Agrisera

# Tips and Tricks for Antibody Production and Validation Process - How to Obtain Good Results -

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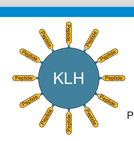
## Three crucial components of successful antibody production

## Antigen – peptide

Covers smaller epitope pool




Peptide-Protein Conjugate

- 7-30 amino acids long peptide, carrier conjugated (KLH, BSA) by Thiol group of cysteine
- Peptide will target: C or N-terminal; should not be located in parts of a protein which are going to be processed, as well as in domains repeated in other proteins
- Internal cysteine should be avoided since epitopes around this amino acid are going to be lost
- Consider if a peptide should target one specific isoform or can be repeated in all isoforms from a given protein family
- A peptide mix can be also used for immunization as it will cover more epitopes
- Immunization with a peptide without a carrier is possible for longer peptides (>30 aa)

**Further reading:**
Peptide synthesis: **www.agrisera.com/peptide-synthesis**
Epitope prediction: **epic.embl.de**

## Antigen – protein

Covers a pool of various epitopes

**Recombinant protein**

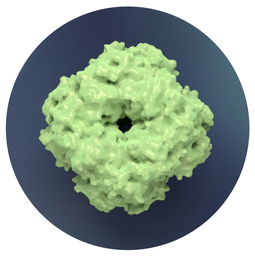
- Can be either tagged or cleaved
- Protein buffer composition should not be harmful to animals
- Ideal protein concentration: ~1 mg/ml
- For protein purity issues, the protein can be excised from a gel
- Required amount for immunization of two animals is 1 mg or less

**Native protein purified from endogenous sample**

- Risk for one or more isoforms present in such preparation

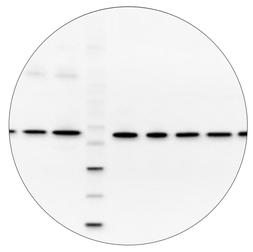
**Further reading:**
FAQ about antibody production process: **www.agrisera.com/faq-antibody-production**
Recombinant protein expression: **www.agrisera.com/recombinant-protein-expression**

## Antigen



## Animal

## Analysis



## Choice of host species

**Polyclonal antibody**
Will recognize a pool of several epitopes

Amount (ml) of produced antibody/animal:
goat > hen > rabbit

**Monoclonal antibody**
Will recognize a single specific epitope (2-15 amino acids)

**Examples of Agrisera immunization protocols**

| | Goat (IgG) | Rabbit (IgG) | Hen (IgY) |
|---|---|---|---|
| Time schedule (weeks) | 15 | 15 | 12 |
| Pre immune product * | 3 ml serum | 3 ml serum | ~ 3 mg IgY |
| Immunizations | 4 | 4 | 3 |
| Antigen ** (µg / program) | 500 | 500 | 500 |
| Immune product | 100-150 ml serum | 65 ml serum | 100-400 mg IgY |

* Pre immune serum/yolk available for background screening
** Less antigen can be used if required.

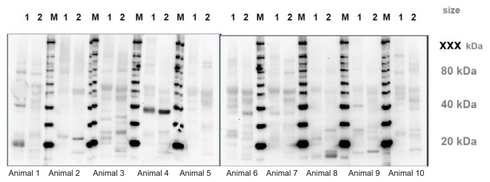
**Further reading:**
Rabbit, goat IgG antibodies: www.agrisera.com/igg
Chicken IgY antibodies: www.agrisera.com/igy

**Important note:** Only 1-10% of the total immunoglobulin IgG or IgY obtained in an immunization process is directed against the specific antigen.

## Before immunization

Why is pre-immune serum screening important?

Some animals may already have antibodies that detect proteins with MW in close proximity to the target protein. Pre-immune serum screening helps with identifying these and choosing the animal with the lowest background.




Sample load: 5-10 µg/well (1- *Arabidopsis thaliana*, 2- *Hordeum vulgare*).
Blocking: 5-10% non-fat milk.
Primary antibody dilution: 1: 1 000 - 1: 10 000, incubation 1h/RT.
Secondary antibody dilution: 1: 25 000, incubation 1h/RT (Goat anti-Rabbit, **AS09 602**)

**Further reading:** Agrisera protocol upon request to **support@agrisera.com**

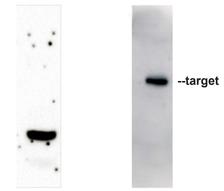
## Sample extraction

Fast and efficient

Bead beater, sonication or mortar and pestle: the chosen method needs to be optimized for a given sample, to allow its prompt and efficient processing.

