EBOOK

Next-Generation Sequencing Assay Validation

A Practical Guide for the Clinical Genomics Laboratory

SeraCare Life Sciences, with Robert D. Daber, Ph.D.

ABOUT THE AUTHOR

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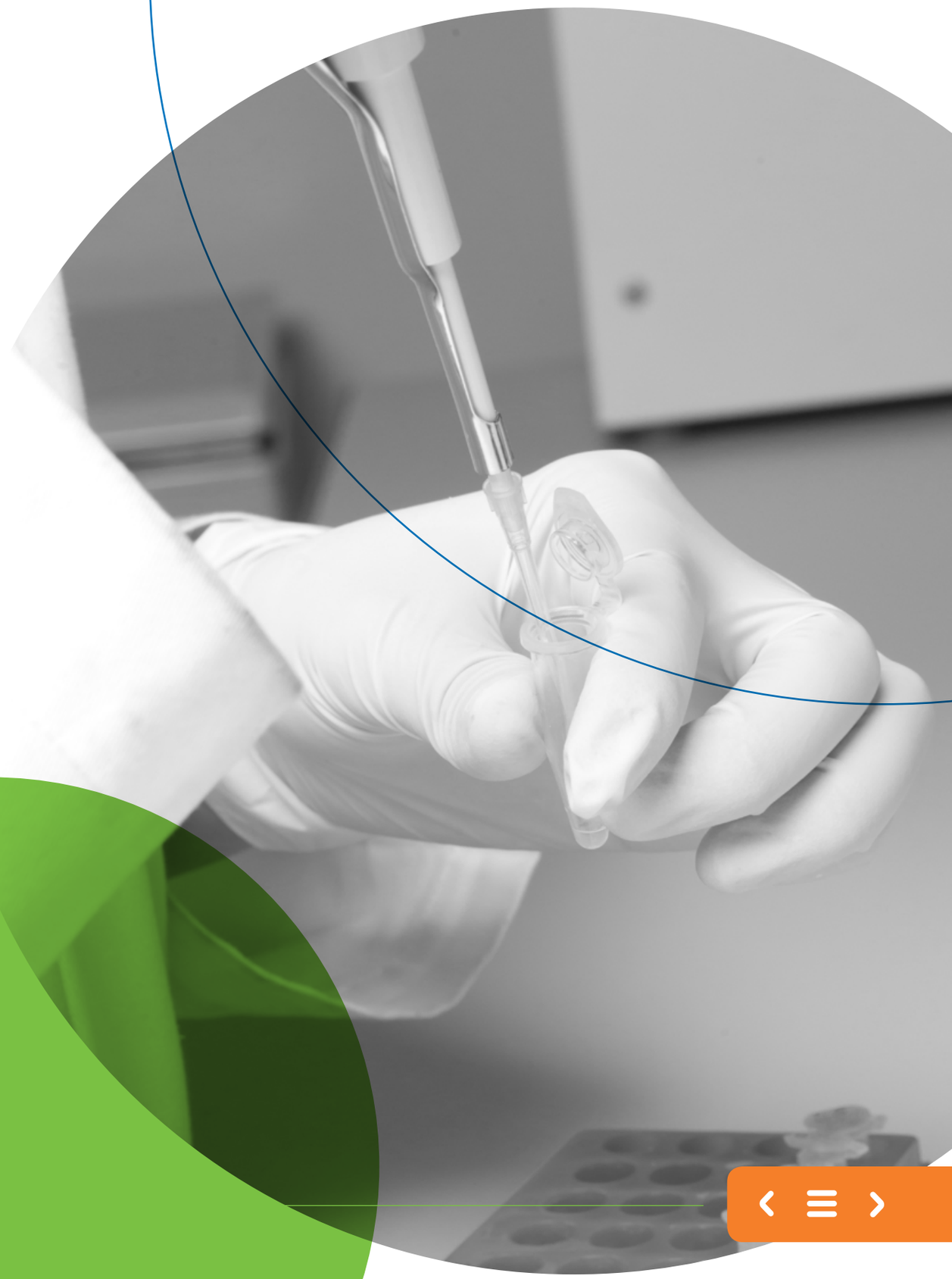
INTRODUCTION

Next-generation sequencing (NGS) has revolutionized the field of genomics and how *in vitro* diagnostic (IVD) test developers, laboratories, and clinicians are diagnosing, treating, and monitoring disease. Before you can successfully launch a clinical assay, platform, or service, you need to be absolutely confident that the test has gone through rigorous development and thorough validation to ensure accuracy of the result.

There are three key phases of bringing a clinical NGS assay into routine operation:

- Development (technology selection and optimization)
- Validation
- Ongoing assay QC protocols

This eBook focuses on the validation process for NGS-based assays and will walk through key considerations and guidelines you can follow to ensure a smooth and successful validation.



What is validation?

Before a diagnostic assay can generate information that will be used to help guide patient care, a clinical laboratory must rigorously prove that all test components are able to function together, as a whole, in order to fulfill pre-defined requirements for a particular intended use. At first glance this seems like a straight-forward concept, but when applied to NGS and its multitude of variables (instrumentation, reagents, processing steps, operators, etc.), things get complex very quickly. Especially since there are so many different approaches to validation that are required or recommended by a diverse group of authoritative bodies.

Here are some assumptions we've made:

- Validation principles are universal, however architecture may differ significantly across different technologies, methods, intended uses, etc. We will illustrate these principals using examples of their application in somatic cancer sequencing. There are two types of validation — clinical validation, which typically requires the use of a certain number of known patient specimens, and analytical validation, which refers to establishing evidence that the method does what it is intended to. We'll focus on analytical validation.
- Validation is required to bring a new test or service to market, as well as part of change-control for an existing test. We'll focus on new test validation as it applies to both IVD and laboratory-developed test (LDT) assays.
- The following types of materials are widely accepted for use in NGS-based clinical genomics assay validation: real-world samples — such as remnant patient specimens — and contrived reference materials, namely biosynthetics (like SeraCare's Seraseq™ reference materials) and those derived from cell lines.
- For both IVD and LDT assays, the validation phase can only commence after the completion of all feasibility and development or design controls. In other words, your test must be completely locked down first — including SOPs.

