Anti c-myc-Tag: Hyper-Myc

Rabbit, mouse and human monoclonals with better affinity and stability

Monoclonal recombinant antibody Hyper-Myc is an improved engineered anti-myc tag antibody. It showed a higher affinity and stability in direct comparison to hybridoma antibody 9E10 (anti-c-myc-tag) that shares the same epitope. It is further thoroughly characterised by cross reactivity profiling on 28895 human epitopes showing higher specificity and increased monovalent affinity (KD= 17 nM). Further, it provides the additional choice to be detected with either anti-rabbit Fc, anti-human Fc or anti-Mouse Fc secondary antibodies (see products ABC0501, ABC0502 and ABC0503).

The myc-tag

Since the first revelation of the epitope of hybridoma clone Myc1-9E10[1] recognising the human proto-oncogene c-Myc, the ten amino acid epitope peptide EQKLISEEDL, named „c-Myc tag" or „myc-Tag" became famous for his universal applicability to tag all kinds of fusion proteins. It can be be placed at the N- or C-terminus[2] and excels by providing very low off-target reactions with a wide variety of biologic samples. Therefore, in the last two decades, it became an indispensable tool for a vast variety of applications, from simple verification of successful production of a recombinant protein to immunoprecipitation analysis and *in vivo* protein tagging.

The mode of binding of the antibody to its antigen was elucidated by X ray crystallography (PDB file: 2OR9) and revealed a very interesting and unusual structure of the paratope, i.e. the pocket of the antibody molecule that provides the binding to the antigen. Two antibodies were found in the crystal to contact the epitope peptide from two different sides. Also, the loop structure of the paratope is unusual, in respect that the

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[1] Evan GI et al. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. Molecular and Cellular Biology 5, 3610-3616

[2] Manstein DJ et al. (1995) Cloning vectors for the production of proteins in Dictyostelium discoideum. Gene 162:129-134
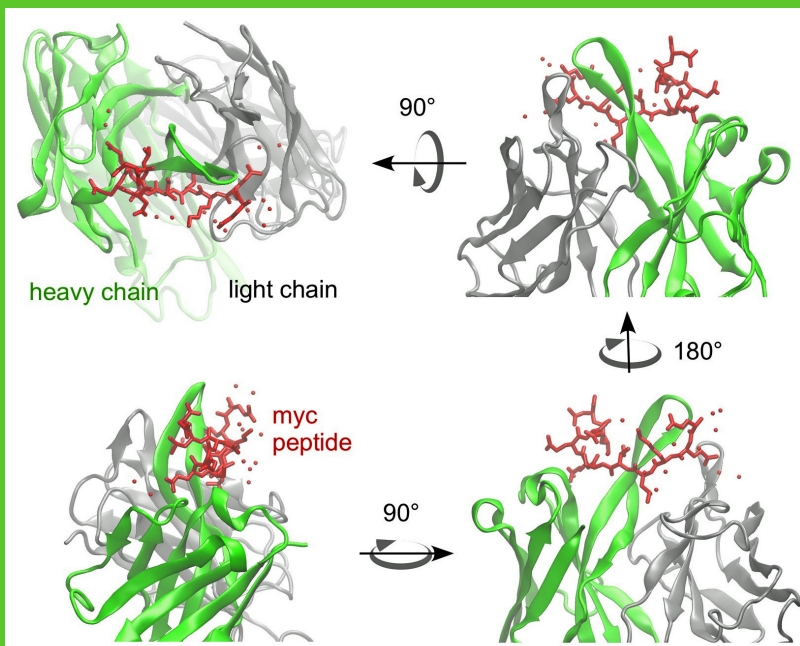
Figure 1: *A molecular structure of monoclonal antibody Myc1-9E10 (green/gray) binding to its epitope peptide (red), as revealed by X-ray crystallography (source: PDB file 2OR9).*

largest loop binds the epitope with his „back" side (Figure 1).

The special for m of the antigen binding site, featuring a large „finger" pointing towards the antigen, can explain the high specificity and affinity of this particular antibody.