Extraction buffer containing LDS, HEPES or TRIS: analysis of freshly extracted samples and optimization of the extraction buffer are highly recommended. For some proteins HEPES may provide much better results.

**Make sure your protein samples are of best quality as no target protein on a membrane means no antibody detection.**

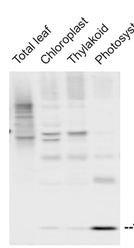



Detection of target protein using the same WB protocol, same primary and secondary antibodies, but with different extraction buffers.

Concentrate your target protein

**Example: fractionation**

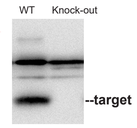
Epitope enrichment by fractionation: 10 µg of total protein from different cellular fractions (leaf, chloroplast, thylakoid and photosystem I) separated on 8-12% gradient gel.




## Antibody validation check list

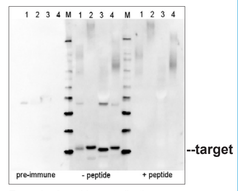
**Direct approach**

- Reliable knockout/knockdown mutant
- Immunoprecipitation and MS of its product confirms protein identity (IP-MS)




**Indirect approach**

- Antibody is recognizing recombinant protein
- Band of the same MW as the target protein is not present in pre-immune
- Peptide neutralization
- Detection of protein at the correct MW (processing sites and modifications can affect apparent MW)
- Low cross-reactivity
- Samples from specific conditions, up or down regulating expression of target protein, are used
- Same band is recognized by two independent antibodies to the same target

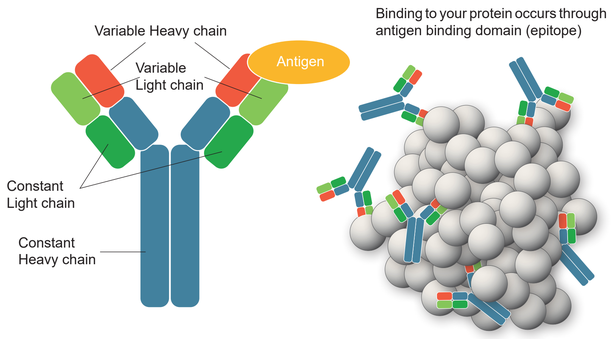



Example of peptide neutralization assay.

Detailed description of peptide neutralization assay:
**www.agrisera.com/neutralization-assay**

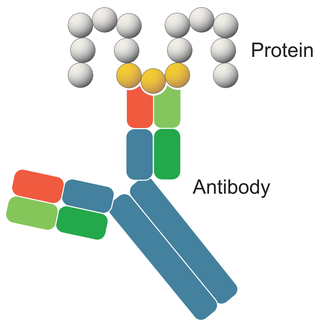
**Every step of Western blotting is important for data quality**

## What an antibody looks like



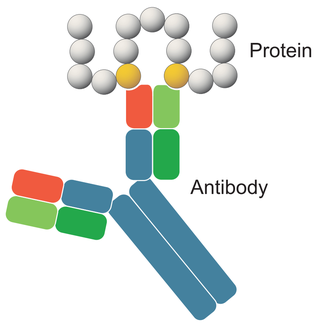

## Linear Epitope

Recognized in WB, IHC (denatured)

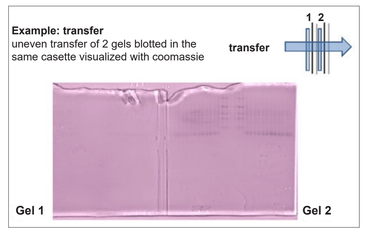



## Discontinuous Epitope

Recognized in IP, WB, ChIP (native)




## Consider MW of your target protein




**Example: transfer**
uneven transfer of 2 gels blotted in the same casette visualized with coomassie

**High MW proteins (>20 kDa)**
Can be retained in a gel
Require longer transfer time

Recommended conditions:
- 0.45 µm membrane
- Lower MEOH (10%)
- Increase SDS
- Wet (tank) transfer

**Low MW proteins (<20 kDa)**
Easily migrates from a gel
Require shorter transfer time

Recommended conditions:
- 0.20 µm membrane
- Increase MEOH (20%)
- Decrease SDS
- Semi-dry transfer

