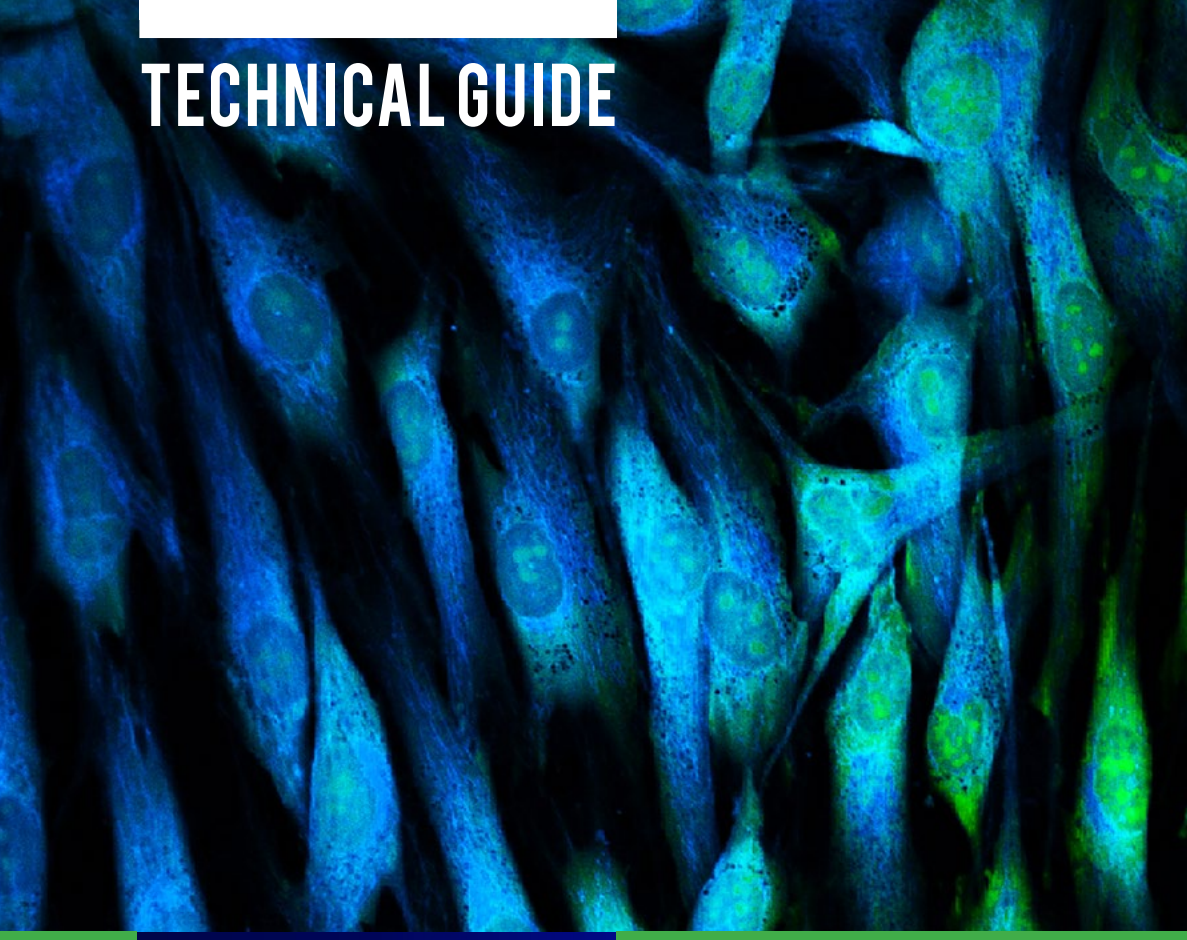
TECHNICAL GUIDE



For Life Science Research

Antibodies

Antibodies

Peptides

Proteins

Eurogentec

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INTRODUCTION

Post-genomic phase and functional proteomics

DNA sequencing of the most important model organisms brought to researchers an information resource about numerous proteins whose existence was not known so far. Though gene expression can be quantified on RNA level, this information does hardly provide any hint on the quantity of the expressed protein, its functionality or its modulation by epiproteomic modifications.

With the advent of RNA interference (RNAi) powerful means have been developed to control the production of proteins *in vivo* either by genuine naturally occurring microRNAs (miRNA), or by their non-natural *in silico* created counter parts, which are referred to as small interfering RNA (siRNA). The accessibility of this type of molecules as tools to knock down protein production specifically enabled researchers to create conditions in their model systems under which a distinct loss of function scenario can be studied.

This loss of function scenario, or the absence or presence of a given protein, are subject to characterisation assays. Enzymatic activities for example can be determined with laborious assays, if available. In contrast to this, antibodies provide tools to characterise the production of a protein and even its epiproteomic modification state by easy-to-establish methods.

Current state-of-the-art immunological research allows us to provide researchers with custom-made antibodies as tailor-made solutions for the different types of protein characterisation studies. Antibodies can be produced by several approaches:

> Use of recombinant proteins as antigens
> Use of peptides as antigens
> DNA immunisation

Eurogentec can help you to produce the required antigen for your immunisation, either by peptide synthesis or protein production in bacterial or eukaryotic production hosts.

Beside the different options for the used antigen, antibodies can be produced in three different modes as well. Here one differentiates between a polyclonal serum, or purified polyclonal antibodies, representing the whole repertoire of antibodies produced by the immunisation host's B-cells. Optimisation of immunisation techniques enables Eurogentec to provide you with polyclonal antibodies after only 28 days of immunisation in rabbits, guinea pigs, rats and goats using the Speedy technology. Evolutionary conserved mammalian antigens are made accessible to antibody production by Eurogentec using chicken as immunisation host. On the other hand are monoclonal antibodies from mice, which are obtained after isolation and immortalisation of splenic B-cells by fusion with an immortal mouse blood cell line. During the subsequent selection of single desired antibody producing cells (clones), the whole cell population becomes diluted to the single cell level, providing one clone of antibody producing cells. This clone produces just one type of antibody specific for one aspect of the used antigen. And, finally, if the antigen is not available (peptide or protein difficult to produce or to purify in sufficient amount), Eurogentec proposes an optimised DNA immunisation approach that enables to obtain polyclonal antibodies starting from the *in silico* DNA sequence.

Experiencing partnership with Eurogentec does not only mean to benefit from decades of experience.
We are also proud to offer you the most complete range of services related to antibody production.

1 GENERAL CONCEPTS IN ANTIBODY PRODUCTION

Antibody classes

The basic structure of all mammalian immunoglobulins involves two pairs of chains – heavy chains and light chains, covalently linked together by disulfide bonds. The type of heavy chains determines the antibody class, while the light chains can be just one of two types, kappa or lambda, and do not play a role in the classification. Based on the different types of heavy chains present in nature, there are five classes of antibodies: IgA, IgD, IgE, IgG and IgM. Each class is composed of two subclasses based on the type of light chains, thus there are two types of IgA, IgD, IgE, IgG and IgM antibodies.

Visually this can be represented as

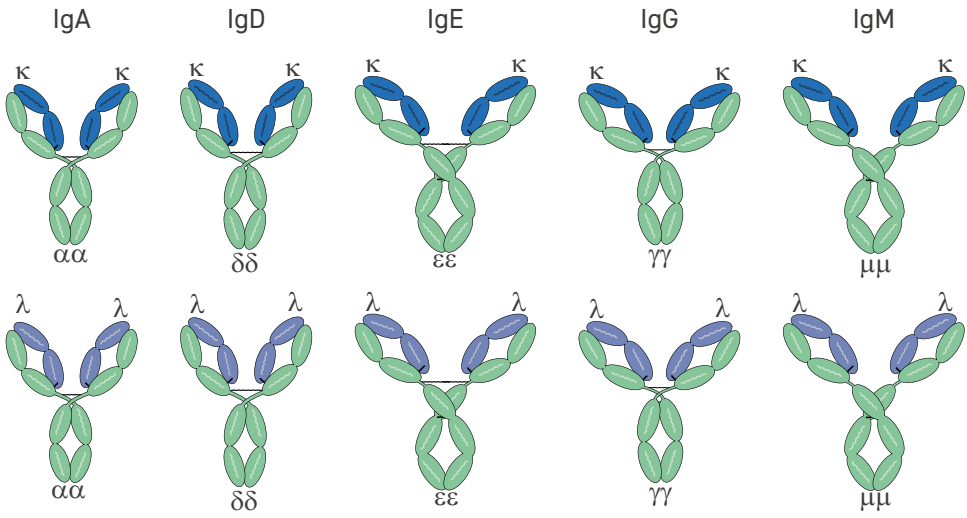


Figure 1: Schematic representation of mammalian antibody classes and subclasses. Each class of antibody (e.g. IgG) comprises two subclasses depending on the type of light chains present (e.g. IgG kappa or IgG lambda subclasses).

Antibody structure

Variable regions

Each chain is made up of a number of domains, formed by intra-chain disulfide bonds. At the N-terminal end of the light chains and the heavy chains the antigen binding Variable domain (V_L and V_H) is located, which consists of approximately 110 amino acids in mammalian antibodies.

Constant regions

The remainder of the antibody is composed of constant regions, found on both the light chains and the heavy chains. Each class of mammalian antibodies has a specific number and type of constant regions associated with its heavy chains, three constant regions for IgG, A and D and four in IgM and E. The constant region on the light chains exists as a single type of domain. Thus an antibody is composed of variable (on the light and heavy chains) and constant portions (on the light and heavy chains).

Functional domains

Immunoglobulins consist of two functional domains. The first one, the Fab fragment (or Fragment Antigen Binding), consists of the two V_H domains and the two V_L domains. The Fc (or Fragment Crystallisable) consists of the C_H domains of both heavy chains. The antigen specificity is associated with the Fab fragment while the Fc region is associated with binding to Fc receptors on other (immune competent) cells. The hinge region provides flexibility for the orientation of the Fab fragment to the Fc fragment.

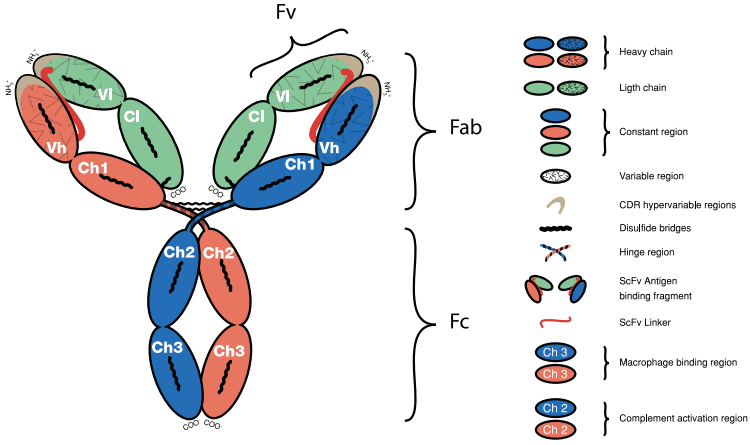


Figure 2: General mammalian antibody structure showing N-termini, C-termini, constant regions and variable regions

Monomeric or multimeric

Some antibodies can exist as monomers or multimers or just multimers. IgA can exist as a four-chain monomer form or as four-chain dimers, IgD, IgE and IgG exist as four-chain monomer forms only, and the IgM class exists as a pentamer or hexamer four-chain structure.

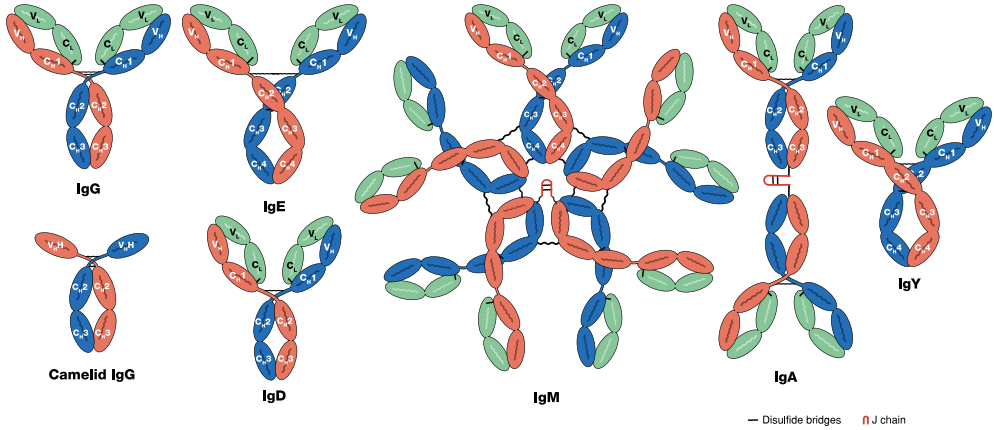


Figure 3: Molecular domain structure of immunoglobulins from each conventional mammalian antibody class, camelid single-chain IgG structure lacking the light chains, and avian IgY. Lines correspond to disulfide bridges; red circles correspond to carbohydrate modifications.

Characteristics of immunoglobulin classes

IgG

This class of antibody represents 70-75 % of the circulating antibodies in mammals. It has a molecular mass of ±150 kDa. IgG is the main immunoglobulin type produced during a secondary immune response and is the only antibody with anti-toxin activity. There are four subclasses of IgG – IgG1, 2, 3 and 4 - each with slight variations in molecular mass, number and position of the disulfide bonds and differences in their functional properties.

IgM

This class of antibody represents 10 % of the circulating antibodies in mammals. It has a molecular mass of 970 kDa and is a pentamer or hexamer of the four-chain units linked via the CH3 domains by disulfide bonds. This antibody is the main player in the primary antibody-mediated immune response.

IgA

This class of antibody represents about 15-20 % of the circulating antibodies in mammals. It exists as two forms, the monomeric (80 %) and dimeric form (20 %) in human, while in other mammals primarily the dimeric form exists. The dimeric form has a molecular mass of 385 kDa and is the main antibody present in the mucous membranes. There are two known subclasses of IgA, IgA1 and IgA2.

IgD

This class of antibody represents about 1% of the circulating antibodies in mammals. It is expressed on the surface membrane of many B-cells and has been associated with the differentiation of pre-B cells into mature B cells.

IgE

This class of antibody circulates in only trace amounts and the majority of these antibodies are found bound to the surface of mast cells and basophils in mammals. Functionally, it is associated with allergic reactions. Along with IgM, IgE antibodies have an extra heavy-chain constant domain.

IgY – Avian IgE-IgG-like antibodies

This class of antibody is found in **chicken and duck** species and is the functional equivalent of both IgG and IgE in mammals. This antibody is found both in the blood and in the egg yolk. IgY antibodies are not recognised by Fc receptors.

Heavy chain - Llama antibodies

Camelids produce single-chain antibodies in addition to conventional ones. This special class of antibody displays the particularity of having heavy chain moieties and no light chains. They are composed of 2 constant regions and a single variable region per heavy chain. The antigen binding domains of these antibodies, called VHH, are the smallest naturally occurring antibody fragments that recognise the antigen. Due to their small size, VHH can bind epitopes that are hidden and are relatively easy to produce in lower eukaryotes. Therefore, llama antibody fragments are good candidates for therapeutics applications requiring small enough molecules to be stable *in vivo*.

