Bridging the Gap: Chromosomal Mutation Analysis and Understanding Risk in Cell and Gene Therapy Development

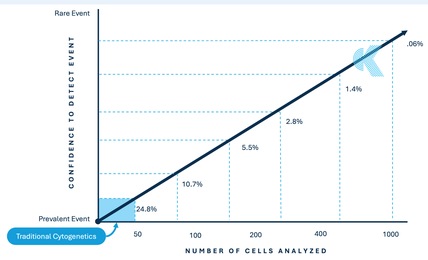
Introduction:

When cells are edited, differentiated, modified, or engineered for therapeutic applications such as CAR-T, accurately measuring mutation rates and chromosomal stability is critical. Comprehensive, accurate analysis of the genome for potentially deleterious or cancer-inducing changes requires a panel of assays. The most robust characterization of genomic and target integrity is provided by combining cytogenetic cell-based analysis, which detects chromosome structural variation and aneuploidy, with targeted sequencing approaches such as NGS and dPCR.

KROMATID's KROMASURE platform of advanced cytogenetic tools enriches the available dataset beyond classic cytogenetic techniques and offers a custom suite of solutions for in-depth single-cell analysis of genomic targets of interest.

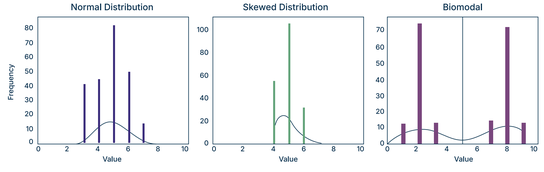
The platform leverages proprietary probe design and hybridization methods, as well as automation and advanced imaging solutions to increase the number of cells analyzed. All platform methods can be paired with custom analytical packages to support a wide variety of study designs, including therapeutic product release testing and comparative analysis.

Next-Gen cytogenetics provides increased confidence in detecting rare-structural events in the genome as well as the advantage of directly visualizing events in every cell and understanding event distribution compared to pooled and extrapolated methods.



Distribution Matters

Average copy number of 5 can look many ways in single cells:



KROMATID

Authors

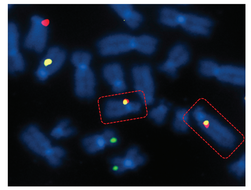
Erin Cross[1], Matthew McGowan[1], Michael Vernich[1], Greg Husar[1], Christopher Tompkins[1], Gretchen Pratt[1]

[1] KROMATID Inc., Longmont CO

Monitoring Edit Sites:
Fit for purpose, flexible solutions

What's New?

Tracking chromosomal rearrangements occurring at or between edit sites:



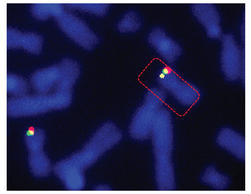
InSite probes (single color) designed to span and bracket the edit site:

Balanced reciprocal translocation between TRAC and B2M:

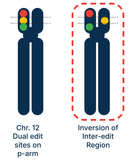
Translocation derivatives produced by an exchange involving a TRAC and a B2M edit site:

Edit-Site Analysis (triple edit): TRAC (red), B2M (yellow), CIITA (green). Reciprocal translocation involving TRAC and B2M present in cell.

Tracking chromosomal rearrangements occurring at or between edit sites on the same chromosome:

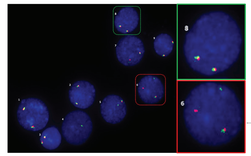


InSite probes designed to track a region between two edits:

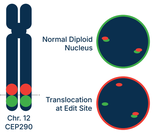


Edit-Site Analysis (dual edit- same chromosome): Edit 1 bracketing probe (red), Inter-edit Region (yellow), Edit 2 bracketing probe (green). Inversion involving the two edit sites present in cell (right).

Tracking chromosomal rearrangements in nuclei:



PPF probes (dual color) designed to bracket the edit site:



Edit-Site Analysis (single edit): Dual-color PPF Assay designed to detect chromosomal rearrangements involving an edit site on Chr 12. 3/8 nuclei in the field of view are positive for large structural rearrangements involving the Chr 12 edit site. Cell 4: deletions flanking both edit sites (two missing red signals, one missing green signal). Cell 5: deletion flanking one edit site (one missing red signal). Cell 6 (enlarged and boxed in red): translocation involving one of the edit sites (red- green signal break apart).

Measuring Structural Variation at the Edit Site:

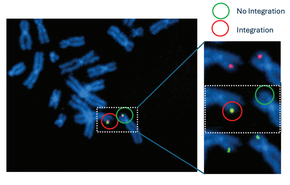
KROMATID's InSite and PPF platform offers a custom suite of solutions for in-depth single-cell analysis of edit site integrity. Assays can be flexibly configured to monitor the effects of single or multiplexed editing strategies, characterizing edit sites on a cell-by-cell basis across hundreds of individual cells.

