教育部暨所屬機關學校九十年度出國研究計畫報告
（出國類別：進修 ）

小兒胸腔醫學之相關研習

吸入性一氧化氮對於暴露於高氧環境下之新生鼠其肺部成長的影響（Effects of Inhaled Nitric Oxide on Lung Growth after Exposure to Hyperoxia in Neonatal Rats)

服務機關：國立成功大學附設醫院小兒科
出國人　職　　稱：主治醫師
　　姓　　名：林毓志
出國地區：美國科羅拉多州丹佛市
出國期間：2001-12-13 至 2002-11-30
報告日期：2003-2-28

系統識別號:C09106091

公 務 出 國 報 告 提 要

頁數: 34　含附件: 是

報告名稱:

小兒胸腔醫學之相關研習

主辦機關:

國立成功大學醫學院附設醫院

聯絡人／電話:

陳秀梅／06-2353535轉2049

出國人員:

林毓志　國立成功大學醫學院附設醫院　小兒部　主治醫師

出國類別: 研究

出國地區: 美國

出國期間: 民國 90 年 12 月 13 日 -民國 91 年 11 月 30 日

報告日期: 民國 92 年 02 月 24 日

分類號/目: J2／西醫　J2／西醫

關鍵詞:　慢性肺部疾病,一氧化氮,血管內皮細胞成長因子

內容摘要: 研究背景的探討- 慢性肺部疾病的病理切片特徵爲肺泡與血管發育停滯，而新生鼠在給予高濃度氧氣後會造成肺泡與肺部血管的損害，該損害會持續至幼兒期。至於對抑制肺部生長的機轉，目前並不瞭解。但最近的研究顯示在發育期的肺部，血管內皮細胞生長因子(VEGF)的訊號傳遞受到抑制及一氧化氮(nitric oxide)的生成減少，會使肺泡與血管發育停滯。 提出研究目的- 探討對於善暴露於高濃度氧氣的新生鼠，吸入性一氧化氮(inhaled NO)是否可改善其受損肺部的組織結構。 研究方法的研習- 實驗的進行時間爲新生鼠出生至三週，實驗的動物首先隨機分配分爲四組-在Sprague Dawley新生鼠出生後兩天時，其中兩組暴露於六天的高氧環境(H組; 氧氣濃度100%)，另外兩組則暴露於六天的一般空氣中(R組)；其後的兩週則將H組與R組中的兩組動物分別給予一般空氣(R)或10 ppm(N)的一氧化氮，因此所有的動物可分爲HR、HN、RR、RN四組。在第三週結束時犧牲動物，測量各組動物的體重、肺部重量、右心室肥厚指數(Right ventricular index; RV/ LV+S右心室重量/左心室+心室中隔重量)，並以MLI (mean linear intercept)與RAC (radial alveolar count)兩種指數來分析其肺泡發展的狀況，此外以Factor 8作爲血管內皮細胞的immunohistochemistry染色，分析其血管發展的狀況(Volume fraction of vessel; Vv)。此外在動物7天與14天時，分別取出其肺部組織磨碎後，以西方蛋白質電泳分析(Western blot)其肺部組織中血管內皮細胞生長因子(VEGF)、其接受器(VEGF receptor)與血管內皮細胞一氧化氮合成媒(eNOS)的變化狀況。

本文電子檔已上傳至出國報告資訊網

摘要

研究背景的探討-
慢性肺部疾病的病理切片特徵為肺泡與血管發育停滯，而新生鼠在給予高濃度氧氣後會造成肺泡與肺部血管的損害，該損害會持續至幼兒期。至於對抑制肺部生長的機轉，目前並不瞭解。但最近的研究顯示在發育期的肺部，血管內皮細胞生長因子(VEGF)的訊號傳遞受到抑制及一氧化氮(nitric oxide)的生成減少，會使肺泡與血管發育停滯。

提出研究目的-
探討對於善暴露於高濃度氧氣的新生鼠，吸入性一氧化氮(inhaled NO)是否可改善其受損肺部的組織結構。

研究方法的研習-
實驗的進行時間為新生鼠出生至三週，實驗的動物首先隨機分配分為四組-在 Sprague Dawley 新生鼠出生後兩天時，其中兩組暴露於六天的高氧環境(H 組; 氧氣濃度 100%)，另外兩組則暴露於六天的一般空氣中(R 組)；其後的兩週則將 H 組與 R 組中的兩組動物分別給予一般空氣(R)或 10 ppm(N)的一氧化氮，因此所有的動物可分為 HR、HN、RR、RN 四組。在第三週結束時犧牲動物，測量各組動物的體重、肺部重量、右心室肥厚指數(Right ventricular index; RV/ LV+S 右心室重量/左心室+心室中隔重量)，並以 MLI (mean linear intercept)與 RAC (radial alveolar count)兩種指數來分析其肺泡發展的狀況，此外以 Factor 8 作為血管內皮細胞的 immunohistochemistry 染色，分析其血管發展的狀況(Volume fraction of vessel; Vv)。此外在動物 7 天與 14 天時，分別取出其肺部組織磨碎後，以西方蛋白質電泳分析(Western blot)其肺部組織中血管內皮細胞生長因子(VEGF)、其接受器(VEGF receptor)與血管內皮細胞一氧化氮合成媒(eNOS)的變化狀況。

目次

目的

1) 研習動物模式使用於兒童胸腔醫學相關疾病的研究(如何建立動物的疾病模式?)- 將新生鼠暴露於高氧環境可造成類似人類新生兒肺支氣管發育不全(Bronchopulmonary dysplasia)的狀況，作為研究探討該疾病的基本動物模式。
2) 研習在封閉的系統中，給予動物吸入性一氧化氮，以作為氣體治療或氣體暴露的動物實驗方法。
3) 學習如何在犧牲動物後，取出心臟與肺臟作病理切片與 morphometrics 的測量。
4) 學習利用磨碎後的肺部組織作 Western blot 分析。

過程

1) 動物模式的建立-

慢性肺部疾病(Chronic lung disease)的病理切片特徵為肺泡與血管發育停滯。根據文憲記載，暴露於高氧環境的新生鼠為類似人類新生兒肺支氣管發育不全(Bronchopulmonary dysplasia)的動物模式。有下列文憲記載:

1. Chronic vascular pulmonary dysplasia associated with neonatal hyperoxia exposure in the rat.
   Pediatr Res. 1987 Jan;21(1):14-20.
   Shaffer SG, O'Neill D, Bradt SK, Thibeault DW.

   Chronic sequelae of neonatal hyperoxia was studied in male rats exposed to 0.96-1.0 FiO2 for the first 8 days of life. At 58 days of age functional and morphologic cardiopulmonary changes were compared with controls. Right ventricular systolic pressure was measured percutaneously under anesthesia and was increased in the O2 group (29.5 mm Hg +/- 3.1 versus 23.2 mm Hg +/- 3.5, p less than 0.001). Lung and heart weights were similar between groups. Right ventricular weights however were increased in the O2 group (0.197 g +/- 0.023 versus 0.175 g +/- 0.020, p less than 0.001). Air pressure-volume curves were similar but in the O2 rats saline deflation curves were shifted left and maximal fluid lung volumes were greater (14.1 +/- 1.2 versus 12.0 +/- 0.7 ml, p less than 0.001). Pulmonary arteries were perfused at 100 cm H2O with a barium-gel mixture and lungs were fixed at 25 cm H2O with formalin. Microscopic examination of lungs revealed dysplastic changes of alveolar architecture which included irregularly enlarged alveoli and incomplete alveolar septation. Morphometric studies of the lungs showed that the O2 rats had an increased volume proportion of parenchyma (0.865 +/- 0.020 versus 0.850 +/- 0.019, p less than 0.05), increased mean linear intercept (72.3 microns +/- 9.5 versus 53.6 microns +/- 5.0, p less than 0.001), decreased number of alveoli per mm2 (207 +/- 34 versus 319 +/- 39, p less than 0.001) and fewer small arteries (20-200 microns) per mm2 (8.7 +/- 1.3 versus 14.9 +/- 2.4, p less than 0.001). The number of small arteries/100 alveoli was similar.

2. Hyperoxia-induced pulmonary vascular and lung abnormalities in young rats and potential for recovery.

Pediatr Res. 1985 Oct;19(10):1059-67.

Wilson WL, Mullen M, Olley PM, Rabinovitch M.

We carried out morphometric studies to assess the effects of increasing durations of hyperoxic exposure on the developing rat lung and to evaluate the potential for new growth and for regression of structural abnormalities on return to room air. From day 10 of life Sprague-Dawley rats were either exposed to hyperoxia (0.8FIO2) for 2-8 wk or were removed after 2 wk and allowed to "recover" in room air for 2-6 wk. Litter mates maintained in room air served as age matched controls. Every 2 wk experimental and control rats from each group were weighed and killed. The heart and lungs were removed, the pulmonary artery was injected with barium-gelatin, and the lung was fixed in formalin in the inflated state. Morphometric assessments were made of right and left ventricular weights, lung volume, axial artery lumen diameter, alveolar number and concentration, and arterial number, concentration and muscularity. Rats continuously exposed to hyperoxia and rats exposed for only 2 wk showed the same degree of impaired parenchymal lung growth, as judged by a decrease in the concentration and number of alveoli. A significant decrease in arterial concentration, increase in muscularization of peripheral arteries, and medial hypertrophy of muscular arteries occurred after 2 wk of hyperoxia. Despite an initial trend toward regression, these features became progressively severe with continued hyperoxic exposure and by 8 wk were associated with a decreased arterial lumen diameter, with right ventricular hypertrophy and with failure to thrive.

3. Oxygen-induced alterations in lung vascular development in the newborn rat.
Pediatr Res. 1983 May;17(5):368-75.

Roberts RJ, Weesner KM, Bucher JR.

Newborn rats were exposed to air or hyperoxic conditions for the first 6 days of life. Resulting effects on the pulmonary vascular bed were determined by analysis of barium angiograms, scanning electron microscopy of methylmethacrylate corrosion casts and whole lung, morphometric estimations of pulmonary arteries/area and capillary number/area, and arterial blood gas measurements. Similar studies were also performed on the lungs of animals allowed to recover in air for 1 and 2 wk. Although the general pattern of the pulmonary arterial bed by barium angiograms appeared similar, diminished branching or underfilling of the distal arterial segments was more frequently encountered in hyperoxic-exposed animals. Morphometric examination and corrosion casts revealed differences in

vascular pattern and density between hyperoxia and air-exposed animals. The number of capillaries/mm2 of lung tissue was less in hyperoxic-exposed pups than controls after 6 days of exposure to hyperoxia but markedly increased to slightly above control levels by 2 wk of air recovery. The number of 20--50 micrometers size vessels/mm2 followed a similar pattern of change. Corrosion casts of lung exposed to 6 days of hyperoxia revealed less microvascular density compared to air controls, but after 1 wk recovery in air, hyperoxic-exposed animal had a more extensive network of microvessels. Maximum PaO2 attained by animals in the various groups closely resembled the patterns of change in microvessel density. These findings support the thesis that a major alteration of lung vascular growth and development occurs subsequent to exposure of the newborn to hyperoxia

4. The development of the newborn rat lung in hyperoxia: a dose-response study of lung growth, maturation, and changes in antioxidant enzyme activities.
Pediatr Res. 1981 Jul;15(7):999-1008
Bucher JR, Roberts RJ