	IgG1	IgG2	IgG3	IgG4	IgM	IgA1	IgA2	IgD	IgE	IgY	Llama
Heavy chain type	γ1	γ2	γ3	γ4	μ	α1	α2	δ	ε	ν	
Light chain	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ	κ or λ		None
N° of constant domains	3	3	3	3	4	3	3	3	4	2	2
N° of variable domains	1	1	1	1	1	1	1	1	1	1	
Molecular mass (kDa) - Total	150	150	165	150	970	160	160	175	190	180	100
Serum concentration (mg/ml)	9	3	1	0.5	1.5	3	0.5	0.04	0.0003	20 mg/ml egg yolk	
Half-life (days)	23	23	7	23	5	6	6	3	2.5	> 30	
Binding to macrophages and other phagocytic cells	+	-	-	+	-	-	-	-	+		
Binding to basophils and mast cells	-	-	-	-	-	-	-	-	+		
Complement activation (classical)	++	+	+++	-	+++	-	-	-	-		
Complement activation (alternate)	-	-	+	-	-	+	-	-	-		

Table 1: Molecular characteristics of mammalian Immunoglobulin isotypes (IgG, IgM, IgA, IgD, IgE), chicken IgY and llama antibodies

Antigen-related factors affecting immune response

Antigen homology

The immune system will attempt to eliminate anything that it considers to be non-self, or non-homologous. Thus a mouse protein injected into a mouse will not elicit an immune response, while the same protein injected into a rabbit generally will. For highly conserved mammalian antigens, chicken hosts often provide a useful alternative to mammals due to phylogenetic differences.

Antigen size

Antigens with molecular masses higher than 100 kDa (or whole bacteria or viruses) are usually excellent immunogens, while those lower than 10 kDa get "lost" and may not stimulate an immune response. Such small molecules (usually referred to as "haptens") may be made immunogenic by coupling to large carrier proteins, as is the case for anti-peptide antibody production.

Route of antigen administration

The route by which an antigen enters the animal will affect the immune response. Thus a pathogen that enters via a wound may cause an immune response, while the digestion after swallowing may destroy the same pathogen. Immunisation is routinely done via subcutaneous injection on multiple sites to receive the best immune response.

Antigen dose

The dose of an antigen, if too high or too low, may or may not elicit an immune response. This absence of immunogenicity is called immunological tolerance.

Adjuvants - Increasing inherent immunogenicity

Adjuvants are a class of compounds that cause, by unknown mechanisms, an increase in the strength and duration of an immune response to a particular antigen. Factors which are presumed to be involved in this phenomenon are (i) increasing the size of the antigen (ii) prolonging the persistence of the antigen (iii) stimulating immune related cells such as macrophages and lymphocytes.

Numerous adjuvants are commercially available. For animal use common adjuvants are Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), Alum, Gerbu, and Titermax.

Freund's Complete Adjuvant : inactivated tuberculosis cells in mineral oil
Freund's Incomplete Adjuvant : mineral oil
Alum : aluminium salts (hydroxide and phosphate)
Gerbu : proprietary adjuvant (www.gerbu.de)
Titermax : proprietary adjuvant (www.titermax.com)

Eurogentec routinely uses Freund's adjuvant for its STANDARD protocols. The SPEEDY protocol uses a non *mycobacterium* containing mixture. Should you prefer that we use an alternative adjuvant, please let us know and we will discuss other options with you.

Antibody titre, affinity and specificity

Antibody titre evolution

Exposure to a specific antigen will initiate the immune response and in the first phase will result in the production of IgM class antibodies. IgM antibodies are responsible for the first attempt of antigen clearance by the host immune system. Initially the specific serum antibody titre is predominantly composed of high avidity, but low affinity, low specificity and low concentrated IgM type antibodies. A second exposure will trigger a faster response over hours or days, and will generate the desired longer lasting IgG type antibodies. IgG antibodies have higher titre, specificity and affinity to the antigen than IgM. Coupled to the generation of IgG antibodies is the generation of memory cells to quickly inactivate the antigen in the case of future exposure.

The efficiency of each immune response is different, and individual. However a general scheme of IgM and IgG antibody titre evolution over time can be represented as below.

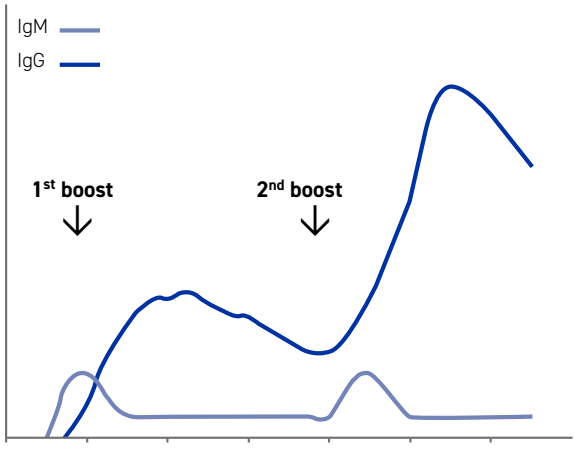


Figure 4: Evolution of IgM and IgG titres as a function of time and immunisation

A lack of titre evolution following immunisation can be due to:

> Homology of the antigen to the host proteins
> Molecular mass of the antigen which is too low
> Insufficient quantities of antigen
> Poor immunogenic properties of the antigen

Antibody affinity evolution

A second immune response to an immunisation is not only characterised by a faster immune reaction but also by antibodies with up to a 10,000 fold increased affinity for the antigen. This increase is due to random somatic hypermutation in the variable region of the light chain and the heavy chain during expansion of memory cells. The diversity of the specificity will increase amongst a single clone. A second mechanism occurs following the antigen binding: isotype class switching. This refers to a rearrangement of the genes coding for the heavy chains in memory cells. The loss of coding region causes a class switch from IgM to IgG, IgA and IgE.

A combination of these two mechanisms, somatic mutation and clonal selection are natural processes that lead to affinity evolution. Affinity evolution can be monitored by a number of methods. Below are results from serum drawn at different time points during Eurogentec's 87-day polyclonal rabbit standard programme. The flatter the plateau of the rising curves the higher the binding affinities.

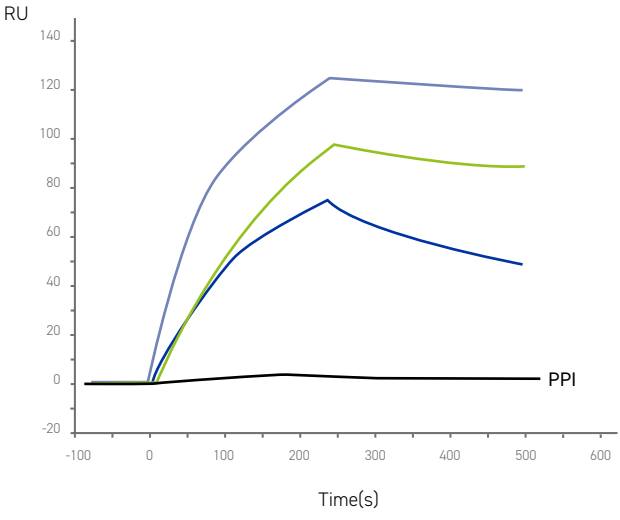


Figure 5: Affinity evolution of a 87-day anti-peptide polyclonal programme (Eurogentec's 3-months standard programme).

Confidentiality

All monoclonal and polyclonal immunisation projects are confidential. All obtained hybridoma, sera and results are and remain the property of the customer. Eurogentec guarantees not to claim any rights on the hybridoma or the antibodies. If desired, confidentiality agreements can be signed.

2 POLYCLONAL ANTIBODY PRODUCTION

Catalogue antibodies

Catalogue antibodies can be mono- or poly-clonal, recognising the full length protein, a domain or a post-translational modification, coupled to a fluorophore, an enzyme (HRP/AP) or not. In contrast to custom antibodies, catalogue antibodies are directly available and less expensive, but they can't be personalised.

The number of catalogue antibodies available rises continually. Some companies offer hundreds of thousands antibodies but their quality control is not always following. To insure the guarantee of high quality, Eurogentec focuses on the most widely used and published ones (e.g.: clone 33D3 to 5-methyl-cytosine, a monoclonal antibody which targets the modified 5-methylcytosine DNA base).

Custom antibodies : choosing an animal host

The most widely used host for immunisation is rabbit.

The New Zealand white rabbit used in our animal facility is easy to reproduce and maintain and is quite resistant to infections, especially when handled under SPF conditions (see page 14). Furthermore, it is able to develop a strong and fast immune response to antigens. Finally, the serum volumes that we can get are quite large and compatible with most of the usual antibody applications in terms of quantity of specific antibody available.

Beside rabbit, Eurogentec also offers immunisation in a wide range of hosts, in order to stick to specific customer requirements. These include :

> Mouse
> Rat
> Guinea pig
> Chicken
> Goat
> Sheep
> Pig
> Llama (provide single chain antibodies)
> Horse

Two major criteria have to be taken into account to choose a non-rabbit host: antigen homology and expected serum volumes.

Antigen homology

The success of an antibody production depends mostly on the immunogenicity of the antigen to the host. Highly conserved secreted mammalian proteins may not result in good antibody titres in rabbits because these mammals may not recognise such proteins as being foreign. In such cases we advise chicken antibody production which can overcome the homology issue. IgY antibodies do not bind to the mammalian Fc receptor, assuming very low background signals when used on mammalian samples containing material from immune competent cells carrying Fc receptors.

Demanding applications

2

If you require antibodies for kit development, you will be interested in having a larger animal host to collect as much antibody at once as possible. It is a known problem that the same antigen when injected into two different hosts from the same species can produce different antibody titres, slightly different specificities, and different affinities. This means that "batch-to-batch" variability is an issue when re-launching a polyclonal antibody production. Three solutions can be suggested : (i) to produce a large batch from a larger animal such as a goat or sheep (ii) to produce the antibody in chicken, which generate highly concentrated amounts of antibodies which are isolated from the eggs (and many eggs can be collected per hen) or (iii) to produce a monoclonal antibody from hybridoma.

	Mammalian IgG	Avian IgY
Antibody sampling	Invasive	Non-invasive
Antibody amount	200 mg total IgG per rabbit	50-100 mg IgY per egg
Amount of antibody per month	200 mg	1500 mg
Amount of specific antibody	5 %	2-10 %
Protein A/G binding	Yes	No
Binding to Fc receptors	Yes	No
Interference with mammalian IgG	Yes	No
Interferences with rheumatoid factor	Yes	No
Activation of mammalian complement	Yes	No

Modified according to : Schade, R., Pfister, C., Halatsch, R. & Henklein, P. (1991). Polyclonal IgY antibodies from chicken egg yolk - an alternative to the production of mammalian IgG type antibodies in rabbits. ATLA 19: 403-419.

Table 2: Comparison of the characteristics of mammalian IgG and avian IgY

Animal maintainance and welfare

Specific Pathogen Free (SPF) and non-SPF environments

The quality of the produced antiserum depends not only on the quality of the animals, but also to a large extent on the animal housing and treatment conditions.

Two housing types exist for research animals, Specific Pathogen Free (SPF) or Bio-Exclusion housing and Non SPF or Pathogen-open housing. Only SPF housing involves the protection of animals from bacterial, viral and pathogenic agents that can infect and negatively influence the animals. Non-SPF (pathogen-open facilities) housing facilitates potential animal cross-contamination with pathogens generating animals that often show high initial background antibody titres. SPF housing maintains animals with a low background antibody titre. SPF housing requires construction of a bio-containment facility.

	SPF Facility Bio-exclusion	Non-SPF Facility Pathogen-open
Personal animal access	Authorised only	Less regulated
Monitoring system – air, humidity, temperature	Controlled 24 / 7	Less regulated
Serological surveillance	Regular	None
Risk of animal contamination	Low	Higher
Animal death rate	Low	Higher
Animal variability	Low	Higher
Background antibody titre	Low	Higher
Immunological response	High	High

Table 3: Differences between SPF and non-SPF facilities

At Eurogentec our SPF animal house is divided into 3 separate parts:

> A green "clean" corridor – to enter into the animal rooms
> The animal rooms
> A red "dirty " corridor – to exit from the animal rooms

Animal welfare

There is an ethical requirement to maintain and improve the research animal's welfare. Not only is it important to minimise animal suffering for welfare and ethical reasons, but it is also imperative to ensure that the psychological and the physiological changes do not contribute to unintentional variables to the experimental design, possibly affecting the accuracy and the reliability of the results.

Different animal welfare standards and guidelines exist. According to country legislation, some certifications and controls are mandatory while others are only voluntary, institution choosing to be accredited or not.

SPF rabbits

Eurogentec breeds and utilises the purebred strain New Zealand white rabbits that are highly stress-insensitive and show high reproducible performances. Besides maintaining a standardised complete diet, and a protected and standardised environment of caging, ventilation, lighting, temperature and humidity, the mental welfare of the animals are also attended to. As rabbits are social animals, they are kept on average up to 4 in the same cage, providing a balance between socialisation and animal space requirements. The cages permit the animal to sit upright or lie at full stretch and the animals are provided with objects to gnaw on to alleviate boredom. Eurogentec strives to ensure that the latest animal housing practices and guidelines are applied.

Eurogentec's New Zealand whites as well as other rodents have been bred and raised in an SPF environment prior to reaching the animal housing facility. Breeding rooms are maintained in SPF conditions to ensure that a constant supply of SPF quality rabbits is available. Prior to transfer to the housing facility the rabbits are quarantined for 1-2 weeks and a daily inspection by our veterinary staff ensures that the SPF nature of the animals is accurate.

FELASA recommended tests for SPF rabbits

Virus	Method	Frequency
Rabbit haemorrhagic disease	ELISA	Quarterly
Rotavirus	ELISA	Quarterly
Bacteria		
Bordetella bronchiseptica	Culture	Quarterly
Clostridium piliformis	Pathology	Quarterly
Dermatophytes	Culture	Quarterly
Pasteurella sp.	Culture	Quarterly
Salmonellae	Culture	Quarterly
Parasites		
Arthropods	Microscope	Quarterly
Helminths	Microscope	Quarterly
Encephalitozoon cuniculi	ELISA	Quarterly
Intestinal protozoa	Microscope	Quarterly
Pathological lesions	Direct inspection	Daily

Table 4: Specific pathogen tests recommended

Advantages of SPF animals

> Cleaner preimmune sera → better negative controls

Due to the minimum contact with bacteria, virus and pathogenic agents, these animals give preimmune sera containing much less antibodies against these microorganisms. This means much lower antisera background than generally observed with classical non-SPF rabbits.

> Increased robustness → higher success rate → higher antibody titre and affinity

As the SPF-animals are exempt from the most usual pathogens, the risk that an animal gets ill before or during the antibody production is greatly reduced. The rate of the animal loss is 10 to 20-fold lower than generally observed with non-SPF rabbits.

