

Overview for Electrophoresis and Western Blotting

Comparison of Buffer Systems for Protein Separations

Dissociating/Non-Dissociating Native Buffer Systems

Many studies employing zone electrophoresis of proteins in a polyacrylamide gel use a buffer system designed to dissociate all proteins into their individual polypeptide subunits. The most common dissociating agent is sodium dodecyl sulfate (SDS), an ionic detergent which denatures proteins by wrapping around the polypeptide backbone. By heating the protein sample at 100°C in the presence of excess SDS and thiol reagent, disulfide bonds are cleaved and the protein is fully dissociated into its subunits. Under these conditions most polypeptides bind SDS in a constant weight ratio (1.4 g of SDS:1 g of polypeptide). The intrinsic charges of the polypeptide are insignificant compared to the negative charges provided by the bound detergent so that the SDS-polypeptide complexes have essentially the same negative charge and shape and migrate through the gel strictly according to polypeptide size. The simplicity and speed of this method, plus the fact that only microgram quantities of protein are required, have made SDS-PAGE the most widely used method for determination of molecular mass in a polypeptide sample. Proteins from almost any source are readily solubilized by SDS so the method is generally applicable.

In contrast, non-dissociating native buffer systems, where proteins are electrophoresed in their native form, are designed to fractionate a protein mixture such that subunit interaction, native protein conformation, and biological activity are preserved. Proteins in non-dissociating native systems are separated based on their charge-to-mass ratio.

Continuous Buffer Systems

In continuous buffer systems, the composition of the gel buffer and running buffer are essentially identical throughout the gel. They consist of a single separating gel and use the same buffer ions at the same pH throughout the sample, gel, and electrode reservoirs.

In continuous systems, molecular charge density and gel pore size are the only factors that have any effect on the stacking, or zone sharpening, of a molecule. The proteins in a sample applied to a continuous system will move faster in the sample well than in the gel. A concentrated band of sample will form where the molecules are slowed down at the interface of the buffer and the restrictive gel. This type of stacking has limitations, particularly when separating molecules which vary widely in size. The smaller molecules have less of a difference between their free solution mobility and their mobility in a gel so the sharpening factor is not as favorable as for the larger molecules.

Discontinuous Buffer Systems

The discontinuous buffer systems employ different buffer ions and pH in the gel and in the electrode reservoirs. Samples are loaded onto a non-restrictive large pore gel, called a stacking gel, which overlays a smaller pore resolving gel.

The major advantages of discontinuous buffer systems are that relatively large volumes of dilute protein samples can be applied to the gel and resolution is much greater than that obtained with continuous systems. This increased resolution is a direct result of the way proteins concentrate, or stack, into narrow zones during migration through the large-pore stacking gel, and destack or resolve, under the given conditions in the small pore resolving gel. The production of thin starting zones by discontinuous systems is described by Ornstein and Davis (1,2).

Ion Mobility and Protein Stacking

Ion mobility is dependent on the charge density of a molecule and the voltage gradient. At a given pH only part of a population of protein molecules will be dissociated (*i.e.*, charged) at any time. The velocity at which these molecules migrate will be dependent upon the effective mobility and the voltage gradient. Therefore, an ion of lower mobility can migrate as fast as one with higher mobility if the products of voltage and effective mobility are equal.

In the Ornstein and Davis system, the sample and stacking gel contain Tris-HCl buffer whereas the upper electrode reservoir contains Tris-Glycine buffer. At the pH of the sample buffer and stacking gel (pH 6.7), glycine is weakly ionized and therefore, its mobility is low. Chloride is completely ionized and has a much higher mobility, while the mobility of proteins are intermediate between that of glycine and chloride. Once a voltage is applied, chloride (leading) ions migrate away from the glycine (trailing) ions leaving behind a zone of lower conductivity, a higher voltage gradient, and higher pH. The zone accelerates the glycine so it keeps up with the chloride ions. As this glycine/chloride boundary moves through the sample and stacking gel, any proteins in front are rapidly overtaken by chloride ions which have a higher velocity. Behind the moving boundary, the proteins have a higher velocity than glycine. Therefore, the moving boundary sweeps up the proteins, concentrating them into thin zones or stacks in order of decreasing mobility.

What Happens Once the Proteins have been Stacked?

First, the stacked proteins may migrate through a low percentage gel, remaining stacked or unresolved behind the moving boundary or dye front. Second, the proteins may unstack or resolve from the moving boundary. Ornstein and Davis described two different methods for unstacking. Unstacking can be achieved by a) slowing down the protein after it has been stacked, or b) by speeding up the trailing ions once the protein has been stacked.

In the first approach, a low percent stacking gel is cast over a higher percentage resolving gel. As the stacked protein enters the higher percentage resolving gel, its mobility is reduced so that it is slower than the glycine ion. Once this occurs, the protein escapes the stack and migrates as if it were in a continuous gel at a similar, lower, local field strength.

