Sample preparation of electrophoresis gels for liquid scintillation counting.

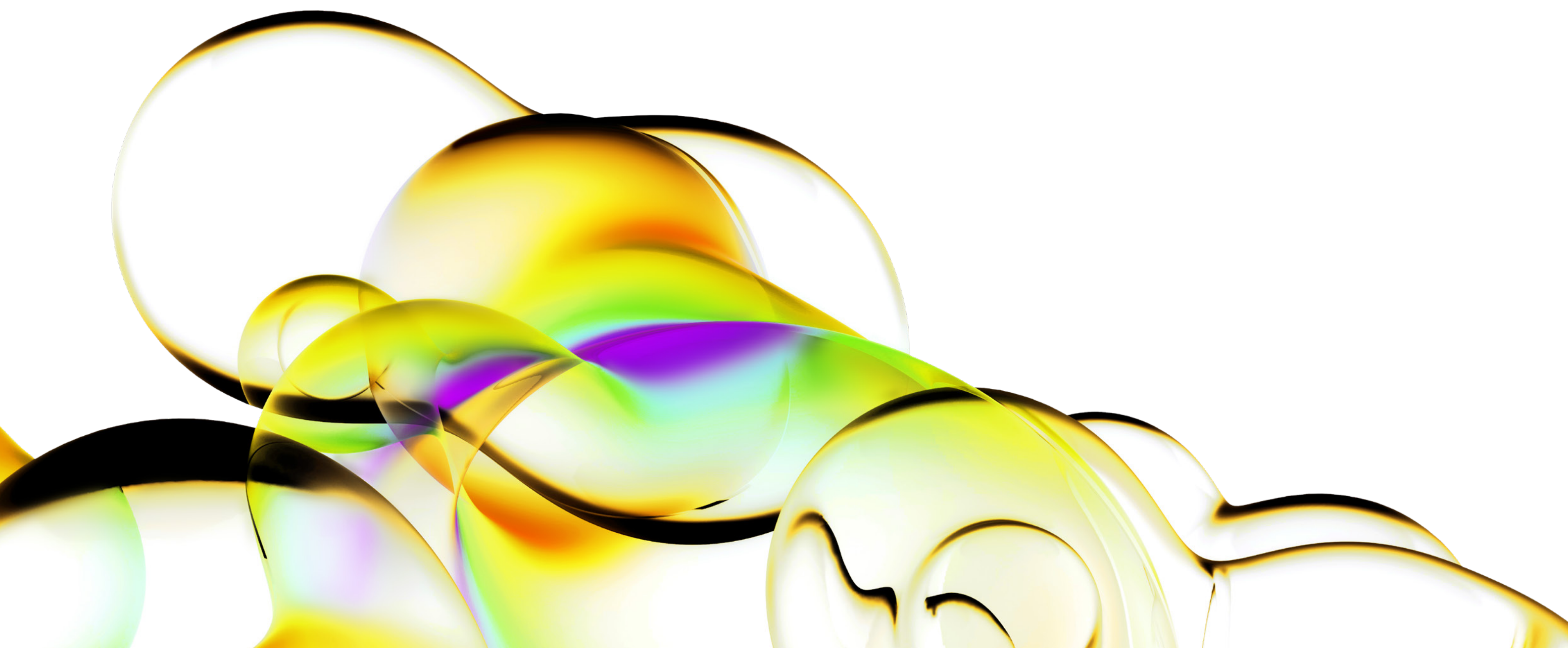
LCS in practice

Polyacrylamide gel electrophoresis (PAGE) is a technique commonly used for the separation and identification of biological species. Electrophoresis can be best described as the movement of small ions and macromolecules in solution under the influence of an electric field. The rate of migration depends on the size and shape of the molecule, the porosity of the gel matrix, the charge carried, the applied current, and the resistance of the medium. Electrophoresis is carried out in gels cast either in tubes or as slabs.

Gel types

A number of gel materials have been used successfully for electrophoresis including agar, agarose and polyacrylamide. Agar and agarose gels are prepared by heating the granular material in the appropriate electrolyte buffer, casting the gels and allowing them to set while cooling.

Polyacrylamide gels are made from acrylamide and N,N′-methylene bisacrylamide (bisacrylamide) mixtures dissolved in electrolyte and polymerized by the addition of chemical catalysts. Cross-linking agents other than bisacrylamide have been used and include DATDA (diallyltartardiamide), BAC (N,N′-bisacrylylcystamine) and ethylene diacrylate.[1] The location of the various compounds within the gel is determined by staining and the presence of radioactivity in discrete zones. Sample preparation for analysis by liquid scintillation counting involves either elution of the sample from the gel or complete dissolution of the gel.