> Better animal welfare

List of available SPF animals

Host Type	SPF	Non-SPF
Mouse	✓	
Hamster	✓	
Rat	✓	
Guinea pig	✓	
Rabbit	✓	
Chicken		✓
Goat		✓
Pig		✓
Sheep		✓
Horse		✓
Llama		✓

Table 5: Available hosts and SPF type

Eurogentec's SPF warranty

We guarantee that our SPF rabbits will withstand our Speedy 28-day or standard 87-day antibody production programme; if ever a customer loses an animal during this period, Eurogentec will replace the rabbit free of charge, except if there is real evidence that the antigen preparation caused the death of the animal.

Eurogentec and your country's requirements

Eurogentec maintains animal housing facilities in compliance with the following association's requirements

> Federation of European Laboratory Animal Science Associations (FELASA)*
> UK Home Office Animals Scientific Procedures Act

Figure 6: Animal housing facility

* FELASA is a consortium of European community government animal ethics committees dedicated to the ethical use of research animals across all EU countries. The website for this organism is www.felasa.org. Eurogentec adheres to FELASA guidelines and continually invests in the improvement of animal housing and treatment as guidelines are updated to maintain the highest standards for animal protection and research quality standards. Eurogentec is regularly audited by customers and national control bodies.

Immunisation protocols

2

Antigen injection

Eurogentec routinely does multi-site subcutaneous injections. The antigen is aliquoted 4 times to inject a minimum volume per site. Other methods of injection (intramuscularly, intraperitoneal or intravenous) are possible on request and with justification. Intravenous injections are conducted without adjuvant and therefore only for booster injections.

Bleeds – Screening, test and terminal bleeds

How bleeds are drawn

All rabbit bleeds are taken using sterile Vacutainer®-tubes; these tubes contain a gel plug, which, upon centrifugation, separates the agglutinated blood cells and the serum, ensuring very clean serum for transfer to the tubes and vials, prior to being shipped to the customer. In addition to the sterility, this sampling system has the important advantage that the serum is obtained without any haemolysis (typical haemoglobin concentrations about 0.3-0.4 g/l). Using the classical "open" bleed method, it is very difficult to avoid lysis of red blood cells, especially as one often has to rub or to treat with xylene the opened vein in order to avoid its closing by agglutination. The result of this treatment is generally a quite red serum (haemoglobin concentration >1 g/l), which can affect the immune staining experiments due to an increase in fluorescent or HRP-reactive background. In contrast, the serum obtained using the Vacutainer®-system is clear and only very slightly coloured.

Pre-immune screening - select the best animal before starting a programme

Animal selection is key to any antibody programme prior to immunisation, thus ensuring that the background antibodies do not cross-react with your antigen or with your assay. Pre-immune screening of serum should be performed on test samples in your application to ensure that there are no cross-reacting antibodies in the host. During pre-immune screening and selection of the suitable animals, Eurogentec will reserve your selected animals for your immunisation programme.

Pre-immune test bleed – your negative control

Once the best animals have been selected from the screening step (optional) and the programme is initiated you will receive pre-immune (PPI) serum as well as test bleeds and a final bleed. These pre-immune test bleeds will serve as negative controls in your experiments since they derive from the same animals that are used to generate the antibodies.

Small test bleed – how is the programme progressing

The small test bleed (PP) allows monitoring the evolution of the antibody titre after one month during a 87-day programme. The small test bleed should be used in combination with the pre-immune test bleed to validate the evolution of titre of the desired antibody. However, the lack of antibodies does not indicate that the programme will not produce antibodies in the following two months.

Large test bleeds – how is the programme progressing

The large test bleed (GP) allows to further monitor the evolution of the antibody titre after two months in a 87-day programme. The large test bleed is pre-aliquoted into two vials, a large stock vial and a smaller (2 ml for rabbits) aliquot tube for testing the antiserum in your applications. The advantage of this pre-aliquoting is to eliminate an additional freeze thaw cycle of the large vial, which is known to potentially damage the antibodies.

Final bleeds – delivery of the desired product

Final bleeds (SAB) provide you with the highest amount of antibody. Typical final serum volumes for one rabbit are 50-70 ml serum (100-140 ml of whole blood).

	Min. antigen quantity / injection		Bleed volumes / animal				
Host	**Antigen MW < 18 kDa**	**Antigen MW > 18 kDa**	**Pre-immune**	**Small Bleed**	**Large bleed**	**Final bleed**	**Comment**
Mouse	40 µg	15 µg	40-70 µl	40-70 µl	40-70 µl	300-500 µl	Good to test antigenicity
Guinea pig	50 µg	30 µg	1 ml	1 ml	2-3 ml	10-15 ml	For small serum volumes
Rat	50 µg	30 µg	± 2 ml	± 2 ml	± 2 ml	5-7 ml	For small serum volumes
Chicken	200 µg	100 µg	1 egg	1 egg	± 8-10 eggs	± 8-10 eggs	For mammalian antigens and large quantities of Antibodies, 4 eggs = 1 rabbit final bleed
Rabbit	200 µg	100 µg	2 ml	2 ml	20-25 ml	50-70 ml	For most applications
Goat	400 µg	200 µg	2 ml	2 ml	250 ml	1000 ml	For large batch volumes
Sheep	400 µg	200 µg	2 ml	2 ml	250 ml	1000 ml	For large batch volumes
Llama	400 µg	200 µg	2 ml	2 ml	250 ml	1000 ml	Single chain antibodies and for large batch volumes
Horse	400 µg	200 µg	2 ml	2 ml	250 ml	1000 ml	For large batch volumes
Other	Contact us for recommendations						

Table 6: Typical bleed volumes and required antigen amounts for immunisation in the most common hosts

Our classcial programme : the 87-day programme (4 injections, 4 bleeds)

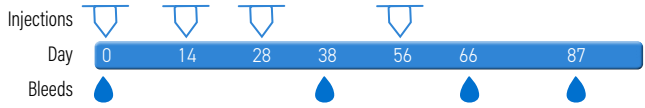


Figure 7: 87-day immunisation schedule

This is our 3-months, 2-animals standard programme. This programme offers the production of polyclonal antibodies with high titres and affinities. It is an excellent programme for the generation of antibodies to be used in applications that require the highest affinity antibodies such as immunohistochemistry. It is particularly well adapted to immunisations with poorly immunognic antigens, which usually require programme prolongations.

Our classical programme is well suited for most requirements. However, we are open to any modifications required concerning the scheme of injections and bleeds.

This protocol is available for all our animal hosts.

Our improved protocol : the Speedy 28-day programme (4 injections, 3 bleeds)

Figure 8: Speedy 28-day immunisation schedule

The Speedy 28-Day programme allows the production of polyclonal antibodies in only 28 days with similar titres and affinities compared to our classical programme by using our **proprietary non-Freund adjuvant combination**.

The Speedy 28-Day is available for rabbit, rat, guinea pig and goat. It is not adapted for programme prolongations.

In addition, the Speedy Mini programme offers anti-peptide antibody production in smaller quantity but is only available for rabbits. It comprises the 28-day production of polyclonal Abs in one rabbit only, with the affinity purification of 10ml serum, allowing direct Ab testing.

Host Type	87-day programme	28-day programme
Mouse	✓	
Guinea pig	✓	✓
Rat	✓	✓
Chicken	✓	
Rabbit	✓	✓
Goat	✓	✓
Sheep	✓	
Llama	✓	
Horse	✓	

Table 7: 28-day Speedy and classical programmes available Hosts

Production of antibodies in chicken

In hen immunisations, the IgY antibodies are collected from the egg yolks and not from the serum. The yolks are pooled per week and per hen and sent in one vial.

IgY must be extracted out of the yolks before affinity purification and for use in Western blots or other immunological experiments. This extraction is performed using a proprietary protocol aimed at removing the lipids and lipoproteins contaminants.

We especially recommend hen immunisation when the antigen homology in mammals is so strong that no immune reaction will occur and/or if the antibody amount expected is high.

Create your own antibody protocol

Eurogentec is ready to adapt its antibody production processes according to your requirements. Should you wish to have antibodies produced over shorter or longer time periods, with a certain number of injections or bleeds, we can help. Just let us know what your programme specifications are and we will work with you to implement them.

To have a specialist help you with the design of your programme, please contact us at:
proteomics.services@eurogentec.com

Antigens

2

Customer-provided protein and other antigen

As long as a protein can be recognised as a foreign compound by the host immune system, immunising directly with a protein is a very efficient way to produce antibodies, due to the huge number of epitopes present at the protein surface.

Antigen Format

Usually we work with the following antigen formats:

Lyophilised

You can send us your freeze-dried protein at ambient temperature. The antigen will be dissolved in 0.9 % salt solution (physiological solution) and aliquoted for the different injections and frozen at –20 °C.
If the antigen is poorly soluble in aqueous solution, we suggest that you avoid lyophilisation and that you send us your protein in solution on dry ice. The addition of adjuvant will help to dissolve even lipophilic proteins. If the antigen does not dissolve, a fine suspension of the antigen will be obtained by thorough mixing. Such fine suspensions can also be useful for antibody production because particles are efficiently phagocyted.

Solutions

You can send us your antigen in solution on dry ice.
We recommend to limit as far as possible the use of detergents and aggressive chemicals such as urea, acetic acid, guanidine hydrochloride, heavy metals and other agents that are toxic to the host animal.

Compound or formulations	Allowed	Not allowed
Water	Yes, keep the volume small (1 ml)	-
PBS	Yes, keep the volume small (1 ml)	-
Physiological buffer solutions	Yes, keep the volume small (1 ml)	-
Metal dyes/heavy metals	-	Risk of toxicity
Salts (KCl, NaCl, $MgCl_2$)	< 1.0 M	> 1.0 M
SDS	< 2.0 %	> 2.0 %
Urea	< 6.0 M (in rabbits)*	> 6.0 M
Guanidinium HCl	-	Risk of toxicity
Digoxin/Digoxigenin	-	Risk of toxicity
Octylglucoside	<1.0 %	> 1.0 %
Triton X-100/Tween-20	< 0.2 %	> 0.2 %
Glycerol	< 20 %	> 20 %
PMSF	-	Risk of toxicity
Pefabloc	< 0.1 mM	> 0.1 mM
Leupeptin/Pepstatin	< 1 µM	> 1 µM
DTT	< 3 M	> 3 M
Mercaptoethanol	-	Risk of toxicity
Imidazole	< 3 M	> 3 M
TFA	-	High risk of toxicity

Table 8: Compounds and formulations that are acceptable for immunisation

* It is possible to immunise animals with an antigen solution containing 8M-urea, but this is more painful for the rabbits. For this reason, we ask our customers to send us the antigen as concentrated as possible so that we can dilute the solution before injection in order to decrease the final urea concentration.
Antigens in solution should be sent in a volume not exceeding 500 µl per injection in case of rabbit, 250 µl for rat and guinea pig, 150 µl for mouse or 1000 µl for goat and sheep.

SDS-PAGE gel pieces

For antibody productions with SDS-PAGE gel fragments, we advise our customers to cut out the band of interest and to aliquot it in separate tubes for each injection. Injection amounts for rabbits depend on the antigen weight: 100 µg per injection for > 18 - 20 kDa proteins; 200 µg per injection for < 18 – 20 kDa proteins. The antigen tubes can be shipped at room temperature. The standard Coomassie staining procedure can be used since the Coomassie staining dyes do not interfere with the antibody evolution. The band should just be washed briefly but thoroughly in water to remove acetic acid and methanol residues, then cut into injection pieces, and aliquoted wet into safe lock tubes to avoid drying. The gel must not be dried or lyophilised, because this would make the fragmentation before injection more difficult. The advantage of antibody production with gel fragments is that the antigen diffuses slowly out of the gel and ensures therefore antigen presentation for a long time. The disadvantage is that the antigen is presented in a denatured form, which can result in difficulties when using the antibodies on native proteins, i.e. in immunohistochemistry or immunoprecipitation.

Please be advised that this immunisation strategy might be subject to ethical discussions and possible restrictions.

This format is not compatible at all with the Speedy 28-Day programmes, nor with mouse, rat and guinea pig when using the classical programme.

Complex Antigens – whole cells and protein mixtures

Under this term, we understand mixtures of proteins, components of the cellular machinery (i.e. ribosomes) or whole cells (i.e. bacteria or parts of tissues) and the lysates thereof. Antisera against complex antigens are often easier to obtain than against single proteins, because the number and variety of epitopes is incomparably higher. On the other hand, good antibody titres are not necessarily obtained against each component of the mixture, this depends on the respective antigenicity and quantity of each single component.

For such antibody productions, we advise to use higher antigen quantities in order to obtain the broadest possible antibody spectrum. As an example, we suggest for whole cells to use around 50 million cells per injection per rabbit. For cell lysates, we advise at least 200 – 500 µg total protein in 500 µl maximum per injection per rabbit.

Please be advised that the formulation of your complex antigen might be critical for the outcome of your project, feel free to discuss with us any precaution that might be appropriate for the handling of your antigen (proteomics.services@eurogentec.com).

Complex Antigens – viruses

This group of antigens comprises viruses that might be pathogenic or non-pathogenic for bacteria, plants, or animals. Whatever pathogenic or non-pathogenic, viruses used for injection must be inactivated. We recommend to use 100 µg of inactivated viruses or $5x10^9$ particles per injection in rabbit. The viruses should be sent aliquoted in solution in a volume not higher than 500 µl per injection.

Please be advised that the formulation of your complex antigen might be critical for the outcome of your project, feel free to discuss with us any precaution that might be appropriate for handling of your antigen.

Haptens

Haptens are small molecules, e.g. short peptides, hormones and a variety of natural products or synthetic molecules whose molecular mass is not sufficient to elicit an immune response upon injection. It is not possible to define an exact minimal molecular mass, starting from which antibody production is stimulated. In general, one can observe however that the direct antibody production with antigens of molecular masses lower than 8-12 kDa is difficult and quite risky.

In order to get antibodies directed against such small molecules, it is necessary to couple (or conjugate) the haptens to a carrier (usually an immunogenic protein) to make them "visible" for the host's immune system.

Due to the immunogenicity of the carrier protein, the immune system will be stimulated and antibodies will be produced against the carrier and almost every time against the co-displayed hapten molecule.
The most critical point with these antibody productions resides often in the covalent coupling of the hapten, because the haptens do not necessarily contain a functional group essential for the conjugation. We are happy to assist our customers in defining the best coupling strategy as well as to carry out the conjugation reaction subsequently.

Other Formats

Please contact us to discuss the immunisation of animals with your intended antigen format.

Antigens provided for purification

Throughout antibody affinity purification, antigens are bound to the column by their amine, sulfhydryl or acid group. Therefore, if you provide your antigen for purification, make sure buffer doesn't contain components with the same functional group as the one to be used for the coupling (e.g. SDS, Thimidine, Urea, anti-proteases, TRIS, DTT, MES, CHOPS, MOPS, Azide, Thimerosal, Mercaptoethanol and Coomassie blue). Otherwise, a dialysis will be performed to eliminate the interfering components and allow antigen fixation to the column. During the manipulation, some material can be lost.

Antigen quantities

Depending on the host species, we require different antigen amounts in order to obtain good antibody titres; the following table summarises recommended quantities per injection per animal. Since the antibody production depends also on the immunogenicity of each protein in the chosen species, it is not possible to indicate precisely the optimal antigen amount and the antibody yield. The table should however help choosing the best-suited animal species with respect to the antigen amount you have.

