

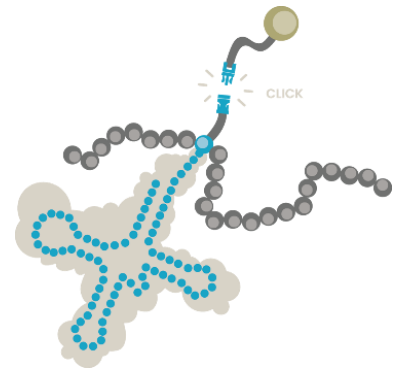
Scalable genetic code expansion platform for multiplex, site-specific protein labelling

White paper



Executive Summary

Multiplex immunofluorescence (mIF) is transforming tissue-based research and diagnostics by enabling simultaneous visualization of multiple biomarkers. However, traditional labeling methods often produce inconsistent results, limiting reproducibility and scalability (Sorelle *et al.*, 2019, Harms *et al.*, 2023).



enGenes and arcoris bio have partnered to address this challenge by combining genetic precision with modular tagging. Using enGenes' **e^xpand technology**, non-canonical amino acids (ncAAs) can be incorporated site-specifically into proteins, creating “ready-to-click” reagents that support flexible, reproducible labeling. These proteins are then paired with **arcoris bio's MUSE® multiplex signal amplification technology**, enabling reliable, high-quality multiplex immunohistochemistry (IHC).

This collaboration allows researchers to:

- Precisely control labeling sites and stoichiometry
- Maintain flexibility in staining strategies and detection chemistries
- Improve reproducibility and signal consistency
- Scale production for industrial and diagnostic applications

Using well-established biomarkers such as TNF- α and HER2, the combined enGenes–arcoris bio approach demonstrates how optimized labeling can significantly enhance multiplex IHC performance, helping customers achieve more reliable, multiplex-ready tissue analysis.

The mIF has become a cornerstone technology for spatial biology, translational research, and precision diagnostics (McGinnis *et al.*, 2021). The ability to visualize multiple biomarkers simultaneously within intact tissue sections is essential for understanding disease heterogeneity, immune contexture, and therapeutic response. However, the performance of mIF assays is fundamentally constrained by the way antibodies and targeting proteins are labelled.

Introduction

The site-specific attachment of functional payloads (e.g., fluorophores, affinity tags, drugs, linkers, or other proteins) is becoming foundational for next-generation diagnostics, imaging, targeted delivery, and protein therapeutics. However, today's dominant bioconjugation workflows—especially for antibodies—often rely on random lysine/cysteine chemistry, leading to heterogeneous products (variable drug-to-antibody ratios, inconsistent labelling sites), a higher development burden, and complex analytics. Site-specific antibody conjugation solutions exist, but many remain costly, workflow-

heavy, or difficult to scale reliably across different proteins and payload combinations (Fan *et al.*, 2025; Junutula *et al.*, 2008).

enGenes-e^xpand addresses this gap by enabling high-yield, high-fidelity incorporation of non-canonical amino acids (ncAAs) at defined positions in recombinant proteins via amber codon suppression, combined with a production concept that decouples recombinant protein production from cell growth, thereby improving yield and consistency relative to conventional *E. coli* expression. The outcome is a “ready-to-click” protein that supports plug-and-play bioorthogonal conjugation under physiological conditions—making modular payload attachment practical at scale (Casas *et al.*, 2020; Wiltshi *et al.*, 2016; Danielewicz *et al.*, 2023). Existing site-specific conjugation strategies offer partial solutions, but each carries significant trade-offs in terms of workflow complexity, protein specificity, or scalability (**Table 1**). None combines precise handle placement with high yield — the gap that the enGenes is designed to address.

Table 1. Conjugation strategies comparison.