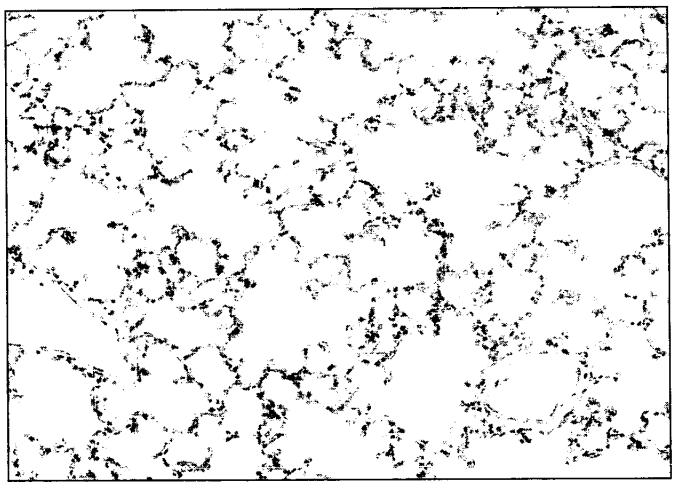
To examine the dose-response relationships of oxygen-induced lung changes, newborn rats were exposed to various patterns of concentrations of hyperoxia (0.4, 0.8, and greater than 0.95 FiO2) for up to 12 days. Prominent findings included microscopic evidence of lung injury and retarded alveolar development (secondary septal development delayed by as much as 88%), lower whole lung DNA (50% of control), lung-to-body-weight ratios (by as much as 18%), and significantly less compliance in the lungs afer exposures of 6 or 12 day duration to all concentrations of hyperoxia. Significant increases in the activities of the lung protective enzymes superoxide dismutase (129 to 160% of control), catalase (112 to 274% of control), and glutathione peroxidase (118 to 256% of control) were noted when activity was expressed on a DNA basis after 12 day exposures to the various patterns of hyperoxia. Lung changes noted after a 7-day recovery period in air included interstitial thickening (117% of control), persistance of the microscopic injury, and retarded alveolar development seen immediately after initial 6-day hyperoxic exposures. At the conclusion of a second wk of recovery in air, the lungs of hyperoxic exposed animals resembled controls in most respects, but a significantly altered compliance was exhibited by the lungs of animals initially exposed to 6 days of 0.4 or greater than 0.95 FiO2. The dose dependency of oxygen-induced lung injury is complex. Straightforward, stepwise dose-response adequately describes the evolution of microscopic injury and slowing of alveolar development in hyperoxia, but the dose dependency is not as

clearly identified in the oxygen-induced retardation of lung growth including DNA content and in changes in antioxidant enzyme activities. Changes in lung compliance clearly do not follow expected dose response relationships

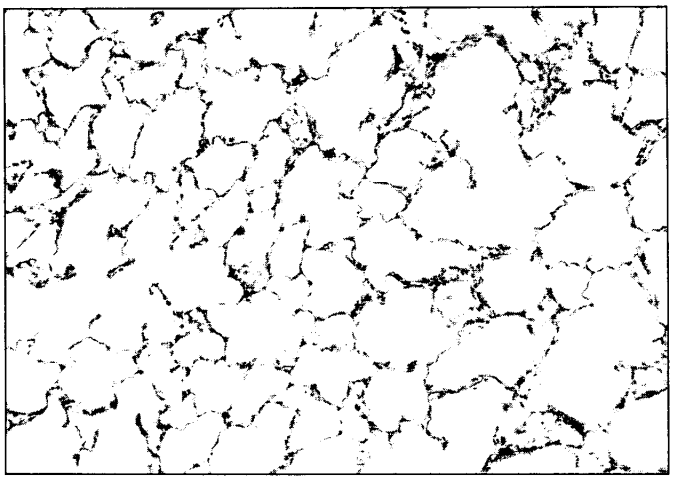
5. Nitric oxide increases the survival of rats with a high oxygen exposure.
Pediatr Res. 1998 Jun;43(6):727-32.
Nelin LD, Welty SE, Morrisey JF, Gotuaco C, Dawson CA

The purpose of this study was to begin to examine the influence of inhaled NO on O2 toxicity. The survival of Sprague-Dawley rats exposed to >95% O2, >95% O2 + 10 ppm NO, >95% O2 + 100 ppm NO, and >95% O2 + 3 ppm NO2 was determined. Survival at 120 h was 2/24 in >95% O2, 2/12 in >95% O2 + 10 ppm NO, and 1/12 in >95% O2 + 3 ppm NO2. Survival at 120 h was 21/30 in >95% O2 + 100 ppm NO ($p < 0.01$ compared with >95% O2). Three additional groups of rats were exposed for 60 h to: 21% O2, >95% O2, or >95% O2 + 100 ppm NO. The lungs were then assayed for total protein, reduced (GSH) and oxidized glutathione (GSSG), and 4-hydroxy-2(E)-nonenal. Both of the high O2 groups had significantly ($p < 0.05$) lower GSH/mg protein and GSH/GSSG ratios compared with the 21% O2 group. The >95% O2 group had a higher 4-hydroxy-2(E)-nonenal/mg of protein than either the 21% O2 group ($p < 0.05$), or the >95% O2 + 100 ppm NO group ($p < 0.05$ compared with >95% O2, not different from the 21% O2 group). Additional groups of rats were exposed to either 21% O2, >95% O2, or >95% O2 + 100 ppm NO for 0, 24, 48, and 60 h. The lungs were examined for neutrophil accumulation, which was increased at 60 h in the two groups exposed to >95% O2, but adding NO had no effect. Thus, the overall result was that 100 ppm inhaled NO improved the survival of rats in high O2

在參考第四篇文憲後，採用下列實驗步驟: 實驗的進行時間為新生鼠出生至三週，實驗的新生鼠首先隨機分配分為兩組-在 Sprague Dawley 新生鼠出生後兩天時，其中一組暴露於六天的高氧環境(H 組; 氧氣濃度 100%)，另外一組則暴露於六天的一般空氣中(R 組)；其後的兩週則將 H 組與 R 組中的兩組動物分別給予一般空氣(R)。在第三週結束時犧牲動物，取出肺部作病理切片。結果可發現暴露於高氧環境的新生鼠，其肺部的生長停滯。(圖一)



Control Group (RR Group)



Hyperoxia Group (HR Group)

Fig. 1- Simplified alveolar structure in hyperoxia exposure group

2) 吸入性一氧化氮的給予-依第一步驟所建立之慢性肺部疾病的動物模式，將實驗的老鼠隨機分為四組，置入氣密的容器中，其中兩組暴露於六天的高氧環境(H 組; 氧氣濃度 100%)，另外兩組則暴露於六天的一般空氣中(R 組)；其後的兩週則將 H 組與 R 組中的兩組動物分別給予一般空氣(R)或 10 ppm(N)的一氧化氮，因此所有的動物可分為 HR、HN、RR、RN 四組。其氣密容器的設計如圖二。

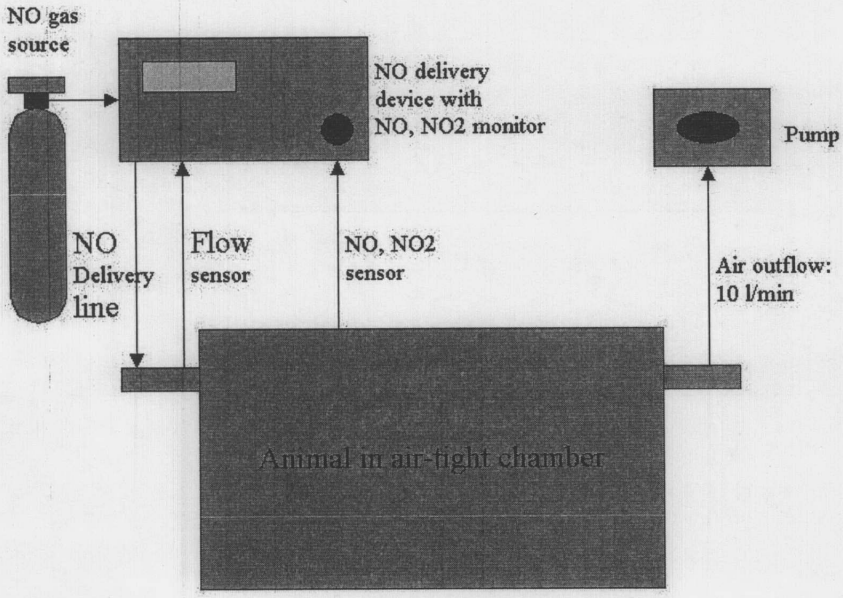


Fig. 2 NO delivery system for animals

3) 肺部病理切片與 morphometrics 的測量- 依附錄 1 的處理程序完成病理切片，此外為分析血管的生長情況，採用 Factor 8 的 immunohistochemistry(IHC)來染血管內皮細胞(附錄 2)，並由顯微鏡上之掃瞄器取得影像。影像取得後，根據 Emery and Mithal 所提出的方法算出其 RAC (Radial Alveolar Count)以評估其 Alveolarization 的程度(圖 3)。而 MLI (Mean Linear Intercept)與 Vv (Volume Fraction of vessel)則以 Stereology Toolbox software (Morphometrix, Davis, CA)來分析(圖 4、圖 5)。

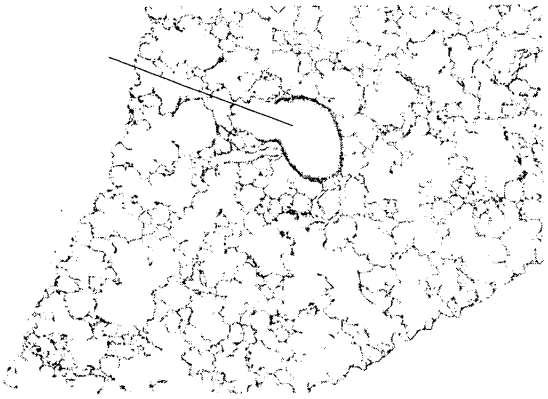


Fig. 3 RAC (Radial Alveolar Count)- a measure of alveolarization of an acinus

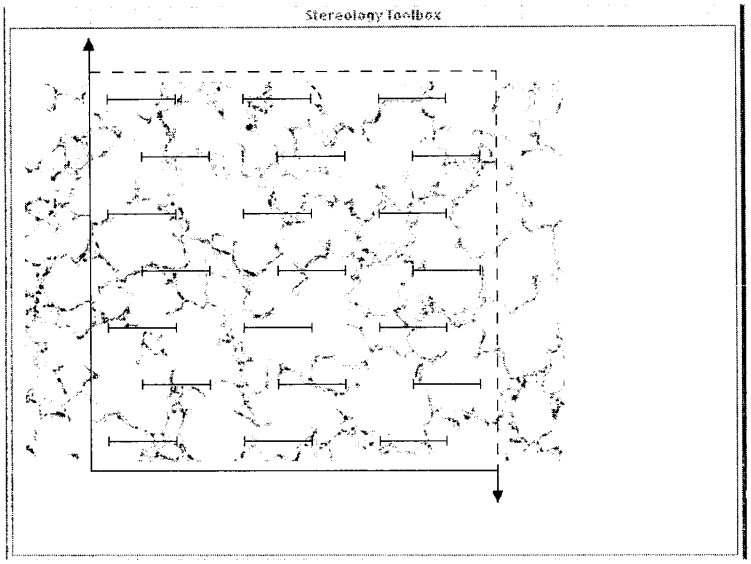


Fig. 4 Mean Linear Intercept (MLI)- Counting the number of intercepts using a 1-mm micrometer gauge placed in the center of the field and study all the slides at the same magnification