As small proteins (< 18 kDa) or coupled peptides (peptide = 1-2 kDa) display a smaller number of possible epitopes, better antibody titres are generally obtained by using higher antigen quantities. For this reason our standard quantity for peptide antibody production is 200 µg of coupled peptide per injection in rabbits.

The administered volume per injection is 500 µl plus 500 µl adjuvant. The amount of antigen in solution should therefore be delivered in a maximum volume of 500 µl.

Immunisation with viruses requires 5 x 10^9 particles per injection in rabbits.

Immunisation with bacteria is usually done without adjuvant and requires 5 x 10^7 cells per injection in rabbits.

	Min. antigen quantity / injection			Bleed volumes				
Host	**Antigen MW < 18 kDa**	**Antigen MW > 18 kDa**	**Max. volume injection**	**Pre-immune**	**Small Bleed**	**Large bleed**	**Final bleed**	**Comment**
Mouse	40 µg	15 µg	150 µl	40-70 µl	40-70 µl	40-70 µl	300-500 µl	Good to test antigenicity
Guinea pig	50 µg	30 µg	250 µl	1 ml	1 ml	2-3 ml	10-15 ml	For small serum volumes
Rat	50 µg	30 µg	250 µl	± 2 ml	± 2 ml	± 2 ml	5-7 ml	For small serum volumes
Chicken	200 µg	100 µg	500 µl	1 egg	1 egg	± 8-10 eggs	± 8-10 eggs	For mammalian antigens and large quantities of Antibodies, 4 eggs = 1 rabbit final bleed
Rabbit	200 µg	100 µg	500 µl	2 ml	2 ml	20-25ml	50-70 ml	For most applications
Goat	400 µg	200 µg	2 ml	2 ml	2 ml	250 ml	1000 ml	For large batch volumes
Sheep	400 µg	200 µg	2 ml	2 ml	2 ml	250 ml	1000 ml	For large batch volumes
Llama	400 µg	200 µg	2,5 ml	2 ml	2 ml	250 ml	1000 ml	Single chain antibodies and for large batch volumes
Horse	400 µg	200 µg	-	2 ml	2 ml	250 ml	1000 ml	For large batch volumes
Other	Contact us for recommendations							

Table 9: Minimum antigen quantities and expected bleed volumes

Shipping antigens

Antigens should be shipped along with a copy of the order form. Should you be shipping additional antigen for an on-going programme please indicate on the accompanying copy of the order form the antibody programme number that your antigen should be used for.

Antigen reception

Once we receive the antigen, the customer receives an acknowledgement of receipt of the antigen, containing the name of the antigen, the ordered animal species, the number of animals and an internal immunisation programme number, which should be referred to when communicating.

After establishing a customised immunisation schedule or offering one of our standard programmes, the programme data are entered into our tracking system allowing to store all important programme related details, like antigen quantities, aliquoting, schedules for injections and bleeding, and the overall structure of the programme. These data are automatically integrated into the daily task list of our immunisation department, and the tracking of these data allows us to find out any detail on the customer's programme.

Protein production services

If you do not have your protein of interest available in a suitable format or in sufficient quantities for immunisation, Eurogentec can provide you with a complete range of services related to protein production.

Eurogentec provides you the possibility to get your protein produced in bacterial cells (*E. coli*) and in eukaryotic cells (insect cells using the baculovirus system, and human embryonal kidney, HEK 293, cells). Eurogentec supports you to choose the correct production host for your protein, and the right scale for final production with an easy to use project definition file. Protein production with Eurogentec comprises the following steps:

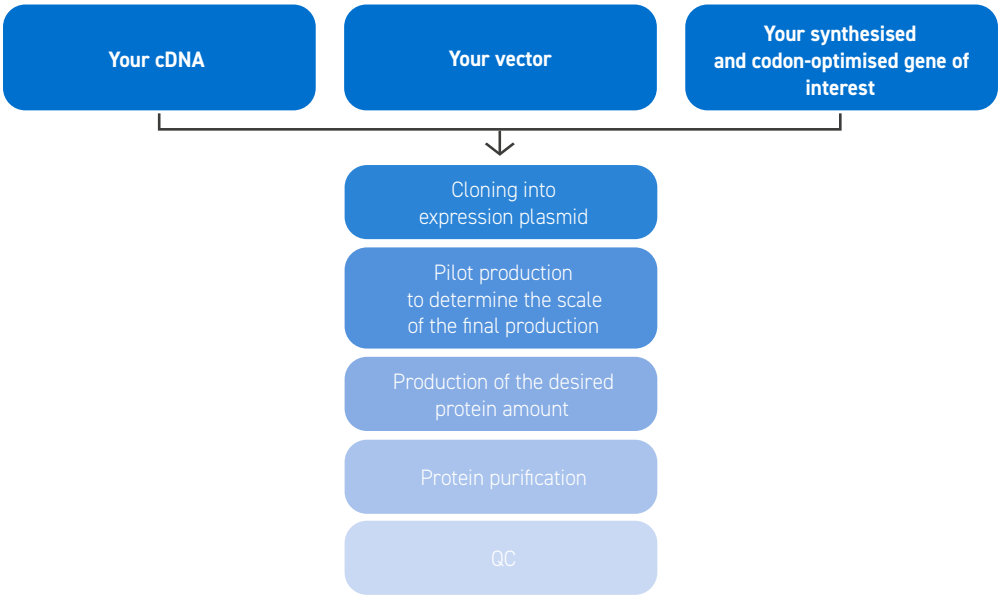


Figure 9: Protein production steps

The following time lines are required until you receive your protein:

Step	Mammalian / insect cell expression	Bacterial expression
Production of your codon optimised gene of interest by gene synthesis	3-4 weeks	3-4 weeks
Cloning of your gene of interest into the expression vector and QC	3 weeks	2 weeks
Pilot production, intermediate QC, and intermediate report	3 weeks	1 week
Final production	1 week	1 week
Protein purification, QC, and final report	1 week	1 week

Table 10: Protein production timelines

Proteins are usually produced in amount of 2-5 mg, but more is also possible. The proteins have usually a purity superior to 80 % and are characterised by:

> Analytical SDS-PAGE
> WB against the purification tag to probe the presence of the target protein
> WB against the target protein (optional)
> Protein quantification
> Purity determination

Proteins which have been produced this way can be used with any protein coupling service offered by Eurogentec:

> Conjugation to enzymes
> Conjugation to biotin
> Conjugation to fluorophores (FITC; rhodamine, phycoerythrine, HiLyte™ fluor dyes, CyLyte dyes,...)
> Binding to magnetic beads

Peptide antigens

When it is not possible to use the original protein of interest for immunisation, one can consider using one or two synthetic peptides whose sequences are judiciously chosen into immunogenic parts of the protein.

This strategy has, among others, the advantage of being much less time consuming and by far less expensive. An additional important advantage of the "anti-peptide" approach is the high specificity of the obtained antibodies, which can be compared nearly to a monoclonal antibody.

The advantages of peptide antibody production are demonstrated clearly by the following problem cases:

> Only the amino acid sequence derived from the corresponding DNA sequence is known
> Only a small part of the protein sequence has been obtained by Edman sequencing (N-terminal sequencing); this sequence information should be used for antibody production
> The protein is available only in small quantity; therefore the obtention of meaningful quantities for antibody production is too difficult or too time consuming
> The protein is toxic for production hosts, or for immunisation hosts (e.g. neurotoxins, or *Botulinus* toxin)
> Antibodies against one member out of a protein family are needed; antibodies against the whole protein would evidently cross-react with other members of the family due to homologies
> Antibodies against a determined phosphorylation, methylation, acetylation, or lipidation site are needed
> Antibodies against one out of several alternative splicing variants are needed
> A specific protein is to be purified by immunoprecipitation or affinity chromatography out of a crude cell extract; the antibodies necessary for this application are best obtained using synthetic peptides due to their extreme specificity

Designing an antigenic peptide

The goal of each peptide-based antibody production is to obtain antibodies that will recognise the corresponding protein. The choice of a well-suited peptide sequence is therefore crucial since the inherent risk underlying every peptide antibody production is that the obtained antibodies recognise the peptide, but not the protein from which the peptide has been derived. In order to minimise this risk, hydrophilic sequences should be chosen, because these have the best chances to reside at the surface of the protein and therefore to be accessible to the antibodies. Antibodies made against hydrophobic peptides by contrast have nearly no chance to bind to the protein, because hydrophobic sequences are most probably found at inner parts of the 3-dimensional protein structure, rendering these parts inaccessible for immunodetection methods. If the recognition of the native protein is necessary, i.e. for immunohistochemical studies, the necessity of free target accessibility of the used peptide is most important for the later detection. Additionally, it is to be noticed that short synthetic peptides are not flexible and are unable to mimic strong secondary structures like those found in proteins. It is therefore also important to take into account secondary structure elements in the protein of interest and to avoid as much as possible alpha-helical regions. With the help of modern algorithms, allowing for calculation of all these parameters, it is possible to limit the risk of failure during peptide antibody production to a large extent, especially if antibodies can be produced against more than only one peptide per protein.

Concerning the optimal length of the peptide, in general it is accepted that best results are obtained with peptides of 13-16 amino acids. Longer peptides have the advantage of containing more possible epitopes, but on the other hand, quite stable secondary structures may form, which not necessarily mimic the secondary structure of the targeted part in the native protein. Also the risk of more unwanted homologies with unrelated proteins is increased with longer peptides.

Shorter peptides can be used upon homology problems; they should however as far as possible not contain less than 10 amino acids in order to keep some chances that the peptide contains at least one good epitope.

Depending on the nature of the projects, part or the whole protein sequence is analysed. Our customers receive a suggestion of several well-suited peptides to check the selected sequences for desired or unwanted homologies to other proteins. The used database for these alignments is stored internally at Eurogentec, so there is no risk of confidentiality breaches, which would be apparent by using free-accessible Internet databases for sequence analysis.

We ask to our customers to send the amino acid sequence of their protein, or its database accession number to us at peptide.design@eurogentec.com.

In our hands, the success rate using only one peptide per protein and after correct sequence analysis turns around 75 % statistically. If the antibody production is carried out with two peptides out of the protein sequence, the failure risk will be reduced from 25 % to 25 %² equalling only 6.25 %. In turn, the chance of success increases from 75 % to about 90-95 % (success = the protein is recognised in Western blot).

Thanks to our long experience in anti-peptide antibody programmes, Eurogentec has optimised the use of a combination of specific algorithms for peptide selection.

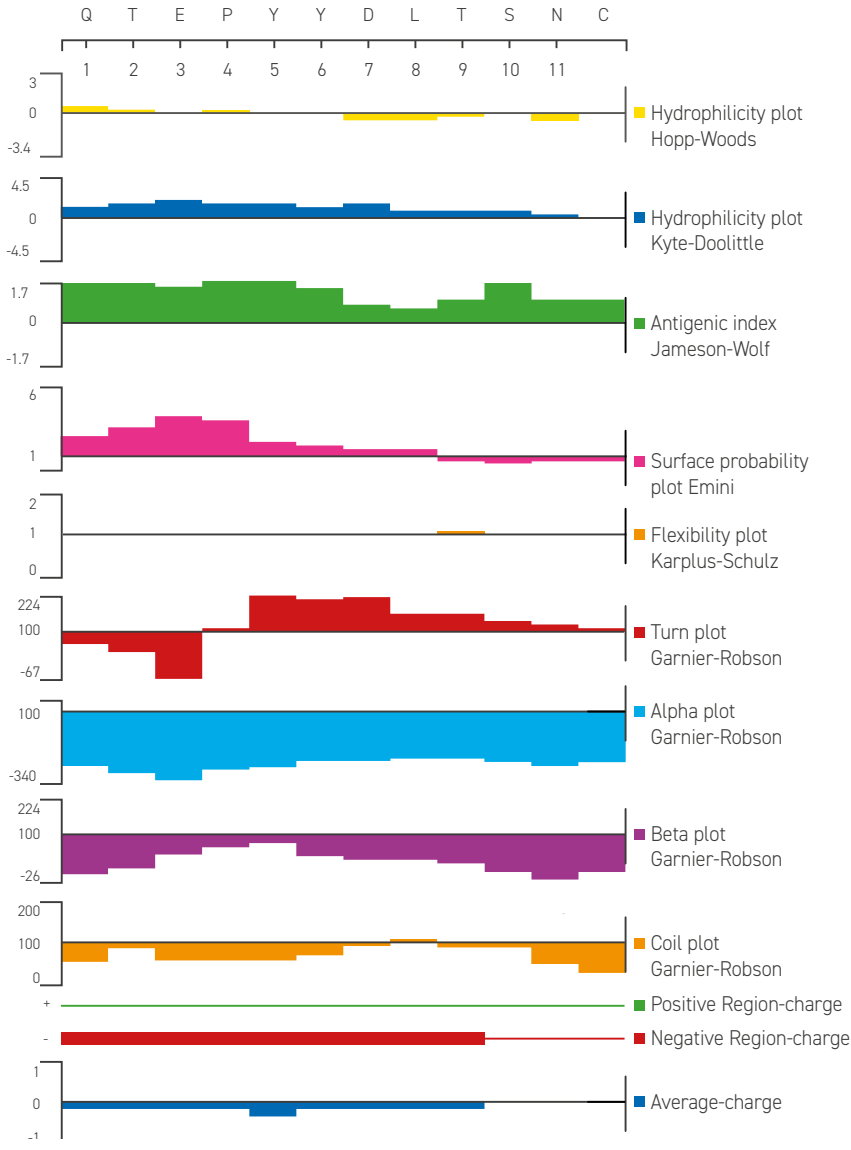


Figure 10: Parameters for peptides choice

Coupling Peptides to Carrier Molecules

Peptides of about 15 amino acids have a molecular mass in the range of 1.5-2 kDa. This molecular mass is generally not sufficient to elicit an immune response. For this reason, synthetic peptides must be converted to higher-molecular mass products.

This can be achieved by using two strategies:

MAP carriers

The first possibility is to synthesise the peptide on a special support, the so-called MAP resin (MAP = Multiple Antigen Peptide). In this approach, 8 copies of the peptide are synthesised on a poly-lysine core (see scheme). After cleavage from the synthesis resin, the resulting MAP peptide reaches a molecular mass of about 13-17 kDa, which is sufficient for a direct antibody production.

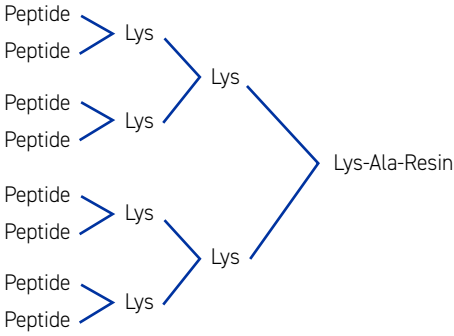


Figure 11: MAP resin illustration

Carrier proteins

The second possibility consists in a covalent coupling of the peptide to a so-called carrier protein. The best-known carrier proteins are BSA (bovine serum albumin), OVA (ovalbumin), THY (bovine thyroglobulin), and KLH (keyhole limpet hemocyanin). These carrier proteins all have a high MW and a good antigenicity.