› **IVD Tests**

"Off-the-shelf" commercial assays that have already received FDA (United States) or CE (Europe) clearance and have already been optimized and validated for analytical performance. New or emerging NGS labs in the US will not perform optimization and will most likely use the assay protocol as-is, and proceed directly to validation. IVD assays are rarely modified, as any modification to an IVD assay renders it an LDT by default, which then requires formal validation.

› **LDT Tests**

According to the FDA, an LDT is *"A type of in vitro diagnostic test that is designed, manufactured, and used within a single laboratory."* This includes commercial assays labeled for research use only (RUO), and custom assays that are based on RUO components (e.g., Illumina's TruSight® Myeloid Sequencing Panel (LDT); a custom cancer panel using Kapa Hyper Kit with IDT xGen capture baits (LDT); or Illumina's Extended RAS Panel (IVD).

OVERVIEW OF AUTHORITATIVE BODIES

While clinical genomics testing is overseen by a variety of authoritative bodies around the world, we'll explore the agencies and regulations in the United States and Europe.

United States

Depending on the type of assay, laboratory, and even location of patients being tested, there are a number of requirements and organizations to consider.

CLIA (Non-New York State)

The Clinical Laboratory Improvement Amendments (CLIA) establish quality standards for clinical labs to ensure accuracy, reliability, and turnaround time for patient testing results. The Centers for Medicare & Medicaid Services (CMS) publishes interpretive guidelines to CLIA regulations in their state operations manual, which state agencies use to certify labs within their jurisdictions. Labs must be CLIA-certified to receive payment through Medicare and Medicaid. Despite this, guidelines for NGS tests are limited.

CAP (Non-New York State)

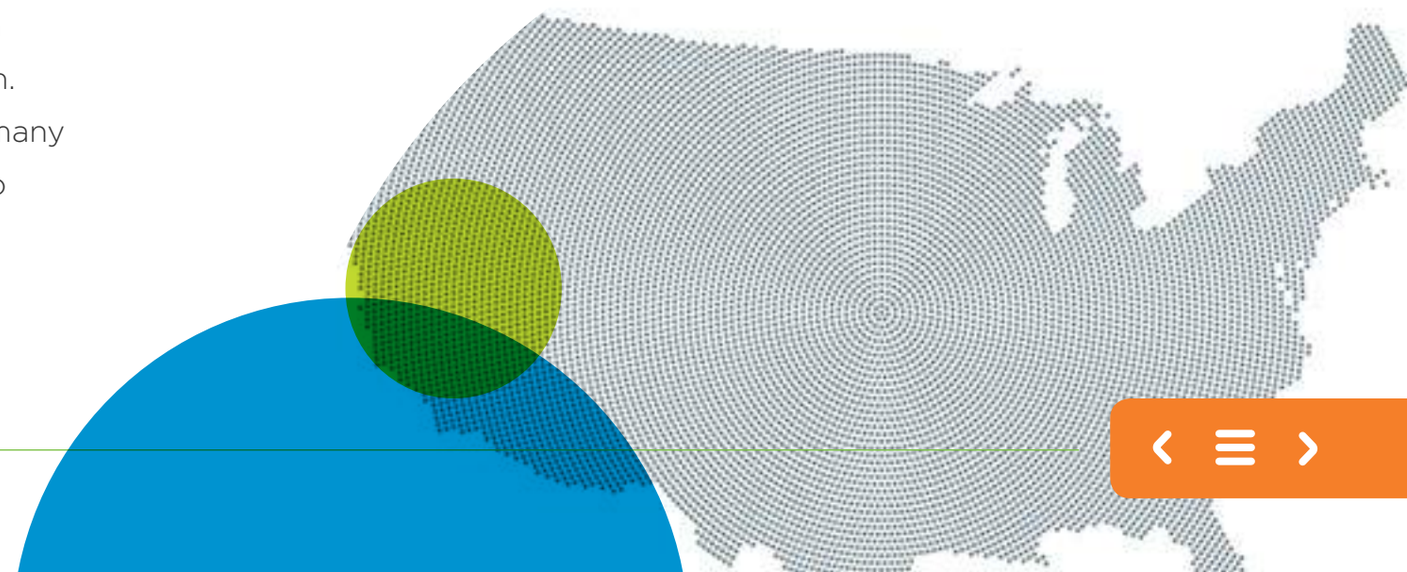
The College of American Pathologists (CAP), founded in 1946, promotes excellence in laboratory testing. CAP accreditation involving programs such as proficiency testing and peer-based inspection helps labs meet CLIA requirements. CMS granted the CAP's Laboratory Accreditation Program deeming authority, which allows CAP inspection in lieu of CMS inspection. It's also recognized by The Joint Commission, and can be used to meet many state certification requirements. The CAP provides a formal framework to perform validations.

New York State

Labs that wish to test patients from the state of New York must obtain permit through the state's Clinical Laboratory Evaluation Program (CLEP). New York employs a stringent interpretation of CLIA guidelines, and provides concrete requirements that tests must meet in order to receive certification. As an example, you can review the current (as of this eBook's publishing) guidelines for NGS tests for somatic mutation detection. CLEP is highly regarded by CMS, which exempts labs holding New York State permits from federal CLIA regulations. The only other waived tests are systems approved by the FDA for home use, and simple, low-risk tests that are approved for CLIA waiver.

US FDA

In 1976, the Medical Devices Amendments to the Food, Drug, and Cosmetic Act established *in vitro* diagnostics as a subset of medical devices that must be cleared or approved by the FDA to be legally marketed. Since then, the FDA's approach has largely been one of discretionary enforcement, which means highest-risk scenarios are given the greatest attention. Because companion diagnostics (CDx) tests are considered very high risk, they have been at the forefront of FDA regulation and policy.



