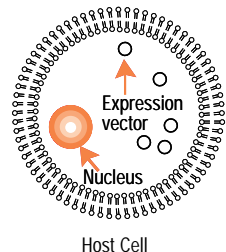


A. Transient transfection



B. Stable transfection

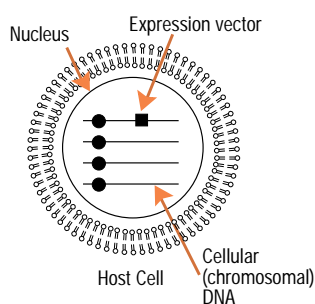


Figure 1C.5: Transient vs. stable transfection. In a transient transfection (panel A), an expression vector makes tagged protein for a brief time in the cell cytoplasm. In a stable transfection (panel B), the expression vector integrates into a chromosome and is replicated, transcribed, and translated indefinitely (like the cell's own DNA).

Depending on the fate of the transfected DNA, a transfection may be:

- ▶ **Transient** (Figure 1C.5, panel A): Transfected DNA remains free within the cell cytoplasm and functions for only a limited time
- ▶ **Stable** (Figure 1C.5, panel B): Transfected DNA recombines with the cell DNA, forming an integrated copy of the vector that the cell will replicate, transcribe, and translate as if it were normal cell DNA

What immunological techniques can I use with tagged proteins?

As Table 1B.1 (page 1.4) shows, epitope-tagging can be an important tool for addressing many research problems. While the literature describes many applications for epitope-tagged proteins, most laboratories use four techniques to analyze or purify tagged proteins:

- ▶ Western blotting
- ▶ Immunofluorescence microscopy
- ▶ Immunoprecipitation
- ▶ Affinity chromatography

Here are some critical factors to consider as you begin to use each of these techniques.

How much antibody reagent will I need?

No exact answer can be given, since different tags and different applications will require differing amounts of tag-specific antibody reagent. However, to estimate how much antibody reagent you will need for each experiment you design, determine:

- ▶ The working concentration of antibody reagent that will give optimal results
Note: Based on Boehringer Mannheim laboratory experience, Table 1C.2 gives some guidelines for the use of our tag-specific antibodies in common techniques.

- ▶ The volume of working antibody reagent solution needed for each sample (as detailed in Chapters 4 and 5 of this manual)
- ▶ The number of samples and controls you will need to include in the experiment

Should I use a direct or indirect detection procedure?

The literature generally refers to two types of detection procedures, direct and indirect. An analytical technique, such as Western blotting, may use either direct or indirect detection. The difference depends upon the type(s) of antibody used and the location of the indicator molecule that allows visualization of the antibody-antigen complexes (Figure 1C.7).

Note on dilution nomenclature

Often in this manual, a procedure may refer to a 1:X dilution (for example, a 1:10 dilution of secondary antibody conjugate). The nomenclature, 1:10, in this manual means "Take 1 part of the concentrated solution and add it to 9 parts of diluent, then mix thoroughly."

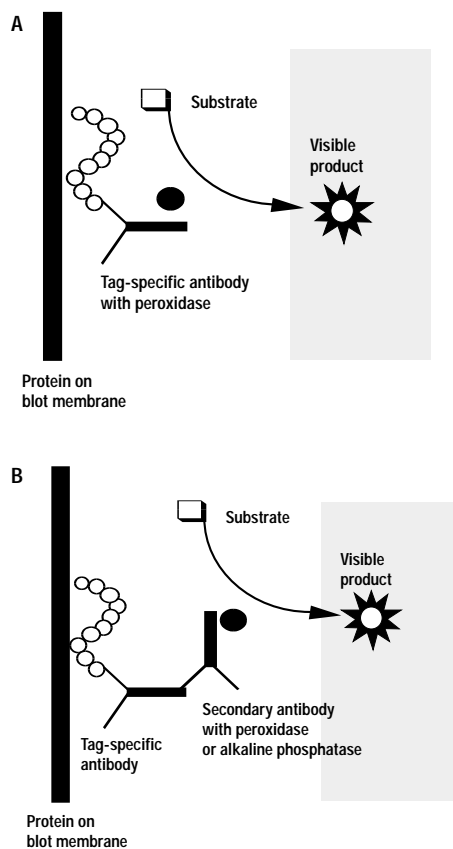


Figure 1C.7: Direct and indirect detection on a Western blot. In a direct detection procedure (panel A), the sample is incubated with a tag-specific antibody that is conjugated with an indicator molecule, such as an enzyme. In an indirect detection procedure (panel B), the sample is incubated with an unconjugated tag-specific antibody (primary antibody), then with a secondary antibody that recognizes the tag-specific antibody and is conjugated with the indicator molecule. In either procedure, the last stage is the visualization of the indicator molecule.