In addition, the carrier proteins contain T-cell epitopes, leading to a proliferation of T-helper cells. As the carrier proteins are recognised as foreign by the immune system of the immunised animal, the whole system becomes stimulated very effectively.

Functional antibodies can be obtained by both coupling strategies; it seems however that the carrier protein method has several advantages over the MAP method and it still remains the most commonly used technique. Although the MAP method often results in better antibody titres, the antibodies seem to recognise less often the protein. An additional disadvantage is that the MAP peptides are not suited for the negatively charged C-terminal peptides (see also below), which are quite often good candidates for antibody production.

One decisive advantage of the carrier protein method, beside the possibility to use C-terminal peptides, is that the remaining quantity of uncoupled peptide can easily be used for testing and especially for affinity purification of the obtained antiserum, which is not the case for MAP-coupled peptides. Moreover, the peptide can be coupled to two different carrier proteins and strongly increased antibody titres can be obtained using alternatively both coupled peptides for booster injections.

In the following, we describe the different methods that are used to couple specifically and covalently synthetic peptides covalently to the carrier protein. A specific coupling means that the peptide is linked to the carrier protein by only one side of the peptide.

In peptide antibody production, by using a short synthetic peptide, one tries to imitate as closely as possible the original protein. If the peptide was linked at its middle part to the carrier, only short "arms" flanking each sides of the coupling site would be presented for antibody production. The antibodies directed to the middle part of the peptide will not work on the protein because they recognise also the carrier.

Therefore, the link of the synthetic peptide to the carrier protein should be placed either at the N-terminus or at the C-terminus of the peptide, ensuring by this way a "good mimicking" presentation of the peptide. This also allows for the presentation of the longest possible part of the peptide sequence not containing any interruption caused by a linker reagent.

It is to be noticed that a peptide corresponding to the N-terminus of the protein must be linked by its carboxy-terminal end to the carrier protein in order to guarantee a natural presentation by the N-terminal positive charge. In the same way, peptides corresponding to the C-terminus of the protein must be coupled to the carrier protein through the N-terminal residue of the peptide sequence, in order to receive the C-terminal negative charge. MAP peptides, which do not have a free carboxyl end, are therefore not suited for antibody productions against peptides from the real C-terminus of the protein.

Internal peptides may be coupled at either end. It is however advised to carry out the coupling at the less antigenic side of the sequence. Another point to be considered in the analysis is the choice of the C- and N-termini of the peptides. If the peptide is chosen from an internal part of the protein, the natural structure is better mimicked by acetylation of the N-terminus (if the coupling is done C-terminally) or by amidation of the C-terminus (if the coupling is done through the N-terminus of the peptide). By this way, one avoids the introduction of a non-natural positive charge at the N-terminus, or negative charge at the C-terminus of the synthetic peptide.

Depending on the amino acid sequence of the chosen peptide, one of the following coupling strategies can be used to link the peptide covalently and in a specific manner to the carrier protein.

The most commonly used carrier protein is **keyhole limpet hemocyanin (KLH)**, a respiratory heme protein from the sea snail *Megathura crenulata*. This protein is commonly used because it is a large protein complex with no homology to vertebrate proteins, and no homology to proteins that are used in the most common laboratory applications for blocking, like BSA. A rabbit will produce antibodies against the carrier KLH; however, this reactivity will not disturb immunological analyses of vertebrate tissues. Since rare cases have been described where KLH reactivity might interfere negatively with experiments carried out on plant samples, you should contact us at info@eurogentec.com to find out if KLH is the suitable carrier for your immunisation project.

For development of antibodies against targets in invertebrates (Drosophila, or Caenorhabditis for instance), the carrier proteins commonly used are **bovine serum albumin (BSA)** or **ovalbumin (OVA)**, which lack homology in species from these phyla.

Thyroglobulin (THY) has also beneficial properties as carrier protein in terms of being highly immunogenic, which has to be considered carefully. Also an advantage of THY is its high MW and dimeric structure, which is similar to KLHs account for more potential peptide conjugation sites on the molecule such as BSA and OVA. THY like KLH does not belong to the group of proteins that are used in immunological applications as blocking compounds, therefore the THY reactivity of serum does not interfere with down stream applications. A negative point of using of THY might be the creation of auto antibodies over extended immunisation periods, leading to autoimmune diseases such as Grave's disease or Hashimoto thyroiditis in the immunised mammalian host. For prolonged immunisations THY as carrier can be used in chicken without further problems, but in rodents THY should be replaced by KLH.

Carrier	Advantages	Disadvantages
KLH (Key hole limpet hemocyanin)	> Not used in ELISA or Western blotting > Suggested for vertebrate > High number of peptide conjugation sites	> Might cause problems if invertebrate tissues, or certain plant tissues should be examined with crude serum
BSA (Bovine serum albumin)	> Better suited for monoclonal production than KLH > Can be used as alternative carrier to KLH in chicken	> Reduced number of peptide coupling sites compared to THY or KLH > Lower number of peptide conjugation sites
OVA (Ovalbumin)	> Can be used as alternative carrier to KLH in mammalian hosts	> Suggested for invertebrates > Lower number of peptide conjugation sites
THY (Thyroglobulin)	> Not used in ELISA or Western blotting > Can be used in chicken and mammals instead of KLH > High number of peptide conjugation sites	> Prolonged immunisation might cause auto immunity to THY of the mammalian host (induction of thyroiditis)

Table 11: Advantages and disadvantages of the different carrier proteins

Single peptide immunisation protocol

Over years Eurogentec has made a lot of efforts to optimise the peptide selection, synthesis, coupling and immunisation protocols, retaining in the same time the greatest possible flexibility concerning special wishes from our customers. Our well-proven classical and Speedy 28-day programmes for peptide antibody production reflect the needs of most customers; the complete package consists of the following services (example for rabbit immunisation) :

> Assistance in peptide selection from the protein sequence as well as determination of the best possible coupling strategy
> After agreement on the peptide sequence, synthesis of 15-25 mg of the peptide, HPLC purified and MS quality controlled
> Coupling of 5 mg peptide to a carrier protein (KLH or BSA, OVA, THY) or synthesis of a MAP peptide
> Antibody production in 2 rabbits during 3 months according to our classical programme (4 injections, 4 bleedings) or 28 days according to our Speedy 28-Day prggramme (4 injections, 3 bleedings)
> Delivery of the remaining quantity of free peptide to the customer; this peptide can be used for testing as well as for affinity purification of the antiserum
> Shipments of antiserum on dry ice

	Min. antigen quantity / injection	Amount needed for standard immunisation	Residual amount for additional injections	Additional injections (please contact us)
Host (hosts/programme)	**Coupled peptide**	**Coupled Peptide / no. injections**	**Coupled peptide**	**Injection/animal**
Mouse (3)	40 µg	480 µg/12	4.5 mg	37
Guinea pig (2)	50 µg	400 µg/8	4.6 mg	46
Chicken (2)	200 µg	1600 µg/8	3.4 mg	8
Rabbit (2)	200 µg	1600 µg/8	3.4 mg	8
Goat	400 µg	Please contact us		
Sheep	400 µg	Please contact us		
Llama	400 µg	Please contact us		
Horse	400 µg	Please contact us		

Table 12: Amount of coupled peptide for injection

Amount of peptide synthesised	Amount of peptide to couple to carrier	Amount of coupled peptide to inject	Peptide used for further test or purification: required peptide	Remaining free peptide for your applications
15 - 25 mg	5 mg	200 µg per injection	ELISA test/ plate: 10 µg	10 - 20 mg
15 - 25 mg	5 mg	200 µg per injection	Affinity purification 50 ml AS-PURI-01: 10 mg	0 - 10 mg
15 - 25 mg	5 mg	200 µg per injection	Affinity purification 5ml AS-PURI-SM: 5 mg	5 - 15 mg
15 - 25 mg	5 mg	200 µg per injection	Affinity purification 20 ml AS-PURI-MED: 8 mg	2 - 12 mg

Table 13: Peptide consumption of immunisation (typical programme), optional additional services and remaining free peptide

Double-peptide strategy (DoubleX)

The inherent risk of each peptide antibody production is that the antibodies, although recognising the peptide used for immunisation, do not recognise the protein from which the sequence has been derived. This risk has a likelihood of about 25 % despite the peptide design and selection being carried out correctly. We therefore advise our customers, especially those who are working on less characterised proteins or need good working antibodies, to work with more than only one peptide during immunisation.

If the antibody production is carried out with two instead of one peptide out of the protein sequence, the failure risk will be reduced from 25 % to 6.25 %. In turn, the chance of success increases from 75 % to about 90-95 % for Western blot as application using two peptides derived from the same protein for immunisation.

To provide our customers this benefit as immunisation option, Eurogentec has created the DoubleX programmes as complete package, containing (rabbit immunisation is taken as example) :

> Design of two peptides
> Synthesis of 15-25 mg of each peptide

> Coupling of 5 mg of each peptide to the desired carrier molecule (the standard would be KLH)
> Antibody production in 2 rabbits during 3 months according to our classical programme (4 injections, 4 bleedings) or 28 days according to our Speedy 28-Day programme (4 injections, 3 bleedings) : a mix of the 2 peptides is used as antigen
> Delivery of the free remaining peptide quantities to the customer for further applications or services
> Shipments of antiserum on dry ice

In principle, these programmes can be considered to replace two normal programmes with the difference being the use of just two rabbits instead of four, resulting in a considerable cost reduction for customers. On the level of the antisera the advantage lies in the fact that the sera from both animals should contain polyclonal antibodies against both peptide epitopes.

Detection of post-translational modifications (PTMs)

Post-translational modifications of proteins and processing alter the biological activity of nearly every protein after their translation. These molecular modifications are responsible to control the activity of the proteins, their localisation and their degradation. Modification specific antibodies are useful tools in the study of cell signalling, protein maturation, protein localisation, protein activity etc.

Eurogentec is capable of producing antibodies against several types of modifications mentioned below. If your desired modification is not amongst those, contact us at info@eurogentec.com to discuss the generation of an antibody against your post-translational modification of interest.

Such type of immunisation programmes is available for both standard (87-day) and Speedy (28-day) programmes.

Phosphospecific antibodies

Our phospho-specific anti-peptide programmes are designed to detect the presence of phosphorylated serine, threonine, or tyrosine in a sequence and protein specific context. The immune response evolution during a phosphospecific programme is monitored by ELISA screening.
The antibody is double affinity purified from serum to ensure specificity (see figure p.35). The crude serum is passed through a first column coupled with the phospho-peptide. The sequence specific and phosphate sequence specific antibodies are captured. The unrelated antibodies are in the flow-through of the column and the specific antibodies are recovered (programme specific: Peak 1). The second column is coupled with the non-phosphorylated peptide. The sequence specific and phosphate sequence specific antibodies recovered from the first purification are applied, only the non-phosphospecific antibodies are captured, the desired phosphospecific antibodies are recovered in the flow through (programme specific: Flow through 2).

Methyl specific antibodies

Our methyl-specific anti-peptide programmes are designed to detect the presence of mono-, di-, or tri-methylated lysine, or mono-, symmetrical and asymmetrical di-methylated arginine in a peptide sequence and protein specific context. The immune response evolution during a methylspecific programme is monitored by ELISA screening.
The antibody is double affinity purified from serum as explained in the following figures.

Double purification

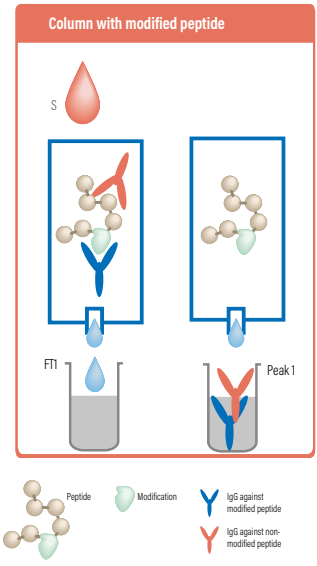


Figure 12: Affinity purification 1

Isolation of all antibodies specific to the peptide carrying the modified amino acid

After selection of the best responding animal upon immunisation, ~ 50 ml of serum (S) are applied to an affinity column 1 with the immobilised modified peptide. During this step, the antibodies that will be retained are:

A) Sequence specific for the peptide

B) Specific for the modification in the peptide

Unrelated antibodies will be separated from the desired antibodies by flow through (FT1), and the washing steps after binding.

After elution of the mixture of peptide sequence specific antibodies (A) and modification specific antibodies (B) from column 1 (Peak 1), the antibody mix will be characterised by ELISA, and applied to a second affinity column carrying the immobilised non-modified peptide.

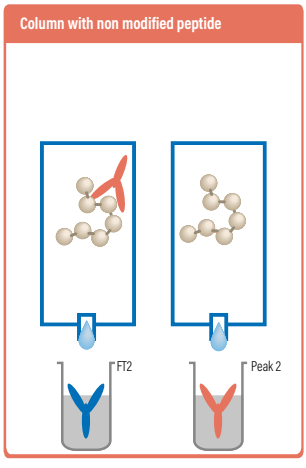


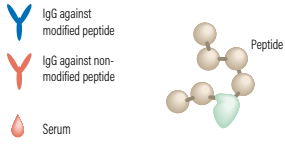
Figure 13: Affinity purification 2

Isolation of modification specific antibodies

Column 2 will retain the antibodies, which are specific to the non-modified peptide (Peak 2).

The antibodies of type B, which are specific to the desired modification, will be harvested from the flow through, and characterised by ELISA (Flow through 2, FT2).

The antibodies of type A, which are non-specific to the desired modification are eluted (Peak2).



Detection of other modifications

The detection of any modification depends on the production of the peptide carrying the modification. Beside phosphorylation and methylation specific antibodies, Eurogentec can also produce antibodies against the following post-translational modifications :

> Acetylation
> Proteolytic processing (neo-epitopes)
> Sulphatation
> Citrullination
> Nitrotyrosine
> Met/Cys sulfoxide
> Lipidation
> SUMOylation

3 MONOCLONAL ANTIBODY PRODUCTION

Monoclonal antibodies are monospecific antibodies that are produced by one type of B-cell. The produced antibodies are all identical, because all antibody producing B-cells are derived from one selected clone. Monoclonal antibodies can be produced with almost any given antigen. Monoclonal antibodies have been evolved to an important tool in biochemistry, molecular biology, and medicine in order to fulfill tasks where high accuracy and efficiency of detection is required, like in diagnostic and therapeutic applications.

Since 1996, Eurogentec has been offering the completely customised production of monoclonal antibodies and has gained a profound know-how and experience in this field.

Every project concerning the development of a monoclonal antibody should be discussed in detail before start in order to make sure that the important parameters will be accordingly addressed during the production. Our specialists are open to all customer needs, which may strongly differ from our standard procedures. Our constant aim is to stay as flexible as possible and to secure the best possible success rate for your products.

The standard programme of monoclonal antibody production is divided into 4 different phases, our customers being informed and keeping control at any time on the production progress, this to keep the door open to any programme modification that would be needed along the project.