Elution

The complete solution of the gel is not required for satisfactory counting. This is indicated by the observation that when acrylamide gels are treated with a solubilizer, such as SOLVABLE™, Soluene® 350 or their equivalents, the gel swells rapidly and the entrapped macromolecules diffuse out into the solubilizer. Addition of an appropriate cocktail results in a suitable counting medium. During this diffusion process, it is presumed that the macromolecules (proteins) are partially hydrolyzed by the strong organic base.[2,3] After 10 to 20 hours of digestion with the solubilizer at ~45 °C of 1 mm gel slices, the undissolved gel can be removed after being counted and no perceptible loss of either ^{3}H or ^{14}C counts or changes in the isotope ratios of the samples are observed.[2] Another method of preparing the gel slices for counting is to add the 1 to 2 mm gel slice to 5 mL water and macerate the slice with the aid of a stirring rod. This mixture is allowed to stand at 50 °C for about two hours, cooled, and then 10 mL of a suitable cocktail, such as Insta-Gel® XF or equivalent, is added.

Dissolution

The alternative to elution is the complete dissolution of gels. This is possible using a variety of reagents including hydrogen peroxide,[4] periodic acid,[5] 2-mercaptoethanol,[6] piperidine (containing EDTA), alkalis and ammonia. The selection of a suitable reagent depends upon the gel, and the choice of crosslinker dictates the required dissolving reagent.

- Acrylamide gels cross-linked with DATDA are dissolved in 0.5 mL of 2% periodic acid in two hours at room temperature[7] and the digest can be counted in 10 mL of a suitable cocktail such as Insta-Gel XF, ULTIMA Gold™ or equivalents.
- Acrylamide gels cross-linked with BAC have previously been reported to be dissolved in 2-mercaptoethanol.[6]
- Polyacrylamide gels that are soluble in alkalis can be constructed using ethylene diacrylate as the crosslinker instead of bisacrylamide.
- Polyacrylamide gels cross-linked with bisacrylamide can be dissolved by adding the 1 to 2 mm gel slice to 0.5 mL of 30% (100 volume) hydrogen peroxide and heating at 50 °C until the gel dissolves. Some authors use a one hour digestion at 55 °C,[3] overnight at 40 °C,[8] and overnight at 60 °C.[9] The hydrogen peroxide also decolorizes the stained gels.

Practical considerations

When using hydrogen peroxide, cocktail selection is extremely important due to the potential for chemiluminescence. When peroxides are present in an alkaline medium, the chances of chemiluminescence are high. Consequently, after dissolving the gel slice in hydrogen peroxide, it is advisable to add a small amount of 1 to 2 M HCl to keep the mixture neutral or slightly acidic. In addition, traces of copper ions have sometimes been added to aid the decomposition of the excess hydrogen peroxide and the presence of these ions intensifies the chemiluminescence in an alkaline medium. Suitable cocktails include Hionic-Fluor™ and Aquasol™-2 (both acidified with 0.5 mL 2 M HCl per 10 mL cocktail). In one instance, a researcher was confronted with this particular problem and the results of the investigation are shown in the following section, titled Sample Preparation Methods.

Certain researchers[10] have criticized the use of hydrogen peroxide and heat on the grounds that during digestion, radioactive carbon dioxide and water may be lost. They proposed the digestion of 1 mm gel slices using 0.25 mL of a mixture containing one part concentrated ammonium hydroxide and 99 parts 30% hydrogen peroxide at room temperature for four to eight hours. This method is reported to be free from the danger of loss of radioactivity because of the low temperature digestion.

Agarose gels can be rapidly and easily dissolved by treatment with sodium hypochlorite solution for about one hour at ~45 °C. In this instance, cocktail selection is very important as any residual hypochlorite solution has a tendency to decompose under acidic or slightly acidic conditions and produces chlorine, which can lead to chemiluminescence. This chemiluminescence only becomes apparent after standing for longer than 24 hours and therefore, cocktail selection is important. At present, the recommended cocktail for this application is Hionic-Fluor.

All the methods mentioned previously are suitable for the isotopes normally encountered in gel electrophoresis with one modification; ^{32}P can be detected and counted in wet gel slices, either alone or covered in buffer, by Cerenkov counting.

