# Why Your MRD Assay Is Only as Good as the Adapters Behind It

Minimal Residual Disease (MRD) testing is one of the most demanding applications in all of oncology diagnostics. When a patient completes cancer treatment, the question clinicians need answered is precise and unforgiving: are there any cancer cells left, even at concentrations that imaging and clinical assessment cannot detect?

NGS-based MRD testing answers that question — but only if every component in the workflow is operating at the very edge of what the technology can deliver. The sequencing instrument, the bioinformatics pipeline, the library preparation chemistry — all of it matters.

Yet one component is routinely overlooked: the adapters and index primers used to build the sequencing library.

That can be a mistake with real clinical consequences.

## The Stakes in MRD Testing

In MRD testing, clinicians are looking for ctDNA signals that may represent as little as 0.01– 0.1% of circulating cell-free DNA. The analyte is rare. The background noise has to be near zero. Any artefactual signal — whether from cross-contaminated reagents, miscalled index assignments, or PCR duplicates that inflate apparent variant frequencies — does not just reduce assay sensitivity. It can produce false positives that alter treatment decisions.

This is not a context where "good enough" adapter quality is acceptable.

# Cross-Contamination: The Hidden Risk in Co-Synthesised Reagents

Illumina-based MRD libraries require paired adapters and index primers. For multiplexed sequencing, these are almost always co-synthesised by oligo providers in batches – which introduces the possibility of cross-contamination between adjacent oligos during synthesis, purification, and plate handling.

Even at low levels, contamination between index sequences can contribute to index hopping and barcode bleeding; reads assigned to the wrong sample, inflating apparent variant frequencies or – more problematically in MRD – masking true signal with background noise from other samples on the same run.

The threshold that matters here is targeting a cross-contamination rate <0.1% and even <0.01% between co-synthesised adapter and index primer pairs. This is not an arbitrary specification. It sits below the variant allele frequencies that MRD assays are designed to detect, meaning contamination at this level will not artefactually drive a positive call.

Achieving and verifying this specification can be a significant challenge in phosphoramidite synthesis. During oligonucleotide manufacture, contamination can potentially arise from several potential sources, such as; - residual phosphoamidites in synthesis lines, incomplete equipment cleaning, purification carryover, physical handling and dispensing errors.
With dedicated equipment, validated cleaning and operating procedure reputable manufacturers can address these challenges. However, there is also the requirement for oligo providers to consider utilizing stringent QC methods, such as NGS-based QC assays, – not just rely on traditional oligo QC considerations such as absorbance measurements or HPLC traces.

By sequencing the product directly during the oligo production cycle, it can be confirmed that contaminating sequences from neighbouring synthesis reactions are absent at the frequencies that matter for MRD applications and provide meaningful reassurance to users.

Oligo suppliers who rely on conventional QC techniques alone cannot provide this assurance.

# Index Design: Why longer UDIs Are the Right Choice

Unique Dual Indexing (UDI) is now the standard for any application where sample misassignment carries significant risk. The use of UDI means every sample carries a combination of two independent indexes – i5 and i7 – and reads are only assigned to a sample if both indexes match. A single index hop, by definition, cannot reassign a read.

For MRD, UDI should not be considered optional.

But the length of those indexes also matters. Longer indexes reduce the probability of index collision (it increases the number of unique index values that can be generated exponentially with each additional base) and improve demultiplexing accuracy when running large sample batches – which is the norm in clinical and CRO settings where MRD testing is deployed.

A 10-base UDI perhaps represents the optimal balance for Illumina sequencing workflows:

- Long enough to provide collision-free demultiplexing across hundreds of samples per run
- Compatible with standard Illumina read configurations without sacrificing per sample sequencing depth
- Designed with sufficient edit distance between indexes to tolerate single-base sequencing errors without misassignment

Careful index design also means avoiding sequences with homopolymer runs, secondary structure potential, or GC content extremes that compromise cluster generation and basecalling accuracy. Poorly designed indexes introduce systematic errors at the library preparation stage that no downstream bioinformatics correction can fully recover.

## UMIs: Separating True Variants from PCR Noise

Unique Molecular Identifiers (UMIs) are short, random sequences incorporated into the adapter at ligation. Each original ctDNA molecule receives a unique UMI tag before PCR amplification. After sequencing, reads sharing the same UMI can be collapsed into a single consensus sequence — effectively erasing the duplication introduced by PCR.

In MRD testing, this matters for two reasons.

First, PCR amplification is inherently error prone. Polymerase induced substitution errors at low frequency positions are indistinguishable from true somatic variants unless read families are consensus-called. Without UMIs, the effective sensitivity floor of an NGS MRD assay is limited by PCR fidelity, not sequencing chemistry.

Second, quantitative accuracy suffers without UMIs. If a ctDNA molecule with a pathogenic variant is amplified 20 times and a molecule without it is amplified 10 times, the apparent variant allele frequency is inflated. UMI-based deduplication restores the true molecular ratio — which is the number clinicians need for accurate MRD quantification and longitudinal monitoring.

Well-designed UMI sequences are randomised enough to distinguish independent molecules, but short enough not to consume excessive sequencing bases. They are typically 8–12 bases, incorporated inline in the adapter sequence so that they do not compromise the adapter's hybridisation performance or ligation efficiency.

# How Biolegio Rebuilt Its NGS Pipeline to Meet This Standard

Specifying < 0.01% cross-contamination is straightforward. Reliably achieving and verifying it across every production batch is a different challenge entirely — one that requires a systematic understanding of where contamination can and does originate in an oligonucleotide synthesis workflow.

