Synthesis and Purification of Various Acyl-ACP Derivatives

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I. **Specific Aim**

Lipoyl synthase is the primary enzyme responsible for the synthesis of lipoic acid, a unique biological cofactor that is involved in cellular respiration. The substrate for lipoyl synthase is the acyl carrier protein (ACP) containing an octanoyl chain tethered to its phosphopantetheine prosthetic group. In order to study the lipoyl synthase reaction, efficient methods for preparing the substrate must be established. Octanoyl-ACP can be synthesized using acyl-ACP synthetases from *Vibrio harveyi* or *Escherichia coli*, or by a chemical method. The objective of this research is to establish an efficient means of preparing octanoyl-ACP using *Escherichia coli* acyl-ACP synthetase.

II. **Background and Significance**

Lipoic acid is an eight-carbon fatty acid with a unique 1,2-dithiolane ring that comprises carbons 6, 7 and 8 (numbering from the carboxylate), and can exist in the oxidized or reduced state (dihydrolipoic acid) (Figure 1). It is found covalently attached *via* an amide linkage with conserved lysine residues on enzymes in which it functions. Lipoic acid is a key cofactor in a number of multienzyme complexes responsible for the oxidative decarboxylation of α-keto acids, including the pyruvate dehydrogenase complex, the branched-chain oxo-acid dehydrogenase complex, the α-ketoglutarate dehydrogenase complex, and the glycine cleavage system.[1]

These complexes play an essential role in cellular energy metabolism; therefore, genetic defects in them are known to produce severe disease states, such as maple urine disease, which results from defects in the branched-chain oxo-acid dehydrogenase complex[8]. The inability to incorporate lipoic acid in any of these complexes would result in non-viable offspring. Lipoic acid is also known to be an important free radical scavenger in the cell. It is used to treat a number of diseases, including heavy metal poisoning, liver disease, and diabetes.[2]

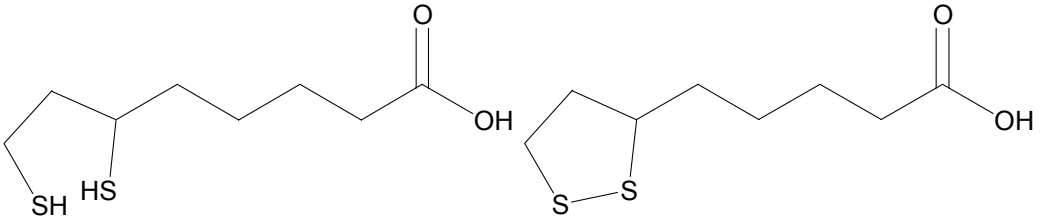

Figure 1: The oxidized and reduced forms of lipoic acid.

III. **Preliminary Studies**

There are several means by which octanoyl-ACP, the substrate for lipoyl synthase, can be generated. The enzyme, holo-ACP synthase, is used to append the phosphopantetheine prosthetic group on to apo-ACP, and acyl-ACP synthetases from either *E. coli* or *Vibrio harveyii* are used to attach an octanoyl group on to the phosphopantetheine group. The goals of the research described herein were to establish a method for isolating the *E. coli* acyl-ACP synthetase and for using it to synthesize octanoyl-ACP. The desire to develop methods to use the *E. coli* acyl-ACP synthetase was fueled by shortcomings associated with employing the enzyme from *Vibrio harveyii*, as well as shortcomings associated with the chemical procedure**.** A major drawback associated with the *Vibrio harveyi* enzyme is that the enzyme is not cloned and overexpressed, making its purification to homogeneity tenuous and tedious. In addition, the *Vibrio harveyi* enzyme copurifies with contaminating thioesterase activity, which degrades the product as it is being synthesized. The difficulty associated with the chemical synthesis is one of specification. It is a complicated task to place a group in a specific location on a large protein using chemical methods, because of the large number of side reactions that invariably take place.