This report describes the development of the new monoclonal antibody Hyper-Myc which expands the versatility of the myc-tag system further by providing a stable monoclonal reagent with higher affinity. In addition, the improved anti-myc-tag antibodies are available with different Fc parts for their detection, namely rabbit or human (in addition to mouse), thus giving the user a

thus giving the user a new choice for secondary antibodies to detect myc-tag as well as the opportunity to combine myc-tag detection with other mouse monoclonal antibodies in double staining experiments typically used in colocalisation studies (Figure 2, right).

Generation of Hyper-Myc

In early days of hybridoma technology, clone Myc1-9E10 showed problems in cultivation, and efforts were undertaken to produce a recombinant form in a different cell host. Shortly after the sequence of the monoclonal antibody secreted by hybridoma clone Myc1-9E10 was determined[3], it became evident that it was not easy to produce the single chain fragment of the antigen binding regions (V regions) as recombinant antibody in *E. coli*, the
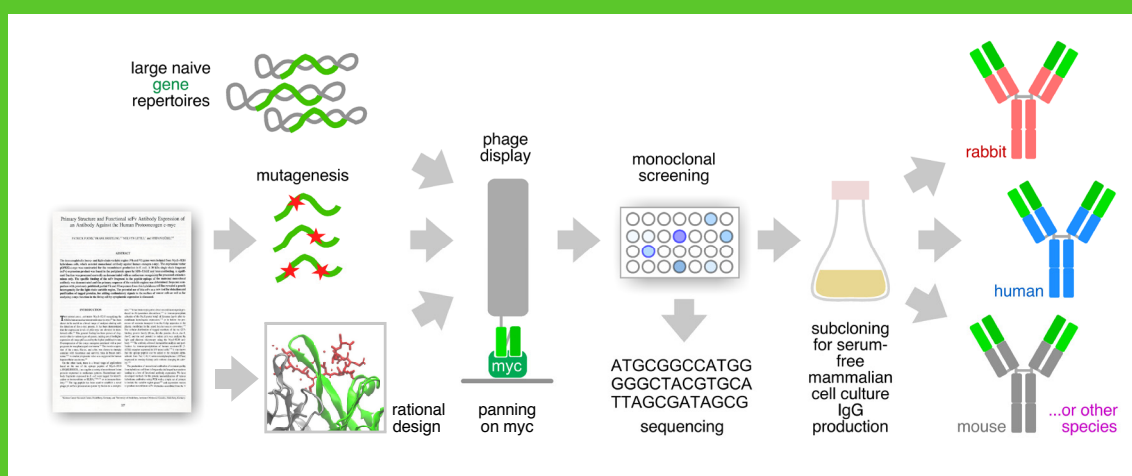

Figure 2: *Generation of rabbit, mouse and human Hyper-Myc variants with improved affinity and stability by in vitro evolution and re-cloning for recombinant production.*

[3] Fuchs P et al. (1997). Primary structure and functional scFv antibody expression of an antibody against the human protooncogen c-myc.
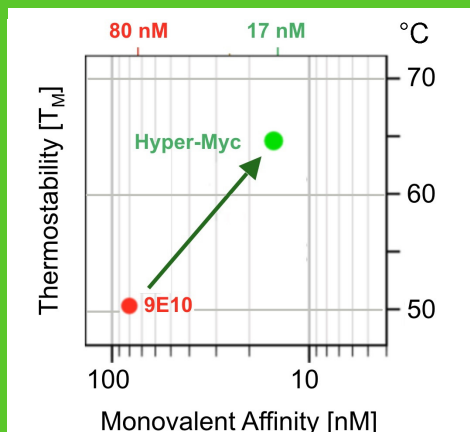
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Figure 3: *Improvement of affinitiy and Fv-region thermal stability of Hyper-Myc compared to hybridoma antibody Myc1-9E10 recognizing the myc-epitope.*

dominant expression system at that time. Consequently, efforts to improve production, stability and shelf life were undertaken.