The second means of unstacking is to use a shift in pH to accelerate the trailing ions. For the glycine/chloride system, a stacking gel is cast so that the glycine zone will regulate to a lower pH than the resolving gel. When the glycine enters the higher pH resolving gel, its net negative charge will increase its effective mobility. Consequently, the glycine overtakes some of the proteins and now migrates directly behind the chloride ions, causing these proteins to resolve.

Ornstein and Davis combined both of these unstacking methods in their Tris-chloride/Tris-glycine discontinuous buffer systems by using a low percentage stacking gel at pH 6.8 over a higher percentage separation gel at pH 8.8. Laemmli later adapted the system for SDS electrophoresis of proteins, keeping essentially the same features (3).

The Relative Importance of Stacking and pH in SDS PAGE Systems

We frequently are asked if the Novex® Pre-Cast Gels have a stacking gel and, if so, what is its pH. The answer is yes, there is a stacking gel (for all NuPAGE®, Tris-Glycine, and Tricine gels), but the pH is the same between the stacking gel and the separating gel. The pH varies depending on the formulation of the gel. Please refer to individual products for the pH value.

We have found that the pH discontinuity of the SDS system plays a much smaller role in stacking proteins than does the ion species discontinuity of the system. In the Laemmli system, the stacking gel pH of 6.8 may serve as an extra boost for the stacking system but is not actually required to stack SDS/protein complexes.

Even at the separating gel pH of the Laemmli system (pH 8.8), glycine is slow enough so that proteins smaller than about 70 kDa will stack in a 4% gel just as well as they would in a pH 6.8 stacking gel. It should be noted that the Ornstein and Davis system was originally developed for separating native proteins. They found that a low pH stacking gel was required to effectively stack some of these proteins. SDS-bound proteins, however, have a considerably greater negative charge density than native proteins. Consequently, the glycine mobility is low enough to stack SDS-bound proteins ahead of the glycine zone even when the stacking gel is set up at the same pH as the resolving gel. Proteins as large as 70 kDa will remain stacked in a 4% gel at pH 8.6–8.8 just as well as in a 4% gel at 6.8–7.2.

The larger proteins will also be separated into discrete bands. Stacking occurs at the interface between the free sample solution and the stacking gel, which promotes band sharpness for the larger proteins. Small and large proteins migrate equally in free solution, but when the larger proteins contact the stacking gel they are slowed considerably more than the smaller proteins and are effectively concentrated. With typical sample volumes, this concentrating effect produces bands over the entire range which are as sharp as those obtained with a pH 6.8 stacking gel.

References:

1. Ornstein, L. (1964) *Ann. NY Acad. Sci.*, **121**: 321.
2. Davis, B.J. (1964) *Ann. NY Acad. Sci.*: **121**: 404.
3. Laemmli, U.K. (1970) *Nature* **227**: 680-685.

Comparison of Discontinuous Buffer Systems

SDS-PAGE utilizes a discontinuous buffer system to concentrate, or “stack,” samples into a very sharp zone in the stacking gel at the beginning of the run. In a discontinuous buffer system, the primary anion in the gel is different (or discontinuous) from the primary anion in the running buffer. Both the NuPAGE® systems (Bis-Tris and Tris-Acetate gels) and the Laemmli (Tris-Glycine) system are examples of discontinuous buffer systems and work in a similar fashion. However, the NuPAGE® system operates at a lower pH as a result of different ions in the system.

In the case of the **Tris-Glycine system** (Figure 1), three ions are primarily involved:

- Chloride (-), supplied by the gel buffer, serves as the leading ion because it has the highest attraction to the anode relative to other anions in the system.
- Glycine (-), the primary anion provided by the running buffer, serves as the trailing ion, because it is only partially negatively charged and remains behind the more highly charged chloride ions in a charged environment.
- Tris Base (+) is a common ion present in both the gel and the running buffers. During electrophoresis, the gel and buffer ions in the Tris-Glycine system form an operating pH of 9.5 in the separating region of the gel.

In the case of the **NuPAGE® Bis-Tris system** (Figure 2), three ions are primarily involved:

- Chloride (-) supplied by the gel buffer, serves as the fast-moving leading ion.
- MES or MOPS (-) (depending on the running buffer choice) serves as the trailing ion.
MES: 2-(N-morpholino) ethane sulfonic acid
MOPS: 3-(N-morpholino) propane sulfonic acid
- Bis-tris (+) acts as the common ion present in the gel while Tris(+) is provided by the running buffer.

The combination of a lower pH gel buffer (pH 6.4) and running buffer (pH 7.3 - 7.7) leads to a significantly lower operating pH (pH 7.0) during electrophoresis.

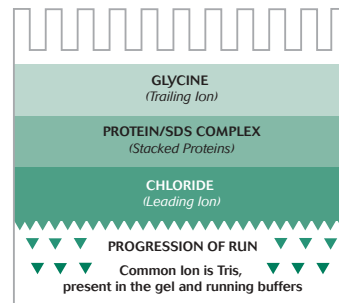
In the case of the **NuPAGE® Tris-Acetate system** (Figure 3), three ions are primarily involved:

- Acetate (-), the leading ion from the gel buffer
- Tricine (-), the trailing ion from the running buffer
- Tris (+), the common ion (in both gel and running buffer)

This system also operates at a significantly lower pH than the Tris-Glycine system.