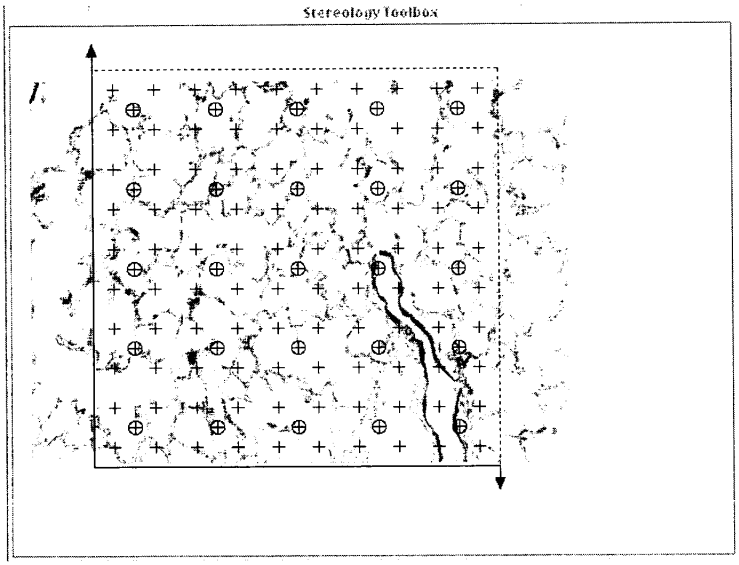


Fig. 5 Vv-

- A point-counting method
  - Lung parenchymal tissue serve as the volume of reference
  - Factor 8 (vWF) as an endothelial marker (IHC)
- A grid of 100 points was superimposed on the slides
- The number of points falling on Factor 8 (+) sites and on lung parenchyma was recorded
- Vv = (Positive stain profile / Parenchymal profile)

4) 肺部組織的 Western blot 分析- 犧牲動物後先將其肺部組織置入試管內並標示檢體，而後立刻放入液態氮中。最後則將全部檢體收集，儲藏於-70 度冰箱。最後依照附錄 3、4 進行 Western Blot 分析。

心得

學習以上的方法並應用於動物的疾病模式，並將成果送至美國 2003 年 Society of Pediatric Research 國際會議。經其大會接受為 platform presentation。回國後將運用該動物的疾病模式探討其治療方法，並利用分子生物學的實驗室方法研究其致病原因。

First Author: Yuh-Jyh Lin
Abstract Number: 75318477
Subspecialty: Neonatal Pulmonology
Theme: Pulmonary Vascular Biology
2003 Pediatric Academic Societies'
Annual Meeting Abstract Submission Form
Contact Person: Yuh-Jyh Lin MD
Department/Institution/Address: Pediatrics, National Cheng-Kung University Hospital, 138
Sheng-Li Road, Tainan, Taiwan, Taiwan
Phone: 886-6-2766186 **Fax:** 886-6-2753083 **E-mail:**
PED1@MAIL.NCKU.EDU.TW
Awards:
Travel Grant Applied for: No
Special Interest Groups, Committees or Regions:
Keywords: BPD; VEGF; Nitric Oxide;
Is the First Author a Trainee? No, Not a Trainee
Title: Inhaled Nitric Oxide Therapy Enhances Distal Lung Growth after Exposure to Hyperoxia in Neonatal Rats.
Authors: Yuh-Jyh Lin MD[1], Neil E. Markham BS[2], Vivek Balasubramaniam MD[2], Jen-Ruey Tang MD[2], John P. Kinsella MD[2], Steven H. Abman MD[2],
Institutions: [1]Pediatrics, National Cheng-Kung University Hospital, Tainan, Taiwan, Taiwan and [2]Pediatric Heart Lung Center, University of Colorado Health Science Center, Denver, CO.
Background: Lung structure in bronchopulmonary dysplasia (BPD) is characterized by decreased alveolarization with dysmorphic vascular growth. Exposure of newborn rats to hyperoxia impairs alveolarization and vessel growth, which persist during infancy. Mechanisms that inhibit distal lung growth are poorly understood, but recent studies suggest that impaired vascular endothelial growth factor (VEGF) signaling and reduced NO production can decrease alveolar and vessel growth in the developing

lung.

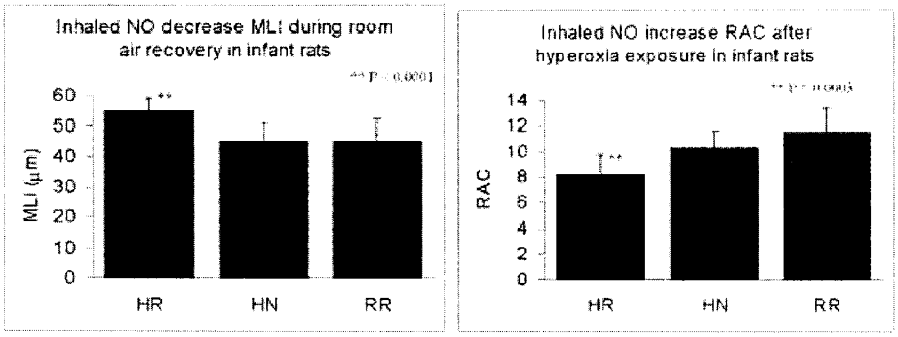
Objective: To determine whether inhaled NO improves lung structure in infant rats that were exposed to hyperoxia in the neonatal period.

Design/Methods: Beginning on the second day of life, Sprague Dawley rat pups were randomly exposed to hyperoxia (H; FiO2:1.00) or room air (R) for 6 days, and then placed in room air, in the presence or absence of low dose inhaled NO (10 ppm) for 2 weeks. Rats were then killed for studies, which included measurements of body weight, lung weight, right ventricular hypertrophy (the ratio of right ventricular to left ventricular plus septum weights; RV/LV+S), and morphometric analysis of alveolarization (by mean linear intercept; MLI; radial alveolar counts (RAC); and vascular volume (Vv)). Western blot analyses for VEGF, VEGF receptors, and endothelial NO synthase (eNOS) were measrued from lung tissue homogenates.

Results: In comparison with room air controls, neonatal hyperoxia reduced body weight, increased MLI, and reduced RAC and Vv in infant rats. RV/LV+S did not differ from controls. NO treatment after hyperoxia increased body weight, and improved distal lung growth, as demonstrated by increased RAC and Vv, and decreased MLI ($p<0.01$ for each parameter; see Figure). Lung VEGF, VEGF receptors and eNOS contents were not different between study groups.

Conclusions: Inhaled NO after hyperoxia enhanced the recovery of distal lung growth in infant rats after neonatal hyperoxia.

Disclosure: Drs. Abman and Kinsella serve as advisors to INO Therapeutics.



建議

1. 宜加強英文的聽讀說寫方面的能力與表達方式，將有助於獲得新知與人際溝通，另外對一些問題的探討也有所助益。普遍來說，英文的說與寫兩方面是更須加強的部分。

2. Dr. Abman 的實驗室中，有一位 Ph D. degree 的成員幫助其實驗的設計與進行，同時每週均進行實驗進度的討論，對於實驗過程中產生的問題可予以指導，建議成立該種諮詢制度，以利實驗進行。

3. 對於一些須大量投資方能進行的實驗，該醫學中心採取成立數個 Core lab. 的作法。由專人負責機器的操作與維護，以控制其實驗結果的品質。建議國內各個貴重儀器中心可比照辦理。

4. 研究助理人員的福利與待遇較國內為佳，所以大部分的助理持續數年的研究經驗後，無須每年重新訓練。

5. 建議成立相關領域的人才資料庫，以利從事同類研究的人員互相溝通，並作相關科技的整合。

6. 美國的醫療費用過高，目前所給予的出國保險費用與實際的醫療保險費用差距太大，建議提高醫療保險的費用。

附錄 1

Rat lung for tissue fixation and biochemical assay

Rat lung for tissue fixation

1. IP pentobarbital (100 mg/ml): 2 ml
2. Dissect the trachea
3. Expose lung and heart
4. Heparin 0.1 cc (1:10000) in RV
5. Incise LA
6. PBS into RV then PA
7. Canulation of trachea
8. Trachea inflation with Zinc Formalin (10 cm H2O for within 1 week, 20 cm H2O for 2 week) for 5 minutes after the inflation
9. Ligate the trachea and remove canulation tip
10. Dissect the heart and lung block (Remove the chest cages first)
11. Put the tissue for fixation into formalin for 24 hrs
12. Change formalin to 70% EtOH after 24 hrs (Put formalin in a special disposal container)

Rat tissue for biochemical assay

1. Prepare liquid Nitrogen
2. IP pentobarbital (100 mg/ml): 2 ml
3. Dissect the trachea
4. Expose lung and heart
5. Heparin 0.1 cc (1:10000) in RV
6. Incise LA
7. PBS into RV then PA
8. Collect lung tissue in the labeled tube and put into liquid nitrogen
9. Collect the tube to –70C refrigerator
10. Return the liquid nitrogen if still available

Factor 8 IHC on paraffin tissue

Factor 8 primary ab from Vector (Rabbit)

Important: Always avoid dryness of slide during the process

1. **Deparaffinization:** 4μ sections are deparaffinized in CitriSolv (Hemo-D), 3x 10 min. ea. Sections are then hydrated through graded ethanols to water. Sections are then placed in coplin jar with ~50 ml PBS. Rinse 3x3 min on rotator and circle sections with hydrophobic marker.

Note: Don't use too much force, otherwise the dye in marker will leak. Also make a complete circle on each block.

2. **Proteinase K Digest:** Sections are digested with PK to expose antigen following formalin fixation. (in slide chamber) Working concentration is 500μg/ml for 10.0 min at room temp. Use 100 ul per slide. Rinse immediately with 3 changes PBS, followed by 3 min in PBS.

Note: Always cover the lid of slide chamber to prevent dryness of slide.

3. Endogenous Peroxidase Block: 3% hydrogen peroxide diluted in cold methanol (in coplin jar) for 20.0 min. (stock hydrogen peroxide is 30%, be sure to wear gloves here). 50 ml is usually enough. Rinse 3x3 min in PBS.

4. Blocking: We do a serum block with 10% serum from host of secondary ab (goat serum), this is diluted in the primary ab diluent. Serum block is for 30.0 min in slide chamber. **Blot sections on some towels, don't rinse.**

6. Primary Ab incubation: Dilute Factor 8 Ab (Rabbit, 1:1000) and Rabbit IgG (1:2000) in primary diluent. Rabbit IgG is added on 2nd block on the same slide as a negative control. 100 ul of each per slide is usually sufficient. Incubate at rm temp. in slide chamber for 60-90 min. Rinse 3x3 min in PBS.

Note: In this step, this incubation can stay overnight at 4 degree.

7. **Prepare AB complex:** Add 2 drops of REAGENT A to 5 ml of PBS. Then add

exactly 2 drops of REAGENT B to the same mixing bottle, mix immediately, and allow to stand for at least 30 min before use.

8. Biotinylated Secondary Ab. Incubation: Dilute appropriate secondary (Vector) 1:200 in PBS with 2% serum from secondary. (5 ul of secondary Ab + 20 ul of serum from secondary to make 1 ml of sol'n). Incubate at rm temp. for 30 min. Rinse 3x3 min in PBS.

9. AB Complex: (Vector) This must be made up at least 30 min. prior to use. Try to make it at the start of the experiment. Incubate ABC for 30 min at rm temp. Rinse 3x3min in PBS.

DAB: (Vector) This is a carcinogen, wear glove. Use about 100 – 200 ul a slide. Monitor reaction under the scope, usually 10.0 min will yield a nice dark brown signal in rat tissue. If using sheep tissue you may want to let it go for 15.0 min. Stop reaction by immersing slides in H20, followed by about a 30 sec rinse in the DI H20. Do this gently, as not to tear the tissue off the slide.

Counterstain: The most common counterstain used in combination with DAB is Hematoxylin. If a diluted 50 ml tube is not on the shelf above the IHC area then dilute the Richard Allen Scientific Hematoxylin 1:10 with H20. Put slides in a empty coplin jar and then quckly pour in the hematoxylin, then pour it back into the tube. Thoroughly rinse slides in H20. Dehyrate through ethanols and mount.

