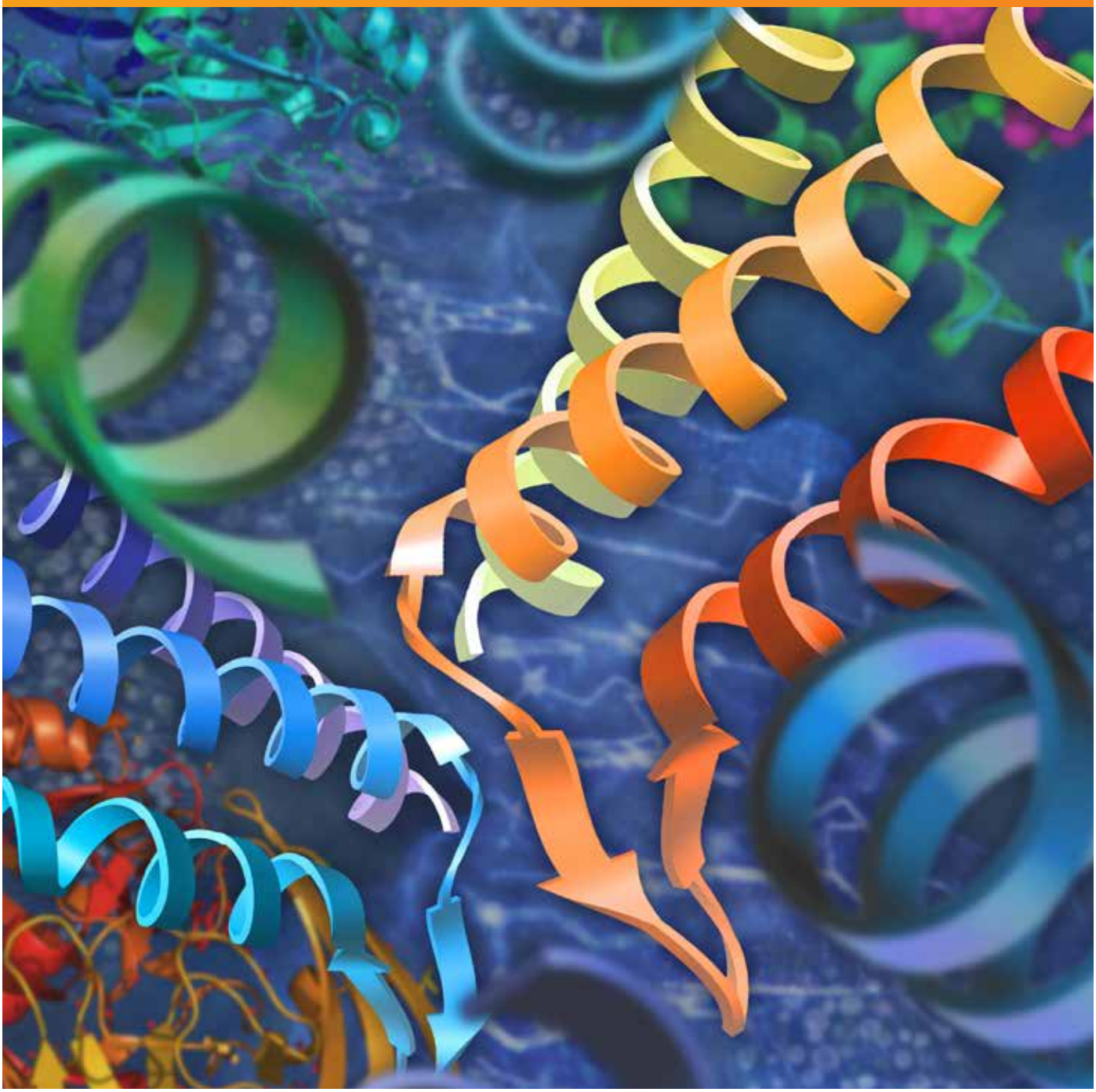
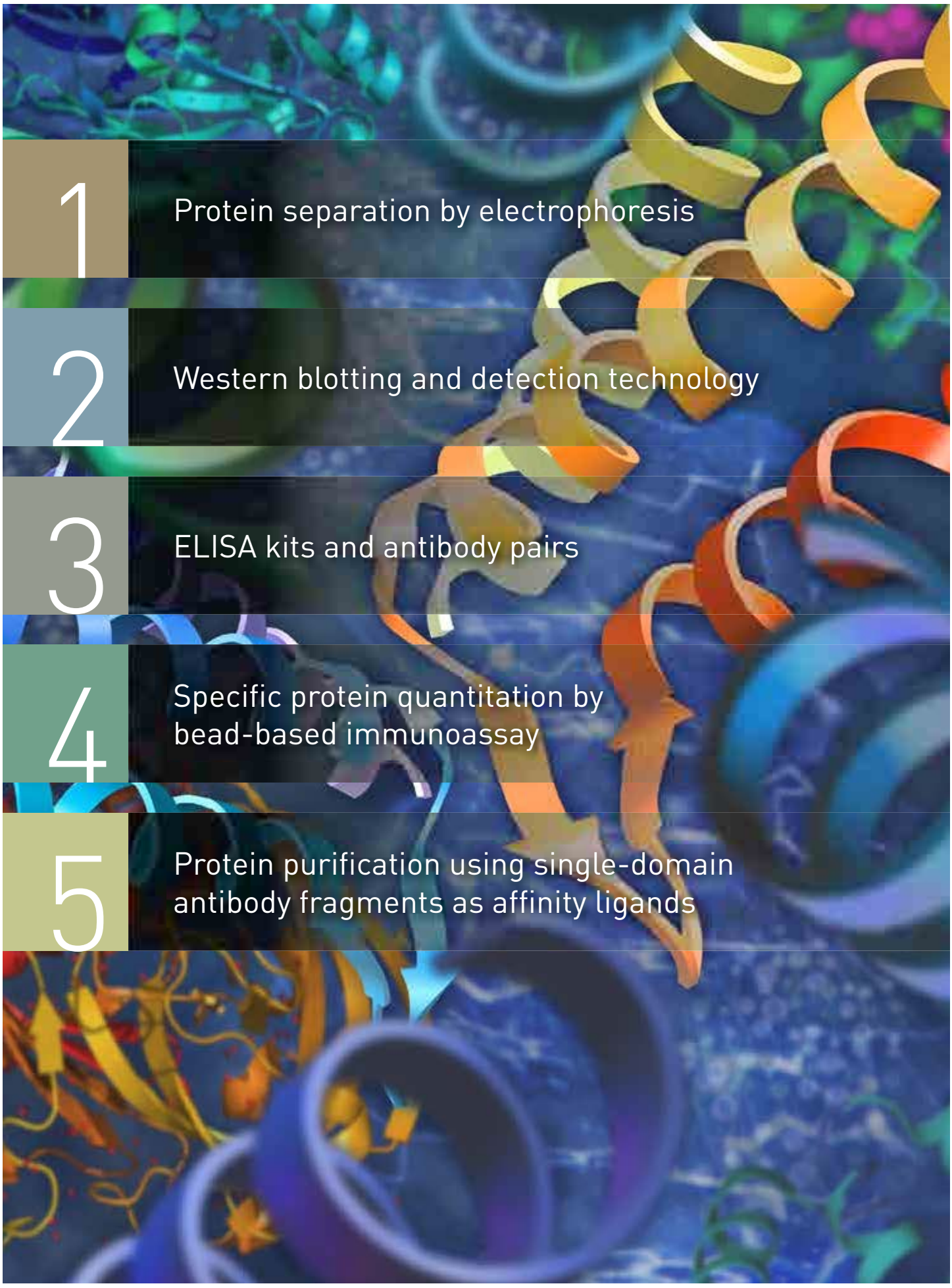
Novex®



# Protein Handbook 2013

life technologies™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## 2013 Life Technologies

# Protein handbook

This online Life Technologies resource offers published, high-performance solutions across the breadth of protein detection, protein quantitation, and protein purification.

The chapters in this book contain technology overviews, protocols, data, tips and tricks, and references to peer-reviewed publications featuring trusted Novex® products for protein research.

This book is designed to be viewed online. Included are:

- Links to more than 20 videos showing these protein research products in action
- Links to detailed protocols, product selection guides, technical guides, simulation tools, application notes, and selected products
- Direct links to our customer service and technical support teams, training courses, and webinars

# Protein separation by electrophoresis



## Electrophoresis: overview and variations


Electrophoresis, the migration of molecules in the presence of an electric field, is commonly used to separate proteins in complex mixtures. Protein electrophoresis is a critical step in many of the workflows that isolate, identify, and characterize proteins, and many of the techniques that follow changes in protein expression during normal development and disease progression. Life Technologies provides everything you need for rapid, reproducible protein electrophoresis, whether it is the first or last step in your process.

Here we present an overview of protein separation technologies, including some of the basics of electrophoresis procedures, the Life Technologies products that can specifically address your situation, some specific application notes, and, finally, tips and tricks from our scientists that will speed your success.

### What is protein electrophoresis?

Protein electrophoresis is the process of separating proteins by placing them in a gel matrix and then observing protein mobility in the presence of an electrical field. The most commonly used technique is sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In these gels, protein mobility is a function of the protein's length and charge. Because proteins are normally folded and the amino acids in the polypeptide chain have different charges, it is important to make all proteins in a mixture have the same charge per unit length and the same shape if you want to compare their sizes by PAGE. This uniform protein shape and charge proportional to size is achieved by adding SDS detergent to remove secondary and tertiary protein structures. The anionic SDS coats the proteins, mostly in proportion to their molecular weight, and confers the same negative electrical charge relative to size across all proteins in the sample. Glycosylated proteins may not migrate at their expected molecular weight because their migration is based more on the mass of their polypeptide chains, not the sugars that are attached (Sambrook J et al. (1989) *Molecular Cloning: A Laboratory Manual*, vol I. Ed 2. Cold Spring Harbor Laboratory Press).

The most widely used gel system for separating a broad range of proteins by SDS-PAGE is the Laemmli system (*Nature* 227:680 (1970)), which uses Tris-glycine gels comprising a stacking gel component that helps focus the proteins into sharp bands at the beginning of the electrophoretic run and the resolving gel, where higher gel percentages separate the proteins based on their size. This classic system uses a discontinuous buffer system where the pH and ionic strength of the buffer used for running the gel (Tris, pH 8.3) is different from the buffers used in the stacking gel (Tris, pH 6.8) and resolving gel (Tris, pH 8.8).

Novex® Gel Cassettes: easy, simple solutions that make a big difference

The highly alkaline operating pH of the Laemmli system may cause band distortion, loss of resolution, or artifact bands. The major causes of poor band resolution with the Laemmli system are:

- Hydrolysis of polyacrylamide at the high gel-casting pH, resulting in a short shelf life of 8 weeks
- Chemical alterations such as deamination and alkylation of proteins due to the high pH of the separating gel
- Reoxidation of reduced disulfides from cysteine-containing proteins, as the redox state of the gel is not constant
- Cleavage of Asp-Pro bonds of the proteins when heated at 100°C in the Laemmli sample buffer, pH 5.2

Unlike traditional Tris-glycine gels, NuPAGE® and Bolt™ Bis-Tris Plus gels are Bis-Tris HCl–buffered (pH 6.4) and have an operating pH of about 7.0. This neutral pH paired with a unique, gentle sample preparation protocol means your protein samples are in mild, nonacidic conditions, preserving the integrity of your proteins and minimizing protein alterations (Figure 1).

Novex® precast protein gels: the beauty of a straight line

As discussed in Chapter 2, a key use of protein gels is to separate proteins for subsequent transfer onto a membrane for interrogation with antibodies, a process called western blotting. The NuPAGE® Transfer Buffer maintains neutral pH and prevents reoxidation of reduced samples during protein transfer to a membrane. This avoids sample modifications that can occur at the alkaline pH of traditional transfer buffers and maintains sample antigenicity. NuPAGE® Bis-Tris gels are able to separate proteins using lower acrylamide concentrations than are required for Tris-glycine gels. This more open gel matrix allows for more efficient transfer of proteins to membranes during western blotting.

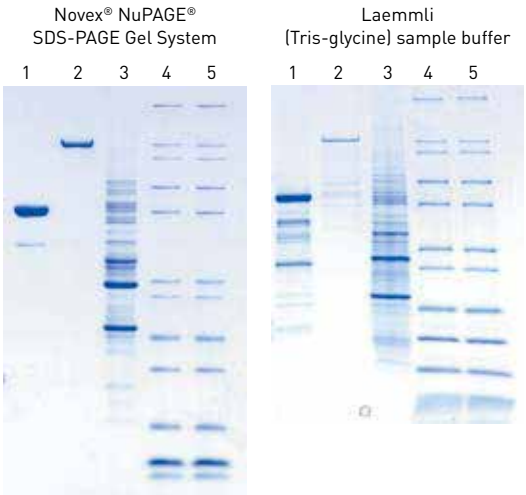
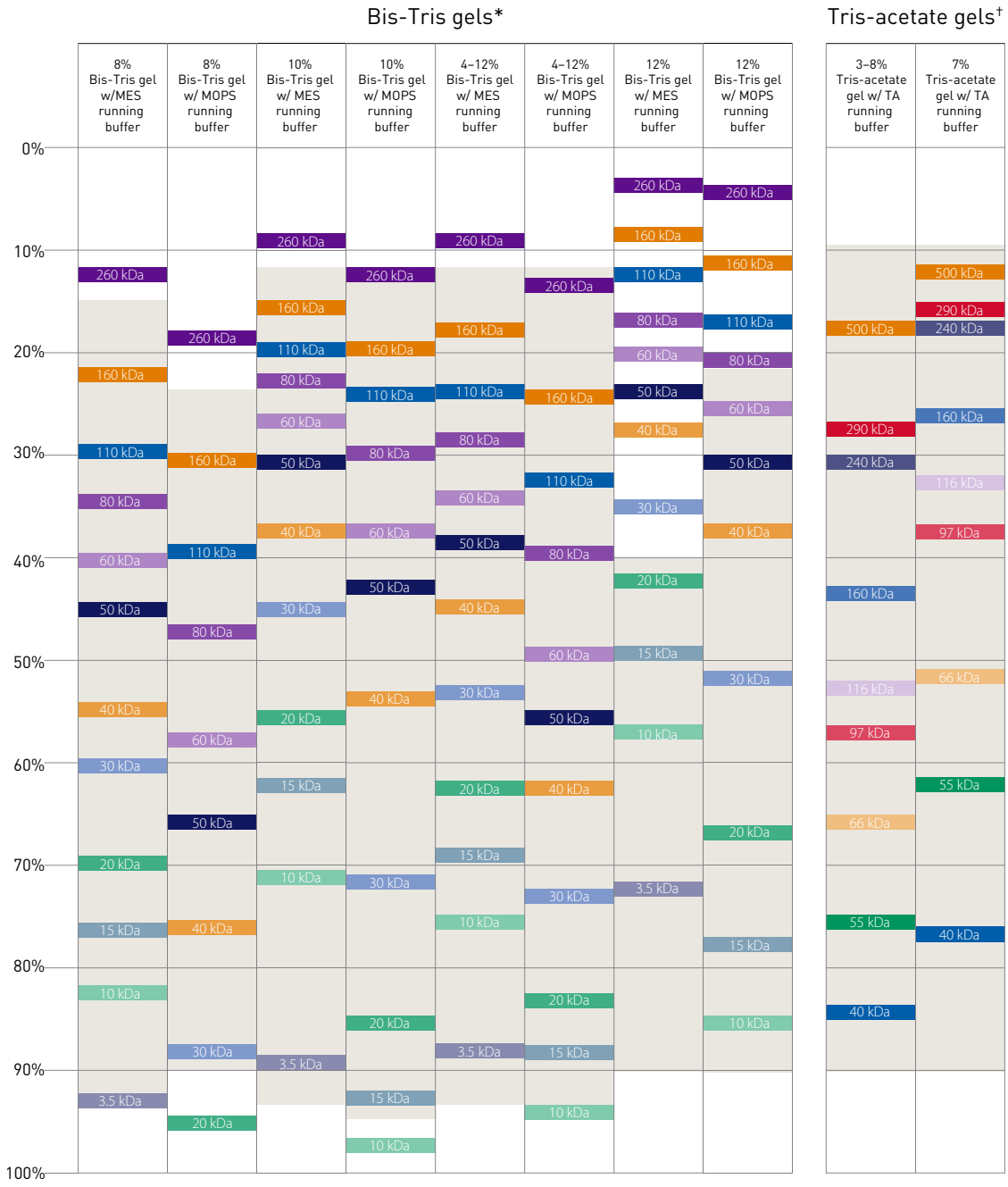



**Figure 1. Novex® NuPAGE® SDS-PAGE Gel System compared with standard Laemmli system.** Integrity of samples is maintained throughout electrophoresis with the Novex® NuPAGE® SDS-PAGE Gel System (left), compared to samples prepared with Laemmli (Tris-glycine) sample buffer (right).

## Factors influencing how SDS-PAGE separates proteins

SDS-PAGE separates proteins primarily based on their size. As proteins can have a wide range of molecular weights, researchers frequently will wish to observe specific proteins more clearly by enhancing the separation of the proteins within a particular molecular weight range. This increased resolution can be achieved by use of different percentages of acrylamide when casting a gel. Additionally, different buffers used to run the gel can affect the protein mobility. Figures 2 and 3 show the migration of proteins in NuPAGE® and Bolt™ gels using different buffers.




* Migration patterns of Novex® Sharp Protein Standards (Cat. No. LC5800, Prestained; Cat. No. LC5801, Unstained) on NuPAGE® Bis-Tris gels.
† Migration patterns of HiMark™ Unstained Standard (Cat. No. LC5688) on NuPAGE® Tris-acetate gels.

Figure 2. Migration patterns of protein standards on NuPAGE® Novex® gels.

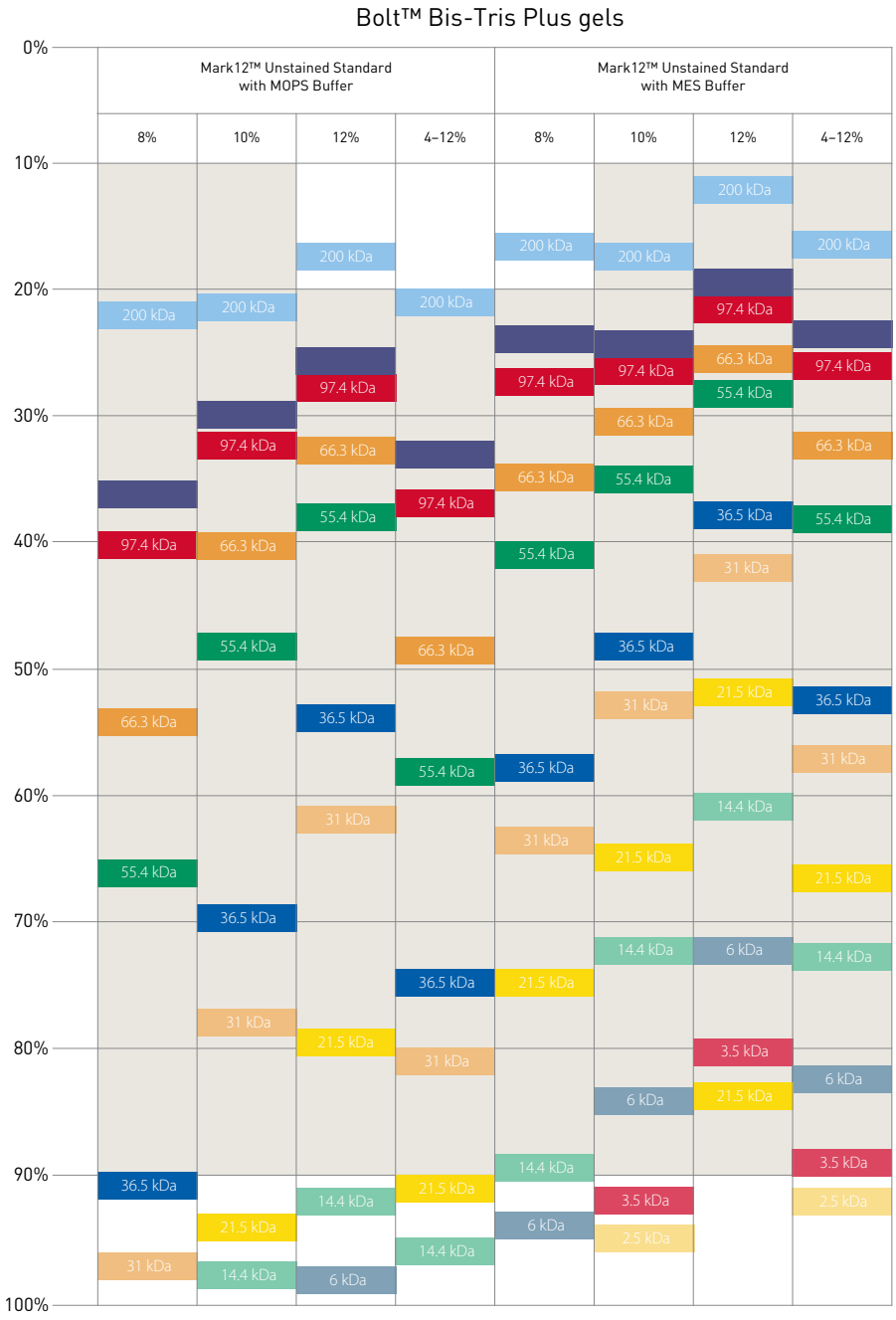



Figure 3. Migration patterns of protein standards on Bolt™ Bis-Tris Plus gels.

## Linear vs. gradient gels

In the preceding figures, some gels list a single acrylamide percentage, whereas other gels show a range of acrylamide concentrations (e.g., 4–12%). Gels that have a single acrylamide percentage are referred to as linear gels, and those with a range are referred to as gradient gels. The advantage of using a gradient gel is that it allows the separation of a broader range of proteins than does a linear gel.

## Continuous vs. discontinuous gels

Researchers occasionally refer to gels as continuous or discontinuous. A continuous gel is a gel that has been formed from a single acrylamide solution in the entire gel cassette. A discontinuous gel is actually formed from two acrylamide solutions, a small, low percentage stacking gel where the protein wells reside, and a larger portion of gel that separates the proteins. In the traditional Tris-glycine protein gel system, the proteins are stacked in the stacking gel between the highly mobile leading chloride ions (in the gel buffer) and the slower, trailing glycine ions (in the running buffer). The reason for using the stacking gel is to improve the resolution of the bands in the gel. These stacked protein bands undergo sieving once they reach the separating gel. However, the resolution of smaller proteins (<10 kDa) is hindered by the continuous accumulation of free dodecyl sulfate (DS) ions (from the SDS in the sample and running buffers) in the stacking gel. This zone of stacked DS micelles causes mixing of the DS ions with the smaller proteins, resulting in fuzzy bands and decreased resolution. The mixing also interferes with the fixing and staining of smaller proteins.