The improved recombinant monoclonal antibody Hyper-Myc is a result of extensive molecular redesign mainly employing *in vitro* evolution (Figure 1). For this purpose, mutation libraries of synthetic genes of the V regions were employed in stringent selections using phage display[4]. This method is based on the enrichment of a gene packaged into a bacterial virus, the phage, via the molecular interaction of the protein encoded by that particular gene. This is achieved by fusing this gene to that of the phage surface protein pIII. This leads to a phage particle that presents the gene product on its surface. By an affinity chromatography-like process named „panning", the best binders can be isolated from a vast repertoire of candidate genes. Most importantly, the biochemical milieu during that very moment of selection can be completely controlled. In addition to just binding its antigen, this allows to employ many different ways of preselection of the antibodies other biochemical properties. For example, crossreactivity patterns of the resulting antibodies can be manipulated by preabsorption or addition of soluble competitors during panning. Compatibility of the selected antibody with special buffer conditions can be assured by using the finally needed buffer for the panning process itself[5].
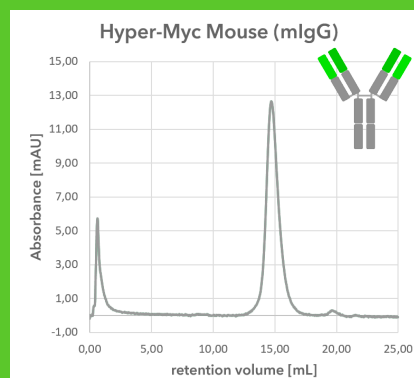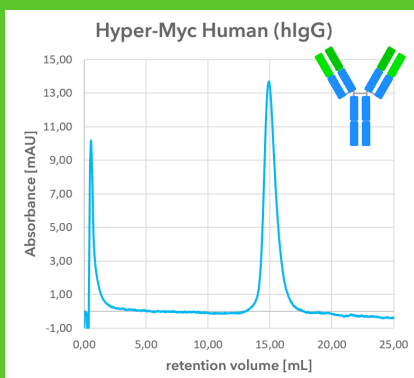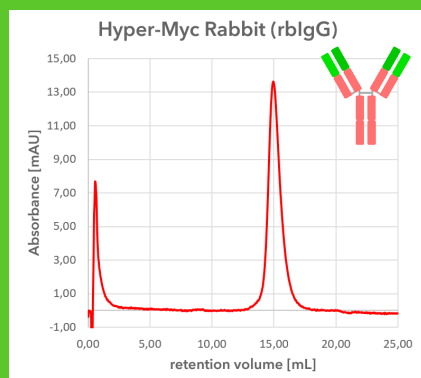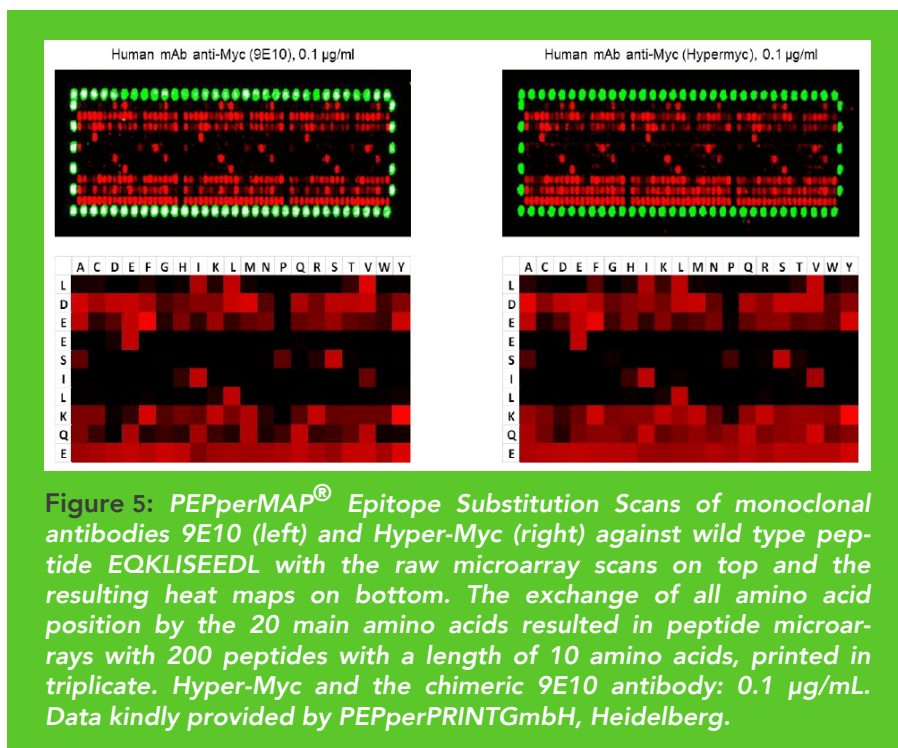