Approach			Strengths	Key limitations
Random labeling	lysine/cysteine		Simple, low cost, familiar	Heterogeneous products, poor multiplex control
Engineered	cysteines	(e.g. THIOMAB-like)	Improved site control	Protein-specific engineering, disulfide complexity
Enzyme-mediated conjugation			Site-directed	Extra steps, substrate constraints, scale variability
Glycan-based conjugation	antibody		Fc-specific precision	Antibody-only, complex processing
Genetic	Code	Expansion (classic)	Precise handle placement	Historically limited yields, scale challenges

To further address the specific requirements of multiplex immunofluorescence, the e^xpand technology has been combined with MUSE®, a multiplex and multimodal signal amplification technology developed by **arcoris bio that requires oligo barcodes on detection proteins**. In this collaborative approach, enGenes enables the site-specific incorporation of chemical handles into recombinant proteins, while arcoris bio applies the MUSE® as a standardized interface for downstream labeling and detection in IHC workflows (**Figure 1**). This combination allows precise control over labeling site and stoichiometry, while maintaining flexibility in the choice of detection chemistry and staining strategy.

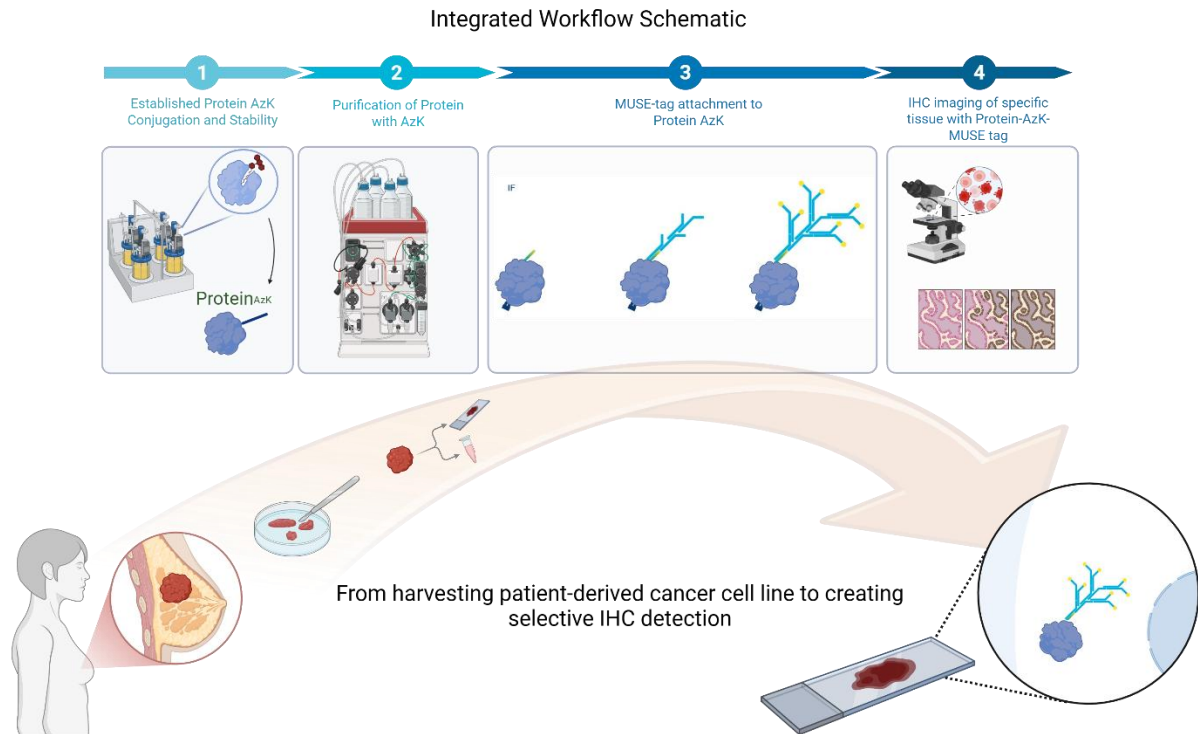
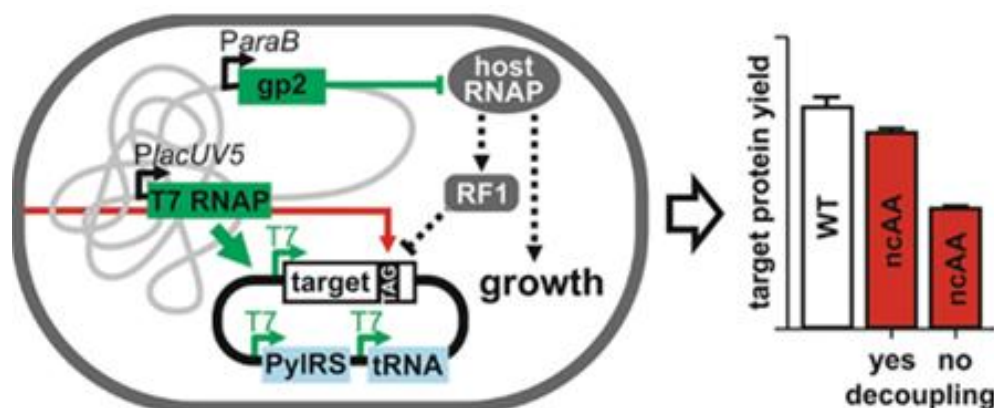