The mAb has long been produced in ascite, the hybridomas being injected into the peritoneum of rodents, proliferating and being stored in ascitic fluid that is subsequently collected. Considered unethical, this method is associated with pain in the animal and since 2010, the European legislation highly recommends the *in vitro* mAb production.

All monoclonal and polyclonal immunisation projects are confidential. All obtained hybridoma, sera and results are and remain the property of the customer. Eurogentec guarantees not to claim any rights on the hybridoma or the antibodies. If desired, confidentiality agreements concerning our production and the customer's purpose can be signed.

Production flow chart

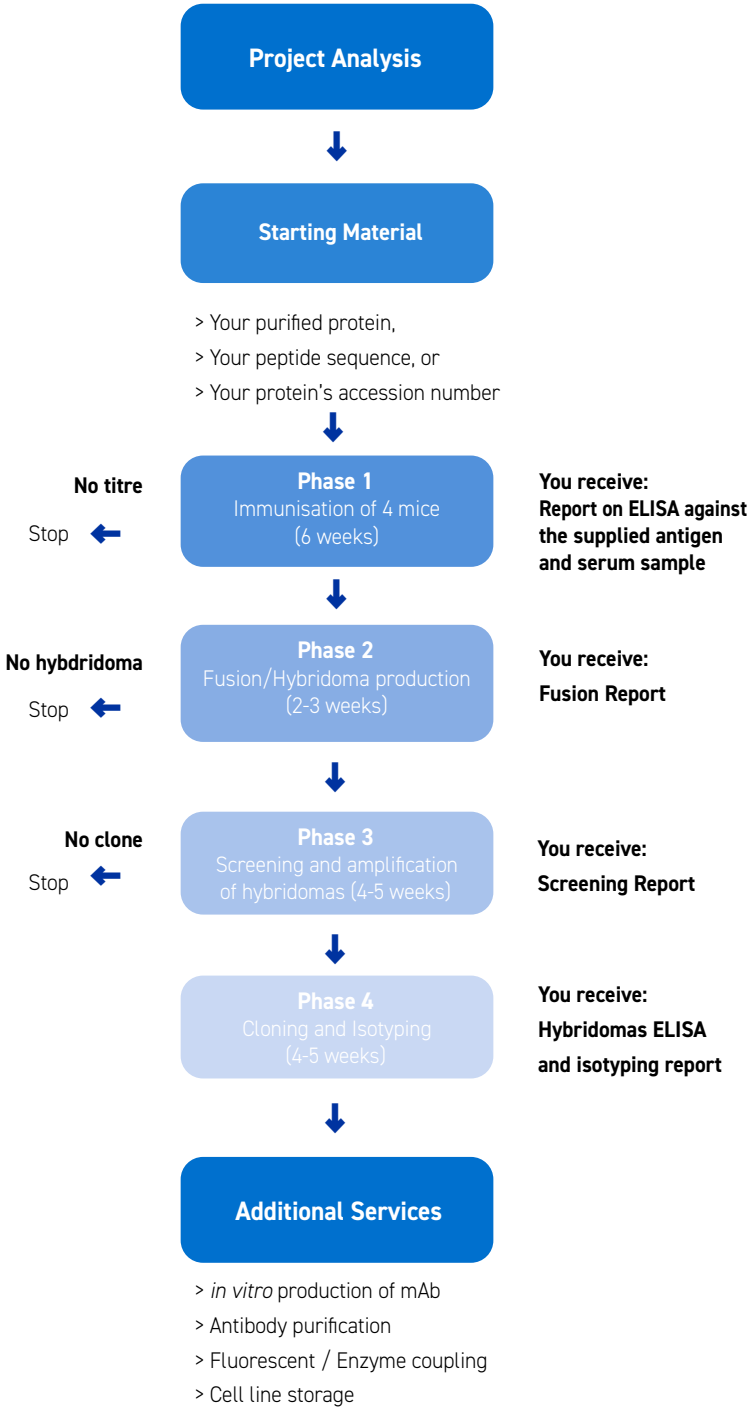


Figure 14: Monoclonal antibody production flow chart

Antigens for monoclonal antibodies production

In contrast to polyclonal antibodies, a monoclonal antibody recognises just one epitope of a given antigen. As described already for polyclonal antibodies, the antigens can be of different molecular size.

Antigen	Provided by Eurogentec	Your antigen	Comments
Compounds (haptens)	Yes, if available	Yes	The feasibility in terms of coupling or toxicity has to be discussed
Peptides	Design, Synthesis, QC, and coupling to carrier	Yes	Sequence information from customer peptide is required for the right coupling strategy
Proteins	Production, purification, and QC	Yes	Please refer to page 22 for formulation guidelines, lyophilised protein can also be used
Blot Membranes or Gel Pieces	No	No	Too harmful for small rodents, both methods might be subject to ethical restriction in the near future

Table 14: Antigens suited for the production of monoclonal antibodies in mice and rats

The antigen should be provided lyophilised or as a concentrated solution (min. 250 µg/ml), if possible in PBS or physiologic salt solution (0.9 % NaCl). It should be shipped frozen on dry ice to the Antibody Production Department at Eurogentec.

Please contact us at monoclonals@eurogentec.com to discuss the nature of your antigen, its shipment, or its suitability for monoclonal production.

Mouse monoclonal antibody production

Mouse monoclonal antibodies have been established in research during the Seventies. Monoclonal antibodies are typically made by fusing the spleen cells from a mouse that has been immunised with the desired antigen with myeloma cells that are immortal and have lost the capability to produce immunoglobulins. The myeloma cells used for hybridoma production is the cell line Sp2/OAg 14.
The production of monoclonal antibodies in mice by Eurogentec can be subdivided into four phases:

> Phase 1: Immunisation
> Phase 2: Fusion
> Phase 3: Screening for positive hybridomas
> Phase 4: Cloning and isotyping

Phase 1 - Immunisation

We use four mice as a minimum for the production of monoclonal antibodies. We generally use 50 micrograms of antigen per animal and per injection. Therefore the total quantity of antigen necessary to get the production done should be at least 1 mg of pure antigen.

Standard immunisation protocol for monoclonal antibody production:

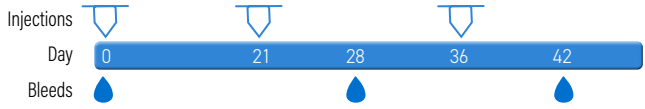


Figure 15: Phase 1 - Immunisation: injection and bleeding scheme

Upon completion of the last test bleed, we perform an ELISA test of the sera obtained against the antigen. The results of these tests are sent together with the remaining sera to the customer, so he can check using his own conditions for the presence of functional antibodies and ask us eventually to perform an additional boost. If the ELISA test showed too low titres or was nearly negative, we advise the extension of phase 1.

After successful immunisation the best reacting animal is selected in accordance with the customer for fusion. A last booster injection is performed 6 days before the fusion step.

Phase 2 - Fusion

After the final bleed, the spleen of two animals (the best responding and one more as a guarantee) are surgically removed. Subsequently, the lymphocytes are isolated and taken up into culture medium. The immunoglobulin-producing lymphocytes cannot be cultured for long time; they will die after only a few cell divisions. In order to keep them alive in culture medium, they have to be fused with a mouse immortal cell line. We use the myeloma Sp2/0Ag 14 (ATCC CRL 8287) and we perform 2 fusions.

The resulting fusion mixture contains both the desired hybrid cells, the non-fused lymphocytes and the myeloma cells, which have to be separated subsequently. This separation is done by a chemical way.
The fusion cocktail is distributed on eight 96-well plates with HAT medium (HAT=Hypoxanthin-Aminopterin-Thymidin). Any remaining fusion mixture is stored frozen in liquid nitrogen. As the used myeloma is hypoxanthine-guanine-phosphoribosyl transferase-negative, non-fused myeloma cells cannot survive in HAT medium. The non-fused primary lymphocytes will die as well during a short time period. The only cells surviving and dividing in this medium contain the immortality (provided by the myeloma) and the capability to produce and secrete IgG (contributed by the spleen B-lymphocytes) and are called **hybridoma**. These have to be selected and grown. This process of selection and cloning of the cells is described in the next phases.

In general about 5-20 % of the obtained hybridoma cells secrete antigen-specific antibodies, around 30 % do not secrete any IgG and the remaining produce antibodies of unknown specificity.

The whole fusion process takes about 2-3 weeks time.

Phase 3 - Screening

The supernatant of the cells cultured in individual wells resulting from phase 2 are tested by ELISA in the presence of antigen. Specific antibodies and hybridoma cells of each positive well will be frozen and stored in liquid nitrogen (two tubes per positive well). The positive hybridomas are grown further and will be tested again for the presence of antigen-specific antibodies.

At this point, Eurogentec can also **test the secreted antibodies against other antigens** than the one used for immunisation, i.e. for excluding unwanted specificities (cross-reaction with other proteins of a family or with compound related molecules) or for selecting antibodies against a certain region of the antigen (a protein domain or peptide). We propose also other tests like Western blots.

At this stage of the project, the customer receives up to 30 positive supernatants (1 ml each) with their ELISA data. We propose to our customer to test them in their experimental conditions to confirm that the secreted antibodies are suitable for their specific application.

This part of the work takes about 4-5 weeks; however there may be some variation in this schedule depending on the growth rate of the individual hybridomas.

Phase 4 - Cloning and isotyping

Up to this phase, work is done with a mixture of hybridomas and part of them only are secreting the desired antibodies. The aim of this phase is to perform serial dilutions of the hybridoma mixture to isolate a single monoclonal antibody-producing hybridoma clone. The method used for this purpose is known as "limiting dilution cloning".

The principle of the method is to dilute the mixture in such a way that, upon distribution in multiwell plates, one can expect statistically only one cell per well. The resulting cell population should therefore be derived from only one "mother cell" and therefore should secrete only one type of antibody – the desired monoclonal antibody.

In practice, the cell culture, after counting the number of cells, is dispensed in ELISA plates in such a way that the first row (A) contains statistically five cells per well, B and C rows 2 cells per well, row D one cell per well, and E and F rows statistically 0 and -1 cell per well. After culturing, the plates are checked under the microscope and wells containing only one colony are marked and selected for further processing. The resulting supernatants from these wells are checked for the presence of functional antibodies and the positive clones are again subdivided until all supernatants of the individual subclones show an equally positive antibody response. Here too, the supernatants can be tested against other antigens for cross-reactions. This phase of cloning is work-intensive and takes several weeks. Depending on customer's wishes and results, one or several monoclonal antibody producing clones are obtained at the end. We store 3 tubes of every clone in liquid nitrogen and send more than 10 ml of each positive supernatant to the customer who can check them for presence of the wanted antibody. In addition we determine at this point the isotype of each monoclonal antibody using ELISA test.

At the end of the project, the customer receives 1-2 positive hybridomas with the whole project report.

In vitro production

Monoclonal antibodies can be produced at any time and in any quantities with constant quality. Eurogentec produces mAbs exclusively *in vitro* in accordance with Belgian animal regulations and the 2010-63-UE directive for animal welfare. This production mode combines the advantages of generating high quality antibodies in a very reproducible manner. Starting from a hybridoma, which can result from our own production service or alternatively be sent to us by the customer, Eurogentec produces monoclonal antibodies in quantities ranging from milligram to gram scales.

The monoclonal antibody is produced by cell culture followed by the purification of the secreted antibodies from the culture medium. The culture can be scaled from flasks up to bioreactor (>1000 litres) formats. Depending on the mAb amount requested and the hybridoma production yield, we select for you the most adapted production system which will guaranty the best production level. For large-scale production batches (>200 mg), a test run is always performed, to assess the clone productivity. The cost for this test run is deduced from the subsequent production run in which the defined parameters are applied.

We use to work with a bioreactor unit which is based technically on the so-called hollow-fibre technology. The hybridoma cells are incubated in a large number of hollow fibres capillaries. The fibres are porous, like tubing used for dialysis, and allow diffusion of molecules. The used hollow fibres are composed of Cell Pharm System 100 membranes of a certain diameter, so that dissolved substances like gases and nutrients can freely pass through, while hybridoma cells or produced antibodies cannot (cut-off = 30 kDa). Using a pump, fresh culture medium is constantly passed though the fibres ensuring the continuous feeding of the hybridoma cells, which are present in the extra capillary space.

The oxygenation of the cells is regulated automatically by an oxygenator, which also regulates the acidity of the culture medium using a pH probe and carbon dioxide gas connection.
The produced antibodies are harvested automatically from the extra capillary medium.

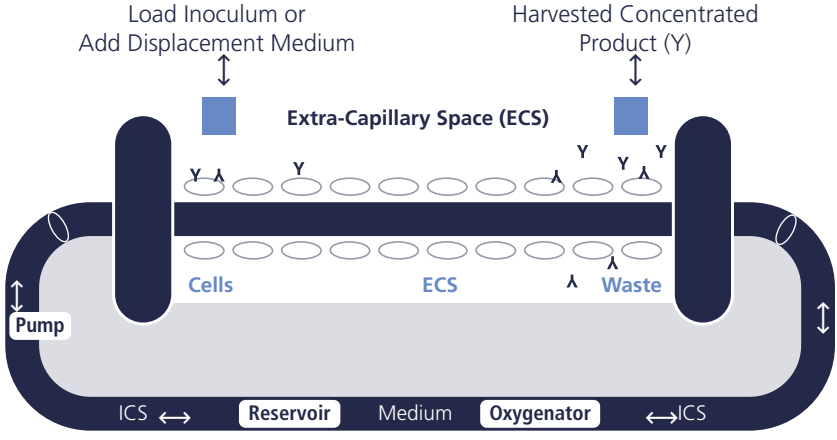


Figure 16: Schematic working of the hollow fiber *in vitro* production system

Cell banking

Beside performing *in vitro* production of your monoclonal antibodies, Eurogentec offers you a banking service of your hybridoma cells.
Please contact us at monoclonals@eurogentec.com to get more information on our cell banking service.

Figure 17: Autoharvester

4 ADDITIONAL SERVICES

Pre-immune sera screening

The preimmune serum represents the only real negative control for antisera in your experiments, since it displays the background signals prior immunisation programme. Rabbits take up with their food a lot of plant proteins, but also some ubiquitously occurring microorganisms, like bacteria, yeast and fungi. It is therefore possible that the serum before injection already contains antibodies against these types of antigens. It is for example well known that many animals produce antibodies against some food borne proteins or against *E. coli* and other bacteria, which they take in with the food or from the excrement.

Such antibodies may be extremely disturbing in some research fields and even render the immune sera nearly useless unless affinity purification is applied. In order to prevent this possible problem, Eurogentec offers the customer the possibility to test sera from a respective number of animals before antibody production. If ordered, Eurogentec collects sera samples of 5, 10 or 20 animals and ship them to the customer who can test each serum (identified by a number) and choose the best-suited animals showing the lowest background for antibody production. During this test period, we keep the animals reserved (for about 2 weeks) for the customer until final choice.

By this easy precaution down stream problems can be avoided in many cases, making for example subsequent affinity purification unnecessary. A simple purification using protein A or protein G generally does not allow reducting background signal since the antibodies responsible for these signals are isolated in the final IgG fraction, too. It is therefore crucial to perform real affinity chromatography procedures, which have the disadvantage that quite large quantities of pure antigen are required; very often such quantities are obtained only with difficulties (e.g.: proteins from inclusion bodies in purification competent form). For this reason, we advise all customers possibly concerned by this type of problems to use this original service of preimmune testing.