Note:

1. Filter the Hematoxylin to filter the particles before use (Film formation on liquid)
2. Use TBS (mounting media) for mounting
3. Use finger to tap the slide in order to keep the air away from slide

Western Blot:

Day 1: Tissue homogenization-

- Prepare TB buffer:
  50 mM Tris, pH 7.4
  a) 1 mM EDTA
  b) 1 mM EGTA

For 50 ml solution of TB buffer (Protocol P.1)

Solution	Amount needed
TB buffer	50 ml
Beta-Mercaptoethanol	50 ul
AEBSF	500 ul
Leupeptin	10 ul
Pepstatin	70 ul

1. Resects 1/2 to 1/3 of lung tissue for western blot

2. Add 4-5 volume of iced-cold TB buffer

3. On ice with a polytron homogenizator for 1 minute. Clean the homogenizer between each sample with milliQ water and then rinse in ETOH (very important). Dry the tip before going to the next sample. (Also if using a lot of samples be sure to change milli-q water and ETOH used to clean the tip after every 3rd to 4th sample)

4. Sonicate the samples for 3, three second bursts. Keep samples on ice as much as possible.

5. Centrifuge the samples at 1,500 X g for 20 minutes at 4C to remove cell debris. If the samples remains cloudy re-spin.

6. Decant the supernatant (do slowly as not to disturb the pellet, also leave a small volume of sample over the pellet to insure no contamination of cell debris) and throw the pellet away.

Day 2: Protein assay

1. Protein assay is from BIO-RAD
2. BSA for std from stratagene at 15 mg/ml. Make up 1 mg/ml solution in sterile H2O with 100 ul of BSA to 1400 ul of sterile H2O
3. Setup standards and blank as follows:

Sample	amt of 1 mg/ml BSA	TB buffer	H2O up to 798 ul
Blank	0	2 ul	798
1 ug BSA	1 ul	2 ul	797
2 ug BSA	2 ul	2 ul	796
4 ug BSA	4 ul	2 ul	794
8 ug BSA	8 ul	2 ul	790
16 ug BSA	16 ul	2 ul	782

4. Setup samples in triplicate. Label every 12 x 75 mm tube
5. Vortex each sample before removing 2 ul aliquot and adding it to a empty glass tube (changing tip between samples). Add 798 ul sterile H2O to bring the final volume of each tube to 800 ul. You want to add the sample first and then the water as not to increase the amount of protein in the assay from the sample found on the outside of the tip. When adding the 2 ul of sample let the tip touch the bottom of the glass tube for easy pipeting.
6. Add H2O up to 800 ul (798 ul for sample)
7. Add 200 ul of BIO-RAD Dye to each tube using repeated pipet. Do this carefully and quickly. Time does matter after this.
8. Vortex each tube 3 times.
9. Incubate at room temp. for 5 minutes.
10. Transfer to labeled cuvettes. Transfer each sample the same way.
11. Read at 595 nm on spectrophotometer. (Beckman DU640)
12. When using the protein reader, ask for help the first time (THIS SHOULD BE KEPT IN MIND). You will start by clicking on the vis light source and then going under application and click on protein. Use the 6 cuvette transport.
    i. First you will put in your standards (following the protocol) and blank the first one to give you a zero reading.
    ii. Next you will hit read sample 1 (not read samples at the top left hand corner) and it will read the rest of the standards (make sure it is only 1 replication). Print out screen.
    iii. You will then print out the screen for future use and then look at the disstd curve to see how the r value looks. You want an r value around

0.99--- if it is not that accurate, I would suggest re-doing the standards (i.e. r=0.98---). Print out this screen.

iv. Next go to the samples, load in your samples in triplicate, check to make sure it will read your samples in triplicate and the dilution factor is correct. Hit read samples and it will read your samples straight down. Repeat until all your samples are done. When you have finished, print screen and then quit out of program. Be sure to shut off the vis light source.

v. When figuring out the amount of protein per sample use the mean values for each sample. Try to keep the error between the three samples less then 20% if not either redo samples or just use the two closest numbers and get a new mean value.

13. Aliquot a known amount of sample into a clean 0.65 ml microfuge tube
    i.e. 25 ug/tube for all westerns
14. When making up samples want to decide which gel to use, if want 10 well or 15 well (we usually use 4-12% gradient gels) gels.
    i. so if decide to use the 10 well gel- load a total of 30 ul (but maximum amount is 50 ul if in a bind (i.e. low protein concentration))
    ii. if using the 12 well gel, maximum amount to load is about 25 ul
    iii. If using the 15 well gel, maximum amount to load is about 20 ul

    from this can decide how to make up samples

Day 2 or 3: Gel Running

1. Use Excel: Western workbook
   i. Total volume from 10, 12 or 15 wells
   ii. 4X sample buffer = Total volume / 4
   figure out how much of the 4X dye need to add
   iii. Protein volume = 25 / protein conc.

   calculate how much protein need to add to make a final concentration of 25 ug
   iv. DTT (Reducing agent) = Total volume / 10
   v. H2O = Total volume – above 3
2. I usually add the dH2O first so I don't have to change tips and then I add my samples (vortex and change tips between each sample), put on ice and then I add the 4X sample buffer (blue dye). Then right before I heat the samples I add the 10% reducing agent. Try to make duplicate copies of each sample incase you have to re-run gels. Keep the duplicates in a box in the -80 or the -20 freezer.
3. Add reducing agent (which is kept in the fridge by Michelle's desk).
   Heat the samples in the heating block in the back of the room at 70 degrees for 10 minutes. After you heat the samples you do not have to put them back on ice.
4. Spin down samples in microcentrifuge for a few seconds. Then load samples onto the gel and always include one molecular weight standard per gel (5 ul of pre-made molecular marker from Amersham and then figure out the rest according to the recipe set up for the 10 or 15 well gel)
5. Setting up the gel (See next page)
6. Use gel loading tips to load gels and take your time. I usually mark the lanes in black pen so I can see them better when I load my sample.
7. Run proteins into gel at 200 V for 1:30 to 2 hours.
8. When dye front gets to the bottom of the gel and you can see the markers spread out well, figure out where you protein should be and decide if you want to run the gel longer or stop it to get the best picture. Turn off power source , remove the gel, crack open the cassette with the knife (the knife that came with the set up), cut off the wells and the edge at the bottom of the gel.
9. Set up gel for transfer of protein onto the Nitrocellulose Paper (NCP). Cut off a piece of NCP from the stock (8 1/2 cm by 6 1/2 cm) along with some filter paper to (2 pieces). Label the NCP on one side so that know which gel is which. Keep the label side up the protein side up.
   Transfer the protein to the NCP at 35 V for 2 hours.

SETTING UP THE GEL

ASK SOMEONE FOR HELP THE FIRST FEW TIMES

1. Make up running buffer from the stock of 20 X
   i. usually make up a liter of it (Anode buffer)
   ii. from the liter remove 200 ml and to this add 500 ul of anti-oxidant agent (Cathode buffer)

   Running Buffer 20 X for 500 ml
   1. MOPS- 104.6 g
   2. Tris-Base- 60.6 g
   3. SDS - 10 g
   4. EDTA (powder)- 3.0 g
      A. (0.5 M solution)- 20.5 ml
   5. dH2O up to 500 ml

2. Setup gel apparatus
   A. Follow picture in book sent by NOVEX.
   B. Face the box so that the electrode is closet to you
   C. Put in the Place holder-bottom wedge (the wedge with the hole in the center of it). Put the wedge in so that the flat face is closet to you.
   D. Put in the white electrode making sure the copper piece is in the slot.
   E. Put the gels on either side of the white electrode or if only running one gel put the glass plate on the other side. Put the gels in so that the smaller plate is facing inward.
   F. Then put the Upper wedge in and making sure the screw is lose and then tighten
   G. Then place the cathode buffer in first in the upper chamber making sure there are no leaks
   H. Then add the anode buffer to the outside area.
   I. Load wells
   J. Run at 200 V for 1-2 hours
3. After done running the gel. Remove the gel from the apparatus, crack away cassette using the flat knife, and then remove the wells along with the up-rise crest at the bottom of the gel.

Transfer

ASK SOMEONE FOR HELP THE FIRST FEW TIMES

1. Make up the transfer buffer from the stock of 20X
   A. Usually make up 1 liter:
   50 ml 20X stock
   1 ml NOVEX antioxidant
   200 ml MeOH (to transfer 2 gels, 100 for one)
   1 ml 10% SDS
   748 ml dH2O (848 if transferring one gel)

   Transfer buffer (20 X for 125 ml)
   1) Bicine 10.2 g
   2) Bis-Tris 13.08 g
   3) EDTA (powder)- 0.75 g
   (0.5 M solution)- 5.125 ml
   4) dH2O up to 125 ml

2. Pre-soak the sponges in the transfer buffer, make sure all the bubbles of air are out of them
3. Set up the transfer apparatus in the container of transfer buffer so that it stays wet constantly and that way you don't introduce any air.
4. Place the bottom piece of the sandwich (the piece without the electrode on the bottom)

   For 1 gel
   1) put down 2 blotting pads
   2) then put pre-soaked filter paper
   3) then gel
   4) then NCP membrane
   5) then filter paper
   6) then again 2 blotting pads
   there is no air in them)

   (make sure no air in any of these steps)

For 2 gels
1) put down 2 blotting pads
2) then put pre-soaked filter paper
3) then gel
4) then NCP membrane
5) then filter paper
6) then 1 blotting pads
7) then filter paper
8) then NCP membrane
9) then filter paper
10) and finally 2 pieces of blotting pads

there is no air in them

5. Then place the top part of the sandwich on top (make sure this is all done in the container of transfer buffer so that you don't introduce air in it at any time)
6. Set up transfer apparatus
7. Put in the Place holder- bottom wedge (the wedge with the hole in the center of it). Put the wedge in so that the flat face is closet to you.
8. Get another person to help you now
9. Very tightly but gently place the transfer gel sandwich into the apparatus with the electrode on the left side. Hold this together tightly so that no air gets introduced. Then have the other person place the top wedge in and tighten it until it is fit snuggly.
10. Place the transfer buffer inside the sandwich as the cathode buffer
11. Use dH2O as the anode buffer
12. Run the transfer for 2 hours at 35 V

Stripping Gels

Stock	for 50 Ml	
100 mM Beta-Mercato	14.4 mM	347 ul
62.5 mM Tris pH 6.7	100 mM	31.25 ml
2% SDS	10%	10.0 ml
Depc		8.5

IMMUNODETECTION

After you Ponceau S stain the gels and remove it with milli-q water and then the appropriate TBS-T buffer you want to detect for the following. I use 50 ml conicals to make up all my solutions.