Figure 1. This pipeline describes the site-specific incorporation of azidolysine (AzK) into a probe-protein of interest, enabling precise bioorthogonal conjugation. Following expression, the Protein-AzK is purified by chromatography and functionalised with a MUSE[®]-activator via strain-promoted azide-alkyne cycloaddition (SPAAC). The resulting Protein-AzK-MUSE[®] conjugate is employed as a probe for tissue immunofluorescence with MUSE[®] signal amplification, generating spatially resolved, potentially-multiplexed protein expression maps at single-cell resolution. Results feed back into the production and workflow, supporting iterative optimization of probes for biomarker discovery and the development of targeted diagnostic or therapeutic IHC applications. (Image generated with Biorender).

Importantly, the enGenes–arcoris bio collaboration extends beyond reagent design to functional validation. The **arcoris bio performed in-vitro analyses of MUSE[®] constructs for tissue immunofluorescence stainings**, demonstrating that site-specific labeling enabled by e^xpand is compatible with downstream tissue imaging applications. This integrated workflow illustrates how genetically encoded chemical precision and modular tagging can jointly improve reproducibility and multiplex compatibility in tissue-based assays. In this white paper, **TNF- α (tumour necrosis factor alpha) and HER2 (human epidermal growth factor receptor 2) are used as model targets** to illustrate how improved labeling strategies facilitate the production of antibody alternatives for multiplex immunofluorescence. These targets were selected because they are well validated, widely used in tissue analysis, and representative of inflammatory and oncological biomarker panels. The intent is not to replace antibodies, but to enable **alternative, multiplex-ready labeling reagents** that build on established binders and are compatible with industrial production and diagnostic workflows.

enGenes-e^xpend - site-specific incorporation of ncAAs

The use of ncAAs in protein engineering has been a significant area of research, with applications in various fields. Galindo *et al.*, 2020 and Wiltschi *et al.*, 2016 both highlight the potential of ncAAs, which can be incorporated into proteins using amber codon suppression. This technique allows for the site-specific integration of ncAAs, enabling the addition of unique chemical functionalities to proteins. This approach uses an amber suppressor tRNA_{CUA} to read the amber codon and an aminoacyl-tRNA synthetase to charge it with the ncAA (**Figure 2**). A major drawback is the low yield of mutant proteins compared to the wild type, due to competition from the release factor. Efforts to improve ncAA incorporation efficiency have had moderate success. In search of increased efficiency for scalable industrial applications, the enGenes-e^xpress technology was introduced, which decouples recombinant protein production from cell growth and significantly increases ncAA incorporation and protein yields compared to conventional *E. coli* BL21(DE3). The target proteins were expressed at high levels with excellent fidelity and preserved function (Galindo *et al.*, 2020). The resulting modified proteins can then be used in a range of applications, including drug delivery (Danielewicz *et al.*, 2023) and structural biology studies (Tyagi *et al.*, 2015). These studies collectively underscore the versatility and potential of precision protein engineering using amber codon suppression and the ncAAs.