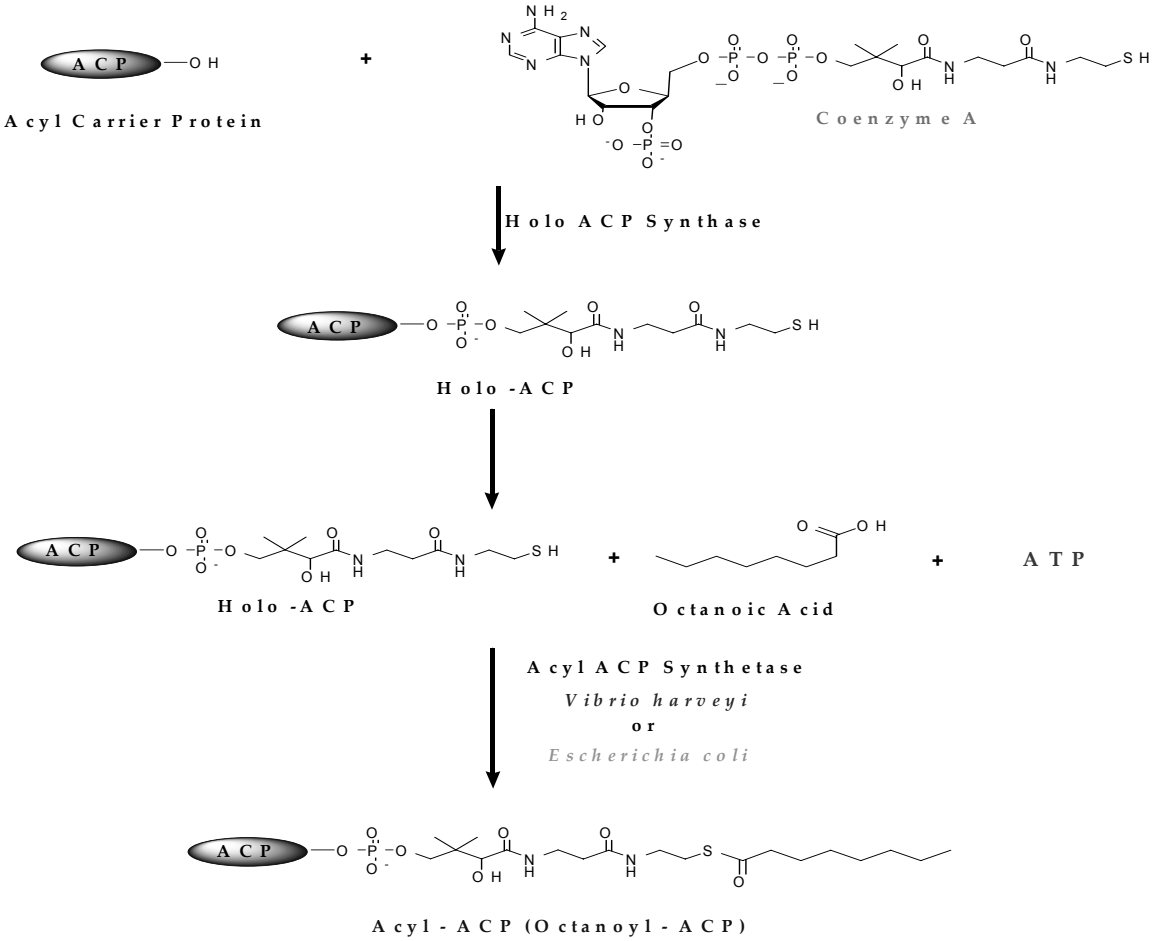

Figure 2: Octanoyl ACP synthesis

The purification and separation of various acyl-ACP derivatives may be achieved by more than one method of experimentation. Our particular research design was included both gel filtration and gel electrophoresis as means of separating reactants from products. The gel electrophoresis system of purification included the use of the Model 491 Prep Cell by Biorad (Hercules, MA)**.** The Model 491 Prep Cell uses continuous-elution electrophoresis to purify proteins from heterogeneous mixtures.[3] The gel filtration method of purification employs size exclusion chromatography under partially denaturing conditions. This method of chromatography separates molecules based on their Stokes radii.