IVDs are classified using a risk-based approach, which determines (among other things) the scope of analytical validation that will be required before the test can be offered on the market:

- **Class I: Lowest risk** — Assays for which "general controls" are usually sufficient.
- **Class II: Moderate risk** — Assays which generally require demonstration of "substantial equivalence" to another legally marketed ("predicate") device. A premarket notification known as a 510(k) must be submitted to the FDA for clearance. This class also covers de novo devices (those for which there is no predicate device) that present low-to-moderate risk.
- **Class III: Highest risk** – These tests require FDA approval through the premarket approval (PMA) process which requires demonstration of safety and efficacy for intended use.

Whether and how the FDA should regulate LDTs is currently being debated. Several draft guidances have been issued, but no final guidance, yet. So far, CDx LDTs that have been approved by the FDA have been treated similarly to IVDs. FDA clearance or approval of NGS tests is accelerating, and so far includes two Illumina MiSeqDx® assays for cystic fibrosis, the FoundationFocus™ CDx BRCA (an LDT performed only at Foundation Medicine), Thermo Fisher's Oncomine® Dx Target Test, and the Illumina Praxis™ Expanded RAS Panel. In each case, extensive validation studies were performed including accuracy, reproducibility, guardbanding (stress testing of key parameters), various stability studies, and many others. While we won't get into the full process for FDA approval of NGS clinical genomics tests, many of the details covered in this eBook could apply for FDA non-clinical studies.

Europe

Unlike in the United States, clinical genomics testing in Europe isn't regulated by a single government, but instead by sets of international standards and agencies.

International Organization for Standardization (ISO) ›

In Europe, local regulators encourage ISO accreditation for genetic testing laboratories. They typically conform to ISO 15189:2012, which specifies requirements for quality and competence in medical laboratories. This is an international standard which goes beyond assay performance and lab personnel training. ISO 15189:2012 is the foundation for CAP 15189 accreditation.

In Vitro Diagnostic Medical Devices, Regulation (EU) 2017/746 ›

In mid-2017, the new European Diagnostic Regulation (IVDR) went into effect, replacing the IVD Directive and reclassifying regulated assays. Genetic testing — including that for oncology — is reclassified to Class C, which is defined as high patient risk or no-or-moderate public health risk. After a five-year transition period, CE-IVD-cleared genetics assays need to undergo certification under IVDR, as self-certifying is no longer allowed. The IVDR certification process puts an emphasis on external assessment, performance evaluation, and clinical evidence.

CLINICAL GENOMICS ASSAY VALIDATION BEST PRACTICES

As you've read, there are myriad criteria for getting a clinical genomics assay approved. But one common thread is the requirement for a rigorous and comprehensive validation. A main challenge for today's laboratories that are increasingly adopting RUO assays as LDTs under CLIA is the lack of standardized validation guidelines. This can result in uneven application of core validation principles, or differences in the depth and rigor of a given validation from one lab to another. While there are many ways to validate a test, the following best practices can give you a more thorough understanding of your assay, greater confidence in your results, and ease inspections and audits.

Accuracy Studies on Known-Negatives to Determine Background Error Rate for Non-FDA-Approved or Cleared Assays

This type of accuracy study requires at least one, but ideally three well-characterized cell lines or biosynthetic controls (New York State requires three samples). These samples must have known variant negative sites, but may also contain known-positive variants, therefore allowing full concordance to be determined at the assay's desired limit of detection (LOD), which could be down to a 5% allele burden or lower based on exact application. A good source of this type of sample is the Seraseq family of multiplexed biosynthetic reference materials which contain known variants and negative regions in well-characterized GM24385 background. Though not required, validation may be further strengthened with a supplement of 5-15 clinically negative real-world patient samples. These could be blood samples from healthy individuals for liquid tumor (blood cancer) panels, or non-tumor FFPE tissue such as tonsils for solid tumor panels. By processing these samples, the laboratory can gain a level of comfort with variant-calling down to their desired LOD, without false positives.

Practical Application Guide

Application	Use Case	Reference Material	Number of Samples
Somatic cancer	Accuracy study on known negatives to determine background error rate	• Seraseq™ Tumor Mutation DNA Mix v2 AF10 HC (material # 0710-0094) • Seraseq Tri-Level Tumor Mutation Mix v2 HC (material # 0710-0097) • Seraseq ctDNA Mutation Mix v2 AF2% (material # 0710-0139) • Seraseq Inherited Cancer DNA Mix v1 (material # 0730-0003) • Seraseq Myeloid Mutation DNA Mix (material # 0710-0408)	Minimum of one, ideally three

Accuracy Studies on Known-Positives to Determine Sensitivity

While differences in requirements exist depending on the CLIA jurisdiction, New York State requirements provide a thorough framework for developing a validation strategy. Please consult the appropriate regulatory agency at the time of your validation to ensure compliance with the specific requirements or recommendations for your lab or assay.

Instead, using highly characterized biosynthetic reference materials can help you avoid a burdensome search for suitable real-world samples while ensuring a thorough validation.

Non-New York State

If you won't be accepting samples from patients in New York State, then your validation sample set should include a minimum of ten samples for each tissue type being validated (e.g., FFPE, blood, bone marrow). The majority of these samples should have a known mutation determined by previous testing or pre-validation screening and confirmation with an orthogonal method. Ideally, the validation set should consist of more than 20 samples total, unless the assay method and performance are well-characterized and have already been validated for a previous version of the same test, or for a similar test that interrogates different disease targets. Alternatively, samples may be obtained from a "friendly" laboratory that characterized the samples with a validated assay, and is willing to share concordance data as well as attest to the accuracy of the shared results. But keep in mind that these are often difficult, time consuming, and expensive to obtain. Instead, using highly characterized biosynthetic reference materials can help you avoid a burdensome search for suitable real-world samples while ensuring a thorough validation.