Sample preparation methods

Method one – Acrylamide (Bisacrylamide)

- Add 1 to 2 mm gel slice to 0.5 mL Soluene-350 or SOLVABLE.
- If the gel piece has been dried, allow the gel to rehydrate for approximately 15 to 30 minutes.
- Ensure the gel piece is free floating for best results.
- Stand at 50 °C for about three hours, during which time the gel will swell.
- Cool to room temperature and add 10 mL HionicFluor or ULTIMA Gold and vortex. As the material is eluted out of the gel, the gel will collapse.
- Allow to temperature and dark adapt before counting.
- The samples may be counted immediately with consistent results; however, for maximum recovery, allow the samples to sit overnight at room temperature. Occasional vortexing will speed diffusion.

Method two – Acrylamide (Bisacrylamide)

- Add 1 to 2 mm gel slice to 0.5 mL water.
- Macerate the gel slice with a glass rod.
- Stand at 50 °C for about two hours.
- Add 5 to 10 mL Insta-Gel XF.
- Allow to temperature and dark adapt before counting.

Method three – Acrylamide (Bisacrylamide)

- Add 1 to 2 mm gel slice to 0.5 mL of 30% (100 volume) hydrogen peroxide.
- Stand at 50 °C until the gel dissolves.
- During this time, the hydrogen peroxide decolorizes the stained gel slice.
- Cool to room temperature.
- Add either 10 mL Hionic-Fluor or 10 mL Aquasol™-2.
- Allow to temperature and dark adapt before counting.

Method four – Acrylamide gels (DATDA)

- Add 1 to 2 mm gel slice to 0.5 mL of 2% periodic acid.
- Stand for two hours at room temperature, or until dissolved.
- Add 10 mL of Insta-Gel XF or Poly-Fluor™.
- Allow to temperature and dark adapt before counting.

Method five – Agarose gels

- Add 1 to 2 mm gel slice to 1.0 mL sodium hypochlorite solution.*
- Stand at 50 °C until the gel dissolves (usually about 45 minutes).
- Cool to room temperature.
- Add 10 mL Hionic-Fluor.**
- Allow to temperature and dark adapt before counting

* Sodium hypochlorite, more commonly known as bleach, is available from most lab supply houses, and should have greater than 5% available chlorine.

** In some cocktails, sodium hypochlorite decomposes and releases traces of chlorine, which causes chemiluminescence. Currently, Hionic-Fluor is the only suitable cocktail to overcome this.

Acrylamide (Bisacrylamide) With hydrogen peroxide/copper sulfate

Typically, copper is added to hydrogen peroxide to promote rapid catalytic decomposition (other ions, such as iron and chromium also promote decomposition). Unfortunately there is another reaction which can occur in addition to decomposition and this causes luminescence. If the pH is at or above 7.0, these metal ions react with peroxide to produce a brown coloration and chemiluminescence. If the medium is kept acidic, the undesired reaction is suppressed and no chemiluminescence is seen. To confirm this, various samples were tested with selected cocktails:

Samples:

- 0.4 mL 30% hydrogen peroxide heated to 60 °C (two hours).
- 0.4 mL 30% hydrogen peroxide with 4 mM copper sulfate heated to 60 °C (two hours).

Table 1: Background results with various combinations of reagents and cocktails.

Cocktail	Sample	2 minutes	15 minutes	30 minutes	Observations
Formula-989	0.4 mL H_2O_2	56 CPM	22 CPM	24 CPM	Colorless
Aquasol-2	0.4 mL H_2O_2	38 CPM	22 CPM	24 CPM	Colorless
Pico-Fluor 40	0.4 mL H_2O_2	23 CPM	17 CPM	19 CPM	Colorless
Hionic-Fluor	0.4 mL H_2O_2	27 CPM	21 CPM	23 CPM	Colorless
Formula-989	0.4 mL $H_2O_2/CuSo_4$	374 CPM	41 CPM	33 CPM	Yellow/brown
Aquasol-2	0.4 mL $H_2O_2/CuSo_4$	444 CPM	135 CPM	91 CPM	Colorless
Pico-Fluor 40	0.4 mL $H_2O_2/CuSo_4$	273 CPM	52 CPM	44 CPM	Yellow/brown
Hionic-Fluor	0.4 mL $H_2O_2/CuSo_4$	394 CPM	69 CPM	49 CPM	Yellow/brown

Cocktails

- 6.0 mL Formula-989.
- 6.0 mL Aquasol-2.
- 6.0 mL Pico-Fluor™ 40.
- 6.0 mL Hionic-Fluor.