ENOS - use TBS-T Buffer #2

1. Load and run gels (use 4%-12% gradient gels) and transfer as specified by the Abman western protocol
2. Ponceau S stain for 5 minutes (re-use this so pour back into bottle)
3. Wash 2-3 times in milli-q water to be able to see accuracy of loading and transfer of the protein bands
4. Rinse 2x 5 minutes in 0.1% TBS-T (pH 7.5) Buffer to remove the Ponceau S stain
5. Then block 1 hour to overnight in 5% NFDM + 0.1% TBS-T Buffer (pH 7.5)-known as blocking buffer (total volume of solution 50 ml)
6. Rinse briefly 2X 2 minutes with 0.1% TBS-T (pH 7.5) ÐDO NOT THROW AWAY BLOCKING BUFFER-SAVE IN A 50 ML CONICAL TUBE
7. Primary Antibody [(Transduction EcNOS) in the white Ð20 fridge in the back of the room] 1:500 diluted in the blocking buffer (that was saved) for 1 hour. Don't use more then 10 ml total for you do not want to use up too much of the primary antibody at one time. Do 2 quick washes with washing buffer. Then wash the membrane 3 X 5 and then 2 X 15 minutes in 0.1% TBS-T buffer (pH 7.5).
8. Secondary antibody is Donkey anti Mouse HRP [in the 4C fridge by the door][stocks in minus 70 freezer, Marilee's shelf] and it is used at 1:17000 diluted in the blocking buffer (rest of buffer that was saved) for 1 hour
9. Do 2 quick washes with the washing buffer. Then wash the membrane 3 X 5 minutes and then 2 X 15 minutes in 0.1% TBS-T buffer (pH 7.5)
10. Use ECL (+) to develop the membrane. You use 2 ml of reagent A and 50 ul of reagent B (adjust volume as needed for number of blots, 25 ul of reagent B for every ml of reagent A). Make sure you warm up the reagents to room temp before use (I usually take them out during the last 5 minute TBS-T wash). Combine them together right before use, remove membrane from wash, blot it off and then add to ECL (+). Incubate for 5 minutes (make sure it covers blot) and then remove and blot of excess.
11. Place in either saran wrap or sheet protector (if use phosphoimager) and go develop
12. Start developing at 30 seconds and adjust for best image

PECAM (Use TBS-T Buffer #2)- DO A LOT OF WASHING IN BETWEEN WITH THIS PROTOCOL, AN EXTRA 3 X 5 MINUTES HERE AND THERE

1. Load and run gels (use 4%-12% gradient gels) and transfer as specified by the Abman western protocol
2. Ponceau S stain for 5 minutes (re-use this so pour back into bottle)
3. Wash 2-3 times in milli-q water to be able to see accuracy of loading and transfer of the protein bands
4. Rinse blots 2x 5 minutes with 0.1% Tween TBST to remove the Ponceau S stain
5. Block non-specific proteins using 5.0% Bovine Serum Albumin (BSA) in 0.1% TBST.
   Use 2.5 grams of BSA (in Tan fridge) and add to 50ml of 0.1% TBST, shake until all BSA is dissolved. Add volume (TBST) to 50ml.
   Block overnight.
6. When blocking is finished. Rinse blots quickly 2 times with 0.1% Tween TBST. Then rinse blots 3 x 5 minutes 3x 15 minutes with 0.1% Tween TBST. Important: Be sure that enough volume is used. Fill yellow container to just below the top. Incubate with Primary antibody for precisely 1 hour.
7. Use 1:200 dilution of the Santa Cruz Pecam Antibody (M-20) and incubate for 1 hour.
8. After incubation rinse blots quickly 2 times with 0.1% Tween TBST. Then rinse blots 3 x 5 minutes 3x 15 minutes with 0.1% Tween TBST. Important: Be sure that enough volume is used. Fill yellow container to just below the top. Incubate with Secondary antibody for precisely 1 hour.
9. Use 1:20000 dilution of Santa Cruz anti-Goat {in tan fridge} and incubate for 1 hour. Important: Do not mix with blocking solution. Use new TBST as solution for antibody.
10. After incubation with secondary antibody, rinse blots quickly 2 times with 0.1% Tween TBST. Then rinse blots 3 x 5 minutes 3x 15 minutes with 0.1% Tween TBST.
    Important: Be sure that enough volume is used. Fill yellow container to just below the top. During this process remove ECL detection kit {from fridge next to Tim's desk top shelf} and allow solutions to acclimate to room temperature.

ImmunoDetection. When finished rinsing, prepare ECL solution. Mix 3mls of each ECL solution into a 15 ml conical. Remove blot from TBST and allow any excess solution on blot to drain off. Place blot on a large piece of saran wrap. Apply ECL mixture directly to protein side of membrane, and allow surface tension to keep

solution covering the membrane. Immunodetect for exactly 1 minute. Drain off excess ECL mixture and place in paper protector to expose under film. Try 5, 10, 15, 20, and 30 sec exposures.

NNOS-Use TBS-T Buffer #1

1. Load and run gels (use 4%-12% gradient gels) and transfer as specified by the Abman western protocol
2. Ponceau S stain for 5 minutes (re-use this so pour back into bottle)
3. Wash 2-3 times in milli-q water to be able to see accuracy of loading and transfer of the protein bands
4. Rinse 2x 5 minutes in 0.05% TBS-T (pH 8) Buffer to remove the Ponceau S stain
5. Then block overnight in 5% NFDM + 0.05% TBS-T Buffer(pH 8)-known as blocking buffer (total volume of solution 50 ml)
6. Rinse briefly 2X 2 minutes with 0.05% TBS-T (pH 8) ÐDO NOT THROW AWAY BLOCKING BUFFER-SAVE IN A 50 ML CONICAL TUBE
7. Primary Antibody [(Transduction) in the white Ð20C fridge at the back of the room] 1:3000 diluted in the blocking buffer (that was saved) for 4 hours. Don't use more then 10 ml total for you do not want to use up too much of the primary antibody at one time
8. Do 2 quick washes with the washing buffer. Wash the membrane 3 X 5 minutes and then 3 X 15 minutes in the 0.05% TBS-T buffer (pH 8)
9. Secondary antibody is anti-rabbit [in the 4C fridge by the door] used at 1:15000 diluted in the blocking buffer (rest of buffer that was saved) for 1 hour
10. Do 2 quick washes with washing buffer. Then wash the membrane 3 X 5 minutes and then 3 X 15 minutes in 0.05% TBS-T buffer (pH 8)
11. Use ECL (+) to develop the membrane. You use 2 ml of reagent A and 50 ul of reagent B (adjust volume as needed for number of blots). Make sure you warm up the reagents to room temp before use (I usually take them out during the last 5 minute TBS-T wash). Combine them together right before use, remove membrane from wash, blot it off and then add to ECL (+). Incubate for 5 minutes (make sure it covers blot) and then remove and blot of excess.
12. Place in either saran wrap or sheet protector (if use phosphoimager) and go develop
13. Start developing at 30 seconds and adjust for best image

INOS- use TBS-T Buffer #1

1. Load and run gels (use 4%-12% gradient gels) and transfer as specified by the Abman western protocol
2. Ponceau S stain for 5 minutes (re-use this so pour back into bottle)
3. Wash 2-3 times in milli-q water to be able to see accuracy of loading and transfer of the protein bands
4. Rinse 2x 5 minutes in 0.05% TBS-T (pH 8) Buffer to remove the Ponceau S stain
5. Then block overnight in 5% NFDM + 0.05% TBS-T Buffer(pH 8)-known as blocking buffer (total volume of solution 50 ml)
6. Rinse briefly 2X 2 minutes with 0.05% TBS-T (pH 8)- DO NOT THROW AWAY BLOCKING BUFFER-SAVE IN A 50 ML CONICAL TUBE
7. Primary Antibody [(Santa Cruz) in the white Ð20C fridge in the back of the room] 1:200 diluted in the blocking buffer (that was saved) for 2 hours. Don't use more then 10 ml total for you do not want to use up too much of the primary antibody at one time
8. Do 2 quick washes with the washing buffer. Wash the membrane 3 X 5 minutes and then 3 X 15 minutes in the 0.05% TBS-T buffer (pH 8)
9. Secondary antibody is anti-rabbit [in the 4C fridge by the door] used at 1:10000 diluted in the blocking buffer (rest of buffer that was saved) for 1 hour
10. Do 2 quick washes with washing buffer. Then wash the membrane 3 X 5 minutes and then 3 X 15 minutes in 0.05% TBS-T buffer (pH 8)
11. Use ECL (+) to develop the membrane. You use 2 ml of reagent A and 50 ul of reagent B (adjust volume as needed for number of blots). Make sure you warm up the reagents to room temp before use (I usually take them out during the last 5 minute TBS-T wash). Combine them together right before use, remove membrane from wash, blot it off and then add to ECL (+). Incubate for 5 minutes (make sure it covers blot) and then remove and blot of excess.
12. Place in either saran wrap or sheet protector (if use phosphoimager) and go develop
13. Start developing at 30 seconds and adjust for best image

VEGF Western Protocol-Gresh

Prior to Detecting:

Load and run gels **(use 10 well 10% gels with MES Running Buffer not MOPS for 40 minutes at 200 volts and transfer for 1:30 at 35 volts)**

Use Ponceau S to detect efficiency of transfer (5 minutes).

Rinse Ponceau S off membrane 3 times with distilled H2O.

Detecting:

Rinse blots 2x 5 minutes with **0.05% Tween TBST**.

Block non-specific proteins using 5.0% Non-fat Dried milk (NFDM) in 0.05% TBST.

Use 2.5 grams of NFDM (in Tan fridge) and add to 50ml of 0.05% TBST, shake until all NFDM is dissolved. Add volume (TBST) to 50ml.

Block for 1 hour or overnight.

When blocking is finished. Rinse blots quickly 2 times with 0.05% Tween TBST. Then rinse blots 2x 2 minutes with 0.05% Tween TBST. Important: Be sure that enough volume is used. Fill yellow container to just below the top.

Incubate with Primary antibody for precisely 1 hour.

Use 1:1000 Santa Cruz VEGF antibody (rabbit polyclonal Antibody (A-20)) {in tan fridge}. Incubate for 1 hour

After incubation rinse blots quickly 2 times with 0.05% Tween TBST. Then rinse blots 3x 5 minutes followed by 3x 15 minutes with 0.05% Tween TBST. Important: Be sure that enough volume is used. Fill yellow container to just below the top.

Incubate with Secondary antibody for precisely 1 hour.

Donkey anti-rabbit IgG 1:10000{in tan fridge}. Incubate for 1 hour.

After incubation with secondary antibody, rinse blots quickly 2 times with 0.05% Tween TBST. Then rinse blots 3 x 5 minutes followed by 2 x 15 minutes with 0.05% Tween TBST. Important: Be sure that enough volume is used. Fill yellow container to just below the top. During this process remove ECL + detection kit and allow solutions to acclimate to room temperature.

KDR also known as FLK-1 (Use Buffer #1)- WASH A LOT WHEN DOING THIS PROTOCOL, A FEW EXTRA WASHES (3 X 5 MINUTES) HERE AND THERE

1. Load and run gels (use 4%-12% gradient gels) and transfer as specified by the Abman western protocol
2. Ponceau S stain for 5 minutes (re-use this so pour back into bottle)
3. Wash 2-3 times in milli-q water to be able to see accuracy of loading and transfer of the protein bands
4. Rinse 2x 5 minutes in 0.05% TBS-T (pH 8) Buffer to remove the Ponceau S stain
5. Then block at least 1 hour to overnight at 4C in 5% NFDM + 0.05% TBS-T Buffer (pH 8)-known as blocking buffer (total volume of solution 50 ml)
6. Rinse briefly 2X 2 minutes with 0.05% TBS-T (pH 8) ĐDO NOT THROW AWAY BLOCKING BUFFER-SAVE IN A 50 ML CONICAL TUBE
7. Primary Antibody [(Santa Cruz) in the white 4C fridge by the door] 1:200 diluted in the blocking buffer (that was saved) for 2 hours. Don't use more then 10 ml total for you do not want to use up too much of the primary antibody at one time
8. Do 2 quick washes. Then wash the membrane 3 X 5 minutes and then 3 X 15 minutes in the 0.05% TBS-T buffer (pH 8)- not blocking buffer
9. Secondary antibody is anti-rabbit [in the 4C fridge by the door] used at 1:10000 diluted in the blocking buffer (rest of buffer that was saved) for 1 hour
10. Do 2 quick washes with washing buffer. Then wash the membrane 3 X 5 minutes and then 3 X 15 minutes in 0.05% TBS-T buffer (pH 8)-not blocking buffer.
11. Use ECL (+) to develop the membrane. You use 2 ml of reagent A and 50 ul of reagent B (adjust volume as needed for number of blots). Make sure you warm up the reagents to room temp before use (I usually take them out during the last 5 minute TBS-T wash). Combine them together right before use, remove membrane from wash, blot it off and then add to ECL (+). Incubate for 5 minutes (make sure it covers blot) and then remove and blot of excess.
12. Place in either saran wrap or sheet protector (if use phosphoimager) and go develop. Start developing at 30 seconds and adjust for best image.