Practical Application Guide

Application	Use Case	Reference Material	Number of Samples
Somatic cancer	Accuracy study on known positives to determine sensitivity	• Seraseq Tumor Mutation DNA Mix v2 AF10 HC (material # 0710-0094) • Seraseq Tri-Level Tumor Mutation Mix v2 HC (material # 0710-0097) • Seraseq ctDNA Mutation Mix v2 AF2% (material # 0710-0139) • Seraseq ctDNA Reference Material v2 AF2% (material # 0710-0203)	Minimum of ten, ideally 20 or more positive samples

New York State Initial Validation

As New York State is very clear on its requirements, a minimum of 50 real-world samples across all primary sample types to be tested (e.g., FFPE, bone marrow, blood) are required. In addition, for NGS panels that are intended to be used primarily on formalin-fixed samples, at least 75% of the samples used in the accuracy study must also be formalin-fixed. Since the quality and quantity of DNA can vary significantly between the various formalin-fixed sample sources (e.g., needle biopsies, cell pellets, and resection specimens), the validation sample set is best designed when it includes a wide range of DNA qualities. This ensures that the assay is validated using samples which represent the expected mix of real-world sample types. Reportable variants must be confirmed with an orthogonal method, which may be an alternative NGS method if run in the same lab, or the same method if run by an external lab that is CLIA-certified (the external lab does not need to be New York State-approved, though this is preferred). It is a best practice for the validation samples to include a variety of mutation types across as many genes or targets as possible, with an assortment of allele frequencies.

It is a best practice for the validation samples to include a variety of mutation types across as many genes or targets as possible, with an assortment of allele frequencies.

But finding samples that satisfy all of these recommendations can be a tall order. The most efficient way to evaluate a diverse range of mutation types is to use contrived reference materials — cell-line or biosynthetic — in addition to the 50 real-world specimens. Another consideration when selecting known positives is to identify samples with mutations across a range of allele frequencies. If you can't identify a sufficient number of mutations across the most relevant range for variant allele frequencies (VAFs), real-world sample mixtures may be created. But for greater confidence, biosynthetic reference materials have allelic burdens that are more precisely determined compared to sample admixtures. Biosynthetic reference materials, such as Seraseq clinical genomics products, are highly consistent and available in abundant, sustainable supply, supporting more extensive or repetitive validation studies.

Practical Application Guide

Application	Use Case	Reference Material	Number of samples
Somatic cancer	Accuracy study on known positives to determine sensitivity	Real-world samples	Minimum of 50. *Note: For FFPE applications, at least 75% of samples must also be formalin-fixed.*
Solid tumors	Supplementary studies to assess assay performance across a broad range of mutation types	• Seraseq Tumor Mutation DNA Mix v2 AF10 HC (material # 0710-0094) • Seraseq Tri-Level Tumor Mutation Mix v2 HC (material # 0710-0097)	As needed (beyond the 50 real-world samples). Each sample is highly multiplexed to provide 40 clinically relevant and/or analytically challenging mutations across 28 different oncogenes and tumor suppressors (from 10% down to 4% allele frequency): • 25 SNVs • Eight insertions • Five deletions • Two DNA translocations
Liquid biopsy	Supplementary studies to assess assay performance across a broad range of mutation types	• Seraseq ctDNA Mutation Mix v2 AF2% (material # 0710-0139) • Seraseq ctDNA Mutation Mix v2 AF0.125% (material # 0710-0143) • Seraseq ctDNA Reference Material v2 AF2% (material # 0710-0203) • Seraseq ctDNA Reference Material v2 AF0.125% (material # 0710-0207)	As needed (beyond the 50 real-world samples). Each sample is highly multiplexed to provide 40 clinically relevant and/or analytically challenging mutations across 28 different oncogenes and tumor suppressors (from 2% down to 0.125% allele frequency): • 25 SNVs • Eight insertions • Five deletions • Two DNA translocations
Myeloid cancers	Supplementary studies to assess assay performance across a broad range of mutation types	• Seraseq Myeloid Mutation DNA Mix (material # 0710-0408)	As needed (beyond the 50 real-world samples). Each sample is highly multiplexed to provide 23 clinically relevant and/or analytically challenging mutations across 16 different genes (between 5% and 15% allele frequency): • 13 SNVs • Four insertions • Four deletions • Two FLT3 internal tandem duplications (ITDs)

New York State Full Validation

For full New York State validation, ten different samples for each gene and mutation type must be orthogonally confirmed. Note that a full validation is not required for the initial submission, so long as the other initial requirements are met. New York State doesn't require full validation of every gene for panels with more than 20 targets, just validation of both SNV and INDEL mutation types for those 20 targets (further confirmations are not necessary).

It is worth noting that copy number variations (CNVs) must always be fully validated for each gene. Though ongoing validation is acceptable, this means that ten CNVs must be confirmed for every gene in the panel. For solid tumor tests that assay for dosage, **Seraseq CNV mixes** for breast, lung, and brain cancers can challenge your ability to detect even subtle increases in copy number, while easing the considerable burden of searching for suitable specimens.

Practical Application Guide

Application	Use Case	Reference Material	Number of Samples
Somatic cancer	Analysis of SNV and INDEL detection for full validation	Real-world samples	Ten for each gene and variant type. *Note: For panels with >20 targets, validation not required for every target (limit to 20 targets).*
Somatic Cancer	Analysis of CNVs for full validation	If real-world samples are not available, use the Seraseq Lung and Brain CNV Mix, +12 (material # 0710-0416) or the Seraseq Breast CNV Mix, +12 (material # 0710-0413)	Ten CNVs for every gene. *Note: Each target gene in the Seraseq CNV Mix is offered at +3, +6, and +12 copies (above wildtype of two copies).*

New York State RNA Fusion Assay Accuracy Studies

Unlike assays designed to identify SNV and INDEL mutations, RNA-based fusion assays require each translocation partner to be validated with three samples. Once three rearrangements are identified for each target (such as ALK or ROS1), the gene is considered fully validated and does not require additional confirmation of positive findings. Initial validation of fusion assays is often the most challenging due to the scarcity of real-world samples, as well as the relatively low nucleic acid abundance once samples are identified. Therefore, initial validations can be supplemented with commercial cell lines or biosynthetic reference material samples to ensure confidence in the assay's sensitivity.

