CHOgro® Expression System

Protocol for MIR 6260

SDS and Certificate of Analysis available at mirusbio.com/6260

INTRODUCTION

The CHOgro® Expression System is an optimized platform for transient, high titer protein production in suspension CHO derived cells. This system consists of CHOgro® Expression Medium, L-Glutamine and Poloxamer 188 medium supplements, *Trans*IT-PRO® Transfection Reagent and CHOgro® Complex Formation Solution.

CHOgro® Expression Medium is a chemically defined, hydrolysate-free and animal-origin-free medium. CHOgro® is formulated to provide high density cell growth, and many suspension CHO cells (e.g. Freestyle™ CHO-S) can easily and quickly grow in CHOgro® Medium with minimal adaptation.

*Trans*IT-PRO® Transfection Reagent was developed by empirically testing proprietary lipid and polymer libraries for high transfection performance in suspension CHO and 293 cell types. It is free of animal-derived components and manufactured at Mirus Bio LLC in Madison, Wisconsin, USA.

The combination of CHOgro® Expression Medium and *Trans*IT®-PRO Transfection Reagent enables robust cell growth and high efficiency transfection that streamlines the transient protein expression process. With this platform, high yields of therapeutic candidates for preclinical studies are achieved.

Customer Notice:
The CHOgro® Expression System ships as multiple components. Store at the temperature listed on the product label.

SPECIFICATIONS

Storage	Store *Trans*IT-PRO® Transfection Reagent at -20°C. ***Before each use***, warm to room temperature and vortex gently. Store CHOgro® Expression Medium and Complex Formation Solution at 4°C, protected from light. Store Poloxamer 188, 10% Solution at room temperature. Store L-Glutamine at -20°C and avoid multiple freeze/thaw cycles.
Stability/ Guarantee	*Trans*IT-PRO® Transfection Reagent is guaranteed for 1 year from the date of purchase. Other components are guaranteed as noted on the product label when properly stored and handled.

CAUTION: Standard safe laboratory practices should be maintained when using all chemical transfection reagents. ***Please refer to product SDS for full safety precautions.***

MATERIALS SUPPLIED

The CHOgro® Expression System (MIR 6260) is supplied in the following format. For bulk quantities of the kit or individual components, please inquire about a custom quote.

Product No.	Component	Volume
MIR 5740	*Trans*IT-PRO® Transfection Reagent	1 × 1 ml
MIR 6200	CHOgro® Expression Medium	2 × 1000 ml
MIR 6210	CHOgro® Complex Formation Solution	1 × 100 ml
MIR 6230	Poloxamer 188, 10% Solution	1 × 100 ml
MIR 6240	L-Glutamine, 200 mM Solution in 0.85% NaCl	1 × 100 ml

CHOgro® Medium is also available in 10 L Powder (MIR 6201) and 10 L Polybag (MIR 6202) formats.

Materials Required, but Not Supplied

- Suspension CHO cell line (e.g. FreeStyle™ CHO-S Cells, Life Technologies® Cat. No. R800-07)
- Erlenmeyer shake flasks (e.g. Corning® Cat. No. 431143 or Thomson Cat. No. 931110)
- Purified, endotoxin-free DNA
- Sterile tube for transfection complex preparation
- Orbital shaker (e.g. New Brunswick Innova 2000)
- Reporter Assay, as required

For Research Use Only

Adaptation and Growth in CHOgro® Media

Media Preparation

Prior to use, CHOgro® Expression Medium (MIR 6200) requires supplementation with L-Glutamine (4 mM final concentration, MIR 6240) and Poloxamer 188 (0.3% final concentration, MIR 6230), as described in Table 1 (below):

Table 1. Supplementation required for CHOgro® Expression Medium

Media Supplements	Per 1000 ml
L-Glutamine, 200 mM Solution (MIR 6240)	20 ml
Poloxamer 188, 10% Solution (MIR 6230)	30 ml

NOTE: Store supplemented media at 4°C, protected from light.

Adaptation to CHOgro® Expression Medium:

CHOgro® Expression Medium is a chemically defined, serum-free growth medium that permits high density growth and large-scale transfection of suspension CHO cells. Many suspension CHO cells (e.g. FreeStyle™ CHO-S and ExpiCHO-S™) readily adapt to supplemented CHOgro® Expression Medium, thus eliminating the time and labor typically required for a sequential adaptation process.

