The Journal of Molecular Diagnostics, Vol. 18, No. 1, January 2016

the Journal of Molecular Diagnostics
jmd.amjpathol.org

TECHNICAL ADVANCE

Ultrasensitive Detection of Multiplexed Somatic Mutations Using MALDI-TOF Mass Spectrometry

Michael J. Mosko,* Aleksey A. Nakorchevsky,* Eunice Flores,* Heath Metzler,* Mathias Ehrich,† Dirk J. van den Boom,† James L. Sherwood,‡ and Anders O.H. Nygren*

From Agena Bioscience, San Diego, California; Sequenom,† San Diego, California; and AstraZeneca,‡ Cambridge, United Kingdom*

Accepted for publication August 28, 2015.

Address correspondence to Anders O.H. Nygren, Ph.D., 3565 General Atomics Ct, San Diego, CA 92121. E-mail: anders.nygren@agenabio.com.

Multiplex detection of low-frequency mutations is becoming a necessary diagnostic tool for clinical laboratories interested in noninvasive prognosis and prediction. Challenges include the detection of minor alleles among abundant wild-type alleles, the heterogeneous nature of tumors, and the limited amount of available tissue. A method that can reliably detect minor variants <1% in a multiplexed reaction using a platform amenable to a variety of throughputs would meet these requirements. We developed a novel approach, UltraSEEK, for high-throughput, multiplexed, ultrasensitive mutation detection and used it for detection of mutant sequence mixtures as low as 0.1% minor allele frequency. The process consisted of multiplex PCR, followed by mutation-specific, single-base extension using chain terminators labeled with a moiety for solid phase capture. The captured and enriched products were then identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. For verification, we successfully analyzed ultralow fractions of mutations in a set of characterized cell lines, and included a direct comparison to droplet digital PCR. Finally, we verified the specificity in a set of 122 paired tumor and circulating cell-free DNA samples from melanoma patients. Our results show that the UltraSEEK chemistry is a particularly powerful approach for the detection of somatic variants, with the potential to be an invaluable resource to investigators in saving time and material without compromising analytical sensitivity and accuracy. *(J Mol Diagn 2016, 18: 23–31; http://dx.doi.org/10.1016/j.jmoldx.2015.08.001)*

Molecular targets that indicate the presence of low-level driver mutations and therapeutic escape variants can provide invaluable information for cancer treatment options. The detection of these genetic aberrations is difficult, because the minor mutant allele may differ from the highly abundant wild-type sequence by only a single nucleotide. The heterogeneous nature of most tumor tissue and the limited amount of clinical sample (eg, circulating cell-free DNA) further complicate detection.[1] Therefore, a method for multiplexed ultrasensitive detection of low-level driver mutations and therapeutic escape variants is essential for diagnosis, prognosis, and/or monitoring of metastatic potential of tumors.

Currently, a variety of different PCR-based methods are available for minor allele detection. The most common strategies include mutant enrichment through allele-specific PCR with specificity toward the minor allele established via the terminal 3′ residue of PCR primers, or wild-type suppression with specific probes.[2–5] These methods have shown a varying degree of sensitivity and specificity in the detection of minor somatic variations.[2,6–8] The limited multiplexing ability of the allele-specific approach represents a major limitation when dealing with clinical samples.[9] Alternate technologies, such as next-generation sequencing (NGS) and droplet digital PCR (ddPCR), do not enrich for minor variants but rely on the sensitivity of

Supported by Agena Bioscience and AstraZeneca.

Disclosures: M.J.M., A.A.N., E.F., H.M., and A.O.H.N. are employees of Agena Bioscience and hold company stock options. M.J.M., D.J.v.d.B., and A.O.H.N. are inventors on patent applications owned by Agena Bioscience. M.E. and D.J.v.d.B. are employees of Sequenom and hold company stock and stock options. J.S. is an employee of AstraZeneca and holds company stock. Agena Bioscience and AstraZeneca provided reagents and clinical samples.

http://dx.doi.org/10.1016/j.jmoldx.2015.08.001

the detection mechanism.[10,11] ddPCR offers enhanced sensitivity over allele-specific PCR methods, but the multiplexing capability of ddPCR is also limited.[12] Sequencing technologies offer *de novo* detection of variants, but are restricted by errors introduced in the sample preparation and sequencing process.[13] Although publically available analysis pipelines can overcome this difficulty and detect minor variants down to 2%, ultrasensitive detection at 1% and below remains a technical challenge.[1] Sampling and error rates can be overcome with deep bidirectional sequencing, and identifying PCR duplicates requires molecular barcodes.[14] The additional cost for increased sequencing and data storage will likely become a budgetary constraint for smaller laboratories using NGS systems.