Post-validation note: It's critical to include a known-positive in each batch for any NGS test that detects RNA fusions. As difficult as it can be to source enough relevant real-world samples for validation studies, this presents an ongoing problem for routine QC monitoring. Biosynthetic reference materials, such as the Seraseq FFPE Tumor Fusion RNA Reference Material v2 and the Seraseq Fusion RNA Mix v3, are excellent solutions.

Initial validation of fusion assays is often the most challenging due to the scarcity of real-world samples, as well as the relatively low nucleic acid abundance once samples are identified.

Practical Application Guide

Application	Use Case	Reference Material	Number of Samples
RNA sequencing (RNA-seq)	RNA fusion accuracy studies	Real-world samples	Three samples for each translocation partner. *Note: Maximum of three rearrangements required for each target gene.*
RNA-seq (for solid tumors)	Supplementary studies to assess RNA fusion detection (when real-world samples are not available)	Seraseq Fusion RNA Mix v3 (material # 0710-0431)	As needed. Each sample is highly multiplexed to provide 16 clinically relevant RNA fusions: • 12 unique fusion partners • Including a MET exon 14 skipping transcript as well as EGFR variant III
RNA-seq (for myeloid cancers)	Supplementary studies to assess RNA fusion detection (when real-world samples are not available)	Seraseq Myeloid Fusion RNA Mix (material # 0710-0407)	As needed. Each sample is highly multiplexed to provide nine clinically relevant RNA fusions.

Intra-Run Precision Recommendations (Non-New York State) and Requirements (New York State)

A typical validation strategy includes a minimum of three independent batches or runs. One of these must include replicate testing for 3-5 samples. While you're required to run three known-positives with variants near the assay's limit of detection (LOD), it is best practice to include one known negative, one positive contrived sample (biosynthetic reference materials are ideal), and three real-world samples (ideally clinical samples or mixtures of clinical samples). SNV and INDEL variants are treated independently, so three tissue samples containing an SNV and three tissue samples containing an INDEL are required. The best way to meet this requirement is to include samples that have both SNV and INDEL variants near the assay's LOD. Since obtaining a tissue-derived nucleic acid sample with both mutations at or near the LOD is challenging, a single chimeric sample made of an admixture of an SNV-containing sample and an INDEL-containing sample can satisfy the requirement for both SNV and INDEL validation. Creating three mixtures of real-world samples that contain both an INDEL and an SNV at the LOD reduces the total number of samples that need to be run from six to three, saving time and money. While New York State requires the use of three real-world samples, validations in other jurisdictions may proceed with contrived samples (such as Seraseq biosynthetic reference materials) to look at intra-run precision near the assay's LOD. All replicates should leverage different barcodes, which also allows the laboratory to validate the integrity and performance of the molecular barcode sequences.

Inter-Run Reproducibility Recommendations (Non-New York) and Requirements (New York)

Inter-run reproducibility has the same requirements as described in the intra-run precision requirements. It is ideal to identify 3-5 samples for which sufficient nucleic acid is available to perform both intra- and inter-run experiments. In total, five different repeats are required for each of these samples. If possible, having the additional two batches performed by different technologists provides the laboratory the ability to ensure equivalent performance across its staff, as well as provide staff with an opportunity to display competency with the assay.

Practical Application Guide

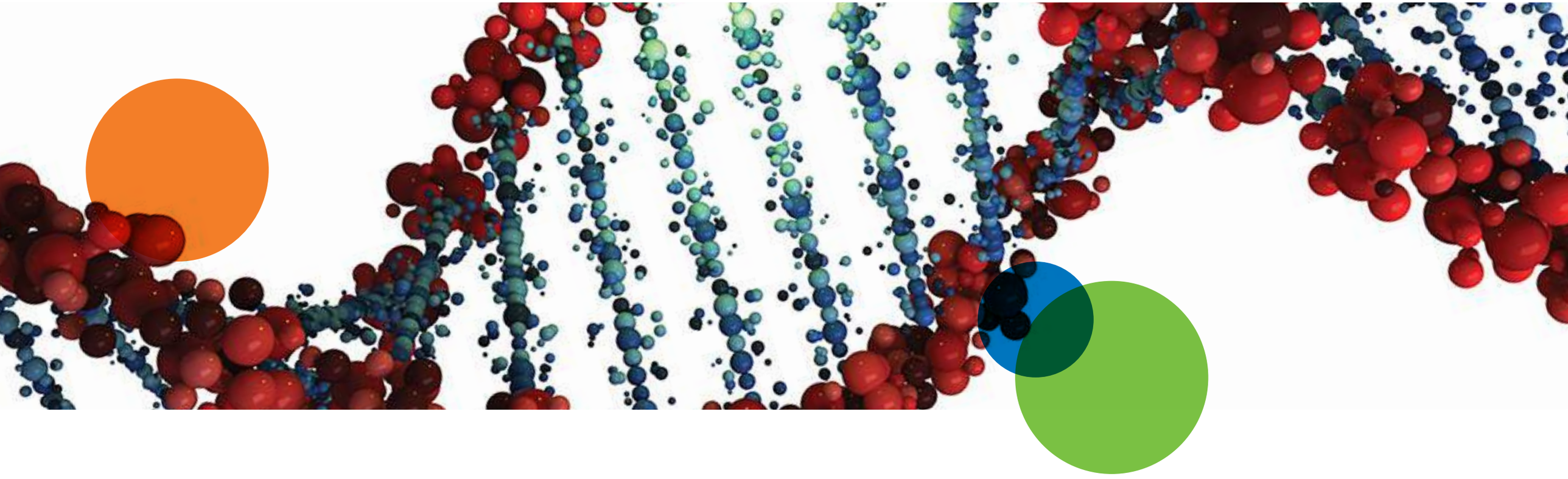
Application	Use Case	Reference Material	Number of Samples