Suspension CHO cells grown in CHOgro® Expression Medium often divide at a faster rate (i.e. doubling every ≤ 24 hours) compared to many typical growth media formulations.

A. Cryopreserved Cell Stock

When bringing suspension CHO cells out of cryopreservation, use complete CHOgro® Expression Medium (i.e. supplemented with L-Glutamine and Poloxamer 188) to dilute cells immediately post-thaw, typically at a density of 0.5 - 1 × 10^6 cells/ml. Incubate cells in a shake flask at an appropriate rpm (e.g. 125 rpm for a 1.9 cm orbital throw) at 37°C, 8% CO_2. Monitor cell growth and viability daily. When viability reaches ≥ 98% and the cells are doubling every ≤ 24 hours, the cells are fully adapted.

B. Ongoing Culture

If cells are cultured in an alternate media formulation, cells must be adapted to complete CHOgro® Expression Medium prior to transfection with the CHOgro® Expression System. For adaptation, seed cells at a density of 3 - 5 × 10^5 cells/ml in a mix of 75% current media and 25% CHOgro® Medium (supplemented with L-Glutamine and Poloxamer) for 2-4 passages or until the cells are doubling normally and viability is > 95%. Increase the ratio of CHOgro® Media in 25% increments and monitor cell health and viability. Cells are fully adapted when viability reaches ≥ 98% and cells are doubling every ≤ 24 hours in 100% CHOgro® Medium. Continue to culture cells in a shake flask at an appropriate rpm (e.g. 125 rpm for a 1.9 cm orbital throw) at 37°C, 8% CO_2 and monitor cell growth and viability frequently.

Do NOT allow CHO suspension cells to grow above 1 × 10^7 cells/ml or below 2.5 × 10^5 cells/ml during continuous culture.

Maintenance of Suspension CHO Cells in CHOgro® Expression Medium:

For best results, subculture CHO suspension cells to a density of 1 - 3 × 10^6 cells/ml. DO NOT allow cells to grow to a density higher than 1 × 10^7 cells/ml or lower than 2.5 × 10^5 cells/ml during continuous culture. Subculture every 1-4 days to maintain desired cell density. Monitor cell density and viability frequently (i.e. daily if possible).

Transfection with *Trans*IT-PRO® Reagent

BEFORE YOU START:

Important Tips for Optimal Plasmid DNA Transfection

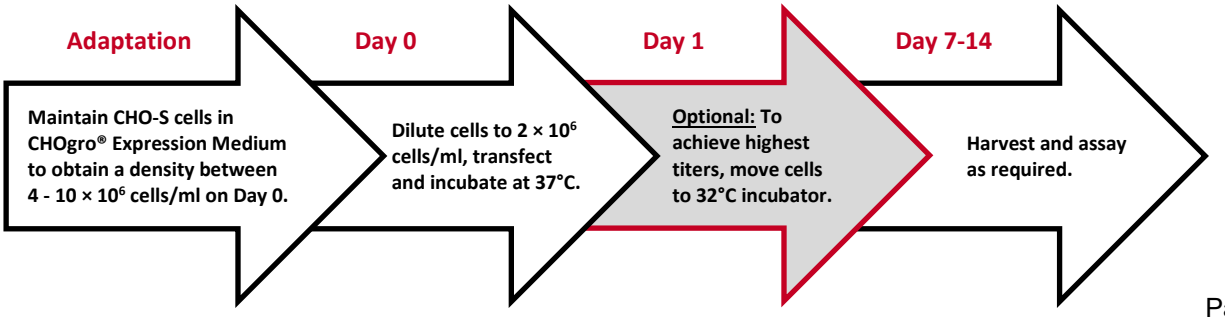
The CHOgro® Expression Medium supports high density growth of suspension CHO cells. When combined with *Trans*IT-PRO® Transfection Reagent, multi-fold increases in protein titer can be obtained. To ensure the most successful transfection, we recommend optimizing reaction conditions for each CHO cell subtype.