Novex® gel simulation

## Mini vs. midi protein gels

Commercial gels are available in two size formats, mini gels and midi gels. Both gels have similar run lengths, but midi gels are wider than mini gels, allowing midi gels to have more wells or larger wells. The additional wells in the Midi gels permits more samples or large sample volumes to be loaded onto one gel. Life Technologies midi gels fit into the XCell4 SureLock® Midi-Cell Runner, as well as into the Bio-Rad Criterion® gel box with adapters.

## Separation of low- and high-molecular-weight proteins

Although the separation of low- and high-molecular-weight proteins uses the same method as traditional SDS-PAGE, the sizes of these molecules require different gel chemistries to achieve optimal resolution.

Traditional Tris-glycine gel systems use a discontinuous buffer system specifically designed for the resolution of low-molecular-weight proteins. The Novex® Tricine gel system has significant advantages over traditional Tris-glycine gel systems for resolving proteins in the molecular weight range of 2–20 kDa.

The advantages of Novex® Tricine gels over traditional Tris-glycine gels include:

- Increased resolution of proteins with molecular weights as low as 2 kDa
- Improved compatibility with direct protein sequencing applications after transferring to PVDF
- Minimized protein modification due to the lower pH of the Tricine buffering system

The Novex® Tricine gel system is based on the Tris-glycine system developed by Schaegger and von Jagow (*Anal Biochem* 166:368-379 (1987)). This modified system uses a low pH in the gel buffer and substitutes Tricine for glycine in the running buffer. The smaller proteins and peptides that migrate with the stacked DS micelles in the Tris-glycine protein gel system are now well-separated from DS ions in the Novex® Tricine gel system, resulting in sharper bands and higher resolution.

Like low-molecular-weight proteins, the separation of high-molecular-weight proteins presents a unique set of challenges. Because gel integrity can be adversely affected by reducing the acrylamide concentration, researchers need alternative methods to study high-molecular-weight proteins. The NuPAGE® Novex® Tris-acetate gel/SDS buffer system is a neutral-pH polyacrylamide minigel system specifically designed for clear separation of high-molecular-weight proteins. The system includes two percentages of NuPAGE® Tris-acetate gels—3–8%, and 7%—and an optimized NuPAGE® Tris-Acetate SDS Buffer Kit. The NuPAGE® Tris-acetate gels allow the separation of the Tris and acetate molecules to act as the leading and trailing ions in the gel, creating a different separation range than do traditional gels. The separation range of these types of gels can be seen in Figures 2 and 3.

Meet the inventor: NuPAGE® system

## Specialty gels

Although SDS gels are the most common gels, Life Technologies provides a wide variety of other gels for researchers with specialized applications.

## Native gels

Like SDS-PAGE, native gels are prepared using a solution of acrylamide. However, in native gels, denaturing detergents and reducing agents are omitted so that proteins retain their native structures and charge. These gels can be used to see how protein interactions with other biomolecules, including other proteins and DNA, affect the mobility of a protein. One variation of this method is called blue-native gel electrophoresis.

The blue-native gel electrophoresis technique (using the NativePAGE™ Bis-Tris gel system) uses Coomassie G-250 as a charge-shift molecule. In SDS-PAGE, the charge-shift molecule is SDS (it binds to proteins and confers a negative charge, while at the same time denaturing the proteins). In blue-native gel electrophoresis, the G-250 binds to proteins and confers a negative charge without denaturing. The G-250 is added to samples that contain nonionic detergents before loading them onto the gels, and it is added to the cathode buffer in the system, providing a continuous flow of dye into the gel. The gels themselves do not contain G-250, so they appear as any other polyacrylamide gel before they are run.

The binding of Coomassie G-250 to protein molecules provides two key benefits:

- Proteins with basic isoelectric points that would normally have a net positive charge are converted to having a net negative charge so that they migrate in the preferred direction—to the anode
- Membrane proteins and other proteins with significant surface-exposed hydrophobic area are less prone to aggregation when G-250 binds nonspecifically to the hydrophobic sites, converting them to negatively charged sites (*Meth Cell Biol* 65:231 (2001)).

Figure 4 shows the migration of the proteins of NativeMark™ unstained native protein standard on several Life Technologies gels that are compatible with native PAGE. NativeMark™ unstained protein standard is provided as ready-to-use liquid, and is compatible with multiple native gel chemistries, e.g., Tris-glycine, Tris-acetate and NativePAGE™ systems. The marker offers a very wide molecular weight range of 20 kDa to over 1,200 kDa, and the 242 kDa B-phycoerythrin band is visible as a red band after electrophoresis (prior to staining) for reference.

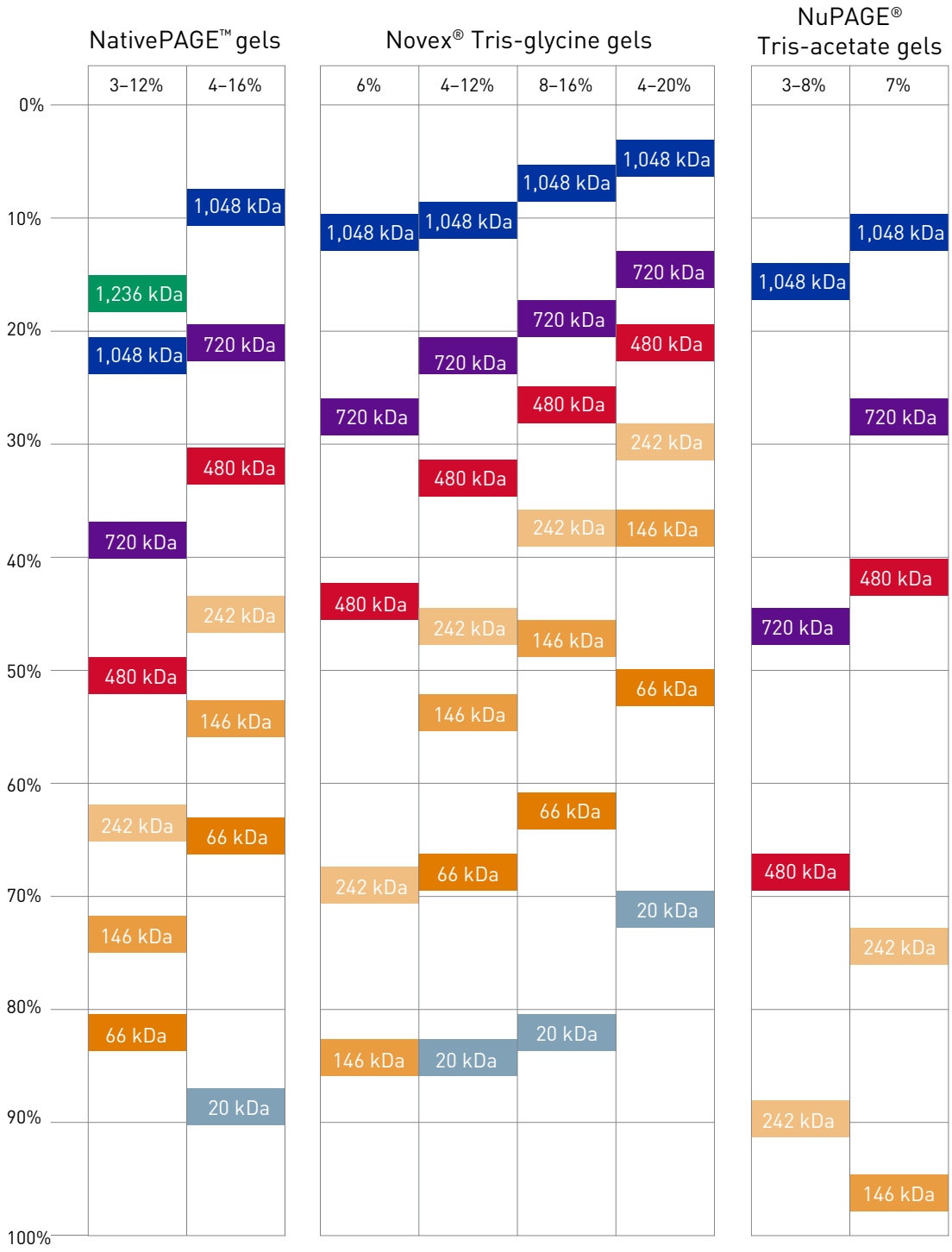



Figure 4. Migration patterns of NativeMark™ unstained native protein standards on various gels.

**Isoelectric focusing (IEF)**

IEF is an electrophoretic technique for the separation of proteins based on their isoelectric point (pI). In IEF, proteins are applied to polyacrylamide gels (IEF gels) or immobilized pH gradient (IPG) strips containing a fixed pH gradient that is generated by the presence of ampholytes (small, soluble molecules with both positive and negative charge groups). An electrical field is applied and the protein sample containing a mixture of proteins migrates through the pH gradient. Individual proteins are immobilized in the pH gradient as they approach their specific pI, the pH at which a protein has no net charge.

IEF gels can be used to determine the pI of a protein and to detect minor changes in the protein due to post-translational modifications such as phosphorylation and glycosylation. After staining the IEF gel and documenting the results, proteins separated by pI can be separated by mass using SDS-PAGE (a process called 2D gel electrophoresis).

The ZOOM® IPGRunner™ System is an oil-free platform for focusing up to 12 samples in as little as 3 hours.

The 7 cm ZOOM® Strips are immobilized pH gradient (IPG) gels available in wide pH range formats (3–10, 4–7, and 6–10) for broader analysis, and in one-unit narrow pH range strips (4.5–5.5, 5.3–6.3, and 6.1–7.1) for expanded protein separation within a specific region of interest.

The ZOOM® Carrier Ampholytes help to stabilize the pH gradient and current in IPG strips and aid in protein solubility, resulting in reproducible IEF resolution.

The ZOOM® IEF Fractionator also reduces highly complex protein samples into fractions, based on isoelectric point, for analysis by two-dimensional gel electrophoresis (2DE), one-dimensional gel electrophoresis (1DE), or two-dimensional liquid chromatography/mass spectrometry (2D LC/MS). The ZOOM® IEF Fractionator offers a versatile range of separation (fractionation) options, and works in as little as 3 hours. Unlike the gels mentioned previously, the ZOOM® IEF Fractionator uses solution-phase isoelectric focusing to provide reproducible and cost-effective separations that:

- Allow for loading of increased amounts of protein for downstream applications
- Enrich low-abundance proteins and increase the range of detection
- Reduce precipitation and aggregation artifacts associated with high protein-load samples

The fractionator uses ZOOM® Disks, which are precast polyacrylamide gels, eliminating the need for manual preparation and minimizing the chance of cross-contamination. These immobilized buffered disks are prelabeled, disposable, and designed for single use, ensuring consistent and reproducible fractionation. Using seven ZOOM® Disks, of specific pH, protein samples can be resolved into six fractions, from pI 3 to 12.

IEF separation is frequently followed by SDS-PAGE to further separate the proteins into a second dimension (size). This separation of proteins by both charge and size is referred to as 2D electrophoresis, and is frequently used to separate proteins in a complex sample. Figure 5 illustrates the use of the ZOOM® Fractionator, ZOOM® IPG strips, and SDS-PAGE gels to separate proteins, first by their isoelectric points and then by their molecular weights.

ZOOM® immobilized pH gradient gel system demonstration

1. Resuspend protein in 2D Protein Solubilizer and load into ZOOM® IEF Fractionator.
2. Fractionate proteins by isoelectric point.
3. Load ZOOM® strips with pre-fractionated proteins in ZOOM® cassettes by rapid rehydration.

   This allows the researcher to take advantage of the integrated design of ZOOM® strip limits to maximize resolution.
4. Focus proteins with the ZOOM® IPGRunner™ System.
5. Equilibrate strips in LDS for separation by MW.
6. Separate by MW with ZOOM® gels using the SureLock® mini-cell and PowerEase® 500 power supply, or XCell6™ cell and ZOOM® Dual Power supply.
7. Stain or blot gels using our kits and standards.
8. Extract, digest, analyze by mass spec using our reagents and standards.

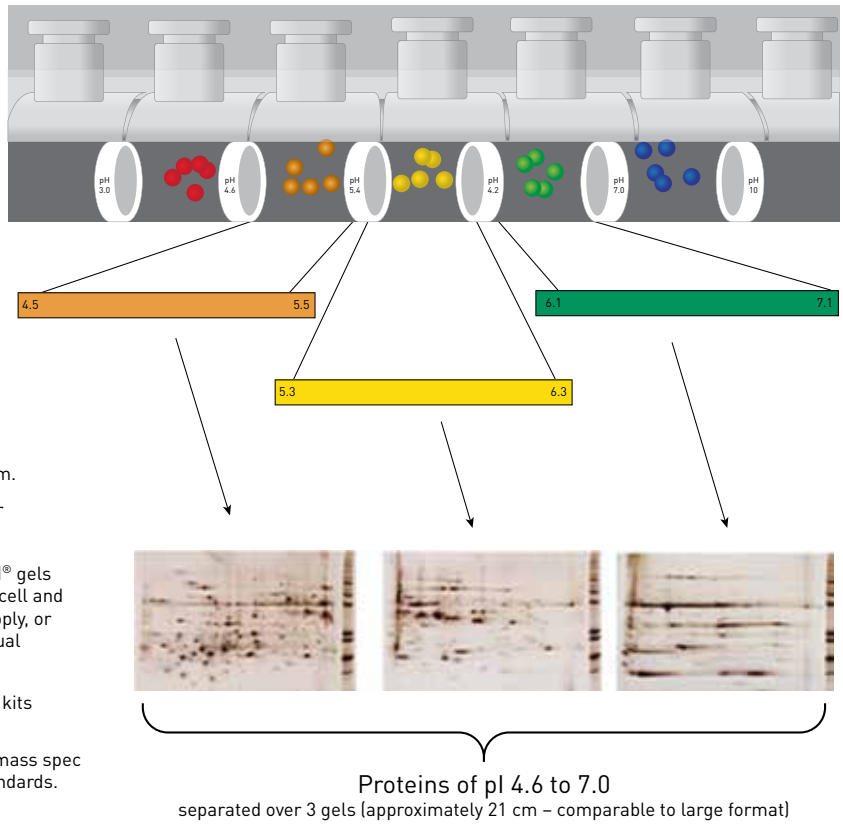



Figure 5. The use of the ZOOM® IEF Fractionator, ZOOM® IPG strips, and SDS-PAGE gels for 2D electrophoresis.

### Novex® zymogram gels

Novex® zymogram gels are excellent tools for detecting and characterizing proteases that use casein or gelatin as a substrate (Figure 6). Casein and gelatin are the most commonly used substrates for demonstrating the activity of proteases. Novex® zymogram gels have been used to analyze a variety of enzymes, including lipases, matrix metalloproteinases, and other proteases.

To use a zymogram gel, protease samples are denatured in SDS buffer under nonreducing conditions and without heating, and run on the gel. After electrophoresis, the enzyme is renatured by incubating the gel in zymogram renaturing buffer containing a nonionic detergent. The gels are then equilibrated in zymogram developing buffer (to add divalent metal cations required for enzymatic activity), and then stained and destained. Regions of protease activity appear against a dark blue background as clear bands where the protease has digested the substrate (Figure 6).

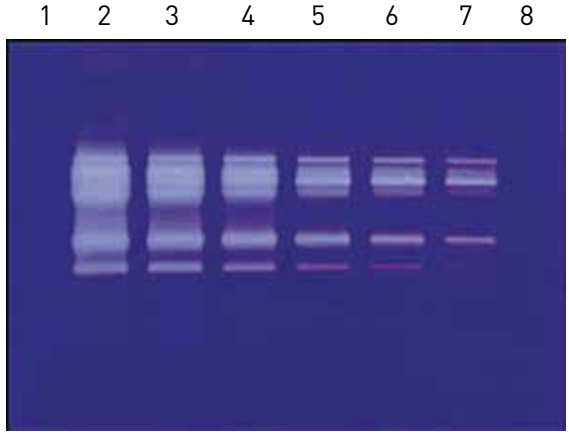



**Figure 6. Zymography of proteins after gel electrophoresis.** Following separation on a Novex® 10% Zymogram (Gelatin) Gel, proteins are renatured using Novex® Zymogram Renaturing Buffer to allow substrate cleavage. Coomassie Blue staining of gel results in clear areas where substrate was digested by protease. Lanes 2–7: Serial dilution of Type IV collagenase, 1.5 x 10–5 units (7.8 ng).

**E-PAGE™ gels**

The E-PAGE™ high-throughput (HTP) precast gel system is designed for fast, buffer-less medium- and high-throughput protein analysis. The self-contained E-PAGE™ *48* or *96* Precast Gels consist of a buffered gel matrix and electrodes packaged inside a disposable, UV-transparent cassette. The gels can be loaded by multichannel pipettor or automated loading system. Electrophoresis of the samples is performed with the E-Base™ Electrophoresis Device which consists of a base for electrophoresis, and an integral power supply. This specialized dry gel electrophoresis system is meant for people studying a large number of samples in parallel or using robotic automation in their work.

## Protein standards

Because the purpose of a protein gel is to separate a mixture of proteins based on a biophysical property (e.g., size), researchers will frequently add predefined mixtures of proteins to their gels to serve as standards in their experiment. By using these standards, researchers are able to estimate the size or other properties of their proteins.

It is important to remember that the same molecular weight standard may have a different mobility and therefore a different apparent molecular weight when run in different SDS-PAGE buffer systems. Each buffer system has a slightly different pH which affects the charge of a protein and its binding capacity for SDS. This can be pronounced in chemically modified proteins, such as prestained standards, and can affect their apparent molecular weights significantly. Therefore, it is important to use the correct calibration values for the buffer system being used (see "Tips, tricks, troubleshooting, and support", later in this chapter, for information on calibration of standards).

## Protein stains

Protein stains are solutions that are applied directly to a gel to visualize proteins once electrophoresis is completed. Some stains, such as SimplyBlue™ Safe Stain, which uses Coomassie G-250, and SilverQuest™ Silver Stain, are able to stain most proteins in gels and be viewed in visible light. There are also total proteins stains, such as SYPRO® Ruby and Coomassie Fluor Orange Stains, that are able to stain gels for visualization on fluorescence detection platforms.

Additionally, there are selective protein stains, such as the Pro-Q® Diamond and Emerald stains, that are able to recognize specific post-translational modifications (phosphoproteins or glycoproteins, respectively). Finally, stains such as the InVision™ and Lumio™ stains can recognize proteins that carry specific fusion tags.

## Drying gels

Frequently, there is a need to preserve a stained gel for documentation and later reference. To accomplish this, the gel can be sandwiched between two pieces of cellophane with gel drying solution in an open frame. The open frame and cellophane allow the gel to dry evenly. After the gel has dried, it can be stored in a laboratory notebook or scanned for documentation. Life Technologies offers frames, cellophane, and gel drying solutions for drying gels.

# General electrophoresis procedures

## SDS-PAGE sample preparation

Prior to loading samples on a PAGE gel, it is necessary to first add sample buffer to the proteins. The sample buffer contains the detergent and buffers needed to effectively prepare a protein sample for separation by PAGE. The exact buffer to be used depends on the sample, the gel, and the conditions that will be used. Please see "Tips, tricks, troubleshooting, and support", later in this chapter, for more helpful hints about preparing samples for gel electrophoresis.

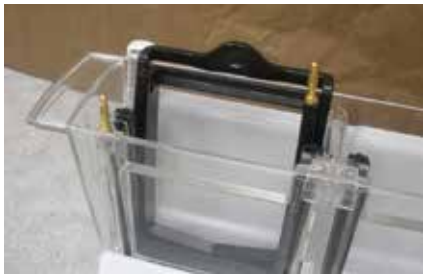


The Bolt™ gel tank in action

## Setting up and running a Bolt™ Bis-Tris Plus gel

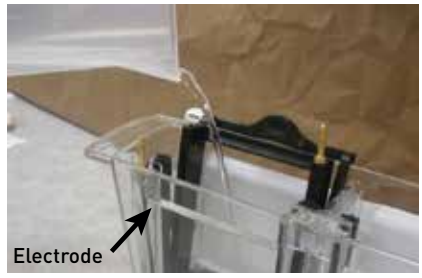
The short workflow summary in Figure 7 shows how to set up the Bolt™ gel tank and Bolt™ Bis-Tris Plus gels. Similar procedures are used for setting up and running other PAGE apparatus.

1. Place the base on a flat surface, and snap the electrophoresis tank into the base.

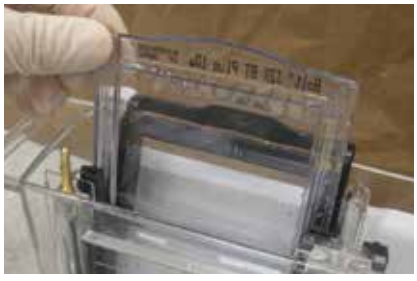


2. Place the cassette clamp(s) into the electrophoresis tank.

   The cassette clamps are directional, so make sure they are placed in the appropriate chamber.

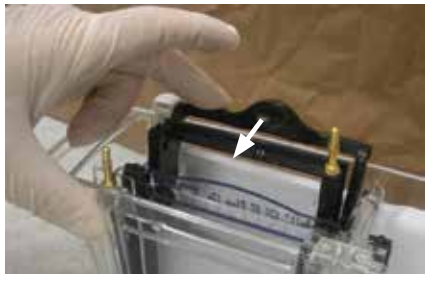



3. Fill the chamber(s) with buffer to just above the level of the electrode.



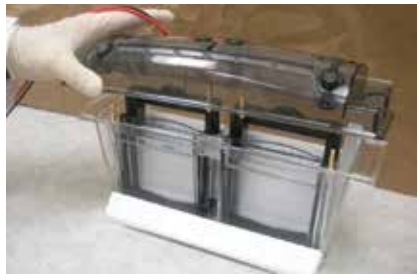
4. Remove the comb, and remove the tape from the bottom of the gel cassette(s).

   Rinse the wells with 1X running buffer, and place the cassette into the electrophoresis tank.



5. Close the cassette clamp by moving the lever forward so that the gel is secured firmly in place.

   Make sure the wells are completely filled with buffer. Load your samples and markers.