Cancer sequencing	Intra-run precision and inter-run reproducibility	Real-world samples and appropriate Seraseq biosynthetic NGS reference materials based on sample type and tumor location	• Minimum of three independent runs (or batches).
			• At least one of the runs with 3-5 samples.
			• Three positives near assay LOD
			• Best practice:
			  • One negative
			  • One positive (commercial reference material)
			  • Three real-world samples with SNVs
			  • Three real-world samples with INDELs

As you've likely experienced, the primary challenge for reproducibility validation studies is having enough of each sample to perform a sufficient amount of replicate testing. Nothing is more frustrating than finding a sample that is positive for a relevant variant but cannot be tested multiple times due to sample depletion.

Not only do all Seraseq biosynthetic NGS reference materials contain valuable mutations that are highly relevant for precision and reproducibility studies, but these products are a sustainable, abundant source of material that allows for a large amount of replicate testing and long-term quality monitoring.

Nothing is more frustrating than finding a sample that is positive for a relevant variant but cannot be tested multiple times due to sample depletion.



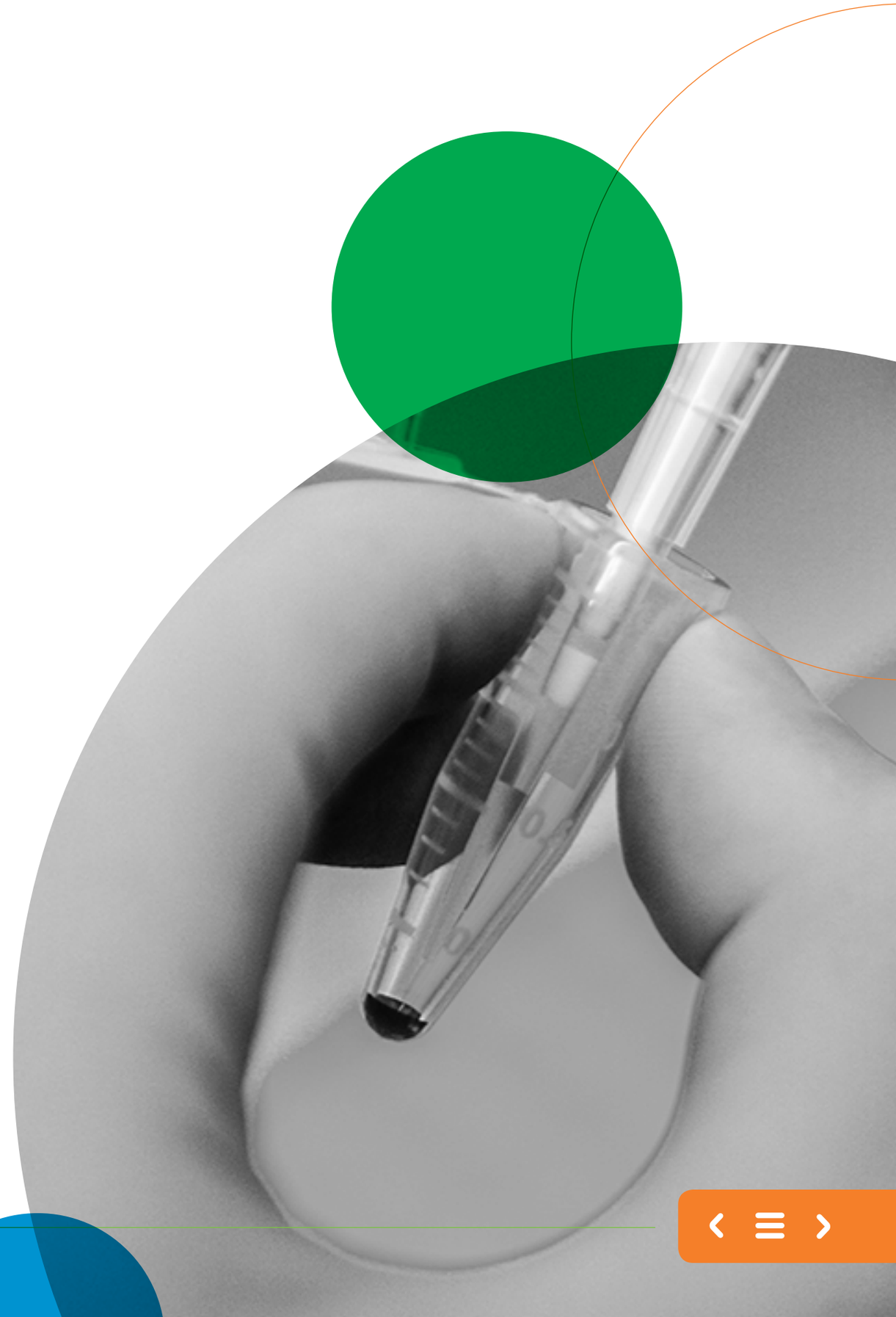
Considerations for Additional Analytical Validation Studies

Sample Input Amount

Most NGS oncology assays are optimized for a specific DNA input amount, but may also be required to work for samples that yield DNA below the nominal level. For non-FDA-regulated assays, rigorous evaluation of the acceptable range of DNA input is not required, but this is still a valuable study to perform for greater confidence across the anticipated range of DNA concentrations for which the validated test will be utilized. One possible DNA input study design involves additional replicates for the precision and reproducibility samples at the lowest possible input amount (i.e., the level below which an unacceptable amount of assay failures, or "no-results" are generated). Running samples with low-frequency variants at the assay's lowest possible DNA input amount allows you to establish sensitivity and specificity at the LOD for low-diversity samples. These metrics are critical to establish since lower DNA amounts represent fewer nuclear equivalents, making sensitivity for low-frequency events mathematically challenging.