ET-B (Use Buffer #2)

1. Load and run gels (use 4%-12% gradient gels) and transfer as specified by the Abman western protocol
2. Ponceau S stain for 5 minutes (re-use this so pour back into bottle)
3. Wash 2-3 times in milli-q water to be able to see accuracy of loading and transfer of the protein bands
4. Rinse 2x 5 minutes in 0.05% TBS-T (pH 7.5) Buffer to remove the Ponceau S stain
5. Then block at least 1 hour to overnight at 4C in 5% BSA + 0.05% TBS-T Buffer(pH 7.5) (total volume of solution 50 ml)
6. Rinse briefly 2X 2 minutes with 0.05% TBS-T (pH 7.5) - DO NOT THROW AWAY BLOCKING BUFFER-SAVE IN Λ 50 ML CONICAL TUBE
7. Primary Antibody [(Alexis) in the white Ð20C fridge in the back of the room] 1:1000 diluted in 0.05% TBS-T (pH 7.5) for 1 hour. Don't use more then 10 ml total for you do not want to use up too much of the primary antibody at one time
8. Do 2 quick washes with the washing buffer. Then wash the membrane 3 X 5 minutes and then 3 X 15 minutes in the 0.05% TBS-T buffer (pH 7.5)
9. Secondary antibody is anti-rabbit [in the 4C fridge by the door] used at 1:15000 diluted in 0.05% TBS-T (pH 7.5) for 1 hour
10. Do 2 quick washes with washing buffer. Then w3.4.99ash the membrane 3 X 5 minutes and then 3 X 15 minutes in 0.05% TBS-T buffer (pH 7.5) and then do a final rinse in the TBS minus the Tween (up on the top shelf where the buffers are)
11. Use ECL (+) to develop the membrane. You use 2 ml of reagent A and 50 ul of reagent B (adjust volume as needed for number of blots). Make sure you warm up the reagents to room temp before use (I usually take them out during the last 5 minute TBS-T wash). Combine them together right before use, remove membrane from wash, blot it off and then add to ECL (+). Incubate for 5 minutes (make sure it covers blot) and then remove and blot of excess.
12. Place in either saran wrap or sheet protector (if use phosphoimager) and go develop. Start developing at 30 seconds and adjust for best image

Stripping Blots:

Follow the Amersham ECL Plus insert protocol:

1. Submerge the membrane in 50 ml stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH6.7.) and incubate at 50 degrees C with occasional agitation. We use a bottle in a hyb oven at 70 degrees C to reduce odor and improve completeness of stripping.
2. Wash the membranes for 2 x 10 minutes in large volumes of TBS-T at room temperature.
3. Block with TBST / 5% NFDM one hour at room temp.
4. Begin primary antibody OR use ECL plus to verify strip.

VEGF #1 buffer (Non-reducing gel)	VEGF #1 buffer (DTT x 2)	PECAM #2 buffer	KDR #1 buffer
□ After blocking □ Rinse (1) x 2 min □ Rinse (2) x 2 min	□ After blocking □ Rinse (1) x 2 min □ Rinse (2) x 2 min	□ After blocking □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 15 min □ Rinse (5) x 15 min □ Rinse (6) x 15 min	□ Blocking x 30 min □ Rinse (1) x 2 min □ Rinse (2) x 2 min
□ 1" Ab x 1 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 15 min □ Rinse (5) x 15 min □ Rinse (6) x 15 min	□ 1" Ab x 1 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 15 min □ Rinse (5) x 15 min □ Rinse (6) x 15 min	□ 1" Ab x 1 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 15 min □ Rinse (5) x 15 min □ Rinse (6) x 15 min	□ 1" Ab x 2 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 5 min □ Rinse (5) x 5 min
□ 2" Ab x 1 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 15 min □ Rinse (5) x 15 min □ Rinse (6) x 15 min	□ 2" Ab x 1 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 15 min □ Rinse (5) x 15 min □ Rinse (6) x 15 min	□ 2" Ab x 1 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 15 min □ Rinse (5) x 15 min □ Rinse (6) x 15 min	□ 2" Ab x 1 hr □ Rinse (1) x 5 min □ Rinse (2) x 5 min □ Rinse (3) x 5 min □ Rinse (4) x 5 min □ Rinse (5) x 5 min
□ ECL (+) x 5 min	□ ECL (+) x 5 min	□ ECL: A x 1.5 ml and B x 1.5 ml □ Immunodetect x 1 min □ 5,10,15,20,30 sec exposure	□ ECL (+) x 5 min

附錄 4

XCell *SureLock*™ Mini-Cell

INSTRUCTIONS

Invitrogen™
life technologies

TABLE OF CONTENTS

The XCell *SureLock*™ Mini-Cell is manufactured in the U.S.A. from domestic and International components.

To ensure safe, reliable operation, always operate the XCell *SureLock*™ Mini-Cell according to instructions. Wear protective gloves and safety glasses when working in a laboratory environment.

Invitrogen products are intended for *in vitro* use only.

Invitrogen is not responsible for injuries or damages caused by improper use.

I. INTRODUCTION

The XCell *SureLock*™ Mini-Cell's unique cam design lets you run gels quickly, easily and leak-free without awkward clamps or grease. Set-up time is only 30 seconds; just drop the core and the cam into the buffer chamber, insert the gels, and pull the cam forward. The perfect no-leak seal means no mess and consistent performance. Tough polycarbonate construction boosts durability. Retractable plugs, recessed jacks and a specially designed lid enhance user safety. You can transfer with the optional XCell II™ Blot Module (Cat. No. EI9051), in the same chamber.

II. SAFETY

During operation, the XCell *SureLock*™ Mini-Cell must be used with an external DC power supply designed specifically for electrophoresis applications. This power supply must be isolated from ground so that the DC output is floating. The PowerEase™500 Programmable Power Supply (EI8600) meets these requirements. The maximum electrical operating parameters for the XCell *SureLock*™ Mini-Cell are:

Maximum Voltage Limit:	1500 VDC*
Maximum Power Limit:	75 Watts*
Maximum Operating Temperature Limit:	70°C

The XCell *SureLock*™ Mini-Cell's lid is designed in such a way that if the lid is removed, the electrical connection to the unit will be broken. **Do not attempt to use the cell without the cell lid. Do not use lids from other mini-cells.**

The XCell *SureLock*™ Mini-Cell is designed to meet EN61010-1 Safety Standards. This product is safe to use when operated in accordance with this instruction manual. If this unit is used or modified in a manner not specified in this manual then protection afforded by the unit will be impaired. Alteration of this unit will:

- Void the warranty.
- Void the EN61010-1 safety standard certification.
- Create a potential safety hazard.

Invitrogen is not responsible for any injury or damage caused by use of this unit when operated for purposes which it is not intended. All repairs and service should be performed by Invitrogen or an authorized agent.

The XCell *SureLock*™ Mini-Cell is classified as Class II of IEC 536 for protection against electrical shock.

**** The XCell SureLock™ is rated at 1500 VDC, 75 Watts, but for running protein gels, the maximum voltage/wattage recommended is 500 VDC/50 Watts.***

II. INSTRUCTIONS DE SECURITE

La Cellule d'Electrophorése SureLock™ doit être uniquement une utilisation avec une alimentation en courant contimu DC externe et spécialement conçue pour en électrophorése. Cette alimentation doit être avec séparation galvanique de façon que la sortie DC du secondaire ai un potentiel électrique flottant. Le domaine d'utilisation de la Celleule d'Electrophorése SureLock™ est limité par les paramétres de fontctionnement suivants:

Tension maximum:	1,500 V continu
Puissance maximum:	75 W
Tempèrature maximum:	70°C

La Cellule d'Electrophorése SureLock™ est conçue de façon á ce que lorsque le couvercle de la cuve est ouvert, la connection électrique avec l'unité est interrompue. Il ne faut en aucun cas essayer de faire fonctionner la cuve sans le couvercle.

La Cellule d'Electrophorése SureLock™ satisfait aux normes de sécurité EN 61010-1. La sécurité est assurée dans tous les cas d'une utilisation conforme au manuel d'utilisation. Dans le cas ou la cellule serait modifiée ou utilisée de manière non spécifiée dans le manuel d'utilisation, la sécurité de l'utilisateur pourrait être remise en cause. En cas d'utilisation non conforme toutes les garanties sinsi que les certifications de conformité aux normes de sécurité sont abrogeès.

La responsabilité de Invitrogen ne péut être mise en cause en cas d' accident et dommages causés par une utilisation non conforme de la Cellue d'Electrophorése. Toute intervention de service ou de réparation doit être unquement effectuée par Invitrogen ou par un agent agrée de Invitrogen.

La Cellule d'Electrophorése SureLock™ est classifié par rapport à la norme d'isolation électrique IEC 536 en classe II.

INFORMATIONAL SYMBOLS

Used on the XCell *SureLock*™ Cell to indicate an area where a potential shock hazard may exist.

Used on the XCell *SureLock*™ Cell to indicate a warning. The operators manual should be consulted to avoid possible personal injury or instrument damage.

SYMBOLERKLÄRUNG

Weist auf eine Zone hin, wo Gefährdung durch Stromschlag besteht.

Lesen Sie die Bedienungsanleitung, um Verletzungen oder Beschädigung des Gerätes zu vermeiden.

SIGNIFICATION DES PICTOGRAMMES

Sur le Cellule d'Electrophorése XCell *SureLock*™, signale les zones où un risque de choc électrique peut exister.

Sur le Cellule d'Electrophorése XCell *SureLock*™, signale un risque potentiel pour l'utilisateur ou pour l'equipement. Veuillez consulter le mode d'emploi.

III. SPECIFICATIONS

XCell *SureLock*™ Mini-Cell Specifications:

Dimensions:	11 x 12 x 16 cm
Upper Buffer Chamber Capacity:	200 ml
Lower Buffer Chamber Capacity:	600 ml
Material:	Polycarbonate
Chemical Resistance:	The XCell *SureLock*™ Mini-Cell is impervious to alcohol, but not compatible with chlorinated hydrocarbons (eg. chloroform), aromatic hydrocarbons (eg. toluene, benzene) or acetone.
Electrode Wire:	Platinum, 0.010" diameter
Electrical Limits:	1500 VDC or 75 Watts*
Temperature Limit:	70°C

Gel Specifications:

Gel cassette:	10 cm x 10 cm
Thickness:	1.0 mm or 1.5 mm
Gel size:	8 cm x 8 cm
Combs:	1 well, 2D well, 5 well, 10 well, 12 well and 15 well

Sample Well Capacity:

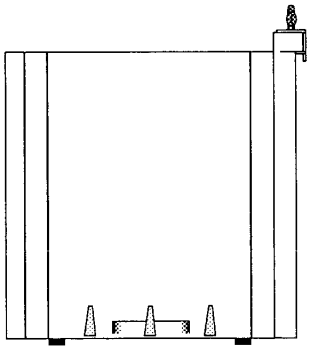
Gel thickness	1 well	2D	5 well	10 well	12 well	15 well
1.0 mm	700 µl	400 µl	60 µl	25 µl	20 µl	15 µl
1.5 mm	N/A	600 µl	N/A	37 µl	N/A	25 µl

Maintenance:	Wash with a mild detergent and rinse with deionized water after each use.