IV. **Materials and Methods**

The purification and handling of all proteins was carried out in a 4ºC cold room, unless otherwise stated.

Overexpression and Purification of Escherichia coli ACP

ACP was overexpressed and purified as described by Haas[4] with slight modifications. A single colony of *E. coli* BL21(DE3)pLysS / pBHF-5 was used to inoculate 200 mL of Luria-Bertani (LB) media containing 50 μg mL^{-1} kanamycin. The culture was incubated at 37 °C for 7-8 hours with shaking at 200 rpm. This culture was used to inoculate 16 L of LB media containing 50 μg mL^{-1} of kanamycin. The culture was grown at 37°C and shaken at 200 rpm until an optical density at 600 nm (OD_{600}) of ~0.6 was attained. Expression was induced by the addition of 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG), 2g L^{-1} casamino acids, and 0.05 g L^{-1} pantothenic acid. Expression was carried out for three hours at 30°C, upon which cells were harvested at 10,000 x g for 6 min and the cell paste was frozen in liquid nitrogen and stored at -80 °C.

Frozen cells were resuspended in 100 ml of 100 mM Tris, pH 8.0, containing 0.15 mg mL^{-1} of lysozyme, DNase, and RNase. The mixture was sonicated on ice in twelve 30-second bursts and centrifuged for one hour at 39,000 x g. After centrifugation the supernatant was diluted with one volume of 25 mM 4-morpholineethanesulfonic acid monohydrate (MES), pH 6.1 (buffer A). The mixture was then loaded onto a diethylaminoethyl cellulose (DE52) (Whatman, International Ltd Mainstone, England) column (50 x 300 mm) equilibrated in buffer A, and the column was washed with 750 ml of buffer A. The protein was eluted from the column in 15-ml fractions with a 1.6-L linear gradient from 0 to 0.85 M sodium chloride (NaCl) in buffer A. The fractions were analyzed using Ultraviolet (UV) visible spectroscopy (monitoring the absorbance at 280 nm) using a Cary 50 Bio spectrophotometer (Varian, Walnut Creek, CA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing the protein were concentrated by ultrafiltration using an Amicon stir cell (Millipore Bedford, MA) fitted with a membrane of 3000 kDa pore size. The concentrated fractions were loaded onto a 30 x 1200 mm Sephacryl S-300 (Amersham Biosciences Corp Piscataway, NJ) column equilibrated in buffer A, containing 100 mM NaCl. The apo-ACP was eluted in the same buffer and analyzed as previously described. Fractions containing apo-ACP were concentrated as previously described. Aliquots of 100 μl were frozen on liquid nitrogen and stored at -80ºC. A DTNB (Dithio-bis(2-Nitrobenzoic Acid)) assay for free sulfhydryl groups was preformed to determine how much ACP was in the holo form.[5]

Preparation of ACP Synthase

Using the method of Lambalot[5], a single colony of *E. coli* containing pACPSI was grown in 25 ml of LB containing 50-μg mL^{-1} of ampicillin at 37 °C for 7-8 hours. This culture was harvested at 3,000 x g for ten minutes and the pellet was resuspended in fresh LB media containing 50 μg mL^{-1} of ampicillin. The culture was then used to inoculate 4

L of LB media (50 μg mL^{-1} ampcillin), which was incubated at 37ºC with shaking at 200 rpm and grown to an OD_{600} of ~0.5-0.6**.** Expression was carried out by the addition of 1 mM IPTG followed by incubation for 3 hours at 30ºC**.** The cells were harvested and stored as described for *E. coli ACP*. Harvesting took place at 10,000g for 6 minutes. The cell paste was frozen in liquid nitrogen and stored at -80°C.

