EXAMPLE OF SECTION I EXPERIMENTAL PROCEDURES

This project will utilize cDNA clones of murine *dumb* and *dumber* genes for heterologous protein expression in cultured cells and non-pathogenic strains of *E. coli*. These gene products are normal components of the vertebrate hair cell and are not considered infectious or biohazardous at any concentration. Clearly labeled bleach, biohazard autoclave bags, and biohazard sharps collectors will be available both in the tissue culture facility, and in the applicant's laboratory for decontamination and disposal. A sink and disinfectant soap will be available for handwashing. Personal protective clothing, including safety goggles, laboratory coats, and gloves will be utilized by all personnel. The applicant's laboratory will be clearly labeled with biohazard warning notices. All laboratory workers will be trained to respond to a spill or laboratory accident.

USE OF BACTERIA
Commercially available *E. coli* K-12 strains will be utilized for plasmid construction and propagation. Commercially available *E. coli* B strains will be used for plasmid propagation and heterologous protein expression. Cultures of less than 1 liter will be grown and manipulated in the applicant's laboratory (530 WH), and a nearby Core laboratory (204 WH) using BSL1 procedures. Centrifugation supernatants collected after disruption of the bacteria by lysis solutions containing at least 2% SDS at pH >11 will be considered free of biohazardous material.

USE OF RECOMBINANT DNA
The expression vectors utilized are widely used, well-characterized, and commercially available. Specifically, pcDNAI/AMP and/or pcDNA3 (Invitrogen, Inc) will be used for the transient transfection of COS-1 cultured cells, and pGEX-2T (Roche) will be used for the expression of glutathione-S-transferase (GST) fusion proteins in *E. coli*. GST has no known toxicity in vertebrates. We will employ the pBSII series of cloning vehicles (Stratagene, Inc.) for the routine propagation and construction of insertional mutations. Operations involving rDNA will be performed in the applicant's laboratory (530 WH), an adjacent Core laboratory (204 WH), and the BSL2 tissue culture facility (203 WH). Transport of rDNA between the main laboratory, Core lab and/or culture facility will utilize sealed polyethylene tubes, inside a sealed secondary plastic container to minimize the possibility of spillage.

USE OF CULTURED CELLS
Revival, passaging, maintenance and cryopreservation of all cell lines will be performed in a BSL2 tissue culture facility dedicated to this purpose and located in 203 WH. Cultured cells will be handled using BSL2 practices and containment.

Hybridoma cell stocks in silicon-gasketed cryovials will be maintained in liquid nitrogen filled Dewar containers, located in 203 WH. These antibody-secreting

hybridomas are not commercially available; however, they were derived from the widely used NS-1 line (ATCC #TIB-18). Hybridoma line H6 was created by fusing mouse spleenocytes with NS-1 cells, and was obtained from Dr. Jack Inthebox (University of Phoenix); it secretes a monoclonal antibody (IgG) against the Dumber protein. Hybridoma line D12 was also created by fusing mouse spleenocytes with NS-1 cells, and was obtained from Dr. Iam Contingent (Macomb Community College); it secretes a monoclonal antibody (IgG) against an unidentified administrative antigen. Hybridoma lines will be revived and maintained as required for the production of monoclonal antibodies. Typically, this is expected to occur four to six times each year over the course of the project period.

COS-1 cells (typically 4-6 - 100 ml dishes) will be maintained continuously over the course of the proposed project period; passaging will usually occur three times per week. COS-1 cell transfections will be performed in a certified Biosafety Cabinet located in 203 WH, twice weekly on average, as follows:

1) Plates of sub-confluent COS-1 will be trypsinized, seeded into fresh plated, and grown O/N at 37oC, 5%CO_2
2) Aliquots of the desired expression plasmids will be combined with the Fugene-6 transfection reagent and added dropwise to the plates. Cells will be returned to the incubator and grown O/N at 37oC, 5%CO_2
3) Media will be aspirated into a “double-flask” setup, and cells will be washed three times with sterile PBS.
4) Cells will be scraped from plates using a rubber policeman and collected into microfuge tubes. Cells will be disrupted by shearing 5-10 times through a 29ga syringe needle (biohazard sharps container will be available for disposal). Microfuge tubes (total liquid volume approx 10 ml) will be sealed into a secondary plastic container for transport to the PI’s laboratory (530 WH). Unbroken cells and nuclei collected by centrifugation will be treated as biohazardous waste and will be autoclaved prior to disposal. The post-nuclear supernatant (lacking viable cells) will be utilized for a variety of biochemical analyses using BSL1 standards.
5) The laminar flow hood will be decontaminated with 70% EtOH after use.