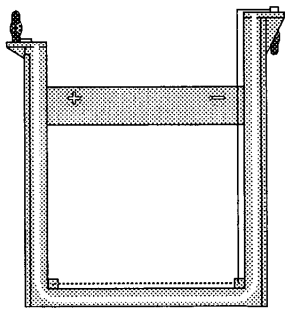
**** The XCell SureLock™ is rated at 1500 VDC, 75 Watts, but for running protein gels, the maximum voltage/wattage recommended is 500 VDC/50 Watts.***

IV. XCELL *SureLock*™ MINI-CELL PARTS

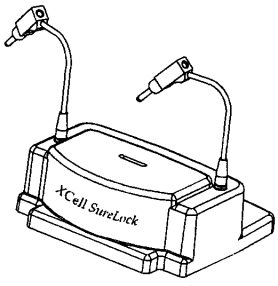
Lower Buffer Chamber
Cat. No. EI0013 (1 ea)



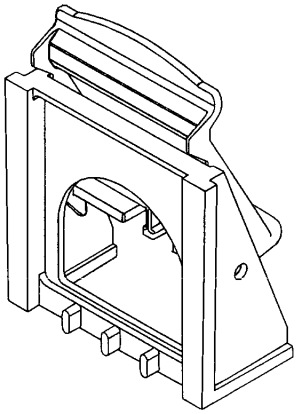
Buffer Core with Electrodes
Cat. No. EI9014 (1 ea)
platinum electrodes, gold terminals
and silicone gaskets



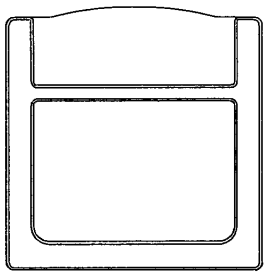
Cell Safety Lid with Cables
Cat. No. EI0010 (1 ea)



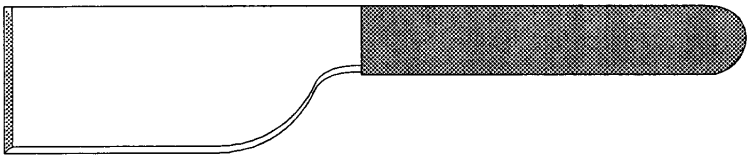
Gel Tension Wedge
Cat. No. EI0011



Buffer Dam
Cat. No. EI0012 (1 ea)



Gel Knife
Cat. No. EI9010 (1 ea)



V. ASSEMBLY OF THE XCELL *SureLock*™ MINI-CELL

Removing Gel Cassette from Pouch

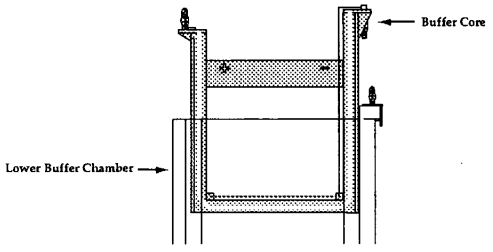
1. Cut open the gel cassette pouch with scissors and remove cassette.
2. Drain away the gel packaging buffer.
3. Remove the gel cassette from the pouch and rinse with D.I. water.
   NOTE: Always handle the cassette by its edges only.

> **Warning**
>
> Always wear protective clothing when performing laboratory experiments.
>
> To avoid contamination from possible residual acrylamide, wear protective gloves when loading and unloading the Mini-Cell and when preparing, staining/destaining, and drying gels.

Preparation for Sample Loading

1. Peel off the tape covering the slot on the back of the gel cassette.
2. In one fluid motion, pull the comb out of the cassette. This exposes the gel loading wells.
3. Use a pipet to gently wash the cassette wells with 1x running buffer. Invert the gel and shake to remove buffer. Repeat twice. Fill the sample wells with running buffer. **NOTE:** Be sure to displace all air bubbles from the cassette wells as they will affect sample running.
4. Lower the buffer core into the lower buffer chamber so that the negative electrode fits into the opening in the gold plate on the lower buffer chamber.
5. Insert the Gel Tension Wedge into the XCell SureLock™ behind the buffer core. Make sure the Gel Tension Wedge is in its unlocked position (see page 9), allowing the wedge to slip easily into the XCell SureLock™ unit. The Gel Tension Wedge should rest on the bottom of the Lower Buffer Chamber.

Insert Buffer Core into Lower Buffer Chamber



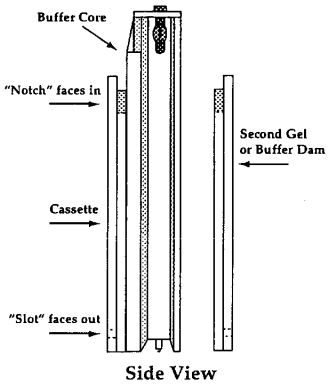
Lower Buffer Chamber and Buffer Core
Front View

V. ASSEMBLY OF THE XCELL *SureLock*™ MINI-CELL

(cont'd)

6. Insert the gel cassettes into the lower buffer chamber. Place one cassette behind the core and one cassette in front of the core. **For each cassette, the shorter "well" side of the cassette faces in towards the buffer core.** The slot on the back of the cassette must face out towards the lower buffer chamber.

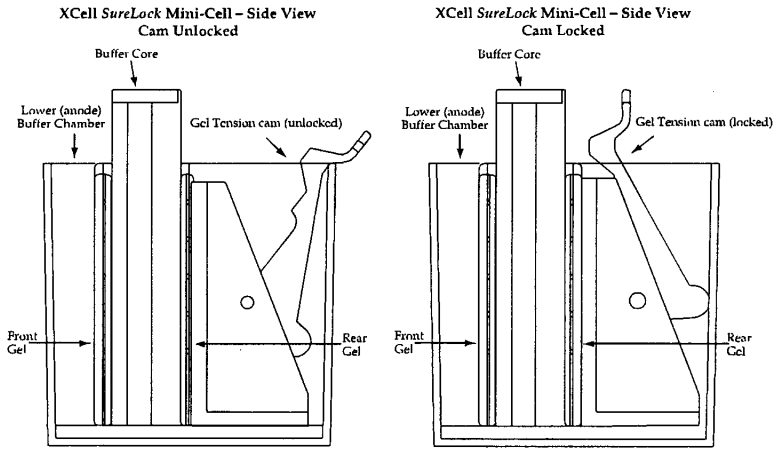
   NOTE: If you are running only one gel, the molded Buffer Dam replaces the rear gel cassette.



Side View

7. Pull foward on the Gel Tension Lever in a direction towards the front of the XCell *SureLock*™ unit until lever comes to a firm stop and the gels or gel/buffer dam appear snug against the buffer core.

8. When fully assembled, cassettes and buffer core are in place and Gel Tension Wedge is locked into position.



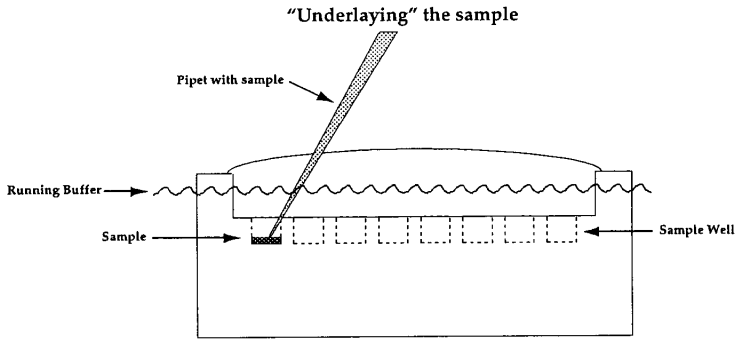
VI. SAMPLE LOADING PROCEDURE

1. The Upper Buffer Chamber (cathode) is the void formed between the two gel cassettes (or one cassette and the buffer dam) on each side of the buffer core.
2. Fill the Upper Buffer Chamber with running buffer (~200ml).

   NOTE: Use enough running buffer to ensure that the sample wells are completely covered.
3. Ensure that the Upper Buffer Chamber is not leaking. If the level of running buffer drops, the electrophoresis core and cassettes are not properly seated. Repeat steps 6 & 7 on page 9.
4. Use the pipet equipped with a round sample loading tip (Catalog No. LC1001) to underlay the samples into the gel wells (see below). Carefully lower the tip to the bottom of the sample well and gently pipet sample into well. Care should be taken so as not to let the sample flow out and contaminate another well.

   If you have difficulty distinguishing the sample wells when loading the sample, please see the Troubleshooting Guide on page 14.

   NOTE: Best results will be obtained by loading sample buffer in all the wells, whether or not they contain samples. This promotes a uniform running of the stacking front.



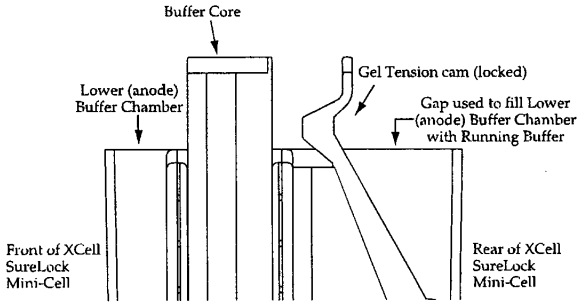
VII. GEL RUNNING

Running Procedure

1. Fill the Lower (anode) Buffer Chamber by pouring approximately 600ml of running buffer through the gap between the Gel Tension Wedge and the back of the lower buffer chamber.

   NOTE: For use with the Tricine and NuPAGE® Precast Gel Systems it is recommended that you fill the lower buffer chamber completely (600ml) as this will help dissipate heat during the run. For other gel types, a filled lower buffer chamber is recommended, but not required. The XCell *SureLock*™ Mini-Cell will function as long as the lower buffer chamber is filled enough to cover the slot at the bottom of the cassette.

Filling Lower Buffer Chamber



2. Align the lid on the buffer core. The lid can only be firmly seated if the (-) electrode is aligned over the banana plug on the right.

   NOTE: If the lid is not properly seated, no power will go through the mini-cell.

3. With the power **OFF**, connect the electrode cords to power supply (red to (+) jack, black to (-) jack).

> **Warning**
>
> Power must be off before connecting the XCell *SureLock*™ Mini-Cell to the power supply.

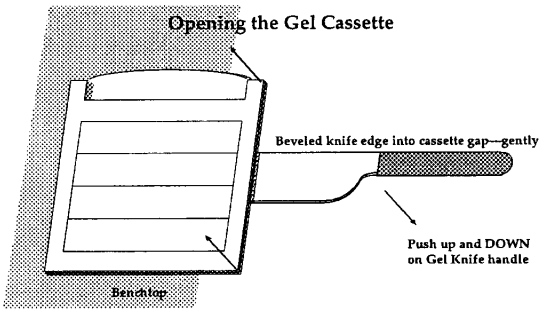
4. Turn on the power.

Refer to Appendix 1 for recommended power conditions.

VIII. DISASSEMBLY OF THE XCELL *SureLock*™ MINI-CELL

1. Upon completion of the run, **turn off the power** and disconnect the cables from the power supply.
2. Remove the lid and unlock the Gel Tension Lever. Ther is no need to remove the Gel Tension Wedge.
3. Remove the gel cassettes from the mini-cell. Handle gel cassettes by their edges only.
4. Lay the gel cassettes (well side up) on a flat surface, such as the benchtop. Allow one edge to hang ~ 1cm over the side of the benchtop.
5. Carefully insert the Gel Knife's beveled edge into the narrow gap between the two plates of the cassette.

   NOTE: Do not push the knife forcefully between the cassette plates or you may cut into the gel.
6. Push up and down gently on the knife's handle to separate the plates. You will hear a cracking sound which means you have broken the bonds which hold the plates together. Repeat until you have broken the bonds on one side.
7. Rotate the cassette and repeat steps 5 through 6, until the two plates are completely separated.



VIII. DISASSEMBLY OF THE XCELL *SureLock*™ MINI-CELL (cont'd)

8. Upon opening the cassette, the gel may adhere to either side. Carefully remove and discard the plate without the gel, allowing the gel to remain on the other plate.

8a. If blotting, proceed to the Western Transfer Protocol **without** removing the gel from the plate. (Please see our Western Transfer Protocol, EP040 for further details.)

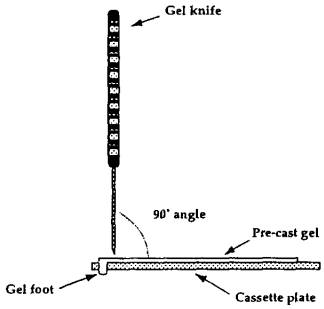
NOTE: Trying to remove the gel at this point may result in the gel tearing. Proceed to step 9 to remove the gel.