The cell paste was resuspended in 3 mL of 50 mM Tris(hydroxymethylaminomethane) (Tris), pH 8.1, 10 mM magnesium chloride ($MgCl_2$), 2 mM dithiothreitol (DTT) and 5% glycerol per gram of cell paste, sonicated on ice in four 1-minute bursts, and centrifuged at 20,000 x g for 30 minutes. The supernatant was decanted into pre-chilled 50-ml tubes. DE-52 slurry equilibrated in Tris buffer (1 mL per mL of extract) was added to the clarified extract and mixed gently for 15 minutes followed by centrifugation at 5,000 x g for 15 minutes. The supernatant was decanted into pre-chilled 50-mL tubes and the DE-52 extraction was repeated. After the second extraction, the supernatant was decanted and the pH was adjusted to 6.5 using 1 M MES. The mixture was centrifuged at 20,000 x g for 15 minutes and the supernatant was applied to a 30 x 30 cm SP-Sepharose (Amersham Biosciences AB Uppsala, Sweden) column equilibrated in MES buffer (50 mM MES, 10 mM $MgCl_2$, 2 mM DTT, 5% glycerol, pH 6.). The column was washed in 250 mL of MES buffer and the protein was eluted using a 500-mL linear gradient of 0 to 1M NaCl in MES buffer.[4]

Synthesis of holo-ACP from apo-ACP

A reaction mixture consisting of a final concentration of 50 mM Tris-HCl**,** pH 8.8, 1.0 mM Coenzyme A, 5.0 mM DTT, 10 mM $MgCl_2$, and 207 μM apo-ACP was pre-incubated in a 37°C water bath. A final concentration of 200 nM of holo-ACP Synthase was then added to initiate the reaction, which was run for two hours at 37°C. The reaction was diluted to one liter with 25 mM MES, pH 6.1 (buffer A) and loaded onto a DE-52 column (2.5 x 20 cm) equilibrated in buffer A. The column was washed with 300 ml of buffer A, and holo-ACP was eluted with 25 mM MES, pH 6.1, containing 0.85 M NaCl. Fractions were analyzed by for protein using the Coomassie Blue dye procedure of Bradford. Fractions containing the protein were pooled and concentrated by Centriplus (MW 3,000) to approximately 1-2 ml. The protein was loaded onto a Sephadex G-25 (Amersham Biosciences AB) column (2 x 15 cm) equilibrated in buffer A. The holo-ACP synthase was eluted from the column with 100 mM Tris pH 8.0. Fractions were collected, analyzed, and concentrated using the methods previously described.[6]

Purification of Acyl-ACP Synthetase from Escherichia coli

The expression of Acyl-ACP Synthetase was carried out by growing cells containing pAaSH with vigorous aeration at 275 rpm in LB media as described.[7] The cells were grown to an OD_{600} of 0.5, and induced by addition of IPTG to 0.4 mM. Expression was carried out for 4.0 hours at 37˚C before harvesting. After harvesting the cell were frozen in liquid nitrogen and stored at -80°C.

In 60 ml of 50 mM Tris-HCl pH 8.0, 26.1g of cell paste was resuspended, followed by the addition of 0.2 mg mL^{-1} of lysozyme. The mixture was allowed to stir and then the bacteria were ruptured using a French press. The supernatant was diluted with an equal volume of 2X column buffer (100 mM Tris, pH 8.0, 40 mM $MgCl_2$, 4% Triton), and was loaded on a Nickel-Nitrilotriacetic acid (Ni-NTA) (Qiagen Valencia, CA) column (equilibrated in 1X column buffer. The column was washed with 1X column buffer and the protein was eluted using Ni-NTA elution buffer (1X column buffer containing 250 mM imidazole). The protein was concentrated using an Amicon (30,000 membrane) and dialyzed against column buffer containing 5 mM ATP to remove excess imidazole and to stabilize the protein. The protein was aliquoted and stored at -80C.