Monitoring of the immune response by ELISA

ELISA testing is a semi-quantitative method to measure the reactivity of a particular antibody against its dilution, and to follow the evolution of an immune response during an immunisation. This is an excellent option to determine if an antibody programme can be terminated or should be extended, or for the selection of the best responding animal.

ELISA tests are also of prior importance during the anti-peptide programmes. Our large experience on this matter showed us that the reactivity increases with the different booster injections up to a certain maximum value after which it often starts to decrease suddenly and irreversibly. This is the time point when the final bleed of the animals should be carried out in order to avoid the risk of loosing the generated immune response. In most cases this sudden decrease in response can be observed after 5-7 booster injections, sometimes it happens earlier, but it is detectable by ELISA screening.

ELISA screening

ELISA screening can be combined with any immunisation strategy offered by Eurogentec.

The ELISA testing of the antisera can be done against your antigen or the free peptide we produce for the anti-peptide projects.

The ELISA tests are carried out in one 96-well plate per animal. We test in parallel, per ELISA, dilutions from:

> Preimmune bleed
> Small bleed
> Large bleed

Against

> The free peptide
> The carrier protein
> Positive and negative controls

The test report includes the experimental parameters, the signal intensity values and the graphical plot of signal intensity against dilution. It is sent to the customer together with the free peptide and all the tested antisera. This will allow the customer to check directly the activity of the antisera against his protein in his application having consolidation by the corresponding ELISA results. This parallel testing not only allows to have a good view of the antibody evolution with the last boost, but it also gives a relatively good decision facilitation for programme prolongation with additional boosts and bleeds, scheduled termination, or immediate termination.

In case a good increase is observed between the first and the second test bleed, the chances to get still higher titres with additional injections are likely. If however both bleeds (first and second test bleeds) show more or less the same titre, an additional injection will most of the time result in only slight or no titre increase of specific antibodies.

Example of results

ELISA plots of successful immunisations show flat curves for the pre-immune bleeds, and sigmoid shaped curves for the large bleeds as depicted in the plot below.

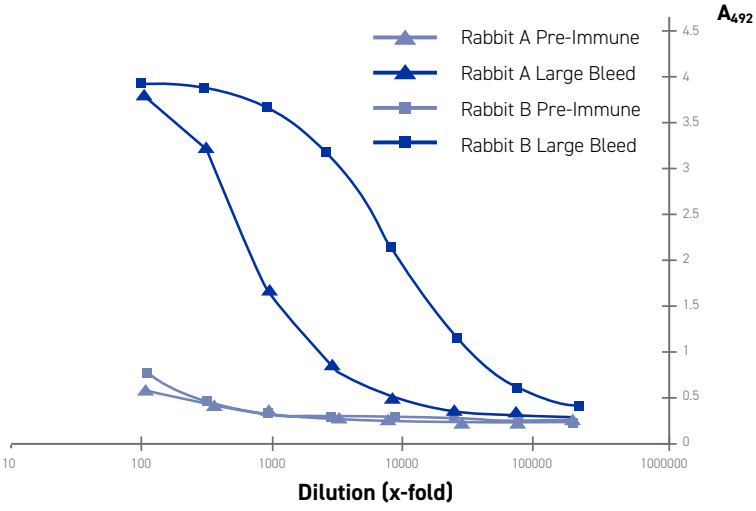


Figure 20 : Sample ELISA results for the pre-immune and large bleeds from two rabbits. This example shows a difference in animal response to the immunisation programme. An ELISA test allows you to select the best responding animal.

Antibody yields in different types of sera

As a statement one can generalise that the same antigen in different animals of one species does not necessarily produce the same immune response. A similar variety is also applicable for the serum concentration of antibodies between different individuals of one species, and there are also striking differences between different species. The following table reflects the concentration ranges of different immunoglobulins that are typically found in the sera from different species.

Species	IgG (mg/ml)	IgM (mg/ml)	IgA (mg/ml)	% κ λ
Human	7.5 – 22	0.2 – 2.8	0.5 – 3.4	67/33
Mouse	2 – 5	0.8 – 6.5	1.0 – 3.2	99/1
> Monoclonal: Cell culture supernatant*	0.01 – 0.05	N/A	-	N/A
Rat	5 – 7	0.6 – 1.0	0.1 – 0.2	99/1
Rabbit	12 – 14.5	0.3 – 0.6	0.4 – 0.8	90/10
Goat	18 – 24	0.8 – 2.0	0.1 – 0.9	1/99
Sheep	18 – 24	0.8 – 1.8	0.1 – 1.0	1/99
Pig	17 – 24	1.0 – 3.4	1.3 – 2.6	50/50
Cow	9 – 24	1.9 – 3.9	0.5 – 1.2	1/99
Horse	11.5 – 21	1.0 – 3.0	1.0 – 4.0	1/99

Table 15: Typical immunoglobulin concentration ranges in sera and cell supernatants.
*The yield depends on the culture format and the duration of production.

Antibody purification

Total IgG purification

The serum from immunised animals contains in addition to the antigen-specific antibodies a lot of non-specific antibodies as well as large quantities of other proteins, such as albumin. In some cases the crude serum is usable in Western blots simply after proper dilution, but, in general, the crude serum produces non-specific reactions in the desired application and thus requires a purification process.

With respect to monoclonal production it is to mention that monoclonal antibodies from culture supernatant are generally present in quite low concentrations and a purification is almost always required to concentrate them. In the case the produced antibody is intended to be coupled to a fluorophore, biotin, or an enzyme, it is crucial to purify the crude serum in order to eliminate non-specific reactions and to avoid a labelling of all the serum proteins.

There are several possibilities for purification; a discussion of the advantages and inconveniences of each would go beyond the frame of this document. In general, the best purification method for culture supernatants and serum is the purification over protein A or protein G columns depending on the affinity of the immunisation host's IgG. Protein A (from *Staphylococcus aureus*) and protein G (from *Streptococcus*) bind to IgG with high affinity at two distinct places on the constant region of the heavy chain (Fc-fragment of the antibody). This means that this binding does not modify at all the antigen binding sites, which are found at the variable regions. The non-covalent association of the antibody to and G can be disrupted by acidic buffers (pH 3-4) allowing non-denaturing elution conditions.

These characteristics, as well as the possibility to use the protein A and G column several times (it can be regenerated easily), allow for a cost-effective and quite efficient purification of IgG antibodies from crude serum. For the purification of culture supernatants, we use generally a protein G column because the protein A has only low affinity for mouse IgG1 subclass which is produced by quite a lot of hybridoma cell lines.
The following table gives an overview of the relative affinities of IgG from different species for protein A and protein G. IgY does not bind proteins A and G.

Origin of Immunoglobulins	Protein A	Protein G
Mouse	+++	++++
Rat	-	+++
Guinea pig	++++	++
Rabbit	++++	+++
Goat	+/-	++
Sheep	-	++
Pig	+++	+++
Horse	++	++++
Hen (IgY)	-	-

Table 16: Binding capacities of immunoglobulins from different species to protein A and G

A quality control by SDS-PAGE is carried out after our protein A or G purification process, this to evaluate the purity of the antibodies.

Total IgY purification

After hen immunisation we send to our customers different vials of egg yolks reflecting pre-immunisation, days 14 – 38, days 39 – 52, and the final collection. Since the isolation of IgY from egg yolk, and the purification of specific IgY are labour-intensive, Eurogentec offers to its customers the purification of IgY by affinity chromatography as an option to the hen immunisation programme.

Affinity purifications

It might happen during antibody production that non-specific antibodies in the serum of the animals lead to background signals in the customer assay. This background can be minimised using SPF-rabbits and pre selecting animals for the production, but despite these precautions, non-specific signals are sometimes detected with the crude serum. Another source of non-specific signals can be due to insufficient purity of the antigen. IgG purification, as described here above, will not decrease these disturbing signals because the unspecific antibodies are purified together with the specific ones using this technique. Affinity purification against the antigen provides a solution to get rid of non-specific reactivity.
This technique is based on the covalent attachment of the antigen to a suitable activated matrix, i.e. sepharose, in a column. Subsequently, serum is passed through the column containing the antigen-carrying matrix, with only the antigen-specific antibodies binding to the column and the non-specific antibodies as well as all other serum proteins flowing through.

This method has proven especially useful for peptide antibody production. Care must however be taken when choosing the elution conditions in order to avoid as much as possible activity losses of the antibodies due to denaturing elution buffers. Eurogentec has gained substantial know-how in the field of affinity purification, i.e., the choice of the suitable sepharose as well as the coupling chemistry and elution conditions.

The disadvantage of affinity purification is that a relatively large antigen quantity (several milligrams of protein) must be available. This can be overcome with peptide antibody productions, because in these programmes quite large quantities of free peptide are available. In order to use the peptide in the same fashion as it has been used for immunisation, the coupling is carried out in the same way as the carrier protein attachment prior to immunisation. If the conjugation was done with a different amino acid, there might be the risk that at least part of the peptide-specific antibodies will not bind due to the change in the epitope resulting from this conjugation reaction to the sepharose.

Antibody labelling and assay development

More and more scientists are using labelled antibodies for indirect labelling experiments. Here a primary antibody recognises the target, and a secondary antibody recognises species-specific epitopes on the primary antibody. The secondary antibody is labelled for detection purposes. The classical labels of antibodies are the following:

> Fluorophores
> Horseradish Peroxidase
> Alkaline Phosphatase
> Streptavidin
> Biotin

For some experiments however it is necessary to use just primary antibodies that are labelled (flow cytometry, or electron microscopy for instance), or if you require multiple labelling experiment with the same sample, it is crucial to use antibodies carrying different conjugates. To respond to this demand Eurogentec offers to its customers the most classical modifications of antibodies, and proteins in general.

Eurogentec offers a broad range of fluorophores covering the various colour ranges of the optical spectrum that are compatible with the antibody labelling.

Horseradish peroxidase and alkaline phosphatase antibody conjugates allow customers to enhance their detection method by enzymatic signal amplification. This is crucial for ELISA screening experiments where lowest amounts of antigens have to be detected accurately.

The conjugation of antibodies with streptavidin or biotin allows customers to benefit from the strong binding forces of both molecules to each other in applications where a strong and stable association of an antibody to a solid support is critical e.g. for the affinity purification of a protein from biological samples.

Eurogentec has developed an excellent alternative to dye labelled secondary antibodies based on the ability of Protein A to bind the FC region of most IgGs (for affinity information see p. 24) This range of universal detection reagents allows the detection via streptavidin labels, or provides a super bright signal thanks to the HiLyte™ fluorophores conjugated to the Protein A.

Please be advised that primary antibodies can be labelled for direct detection, or protein immunoprecipitation purposes, too.

Please contact us at info@eurogentec.com to learn if we have your desired antibody or protein conjugation reagent, or how we might help you if you want to perform immunoprecipitation with biotinylated antibodies or set up an immunoprecipitation.

Antibody fragmentation

Many cells have receptors for the Fc fragment of antibodies. This may sometimes lead to unwanted antibody binding and result in background signals in immunohistology experiments. It is possible to avoid such background signals by working with antibody fragments lacking this constant region.

Two different fragmentation methods can be used for this purpose; the following scheme illustrates both possibilities. The digestion of antibodies with papaìn leads in addition to the Fc fragment to two so-called Fab-fragments. The pepsin digestion by contrast leads to the so-called $F(ab')_2$-fragment, in which both antigen-binding regions are still linked together by a disulfide bridge and an extensively degraded Fc portion.

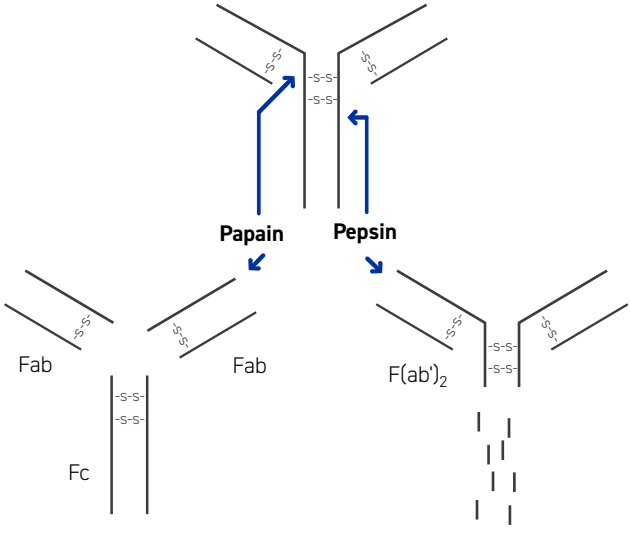


Figure 21: Fragmentation of IgG using enzyme digestion

Both variants can be equally used if background due to the Fc fragment is to be avoided. If in addition a multivalent fixation of the antibody must be avoided, the best solution is the production of the Fab fragments.

Rabbit-IgG contains only a few sites where pepsin or papain is able to cleave; these antibodies are therefore quite easy to fragment.

For monoclonal antibodies however, the efficiency of the fragmentation reaction is strongly dependent on the subclass of the antibody, because some of these contain multiple cleavage sites.
One can classify these subclasses in the following order in function to their resistance to digestion at other sites than the wanted ones: **IgG1 > IgG2e > IgG3 > IgG2b.**

Before fragmenting a large batch of antibodies, Eurogentec carries out a pilot digestion to determine the best possible digestion conditions. Especially the incubation time with the considered enzyme must be precisely defined in order to obtain the best possible yield of antibody fragments. After fixation of the best conditions, the production fragmentation is carried out and the antibody fragments are purified subsequently. This is best done using a protein A/G column, which will retain the remaining undigested antibodies as well as the Fc fragments. If the antibodies has a low affinity for protein A or protein G, a good purification can also be done by gel filtration followed by ion exchange chromatography.

A final quality control by SDS-PAGE and ELISA (if antigen can be delivered) is performed at the end of the process.

Magnetic beads coupling

Eurogentec offers antibody-magnetic bead complexes, ready-to-go for your application.

The coupling principle involves the biotinylation of your antibody followed by linking to streptavidin-coated beads. Example of basic downstream process is described below.

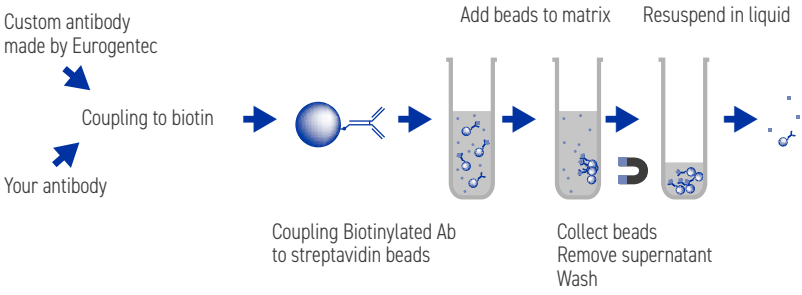


Figure 22: Example of basic downstream process

Technical features

> We use 1 µm beads with the most narrow size distribution
> High and constant magnetic content
> Perfect compromise between size, magnetic content and sedimentation property
> Optimised binding surface
> Allows easy and gentle handling of proteins

Coupling of your antibody to magnetic beads includes:

> Sample preparation
> Antibody biotinylation
> Coupling of 60 µg of biotinylated Ab to 3-8.10^8 1µm magnetic beads

If our standard coupling conditions don't fit to your needs, please contact us. We will be pleased to design a tailor-made solution.