We therefore developed a novel multiplex minor variant detection technique. The UltraSEEK chemistry (Figure 1) is able to interrogate multiple informative variants within a single reaction. This approach does not require allele-specific PCR or annealing temperature constraints. UltraSEEK uses a mass spectrometer for detection, and does not require the accessory equipment and support often needed with NGS-derived data. Like allele-specific PCR and ddPCR, a priori knowledge is required for target selection and design. UltraSEEK is amenable to a manual workflow, but is also compatible with high-throughput processes using various automated liquid dispensing platforms. UltraSEEK differs from similar biochemistries[15] in that it enriches the minor alleles by probing them specifically in a post-PCR primer extension step that omits the wild-type allele. We achieve this by using only a biotin-labeled minor variant-specific terminating nucleotide, which is only incorporated when the minor allele is present. Extended products are subsequently captured to a solid support and washed to eliminate all other components. Previous attempts to release the captured extension products required additional steps using formamide, ethanol precipitation, and resuspension,[15] or photocleavable nucleotides.[16] The method we introduce herein requires only a single elution step and avoids the use of highly modified substrates that are not readily available. Last, the eluted products are dispensed onto bioarrays for detection using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Materials and Methods

Analytical Sensitivity and Specificity

The UltraSEEK Oncogene Panel assay validation used a model system developed to simulate samples harboring low-frequency somatic mutations. Wild-type DNA (Coriell Cell Repositories, Camden, NJ) was spiked with different amounts of characterized cell lines (Horizon Diagnostics, Cambridge, UK) harboring engineered mutations (Supplemental Table S1). The mixtures represented a 0.5% and a 1% mutant allele frequency, while keeping the total number of DNA molecules constant. Each dilution was analyzed in four replicates. Each cell line harboring a mutation for a specific assay was considered wild-type for all other assays in that plex. Some cell lines carried endogenous mutations for other assays in the same plex and were counted as another replicate of 1% and 0.5% (*BRAF*^{V600E}, *EGFR*^{G719S}, *KRAS*^{G13D}, *PIK3CA*^{H1047R}).

ddPCR Verification/Limit of Detection

Cell line–derived genomic DNA harboring a 50% *BRAF*^{V600E} mutation (Horizon Diagnostics) was used as an additional model to determine the analytical limit of detection of the UltraSEEK chemistry. This model was also used as a comparative measure against the QX100 Droplet Digital PCR System (Biorad, Hercules, CA). This cell line was diluted with a mixed population of wild-type DNA

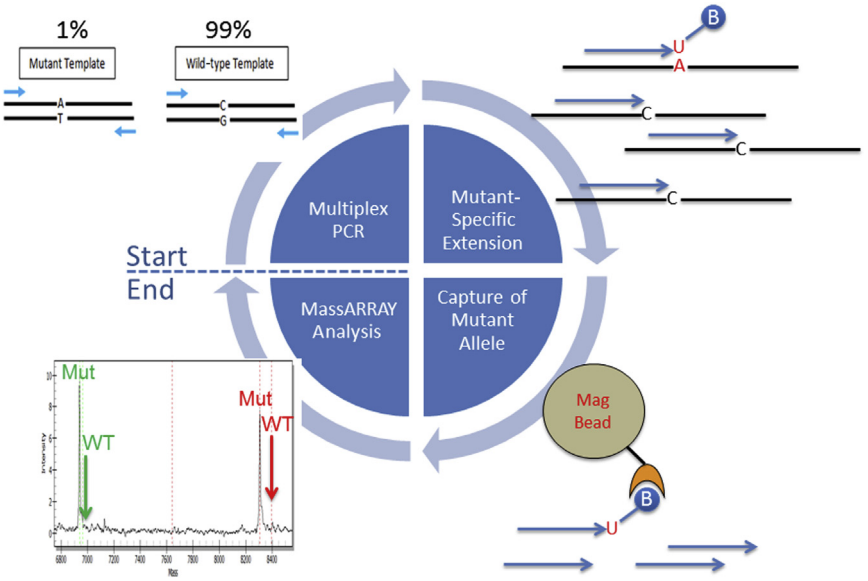

Figure 1 The UltraSEEK chemistry consists of multiplex PCR followed by a mutation-specific single-base extension reaction. The extension reaction uses a single mutation-specific chain terminator labeled with a moiety for solid phase capture. Captured, washed, and eluted products are interrogated for mass, and mutational genotypes are identified and characterized using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Mag, magnetic; Mut, mutation; WT, wild type.