Synthesis of Octanoyl-ACP

The reaction mixture, which consisted of 50 mM [4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid] (EPPS), pH 8.0, 3 mM DTT, 0.4 M lithium chloride (LiCl), 10 mM $MgCl_2$, 5 mM ATP, 2% Trition X-100, 50 µM Holo-ACP, 50 µM octanioc acid, acyl-ACP synthetase from *Escherichia coli*, and H_20 were combined to a final volume of 100.3 ml. The reaction was allowed to proceed for approximately 5.0 hours at 37ºC. The reaction was diluted to one liter in cold 25 mM MES, pH 6.1 (buffer A), titrated to pH 6.1, and loaded onto a DE-52 column (2.5 x 10 cm) equilibrated in buffer A. The column was washed with 500 ml of buffer A, and the octanoyl-ACP was eluted from the column with 0.8 M NaCl in buffer A. Fractions containing protein (method of Bradford) were pooled and titrated to pH 3.9 with concentrated acetic acid. The octanoyl-ACP was precipitated overnight at 0ºC (ice water bath). The precipitate was pelleted at 20,000 x g for 30 minutes, and then dissolved in a minimal volume of 1 M 2-[N-cyclohexylamino] ethane sulfonic acid (CHES), pH 9.0, containing 1 M urea (buffer B). The dissolved protein was loaded onto an S-300 column (30 x 1200 mm) equilibrated in buffer B. The protein was eluted from the column in a fraction size of approximately 2-3 ml. Fractions displaying an absorbance maximum at A_{280} and reacting with Bradford reagent were analyzed by native gel electrophoresis.

Purification of Acyl-Acyl Carrier Protein by gel electrophoresis

In preparation of the gel electrophoresis unit a solution with a final concentration of 13% acrylamide / 5% bis -crylamide, 0.37 M Tris, and 1M Urea was mixed and degassed to form the running gel. To this mixture 100 µL of ammonium persulfate (APS) and 10 µl of *N,N,N',N'*-tetramethylethylenediamine (TEMED) were added to facilitate polymerization which was allowed to occur overnight. A stacking gel containing 6M Urea, H_2O, Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8), acrylamide / bis-acrylamide, 50 µL APS, 10 µL TEMED was poured one to two hours prior to loading the gel. The gel electrophoresis unit was filled with electrophoresis buffer (0.025M Tris, 0.192 M glycine, 0.1% SDS), 500 ml in the upper electrophoresis buffer reservoir and 750 ml in the elution buffer reservior. The lower buffer reservoir was filled with enough buffer to cover the gel. The apo-ACP sample was diluted 10-fold in deionized H_2O and

10 mL of it was mixed with 3.3 mL of sample loading buffer (0.1 M Tris pH 6.8, 30% glycerol, 0.03% bromophenol blue). The protein-dye sample was loaded into the gel tube of the electrophoresis unit. The gel was run at a continuous 7W, with an elution rate of 1 ml min^{-1}. Fractions were collected and analyzed using UV-visible spectroscopy. See appendix.[3]

V. Results and Discussion

Assessment of the activity of *Escherichia coli* acyl-ACP synthetase was preformed through monitoring of the production of radioactive octanoyl-ACP, using [8-^{3}H] octanoic acid as one of the substrates. [8-^{3}H] octanoyl-ACP was easily separated from [8-^{3}H] octanoic acid by gel filtration using calibrated Nick^{TM} columns (Amersham Biosciences AB). Control experiments established that the time-dependent formation of [8-^{3}H] octanoyl-ACP is absolutely dependent upon the *E. coli* acyl-ACP Synthetase (Figure 3). The specific activity of the enzyme was determined to be 2.1 x 10^{-5} U mL^{-1}. The purified *E. coli* acyl-ACP synthetase was used to synthesize tritiated, deuterated, and protiated octanoyl-ACPs. The properties of octanoyl-ACP are not significantly different from those of holo-ACP, making the purification of the product from the substrate difficult. Acyl carrier proteins are protected from denaturation at high pH by long-chain acyl groups (>C-8). This denaturation results in an increase in the Stokes radius of the protein, causing it to