6. Place the cover on the tank. Make sure the power supply is off, and plug the power leads into your power supply.

   Turn the power supply on to begin electrophoresis.

Figure 7. Setting up and running a Bolt™ Bis-Tris Plus gel.

## Selecting settings to run an SDS-PAGE gel

SDS-PAGE gels are typically at a constant voltage. Increasing the voltage will increase protein mobility, decreasing the time needed to run the gel. However, increasing the voltage can also lead to bands that are more diffuse, and it increases the heat in the tank, which can melt the gel matrix. Please refer to the instructions for your particular gel for the optimal running conditions. Tables 1–3 provide information on the voltage settings recommended for Life Technologies gels.

Table 1. Recommended electrophoresis conditions for a variety of Life Technologies gel types.

| Gel type | Voltage | Current* | Run time |
|---|---|---|---|
| Bis-Tris gels* (see next table for more details) | 165 V constant | Start 130 mA<br>End:60 mA | • 35–45 min, dependent on buffer type<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |
| Tris-glycine gels (SDS-PAGE) | 125 V constant | Start: 30–40 mA<br>End: 8–12 mA | • 90 min<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |
| Tris-glycine gels (native PAGE) | 125 V constant | Start: 6–12 mA<br>End: 3–6 mA | • 1–12 hr |
| Tricine gels | 125 V constant | Start: 80 mA<br>End: 40 mA | • 90 min<br>• Run the gel until the phenol red tracking dye reaches the bottom of the gel. |
| Zymogram gels | 125 V constant | Start: 30–40 mA<br>End: 8–12 mA | • 90 min<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |
| IEF gels | 100 V constant: 1 hr<br>200 V constant: 1 hr<br>500 V constant: 30 min | Start: 5 mA<br>End: 6 mA | • 2.5 hr |
| TBE gels | 200 V constant** | Start: 10–18 mA<br>End: 4–6 mA | • 30–90 min, dependent on gel type<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |
| 6% TBE-urea gels | 180 V constant** | Start: 19 mA<br>End: 14 mA | • 50 min<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |
| 10% TBE-urea gels | 180 V constant** | Start: 15 mA<br>End: 8 mA | • 60 min<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |
| 15% TBE-urea gels | 180 V constant** | Start: 13 mA<br>End: 6 mA | • 75 min<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |
| DNA retardation gels | 100 V constant | Start: 12–15 mA<br>End: 6–15 mA | • 90 min<br>• Run the gel until the bromophenol blue tracking dye reaches the bottom of the gel. |

* Expected start and end current values are for single gels.
** Voltages up to 250 V may be used to reduce the run time.

Table 2. Recommended electrophoresis conditions for NuPAGE® Novex® Bis-Tris and Tris-acetate gels.

| Gel type | Voltage | Expected current* | Run time |
|---|---|---|---|
| NuPAGE® Novex® Bis-Tris gels with MES SDS running buffer | 200 V constant† | Start: 110–125 mA/gel<br>End: 70–80 mA/gel | 35 min |
| NuPAGE® Novex® Bis-Tris gels with MOPS SDS running buffer | 200 V constant† | Start: 100–115 mA/gel<br>End: 60–70 mA/gel | 50 min |
| NuPAGE® Novex® Tris-acetate gels | 150 V constant | Start: 40–55 mA/gel<br>End: 25–40 mA/gel | 1 hr |
| NuPAGE® Novex® Tris-acetate native gels | 150 V constant | Start: 18 mA/gel<br>End: 7 mA/gel | ~2 hr<br>(run times may vary) |

† Recommended voltage for 9- and 17-well gels is 150–175 V.

**Table 3. Electrophoresis conditions for Bolt™ mini gels.** We recommend running Bolt™ mini gels at constant voltage (1 or 2 mini gels).

| Running buffer | Recommended voltage, standard run | Expected current | Run time* | Recommended voltage, fast run | Expected current | Run time* |
|---|---|---|---|---|---|---|
| MES | 165 V constant | 130 mA to 60 mA | 35 min | 200 V | 180 mA to 90 mA | 22 min |
| MOPS | 165 V constant | 125 mA to 40 mA | 45 min | 200 V | 160 mA to 60 mA | 32 min |

* Run time may vary depending on the gel type and power supply used for electrophoresis.

NuPAGE® Novex® gel system demonstration

Novex® Bis-Tris gel system demonstration

## Selecting and preparing protein standards

In general, unstained standards provide a better estimation of size than do prestained standards. However, prestained standards are ideal for confirming the electrophoresis run and estimating the efficiency of transfer onto a membrane. In both cases, the proteins in Life Technologies protein standards have been modified and stabilized for use in electrophoresis. This often means that their apparent molecular weights are different from that of the proteins in their unmodified state. With prestained standards, the difference between native and apparent molecular weights may be greater, as proteins have been saturated with dye to ensure consistent migration. In addition, bands formed by prestained standards may also appear less sharp than those formed by unstained standards.

Therefore, we recommend the BenchMark™ Protein Ladder, Mark12™ Unstained Standard, HiMark™ HMW Unstained Standard, or Novex® Sharp Unstained Standard for molecular weight estimation, and our prestained standards for confirming migration and estimating blotting efficiency. All of Life Technologies standards are provided in ready-to-use formulations that can be directly loaded onto a gel. If you are using a standard that does not include loading buffer, add the appropriate amount of loading buffer prior to loading the sample on the gel. If you are using a protein standard that has not already been calibrated, it will be important to do this so you can accurately predict the size of your proteins. Please see "Tips, tricks, trouble-shooting, and support", later in this chapter, for more information on calibration of standards.

## Selecting protein stains

As mentioned above, protein gel stains can be total stains, which label all proteins, or selective for particular modifications to a protein, such as posttranslational modifications or epitope tags. The correct stain for your experiment will depend on your target samples and their abundance. Table 4 summarizes the sensitivity of several stains available from Life Technologies.

Follow the instructions for your stain to obtain optimal results. Aside from the stain, you will need a tray, such as the StainEase Staining Tray, to hold the gel and stain. It is advisable to use clean or new supplies when using high-sensitivity stains, such as silver stain, to avoid contamination of your gel.

Table 4. Protein stains available from Life Technologies.

| Stain type | Sensitivity | Gel type compatibility | Application |
|---|---|---|---|
| Coomassie Blue | 100–500 ng | Tris-glycine, Bis-Tris, Tricine, native | General |
| Coomassie Fluor™ Orange | 8–16 ng | | |
| Colloidal Coomassie Blue | <10 ng | | |
| SimplyBlue™ SafeStain | 5 ng | | |
| SilverXpress® | 1 ng | Tris-glycine, Bis-Tris, Tricine, TBE | Low sample quantity, nucleic acid |
| SilverQuest™ | 0.3–2.5 ng<br>0.3–0.9 ng (50 bp) | Bis-Tris, Tricine, TBE | |
| SYPRO® Ruby | 0.25–1 ng | Tris-glycine, Bis-Tris, Tricine, native | Low sample quantity, nucleic acid, mass spec |
| Pro-Q® Diamond | 1–16 ng | Tris-glycine, Bis-Tris | Phosphoprotein |
| Pro-Q® Emerald | 0.5–3 ng | Tris-glycine | Glycoprotein |
| Ethidium bromide | 10 ng (50 bp) | TBE | Nucleic acid |
| SYBR® Green | 60 pg (dsDNA)<br>100–300 pg (ssDNA)<br>1–2 ng (24 bp) | TBE | Nucleic acid |

## Gel electrophoresis demonstration and protocol videos

- Novex® gel simulation
- Novex® gel cassettes: easy, simple solutions that make a big difference
- Novex® precast protein gels: the beauty of a straight line
- NuPAGE® Novex® gel system demonstration
- Novex® Bis-Tris gel system demonstration
- Bolt™ gel tank animation video
- Meet the inventor: NuPAGE® system
- ZOOM® IPG video
- Detection of functional matrix metalloproteinases by zymography

## Online protocols

- Precast gels for medium-/high-throughput nucleic acid analysis
- Novex® zymogram gels
- BenchMark™ Protein Ladder
- Electrophoresis of Novex® Tricine gels—quick reference
- Gel drying
- One-dimensional SDS gel electrophoresis of peptides and small proteins with Novex® Tricine precast gels
- One-dimensional SDS gel electrophoresis of proteins with NuPAGE® Novex® precast gels
- One-dimensional SDS and nondenaturing gel electrophoresis of proteins
- Prestained protein standards—SeeBlue® Plus2 Protein Standard
- Unstained protein standards—Mark12™ Protein Standard
- Silver staining
- Coomassie staining

## Protein electrophoresis product lists and selection tools

- Protein gel selection guide
- Protein gel casting
- Novex® Tris-glycine gels
- Bolt™ gels and mini gel tank
- NuPAGE® SDS-PAGE gel system
- Novex® precast midi gels
- Specialized protein gels
- Protein standards and ladders
- Protein gel staining and imaging
- Electrophoresis buffers and reagents
- Electrophoresis chambers, power supplies, and accessories

## Selected application notes and white papers

- NuPAGE® Novex® precast gels: green features
- Bolt™ brochure
- NativePAGE™ Bis-Tris gels and buffers for blue-native electrophoresis
- Novex® NuPAGE® Bis-Tris Electrophoresis System: Performance comparison with the Mini-PROTEAN® TGX™ System
- Better separation, better results: the NuPAGE® Bis-Tris gel system
- Running MagicMark™ and SeeBlue® Protein Standards together in the same lane
- Western blotting NativePAGE™ Bis-Tris gels using the iBlot® Dry Blotting System

# Tips, tricks, troubleshooting, and support

## Sample preparation

### Reducing agent

When preparing samples for reducing gel electrophoresis, any of the following reducing agents may be used:

- NuPAGE® Reducing Agent
- Dithiothreitol (DTT), 50 mM final concentration
- β-mercaptoethanol, 2.5% final concentration
- Tris(2-carboxyethyl)phosphine (TCEP), 50 mM final concentration

We recommend only adding the reducing agent to the sample up to an hour before loading the gel. Avoid storing reduced samples for long periods, even if they are frozen. Reoxidation of samples occurs during storage and produces inconsistent results. For optimal results, we do not recommend running reduced and nonreduced samples on the same gel. If they must be applied to the same gel, do not run reduced and nonreduced samples in adjacent lanes; the reducing agent from the reduced samples may affect the nonreduced samples if they are in close proximity.

### Heating samples

Heating the sample at 100°C in SDS-containing buffer results in proteolysis (*Anal Biochem* 225:351 (1995)). We recommend heating samples for denaturing electrophoresis (reduced or nonreduced) at 85°C for 2–5 minutes for optimal results. Do not heat the samples for nondenaturing (native) electrophoresis or zymogram gels.

### High salt concentrations in samples

High salt concentrations result in increased conductivity that affects protein migration, and can result in gel artifacts in adjacent lanes containing samples with normal salt concentrations. Perform dialysis, or precipitate and resuspend samples in lower-salt buffer prior to electrophoresis.

### Guanidine-HCl in samples

Samples solubilized in guanidine-HCl have high ionic strength and produce increased conductivity similar to the effects of high salt concentrations. In addition, guanidine precipitates in the presence of SDS, leading to various types of gel artifacts. If possible, change the solubilization agent by dialysis prior to electrophoresis.

### Cell lysates

Consider the following when performing electrophoresis of cell lysates:

- Genomic DNA in the cell lysate may cause the sample to become viscous and affect protein migration patterns and resolution. Shear genomic DNA to reduce viscosity before loading the sample.
- Cells lysates contain soluble and insoluble fractions. The size of each fraction depends on the type of sample being analyzed. The nature of the insoluble fraction may result in altered protein migration patterns and resolution. Separate the two fractions by centrifugation and load them on separate lanes for electrophoresis.
- If radioimmunoprecipitation assay (RIPA) buffer is used in cell lysis, subsequent blotting of proteins less than 40 kDa may be inhibited due to the presence of Triton® X-100 in the buffer.

## Calibrating protein molecular weight

The molecular weight of a protein can be determined based on its relative mobility, by constructing a standard curve using proteins of known molecular weights.

The protein mobility in SDS-PAGE gels is dependent on:

- Length of the protein in its fully denatured state
- Extent and types of protein glycosylation
- SDS-PAGE buffer systems
- Secondary structure of the protein

The same molecular weight standard may have slightly different mobility, resulting in different apparent molecular weight when run in different SDS-PAGE buffer systems.

If you are using the Novex® protein molecular weight standards, use the apparent molecular masses of these standards in the gels listed in Tables 5 through 8 to determine an apparent molecular weight of your protein.

Table 5. Apparent molecular masses of Mark 12™ Unstained Standards and Novex® Sharp Prestained Protein Standards under various gel and buffer conditions.

| Mark 12™ Unstained Standard | NuPAGE® (4–12%) Bis-Tris/MES | NuPAGE® (4–12%) Bis-Tris/MOPS | NuPAGE® (3–8%) Tris-Acetate |
|---|---|---|---|
| Myosin | 200 kDa | 200 kDa | 200 kDa |
| β-Galactosidase | 116.3 kDa | 116.3 kDa | 116.3 kDa |
| Phosphorylase B | 97.4 kDa | 97.4 kDa | 97.4 kDa |
| Bovine serum albumin | 66.3 kDa | 66.3 kDa | 66.3 kDa |
| Glutamic dehydrogenase | 55.4 kDa | 55.4 kDa | 55.4 kDa |
| Lactate dehydrogenase | 36.5 kDa | 36.5 kDa | 36.5 kDa |
| Carbonic anhydrase | 31 kDa | 31 kDa | 31 kDa |
| Trypsin inhibitor | 21.5 kDa | 21.5 kDa | NA |
| Lysozyme | 14.4 kDa | 14.4 kDa | NA |
| Aprotinin | 6 kDa | 6 kDa | NA |
| Insulin B chain | 3.5 kDa | NA | NA |
| Insulin A chain | 2.5 kDa | NA | NA |
| Novex® Sharp Prestained Protein Standard | NuPAGE® (4–12%) Bis-Tris/MES | NuPAGE® (4–12%) Bis-Tris/MOPS | NuPAGE® (3–8%) Tris-Acetate |
| Band 1 | 260 kDa | 260 kDa | 260 kDa |
| Band 2 | 160 kDa | 160 kDa | 160 kDa |
| Band 3 | 110 kDa | 110 kDa | 110 kDa |
| Band 4 | 80 kDa | 80 kDa | 80 kDa |
| Band 5 | 60 kDa | 60 kDa | 60 kDa |
| Band 6 | 50 kDa | 50 kDa | 50 kDa |
| Band 7 | 40 kDa | 40 kDa | 40 kDa |
| Band 8 | 30 kDa | 30 kDa | 30 kDa |
| Band 9 | 20 kDa | 20 kDa | NA |
| Band 10 | 15 kDa | 15 kDa | NA |
| Band 11 | 10 kDa | 10 kDa | NA |
| Band 12 | 3.5 kDa | NA | NA |

Table 6. Apparent molecular masses of SeeBlue® and SeeBlue® Plus2 Prestained Protein Standards under various gel and buffer conditions.

| SeeBlue® Prestained Standard | NuPAGE® (4–12%) Bis-Tris/MES | NuPAGE® (4–12%) Bis-Tris/MOPS | NuPAGE® (3–8%) Tris-Acetate |
|---|---|---|---|
| Myosin | 188 kDa | 191 kDa | 210 kDa |
| BSA | 62 kDa | 64 kDa | 71 kDa |
| Glutamic dehydrogenase | 49 kDa | 51 kDa | 55 kDa |
| Alcohol dehydrogenase | 38 kDa | 39 kDa | 41 kDa |
| Carbonic anhydrase | 28 kDa | 28 kDa | NA |
| Myoglobin | 18 kDa | 19 kDa | NA |
| Lysozyme | 14 kDa | 14 kDa | NA |
| Aprotinin | 6 kDa | NA | NA |
| Insulin | 3 kDa | NA | NA |
| **SeeBlue® Plus2 Prestained Standard** | **NuPAGE® (4–12%) Bis-Tris/MES** | **NuPAGE® (4–12%) Bis-Tris/MOPS** | **NuPAGE® (3–8%) Tris-Acetate** |
| Myosin | 188 kDa | 191 kDa | 210 kDa |
| Phosphorylase B | 98 kDa | 97 kDa | 111 kDa |
| BSA | 62 kDa | 64 kDa | 71 kDa |
| Glutamic dehydrogenase | 49 kDa | 51 kDa | 55 kDa |
| Alcohol dehydrogenase | 38 kDa | 39 kDa | 41 kDa |
| Carbonic anhydrase | 28 kDa | 28 kDa | NA |
| Myoglobin | 17 kDa | 19 kDa | NA |
| Lysozyme | 14 kDa | 14 kDa | NA |
| Aprotinin | 6 kDa | NA | NA |
| Insulin | 3 kDa | NA | NA |

Table 7. Apparent molecular masses of Novex® Sharp Prestained Protein Standards and Mark 12™ Unstained Standards on Tris-glycine and Tricine gels.

| Novex® Sharp Prestained Protein Standard | Tris-glycine gels (4–20%) | Tricine gels (10–20%) |
|---|---|---|
| Band 1 | 260 kDa | 260 kDa |
| Band 2 | 160 kDa | 160 kDa |
| Band 3 | 110 kDa | 110 kDa |
| Band 4 | 80 kDa | 80 kDa |
| Band 5 | 60 kDa | 60 kDa |
| Band 6 | 50 kDa | 50 kDa |
| Band 7 | 40 kDa | 40 kDa |
| Band 8 | 30 kDa | 30 kDa |
| Band 9 | 20 kDa | 20 kDa |
| Band 10 | 15 kDa | 15 kDa |
| Band 11 | 10 kDa | 10 kDa |
| Band 12 | NA | 3.5 kDa |
| **Mark 12™ Unstained Standard** | **Tris-glycine gels (4–20%)** | **Tricine gels (10–20%)** |
| Myosin | 200 kDa | 200 kDa |
| β-Galactosidase | 116.3 kDa | 116.3 kDa |
| Phosphorylase B | 97.4 kDa | 97.4 kDa |
| Bovine serum albumin | 66.3 kDa | 66.3 kDa |
| Glutamic dehydrogenase | 55.4 kDa | 55.4 kDa |
| Lactate dehydrogenase | 36.5 kDa | 36.5 kDa |
| Carbonic anhydrase | 31 kDa | 31 kDa |
| Trypsin inhibitor | 21.5 kDa | 21.5 kDa |
| Lysozyme | 14.4 kDa | 14.4 kDa |
| Aprotinin | 6 kDa | 6 kDa |
| Insulin B chain | Unresolved insulin | 3.5 kDa |
| Insulin A chain | | 2.5 kDa |

Table 8. Apparent molecular masses of SeeBlue® and SeeBlue® Plus2 Prestained Protein Standards on Tris-glycine and Tricine gels.

| SeeBlue® Prestained Standard | Tris-glycine gel (4–20%) | Tricine gel (10–20%) |
|---|---|---|
| Myosin | 250 kDa | 210 kDa |
| BSA | 98 kDa | 78 kDa |
| Glutamic dehydrogenase | 64 kDa | 55 kDa |
| Alcohol dehydrogenase | 50 kDa | 45 kDa |
| Carbonic anhydrase | 36 kDa | 34 kDa |
| Myoglobin | 30 kDa | 23 kDa |
| Lysozyme | 16 kDa | 16 kDa |
| Aprotinin | 6 kDa | 7 kDa |
| Insulin | 4 kDa | 4 kDa |
| **SeeBlue® Plus2 Prestained Standard** | **Tris-glycine gel (4–20%)** | **Tricine gel (10–20%)** |
| Myosin | 250 kDa | 210 kDa |
| Phosphorylase B | 148 kDa | 105 kDa |
| BSA | 98 kDa | 78 kDa |
| Glutamic acid dehydrogenase | 64 kDa | 55 kDa |
| Alcohol dehydrogenase | 50 kDa | 45 kDa |
| Carbonic anhydrase | 36 kDa | 34 kDa |
| Myoglobin | 22 kDa | 17 kDa |
| Lysozyme | 16 kDa | 16 kDa |
| Aprotinin | 6 kDa | 7 kDa |
| Insulin | 4 kDa | 4 kDa |

### Protein secondary structure

When using SDS-PAGE for molecular weight determination, slight deviations from the calculated molecular weight of a protein (calculated from the known amino acid sequence) can occur due to the retention of varying degrees of secondary structure in the protein, even in the presence of SDS. This phenomenon is observed in highly organized secondary structures (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.

### Buffer systems

Slight differences in protein mobilities also occur when the same proteins are run in different SDS-PAGE buffer systems. 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 more pronounced with "atypical" or chemically modified proteins, such as prestained standards.

## Selected online electrophoresis support

- Protein gel selection guide
- NuPAGE® gel conversion guide
- Bolt™ gel simulation tool
- Protein analysis tips and tricks from Novex®
- NuPAGE® technical guide
- Novex® gel electrophoresis manual
- Migration patterns of proteins on Novex® Tris-glycine gels
- Migration patterns of proteins on Bolt™ Bis-Tris gels
- Migration patterns of proteins on NuPAGE® SDS-PAGE gels
- NuPAGE® precast gel system FAQs
- E-PAGE™ gel technical guide
- E-Gel® precast agarose gel technical guide
- Migration patterns of protein markers on Novex® Tricine, IEF, and zymogram gels

## We are always here to help: technical and customer support

To serve our customers who work around the globe and around the clock, Life Technologies offers technical support by email, telephone, live chat, and many other avenues. Stay in touch with us through Facebook, Twitter, and on our YouTube channel.

If you have questions about product selection or use, assay or experimental design, data analysis, or trouble-shooting, contact our team of technical support scientists or use our comprehensive portfolio of online product and application support tools.

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We look forward to continuing to provide you with the products and support you need.

# Western blotting and detection technology

## Protein identification by western blot

Western blotting uses antibodies to identify proteins after the proteins have been separated by gel electrophoresis. In order to make the proteins accessible to antibody detection, they are moved from within the gel onto a membrane composed of nitrocellulose or polyvinylidene difluoride (PVDF). In a basic transfer protocol, the membrane is placed on top of the gel, and the assembly is sandwiched between stacks of filter papers. The gel side of the stack is placed in a buffer solution which moves up the paper by capillary action, through the stack, bringing the proteins with it. The proteins are carried through the gel and bind to the transfer membrane. Another method for transferring the proteins is called electrophoretic blotting (or electroblotting) and uses an electric current to pull proteins from the gel onto the blotting membrane. With either method, the proteins move from within the gel onto the membrane while maintaining the spatial separation they had within the gel. Once transferred to the membrane the proteins are accessible for detection.