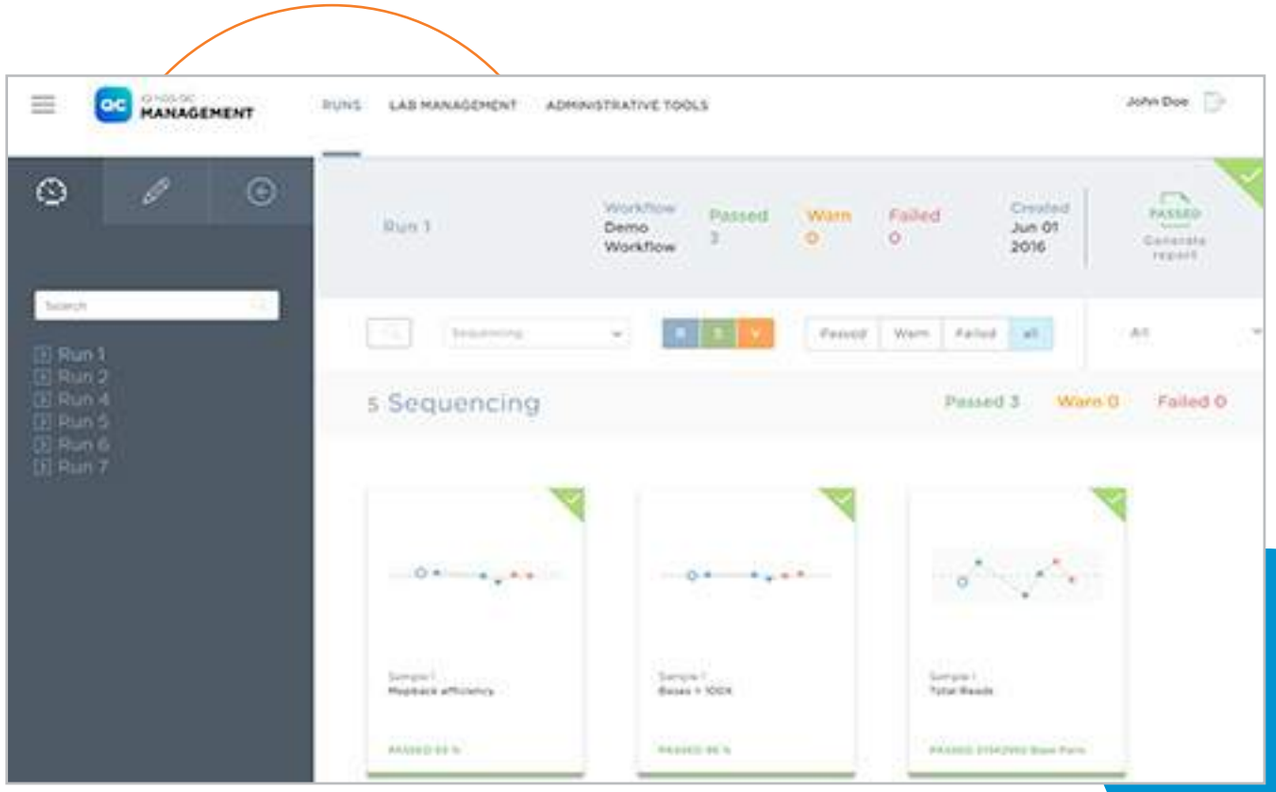
Linear Range and Quantitation Validation

Though the intra- and inter-run experiments described earlier can satisfy the New York State requirement for analytical sensitivity if designed properly, additional titration experiments may be required. By mixing two samples at different molar ratios, you can generate an allele frequency titration curve to measure the precision of allele frequency determination. Since there is no requirement to perform these studies on real-world samples, biosynthetic reference materials such as the Seraseq Tri-Level Tumor Mutation DNA Mix v2 HC (which contains 40 variants across a range of allele frequencies in a single sample) can offer many advantages. One advantage is having a sample with a precisely quantitated allele frequency by which to measure the precision of the NGS methodology.



Calculation of Acceptable Variation

While not required by all regulatory agencies, calculating your assay's acceptable variation is part of a thorough validation. One way to do this is by using known, orthogonally confirmed allele frequencies by performing extensive repeats on the same samples used for the reproducibility and precision studies. These values can establish a baseline for determining acceptance criteria for a known-positive that is run in clinical batches. The best way to calculate variance as it relates to genomic position (gene and location in gene) and mutation type (e.g. SNV, small INDEL, large INDEL) is to use biosynthetic reference materials such as Seraseq clinical genomics products, which contain a diverse set of representative mutations. These biosynthetic materials are abundant and do not change over time, in contrast to residual patient samples. This allows long-term use for ongoing quality monitoring of the assay. Additionally, tools such as SeraCare's iQ™ NGS QC Management software can then be used to easily visualize meaningful QC data to quickly distinguish problematic process drift from the expected day-to-day variation in the assay.



Sample screenshot from the iQ NGS QC Management software showing critical QC information about NGS runs.

Instrument Equivalence

If you plan to run a test on more than one sequencing platform, it is important to validate the assay on each type of instrument. For example, if a test may run on both an Illumina NextSeq® 500 and a MiSeq®, then experimental batches containing the same samples should be sequenced on both instruments during the course of validation. You should then compare the resulting data and measure concordance. Since the NextSeq in this example produces so much more data, you would potentially need to spike-in PhiX Control DNA to ensure the samples are not dramatically over-sequenced relative to the MiSeq run.

If you have multiple instruments of the same type in your lab, you're not required to validate non-FDA-regulated assays on each individual, identical machine, so long as you have an ongoing six-month machine qualification plan, and each instrument is validated prior to its first clinical use. But for a truly thorough validation, it is best practice to run each experimental batch from the various studies detailed earlier on a different machine. That way, any instrument that could be used to run the assay with actual clinical samples is involved in the assay's validation.