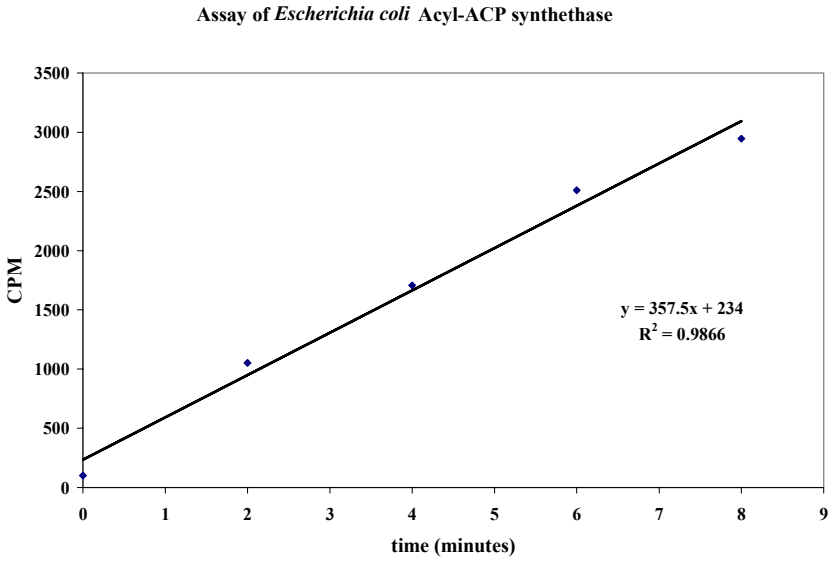

Figure 3: Production of octanoyl-ACP as a function of time.

migrate faster on gel-filtration columns, but slower by native gel electrohporesis. Thus, long chain acyl-ACPs migrate slower than holo or apo-ACP by gel-filtration.

This research was found to be a successful assessment of whether the *Escherichia coli* acyl-ACP synthetase could synthesize octanoyl-ACP, despite the fact that previous research has shown that this is not the enzyme's preferred substrate. It also has been

assessed that the presence of 1 M urea in the gel-filtration buffer may be a resource in the separation of ACPs.