Over the last several years, Biolegio has undertaken a comprehensive rebuild of its NGS oligo synthesis pipeline, driven directly by customer collaborations and

feedback. As NGS-based MRD testing moved from research into clinical and diagnostic settings, customers came back to us with a consistent message: they needed not just a specification, but evidence – batch level NGS QC data demonstrating that the product they were receiving could not introduce contamination at frequencies relevant to MRD detection.

Meeting that requirement meant going further than adjusting a single step. It meant mapping the entire production process and asking, at every stage, where cross contamination risk existed and what could be done to eliminate or control it.

The answer was that risk was present across the pipeline – not concentrated in one place.

Starting with on-platform synthesis, we examined how co-synthesised sequences in adjacent wells could generate contamination through reagent carryover, aerosol generation during dispensing cycles, and instrument-level crosstalk. Synthesis parameters were reviewed and optimised to minimise these events, and platform configurations were assessed for their contribution to inter-well contamination profiles.

Operator handling was identified as a significant and underappreciated risk factor. Manual steps in high-throughput NGS oligo production – transfers, plate moves, capping and sealing operations – each represent an opportunity for low-level mixing of adjacent samples. We mapped these touchpoints systematically and implemented handling protocols and physical controls specifically designed to reduce contamination risk at each one, drawing on our ISO 13485 quality management framework to ensure these controls were documented, validated, and consistently applied.

Post-synthesis processing and purification introduced its own contamination vectors.

Desalting, precipitation, and purification steps each involve liquid handling where carry across of low-level contaminants is possible. We reviewed and redesigned these workflows with cross-contamination as an explicit design criteria – not an afterthought.

Dispensing – the final step before a product leaves the facility – was treated with the same rigour. Reformatting from synthesis plates to customer-format plates using automated liquid handling systems introduces contamination risk through tip

use, aerosol, and positional errors. Dispensing protocols were validated specifically against cross-contamination metrics, not just volumetric accuracy.

What this risk mapping revealed was that process optimisation alone would not be sufficient. Some of the contamination hotspots identified could not be adequately controlled within the constraints of existing equipment and workflows. Addressing them properly required a more fundamental commitment: new standard operating procedures, new instrumentation, and significant capital investment in automation.

New SOPs were developed from the ground up for the NGS adapter pipeline — not adapted from existing oligo synthesis procedures but written specifically around the contamination risk profile of co-synthesised adapter and index primer sets. These SOPs codified not just what to do, but the contamination rationale behind each step, ensuring that operators understood the consequence of deviations and that the procedures remained fit for purpose as the pipeline evolved.

Where the risk mapping identified synthesis as a root-cause hotspot, Biolegio went further still: designing and building new synthesisers configured specifically for NGS oligo production. Rather than working around the contamination characteristics of existing platforms, the approach was to engineer the instrumentation to the contamination specification — with fluidic architectures, well geometries, and reagent delivery systems designed with inter-well cross-contamination as a primary engineering constraint.

Automation and liquid handling received significant investment across multiple stages of the pipeline. Automated platforms were introduced to replace manual steps identified as contamination risks, including post-synthesis processing, normalisation, and plate reformatting for dispensing. Automation reduces operator-to-operator variability, eliminates the handling errors that manual pipetting introduces at scale, and — critically— allows contamination-relevant parameters to be monitored and controlled with a consistency that manual workflows cannot match.

None of this infrastructure would be meaningful without a way to measure its performance with the sensitivity the application demands. Biolegio designed a dedicated NGS QC assay and accompanying bioinformatics analysis pipeline specifically for cross-contamination quantification in adapter and index primer products. This is not a repurposed research assay — it was built to detect contaminating sequences at sub-0.01% frequencies, with the analytical sensitivity,

specificity, and data interpretation framework needed to generate a binary release decision for every production batch.

The QC pipeline produces sequence-level contamination data across all index

combinations present in a synthesised set – identifying not just whether contamination is present, but precisely which well-to-well transfers are responsible when it is. That resolution is what makes the data actionable: it feeds directly back into process improvement, closing the loop between measurement and manufacturing.

Critically, none of these process changes were accepted on the basis of theoretical risk reduction alone. Each was validated by sequencing, and the NGS QC assay remains the final arbiter of batch release. That closed loop – process change, NGS verification, feedback into further optimisation – is what allowed us to drive cross-contamination rates to levels that are genuinely fit for MRD applications.

The outcome is a synthesis pipeline where cross-contamination control is not a single quality checkpoint at the end of production, but a designed property of every step within it– backed by the capital investment, procedural rigour, and measurement infrastructure to prove it.

## Why Adapter Quality Is a Clinical Variable

Put these three requirements together and the picture is clear. In NGS-based MRD testing:

• Cross-contamination < 0.01%, verified by NGS QC, ensures that no artefactual signal migrates between co-synthesised index pairs and confounds variant calling.

• 10-base UDIs, carefully designed with optimal edit distances, ensure that every read is assigned to the correct sample – even in large multiplexed clinical runs.

• UMI incorporation ensures that variant frequencies reflect true molecular ratios, not PCR amplification artefacts.

Each of these is a specification that must be built into the adapter design and synthesis process from the start. They cannot be retrofitted through bioinformatics alone.

Clinicians and laboratory directors evaluating NGS-based MRD platforms rightly focus on sensitivity, analytical validation, and clinical utility data. But the adapter reagents sitting upstream of all of that data are a fundamental determinant of whether the assay can achieve its stated performance. Specifying adapters and index primers to clinical-grade standards — and insisting on NGS-based QC data to back those specifications — is not over-engineering. It is basic assay integrity.

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*Biolegio's LegiSeq NGS Ready-to-Use Oligos are designed and manufactured to meet exactly these requirements — including NGS-verified cross-contamination rates <0.01% for every batch, optimally designed 10-base UDIs, and UMI-incorporated adapter architectures. Manufactured under ISO 13485, every product leaves our facility with the quality documentation your workflow demands.*