Read
more
at

<https://www.engenes.cc/post/precision-protein-engineering-using-non-canonical-amino-acid-incorporation>.

Figure 2 provides an overview of the noncanonical amino acids (ncAAs) and the genetic components used in the study. It shows the chemical structures of pyrrolysine (Pyl) and tested Pyl-derived ncAAs, and summarizes the expression/plasmid design for amber (TAG) suppression in *E. coli*—i.e., an orthogonal tRNA_{CUA} / aminoacyl-tRNA synthetase system paired with model protein expression constructs. Overall, the figure sets up how the authors generate “ncAA-ready” expression systems before evaluating production performance in later figures.

arcoris bio’s MUSE® as a modular technology for high-sensitivity multiplexing

Modern multiplex immunofluorescence increasingly depends on reagent architectures that are not only specific but also modular and standardized. As staining panels grow in size and complexity, the bottleneck often shifts from binder discovery to practical considerations: how reliably a reagent can be labeled, how reproducibly signal output can be tuned across lots, and how quickly new markers can be added to an existing panel without redesigning the entire workflow.

MUSE®, developed by **arcoris bio**, addresses this need by providing a multiplex and modular signal amplification technology intended to support consistent downstream functionalization. Rather than relying exclusively on direct antibody labeling—where conjugation density and attachment sites are often variable—the use of a dedicated site-specific MUSE®-activator oligonucleotide can create a more controlled “interface” for subsequent reagent enhancement. In multiplex IF workflows, this can translate into more predictable assay development because detection chemistry can be applied in a standardized manner, reducing the iterative optimization typically required when every binder is labeled independently through random chemistry.

Within the framework of this white paper, TNF- α and HER2 are used as model targets to illustrate how improved reagent architecture and labeling strategy can elevate multiplex performance (**Figure 3**). MUSE® activated constructs provide a practical example of how standardization at the design level can support scalable assay development. In particular, when combined with site-specific chemical handle installation via genetic code expansion, an activator-tag-based approach aligns well with a two-stage workflow: (i) produce a well-defined recombinant binder intermediate, and (ii) apply modular labeling or functionalization in a controlled, reproducible downstream step.

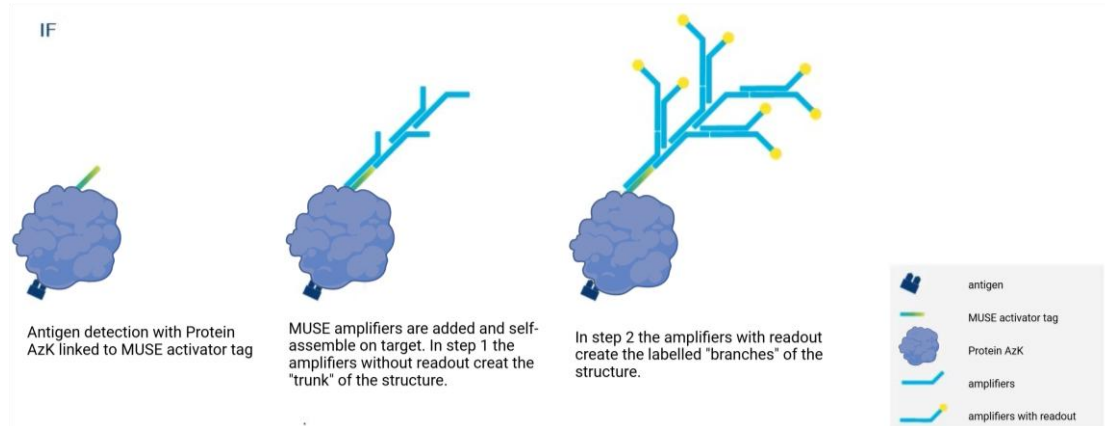


Figure 3. MUSE® technology schematics- Multiplex Universal Signal Enhancement (Image generated with Biorender).