Figure 4: *Size exclusion chromatography of Hyper-Myc IgG on a Superdex 200 column shows high purity and no aggregation for the three variants with Fc parts of different species.*

To generate Hyper-Myc, panning conditions were tuned such that variants with both higher affinity and better thermostability could be selected. Affinity of the binding region of Hyper-Myc was determined in equilibrium by microscale thermophoresis. The best variant showed both a significantly improved resistance against heat denaturation and an affinity increase (Figure 3). A KD of 17 nM was found for the monovalent binding arm. Effective affinities in many typical user assays (e.g. immunoblot, ELISA) are substantially higher due to the avidity effect provided by the bivalent IgG format of Hyper-Myc. The significance of better stability for a lab tool is obvious, and higher affinities are of advantage since they allow the use of lower amounts of antibodies in an experiment to obtain the same specific signal, while lowering unspecific background. Last but not least, higher dilutions directly save money. Hyper-Myc adds another new feature to the already versatile myc-tag system.

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[4] Breitling F et al. (1991). A surface expression vector for antibody screening. Gene 104, 147-153

[5] Frenzel A et al. (2017). Designing human antibodies by phage display. Tranfusion Medicine and Hemotherapy 44: 312-318

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Figure 5: *PEPperMAP® Epitope Substitution Scans of monoclonal antibodies 9E10 (left) and Hyper-Myc (right) against wild type peptide EQKLISEEDL with the raw microarray scans on top and the resulting heat maps on bottom. The exchange of all amino acid position by the 20 main amino acids resulted in peptide microarrays with 200 peptides with a length of 10 amino acids, printed in triplicate. Hyper-Myc and the chimeric 9E10 antibody: 0.1 µg/mL. Data kindly provided by PEPperPRINTGmbH, Heidelberg.*

The improved myc-epitope binding V regions were recloned to a set of different vectors allowing mammalian cell culture production. These vectors introduced the constant regions, including the Fc-parts, i.e. the end of the immunoglobulin molecule which is usually recognised by the secondary antibodies used for the visualisation of primary antibody. Here, Fc-parts are available not only from the mouse, but also from human and rabbits (Figure 2). Other species are also possible. The delivered products therefore consist of V regions with human sequence origin, combined with constant regions of the indicated species.

It is therefore advised to use Fc-specific anti IgG secondary antibodies recognising the corresponding species. The availability of Hyper-Myc IgG with different Fc regions provides an additional choice and allow to employ the same myc-tag specificity with different detection systems established at the user's lab. Further, this allows to combine myc-tag detection with the use of another mouse monoclonal antibody, e.g. from a hybridoma, in the same staining. This enables colocalisation studies of a myc-tagged protein and another protein identified by the vast repertoire of monoclonal mouse research antibodies.

Hyper-Myc is made without the use of animals and comes as highly purified IgG (Figure 4).

Hyper-Myc recognises the same epitope as 9E10

Extensive studies using microarrays of peptide variants of the epitope were done to assure that specificity for the myc tag was not compromised by the process leading to increased affinity and stability. This was done using PEPperMAP® Epitope Substitution Scan arrays of printed peptides, custom designed by PEPperPRINT GmbH, Heidelberg.

Very similar epitope substitution patterns were obtained for both 9E10 and Hyper-Myc with conserved (few spots in a row) and variable (continuous row of spots) amino acid positions (Figure 5). A slight cross reactivity with the peptide motiv AKLVSE/LVSE of the myc-antigen was detected for both antibodies, this has already been observed before for 9E10[6].