## Include loading controls

**Cytoplasm**
Anti-ACTIN
AS13 2640 (polyclonal)
AS16 4111 (monoclonal)

**Nuclei**
Anti-H3
AS10 710-100 (rabbit)
AS15 2855 (chicken)

**Chloroplasts**
Anti-RbcL (Rubisco large subunit)
AS03 037 (rabbit)
AS01 017 (chicken)

**Important note:** These controls are not universal to ALL experimental conditions

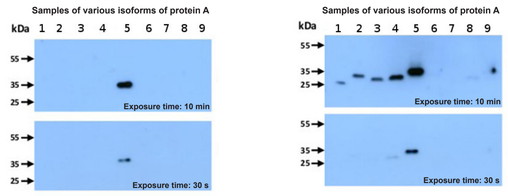
**Further reading:** www.agrisera.com/loadingcontrols

## Testing - variability in obtained results

Why more than one animal should be immunized

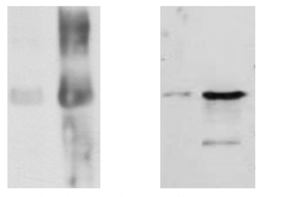
**Animal 1**
Immunized with protein A

**Animal 2**
Immunized with protein A




Each animal, injected with the same antigen will produce a unique set of antibodies, in this case to various isoforms of the same protein.

## Primary antibody incubation time



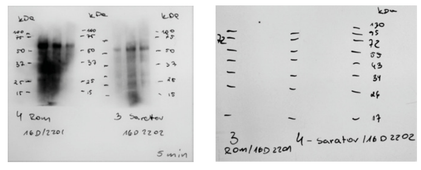

Same sample, primary antibody and Western blot protocol

In case of background: shorten incubation time with primary antibody.

## Blocking reagent

Comparison

www.agrisera.com/blocking



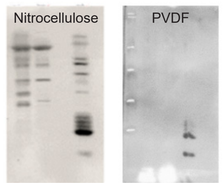

In both cases the same sample, primary and secondary antibodies were used, and the same western blot protocol was applied, except a change in a blocking reagent from 5 % BSA to 5 % non-fat milk.

In case of background problems, increase incubation time and change percentage and type of blocking reagent.

## Membrane type

Does it matter?

The same sample and primary antibody was used on two different membranes




Various membranes have different protein retention. PVDF membrane can be dried in air for a few hours to increase protein binding. In case of increased background signal, a change of membrane type is recommended.

## Antibodies and Good Laboratory Practice

- Store antibodies in 4°C/-20°C (short-term) or -20°C/-80°C (long-term)
- Store chicken antibodies (IgY) in 4°C with preservative of bacterial growth
- Prepare aliquots
- Primary and secondary antibodies should not be re-used
- Affinity purification is recommended for techniques like ChIP, IP, IHC (should never be done on whole material at once)
- Optimize used protocols. Each antibody-antigen interaction is unique




## Rapid WB - Simultaneous incubation

**Rapid Western blot protocol with Agrisera IncuBlocker**

**Standard Western blot protocol**




**Primary and secondary antibody incubation time**
Primary antibody used: **AS05 084**

**Primary antibody incubation time**

- Shortens Western blot protocol by several hours
- Blocking, primary and secondary antibody incubations are all done in one step
- Suitable for proteins of high and moderate expression

www.agrisera.com/rapid-wb

## Protocol for unknown / not working antibody

- Check sequence used to elicit the antibody you are working with. Is it conserved in your target protein? For catalogue antibodies, contact the supplier
- Include positive and negative controls for Western blot procedure
- Use cellular fraction/organ in which the protein of interest is expressed
- Protein load/well, min. 50 µg
- Blocking: 5-10 % non-fat milk, 1h/RT or ON/4°C
- Blocking: non-protein blocker, 0.1-1% PVP40, 1h/RT
- Primary antibody affinity purified
- Primary antibody starting dilution: 1: 500-1:1 000/ON/4°C incubation
- Use sensitive detection: chemiluminescence or fluorescence is preferred over chromogenic
- Overexpose to make sure if a band of interest does not require longer time to become visualized

## If your antibody DOES NOT WORK

Contact Agrisera support

**Live chat** www.agrisera.com
**E-mail:** support@agrisera.com
**Phone:** +46 935 33 000

Or use Agrisera's Western blot resources!
www.agrisera.com/western-blot



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