From an industry perspective, the value of modular labeling concepts such as MUSE® lies in operational advantages that matter for multiplex tissue assays: reproducibility, comparability across batches, and the ability to adapt labeling strategy without repeatedly re-engineering the binder itself. As multiplex immunohistochemistry advances toward higher plex levels and more quantitative interpretation, reagent platforms that enable repeatable assembly of labeled binders will be increasingly important for both research and translational settings.

Functional validation of site-specific labeling for multiplex immunohistochemistry

To move from concept to evidence, we applied this integrated approach to two clinically relevant biomarkers: TNF- α and HER2. The following section presents functional validation of MUSE®-activated constructs produced via e^xpand, demonstrating that site-specific labeling preserves target recognition and enables high-contrast detection in FFPE (Formalin-Fixed Paraffin-Embedded) tissue under standard IHC conditions. Both targets were selected because they represent demanding and widely used benchmarks, making them a stringent test of the platform's practical utility.

To enable TNF- α detection, an anti-TNF- α antigen-binding fragment (Fab) was produced following the approach described by Hanaee-Ahvaz et al. (2024). The purified Fab contained a site-specifically incorporated non-canonical amino acid at position 133. The selected ncAA was *N* ϵ -((2-azidoethoxy)carbonyl)-L-lysine (AzK), introduced via an orthogonal pyrrolysyl-tRNA synthetase/suppressor tRNA^{Pyl}/_{CUA} pair derived from *Methanosarcina mazei*. Anti-TNF- α Fab was attached to the M8 activator.

Figure 4 shows an immunofluorescence staining of a human tonsil FFPE section, highlighting the spatially resolved detection of TNF- α using an e^xpand-engineered anti-TNF- α Fab fragment conjugated a MUSE® activator and with MUSE® signal amplification.

Tonsil tissue represents a highly relevant model for inflammatory marker detection due to its dense and heterogeneous immune cell composition, providing a stringent test for specificity and signal-to-noise performance.

In the upper panels, staining with anti-TNF- α Fab-MUSE®-ATTO647 reveals discrete TNF- α -positive signals (**red**) distributed throughout the tissue, predominantly localized within immune-cell-rich regions. These signals appear as punctate and cell-associated fluorescence, consistent with localized TNF- α expression rather than diffuse background staining. DAPI nuclear staining (**blue**) clearly delineates tissue architecture and cell density, while the autofluorescence channel (**green**) highlights intrinsic tissue fluorescence, allowing visual assessment of signal separation and spectral specificity.

Importantly, the TNF- α -specific signal shows minimal overlap with tissue autofluorescence, demonstrating effective spectral discrimination and confirming that the observed red signal is not driven by endogenous background. The clear contrast between TNF- α -positive regions and surrounding tissue underscores the benefit of defined, site-specific labeling in complex FFPE samples.

The lower panels show the negative control, in which tissue sections were incubated with M8 reagents in the absence of MUSE®-tagged TNF- α -specific Fab. Under identical imaging conditions, no detectable red signal is observed, and only nuclear staining and autofluorescence remain visible. This absence of nonspecific ATTO™ 647N signal confirms that the staining observed in the upper panels arises from specific TNF- α recognition rather than from nonspecific binding of the fluorophore, MUSE® activator, or protein scaffold.

Together, these images demonstrate that the site-specific ncAA incorporation and subsequent MUSE®-activator-mediated labeling preserve antigen recognition and enable high-contrast, specific detection of TNF- α in FFPE tissue. The clear separation of TNF- α signal from autofluorescence and the clean negative control highlight the suitability of this approach for multiplex immunofluorescence.