Methods of transfer include wet, semi-wet, semi-dry, and dry blotting. Semi-dry blotting can be performed with the Novex® Semi-Dry Blotter. Dry blotting can be performed with the iBlot® Gel Transfer Device. Semi-wet blotting can be performed with the XCell II™ Blot Module. Transfer efficiency can be checked using reversible membrane stains. After the transfer, the membrane is probed for the presence of specific proteins using primary antibodies. The probing of the membrane with antibodies can be conducted manually, or it can be automated using the BenchPro® 4100. Figure 1 outlines the western blotting workflow. The presence of the target protein(s) is typically visualized using chromogenic, chemiluminescent, or fluorescent detection reagents (i.e., goat anti-rabbit HRP).

The iBlot® Western Blotting System

Meet the inventor of the iBlot® 7-Minute Blotting System

iBlot® Gel Transfer Device

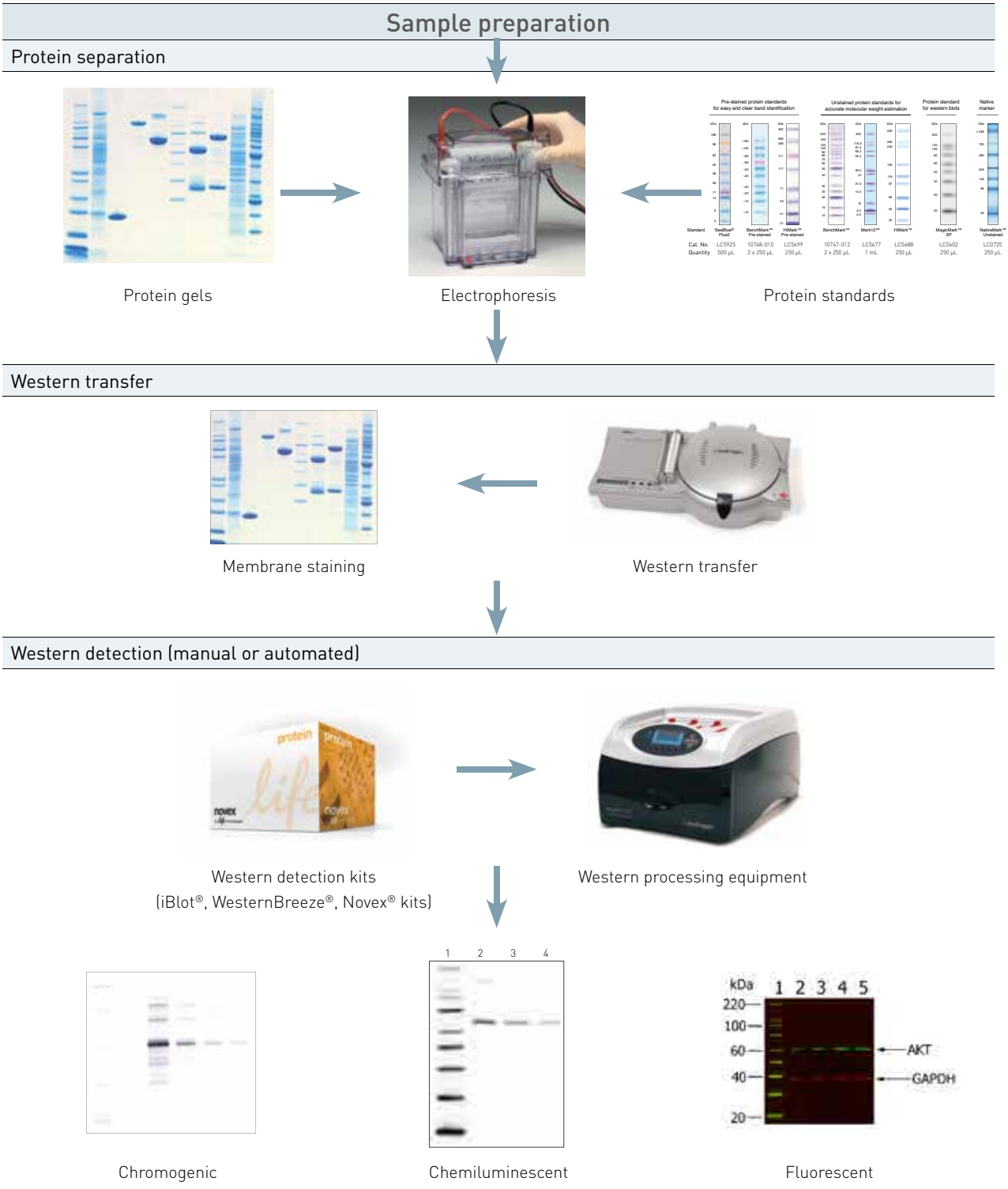



Figure 1. The western blotting workflow.

# Factors influencing western blot results

There are many factors that influence the quality of results observed when performing western blotting. These factors include the membrane used during the transfer, the type of transfer method, and the size of the proteins being transferred. Additionally, the blocking buffer and antibodies used during the probing of the membrane, the detection reagents used, and the probing technique can affect the results.

## PVDF vs. nitrocellulose membranes

A protein's properties (i.e., charge, hydrophobicity, etc.) affect its ability to bind to membrane surfaces, so finding the optimal membrane may require trying the protein of interest on different membranes. The two most commonly used membrane materials are PVDF and nitrocellulose. Both varieties of membrane are chosen for their nonspecific protein binding properties (i.e., they bind most proteins equally well). Protein binding is based upon hydrophobic interactions, as well as charged interactions between the membrane and protein. Nitrocellulose membranes are less expensive than PVDF, but are far more fragile and do not stand up well to repeated probing. PVDF has a higher overall binding capacity, but also a tendency for higher nonspecific background binding of antibodies.

Researchers, especially those using the electroblotting method, frequently include methanol when transferring proteins onto a nitrocellulose membrane. The inclusion of methanol in the transfer buffer minimizes swelling of the gel due to heat during transfer, and increases the protein binding capacity of a nitrocellulose membrane. However, it also has the effect of reducing the pore size of the gel, which restricts the transfer of some molecules. Methanol also has the effect of removing sodium dodecyl sulfate (SDS) from the proteins, which can also inhibit transfer and may favor renaturation of some proteins. If methanol is left out of the transfer buffer, it is important to pre-equilibrate the gel in transfer buffer for at least 30 minutes prior to electroblotting.

## Wet, semi-dry, and dry electroblotting

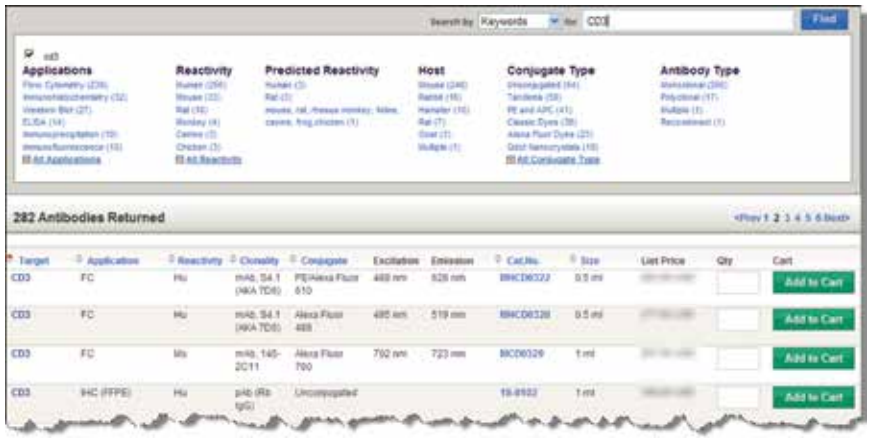
One of the key steps in the western blot workflow is the transfer of proteins from the polyacrylamide gel after electrophoresis to the nitrocellulose or polyvinylidene difluoride (PVDF) membrane so that specific proteins can be detected using immunodetection techniques. As previously discussed, methods for achieving this protein transfer include capillary transfer and electrophoretic transfer. Life Technologies has electrophoretic transfer systems using wet, semi-dry, and dry methods outlined below.

The key difference between these systems is the amount of buffer used during the setup of the transfer sandwich. In traditional wet and semi-wet transfer systems, the membrane-gel sandwich is submerged into a tank that contains transfer buffer. A current is passed through the buffer to move proteins from the gel onto the membrane. For semi-dry transfer, the membrane-gel sandwich is flanked by filter paper soaked with blotting buffer. Charge is driven through the filter paper to move the proteins from the gel to the membrane. In dry transfer systems, the membrane-gel sandwich is placed between gel matrices that contain ions. These ions move when current is applied, resulting in transfer of the proteins from the separation gel to the membrane.

Western transfer showdown: wet blot vs. dry blot

## Protein size

Transfer efficiency is influenced by molecular weight; proteins smaller than 60 kDa are transferred more efficiently than larger ones, irrespective of transfer buffer. The addition of 0.1% SDS has been reported to improve transfer of larger proteins, but may cause a general reduction in protein binding to a nitrocellulose membrane, due to interruption of hydrophobic interactions. In addition, the presence of detergent and the heating caused by high transfer current may have adverse effects on epitope stability, and consequently adversely affect the antibody-based detection of transferred proteins.




**Quickly and easily find exactly the right primary antibody.** Life Technologies offers over 2,800 antibodies, and we are adding new ones every day. For the fastest and easiest way to find the primary antibody you need, use the Primary Antibody Search Tool.

## Membrane stains

The uniformity and overall effectiveness of the transfer of protein from the gel to the membrane can be checked by staining the membrane with a reversible dye, such as the Novex® Reversible Membrane Stain. The reversible nature of this stain allows you to subsequently destain the membrane without affecting downstream probing of the membrane with antibodies.

## Blocking conditions

Because membrane materials are selected for their ability to bind protein, and both antibodies and their targets are proteins, steps must be taken to prevent interactions between the membrane and the antibody used for detection of the target protein. Blocking of nonspecific binding is achieved after transfer by placing the transfer membrane in a blocking solution such as WesternBreeze® Blocker. The blocking solution binds to any part of the membrane where transferred proteins have not already attached. Then, when the membrane is probed with antibody, the nonspecific protein-binding sites are already occupied and the antibody binds only to the specific target protein. This reduces "noise" in the final product of the western blot, enabling clearer results and minimizing false positives.

## Primary antibodies

Antibodies are critical to the success of the western blot technique. They allow for the selective detection of the protein of interest amid a vast array of other proteins. Typically a primary antibody is used to specifically bind the protein of interest and a labeled secondary antibody is used for detection. The primary antibody you choose for western blot needs to bind to a denatured form of the target protein, usually to a unique portion of its primary amino acid sequence. An antibody that effectively binds a protein on the surface of a cell may work very well for flow cytometry because it recognizes the protein's folded, native structure, but fail to detect it on a western blot for the same reason. However, western blotting is the most common application for antibodies, so commercially available primary antibodies are often produced to detect proteins on western blots (and then tested and certified for western blotting applications).

Primary antibodies are typically diluted from their stock concentration prior to use, and each antibody requires some optimization in order to perform at its best. The typical dilution range for a Life Technologies primary antibody used for a western blot is 1:500 to 1:5,000 (check the antibody manual; some antibodies may require more or less dilution).

After the blot is incubated with the primary antibody solution, the blot is washed, and if the primary antibody is not labeled with a detection molecule, a secondary antibody or other secondary detection reagent is added to the blot.

## Secondary antibodies

The secondary antibody can be conjugated to a number of different molecules for detection, such as enzymes, fluorophores, dyes, and haptens for signal amplification. The most common means of detection is to use a secondary antibody conjugated to horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzyme. Recently, however, there has been a move toward using fluorescently labeled secondary antibodies that can be imaged on a scanner designed to detect fluorescence. Fluorescence-based detection provides sensitivity similar to that of chemiluminescence detection but allows for the detection of multiple fluorophores at the same time, to give comparative data for two or more different proteins.

The use of secondary antibodies can greatly increase sensitivity compared to the use of a labeled primary antibody. Directly conjugated primary antibodies usually have a relatively small number of labels conjugated per antibody. Secondary antibodies are designed to bind the primary antibody in more than one place, which results in several secondary antibodies being bound to the primary antibody, each with their own labels or enzymes. The resulting 3- to 5-fold increase in the number of labels or enzyme present results in a significant amplification in signal. Further amplification can be achieved by using a biotinylated secondary antibody followed by incubation with streptavidin HRP. Although amplification strategies allow you to detect low-abundance targets, they also introduce new variables. Each reagent needs to be titrated to determine the concentrations which provide the best signal-to-noise ratio.



## Colorimetric detection

Colorimetric detection depends on incubation of the western blot with a substrate for a reporter enzyme that is bound to the primary or a secondary antibody used to probe the blot. The most frequently used reporter enzymes are horseradish peroxidase and alkaline phosphatase. The enzymatic reaction converts the soluble substrate dye into an insoluble form of a different color that precipitates next to the enzyme and thereby stains the membrane. Development of the blot is then stopped by washing away the soluble dye. Protein levels are evaluated for stain intensity using densitometry or spectrophotometry. Colorimetric detection is simpler, but less sensitive than other methods. Also, the colored precipitate generally cannot be removed, so these membranes cannot be stripped for reprobing.

## Chemiluminescence detection

Chemiluminescence detection depends on incubation of the western blot with a substrate that will luminesce when exposed to the reporter enzyme that is bound to the primary or secondary antibody. The light is then detected either by photographic film, or by CCD cameras or scanners, which capture a digital image of the western blot. The image is analyzed by densitometry, which evaluates the relative amount of protein staining and quantifies the results in terms of optical density of the stained image. Newer software allows further data analysis such as molecular weight analysis, if appropriate standards are used.

## Fluorescence detection

A fluorescent label on a probe is excited by light, and the emission of light energy from the dye is then detected by a photosensor, such as a CCD camera equipped with appropriate filters. The camera can capture a digital image of the western blot, which can be further analyzed to gain information such as molecular weight and semiquantitative western blot data. Fluorescence is gaining popularity and, when used optimally, can be considered among the most sensitive detection methods for blotting analysis.

## Manual vs. automated probing of western blots

Western blotting of proteins is an essential part of many workflows because of the high sensitivity and specificity that can be obtained. However, the manual process is long and time-consuming. A typical western blotting protocol can take between 6 and 16 hours to perform, and a significant part of the time is spent on processing the western blot for detection. The western blot processing may include more than a dozen individual blocking, washing, incubation, and rinsing steps. This lengthy and tedious process can result in inconsistencies and errors. The BenchPro® 4100 Western Processing System is designed to eliminate the need for manual processing of routine liquid-handling steps. With an on-board CPU and an intuitive interface, the BenchPro® system makes it easy to create and run western protocols reproducibly and with minimal errors.

# General western blotting and detection procedures

Regardless of the type of transfer used, the general setup for an electrophoretic western transfer is similar. Specific methods for setting up wet or semi-wet, semi-dry, and dry transfers are shown in the tables and figures that follow. Additionally, please view the videos (at the links listed later in this section) for instruction on how to set up an electrophoretic gel transfer.

In brief, the transfer consists of a gel and a membrane sandwich, with the gel placed closer to the cathode and the membrane positioned closer to the anode. When current is applied, the negative charge of the proteins (conferred by the SDS applied during electrophoresis) causes them to migrate from the gel onto the membrane. The transfer is conducted under constant voltage, with the time and current settings inversely related (i.e., higher current decreases transfer time). It is important to note that higher currents may overheat the system, resulting in melting of the gel or poor transfer.

## Setting up a wet or semi-wet transfer

Table 1 shows the recommended settings for transferring proteins in Life Technologies gels using the XCell II™ semi-wet blot module. Note that the expected current listed in the table is for transferring one gel. If you are transferring two gels in the blot module, the expected current will double. Figure 2 shows the correct setup of a blot for a wet/semi-wet transfer.

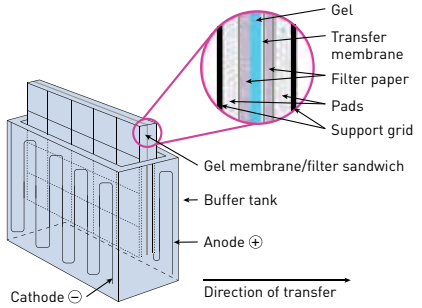



Figure 2. A wet/semi-wet transfer setup for western blotting.

Table 1. Recommended wet transfer conditions for performing western blotting with various Life Technologies gel types.

| Gel | Transfer buffer | Membrane | Power conditions |
|---|---|---|---|
| NuPAGE® Novex® Bis-Tris gel | 1X NuPAGE® Transfer Buffer with 10% methanol<br>0.1% NuPAGE® Antioxidant for reduced samples | Nitrocellulose or PVDF | 30 V constant for 1 hr<br>Expected current<br>Start: 170 mA<br>End: 110 mA |
| NuPAGE® Novex® Tris-acetate gel | 1X NuPAGE® Transfer Buffer with 10% methanol<br>0.1% NuPAGE® Antioxidant for reduced samples | Nitrocellulose or PVDF | 30 V constant for 1 hr<br>Expected current<br>Start: 220 mA<br>End: 180 mA |
| Tris-glycine gel<br>Tricine gel | 1X Tris-glycine transfer buffer with 20% methanol | Nitrocellulose or PVDF | 25 V constant for 1–2 hr<br>Expected current<br>Start: 100 mA |
| IEF gel | 1X Tris-glycine transfer buffer with 20% methanol | Nitrocellulose or PVDF | 25 V constant for 1 hr<br>Expected current<br>Start: 65-85 mA |
| | 0.7% acetic acid, pH 3.0 | Nitrocellulose or PVDF | 10 V constant for 1 hr<br>Expected current<br>Start: 65-85 mA |
| TBE gel | 0.5X TBE running buffer | Nylon | 30 V constant for 1 hr<br>Expected current<br>Start: 39 mA<br>End: 35 mA |
| TBE-urea gel | 0.5X TBE running buffer | Nylon | 30 V constant for 1 hr<br>Expected current<br>Start: 39 mA<br>End: 35 mA |
| DNA retardation gel | 0.5X TBE running buffer | Nylon | 30 V constant for 1 hr<br>Expected current<br>Start: 39 mA<br>End: 35 mA |

## Setting up a semi-dry transfer

Table 2 contains the recommended settings for transferring proteins using the Novex® Semi-Dry Blotter. Figure 3 shows the correct setup of a blot for a semi-dry transfer. It is important that the pressure on the gel stack(s) be even without being too firm.

Table 2. Recommended semi-dry transfer conditions for performing western blotting with various Life Technologies gel types.

| Gel type | Transfer buffer | Membrane | Power conditions |
|---|---|---|---|
| NuPAGE® Novex® Bis-Tris and Tris-acetate gels | 2X NuPAGE® Transfer Buffer with 10% methanol and 0.1% NuPAGE® Antioxidant for reduced samples | Nitrocellulose or PVDF | 20 V constant for 30–60 min |
| E-PAGE™ gel | 2X NuPAGE® Transfer Buffer with 0.1% NuPAGE® Antioxidant for reduced samples | Nitrocellulose or PVDF | 25 V constant for 30–60 min |
| Tris-glycine gel<br>Tricine gel | 2X Tris-glycine transfer buffer with 20% methanol | Nitrocellulose or PVDF | 20 V constant for 30–60 min |

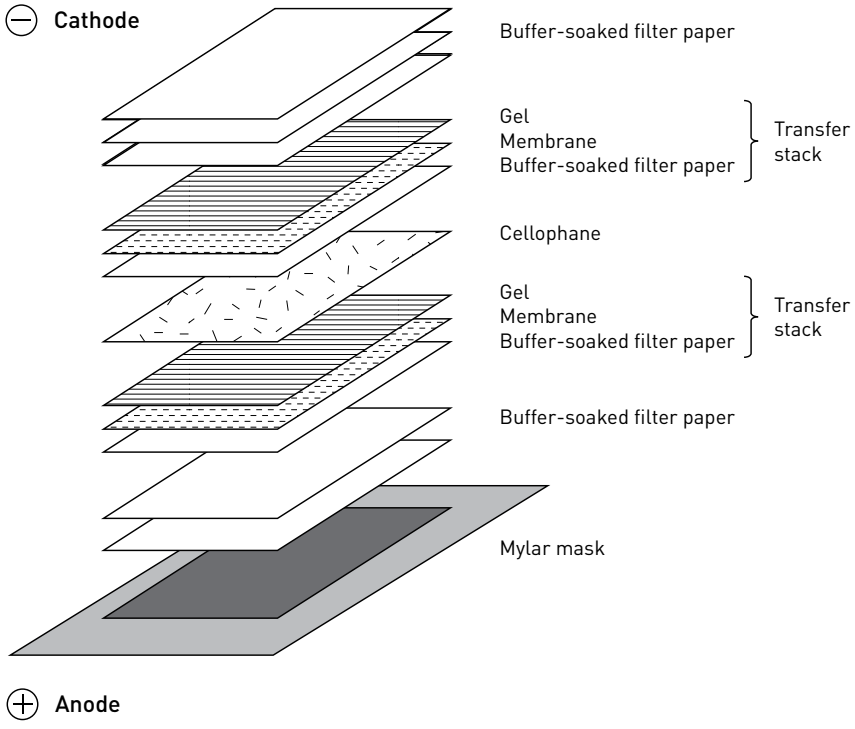



Figure 3. A semi-dry transfer setup for western blotting.

## Setting up a dry transfer with iBlot® Gel Transfer Device

Table 3 contains the recommended settings for transferring proteins in Life Technologies gels using the iBlot® Gel Transfer Device. Figure 4 shows the correct setup of a blot for a dry transfer using the iBlot® Gel Transfer Device.

