA products are added to the N-PLEX plate where the complementary sequence of the mutation-specific ligation products will bind to their respective capture oligos.
- After incubation with SULFO-TAG labeled streptavidin and addition of read buffer, signal levels are read on an MSD instrument and mutation status assigned.

4 Assay Optimization Steps

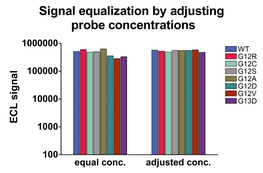
Selection of Ligation Temperature

Assay specificity was tested at ligation temperatures of 64-69°C using synthetic DNA targets. Increasing ligation temperatures resulted in reduced overall ligation efficiency, but they increased the ligation specificity. For future experiments, 67°C was selected as the temperature providing the highest sensitivity and specificity for this assay.



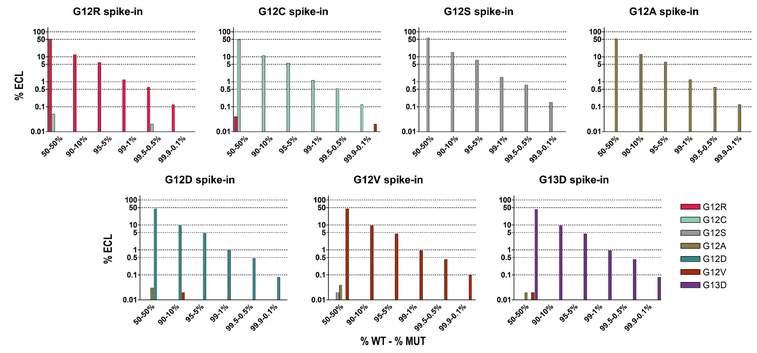
Optimization of Probe Concentrations

DNA ligases show slightly different ligation efficacy depending on the nature of the 3' and 5' bases to be joined at the ligation site. Using equal probe concentrations for all synthetic DNA targets resulted in significant differences in signal levels between the target mutations. Concentrations of individual upstream probes in the OLA probe mix were adjusted to provide uniform signal levels across all target mutations.



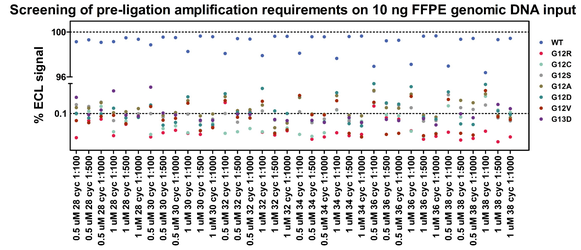
Testing of OLA with Artificial DNA Targets

After the optimization of the ligation protocol, specificity and sensitivity of the ligation reaction was assessed using synthetic double-stranded DNA targets. Target regions carrying the KRAS mutations of interest were mixed with wild-type KRAS constructs at the ratios indicated in the graph below. 30 cycles of denaturation and ligation were carried out followed by hybridization to the plate captures and incubation with SULFO-TAG labeled streptavidin. Mutation burden was calculated based on signal ratios observed between the wild-type and mutant spots of the N-PLEX plate. As the assay aims to detect KRAS mutations in the presence of a wild-type background, background correction was applied by subtracting the non-specific signal observed with a wild-type calibrator from all spiked samples. The ligation reaction was able to accurately identify mutant alleles at spike levels as low as 0.1% and provided a good approximation of the tumor burden of the spiked samples.



Selection of PCR Primer Concentration, Cycle Number and Dilution Factor

Genomic DNA from WT KRAS FFPE reference samples was used to identify the optimal PCR conditions to maximize signal-to-background ratio in the assay. Amplification of the target gene followed by dilution of the amplicon helps enrich for the target region and reduces the non-specific background of the assay. Increasing primer concentration and post-PCR dilution of amplicons increased the sensitivity of the assay. The optimal conditions for this assay were as follows: 36 cycles of amplification using 1µM primer, followed by 1000-fold dilution of the amplicons.



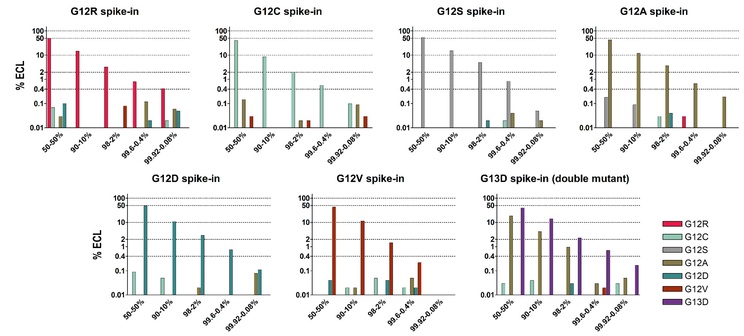
5 Optimized Protocol

The following protocol was established for FFPE sample testing:

1. Amplify 10 ng of FFPE gDNA using Phusion Flash High-Fidelity Mastermix and 1 µM primers with the following cycling parameters: 30 sec at 98°C; 36 cycles of 1 sec at 98°C, 5 sec at 60°C, 15 sec at 72°C; 1 min at 72°C, hold at 4°C.
2. Dilute amplicons 1000-fold in nuclease free water.
3. Combine 10 µL of the diluted amplicons and 10 µL of a probe mix containing all upstream and downstream probes, ligase buffer and Taq DNA ligase and perform OLA using the following protocol: 2 min at 95°C, 30 cycles of 30 sec at 95°C, and 2 min at 67°C.
4. Block N-PLEX plates at room temperature for minimum 30 minutes during OLA cycling.
5. Combine ligation products with blocking oligos and heat the reaction at 95°C for 5 minutes.
6. Wash N-PLEX plate, add the OLA product to the plate in duplicates and incubate for 1 hour at 45 °C.
7. Wash plates, add SULFO-TAG labeled streptavidin detection solution and incubate for 30 minutes at room temperature.
8. Washed plates, add read buffer, and read plate on MSD instrument.

6 OLA Performance Using FFPE Reference Samples

The optimized assay protocol was tested using spike-ins generated from commercially obtained KRAS FFPE reference samples. Genomic DNA was extracted from wild-type FFPE reference samples and from reference samples heterozygous for the mutations of interest. KRAS mutant gDNA samples were diluted with wild-type gDNA to the ratios indicated in the graph below. Mutations were accurately called in spiked samples down to 0.4% mutation rates. The assay also identified a second mutation in the G13D reference sample. The manufacturer was not aware of this second mutation, as they only test their reference samples for the presence of their target mutation using PCR.



7 Conclusions

We developed a multiplex OLA assay for the detection of eight KRAS genotypes in a single reaction. After assay optimization, spike recovery experiments using synthetic target DNA showed successful identification of KRAS mutant genotypes at spike levels as low as 0.1% over wild-type background. Validation experiments with gDNA extracted from FFPE reference samples confirmed that the multiplex assay can accurately differentiate eight KRAS genotypes in samples with ≥0.4% tumor burden. Further assay validation is planned using FFPE samples with known KRAS mutations. These data highlight a novel approach to simultaneously identify eight KRAS genotypes from 10 ng of DNA input in a single reaction within 4-5 hours. The assay is capable of identifying double mutants and can provide a semi-quantitative assessment of tumor burden without the need for follow-up testing.

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