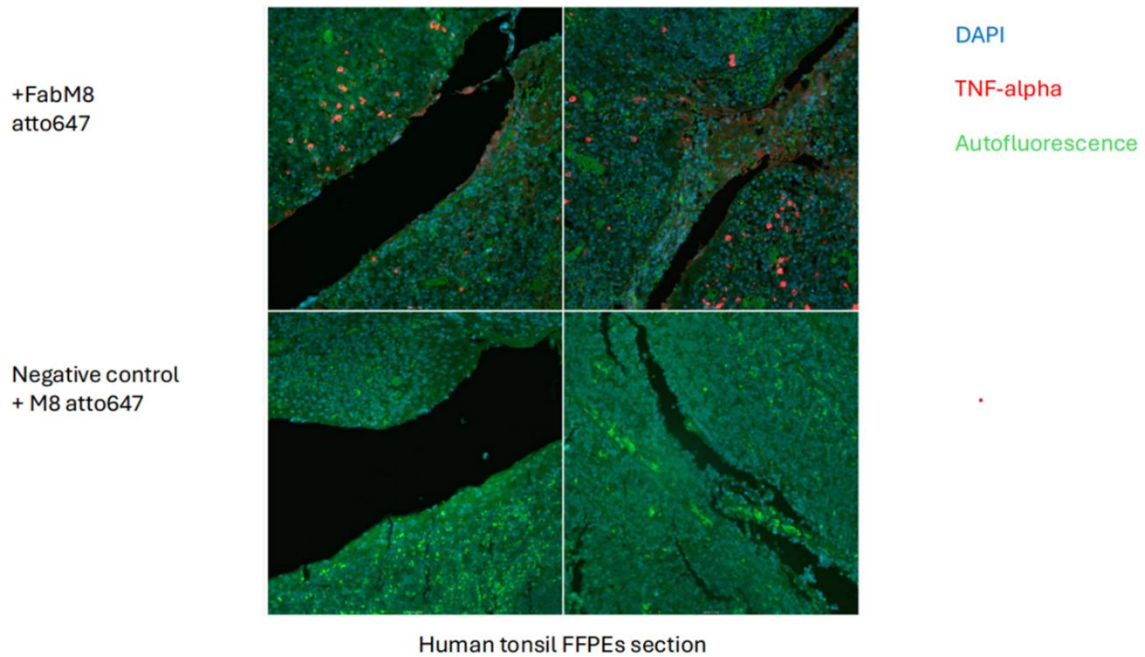


Figure 4. Multiplex immunofluorescence staining of human tonsil FFPE sections using e^xpress-engineered anti-TNF- α Fab conjugated to ATTO™ 647N shows specific TNF- α signal (red) with minimal autofluorescence and no signal in the negative control.

To enable HER2 detection, an anti-HER2 Designed Ankyrin Repeat Protein (DARPin), designated **H10-2-G3**, was produced as previously described (Epa *et al.*, 2013, Plückthun *et al.*, 2015). DARPins are engineered, non-antibody binding proteins characterized by high affinity and specificity, making them suitable for therapeutic, diagnostic, and research applications. The anti-HER2 DARPin was expressed in the enGenes **e^xpress** strain using established protocols (Casas *et al.*, 2020; Stargardt *et al.*, 2020), DARPin selection and ncAA positioning were performed according to enGenes' proprietary optimization framework, details of which are available to partners under a confidentiality agreement. Anti-HER 2 DARPin was attached to M7

Figure 5 shows immunofluorescence staining of human tonsil FFPE sections using the DARPin H10-2-G3 variant site-specifically conjugated to the M7-activator via ncAA-enabled click chemistry. The experiment was designed to functionally validate both DARPin binding activity after conjugation and compatibility of the MUSE®-tagged construct with immunofluorescence workflows under different antigen retrieval and protease treatment conditions.