These combinations were tested by adding the samples to the cocktails in 20 mL glass vials, then monitoring the background for a two minute count time in a 0 to 18.6 keV window using a Revvity Tri-Carb® Model 1900 operated at 19 °C. The results are in Table 1.

Of the cocktails used above, only Aquasol-2 has a pH less than 6.0, but it is not sufficiently acidic to suppress the chemiluminescence. The other three cocktails have a pH of approximately 7.0. This series of experiments shows that the presence of copper ions in a neutral, or slightly alkaline medium, is indeed the cause of the chemiluminescence. To confirm this, the sample preparation with 0.4 mL 30% hydrogen peroxide and 4 mM copper sulfate (heated for two hours at 60 °C), was repeated, and 0.4 mL 2 M hydrochloric acid was added to the samples before adding the cocktail. The results obtained are tabulated in Table 2.

Table 2: Background results with reagents modified with HCl.

Cocktail	Sample	2 minutes	15 minutes	30 minutes	Observations
Formula-989	0.4 mL $H_2O_2/CuSo_4/HCl$	26 CPM	17 CPM	18 CPM	White emulsion unsuitable for counting
Aquasol-2	0.4 mL $H_2O_2/CuSo_4/HCl$	25 CPM	19 CPM	23 CPM	Clear/colorless
Pico-Fluor 40	0.4 mL $H_2O_2/CuSo_4/HCl$	19 CPM	20 CPM	18 CPM	Clears slowly
Hionic-Fluor	0.4 mL $H_2O_2/CuSo_4/HCl$	19 CPM	23 CPM	23 CPM	Clear/colorless
Hionic-Fluor	0.4 mL $H_2O_2/CuSo_4/HCl$	32 CPM	24 CPM	20 CPM	Clear/colorless

These results showed that the addition of HCl stopped the color formation and suppressed chemiluminescence.

Formula-989 could not accept the strong HCl samples and is therefore not suitable, while Pico-Fluor 40 only accepted this strong acid after a period of standing.

Other methods

As an alternative to elution or dissolution, the gel slices can be combusted using a commercially available oxidizer system. Although only applicable to ^{3}H and ^{14}C, combustion offers a rapid and accurate means of sample preparation free from the chemiluminescence problems associated with most acrylamide gel procedures.

A further alternative worthy of consideration is the use of imaging technology. Gels can be imaged either directly using a direct beta counter or indirectly using the Revvity Cyclone™ storage phosphor system. Both of these instruments read radioactivity from the intact gel.

Once an image is acquired, areas of interest can be identified and quantified using the software available with the imagers. InstantImager can be used with wet or dry gels; with the Cyclone, the gels are dried and then exposed to a storage phosphor screen before imaging. Both instruments have the advantage over elution and dissolution methods in which the operator does not have to guess the position of the band. Tightly spaced bands can also be quantified without risk of selecting the wrong portion of the gel. Imaging techniques also have the advantage of being non-destructive, the material from a band can be recovered if necessary after the image is acquired.

Summary

A compilation of recommended cocktails for different gel types is shown in Table 3. This may prove useful in providing a basic guide to cocktail selection

Table 3: Recommended reagents and cocktails for various gels.

Gel type	Solubilizer	Maximum sample size	Suitable cocktails
Bisacrylamide	H_2O_2	1/-2 mm slices	Hionic-Fluor, Aquasol-2, Pico-Fluor 40
	H_2O_2/copper sulfate	1-2 mm slices	Hionic-Fluor + HCl Aquasol-2 + HCl
	Soluene-350	1-2 mm slices	Hionic-Fluor
	SOLVABLE	1-2 mm slices	Hionic-Fluor or ULTIMA Gold
	Water	1-2 mm slices	Insta-Gel XF
DATDA	Periodic acid	1-2 mm slices	Hionic-Fluor or ULTIMA Gold
Agarose	Sodium hypochlorite	1-2 mm slices	Hionic-Fluor

Conclusion

There are a variety of Revvity LSC cocktails, of both safer, high flash-point and classical solvent types, which are suitable for use in counting prepared gels. In addition, there are a variety of reagents which can be used to prepare the gels for LSC. If problems with sample preparation or cocktail selection persist, or help is needed with a particular sample type not mentioned in this publication, please call your local Revvity representative for further applications support.

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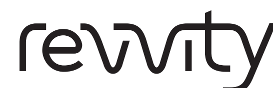

Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com

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