Table 3. Recommended settings for transferring proteins from Life Technologies gels using the iBlot® Gel Transfer Device.

| Gel type | Program | Voltage | Run time |
|---|---|---|---|
| E-PAGE™ *48* gel | P2 | 23 V | 7–8 min |
| E-PAGE™ *96* gel | P2 | 23 V | 7–8 min |
| Novex® midi gel, 1 mm thick | P2 | 23 V | 6 min |
| 2 mini gels (1.0 or 1.5 mm thick) | P2 | 23 V | 6 min |
| 1 mini gel (1.0 or 1.5 mm thick) using mini transfer stacks | P2 | 23 V | 6 min |

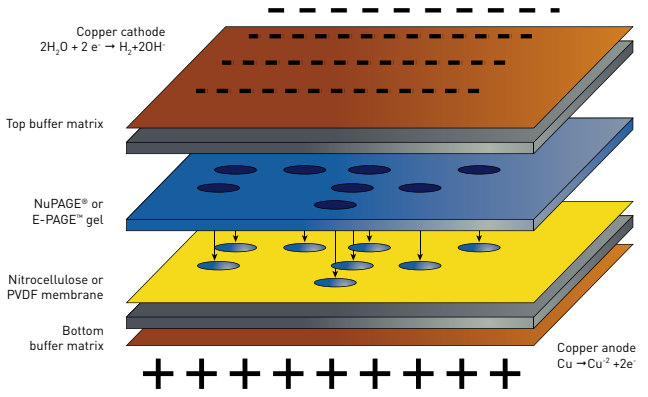



Figure 4. The setup of a blot for a dry transfer with the iBlot® Gel Transfer Device.

## Probing and detection

Once proteins have been transferred onto a membrane, they can be probed with antibodies. The proteins on the membrane are exposed to antibodies either through passive diffusion or through electrically driven binding. Passive diffusion involves submerging the membrane in a solution that contains the diluted antibody, washing of the membrane, and then incubation with a diluted secondary antibody. The extent of dilution that works best will depend on the antibodies used. Electrically driven probing of a membrane is performed using the iBlot® Gel Transfer Device and the iBlot® Western Detection Kits, which have anode and cathode stacks designed to move antibodies toward the transfer membrane for western detection.

After the membrane has been probed with labeled antibodies, the target proteins on the membrane can be detected by the method appropriate to the label. Enzymatic detection involves the addition of an enzyme substrate to the membrane followed by washing unbound reaction products and unreacted substrate away from the membrane. Chromogenic reaction products can be visualized by examining the membrane directly or by analyzing a visible-light image of the membrane. Chemiluminescent reaction products can be detected by exposing film to the membrane or by using a chemiluminescence scanner. Fluorescently labeled antibodies can be visualized directly by placing the membrane in an appropriate scanning instrument that can excite and detect the bound fluorophore.

## Stripping and reprobing membranes

When multiple proteins need to be detected on a single membrane, the traditional approach is to strip the primary and secondary antibodies from the blot and reprobe with different primary and secondary antibodies. One common method of antibody removal is the use of a heated, low-pH solution containing glycine. The combination of heat and low pH causes dissociation of the antibody–antigen complex. After the membrane has been stripped, it can be reprobed with different antibodies.

When planning to strip and reprobe a membrane, it is recommended that you use PVDF for the transfer. Because PVDF is a more durable material, it is less prone to damage during the stripping process than is nitrocellulose.

### Western blotting videos

- iBlot® Western Blotting System
- Meet the inventor: iBlot® 7-Minute Blotting System
- Western transfer showdown: wet blot vs. dry blot
- How to set up a western (part 1)—the ingredients
- How to set up a western (part 2)—running the gel on the XCell SureLock® System
- How to set up a western (part 3)—the blot transfer using the iBlot® Gel Transfer Device
- How to set up a western (part 4)—unboxing the WesternBreeze™ Kit
- How to set up a western (part 5)—unboxing the iBlot® Detection Kit
- How to set up a western (part 6)—demo of detection with the iBlot® Western Detection Kit
- How to set up a western (part 7)—automated western processing with the BenchPro® 4100
- How to perform a traditional wet protein transfer using the XCell SureLock® Blot Module
- How to perform a 7-minute protein transfer with the iBlot® Western Blotting System

### Online protocols

- Blotting Novex® precast gels
- Electrophoresis and blotting overview
- MagicMark™ XP Western Protein Standard
- Novex® chromogenic substrates
- Novex® Tris-glycine midi gels—quick reference
- Western blotting using the iBlot® 7-Minute Blotting System
- Western blotting using nitrocellulose membranes
- Western blotting using the Novex® Semi-Dry Blotter
- Western blotting using polyvinylidene difluoride (PVDF) membranes
- WesternDot™ technology with WesternBreeze® chemiluminescent detection



## Western blotting product lists and antibody selection tools

Life Technologies offers a wide variety of western blot products. We help you navigate the website by presenting starting points and product lists to help you narrow your search.

- Western blot portal
- Western blot main page
- Western blot transfer systems
- Western blot membranes
- Western blot buffers & accessories
- Western processing instruments

Life Technologies also offers a wide variety of antibodies for research purposes. Finding the right one is easy at lifetechnologies.com/antibodies, a page with links to our Antibody Selection Tools. Simply search by keyword and filter the results to find the antibody you need.

- Antibodies main page
- Primary Antibody Selection Tool
- Secondary Antibody Selection Tool
- Monoclonal antibodies
- Polyclonal antibodies
- ABfinity™ antibodies

## Selected publications and application notes

The scientific community uses Life Technologies products to move their research forward. Follow this link to explore literature published by your colleagues.

- iBlot® 7-Minute Blotting System literature citations

The R & D team at Life Technologies has performed thousands of western blots under typical research conditions and with relevant proteins, to ensure that these technologies perform to exact specifications every time. Follow the links below to read our technical publications.

- Running MagicMark™ and SeeBlue® Protein Standards together in the same lane (PDF)
- Western blotting NativePAGE™ Bis-Tris gels using the iBlot® Dry Blotting System (PDF)
- iBlot® Western Detection Kits (PDF)
- A new program and protocol to obtain improved sensitivity protein transfer using the iBlot® 7-Minute Blot (PDF)
- Troubleshooting background issues when using the iBlot® 7-Minute Blot (PDF)
- Glycine reprobing protocol using the iBlot® 7-Minute Blot (PDF)
- Transferring large and small proteins using the iBlot® Dry Blotting System (PDF)
- Rapid northern blotting (RNA transfer) with the iBlot® Dry Blotting System (PDF)
- A new dry blotting system for rapid protein transfer from polyacrylamide gels to membranes (PDF)
- Southern blotting (DNA transfer) with the iBlot® Dry Blotting System (PDF)
- Detection methods and the iBlot® Dry Blotting System (PDF)

## Tips and tricks, troubleshooting, and support

In addition to providing technical support by email, telephone, chat, and social media, Life Technologies makes a variety of resources available online to help speed your success.

- Running MagicMark™ and SeeBlue® Protein Standards together in the same lane (PDF)
- A new program and protocol to obtain improved sensitivity protein transfer using the iBlot® 7-Minute Blot (PDF)
- Troubleshooting background issues when using the iBlot® 7-Minute Blot (PDF)
- iBlot® 7-Minute Blotting System FAQs
- iBlot® Dry Blotting System FAQs (PDF)
- Instrument registration
- iBlot® 7-Minute Blotting System literature citations
- Protein analysis tips and tricks from Novex
- NuPAGE® technical guide (PDF)
- Novex® precast gel electrophoresis guide (PDF)
- Migration patterns of proteins in Novex® Tris-glycine gels
- Migration patterns of proteins in Bolt™ Bis-Tris Plus gels
- Migration patterns of proteins in NuPAGE® Novex® SDS-PAGE gels
- NuPAGE® precast gel system FAQs (PDF)
- E-PAGE™ technical guide (PDF)
- E-Gel® technical guide (PDF)

### We are always here to help: technical and customer support

To serve our customers who work around the globe and around the clock, Life Technologies offers technical support by email, telephone, live chat, and many other avenues. Stay in touch with us through Facebook, Twitter, and on our YouTube channel.

If you have questions about product selection or use, assay or experimental design, data analysis, or troubleshooting, contact our team of technical support scientists or use our comprehensive portfolio of online product and application support tools.

Our service and support teams are eager to help you advance your research. Whether you need assistance with a current order, placing a new order, or finding more information about product availability, please contact your local customer service team.

### How to find us

To find your local customer service or technical support team, go to lifetechnologies.com/contactus.

For additional support, product FAQs, protocols, training courses, and webinars, go to lifetechnologies.com/technicalresources.

Follow us on Twitter: @everydayprotein and @LifeTechSupport

Like us on Facebook: Facebook.com/novexprotein

We look forward to continuing to provide you with the products and support you need.

# ELISA kits and antibody pairs


## Protein quantitation by ELISA

The enzyme-linked immunosorbent assay, or ELISA, is a benchmark for quantitation of proteins. ELISAs are adaptable to high-throughput screening because results are rapid, consistent, and relatively easy to analyze. Life Technologies offers a wide selection of products to assist researchers who need to quantify and analyze proteins in the fields of immunology, oncology, neurobiology, stem cell research, and more.

Here we present an overview of ELISA technology, some of the basic ELISA procedures, the Life Technologies products best for your unique situation, some specific references, and, finally, tips and tricks from our scientists that will speed your success.

Life Technologies offers two options for performing ELISAs: antibody pairs and ready-to-use ELISA kits (Table 1). Novex® Antibody Pairs are matched pairs of detection and capture antibodies for researchers who prefer to coat their own ELISA plates or wish to use another platform for protein analysis. Novex® ELISA kits are complete, ready-to-use kits with precoated plates, buffers, and capture antibodies included.

Table 1. Life Technologies options for ELISAs.

| | Novex® Antibody Pairs | Novex® ELISA kits |
|---|---|---|
| Ready-to-use reagents? | No; need overnight coating process | Yes |
| Optimization required? | Yes | No |
| Total assay time | Overnight plus 4 hr | 2.5–4 hr |
| Readout | HRP-TMB (colorimetric) | Varies (colorimetric or chemiluminescent) |
| Instrumentation needed | Microplate reader | Varies (absorbance or luminescence microplate reader) |

Using Novex® Antibody Pairs to make an ELISA
Learn how to use Novex® Antibody Pairs to set up an ELISA, and find out what pre-matched antibody pairs we offer to help make ELISAs easier and more successful. Visit lifetechnologies.com/protein to learn more.

Novex® ELISA kits for protein analysis
Learn all about running an ELISA to measure target proteins in serum, plasma, supernatants, lysates, and other sample types. The ELISA is a widely accepted method for quantifying selected proteins and is often used in conjunction with western blot to analyze proteins in research samples. Visit lifetechnologies.com/protein to learn more.

Novex® ELISA kits can be further categorized into several different groups (Table 2), based on a number of factors: target protein class, sensitivity, readout method, or ability to detect specific phosphorylation states of the target protein.

Novex® phosphoELISA™ kits enable the specific detection of phosphorylation of key signaling proteins with high specificity, and are often used to supplement western blot data and provide quantitative data.

Novex® Chemi ELISA kits enable protein quantitation across a wide range of sample concentrations, minimizing guesswork for sample dilutions.

Novex® Ultrasensitive ELISA kits use a standard colorimetric readout but enable detection and analysis of proteins to levels as low as 0.5 pg/mL, especially useful with highly diluted samples.

Table 2. Comparison of Novex® ELISA kits.

| | Standard colorimetric ELISA | Ultrasensitive colorimetric ELISA | Chemi ELISA | phosphoELISA™ |
|---|---|---|---|---|
| Analytical sensitivity* | <10 pg/mL | <1 pg/mL | <1 pg/mL | <1 unit/mL |
| Measurement range* | 5–250 pg/mL | 0.5–20 pg/mL | 0.5–2,000 pg/mL | 1.6-100 units/mL |
| Incubation time* | 4 hr | 4–5 hr | 3.5 hr | 4 hr |
| Readout | HRP-TMB (colorimetric) | HRP-TMB (colorimetric) | AP-CSPD (chemiluminescent) | HRP-TMB (colorimetric) |
| Instrumentation needed | Microplate reader | Microplate reader | Microplate reader capable of measuring luminescence | Microplate reader |
| Able to detect phosphorylation state? | No | No | No | Yes |

*Every assay has its own unique specifications. Please consult the manual associated with your specific Novex® ELISA kit.

## Features of Novex® ELISA kits

- Sensitive, accurate, and consistent performance
- Validated on serum, plasma, cell culture supernatant, or cell lysate samples
- Ready-to-use, convenient assay

Our ELISA kits help provide accurate and consistent results (Figure 1). We research each target protein and calibrate our ELISA kits to provide physiologically relevant sensitivity. In addition, kits are validated using common sample types including serum, plasma, and cell culture supernatant. Cell lysates are used to validate kits that detect signaling proteins or phosphorylation.

Novex® ELISA kits must meet rigorous quality-control specifications. Our ELISA kits are manufactured in an ISO 13485 facility with stringent quality controls to ensure excellent quality and reproducibility (Figures 2–9).

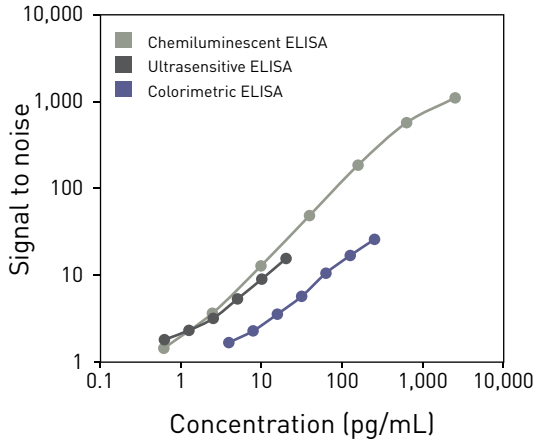



Figure 1. Comparison of sensitivity and range of different types of Novex® ELISA kits: chemiluminescent, ultrasensitive colorimetric, and standard colorimetric.

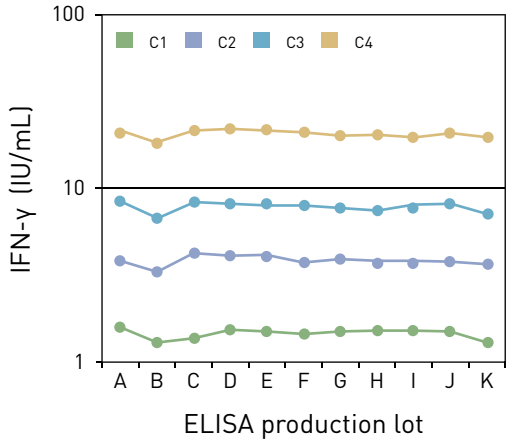



Figure 2. Novex® ELISA kits provide reproducible results from lot to lot. Individual production lots (A–K) of the Novex® IFN-γ Human ELISA Kit were analyzed using 4 concentrations of control target protein (C1–C4). All 11 lots provided uniform sensitivity and low lot-to-lot variation (<20%).

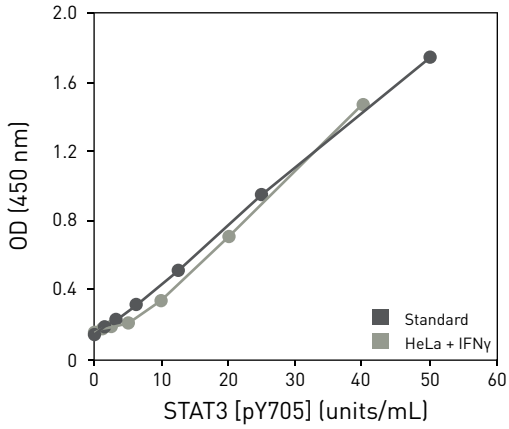



Figure 3. phosphoELISA™ Kit standards are comparable to natural samples. Recombinant standards are tested against cell lysates to ensure correct measurement values of natural samples. Note that the standard is closely aligned to the natural sample used.

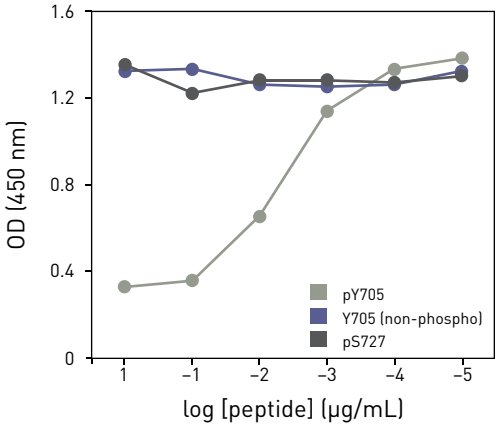



Figure 4. High specificity of the STAT3 [pY705] phosphoELISA™ kit: peptide competition. Peptide blocking is performed on each kit to confirm specificity of the phosphorylation site. The phosphorylated tyrosine 705 blocks the ELISA signal, but the nonphosphorylated peptide sequence and another phosphopeptide do not.

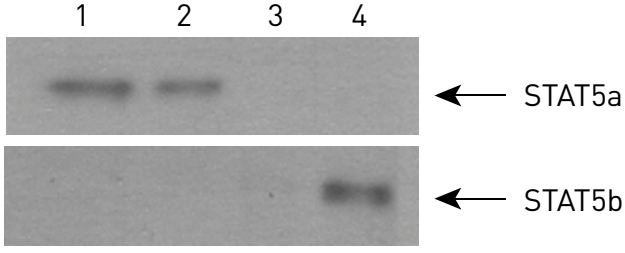



Figure 5. High specificity of the phosphoELISA™ kit: no cross-reactivity. HEL cell lysates were incubated with the capture antibody used in the STAT5a [pY694] ELISA (lane 2). An antibody specific for STAT5a and STAT5b was used as a positive control (lanes 1 and 4). IgG beads were used as a negative control (lane 3). The capture antibody recognizes the a isoform of STAT5 but not the b isoform. Thus, the STAT5a [pY694] ELISA does not cross-react with the STAT5b protein.

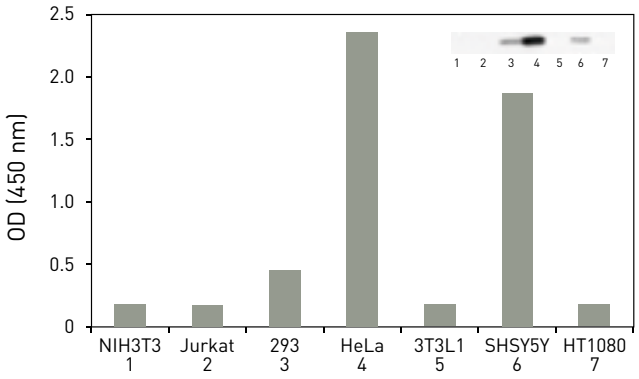



Figure 6. The expression of α-synuclein in various cell lines, detected by α-synuclein ELISA. The α-synuclein ELISA kit is specific for measuring α-synuclein and is consistent with western blotting (inset). The blot was probed with α-synuclein rabbit polyclonal antibody and developed using an alkaline phosphatase–conjugated anti–rabbit IgG followed by a chemiluminescent substrate and autoradiography.

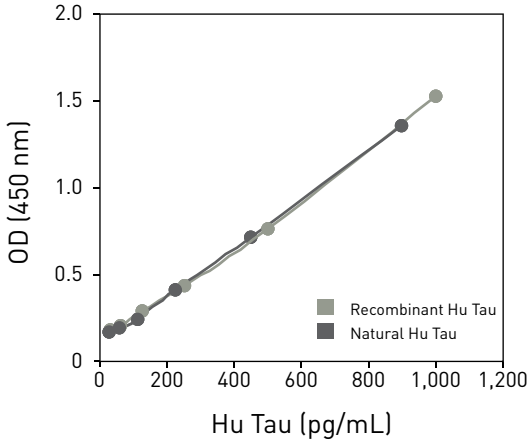



Figure 7. Correlation between recombinant and natural Hu Tau. Natural human tau protein was serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the standard curve obtained using the Tau (Total) Human ELISA Kit. The closely aligned values of the natural and recombinant proteins indicate that the standard accurately reflects natural human tau content in samples.

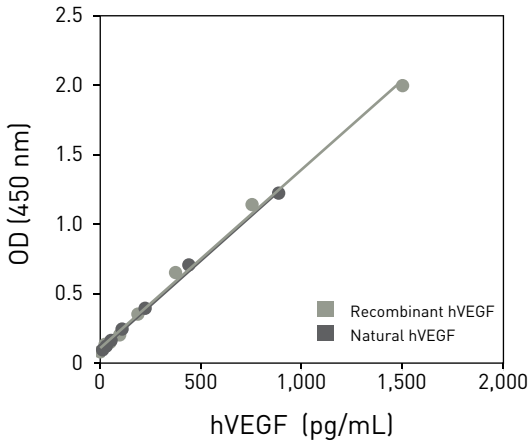



Figure 8. Correlation between recombinant and natural human vascular endothelial growth factor (hVEGF). Natural hVEGF was serially diluted in Standard Diluent Buffer. The optical density of each dilution was plotted against the standard curve. The closely aligned values of the natural and recombinant proteins indicate that the standard accurately reflects natural human VEGF content in samples.

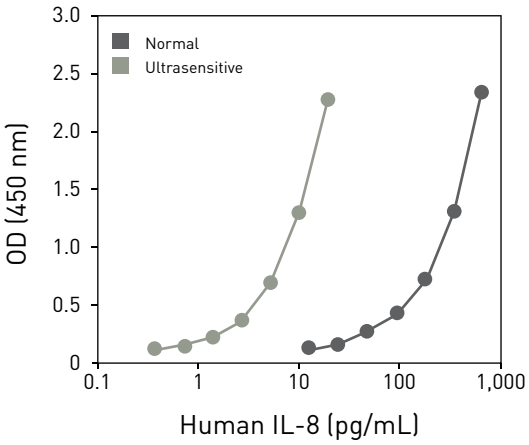



Figure 9. Representative standard curves for human IL-8 ELISA kits. Standard curves generated using the Novex® IL-8 Human ELISA Kit (normal) and the Novex® IL-8 Human Ultrasensitive ELISA Kit (ultrasensitive).

## General ELISA procedures

A detailed protocol is shipped with every Novex® ELISA kit. The general ELISA protocol, however, is quite simple and is shown in Figure 10 in schematic form. The procedure for Chemi ELISA kits is similar, except the readout is chemiluminescent instead of colorimetric.

The assay procedure for phosphoELISA™ kits is similar to our general ELISA procedure with only a few minor differences (Figure 11). The detection antibody for ELISA kits is biotin-labeled and is followed by incubation with streptavidin-HRP, whereas the detection antibody for phosphoELISA™ kits is a rabbit polyclonal antibody and is followed by incubation with HRP-labeled anti–rabbit IgG.

Novex® immunoassay tips and tricks
Learn proper pipetting techniques for immunoassays, keep your plates clog-free, and more. Visit lifetechnologies.com/protein to learn more.