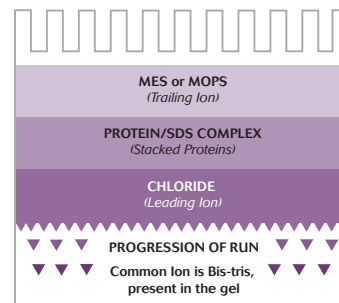
The diagrams to the right summarize the migration differences in the stacking gel for each system.

Figure 1 - Tris-Glycine System



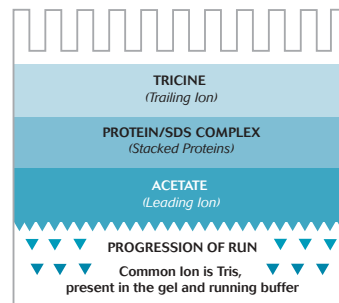
- Gel Buffer Ions are Tris⁺ Cl⁻ (pH 8.7)
- Running Buffer Ions are Tris⁺, Gly⁻ and SDS (pH 8.3)
- Gel Operating pH is 9.5

Figure 2 - NuPAGE® Bis-Tris System



- Gel Buffer Ions are Bis-tris⁺, Cl⁻ (pH 6.4)
- Running Buffer Ions are Tris⁺, MES⁻ or MOPS⁻ and SDS (pH 7.3)
- Gel Operating pH is 7.0

Figure 3 - NuPAGE® Tris-Acetate System



- Gel Buffer Ions are Tris⁺, Acetate⁻ (pH 7.0)
- Running Buffer Ions are Tris⁺, Tricine⁻ and SDS (pH 8.3)
- Gel Operating pH is 8.1

Accurate Calibration of Molecular Weight Standards for Different Buffer Systems

Generally, protein mobility in SDS gels is a function of the length of the protein in its fully denatured state (1). By constructing a standard curve with protein standards of known molecular weights, the molecular weight of a sample protein can be calculated based upon its relative mobility. However, the same molecular weight standard may have slightly different mobility and therefore, different apparent molecular weight when run in different SDS-PAGE buffer systems.

The Effects of Secondary Structure

When using SDS-PAGE for molecular weight calibration, slight deviations from the “true” molecular weight of a protein (definitively calculated from the known amino acid sequence) can occur mostly because of the retention of varying degrees of secondary structure in the protein, even in the presence of SDS. This phenomenon is more prevalent in proteins with highly organized secondary structures (such as collagens, histones, or highly hydrophobic membrane proteins) and in peptides, where the effect of local secondary structure becomes magnified relative to the total size of the peptide (2).

The pH Factor

It has also been observed that slight differences in protein mobilities occur when the same proteins are run in different SDS-PAGE buffer systems (2). Each SDS PAGE buffer system has a different pH, which affects the charge of a protein and its binding capacity for SDS. The degree of change in protein mobility is usually small in natural proteins but is more pronounced with “atypical” or chemically modified proteins, such as pre-stained standards.

Demonstrated Protein Shifts

An example of a situation where there is a shift in protein migration occurs when running the MultiMark® Multi-Colored Standard on a Tris-Glycine Gel in comparison to the same standard run on a NuPAGE® Novex Bis-Tris Gel run with either MES SDS or MOPS SDS Running Buffer. The two bands of myoglobin (red and blue) in the MultiMark® Standard are reversed in the NuPAGE® Bis-Tris buffer system, compared to the Tris-Glycine system. This phenomenon can be addressed by applying the recalculated values for the MultiMark® standard run on the respective NuPAGE® System.

Calculated and Assigned Molecular Weights

The apparent molecular weight values currently provided with the Novex® molecular weight standards were derived from the construction of a calibration curve in the Tris-Glycine SDS-PAGE System. We have calculated and assigned apparent molecular weights for the Novex® pre-stained markers in several electrophoresis gel/buffer systems including the Novex® Tricine gel system and the NuPAGE® Novex systems. Remember to use the one which matches your gel for the most accurate calibration of your sample proteins. You may generate additional calibration curves in your own lab with the Novex® standards run in a buffer system of choice.

Lot-to-Lot Molecular Weight Consistency

The important point to remember is that all the Invitrogen standards are manufactured and quality controlled to migrate consistently from one lot of standards to the next and from one gel to the next, given that the gels are using the same buffer system. This makes each of the Novex® standards equally useful in all the available buffer systems.

References:

1. Rodbard, D. (1976) *Meth. Protein Separation* **2**: 145-218.
2. Patton, W.F. *et al.* (1991) *Anal. Biochemistry*, **200**: 25-30.
3. Wyckoff, M. *et al.* (1977) *Anal Biochem.* **78**: 459-482.