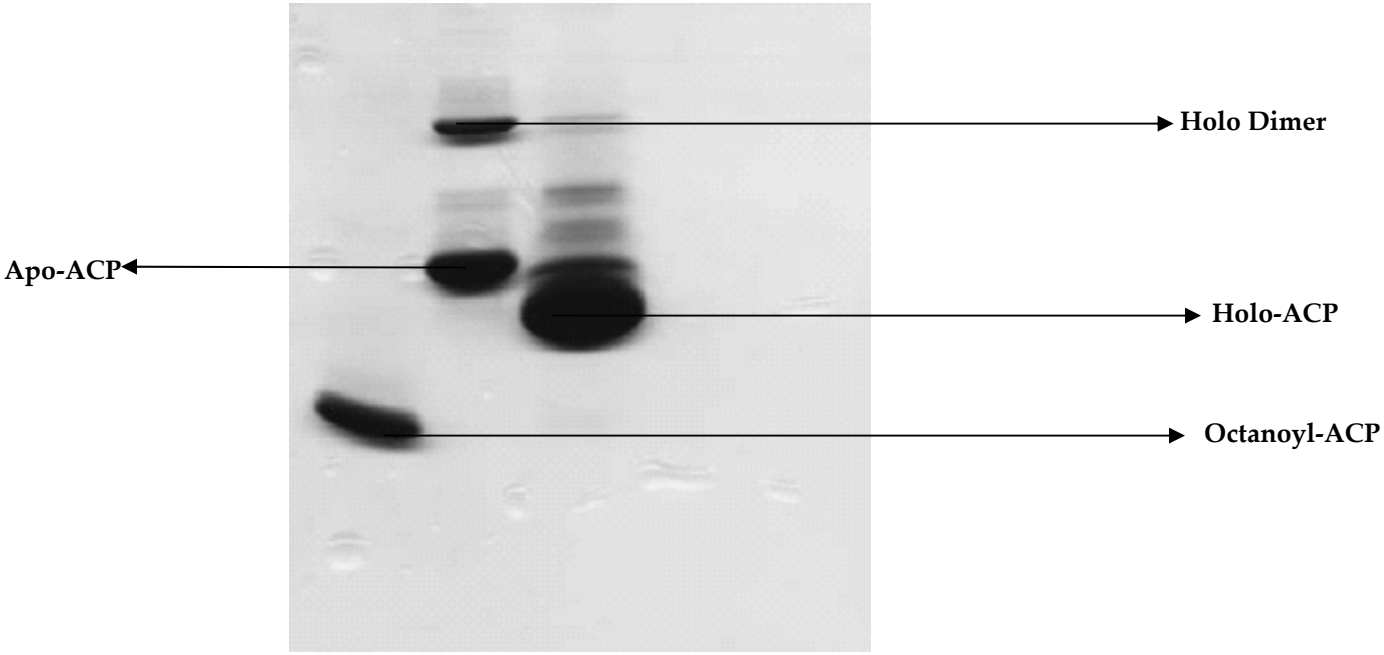

Figure 4: Native Gel Electrophoresis

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Appendix

Native Gel

Running:

Reagent	**Initial**	**Final**	***Amount***
Acrylamide/Bis	30% / 1.12%	13% / 0.5%	8.7 mL
Tris	2 M	.37 M	3.7 mL
Urea	6 M	1 M	3.3 mL
H_2O			4.3 mL

***** Note the above amounts are for a 20 mL gel; therefore, measurements should be multiplied by two.

APS	100 μL
TEMED	10 μL

Stacking:

Reagent	**Amount**
6 M Urea	3.3 mL
H_2O	9 mL
Stacking Gel Buffer	5 mL
Acrylamide/Bis	2.66 mL

APS	50 μL
TEMED	10 μL

Small Native Gel

Running:

Reagent	**Amount**
Acrylamide/Bis	6.7 mL
2 M Tris	2.8 mL
6 M Urea	2.5 mL
H_20	3.3 mL

APS	38.5 µL
TEMED	3.8 µL (5.0)

Stacking:

Reagent	**Amount**
Acrylamide	1.3 mL
Stacking Gel Buffer	1.7 mL
6M Urea	1.25 mL
H_20	1 mL

APS	12.5 µL
TEMED	2.5 µL

***** Note the above amounts are for two small native gels.

30% Acrylamide Stock Solution (30%T/2.67%C)

Acrylamide 29.2g
Bis 0.8g

Dissolve into 70 ml H_2O

Stacking Gel Buffer Stock

0.5 M Tris – HCL, pH 6.8
Dissolve 6 grams Tris base in approximately 60 ml deionized water.
Adjust to 100 mL with deionized water and store at 4˚C

Loading the Sample:

Sample Loading Buffer:

pH 6.8
0.1 M Tris
30% glycerol
0.03% bromphenol blue

*Note samples are loaded 3 parts sample to one part sample loading buffer
Ex: 10 µl sample; 3 µL sample loading buffer

Electrophoresis Buffer (10X Electrode[Running] Buffer):

1 Liter Stock Solution: Tris base 30.3g
Glycine 144.0g
SDS 10.0g
Dissolve and adjust to 1 L with deionized water. DO NOT adjust pH with acid or base.

*Note to run the native gel you use **1X Electrode (Running) Buffer.** To make 1 Liter of **1X Electrode (Running) Buffer** dilute 100 ml of **10X** stock with 900 ml of deionized water.

Native Gel Stain

Stain:

50% Methanol
10% Acetic Acid
0.1% Coomassie Blue

1 Liter Stock
500 ml Methanol
100 ml Acetic Acid
1 g Coomassie Blue
400 ml H_2O

Destain:

10% Methanol
10% Acetic Acid

1 Liter Stock

100 ml Methanol
100 ml Acetic Acid
800 ml H_2O