- **Cell density at transfection.** Prior to large scale production, determine the optimal cell density for each CHO cell subtype to maximize transfection efficiency. Ideally, cells should be passaged 18-24 hours prior to transfection to obtain a next day density of 4 - 10 × 10^6 cells/ml. This allows for a 2- to 5-fold dilution immediately prior to transfection for a final density of 2 × 10^6 cells/ml at the time of transfection. Cultures should be placed at 37°C, 8% CO_2 prior to transfection.
- **Cell culture conditions:** Culture cells in CHOgro® Expression Medium for optimal growth and viability. The CHOgro® Expression Medium requires supplementation with L-Glutamine (4 mM final concentration, MIR 6240) and Poloxamer 188 (0.3% final concentration, MIR 6230).
- **DNA concentration**. Determine the optimal DNA concentration for each cell type. Start with 1 µg of DNA per 1 ml of culture and vary the DNA concentration from 1-2 µg/ml to find the best working DNA concentration. Adjust the reagent volume accordingly.
- **DNA purity.** Use highly purified, sterile and contaminant-free DNA for transfection. Plasmid DNA preparations that are endotoxin-free and have $A_{260/280}$ absorbance ratio of 1.8-2.0 are desired. We recommend using MiraCLEAN® Endotoxin Removal Kit (MIR 5900) to remove any traces of endotoxin from your DNA preparation.
- **Ratio of *Trans*IT-PRO® Reagent to DNA.** Determine the best *Trans*IT-PRO® Reagent:DNA ratio for each cell type. Start with 1 µl of *Trans*IT-PRO® Reagent per 1 µg of DNA. Vary the concentration of *Trans*IT-PRO® Reagent from 1-2 µl per 1 µg of DNA to find the optimal ratio.
- **Complex formation conditions.** Prepare *Trans*IT-PRO® Reagent:DNA complexes in CHOgro® Complex Formation Solution (MIR 6210).
- **Presence of antibiotics.** Antibiotics inhibit transfection complex formation and therefore should be excluded from the complex formation step. Transfection complexes can be added to cells grown in culture medium containing no or low levels of antibiotics (100X stock of penicillin/streptomycin diluted up to 0.1-1X final concentration).
- **Feeds.** No feeds are required for high yield, but an optional feed can be added to prolong cellular viability (see 'Addition of Cell Culture Feeds to Extend Cell Viability' on Page 5).
- **Post-transfection incubation time.** The optimal post-transfection incubation time may vary depending on the experiment goal and the nature of the plasmid used. For secreted antibody constructs, optimal titers are obtained at 32°C at 7-14 days post-transfection in batch culture.

Suspension CHO cells grown in CHOgro® Expression Medium often divide at a faster rate compared to other growth media formulations.

There is no need to perform a media change to remove the transfection complexes. Media additives or supplements can be added 4-24 hours post-transfection.

Process Flow Chart



TRANSFECTION PROTOCOL

The following procedure describes how to perform plasmid DNA transfections in 125 ml Erlenmeyer shake flasks using 20 ml of complete growth medium. Increase or decrease the amounts of CHOgro® Complex Formation Solution, *Trans*IT-PRO® Transfection Reagent and DNA based on the volume of complete CHOgro® Expression Medium used in alternate cell culture vessels (see Table 2 for reference).

Table 2. Calculation worksheet for CHOgro® Expression System scaling

Starting conditions per milliliter of CHOgro® Expression Medium						
	Per 1 ml			Total culture volume		Reagent quantities
CHOgro® Complex Formation Solution	0.1	ml	×	_____ml	=	_____ml
Plasmid DNA (1µg/µl stock)	1	µl	×	_____ml	=	_____µl
*Trans*IT-PRO® Reagent	1	µl	×	_____ml	=	_____µl

Transient Plasmid DNA Transfection Protocol for Cells in 125 ml Erlenmeyer Shake Flask (20 ml Culture Volume)

A. Maintenance of cells

1. Passage cells 18-24 hours prior to transfection to ensure that cells are actively dividing at the time of transfection and to obtain a density of 4 - 10 × 10^6 cells/ml the next day.

   NOTE: Suspension CHO cells grown in alternate media formulations (e.g. FreeStyle™ CHO Expression Medium) can be centrifuged (300 × g for 5 minutes) 18-24 hours prior to seeding for transfection and resuspended in 100% CHOgro® Expression Medium supplemented with 4 mM L-Glutamine and 0.3% Poloxamer 188.
2. Incubate cells overnight at 37°C in 8% CO_2 on an orbital shaker platform.