FDA-Cleared or Approved NGS Assays

There are currently only two FDA-approved NGS oncology tests on the market that are available for independent labs to operate — Thermo Fisher's Oncomine Dx Target Test, and Illumina's Praxis Expanded RAS Panel (note that the FoundationFocus CDx BRCA FoundationOne CDx™ tests are single-site assays and only performed at Foundation Medicine). Because these are IVD assays, the explicit procedures contained in their package inserts must be followed precisely to stay on-label (all optimization has already been performed by the manufacturer). Labs who bring an FDA-cleared or approved NGS test in-house must confirm that the test is working as expected, but there are no specific guidelines on how to do this from New York State, CAP, or other CLIA agencies. Therefore, by default, validation becomes an equivalency study to demonstrate that the assay performs comparably in the hands of the laboratory compared to the manufacturer who produced the test. At minimum, known positives — which can be comprised of contrived samples, such as Seraseq biosynthetic reference materials, and known-positives derived from the approved tissue type — should be tested. Samples that are known to be negative for the selected targets must also be included. Though not a requirement, many lab directors consider it best practice to perform more extensive validation studies on FDA-cleared or approved panels, similar to the validations performed for LDTs. Note that you are only required to show equivalency of performance of the assay within your laboratory.

Practical Application Guide

Application	Use Case	Reference Material	Number of Samples
Tumor profiling	Thermo Fisher Oncomine Dx Target Test	• Seraseq Tumor Mutation DNA Mix v2 AF10 HC (material # 0710-0094) • Seraseq Tumor Mutation DNA Mix v2 AF10 LC (material # 0710-0074)	Confirm if expected (positive) results are obtained for BRAF V600E, EGFR L858R, and EGFR exon 19 deletions
RNA-seq (fusions)	Thermo Fisher Oncomine Dx Target Test	• Seraseq FFPE Tumor Fusion RNA Reference Material v2 (material # 0710-0129) • Seraseq Fusion RNA Mix v3 (material # 0710-0431)	Help confirm expected (positive) results are obtained for ROS1 fusions
Tumor profiling (RAS)	Illumina Praxis Extended RAS Panel	• Seraseq Tumor Mutation DNA Mix v2 AF7 HC (material # 0710-0095)	Help confirm expected (positive) results are obtained for KRAS G12D and NRAS Q61R

Other Areas to Consider

Determining Minimum Read Threshold

Thoroughly validating your assay allows you to define a minimum total number of reads required to establish a sensitivity threshold for a given LOD (i.e., 500x for 5% allele burden vs. 20x for a 30% allele burden), and determine what the minimum number of reads is to ensure a true negative result. You should also specify how to handle positive variant findings in regions with coverage below a cutoff. It is possible in a methods or disclaimer section to validate certain regions or genes in a panel to a different LOD if coverage is not consistently above a minimum threshold.

Characterizing Sensitivity at Different Allele Frequencies

Properly determining what the lowest limit your assay is validated to with a given sensitivity threshold (i.e. 99% sensitivity to 4%, vs. 80% sensitivity to 1%) assuming a 100% specificity is crucial. Note that you may decide to validate an assay to a very low LOD so long as you also establish the sensitivity to that LOD. If the LOD results in false positives (low specificity), it is possible to use a confirmatory workflow to gain an overall higher specificity for the test (as compared to the NGS assay itself) by reporting only those variants which confirm orthogonally.

Strand Bias Analysis

For amplicon assays with overlapping reads from each paired end, or for capture libraries with small insert sizes, you should establish the criteria for allowed strand bias, especially in homopolymer regions where errors typically accumulate at each side, or a large homopolymer region.

Developing a Best-in-Class QC Program

For positive controls run as part of a global quality-control program, criteria should be set for acceptance and accuracy. A good method to do so is one that's strongly endorsed by New York State in which the lab should run a complex positive control, such as the Seraseq Tumor Mutation DNA Mix v2, in all replicate batches during assay validation, and then include it in production as an ongoing control. From this data, you can establish an acceptable allele frequency variance for each mutation in the material required to pass the control sample, and therefore the entire batch. Additionally, use of a quantitative multiplexed reference material and powerful QC tracking software will permit longitudinal performance tracking, and enable labs to more easily monitor for performance drift over time or across sites. This data will also prove indispensable when preparing for audits and inspections, and should you need to troubleshoot your assay.

CONCLUSIONS – WHAT ARE SOME OF THE THINGS THOROUGH VALIDATION OF YOUR ASSAY ENABLES YOU TO DO?

Though there are some critical standards your test must absolutely meet, there are also many recommended guidelines and best practices you should follow before your assay is ready for patient specimens. But regardless of your location or type of test, the more thoroughly you validate your assay, the more confidence you'll have in your results. And biosynthetic reference materials alleviate many of the most time-consuming and burdensome parts of a thorough validation. Here are the key takeaways.

- Biosynthetic reference materials with a broad mix of clinically relevant variants enable deeper insights into your assay's performance characteristics than real-world specimens alone, or cell-line derived samples.
- Proper and thorough validation studies require extensive repeats which can quickly deplete precious real-world samples. Sustainable and highly consistent biosynthetic reference materials nicely meet this challenge, especially for rare mutations.
- Authoritative bodies worldwide recognize the value of contrived samples such as biosynthetic reference materials for clinical genomics assay validation. Biosynthetic reference materials are highly multiplexed, thoroughly characterized, and extremely patient-like which gives them several advantages over material derived from cell lines. While biosynthetic reference materials are available in a comprehensive array of mutations, mutation types, and sample formats, they can also be quickly and easily customized for individual needs.

As the impact and adoption of NGS-based clinical genomics assays increases, it is critical that these tests be thoroughly validated to ensure the accuracy and consistency of results, which ultimately leads to optimal patient care. SeraCare has the most comprehensive clinical genomics QC solutions available, from precisely quantitated biosynthetic reference materials that can be quickly and easily customized for your specific needs, to intuitive QC metric tracking and reporting software. All designed to help you remove doubt and add confidence in your assay.

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