Hyper-Myc shows lower cross-reactivity with human antigens

Typically, research antibodies do not come with a description of their unwanted off-target reactivities, despite every antibody has these. For Hyper-Myc, in contrast, a very thorough crossreactivity profiling was done using the PEPperCHIP® Human Epitome Microarray covering 28.895 different epitopes of the human proteome, but NOT including the

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[6] Validation of a New Recombinant Human anti-c-Myc Tag Antibody, PEPperPRINT GmbH,
https://www.pepperprint.com/downloads/Validation_of_a_Recombinant_Human_anti-Myc_Antibody.pdf
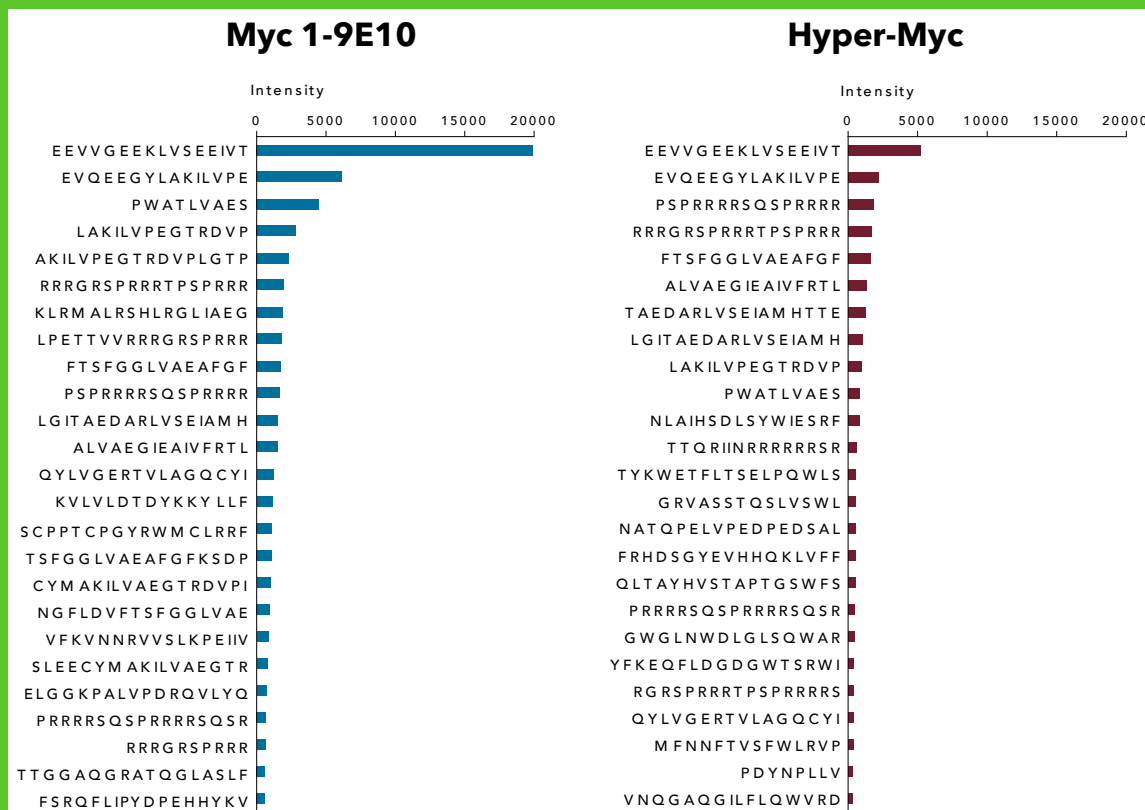
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Figure 6: *The top 25 peptide hits of Myc1-9E10 (left) and Hyper-Myc (right) on a PEPperCHIP® Human Epitome Microarray providing reactivity quantification for 28.895 human peptides. Peptides were sorted by decreasing spot intensities. Among the huge number of different peptides tested on the microarray, only peptide EEVVGEEKLVSEEIVT and EVQEEGYLAKILVPE showed significant cross reactivity with both antibodies. Intensities of crossreactivities were generally lower for Hyper-Myc. Note: the original (correct) c-myc epitope is NOT represented on this array type.*

„correct" wildtype myc-tag epitope. Purified mouse monoclonal Myc1-9E10 and Hyper-Myc were compared to determine their crossreactivity profiles (Figure 6). For both antibodies, only very few crossreactivities were found. This low cross reactivity certainly is an important contributing factor for the huge success of the myc-tag system. Significantly, Hyper-Myc showed an even lower crossreactivity profile than 9E10, despite it's higher binding affinity to the correct sequence. Interestingly, this approach also identified the crossreactivity with the LVSE core motif for both antibodies observed in the PEPperMAP® arrays[7], which seems to be a characteristic feature of the paratope of these antibodies.