The principal advantages of the direct method are:

- ▶ **Time savings**, since the direct procedure has fewer steps and is easier to optimize than the indirect procedure
- ▶ **Lower background**, since, in general, each additional step increases the chance of nonspecific signal
- ▶ **Increased specificity**, since the direct method eliminates potential cross-reactivity associated with the secondary antibody

- ▶ **Lower cost**, since direct procedures
 - 1) require much lower amounts of tag-specific antibodies than indirect procedures, and
 - 2) do not require a secondary antibody

Technique	Tag-specific antibody	Suggested working conc. (µg/ml)	Secondary antibody [§]	Suggested working dilution or concentration
Western blotting				
Direct	Anti-c-myc-peroxidase	0.1–1.0	Not needed	
	Anti-HA-peroxidase	0.1–1.0	Not needed	
	Anti-VSV-G-peroxidase	0.1–1.0	Not needed	
Indirect	Anti-c-myc	1–5	Anti-Mouse IgG (H&L)-peroxidase	1:500–1:1000
	Anti-HA	1–5	Anti-Mouse IgG (H&L), F(ab') ₂ -peroxidase	1:3000–1:10,000
	Anti-HA-biotin	1–5	Streptavidin-peroxidase	0.1–0.25 U/ml
	Anti-VSV-G	1–5	Anti-Mouse IgG (H&L), F(ab') ₂ -peroxidase	1:3000–1:10,000
Immunoprecipitation				
	Anti-c-myc	1–10	Not needed	
	Anti-HA	1–10	Not needed	
	Anti-VSV-G	1–5	Not needed	
Immunofluorescence microscopy				
Direct	Anti-HA-fluorescein	1–10	Not needed	
	Anti-HA-rhodamine	1–10	Not needed	
Indirect	Anti-c-myc	1–10	Anti-Mouse IgG (H&L), F(ab') ₂ -fluorescein	1:10–1:300
			Anti-Mouse IgG (H&L), F(ab') ₂ -rhodamine	1:20–1:500
	Anti-HA	1–10	Anti-Mouse IgG (H&L), F(ab') ₂ -fluorescein	1:10–1:300
			Anti-Mouse IgG (H&L), F(ab') ₂ -rhodamine	1:20–1:500
	Anti-HA-biotin	1–10	Streptavidin-fluorescein	5–40 µg/ml
			Streptavidin-sulfo-rhodamine 101	10 µg/ml
Anti-VSV-G	1–10	Anti-Mouse IgG (H&L), F(ab') ₂ -fluorescein	1:10–1:300	
		Anti-Mouse IgG (H&L), F(ab') ₂ -rhodamine	1:20–1:500	

*These are suggested concentrations for initial experiments. Optimal concentrations should be determined experimentally for each tagged protein.

[§]The working concentration of secondary antibody is applicable only to the listed antibodies from Boehringer Mannheim. Secondary antibodies from different manufacturers or different antibodies from Boehringer Mannheim should be diluted according to the manufacturer's instructions.

Table 1C.2: Suggested working concentrations for Boehringer Mannheim antibodies*

The principal advantages of the indirect method are:

- ▶ **Greater flexibility**, since the indirect method provides a wide choice of detection systems and a secondary antibody can be used to detect a variety of primary antibodies (useful in laboratories that tag with more than one epitope)
- ▶ **Sensitivity**, since the indirect method can be more sensitive than a direct method (secondary antibody often amplifies the detection signal)

Note: *Some direct detection systems, however, are equal in sensitivity to indirect detection systems. (See Figure 4A.3 in on page 4.3 of this manual).*

How can I ensure that I analyze or purify an intact tagged protein?

The most important things that you can do to ensure analysis or purification of a full-sized, biologically active form of your protein are:

- ▶ When breaking open cells containing the tagged protein, use a lysis method that is strong enough to release the protein from the cell, yet gentle enough not to denature the tagged protein. For more information about cell lysis, see “Lysing different types of cells” in Chapter 3 of this manual.
- ▶ Include a “cocktail” of different protease inhibitors in analysis and purification buffers to prevent degradation of the tagged protein by a variety of proteases that may be present. For more information about protease inhibitors, see “Preventing proteolysis during sample purification” in Chapter 3 of this manual.

References

Berkower, C., Loayza, D. and Michaelis, S. (1994) Metabolic instability and constitutive endocytosis of STE6, the **a**-factor transporter of *Saccharomyces cerevisiae*. *Mol. Biol. Cell* 5:1185–1198.

Tyers, M., Tokiwa, G. and Futcher, B. (1993) Comparison of the *Saccharomyces cerevisiae* G₁ cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J.* 12:1955–1968.