Passage cells 18-24 hours before transfection to ensure that cells are actively dividing at the time of transfection.

B. Prepare suspension CHO Cells (immediately before transfection)

1. Prior to transfection, count cells and dilute to a density of 2 × 10^6 cells/ml into a tissue culture vessel (e.g. 20 ml per 125 ml Erlenmeyer shake flask). For optimal protein yields, dilute cells at least 2-fold or centrifuge (300 × g for 5 minutes) and resuspend in 100% fresh CHOgro® Expression Medium. Cultures should be shaking at 37°C in 8% CO_2 prior to transfection.

If cells are split at less than a 1:2 ratio, centrifuge the cells and resuspend in 100% fresh CHOgro® Expression Medium.

C. Prepare TransIT-PRO® Reagent:DNA complexes

1. Warm *Trans*IT-PRO® Reagent to room temperature and vortex gently before using.
2. Place 2 ml of CHOgro® Complex Formation Solution in a sterile tube.
3. Add 20 µg plasmid DNA (20 µl of a 1 µg/µl stock). Mix gently but thoroughly.
4. Add 20 µl *Trans*IT-PRO® Reagent to the diluted DNA solution. Mix gently but thoroughly.
5. Incubate at room temperature for 5 minutes to allow sufficient time for complexes to form.

Incubate complexes for 5 minutes prior to adding to cells.

D. Distribute the complexes to cells in complete growth medium

1. Add the *Trans*IT-PRO® Reagent:DNA complexes (prepared in Step C) to culture vessel.
2. Shake flasks on an orbital shaker (125 rpm when using a shaker with a 1.9 cm orbital throw) at 37°C in 8% CO_2.
3. **Optional:** At 18-24 hours post-transfection, move cells from a 37°C to a 32°C incubator. Up to 2-fold increases in titer can be achieved by culturing cells under mild hypothermal conditions.
4. Incubation time will depend on the culture temperature, nature of the protein and detection method. For secreted antibody constructs, optimal titers are typically obtained at 32°C at 7-14 days post-transfection. For 37°C cultures, shorter incubation times are recommended due to potential protein degradation.
5. Harvest cells and/or supernatant and assay as required.

Place flasks at a lower temperature to increase the specific productivity and decrease protein degradation.

Addition of Cell Culture Feeds to Extend Cell Viability (Optional)

Maximum yields are achieved if cultures are shifted to mild hypothermal conditions (32°C) immediately after transfection with the *Trans*IT-PRO® Reagent (Step D). Cells maintained at 37°C are generally less productive and experience a decrease in viability at earlier timepoints post-transfection. The following steps can be performed to extend the viability of cells post-transfection for cultures incubated at either 32°C or 37°C.

1. Complete Steps A-D as described in the protocol above.
2. At 24-48 hours post-transfection, add 15% (v/v) culture volume of EX-CELL® Advanced CHO Feed 1 (with glucose) (Sigma Cat. No. 24367C), e.g. 3 ml of EX-CELL® Advanced CHO Feed 1 (with glucose) for a 20 ml culture.
   NOTE: Prepare a fresh solution of EX-CELL® Advanced CHO Feed 1 (with glucose) before each use as the prepared solution is unstable. The optimal amount of feed to add may vary from 10-20% (v/v) culture volume and should be empirically determined for each culture system.
3. Maintain cultures at 32°C (or 37°C if necessary) for the duration of the expression experiment. Whenever possible, monitor cultures for viability and protein titers at several timepoints post-transfection (e.g. 5, 7 and 10 days post-transfection), as sufficient titers may be achieved at earlier time points with the CHOgro® Expression System.
4. Harvest cells and/or supernatant and assay as required.

Addition of EX-CELL® Advanced CHO Feed 1 (with glucose) is only beneficial for extending cellular viability post-transfection and may not influence protein yield.