This detailed list of potential crossreacting epitopes (Figure 6) allows the user to check for the occurrence of these motifs and its potential implications upon application of this antibody in his assays.

Application examples

While the epitope determination experiments described above have demonstrated the compatibility of Hyper-Myc with microarray experiments, a variety of other typical lab applications were tested. Hyper-Myc worked well in Microarrays, ELISA and immunoblots, and showed similar or better performance than 9E10 in every assay that was tried.

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[7] http://www.pepperprint.com/applications/antibody-cross-reactivity-analysis

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Quantitative capillary western blots show improved sensitivity of Hyper-Myc

Simple Western Immunoassay™[8] is an automated gel-free and blot–free method to provide quantitative immunoblot results. A myc-tag labelled protein was separated by capillary electrophoresis according to its size, then identified using either monoclonal antibody 9E10 or Hyper-Myc, which were then detected by a HRP-conjugated secondary antibody and visualised chemiluminescent substrate. The resulting chemiluminescent signal allows quantification over a large range of concentrations. Hyper-Myc showed better detection in all dilutions (Figure 7).
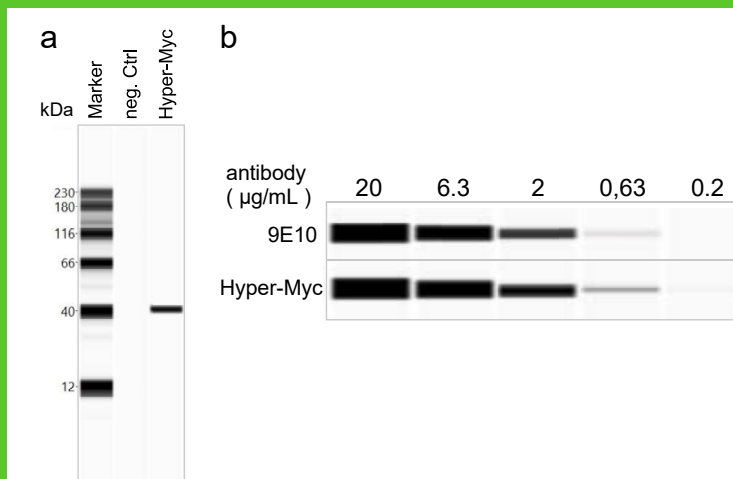

Figure 7: *Quantitative immunodetection of a myc tagged protein using the automated Protein Simple™ Western Immunoassay system. Quantified chemiluminescence was automatically converted to gel-like pictures by the system. a, whole chromatogram, b, comparison of the detected bands, either stained with 9E10 or Hyper-Myc. Hyper-Myc provides improved detection sensitivity compared to hybridoma antibody 9E10.*

Detection of c-myc tag in zebrafish whole mounts

The low crossreactivity profile of Hyper-Myc suggests that it could be used in challenging assays depending on antigen recognition with very high specificity in a dense environment of a vast number of other antigens. The most challenging of these assays are whole mounts of complete organisms, literally containing every possible protein and molecule of an entire animal. To check this, Hyper-Myc was challenged to identify the myc-tag in whole mounts of transparent fishes. The Zebrafish (*Danio rerio*) is a widely used model system for a large variety of studies, ranging from developmental biology to toxicology.