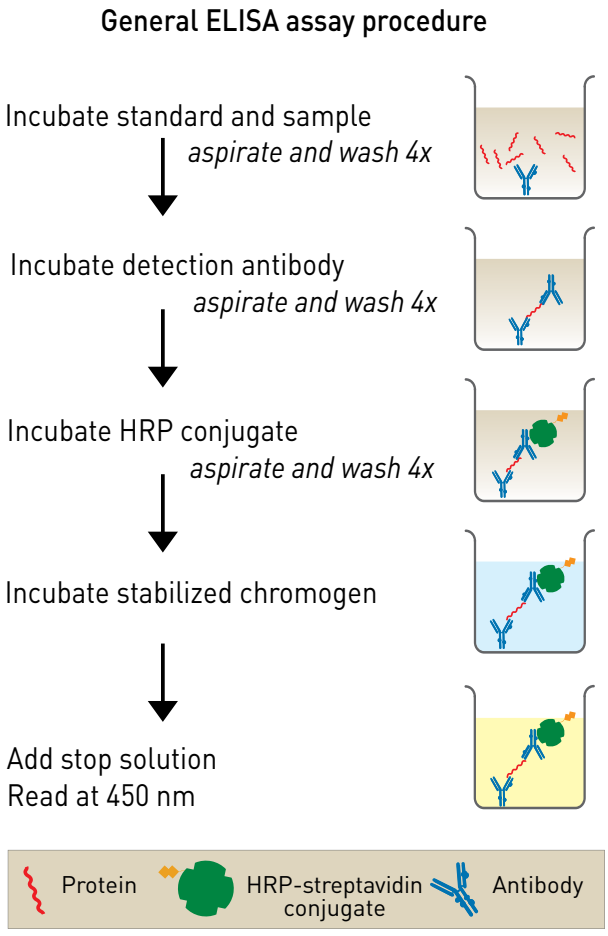



Figure 10. Fast and easy, 4-hour Novex® ELISA kit protocol. Capture antibodies are precoated on the bottom of a 96-well plate. The sample or standard is added to the wells and incubated to allow target proteins to bind. The wells are then washed to remove unbound material, and the detection antibody is added and incubated to form a sandwich around the protein of interest. HRP conjugate is then added and incubated to allow binding via a biotin–streptavidin interaction. Next, the chromogen substrate for HRP is added, and the subsequent enzymatic reaction turns the solution blue. Finally, the reaction is stopped, turning the solution yellow in proportion to the amount of target protein in the sample. Absorbance is read in a microplate reader at 450 nm.

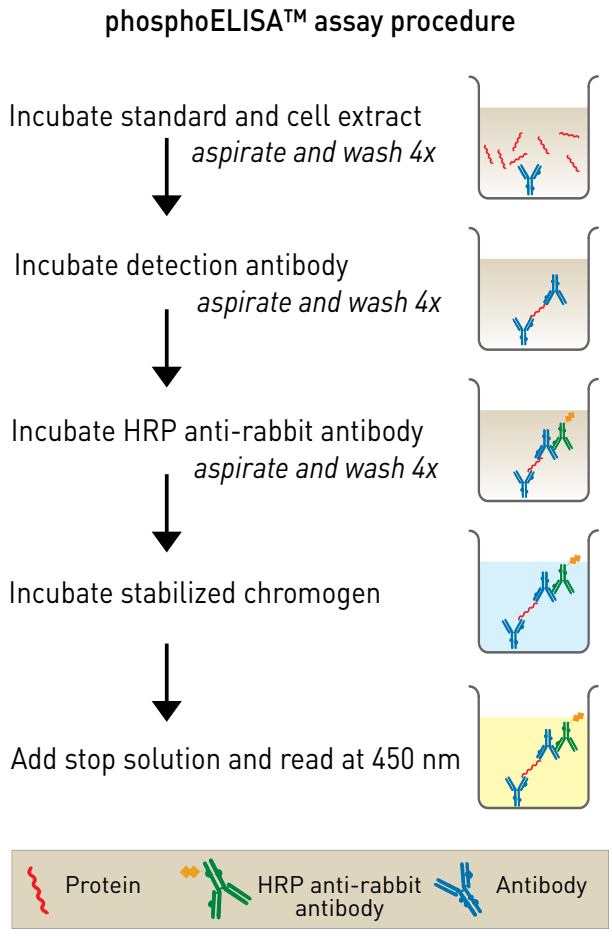



Figure 11. General schematic of phosphoELISA™ protocol. Capture antibodies are precoated on the bottom of a 96-well plate. The cell extract or standard is added to the wells and incubated to allow target proteins to bind. The wells are then washed to remove unbound material, and the detection antibody is added and incubated to form a sandwich around the protein of interest. HRP anti-rabbit antibody is then added and incubated to allow binding to the rabbit-derived detection antibody. Next, the chromogen substrate for HRP is added, and the subsequent enzymatic reaction turns the solution blue. Finally, the reaction is stopped, turning the solution yellow in proportion to the amount of target protein in the sample. Absorbance is read in a microplate reader at 450 nm.

### ELISA sample preparation protocols

Several sample preparation protocols, based on sample type, are available from Life Technologies. Click on the sample type that most closely matches the sample type you wish to analyze.

- 96-well (suspension cells)
- 96-well (adherent cells)
- Cell extraction protocol
- Nuclear extraction protocol
- Plasma and serum preparation
- Rat blood collection protocols
- Rat liver preparation
- Rat lung perfusion

## Novex® ELISA kits and antibody pairs selection guide

Life Technologies offers a wide variety of Novex® ELISA kits for research purposes. Finding the right one is easy at lifetechnologies.com/immunoassays, where you can find our immunoassay selection guide. Simply search by keyword and filter the results to find the assay you need. Keywords you can use include:

- Protein family or protein name
- Assay type
- Species
- Detection method
- Research category
- Sample type

## Selected references

Novex® ELISA kits and antibody pairs are proven performers in a wide variety of applications, for both basic research and drug discovery. In Table 3, we have collected a few examples of recent peer-reviewed publications that make use of these products.

Table 3. Peer-reviewed research publications describing the use of Novex® ELISA kits.

| Kit name | Application | References |
|---|---|---|
| Aβ 40 Human ELISA Kit | Neurology research | Sierksma AS, Vanmierlo T, De Vry J et al. (2012) Effects of prenatal stress exposure on soluble Aβ and brain-derived neurotrophic factor signaling in male and female APPswe/PS1dE9 mice. *Neurochem Int* 61(5):697–701.<br>Choy RW, Cheng Z, Schekman R (2012) Amyloid precursor protein (APP) traffics from the cell surface via endosomes for amyloid β (Aβ) production in the trans-Golgi network. *Proc Nat Acad Sci U S A* 109(30):E2077–E2082.<br>Kofler J, Lopresti B, Janssen C et al. (2012) Preventive immunization of aged and juvenile non-human primates to β-amyloid. *J Neuroinflammation* 9(1):84.<br>Cacquevel M, Aeschbach L, Houacine J et al. (2012) Alzheimer's disease-linked mutations in presenilin-1 result in a drastic loss of activity in purified γ-secretase complexes. *PLoS One* 7(4):e35133. |

Table 3 (continued).

| | | |
|---|---|---|
| Aβ 42 Human ELISA Kit | Neurology research | Kofler J, Lopresti B, Janssen C et al. (2012) Preventive immunization of aged and juvenile non-human primates to β-amyloid. *J Neuroinflammation* 9(1):84.<br>Mao P, Manczak M, Calkins MJ et al. (2012) Mitochondria-targeted catalase reduces abnormal APP processing, amyloid β production and BACE1 in a mouse model of Alzheimer's disease: implications for neuroprotection and lifespan extension. *Hum Mol Genet* 21(13):2973–2990.<br>Martín-Moreno AM, Brera B, Spuch C et al. (2012) Prolonged oral cannabinoid administration prevents neuroinflammation, lowers β-amyloid levels and improves cognitive performance in Tg APP 2576 mice. *J Neuroinflammation* 9:8. |
| IL-6 Human ELISA Kit | Inflammation research | Lambert C, Mathy-Hartert M, Dubuc JE et al. (2012) Characterization of synovial angiogenesis in osteoarthritis patients and its modulation by chondroitin sulfate. *Arthritis Res Ther* 14(2):R58.<br>Ara T, Fujinami Y, Urano H et al. (2012) Protein kinase A enhances lipopolysaccharide-induced IL-6, IL-8, and $PGE_2$ production by human gingival fibroblasts. *J Negat Results Biomed* 11:10.<br>den Dunnen J, Vogelpoel LT, Wypych T et al. (2012) IgG opsonization of bacteria promotes Th17 responses via synergy between TLRs and FcγRIIa in human dendritic cells. *Blood* 120(1):112–121.<br>Chen CA, Carolan PC, Annes JP (2012) In vivo screening for secreted proteins that modulate glucose handling identifies interleukin-6 family members as potent hypoglycemic agents. *PLoS One* 7(9):e44600.<br>Tartibian B, Hajizadeh Maleki B, Kanaley J et al. (2011) Long-term aerobic exercise and omega-3 supplementation modulate osteoporosis through inflammatory mechanisms in post-menopausal women: a randomized, repeated measures study. *Nutr Metab (Lond)* 8:71. |
| IL-8 Human ELISA Kit | Inflammation research | Lambert C, Mathy-Hartert M, Dubuc JE et al. (2012) Characterization of synovial angiogenesis in osteoarthritis patients and its modulation by chondroitin sulfate. *Arthritis Res Ther* 14(2):R58.<br>Ara T, Fujinami Y, Urano H et al. (2012) Protein kinase A enhances lipopolysaccharide-induced IL-6, IL-8, and $PGE_2$ production by human gingival fibroblasts. J *Negat Results Biomed* 11:10.<br>Sarkar A, Hellberg L, Bhattacharyya A et al. (2012) Infection with Anaplasma phagocytophilum activates the phosphatidylinositol 3-Kinase/Akt and NF-κB survival pathways in neutrophil granulocytes. *Infect Immun* 80(4):1615–1623. |
| STAT3 [pY705] ELISA Kit | Oncology research | Shi JG, Chen X, Emm T et al. (2012) The effect of CYP3A4 inhibition or induction on the pharmacokinetics and pharmacodynamics of orally administered ruxolitinib (INCB018424 phosphate) in healthy volunteers. *J Clin Pharmacol* 52(6):809–818.<br>Eghtedar A, Verstovsek S, Estrov Z et al. (2012) Phase 2 study of the JAK kinase inhibitor ruxolitinib in patients with refractory leukemias, including postmyeloproliferative neoplasm acute myeloid leukemia. *Blood* 119(20):4614–4618. |
| Tau (Total) Human ELISA Kit | Neurology research | Kofler J, Lopresti B, Janssen C et al. (2012) Preventive immunization of aged and juvenile non-human primates to beta-amyloid. *J Neuroinflammation* 9:84.<br>Mohamed NE, Zhao Y, Lee JH et al. (2011) Upregulation of AMPA receptor GluR2 (GluA2) subunits in subcortical ischemic vascular dementia is repressed in the presence of Alzheimer's disease. *Neurochem Int* 58(7):820–825.<br>Liliang PC, Liang CL, Weng HC et al. (2010) Tau proteins in serum predict outcome after severe traumatic brain injury. *J Surg Res* 160(2):302–307. |
| TNF-α Human ELISA Kit | Inflammation research | Akcay YD, Sagin FG, Aksu K et al. (2012) A panel of oxidative stress assays does not provide supplementary diagnostic information in Behcet's disease patients. *J Inflamm (Lond)* 9:13.<br>Guerrero F, Montes de Oca A, Aguilera-Tejero E et al. (2012) The effect of vitamin D derivatives on vascular calcification associated with inflammation. *Nephrol Dial Transplant* 27(6):2206–2212.<br>Bouzani M, Ok M, McCormick A et al. (2011) Human NK cells display important antifungal activity against Aspergillus fumigatus, which is directly mediated by IFN-γ release. *J Immunol* 187(3):1369–1376.<br>Karikó K, Muramatsu H, Ludwig J et al. (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res* 39(21):e142. |
| VEGF Human ELISA Kit | Inflammation research | Nagineni CN, Kommineni VK, William A et al. (2012) Regulation of VEGF expression in human retinal cells by cytokines: implications for the role of inflammation in age-related macular degeneration. *J Cell Physiol* 227(1):116–126.<br>Sánchez-Lázaro IJ, Almenar-Bonet L, Reganon-Salvador E et al. (2011) Are there differences in acute phase inflammation markers regarding the type of heart failure? *Heart Int* 6(2):e17. |

# Tips, tricks, troubleshooting, and support

In addition to providing technical support by email, telephone, chat, and social media, Life Technologies periodically publishes helpful material to our website to help our customers succeed.

For additional information on ELISA and antibody pairs, consult our ELISA Technical Guide at lifetechnologies.com/elisaguide (PDF).

## We are always here to help: technical and customer support

To serve our customers who work around the globe and around the clock, Life Technologies offers technical support by email, telephone, live chat, and many other avenues. Stay in touch with us through Facebook, Twitter, and on our YouTube channel.

If you have questions about product selection or use, assay or experimental design, data analysis, or troubleshooting, contact our team of technical support scientists or use our comprehensive portfolio of online product and application support tools.

Our service and support teams are eager to help you advance your research. Whether you need assistance with a current order, placing a new order, or finding more information about product availability, please contact your local customer service team.

## How to find us

To find your local customer service or technical support team, go to lifetechnologies.com/contactus.

For additional support, product FAQs, protocols, training courses, and webinars, go to lifetechnologies.com/technicalresources.

Follow us on Twitter: @everydayprotein and @LifeTechSupport

Like us on Facebook: facebook.com/novexprotein

We look forward to continuing to provide you with the products and support you need.

# Specific protein quantitation by bead-based immunoassay

## Novex® multiplex immunoassays

Traditionally, single-analyte, or singleplex, protein detection methods such as enzyme-linked immunosorbent assays (ELISA) or western blotting have been used sequentially to analyze multiple intracellular and extracellular proteins. Although these are both well-established, validated techniques, they can be time-consuming, sample-depleting, and costly when used to measure numerous markers per sample. Life Technologies offers a range of Novex® assays on the Luminex® xMAP® (multi-analyte profiling) technology platform for detection and quantitation of multiple proteins simultaneously, saving time, sample, and money.

- Efficient—simultaneously analyze multiple proteins using only 50 µL of sample
- Economical—helps save time and costs compared to western blot or ELISA
- Simple—easy operation with streamlined start-up and shutdown protocols

Novex® multiplex immunoassays enable the simultaneous analysis of multiple proteins in single samples from a broad range of biological sources. They combine the efficiencies of multiplexing with the accuracy, sensitivity, reproducibility, and simplicity of ELISA.

Measure multiple proteins simultaneously using the Novex® multiplex bead-based kit on the Luminex® instrument platform

### How xMAP® technology works

Luminex® xMAP® technology enables scientists to measure multiple proteins in a single well. This technology combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology to deliver multiplexed assay capabilities. Featuring a flexible open-architecture design, xMAP® technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately.

Microsphere beads, either polystyrene or paramagnetic, are color-coded into up to 500 distinct sets. Each bead set can be coated with a reagent specific to a particular bioassay, allowing the capture and detection of specific analytes from a sample. Inside the Luminex® analyzer, a light source excites the internal dyes that identify each microsphere particle, and also any reporter dye captured during the assay. Multiple readings are made on each bead set, which further validates the results. Using this process, xMAP® technology allows multiplexing of up to 500 unique bioassays within a single sample, both rapidly and precisely. xMAP® technology is compatible with the following Luminex® analyzers:

- MAGPIX® system—affordable, efficient, and compact
- Luminex® 100/200™ system—versatile, efficient, and widely used in multiplexing
- FLEXMAP 3D® system—high throughput, up to 500 simultaneous assays, and automation compatible

Novex® multiplex immunoassay tips and tricks

Our multiplex kits undergo rigorous quality testing (Table 1) and are calibrated against matching Novex® ELISA kits, if available, so that both protein analysis platforms will provide comparable analytical results. Novex® multiplex kits are:

- Fast and efficient—simultaneous analysis of multiple proteins using only 50 µL of precious sample
- Accessible—broad and expanding menu of Novex® immunoassay kits based on xMAP® technology
- Economical—can significantly reduce time and costs compared to running multiple western blots or ELISA assays

Life Technologies makes the use of Luminex® xMAP® technology simple and reliable by providing a range of Novex® singleplex and multiplex immunoassay kits, a full line of multiplexing Luminex® instruments, xPONENT® data analysis software, and extensive assay and instrument technical support, including on-site demonstrations and personalized technical consultation services.

Table 1. Rigorous assay validation of Novex® multiplex kits helps ensure consistent, reliable results.

| Specification | Description |
|---|---|
| Benchmarking to ELISA (see Figure 1) | Correlates to ELISA data (>90% correlation) |
| Recovery | Tested on serum and plasma |
| Sensitivity | Physiologically relevant levels, <10 pg/mL (based on detectable signal >2 SD above background) |
| Precision (see Figure 2) | Inter-assay CV: <10%<br>Intra-assay CV: <10% |
| Specificity (see Table 2) | Cross-reactivity tests are performed with other analytes and antibodies |
| Linearity of dilution | High coefficient of correlation between sample dilutions and expected concentration over the range of the assay |
| Parallelism to natural samples (see Figure 3) | ($R^2$ > 0.99) Recombinant standards are compared to natural samples to ensure equivalency |

How to prepare your Novex® multiplex assay

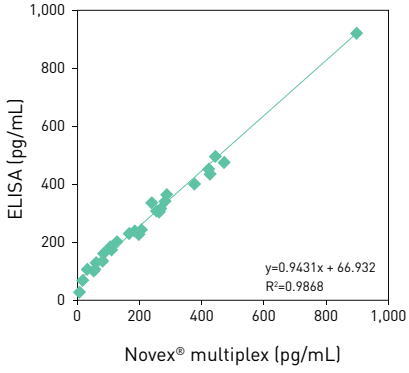



Figure 1. Multiplex assay results correlate to ELISA assay data. Murine GM-CSF in tissue culture supernatant fluid was tested by ELISA and in a Novex® multiplex assay. Correlation of values over 3 logs of sample dilution was 0.9868.

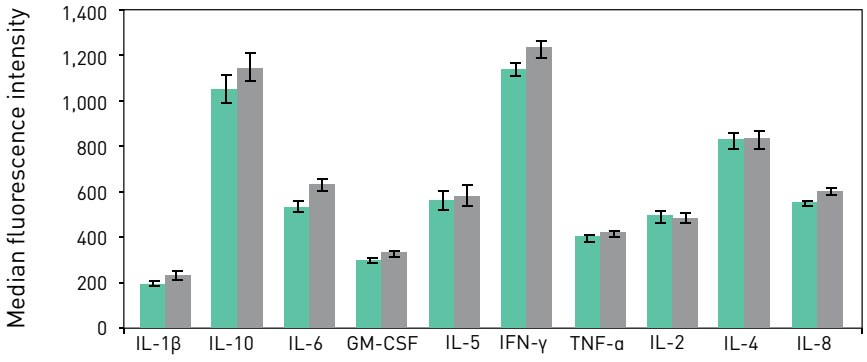



Figure 2. Assay precision. Green and gray bars each represent 24 replicates measured on separate days. CVs in all cases were less than 10%. Data were generated using a Novex® human cytokine 10-plex magnetic assay kit.

How to analyze multiplexed protein data

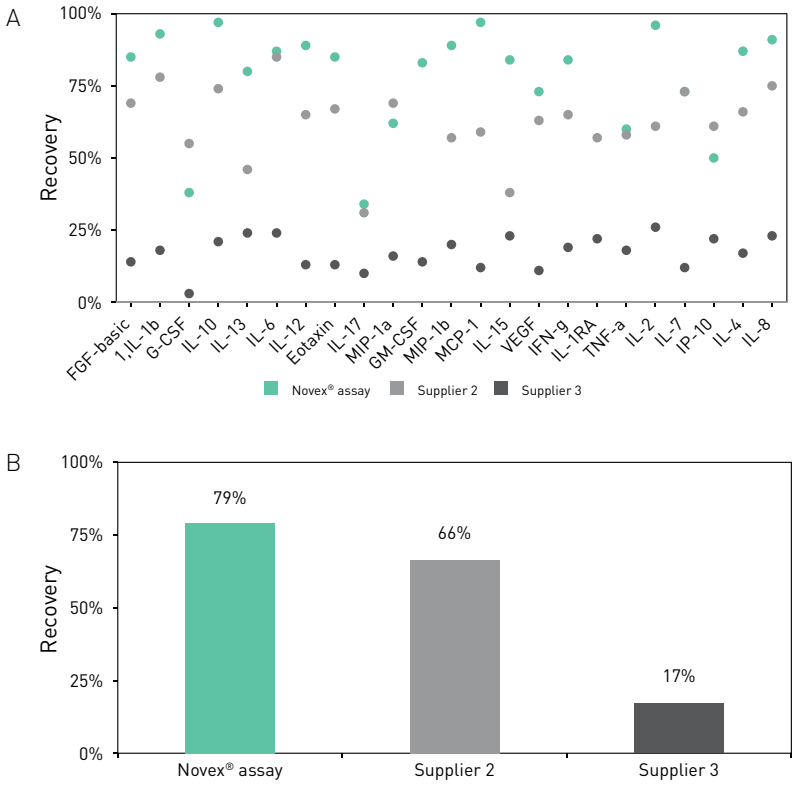


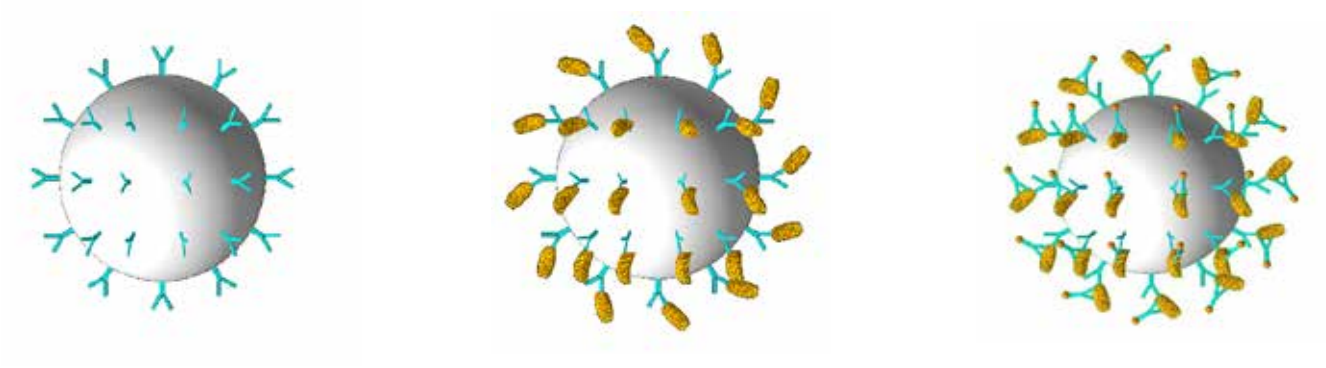

**Figure 3. Parallelism of recombinant standards to natural samples.** To evaluate the Novex® multiplex assays, samples of 23 human protein markers were spiked into a sample of human serum, and the sample was processed using the manufacturer's instructions for the Novex® multiplex assay kit and using a similar kit from two other suppliers. The multiplex sample was quantified on the MAGPIX® system, and percent recovery calculated (A) for the individual markers and (B) as an average for the entire group.