TROUBLESHOOTING GUIDE

LOW PLASMID DNA TRANSFECTION EFFICIENCY

Problem	Solution
Medium formulation incompatible with transfection	Transfection complexes must be formed in serum-free medium without any additional supplements. Mirus recommends CHOgro® Complex Formation Solution (MIR 6210) for transfection complex formation.
	The complete growth medium formulation has profound effects on transfection efficiencies. To test if medium formulation is adversely affecting transfection, try a 50%:50% mix with a compatible medium or adapt cells to a compatible medium formulation. For suspension CHO cells, Mirus recommends CHOgro® Expression Medium (MIR 6200).
	If the cells do not readily adapt to CHOgro® Expression Medium, try a step-wise sequential adaptation protocol. As a general guideline, seed cells at a density of 3 - 5 × 10^5 cells/ml in a mix of 75% current and 25% CHOgro® media for 2-4 passages until the cells return to normal doubling time and viability is > 95%. Do not passage cells if viability is below 95%. Increase the ratio of CHOgro® media (e.g. 50% current and 50% CHOgro® media) stepwise monitoring doubling and viability as outlined above until 100% CHOgro® media is reached. Create a new cell bank in freezing medium (10% DMSO and 90% CHOgro® medium).
Complete growth medium volume too high based on culture vessel size	For standard Erlenmeyer shake flasks, we recommend that the complete growth medium does not exceed one-third the capacity of the flask (e.g. ≤ 40 ml in a 125 ml Erlenmeyer flask). For Thomson Optimum Growth™ flasks the culture volume can be increased per manufacturer's recommendations (e.g. 62 ml in 125 shake flask) without adverse effects on viability or growth.
	For spinner flasks, the maximum complete growth medium is equivalent to the capacity of the flask although cell movement and aeration will vary depending on the culture volume.
Inhibitor present during transfection	Serum and antibiotics inhibit transfection complex formation. Prepare *Trans*IT-PRO® Reagent:DNA complexes in serum-free growth medium; we recommend CHOgro® Complex Formation Solution. Once transfection complexes are formed, they can be added directly to cells cultured in supplemented CHOgro® Expression Medium.
Cells not actively dividing at the time of transfection	Divide the culture 18-24 hours before transfection to ensure that the cells are actively dividing at time of transfection. Determine the optimal cell density for each cell type to maximize transfection efficiency. Typically, a cell density of 2 × 10^6 cells/ml is desired at the time of transfection. Ideally, cells should be maintained 18-24 hours prior to transfection to obtain a density of 4 - 10 × 10^6 cells/ml the next day. If cells are split at less than a 1:2 ratio, they can be spun down and resuspended in 100% fresh CHOgro® Expression Medium. Cultures should be placed at 37°C, 8% CO_2 prior to transfection.

TROUBLESHOOTING GUIDE continued

LOW PLASMID TRANSFECTION EFFICIENCY	
Problem	**Solution**
Low-quality plasmid DNA	Use highly purified, sterile, endotoxin and contaminant-free DNA for transfection.
	We recommend using MiraCLEAN® Endotoxin Removal Kit (MIR 5900) for removal of endotoxin from your DNA preparation. Alternatively, use cesium chloride gradient or anion exchange purified DNA which contains levels of endotoxin that do not harm most cells.
	Do not use DNA prepared using miniprep kits as it might contain high levels of endotoxin.
*Trans*IT-PRO® Reagent was not mixed properly	Warm *Trans*IT-PRO® Reagent to room temperature and vortex gently before each use.
Suboptimal *Trans*IT-PRO® Reagent:DNA ratio	Determine the best *Trans*IT-PRO® Reagent:DNA ratio for each cell type. Titrate the *Trans*IT-PRO® Reagent from 1-2 µl per 1 µg DNA. Refer to "Before You Start" on Page 3.
Suboptimal DNA concentration	Confirm DNA concentration and purity. Use plasmid DNA preps that have an $A_{260/280}$ absorbance ratio of 1.8-2.0.
	The optimal DNA concentration generally ranges between 0.5-2.0 µg/ml of culture medium. Start with a DNA concentration of 1 µg/ml. Consider testing more or less DNA while scaling the amount of *Trans*IT-PRO® Transfection Reagent accordingly.
Incorrect vector sequence	If you do not observe expression of your target insert, verify the sequence of the plasmid DNA.
Post-transfection harvest time	Determine the optimal transfection incubation time for each cell type and experiment. The optimal incubation time will vary depending on the goal of the experiment and the nature of the plasmid used. For secreted antibody constructs, optimal titers are typically obtained at 32°C at 7-14 days post-transfection in batch culture. For 37°C cultures, shorter incubation times of 3-5 days are recommended due to the potential for protein degradation at higher temperature.
Precipitate formation during transfection complex formation	Precipitation may be observed when excess DNA is used during complex formation. This may negatively impact transfection efficiency. As recommended in the protocol, always dilute DNA first in CHOgro® Complex Formation Solution and mix before adding *Trans*IT-PRO® to the diluted DNA mixture during complex formation.
Proper experimental controls were not included	To verify efficient transfection, use *Trans*IT-PRO® Transfection Kit to deliver a positive control such as the Human IgG1 Expression Control (MIR 6250), luciferase, beta-galactosidase or green fluorescent protein (GFP) encoding plasmid.
	To assess delivery efficiency of plasmid DNA, use the Mirus Bio *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kit to label the target plasmid, or use the prelabeled *Label* IT® Plasmid Delivery Controls (please refer to Related Products on Page 8).