Figure 8: *Whole mount of 2 dpf transgenic zebrafish expressing the myc-epitope as a fusion to a fluorescent protein (mblbp-E1b: mScarlet-myc) stained with recombinant Hyper-Myc Mouse (ABC0503 and fluorescently labelled secondary antiy-mouse Fc antibody.*

The transparency of the zebrafish allows to study the process of tissue differentiation and pattern formation of a vertebrate organism in great detail.
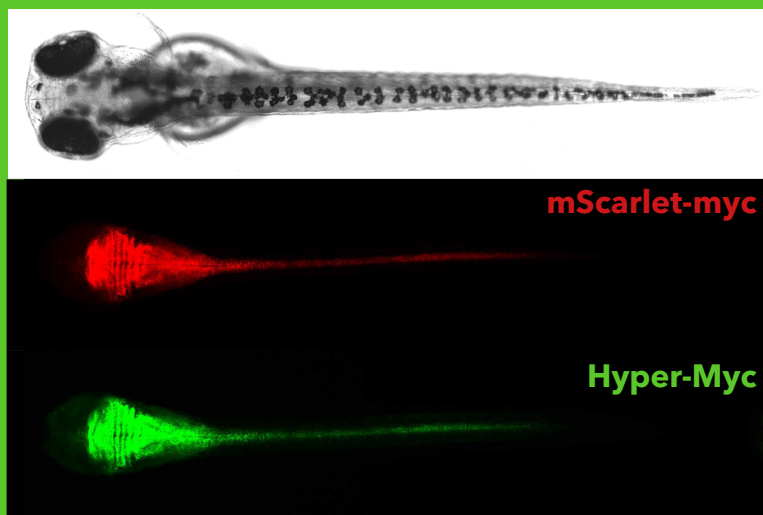

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On the other side, staining of whole mounts with antibodies frequently leads to false positive results due to compromised specificity of available research antibodies.

For example, an antibody frequently used to detect cadherin in zebrafish studies was showed false positive reactions in cadherin knockouts[9]. Here, mutants were generated expressing an artificial fusion of gene *mblbp-E1b* with the red autofluorescent protein mScarlet and a myc-tag[10]. *mblbp* is expected to be expressed specifically in the nervous system, in particular in a fraction of GFAP (glial fibrillary acidic protein)-positive glial cells. The whole mounts of *mblbp-E1b:mScarlet-myc* expressing fish were stained with Hyper-Myc, using an Alexa-488 labelled secondary antibody, detected in the green channel.

If the reactivity of Hyper-Myc is specific in the context of all the other available proteins and other molecules present in a complete fish, the red and green reactivity patterns should colocate. Indeed, the pattern of green (Hyper-Myc) fluorescence matched the expression pattern of myc-tagged protein (red) in a pattern correlated to the central nervous system typical for glial cells (Figure 8), while background staining was very low.

This demonstrates both excellent specificity and negligible off-target reactivity of Hyper-Myc in the very complex molecular environment of whole mount zebrafish preparations.

Featured products:

Hyper-Myc Rabbit
Rabbit anti myc-tag (recombinant monoclonal, purified IgG, rabbit Fc), Order No. ABC0502

Hyper-Myc Human
Human anti myc-tag (recombinant monoclonal, purified IgG, rabbit Fc), Order No. ABC0501

Hyper-Myc Mouse
Mouse anti myc-tag (recombinant monoclonal, purified IgG, rabbit Fc), Order No. ABC0503

Abcalis Animal Use Statement:

Hyper-Myc is an engineered recombinant antibody obtained by in vitro evolution employing phage display of a synthetic gene library inspired by the published sequence of Myc1-9E10. No animal immunisation was involved in its generation, and its production in human cell culture is achieved without the use of fetal calf serum, BSA or other animal derived materials. The genes for the immunoglobulin constant regions of rabbit and mouse were obtained as recombinant DNA from catalogues. Only a small number of zebrafish specimens were used for antibody specificity assessment in whole mount zebrafish immunostaining. Zebrafish larvae where anesthetised in Tricaine 0,05% and incubated 20 min on ice prior to fixation.

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[9] Russo G et al. (2018) Sequence defined antibodies improve the detection of Cadherin 2 (N-Cadherin) during Zebrafish development. N. Biotechnology 45, 98-112
https://doi.org/10.1016/j.nbt.2017.12.008

[10] We thank Reinhard Köster for providing this strain.

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