**Table 2. Specificity data from the human cytokine 10-plex panel shows no measurable cross-reactivity.** Numbers represent mean fluorescence intensity (MFI) units generated when individual recombinant proteins were analyzed independently in a series of 10-plex assays.

| | | Antibody bead kit | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | IL-1β | IL-2 | IL-4 | IL-5 | IL-6 | IL-8 | IL-10 | TNF-α | IFN-γ | GM-CSF |
| Recombinant protein | IL-1β | 6,332 | 41 | 37 | 13 | 9 | 37 | 32 | 27 | 18 | 51 |
| | IL-2 | 38 | 11,324 | 47 | 17 | 26 | 40 | 33 | 27 | 15 | 50 |
| | IL-4 | 38 | 41 | 10,503 | 15 | 25 | 40 | 32 | 25 | 12 | 50 |
| | IL-5 | 48 | 54 | 36 | 8,265 | 11 | 34 | 33 | 25 | 16 | 51 |
| | IL-6 | 44 | 72 | 40 | 11 | 6,827 | 42 | 31 | 22 | 12 | 70 |
| | IL-8 | 39 | 39 | 44 | 12 | 27 | 10,002 | 32 | 27 | 19 | 52 |
| | IL-10 | 41 | 51 | 39 | 11 | 12 | 41 | 8,151 | 23 | 12 | 46 |
| | TNF-α | 32 | 55 | 36 | 10 | 7 | 60 | 30 | 6,642 | 10 | 47 |
| | IFN-γ | 32 | 41 | 39 | 10 | 8 | 70 | 35 | 25 | 3,540 | 51 |
| | GM-CSF | 41 | 40 | 36 | 12 | 8 | 46 | 33 | 25 | 13 | 4,932 |

## How Luminex® technology works

The Luminex® xMAP® technology is based on polystyrene or paramagnetic microspheres, or beads, that are internally dyed with red and infrared fluorophores of differing intensities. Each dyed bead is given a unique number, or bead region, allowing the differentiation of one bead from another. For Novex® multiplex immunoassay kits, individual bead sets are then coated with a capture antibody qualified for one specific analyte (Figure 4). Multiple analyte-specific beads can then be combined in a single well of a 96-well microplate-format assay to detect and quantify multiple targets simultaneously, using one of the Luminex® instruments for analysis. We offer multiplex assays using either polystyrene or paramagnetic beads.



Antigen-specific capture antibodies are bound to microspheres

Antigen from the test sample is bound to the capture antibodies

Signal is generated by attachment of the labeled detection antibodies

Figure 4. Protocol used with bead-based assays (Luminex® Corporation).

## Multiplex assays compared to ELISAs

Novex® multiplex assays are performed in much the same way as ELISAs (Figure 5). The steps are similar, with the exception that antibody-specific capture beads are added to the wells of 96-well microtiter plates, instead of having capture antibodies attached to the wells. Samples are then placed into the microtiter plate wells. Novex® multiplex assay kits are provided with protein standards of known concentration, so that standard curves for the proteins being analyzed can be generated.

After incubation, the beads are washed and resuspended in a solution containing biotinylated detection antibody. Another incubation and wash step is performed, followed by the addition of streptavidin–R-phycoerythrin (R-PE). The beads are then washed and are ready to be analyzed on a Luminex® instrument.

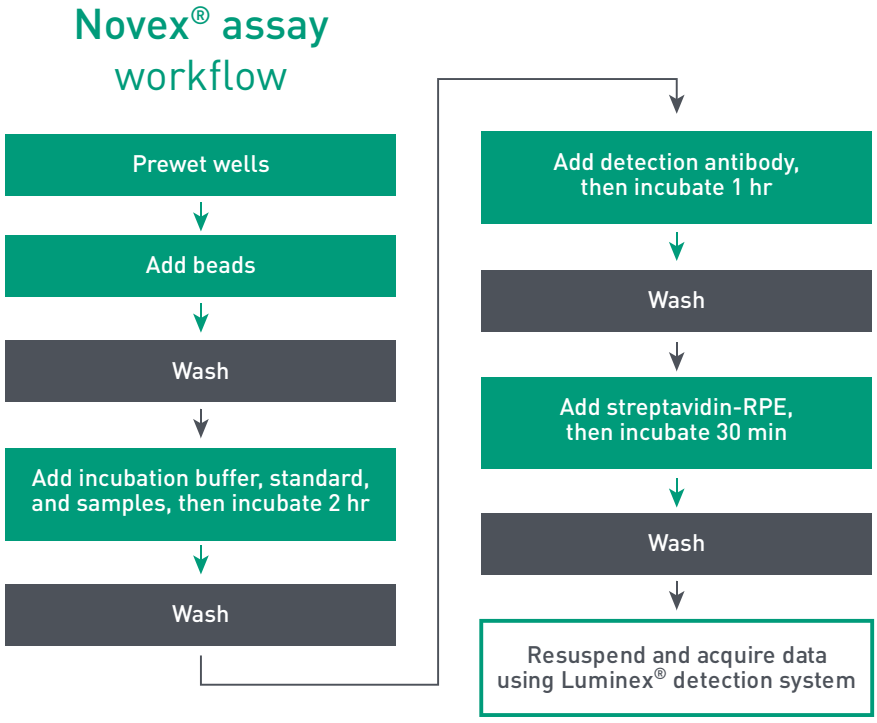



Figure 5. Workflow for using Novex® multiplex assay kits.

## Novex® singleplex and multiplex magnetic assays

Novex® singleplex and multiplex assays, based on Luminex® xMAP® technology, provide a versatile platform that gives users more flexibility and a greater array of options for measuring single or multiple analytes. The new line of paramagnetic bead assays was carefully designed and tested to help ensure that sensitivity, range, and correlation to other assay systems are maximized, using some of the same components as our polystyrene bead assays. This means that the paramagnetic and polystyrene bead–based assays perform with comparable quality and consistency (Figure 6).

Advantages of Novex® paramagnetic bead assay kits include:

- Compatibility with manual or automated handling
- Easy wash steps with the handheld 96-well magnetic separator (Figure 7)
- Automation-enabled wash steps that eliminate vacuum manifold washes, clogging of filter plates, and bead loss

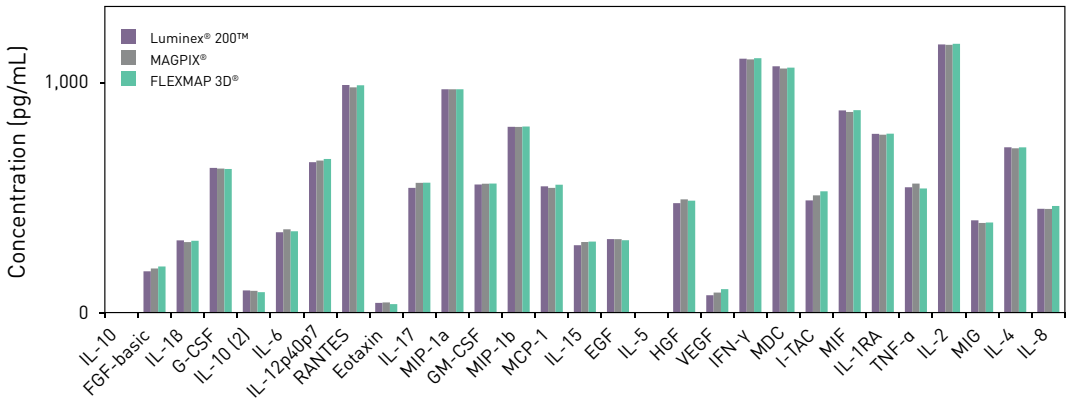



Figure 6. Consistency across assay systems. Monkey peripheral blood mononuclear cells (PBMC) were stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA) and A23187 for 72 hr. Markers were detected using the Monkey Cytokine Magnetic 28-Plex Panel, and the results were analyzed on the Luminex® 200™, MAGPIX®, and FLEXMAP 3D® systems.

How to perform magnetic assay wash steps

How to use the protocol and batch functions with the MAGPIX® system

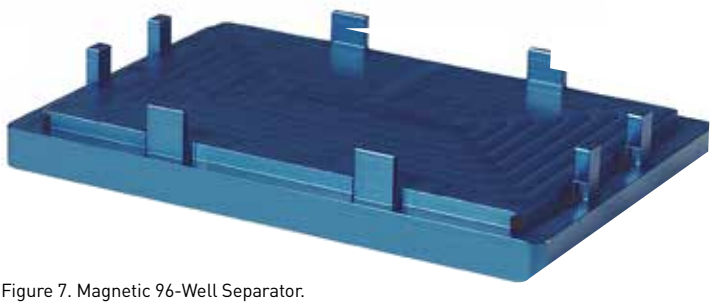


Figure 7. Magnetic 96-Well Separator.

## Luminex® instrument systems

Luminex® systems are built on proven technology—flow cytometry, microspheres, lasers, digital signal processing, and traditional chemistry—combined in a unique way. Featuring a flexible open-architecture design, Luminex® xMAP® technology can perform a wide variety of bioassays quickly, cost-effectively, and accurately. In Table 3, we present an overview of the 3 different Luminex® instruments available for multiplex detection and analysis.

Table 3. Overview of the Luminex® instrument systems.

| | MAGPIX® system | Luminex® 100/200™ system | FLEXMAP 3D® system |
|---|---|---|---|
| Feature | Affordable, efficient, compact size | Versatile and efficient; the standard in multiplexing | High-throughput, high-plex, automation compatible |
| Optics | LED/CCD camera | Lasers/APDs/PMTs | Lasers/APDs/PMTs |
| Hardware | Fluorescence imager | Flow cytometry–based | Flow cytometry–based |
| Bead compatibility | Magnetic beads only | Polystyrene and magnetic beads | Polystyrene and magnetic beads |
| Multiplex capacity | 50 | 100 | 500 |
| Read time for 96-well plate | ~60 min | ~40 min | ~20 min |
| Measurement range | 3.5 logs | 3.5 logs | 4.5 logs |
| Microtiter plate | 96-well | 96-well | 96-well and 384-well |

Multiplex protein analysis: the MAGPIX® system

How to set up the computer on the MAGPIX® system

## The MAGPIX® system

The MAGPIX® system analyzes magnetic beads immobilized with a magnet, excites the beads using light-emitting diodes (LEDs), and then detects and analyzes the beads using a CCD camera (Figure 8). The MAGPIX® system is thus a more compact, robust, and cost-effective multiplexing tool. In addition, streamlined startup and shutdown protocols, and minimal maintenance requirements make the system easy to use—ideal for both new and experienced users.

The MAGPIX® system is a compact benchtop instrument with a multiplex capability of up to 50 analytes. It features simple out-of-the-box setup and interactive software. For more details, please contact your Life Technologies sales representative or visit lifetechnologies.com/magpix.

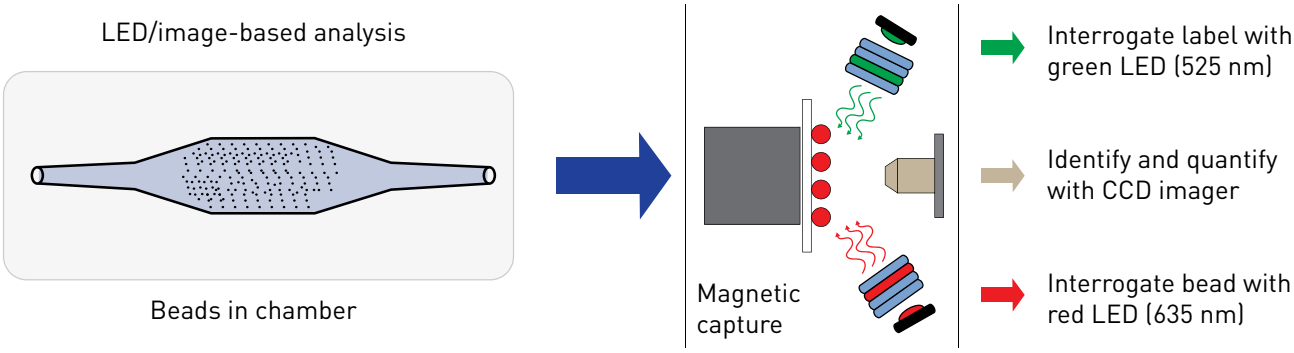



Figure 8. The MAGPIX® instrument uses light-emitting diodes (LEDs) to measure the levels of several (typically 5 to 30) targets in a single sample.



## The Luminex® 100/200™ system

The Luminex® 100/200™ system is a compact analyzer that performs up to 100 assays simultaneously in a single well of a microtiter plate. This flexible system is based on the principles of flow cytometry, integrating key xMAP® detection components to meet the multiplex testing needs of research laboratory professionals. For more details, please contact your Life Technologies sales representative or visit lifetechnologies.com/luminexinstrument.

## The FLEXMAP 3D® system

The FLEXMAP 3D® system combines differentially dyed fluorescent microsphere sets with an innovative instrument design to take bead-based multiplexing technology to the next dimension, and is capable of multiplexing up to 500 analytes simultaneously. For more details, please contact your Life Technologies sales representative or visit lifetechnologies.com/luminexinstrument.

The Luminex® 100/200™ and FLEXMAP 3D® systems have gained wide acceptance for use in applications such as protein expression profiling, focused gene expression profiling, and disease testing. These instruments perform sample analysis using flow cytometry, exciting samples with two lasers, and evaluating the results using real-time digital signal processing and xPONENT® software to distinguish bead color (analyte) and assay signal-strength (R-PE) fluorescence intensity (Figure 9).

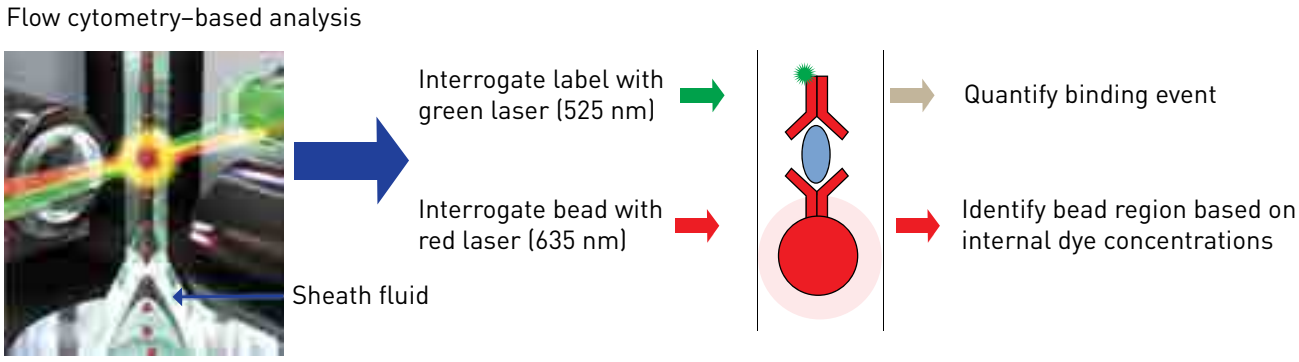



Figure 9. The Luminex® 200™ and FLEXMAP 3D® instruments analyze the results of fluorescent bead–based assays using flow cytometry, exciting samples with two lasers, and evaluating the results using real-time digital signal processing and xPONENT® software.

# General procedures for Novex® singleplex and multiplex assays on the MAGPIX® system

In addition to the procedures at the links that follow, Life Technologies offers in-person training, telephone and email consultations, and detailed user guides to assist with the xMAP® technology protocols.

## Multiplex protein analysis—the MAGPIX® system

The MAGPIX® system is an affordable, compact, fluorescence detection system based on proven Luminex xMAP® technology. Visit lifetechnologies.com/magpix for links to a wide range of information.

For videos on how to multiplex on the MAGPIX® system, take a look at our complete video collection, which includes the following subjects:

- MAGPIX® system—how to prepare your Novex® assay
- MAGPIX® system—how to set up the computer
- How to perform magnetic assay wash steps
- MAGPIX® system—how to use the protocol and batch functions
- MAGPIX® system—how to analyze protein data
- MAGPIX® system—how to generate data reports

## Luminex® instruments and Novex® singleplex and multiplex assays: product lists and selection guide

**Luminex® instruments**

Learn more about our instruments for multiplex assays at lifetechnologies.com/luminex-instrument, where you can also request a demo or have a sales specialist contact you.

**Novex® singleplex and multiplex assays**

Life Technologies offers over one hundred bead-based Novex® singleplex and multiplex assay kits, and can also work with you to design a custom assay.

- Novex® singleplex assay kits can be found at lifetechnologies.com/singleplexkits
- Novex® multiplex assay kits can be found at lifetechnologies.com/multiplexkits
- Custom Novex® multiplex assays can be found at lifetechnologies.com/customnovex
- Our complete immunoassay selection guide can be found at lifetechnologies.com/immunoassays

# Selected references and practical applications of bead-based immunoassays

## Selected references

Novex® singleplex and multiplex assay kits are proven performers in a wide variety applications, for both basic research and drug discovery. In Table 4, we have collected a few examples of recent peer-reviewed publications that describe the use of these products.

Table 4. Selected references.

| Kit name | Application | References |
|---|---|---|
| Cytokine Human 10-Plex Panel | Inflammation, oncology, viral studies | Cohen S, Janicki-Deverts D, Doyle WJ et al. (2012) Chronic stress, glucocorticoid receptor resistance, inflammation, and disease risk. *Proc Natl Acad Sci U S A* 109(16):5995–5999.<br>Hoadley ME, Scarth S, Hopkins SJ (2012) Reconstituting National Institute for Biological Standards and Control (NIBSC) chemokines. *Cytokine* 58(2):162–164.<br>Breen EC, Reynolds SM, Cox C et al. (2011) Multisite comparison of high-sensitivity multiplex cytokine assays. *Clin Vaccine Immunol* 18(8):1229–1242.<br>Söderlund J, Olsson SK, Samuelsson M et al. (2011) Elevation of cerebrospinal fluid interleukin-1β in bipolar disorder. *J Psychiatry Neurosci* 36(2):114–118. |
| Cytokine Human 25-Plex Panel | Inflammation, oncology, viral studies | Pandrea I, Cornell E, Wilson C et al. (2012) Coagulation biomarkers predict disease progression in SIV-infected nonhuman primates. *Blood* 120(7):1357–1366.<br>Björkman L, Brokstad KA, Moen K et al. (2012) Minor changes in serum levels of cytokines after removal of amalgam restorations. T*oxicol Lett* 211(2):120–125.<br>Tsuruyama T, Fujimoto Y, Yonekawa Y et al. (2012) Invariant natural killer T cells infiltrate intestinal allografts undergoing acute cellular rejection. *Transpl Int* 25(5):537–544.<br>Weber C, Müller C, Podszuweit A et al. (2012) Toll-like receptor (TLR) 3 immune modulation by unformulated small interfering RNA or DNA and the role of CD14 (in TLR-mediated effects). *Immunology* 136(1):64–77.<br>Smith AL, St Claire M, Yellayi S et al. (2012) Intrabronchial inoculation of cynomolgus macaques with cowpox virus. *J Gen Virol* 93(Pt 1):159–164. |
| Cytokine Human 30-Plex Panel | Inflammation, oncology, viral studies | Teigler JE, Iampietro MJ, Barouch DH (2012) Vaccination with adenovirus serotypes 35, 26, and 48 elicits higher levels of innate cytokine responses than adenovirus serotype 5 in rhesus monkeys. *J Virol* 86(18):9590–9598.<br>Stanisic M, Aasen AO, Pripp AH et al. (2012) Local and systemic pro-inflammatory and anti-inflammatory cytokine patterns in patients with chronic subdural hematoma: a prospective study. *Inflamm Res* 61(8):845–852.<br>Gillespie EF, Papageorgiou KI, Fernando R et al. (2012) Increased expression of TSH receptor by fibrocytes in thyroid-associated ophthalmopathy leads to chemokine production. *J Clin Endocrinol Meta*b 97(5):E740–E746.<br>Chaudhuri R, McSharry C, Brady J et al. (2012) Sputum matrix metalloproteinase-12 in patients with chronic obstructive pulmonary disease and asthma: relationship to disease severity. *J Allergy Clin Immunol* 129(3):655–663.e8. |
| Cytokine Monkey Magnetic 28-Plex Panel | Inflammation, oncology, viral studies | Teigler JE, Iampietro MJ, Barouch DH (2012) Vaccination with adenovirus serotypes 35, 26, and 48 elicits higher levels of innate cytokine responses than adenovirus serotype 5 in rhesus monkeys. *J Virol* 86(18):9590–9598. |
| Cytokine Mouse 10-Plex Panel | Inflammation, oncology, wound repair | Koch A, Pernow M, Barthuber C et al. (2012) Systemic inflammation after aortic cross clamping is influenced by Toll-like receptor 2 preconditioning and deficiency. *J Surg Res* 178(2):833–841.<br>Dong L, Watanabe K, Itoh M et al. (2012) CD4+ T-cell dysfunctions through the impaired lipid rafts ameliorate concanavalin A-induced hepatitis in sphingomyelin synthase 1-knockout mice. *Int Immunol* 24(5):327–337.<br>Seavey MM, Lu LD, Stump KL et al. (2012) Therapeutic efficacy of CEP-33779, a novel selective JAK2 inhibitor, in a mouse model of colitis-induced colorectal cancer. *Mol Cancer Ther* 11(4):984–993.<br>Seavey MM, Lu LD, Stump KL et al. (2012) Novel, orally active, proteasome inhibitor, delanzomib (CEP-18770), ameliorates disease symptoms and glomerulonephritis in two preclinical mouse models of SLE. *Int Immunopharmacol* 12(1):257–270. |
| Cytokine Mouse 20-Plex Panel | Inflammation, oncology, asthma, wound repair | Sumpter TL, Dangi A, Matta BM et al. (2012) Hepatic stellate cells undermine the allostimulatory function of liver myeloid dendritic cells via STAT3-dependent induction of IDO. *J Immunol* 189(8):3848–3458.<br>Jiang HR, Milovanović M, Allan D et al. (2012) IL-33 attenuates EAE by suppressing IL-17 and IFN-γ production and inducing alternatively activated macrophages. *Eur J Immunol* 42(7):1804–1814.<br>Crilly A, Palmer H, Nickdel MB et al. (2012) Immunomodulatory role of proteinase-activated receptor-2. *Ann Rheum Dis* 71(9):1559–1566.<br>Dangi A, Sumpter TL, Kimura S et al. (2012) Selective expansion of allogeneic regulatory T cells by hepatic stellate cells: role of endotoxin and implications for allograft tolerance. *J Immunol* 188(8):3667–3677.<br>Seavey MM, Lu LD, Stump KL et al. (2012) Novel, orally active, proteasome inhibitor, delanzomib (CEP-18770), ameliorates disease symptoms and glomerulonephritis in two preclinical mouse models of SLE. *Int Immunopharmacol* 12(1):257–270. |
| Cytokine Rat 10-Plex Panel | Inflammation, oncology, wound repair | Kobayashi N, Naya M, Ema M et al. (2010) Biological response and morphological assessment of individually dispersed multi-wall carbon nanotubes in the lung after intratracheal instillation in rats. *Toxicology* 276(3):143–153.<br>Boomer L, Jones W, Davis B et al. (2009) Optimal fluid resuscitation: timing and composition of intravenous fluids. *Surg Infect (Larchmt)* 10(5):379–387.<br>Downing TE, Sporn TA, Bollinger RR et al. (2008) Pulmonary histopathology in an experimental model of chronic aspiration is independent of acidity. *Exp Biol Med (Maywood)* 233(10):1202–1212.<br>Nagata N, Iwata N, Hasegawa H et al. (2007) Participation of both host and virus factors in induction of severe acute respiratory syndrome (SARS) in F344 rats infected with SARS coronavirus. *J Virol* 81(4):1848–1857. |

### Practical applications of bead-based immunoassays

Life Technologies scientists have compiled some practical application information for you:

- Moving from ELISA technology to the MAGPIX® system for multiplex sample analysis (PDF)
- When inflammatory cytokines are unbalanced—investigate the role of inflammation in disease states using immunoassays

## Tips and tricks, troubleshooting, and support

In addition to providing technical support by phone, email, chat, and social media, Life Technologies periodically publishes online material to help our customers succeed.

