FAQ - Why do I see too many bands on my Western blot?

Western blotting (immunoblotting) is a widely used method for detecting specific protein antigens within complex mixtures. Following separation of samples by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), proteins are transferred to a membrane - typically nitrocellulose or PVDF. The membrane is then incubated with an antibody specific to the target protein, which binds to the immobilized band. Detection is subsequently achieved using a secondary reagent specific for the primary antibody that is coupled to an enzyme or fluorophore for visualization.

It is not unusual for Western blots to reveal multiple bands, even when a single band is expected. Although antibody cross-reactivity is one possible explanation, several other factors may contribute:

Alternative splicing

Several bands are not always the result of suboptimal experimental conditions or cross reactivity. Many proteins have several isoforms, and an antibody may detect more than one of them.
Alternative splicing produces mRNA isoforms that encode protein isoforms that often differ in amino-acid length, domain composition or post-translational modification (PTM) sites. When the protein mixture is run on an SDS-PAGE each isoform can migrate to a different apparent molecular weight (MW).
Probeing with an antibody directed against a shared epitope can give rise to multiple bands.

Proteolytic degradation of the antigen

Proteolysis can occur, especially if samples are not freshly prepared, are stored for extended periods, or are subjected to fractionation after homogenization. Proteolytic fragments typically appear as additional bands of lower molecular mass than the full-length protein. Proteins such as synapsins and synaptotagmins are particularly sensitive. Including protease inhibitors such as PMSF, pepstatin, leupeptin, or commercially available cocktails is strongly recommended.

Excess protein loading or detection system too sensitive

Overloading gel lanes is one of the most common causes of *ghost bands*. High protein concentrations on the membrane can create a dense adsorptive surface that promotes nonspecific IgG binding. Similarly, highly sensitive detection methods—such as enhanced chemiluminescence—can reveal nonspecific interactions. Performing a dilution series of the sample can help distinguish true signals from artefacts.

Ineffecient blocking

A variety of blocking agents are described in the literature, including detergents and protein-based blockers. Inadequate or suboptimal blocking may increase nonspecific binding. Adjusting the blocking reagent or blocking duration can often resolve these issues.

Low antigen abundance

SDS-PAGE can resolve only about 50–100 distinct bands. If the target antigen represents less than ~0.2% of total protein, detection may be challenging. For example, synaptobrevin/VAMP co-migrates with histones in crude homogenates, making it difficult to visualize. Attempts to enhance signal intensity may inadvertently produce artificial bands. In such cases, antigen enrichment, such as through fractionation or immunoprecipitation, should be considered.