5 HANDLING AND STORAGE OF ANTIBODIES

Storage Temperatures

Purified antibodies from Eurogentec are delivered in PBS with 0.1 % BSA as stabiliser and 0.01 % thimerosal as preservative. For these formulations we recommend the storage in small aliquots at –20 °C.

The aliquoting of antibodies has the following advantages:

1. Minimisation of the damage due to freezing and thawing of a single vial
2. Exclusion of contamination due to multiple pipetting from a single vial

When customers receive an antibody they should carefully thaw it (in a 4 °C water bath) and centrifuge it at 10 000 g for 20 sec in order to pull down all the solution. Aliquot the antibody in low-protein-binding micro centrifuge tubes for storage. Please adapt the size of the aliquots to your typical experimental consumption. Aliquots should not be smaller than 10 µl to avoid damage of the antibody by evaporation and adhesion to the vessel wall. Aliquots should be thawed once.

Though it is considered in general as optional, we recommend the addition of 50 % sterile glycerol (v/v, final) to your antibodies for additional freezing and evaporation protection for storage at –20 °C.

Customers should only use sterile chemicals and solutions for dilution of their antibodies to prevent down stream microbial growth.

Upon receipt, antibody storage for one or two weeks (time required for experimental establishment) at 4 °C should be acceptable with subsequent aliquoted freezing for long-term storage.

Serum requires immediate storage at –20 °C since it possibly contains proteases.
Please be advised that antisera produced at Eurogentec do not contain any preservative; azide or thimerosal should thus be added before aliquoting for storage at –20 °C after first thawing.

Egg yolks must never be frozen, since they denature into a form that does not allow isolation of IgY and affinity purification anymore. Egg yolks from Eurogentec are shipped at 4 °C with 0.01 % azide added as preservative ready for short-term storage (over several weeks) at 4 °C. Alternatively, Eurogentec can perform IgY extraction from yolks which is recommended for long term storage.

IgY isolates and affinity purified IgY antibodies should be treated like purified antibodies described in the beginning of this section.

Antibody formats that require special attention

- **Enzyme-conjugated antibodies** should not be frozen at all in order to retain the maximum enzymatic activity; storage at 4 °C is therefore mandatory, please refer to the technical datasheet on the solvent formulation, and add if required 0.01 – 0.1 % thimerosal (Azide will inactivate HRP-conjugates).
- **Antibodies conjugated with fluorophore and biotin** should be stored in the dark (e.g. protected by black foil bags of X-ray films or photographic paper, and the corresponding boxing).

> **Fluorophore-conjugated antibodies** can be stored aliquoted at –20 °C with 50 % glycerol (final v/v) – customers should make sure by the data sheet that they are formulated as well with BSA, physiologically concentrated buffer salts (e.g. PBS), and preservatives (azide or thimerosal), furthermore, customers should be sure to use a sterile glycerol formulation, since microbial growth might occur and destroy the antibody. Storage at –80 °C of glycerol containing solutions is not advised since this is below the freezing point of glycerol.
> **Minimisation of aggregate** formation of some IgG isotypes (IgG3 for instance) requires storage at 4 °C.

Contamination prevention

Purified custom-made antibodies from Eurogentec already contain 0.01 % thimerosal to avoid microbial growth. But customers should be advised that any serum, like test sera for animal selection, pre-immune sera (PPI), small bleed sera (PP), large bleed sera (GP), or final bleed sera (SAB) do not contain any preservative.

Customers should be advised of the following prior to use thimerosal or sodium azide for storage:

> Azide and thimerosal are toxic to organisms and should be avoided in applications which are done with living cells or tissues; please advise Eurogentec before starting your immunisation programme of your research purpose in order to avoid any preservative.
> If antibodies should be conjugated or spotted involving amino reactive mechanisms, azide or primary amine carrying buffer salts like Tris will interfere negatively into the process, thimerosal is an acceptable alternative without these unwanted side effects.

Preservatives can be removed from an antibody formulation, by the following ways:

> By dialysis or centrifugal filtration: using a device with a MWCO of 14 kDa, please refer to the manufacturer's recommendations for most efficient use of centrifugal filter devices or dialysis equipment (MW of sodium azide ~ 65 Da, thimerosal ~ 404 Da, IgG ~ 160 kDa, IgM ~ 600 kDa).
> By gel filtration (which is faster than dialysis). Please refer to the manufacturer's recommendations for most efficient use of suited filter cartridges.
> Please be advised that all materials to be used here should be sterile to ensure the subsequent stability of the antibody, and to avoid contamination by purification.

Damage caused by freezing and thawing of antibodies

The antibody's binding capacity can be reduced by aggregate formation due to denaturation following cycles of freezing and thawing.

Antibodies should rather be stored protected from daily routine in the freezer, e.g. rather in the back than in the front, or in drawers than standing free accessible.

Working concentrations of antibodies should not be stored for more than one day at 4 °C, since proteins are more susceptible to degradation if they are stored in lower concentrations. Ideal would be to formulate the antibody with additional protein, like BSA or milk powder to reach concentrations of total protein in the mg/ml range. BSA or milk powder in working solutions also minimise antibody loss due to attachment to the vessel wall.

The addition of stabilising proteins is not recommended for antibodies that are subject to conjugation or array spotting or coating of amino reactive surfaces.

6 PRODUCTION OF SYNTHETIC PEPTIDES

Thanks to the acquisition of AnaSpec Inc. in 2010, Eurogentec now provides premium custom and catalogue peptides to elite pharmaceutical, biotechnology companies and academic institutions. AnaSpec has an international reputation for demanding standards for quality and innovation. Custom peptide services include: long peptides, cyclic peptides, difficult sequences, fluorescent labels, phosphopeptides, and large scale synthesis (grams). AnaSpec possesses the world's largest selection of amyloid-related peptides, kinase/phosphatase substrates, phospho-peptides, FRET peptides, TR-FRET and fluorescent imaging peptides. Our enormous database of catalog peptides is searchable by sequence. All peptides are manufactured in Fremont, California, USA.

Principle of peptide synthesis

The automated peptide synthesis starts from a special resin on which the whole peptide sequence is synthesised sequentially by stepwise addition of the different amino acids. The growing peptide chain stays fixed to the resin until the end of the synthesis. Each single amino acid addition consists of 3 individual reaction steps: deprotection, activation and coupling.

Deprotection

Raw material consists of commercially available protected amino acids in which the alpha amino group is protected by the so-called FMOC group (9-Fluorenylmethyloxycarbonyl) - BOC protection is also possible- , while the reactive side chain is also protected to avoid secondary reactions. The decisive advantage of using FMOC is that it can be cleaved under relatively mild basic conditions using Piperidine.
During the deprotection phase, Piperidine is used to remove the FMOC protection of the last amino acid of the elonging chain, leading to a free and reactive amino group that can react with the next amino acid to be added to the chain.

Activation

The next amino acid to be added, fully protected, is then introduced in large excess. In order to allow for a quick reaction, its carboxyl group is to be activated. The activator transforms the carboxylic acid to a so-called active ester.

Coupling

This active ester in turn reacts on the free amino group of the elonging chain, resulting in the formation of a new amide bond and thus to the addition of one amino acid to the chain.

The whole process is subsequently repeated with the next amino acid.

Completion of synthesis

After addition of all amino acids to the chain, the peptide is present in protected form and still linked to the resin. Piperidine is then used to remove the remaining FMOC protection at the end of the chain and TFA (Trifluoroacetic acid) is applied both to remove side chain protections and to free the peptide from the resin.

The figure below summarises the peptide synthesis process.

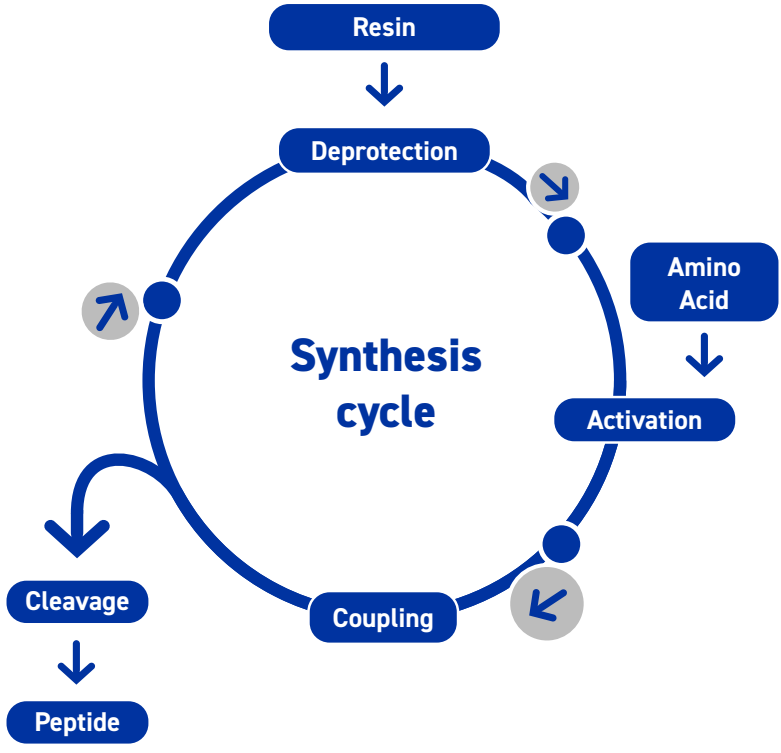


Figure 23: Peptide synthesis process

Purity grades

In order to respond to nearly all kinds of requests, we offer different purity grades (crude, >70 %, >85 %, >90% and >95 % purity) of our synthetic peptides.

For screening purposes, crude peptides are usually used. For antibody production, our immunograde purity (>70 %) is recommended. Peptides for biochemical applications, as i.e. enzyme substrates or blockers, as well as other bioactive peptides should be purified at >85 % or better at >95 % purity. These peptides are free of aborted sequences that are generated during synthesis (small quantities of peptides lacking one or more amino acids).

Quant-peptide : quantify peptide amount

Eurogentec exclusively offers 2 proprietary methods that are employed to measure the net peptide content of a target peptide in a peptide mixture.
> Quant-Peptides with no Quant-Tag are absolutely quantified using a proprietary and highly optimised AAA-MS method. These are universal peptide standards which can be used in any proteomics application. This method applies to peptides containing at least 2 of the following amino acids: F, I, K, L, P, R, V.
> Quant-Peptides with a Quant-Tag are absolutely quantified based on the inherent spectral properties of the proprietary tag. The Quant-Tag is coupled to the C-term of the peptide via an Arginine (R) or Lysine (K) residue and can be released by trypsin digestion. The precise molecular mass of the tag (1356,7 Da) can be used in assessing the cleavage efficacy, and hence in setting the optimal trypsinizations conditions of a sample using i.e. MS:MS methods. To do so, the Quant-Peptide must be spiked into the sample prior to trypsin digestion. The Quant-Tag is then used as a reporter of the trypsin digestion efficacy: a peak corresponding to the tag mass is indicative of trypsin digestion. This option is recommended for peptides that do not contain internal K, R or C residues.

Dispensing

The dispensing service is in line with ISO15189 quality standards and provide your ready to use aliquotes of your peptides. Any size of routine assays to full kitting solutions can be produced with a very high reliability, reproducibility and accuracy. This process saves set-up time and reduces reagent wastage, while keeping format flexibility.

Evaluation of synthesis difficulty and sequence verification

After reception of an order, Eurogentec always carries out an analysis of synthesis difficulty. The most frequent problem occurring during peptide synthesis is the formation of hairpin structures due to the presence of hydrophobic amino acids residues, causing steric hindrance and thus compromising the synthesis process.

This analysis allows our synthesis chemists to adapt right at start the production conditions, in particular the coupling times to minimise the risk of synthesis failure. After analysis, we send an acknowledgement of receipt of the order to the customer, indicating the sequence to be synthesised. This way, the customer has the possibility to check before the start of the synthesis that no error has been introduced.

Thanks to the development of diverse peptide synthesis platforms, AnaSpec can manage very challeging peptide synthesis.

Quality control

An analytical HPLC analysis using a standard water-acetonitrile gradient allows evaluating the peptide purity. If the peptide is ordered in crude or immunograde quality, an additional purification is often not necessary. If however there are large quantities of shorter peptides resulting from uncomplete couplings, the crude peptide is further purified by preparative HPLC.

The HPLC profile of a peptide shows its purity, but gives no indication on the peptide content. It is therefore very important to conduct a QC by mass spectrometry, even for crude peptides. We use for this purpose a MALDI-TOF mass spectrometer, which gives the molecular mass of the obtained peptide. The interpretation of the spectrum is relatively easy, because this ionisation method does not lead to peptide fragmentation in the instrument. Mass spectrometry is the best way to identify the correct peptide.

Delivery

After purification and corresponding quality control, the peptide is lyophilised, weighted and shipped. If ever we were unable to obtain the ordered quantity, a resynthesis is started immediately and the additional quantity delivered afterwards.

The peptide is always shipped together with:

> The HPLC analysis profile
> The mass spectrum
> A data sheet containing useful information (molecular mass, isoelectric point,...)
> Information about solubilization and storage

Peptide dissolution

A vial containing a peptide should never be opened before it has equilibrated at room temperature. First try to dissolve the peptide in a small volume of pure water. If the peptide does not dissolve easily, sonication may help. If necessary, add a small amount of aqueous acetic acid for a basic peptide, or aqueous ammonia for an acidic peptide. Once the peptide has dissolved, the buffer for the experiment can be added.

For some neutral or very hydrophobic peptides, polar organic solvents such as DMSO or DMF can be used. These solvents should be dripped - while stirring - until the peptide has dissolved. Then water or the buffer can be added to reach the final desired concentration. Lyophilised peptides should be stored in a dessicator at 2-6 °C. Once reconstituted, peptides should be used as soon as possible to avoid degradation in solution. If this is not possible, we recommend aliquoting them in the amounts needed for each experiment. These aliquots should then be relyophilised and stored as before. Unused peptide solutions can be stored frozen at –20 °C for a limited period of time. Repeated freezing and thawing must be avoided.

Peptide modifications

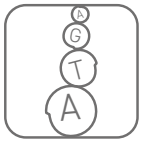
For many applications, modified peptides are needed. Eurogentec has gained a considerable know-how in the synthesis of modified peptides.

The most widespread modifications available are:

> Biotinylation
> Labelling with fluorescent dyes
> Phosphorylation (serine, threonine, tyrosine)
> Disulfide-bridged cyclic peptides
> MAP peptides for antibody production
> Branched peptides
> Cyclic peptides
> Peptides containing non-natural amino acids.

A detailed discussion on all possibilities would go beyond the limits of this booklet but we would be happy to discuss corresponding projects with our customers.

Life Science Research



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