- Recommendations for manual magnetic plate washing and pipetting on the Luminex® MAGPIX® System (PDF)
- MAGPIX® Data Analysis Tips: xPONENT® Software for the Luminex® MAGPIX® System (PDF)

For additional assistance with bead-based immunoassays, visit lifetechnologies.com/luminex.

### We are always here to help: technical and customer support

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# Protein purification using single-domain antibody fragments as affinity ligands

## Protein purification

Protein purification is vital for the characterization of the function, structure, and interactions of proteins. The various steps in the purification process may include cell lysis, separating the soluble protein components from cell debris, and finally separating the protein of interest from product- and process-related impurities. Separation of the protein of interest, in its desired form, from all impurities, is typically the most challenging aspect of protein purification.

Affinity chromatography is an effective technique for protein purification that often enables a single-step purification of proteins to a purity level sufficient for analytical characterization. Affinity chromatography is a separation technique based on molecular conformation—molecules that "fit" one another bind selectively in a "lock and key" fashion (e.g., an antibody may recognize and specifically bind an antigen). The technique can use application-specific chromatography resins that have *antibody ligands* (fragments of antibodies that contain the antigen-binding domain) attached to the resin surface. Most frequently, these ligands function with the target protein in a manner similar to that of antibody-antigen interactions. This highly specific fit between the ligand and its target compound enables affinity column chromatography that is also highly specific. Antigens bind to the resin-bound antibody ligand, while other sample components and impurities do not bind and flow through the affinity column. Bound antigen (typically the protein of interest) can then be eluted, often with a pH change that breaks the molecular antigen–antibody interaction, yielding a single, highly pure elution peak.

Life Technologies offers a variety of products for affinity chromatography through its CaptureSelect® product portfolio. CaptureSelect® products are affinity ligands based on camelid-derived (Figure 1) single-domain antibody fragments ($V_HH$), created using proprietary technology. The affinity ligands are a 12 kDa single-domain fragment that comprises the 3 complementarity-determining regions (CDRs) that form the antigen-binding domain. These affinity ligands are efficiently produced in the yeast *Saccharomyces cerevisiae* and thus are not animal-derived. The affinity ligands are then covalently bound to chromatography beads, generating resins suited for protein purification via column chromatography.

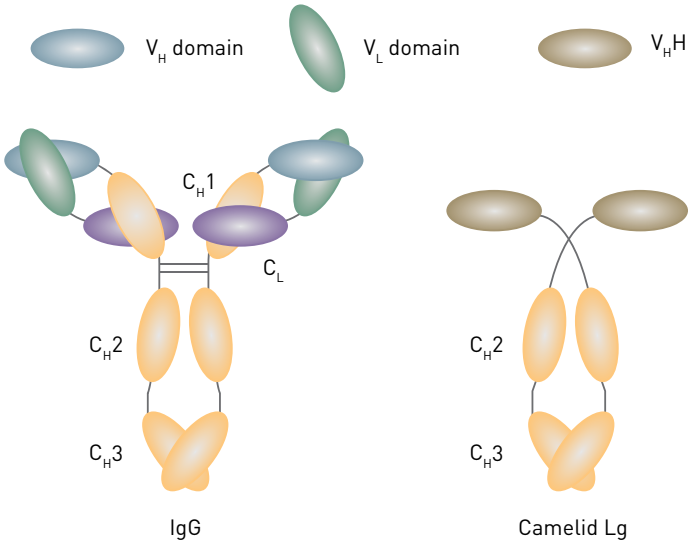



Figure 1. Comparison of overall structure and major domains of human IgG and camelid Ig.

CaptureSelect® products possess a combination of unique properties, including selectivity, affinity, and stability, that provide competitive benefits: single-step purification, ease of use, minimized cost of purification, effective impurity removal, higher-quality product, and increased flexibility in the purification process. These features make CaptureSelect® products the right tools for small-scale protein purification. The products are specific for a wide variety of target proteins and are therefore well suited to a variety of applications:

- Small-scale protein purification—ready-to-use affinity resins, including:
  - Antibody Toolbox® products for the separation of immunoglobulin formats from a wide range of environments, such as plasma, milk from transgenic animals, or supernatant from mammalian cell cultures
  - Proteomics Toolbox® products for protein depletion to support biomarker research applications
- HPLC analytical chromatography columns—rapid, sensitive, and precise quantitation and small-scale sample preparation of proteins from complex mixtures
- Custom ligand design—the development of antibody ligands against a specific protein or impurity, enabling affinity purification tailored to novel applications

Most biomolecules can be purified with one of these products. Unlike the common bacterial coat protein components proteins A, G, and L, CaptureSelect® affinity ligands are developed with defined specificity and high avidity to the target, enabling the creation of affinity chromatography resins well suited for single-step protein purification (Tables 1 and 2).

Table 1. Comparison of CaptureSelect® affinity products.

| | POROS® CaptureSelect® analytical columns | Antibody Toolbox® Products | Protein purification products | Proteomics Toolbox® Products | Conjugated ligands |
|---|---|---|---|---|---|
| Antibody analytics<br>• Quantitation<br>• PTM analysis | ●<br>● | | | | ● |
| Antibody purification<br>• Small scale | | ● | | | |
| Protein purification<br>• Natural/recombinant<br>• Affinity tag | | | ●<br>● | | |
| Biomarker discovery | | | | ● | |
| Instrumentation needed | HPLC/FPLC | FPLC | FPLC | FPLC<br>Spin columns | ELISA<br>western<br>SPR, BLI |

BLI: bio-layer interferometry; PTM: post-translational modifications; SPR: surface plasmon resonance.

Table 2. POROS® CaptureSelect® analytical columns.

| | POROS® protein A, G resins | | | POROS® CaptureSelect® ligands | | | |
|---|---|---|---|---|---|---|---|
| Target type | Prot A | Prot G | IgG Fc | Kappa | Lambda | IgG-CH1 | IgM |
| IgG1, IgG2, IgG4 | ● | ● | ● | ● | ● | ● | |
| IgG3 | | ● | ● | ● | ● | ● | |
| IgG, $C_H2$ domain deleted | | | ● | ● | ● | ● | |
| IgM | | | | ● | ● | | ● |
| IgA, IgD, IgE | | | | ● | ● | | |
| Fab, F(ab´)$_2$ | | | | ● | ● | ● | |
| IgG Fc fusion protein | ● | ● | ● | | | | |
| Free light chains | | | | ● | ● | | |

### Features of CaptureSelect® affinity products

- Tunable specificity—The specificity of the ligand can range from broad to very narrow: species, antibody class, subclass format, glycoform, isomer, idiotype, etc. Specific affinity ligand features can be selected during the ligand development and screening processes. Specific, designable features include binding and elution characteristics and column-regeneration or cleaning conditions. For example, ligands have been isolated that can have their targets eluted at near-neutral pH values, thereby enabling neutral-pH chromatography and the purification of stable, functional biologics. The screening for selectivity and process functionality has proven to be highly effective in identifying antibody ligands that satisfy rigid requirements for specific protein purification processes.
- Optimal affinity—The binding affinity is in the nanomolar range and can be optimized for either capture or scavenging applications. This high affinity enables purification of valuable biotherapeutics that may be present at very low concentrations in the starting material.
- Excellent quality—Because the DNA that codes for each ligand is amplified by PCR and subsequently cloned and expressed in yeast, all ligands are animal origin–free (AOF). This simple and robust production strategy yields superior CaptureSelect® affinity products with consistently excellent quality.

## General procedures and protocols for using CaptureSelect® affinity products

### General affinity procedures

A detailed protocol is designed for the use of each CaptureSelect® affinity product. Each product group is designed to work with a range of standard applications. POROS® CaptureSelect® analytical columns are designed for rapid quantitation and small-scale sample preparation of immunoglobulins, fusion proteins, antibody fragments (Fabs), and bispecific antibodies. Antibody Toolbox® products are used for the purification of antibodies and antibody fragments from serum, plasma, and cell culture supernatants. Proteomics Toolbox® products are used to improve proteomics analysis by depleting human plasma of selected abundant proteins. Biotinylated CaptureSelect® ligands are used for the detection, quantitation, and characterization of specific antibodies in applications such as capture ELISA and other immunoassays, and in label-free detection platforms like bio-layer interferometry (BLI) and surface plasmon resonance (SPR). CaptureSelect® protein purification products are used for the detection and purification of proteins from recombinant sources.

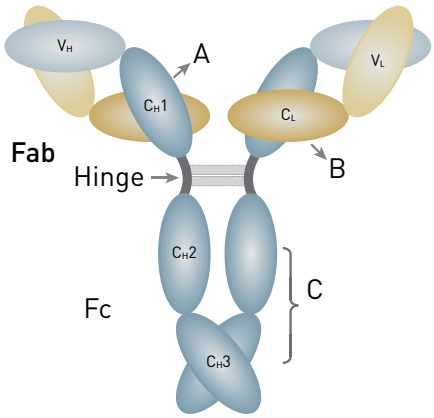
## CaptureSelect® affinity protocols

Here is a complete list of the current products, with general protocol links:

- POROS® CaptureSelect® analytical column general protocol (PDF)
- Antibody Toolbox® general protocol for antibody purification (PDF)
  - CaptureSelect® IgA affinity matrix
  - CaptureSelect® IgA-$C_H1$ (Hu) affinity matrix
  - CaptureSelect® IgG1 (Hu) affinity matrix
  - CaptureSelect® IgG3 (Hu) affinity matrix
  - CaptureSelect® IgG4 (Hu) affinity matrix
  - CaptureSelect® IgG-CH1 affinity matrix
  - CaptureSelect® IgG-Fc (Hu) affinity matrix
  - CaptureSelect® IgG-Fc (MS) affinity matrix
  - CaptureSelect® IgM affinity matrix
  - CaptureSelect® LC-kappa (Hu) affinity matrix
  - CaptureSelect® LC-kappa (mouse) affinity matrix
  - CaptureSelect® LC-lambda (Hu) affinity matrix
  - CaptureSelect® LC-lambda (mur) affinity matrix
  - CaptureSelect® LC-lambda (rat) affinity matrix
  - CaptureSelect® LC-lambda (ung) affinity matrix
- Protein purification general protocol (PDF)
  - CaptureSelect® alpha-1 antitrypsin (AAT) affinity matrix
  - CaptureSelect® antithrombin III (ATIII) affinity matrix
  - CaptureSelect® C-tag affinity matrix
  - CaptureSelect® fibrinogen affinity matrix
  - CaptureSelect® follicle stimulating hormone (FSH) affinity matrix
  - CaptureSelect® human growth hormone (hGH) affinity matrix
  - CaptureSelect® human serum albumin (HSA) affinity matrix
  - CaptureSelect® transferrin affinity matrix
  - CaptureSelect® von Willebrand factor (vWF) affinity matrix
- Proteomics Toolbox® general protocol for sample preparation(PDF)
  - CaptureSelect® human plasma 14 affinity matrix
  - CaptureSelect® human serum albumin (HSA) affinity matrix
  - CaptureSelect® human serum Ig/albumin affinity matrix
  - CaptureSelect® multispecies albumin affinity matrix
- Conjugated ligand general protocol (PDF)
  - Biotin anti-IgA conjugate
  - Biotin anti-IgG-$C_H1$ conjugate
  - Biotin anti-IgG-Fc (Hu) conjugate
  - Biotin anti-IgG-Fc (MS) conjugate
  - Biotin anti-IgG3 (Hu) conjugate
  - Biotin anti-IgG4 (Hu) conjugate
  - Biotin anti-IgM conjugate
  - Biotin anti-LC-kappa (Hu) conjugate
  - Biotin anti-LC-kappa (mur) conjugate
  - Biotin anti-LC-lambda (Hu) conjugate

Abbreviations: Hu, human; Ig, immunoglobulin; LC, light chain; MS, multi-species; mur, murine; ung, ungulate.

Additional material can be found at lifetechnologies.com/captureselect.




(A) **$C_H1$ domain** (heavy chain)

| | | |
|---|---|---|
| CaptureSelect® | IgG-$C_H1$ | - human, all subclasses |
| | IgA-$C_H1$ | - human, all subclasses |
| | IgG1 | - human |

(B) **$C_L$ domain** (light chain)

| | | |
|---|---|---|
| CaptureSelect® | LC-kappa | - human |
| | LC-kappa | - murine |
| | LC-lambda | - human |
| | LC-lambda | - mouse or rat |
| | LC-lambda | - ungulate |

(C) **Fc domain** (heavy chain)

| | | |
|---|---|---|
| CaptureSelect® | IgG-Fc | - human, all subclasses |
| | IgG-Fc | - multi-species |
| | IgG3 | - human |
| | IgG4 | - human |
| | IgA | - human, all subclasses |
| | IgM | - human |

Figure 2. IgG structure and CaptureSelect® products that bind components of IgG structure.

# CaptureSelect® affinity product lists and custom product design service

## CaptureSelect® affinity products

Life Technologies offers a wide variety of CaptureSelect® affinity products for small-scale protein purification. Finding the best product for your application is simple at lifetechnologies.com/captureselect.

Follow the links below to view the CaptureSelect® affinity products that Life Technologies offers for:

- Antibody and antibody fragment purification
- Antibody analytics
- Antibody detection
- Protein and fusion protein purification
- Affinity tag purification
- Biomarker research
- Plasma depletion studies
- Inflammation research

## Custom affinity purification product design service

If you do not see the affinity purification product that meets your specific needs, please contact us to discuss our custom ligand design solutions. We have worked with clients to develop custom affinity purification products that resulted in:

- Minimized clean-room time and maximized yield by significantly reducing the number of purification steps
- Maximized purity through highly specific, targeted protein purification capture and impurity removal
- The ability to elute at pH values that leave the target biomolecule completely intact and active
- Selective separation and purification of proteins that only differ by a single amino acid

# Selected references

CaptureSelect® affinity products are proven performers in a wide variety of purification applications. In Table 3, we have collected a few examples of peer-reviewed publications that highlight the use of these products.

Table 3. Selected references.

| Topic | References |
|---|---|
| Use of CaptureSelect® affinity ligands in chromatography | McCue JT, Selvitelli K, Walker J (2009) Application of a novel affinity adsorbent for the capture and purification of recombinant factor VIII compounds. *J Chromatogr A* 1216(45):7824–7830.<br>Adams H, Brummelhuis W, Maassen B et al. (2009) Specific immuno capturing of the staphylococcal superantigen toxic-shock syndrome toxin-1 in plasma. *Biotechnol Bioeng* 104(1):143–151.<br>Beyer T, Lohse S, Berger S et al. (2009) Serum-free production and purification of chimeric IgA antibodies. *J Immunol Methods* 346(1–2):26–37.<br>Kuroiwa Y, Kasinathan P, Sathiyaseelan T et al. (2009) Antigen-specific human polyclonal antibodies from hyperimmunized cattle. *Nat Biotechnol* 27(2):173–181.<br>Verheesen P, ten Haaft MR, Lindner N et al. (2003) Beneficial properties of single-domain antibody fragments for application in immunoaffinity purification and immuno-perfusion chromatography. *Biochim Biophys Acta* 1624(1–3):21–28.<br>ten Haaft M, Hermans P, Dawson B (2006) Separations in proteomics: use of camelid antibody fragments in the depletion and enrichment of human plasma proteins for proteomics applications. In: Smejkal GB, Lazarev A (editors), *Separation methods in proteomics*. Boca Raton, FL: CRC Press. pp 29–40. |
| Camelid antibody reviews | Muyldermans S (2001) Single domain camel antibodies: current status. *J Biotechnol* 74(4):277–302. |
| Immunogenicity of camelid single-domain antibody fragments | Cortez-Retamozo V, Lauwereys M, Hassanzadeh Gh G et al. (2002) Efficient tumor targeting by single-domain antibody fragments of camels. *Int J Cancer* 98(3):456–462.<br>Revets H, De Baetselier P, Muyldermans S (2005) Nanobodies as novel agents for cancer therapy. *Expert Opin Biol Ther* 5(1):111–124. |

# Tips, tricks, troubleshooting, and support

In addition to providing technical support by email, telephone, chat, and social media, Life Technologies periodically publishes online material to help speed your research.

- POROS® A and G affinity columns and CaptureSelect® affinity columns—operating instructions (PDF)

To find out more, visit lifetechnologies.com/captureselect.

## We are always here to help: technical and customer support

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We look forward to continuing to provide you with the products and support you need to effectively purify proteins and other biomolecules.

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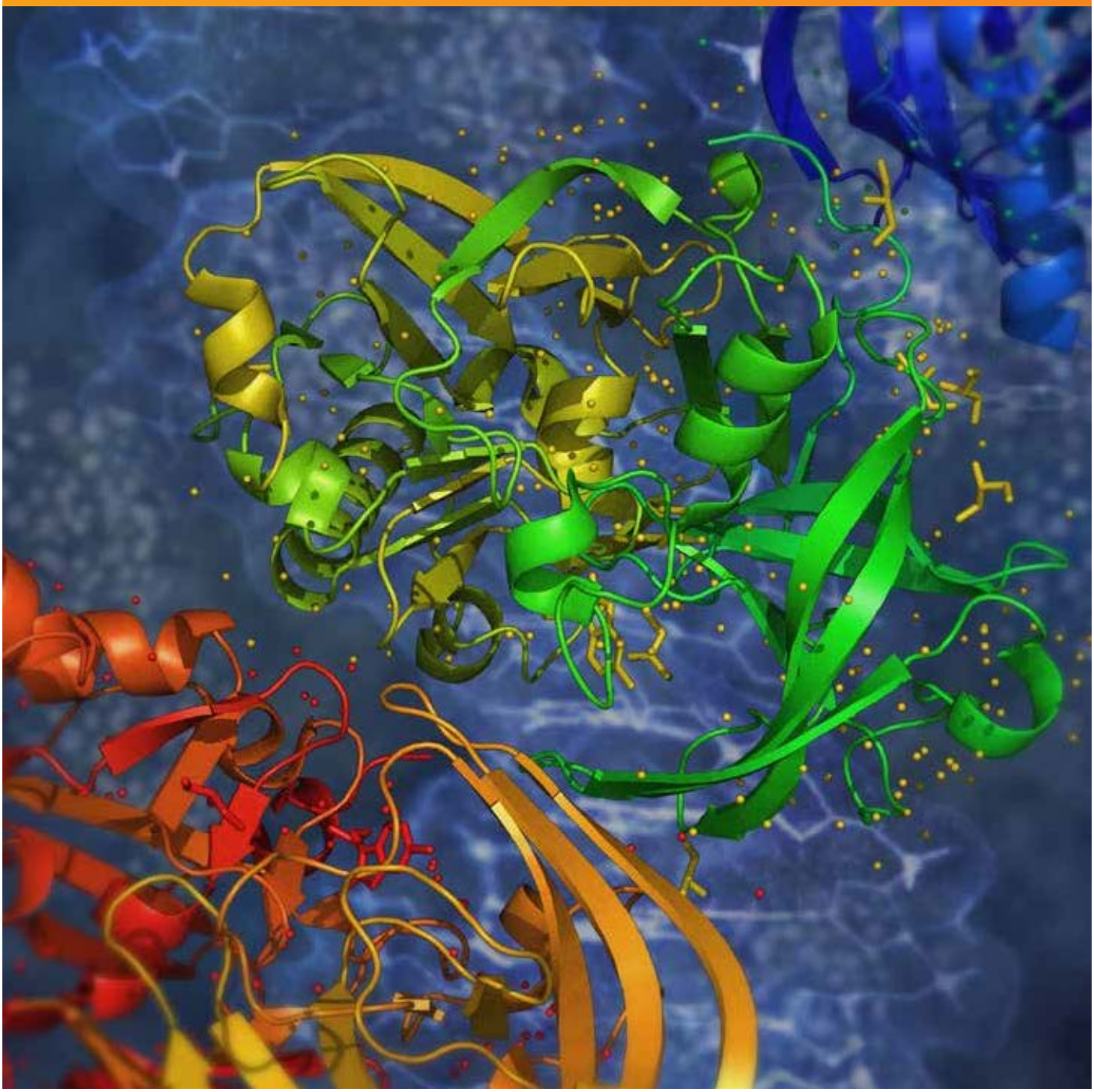

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