TROUBLESHOOTING GUIDE continued

HIGH CELLULAR TOXICITY	
Problem	**Solution**
Shake/spin culture conditions not optimal	Excessive agitation is harmful to cells. Monitor viability of cells using trypan blue exclusion.
Cells not properly adapted to growth culture medium prior to transfection	Check the viability of cultured cells before transfection. Ensure complete adaptation to growth culture medium by verifying consistent doubling times and viability ≥ 95% using trypan blue exclusion.
Cell density not optimal at time of transfection	Determine the best cell density for each cell type to maximize transfection efficiency. Use this cell density in subsequent experiments to ensure reproducibility. For most suspension CHO cell types, a cell density of 2×10^6 cells/ml is recommended at the time of transfection but use of higher or lower densities may be desirable depending on cell type, length of experiment and feeding schedule.
Cell morphology has changed	Mycoplasma contamination can alter cell morphology and affect transfection efficiency. Check your cells for mycoplasma contamination. Use a fresh frozen stock of cells or use appropriate antibiotics to eliminate Mycoplasma. A high or low cell passage number can make cells more sensitive and/or refractory to transfection. Maintain a similar passage number between experiments to ensure reproducibility.
Endotoxin-contaminated plasmid DNA	Use highly purified, sterile, endotoxin and contaminant-free DNA for transfection. We recommend using MiraCLEAN® Endotoxin Removal Kit (MIR 5900) for removal of any traces of endotoxin from your DNA preparation. Alternatively, use cesium chloride gradient or anion exchange purified DNA which contains levels of endotoxin that do not harm most cells. Do not use DNA prepared using miniprep kits as it might contain high levels of endotoxin.
Expressed target gene is toxic to cells	Compare toxicity levels against a cells alone control and cells transfected with an empty vector to assess the cytotoxic effects of the target protein being expressed. If lower levels of target gene expression are desired in your transfection experiments, consider reducing the amount of target plasmid. Maintain the optimal *Trans*IT-PRO® Reagent:DNA ratio by using carrier DNA such as an empty cloning vector.
Cultures not shifted to 32°C following addition of transfection complexes	Maximum yields are achieved if cultures are shifted to mild hypothermal conditions (32°C) immediately after addition of the transfection complexes. Cells maintained at 37°C are generally less productive and experience a decrease in viability at earlier timepoints post-transfection. See '**Addition of Cell Culture Feeds to Extend Cell Viability**' on Page 5.

RELATED PRODUCTS

- CHOgro® High Yield Expression System
- CHOgro® Transfection and Titer Enhancer Kit
- Human IgG1 Expression Control
- *Trans*IT-PRO® Transfection Kit
- MiraCLEAN® Endotoxin Removal Kits
- Ingenio® Electroporation Solution and Kits
- *Label* IT® Tracker™ Intracellular Nucleic Acid Localization Kits
- *Trans*IT-X2® Dynamic Delivery System
- CHOgro® Expression Medium, Dry Powder (10L)
- CHOgro® Expression Medium, 10 L Polybag

For details on the above mentioned products, visit www.mirusbio.com

Reagent Agent®

Reagent Agent® is an online tool designed to help determine the best solution for nucleic acid delivery based on in-house data, customer feedback and citations.

Learn more at:
www.mirusbio.com/ra

Contact Mirus Bio for additional information.

Mirus Bio LLC
5602 Research Park Blvd, Ste 210
Madison, WI 53719
Toll-free: 888.530.0801
Direct: 608.441.2852
Fax: 608.441.2849

Rev1 022222