Images were acquired at 20 $\times$ magnification using a Zeiss LSM 900. The upper row represents the negative, no-DARPin conjugate control in the presence of M7 reagents,

while the lower row shows sections stained with DARPin_ncAA–MUSE®-tagged conjugates.

In the no-DARPin control, no specific fluorescence signal is observed across all conditions, confirming the absence of nonspecific binding from detection reagents or tissue autofluorescence under the applied imaging settings. Tissue architecture remains visible, but no structured or target-associated signal is detected. In contrast, sections incubated with DARPin H10-2-G3–MUSE®-activator display distinct and spatially organized fluorescence patterns, indicating specific tissue binding.

The clear contrast between DARPin-treated samples and the no-DARPin control demonstrates that site-specific ncAA incorporation and MUSE®-activator conjugation do not impair the binding functionality of DARPin H10-2-G3.

Overall, these results confirm that MUSE®-activator–conjugated DARPINS produced via e^xpand-enabled ncAA incorporation are compatible with standard FFPE IF workflows and retain functional target recognition in complex human tissue. This supports their suitability as defined, modular alternatives to antibodies for multiplex and spatially resolved immunofluorescence applications.

E1.89: Conjugation of DARPin variants with MUSE-M7 + IF staining for functional verification 2
Human Tonsil
20X images, Zeiss LSM 900

15min HIER + 5min ProK at RT

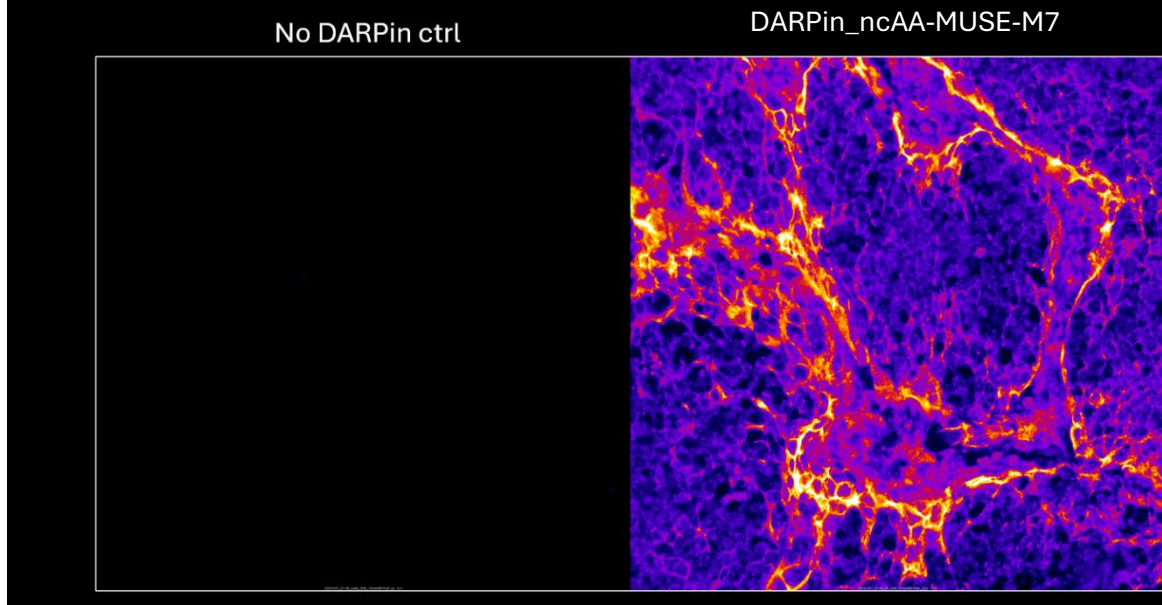


Figure 5. Immunofluorescence staining of human tonsil FFPE sections using DARPin H10-2-G3 conjugated to M7 (MUSE®-activator).

e^xpand: shifting precision upstream into protein expression

In proof-of-concept work, AzK (*azido-lysine*) was incorporated site-specifically into a recombinant protein (e.g., DARPins or Fab), followed by overnight DBCO-based click conjugation to a **MUSE®-activator oligonucleotide** (in phosphate saline buffer), while maintaining target recognition and demonstrating functional cell-based activity. This technology requires no extensive conjugation protocol, with a convenient reaction in physiological conditions.