Mapping Transgenes

Measuring Transgene Integration and Structural Variation at the Edit Site:

KROMATID's InSite and PPF platform offers a custom suite of solutions for in-depth single-cell analysis of transgene integration. Assays can be configured to monitor random and targeted integrations in dividing and non-dividing cell populations, and integrational copy number can be assessed across hundreds to thousands of individual cells.

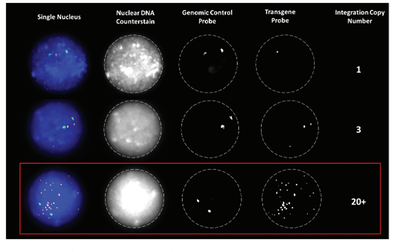
Measuring Site-Specific Integrations:



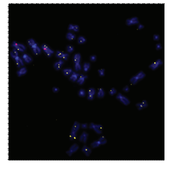
InSite probes (dual color-red/green) designed to bracket the edit site and a transgene probe (yellow):

Targeted Integration Tracking (knock-in): CD38 centromeric bracketing probe (red), Transgene (yellow), CD38 telomeric bracketing probe (green). Mono-allelic knock in (circled in red), and no off-target integrations present in cell.

Measuring Transgene Integrational Copy Number in Nuclei:



Enumeration of Integration Events: PPF assay designed to measure integrational copy number of a transgene in non-dividing cells (lenti-based delivery system). Assay included a Genomic Control Probe (green) to measure genome ploidy, and transgene probe to measure integration events. Bi-modal distribution of copy number observed: 80+% of cells had 0-1 integrations, 10% of cells had 10-20+ integration events. Average copy number was 5- matching ddPCR results.



Measuring Random Integrations in Metaphase Cells:

Enumeration of Integration Events: InSite assay designed to measure integrational copy number of a transgene in metaphase cells. Assay includes a Genomic Control Probe (red) to measure genome ploidy, and transgene probe (yellow) to measure integration events. In this example of a cell with a high-copy integration count, 36 integration events were observed.

GIAB HG008 Matched Tumor-Normal Pair

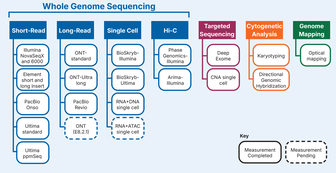
Structural Variant Characterization

- The National Institute of Standards and Technology (NIST) Genome In a Bottle (GIAB) initiative aims to characterize complex structural variants in the pancreatic ductal carcinoma line HG008-T as a benchmark dataset for genome structural variation research.
- KROMASURE SCREEN analysis demonstrated the critical role of direct visualization technologies in complementing sequencing-based approaches for complex event characterization.[1]

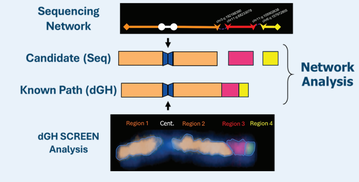
When applied to the HG008 cell line, KROMASURE SCREEN revealed key insights into genome architecture, including the detection of cells with and without whole genome doubling, and the identification of phasing of complex rearrangements difficult to resolve by G-banding and sequencing alone.

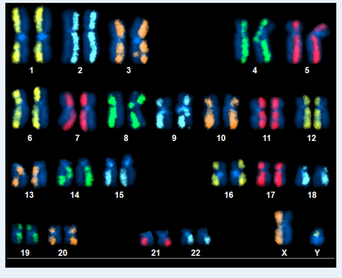
Unlike sequencing methods that rely on pooled DNA and bioinformatic reconstruction, KROMASURE SCREEN directly visualizes chromosomal structure in individual cells, enabling unbiased detection of low-frequency variants and complex events. The technology's unique ability to determine co-occurrence of multiple variants on the same chromosome (phasing) and establish structural orientation provides critical validation data for comprehensive genomic analysis. The integration of cytogenetic and sequencing approaches delivers the most robust characterization of genomic integrity, which is essential for advancing the understanding of cancer genomes and therapeutic applications.

GIAB is utilizing extensive genome-scale measurements for characterizing tumor normal genomes, including KROMASURE SCREEN



Integrating Sequencing and Cytogenetic Data





Whole genome doubling with multiple complex variations observed in HG008-T (28/40 cells at passage 53)

KROMATID is developing network-based methods for integrating sequencing and KROMASURE SCREEN data. This quantitative approach enables cross-method validation, identifying concordant variants between technologies to resolve complex structural rearrangements difficult to fully characterize by either method alone.

[1] McDaniel, Jennifer H et al. "Development and extensive sequencing of a broadly-consented Genome in a Bottle matched tumor-normal pair." *bioRxiv : the preprint server for biology* 2024.09.18.613544. 18 Oct. 2024, doi:10.1101/2024.09.18.613544. Preprint.