The e^xpand technology takes a fundamentally different approach by embedding chemical precision directly into the protein during expression. Using genetic code expansion, specific amber codons are introduced at predefined positions within the protein sequence. During expression, these codons are selectively decoded with a non-canonical amino acid bearing a bioorthogonal functional group, such as an azide.

The resulting protein is expressed and purified as a full-length, functionally intact molecule that already contains a defined chemical handle. This “ready-to-click” intermediate can then be functionalized post-expression using standard click chemistry, without the need for further protein engineering or extensive conjugation optimization.

Crucially, the e^xpand platform is coupled to a production strategy designed to overcome historical yield and scalability limitations of genetic code expansion, enabling yields suitable for industrial applications and supporting target cost levels ~€1.5 per µg, in comparison to standard antibody models reaching more than €5 per µg. Internal production cost modelling based on published and internal yield data (Casas *et al.*, 2020; Hanaee-Ahvaz *et al.*, 2024) confirms the feasibility of these cost targets and validates the commercial viability of the platform at scale. Beyond pricing, the e^xpand platform addresses a massive hidden cost: antibody irreproducibility. Recent estimates suggest **\$350 million to \$1 billion** is wasted annually on unreliable antibodies (Bradbury & Plückthun, Nature 2015). Random lysine-conjugated antibodies can produce an estimated 4.5 million unique molecular species per batch (mAbs 2019), making true lot-to-lot consistency impossible. Site-specific ncAA incorporation at genetically defined positions eliminates this stochastic variability, providing absolute reproducibility between production runs—a critical advantage for diagnostic and translational applications where regulatory scrutiny demands batch consistency

For industry partners, the promise is straightforward:

- **Conjugation readiness:** encode defined chemical handles 1–N payloads with standardized chemistry.
- **Cleaner product definition:** reproducible conjugation sites and analytics compared with random labeling.
- **Economic scalability:** designed for industrial production goals (indicated an internal target cost level of ~€1.5 per 1 µg).
- **Broad applicability:** approach extends naturally to **antibody fragments, DARPins/affibodies, cytokines, enzymes, lectins, and cell-targeting proteins.**

Conclusions

The combination of enGenes-e^xpand genetic code expansion with arcoris bio's MUSE® platform represents a new paradigm for multiplex immunohistochemistry reagent development. By embedding chemical precision directly into protein expression, this integrated approach addresses three critical limitations of conventional workflows:

- **Reproducibility:** Site-specific ncAA incorporation eliminates the stochastic variability inherent in random chemical conjugation, enabling absolute batch-to-batch consistency.
- **Modularity:** the MUSE® technology supports standardized downstream functionalization, allowing detection chemistry to be optimized independently of binder engineering.
- **Scalability:** Growth-decoupled *E. coli* expression achieves ncAA incorporation yields approaching wild-type levels, enabling industrial-scale production at target costs significantly below conventional antibody conjugates.

The functional validation presented here—demonstrating specific, high-contrast detection of TNF- α and HER2 in FFPE tissue—confirms that site-specific labeling preserves target recognition while delivering the reproducibility and signal consistency demanded by quantitative multiplex applications.

Partnership Opportunities

enGenes Biotech and arcoris bio invite inquiries from diagnostic developers, spatial biology platform providers, and pharmaceutical companies seeking defined, multiplex-ready detection reagents. Custom construct development, technology licensing, and collaborative R&D partnerships are available.

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