8b. If staining, remove the gel from the cassette plate by one of the following two methods:

I. If the gel remains on the shorter (notched) plate, use the sharp edge of the Gel Knife to remove the bottom foot of the gel. The Gel Knife should be at a 90° angle to the gel and the slotted cassette plate. Push straight down on the knife to cut the gel. Repeat the motion across the gel to cut the entire foot. Hold the cassette plate and gel over a container with the gel facing downward, use the knife to carefully loosen one lower corner of the gel and allow the gel to peel away from the plate.

II. If the gel remains on the longer (slotted) plate, hold the cassette plate and gel over a container with the gel facing downward. Gently push the gel knife through the slot in the cassette, until the gel peels away from the plate. Cut the foot off the gel after fixing and staining, but before drying.

Removing Bottom Lip of Gel



9. Immediately fix, stain or transfer as desired. Please see Bulletin EP009 for a Coomassie® staining and destaining procedure and EP040 for electrotransfer protocol using the Novex® Blot Module EI9051.

IX. TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	REMEDY
Run taking longer than usual.	A. Buffers are too diluted.	A. Check buffer recipe; remake if necessary.
	B. Upper buffer chamber is leaking.	B. Make sure the buffer core is firmly seated, the gaskets are in place and the gel tension lever is locked.
	C. Voltage is set too low.	C. Set correct voltage.
Current reading on power supply is zero or very low.	A. Tape left on the bottom of the cassette.	A. Remove tape from bottom of cassette.
	B. Connection to power supply not complete.	B. Check all connections with a volt meter for conductance.
	C. Buffer level not sufficient.	C. Make sure the upper buffer (cathode) is covering the wells of the gel. Make sure there is sufficient buffer in the lower buffer chamber to cover the slot at the bottom of the gel.
Run is faster than normal with poor resolution.	A. Buffers are too concentrated or incorrect.	A. Check buffer recipe; dilute or re-make if necessary.
	B. Voltage, current or wattage is set too high.	B. Decrease power conditions to recommended running conditions (see Appendix 1).
Cannot see the sample wells to load sample.	A. There is little contrast between the sample well and the rest of the gel.	A. Mark cassette at the bottom of the wells with a marker pen prior to assembling the Upper Buffer Chamber. Illuminate the bench area with a light source placed directly behind the XCell *SureLock*™ unit.

APPENDIX 1.
BUFFER FORMULATIONS AND RUNNING CONDITIONS

Gel Type	Sample Buffer	Running Buffer	Power Settings Note: current readings are per gel.	Run Time
Tris-Glycine SDS-PAGE (Denaturing, non-reducing)	Tris-Glycine SDS Sample Buffer (LC2676)	Tris-Glycine SDS Running Buffer (LC2675)	Voltage: 125V Constant Expected Current: Start: 30-40mA End: 8-12mA	~ 90 minutes
(for reducing conditions)	**Add** NuPAGE® Sample Reducing Agent (NP0004)			
Tris-Glycine (Native)	Tris-Glycine Native Sample Buffer (LC2673)	Tris-Glycine Native Running Buffer (LC2672)	Voltage: 125V Constant Expected Current: Start: 6-12mA End: 3-6mA	1-12 hours
Tricine SDS-PAGE (Denaturing, non-reducing)	Tricine SDS Sample Buffer (LC1676)	Tricine SDS Running Buffer (LC1675)	Voltage: 125V Constant Expected Current: Start: 80mA End: 40mA	~ 90 minutes
NuPAGE® Bis-Tris SDS-PAGE (Denaturing, non-reducing)	NuPAGE® LDS Sample Buffer (NP0007)	NuPAGE® MES SDS Running Buffer (NP0002) or NuPAGE® MOPS SDS Running Buffer (NP0001)	Voltage: 200V Constant Expected Current: Start: 110-125mA End: 70-80mA	35-50 minutes
(for reducing conditions)	**Add** NuPAGE® Sample Reducing Agent (NP0004)	**Add** NuPAGE® Antioxidant (NP0005)		
NuPAGE® Tris-Acetate SDS-PAGE (Denaturing)	NuPAGE® LDS Sample Buffer (NP0007)	NuPAGE® Tris-Acetate Running Buffer (LA0041)	Voltage: 150V Constant Expected Current: Start: 40-55mA End: 25-40mA	50 minutes
(for reducing conditions)	**Add** NuPAGE® Sample Reducing Agent (NP0004)	**Add** NuPAGE® Antioxidant (NP0005)		

APPENDIX 1.
BUFFER FORMULATIONS AND RUNNING CONDITIONS

Gel Type	Sample Buffer	Running Buffer	Power Settings Note: current readings are per gel.	Run Time
Zymogram	Tris-Glycine SDS Sample Buffer (LC2676)	Tris-Glycine SDS Running Buffer (LC2675)	Voltage: 125V Constant Expected Current: Start: 30-40mA End: 8-12mA	~90 minutes
IEF pH 3-7	IEF pH 3-7 Sample Buffer (LC5371)	IEF pH 3-7 Cathode Buffer (LC5370) IEF Anode Buffer (LC5300)	Voltage: 100V 1 hour 200V 1 hour 500V 30 minutes Expected Current: Start: 5mA End: 6mA	2.5 hours
IEF pH 3-10	IEF pH 3-10 Sample Buffer (LC5311)	IEF pH 3-10 Cathode Buffer (LC5310) IEF Anode Buffer (LC5300)	Voltage: 100V 1 hour 200V 1 hour 500V 30 minutes Expected Current: Start: 5mA End: 6mA	2.5 hours
TBE	Hi-Density TBE Sample Buffer (LC6678)	TBE Running Buffer (LC6675)	Voltage: 200V Constant Expected Current: Start: 10-18mA End: 4-6mA	30-90 minutes
TBE-Urea	TBE-Urea Sample Buffer (LC6876) or Prep TBE-Urea Sample Buffer (LC6877)	TBE Running Buffer (LC6675)	Voltage: 180V Constant Expected Current: Start: 10-20mA End: 6-14mA	50-75 minutes
DNA Retardation	Hi-Density TBE Sample Buffer (LC6678)	TBE Running Buffer (LC6675) at 1/2 X concentration	Voltage: 100V Constant Expected Current: Start: 12-15mA End: 6-15mA	~90 minutes
QuickPoint®	QuickPoint* Sample Loading Buffer (QP9733)	QuickPoint* Running Buffer (QP9732)	Voltage: 1200V Constant Expected Current: Start: 18-25mA End: 10-15mA	15 minutes

APPENDIX 2. ORDERING INFORMATION

Cat. No.	Description
EI0001	XCell *SureLock*™ Mini-Cell
EI9051	XCell II Blot Module

Replacement Parts

EI9010	Gel Knife
EI0013	Lower Buffer Chamber for EI0001 XCell *SureLock*™ Mini-Cell
EI0011	Gel Tension Wedge
EI9014	Buffer Core with Electrodes for EI0001
EI0010	Lid with Cables for EI0001
EI9016	Gaskets for EI0001 and EI9051
EI0012	Buffer Dam for EI0001
EI9021	Repair Kit for XCell Buffer Core: platinum wire, 0.01 in. x 12 in, wire insulation, post , nut, washer
EI9022	Buffer Core replacement wire, platinum, 0.010 in. x 12 in.
EI9052	Sponge Pad for Blotting, 8/pk
EI9053	Anode Core for EI9051
EI9054	Cathode Core for EI9051
EI9055A	Anode Electrode for EI9051
EI9055C	Cathode Electrode for EI9051

Pre-Mixed Liquid Buffers

LA0041	NuPAGE® Tris-Acetate SDS Running Buffer (20x), 500ml
LA0050 Kit	NuPAGE® MES SDS Buffer Kit (for Tris-Acetate Gels) (contains 1ea LA0041, NP0004, NP0005, NP0007) *For native applications with NuPAGE Tris-Acetate gels, Tris-Glycine native buffers (LC2672 and LC2673 should be used.*
LC1675	Tricine SDS Running Buffer (10x), 500ml
LC1676	Tricine SDS Sample Buffer (2x), 20ml
LC1677 Kit	Tricine SDS Buffer Kit (1ea LC1675 & LC1676)
LC2670	Zymogram Renaturing Buffer (10x), 500ml
LC2671	Zymogram Developing Buffer (10x), 500ml
LC2672	Tris-Glycine Native Running Buffer (10x), 500ml
LC2673	Tris-Glycine Native Sample Buffer (2x), 20ml
LC2675	Tris-Glycine SDS Running Buffer (10x), 500ml
LC2675-4	Tris-Glycine SDS Running Buffer (10x), 4 x 1L
LC2676	Tris-Glycine SDS Sample Buffer (2x), 20ml

Pre-Mixed Liquid Buffers con't.

LC2677 Kit	Tris-Glycine SDS Buffer Kit (1 ea LC2675 & LC2676)
LC3675	Tris-Glycine Transfer Buffer for Blotting (25x), 500ml
LC5300	IEF Anode Buffer (50x), 100ml
LC5310	IEF Cathode Buffer (10x) pH 3-10, 125ml
LC5311	IEF Sample Buffer (2x) pH 3-10, 25ml
LC5317 Kit	IEF Buffer Kit pH 3-10 (1 ea LC5300, LC5310 & LC5311)
LC5370	IEF Cathode Buffer (10x) pH 3-7, 125ml
LC5371	IEF Sample Buffer (2x) pH 3-7, 25ml
LC5377 Kit	IEF Buffer Kit pH 3-7 (1 ea LC5300, LC5370 & LC5371)
LC6675	TBE Running Buffer (5x), 1000ml
LC6676	TBE Sample Buffer (6x), 10ml
LC6677 Kit	TBE Buffer Kit (1 ea LC6675 & LC6676)
LC6678	Hi-Density TBE Sample Buffer (5x), 10ml
LC6876	TBE-Urea Sample Buffer (2x), 10ml
LC6877	Prep TBE-Urea Sample Buffer (2x), 20ml
NP0000	NuPAGE® Sampler Kit
NP0001	NuPAGE® MOPS SDS Running Buffer (for Bis-Tris gels only) (20x), 500ml
NP0002	NuPAGE® MES SDS Running Buffer (for Bis-Tris gels only) (20X), 500ml
NP0004	NuPAGE® Sample Reducing Agent (10x) 250µl
NP0005	NuPAGE® Antioxidant, 15ml
NP0006	NuPAGE® Transfer Buffer (20x), 125ml
NP0007	NuPAGE® LDS Sample Buffer (4x), 10ml
NP0050 Kit	NuPAGE® MES SDS Buffer Kit (for Bis Tris Gels) (contains 1ea NP0001, NP0004, NP0005, NP0007)
NP0060 Kit	NuPAGE® MES SDS Buffer Kit (for Bis Tris Gels) (contains 1ea NP0002, NP0004, NP0005, NP0007)

Sample Tips for Gel Loading

LC1001	Gel Loading Tips, (Round), 200/pk
LC1002	Sequencing Gel Loading Tips, (Flat), 0.37mm, 200/pk
LC1010	Gel Loading Tips, (Round, Eppendorf), 200/pk

For a complete listing of Novex® Pre-Cast Gels and electrophoresis accessories, please request Literature No. EP001.

APPENDIX 3. ADDITIONAL LITERATURE

Literature Piece	*Code*
Instruction Manuals	
Pre-Cast Gel Instruction Booklet	IM-6000
XCell *SureLock*™ Instruction Booklet	IM-9003
Western Transfer Instructions	IM-9051
DryEase™ Mini Gel Drying Instructions	IM-2380
StainEase™ Staining Tray Instructions	IM-2400
SilverXpress™ Silver Staining Kit Instructions	IM-6100
Colloidal Blue Staining Kit Instructions	IM-6025
Nitrocellulose Membrane (0.2µm) Instructions	IM-1600
PVDF Membrane Instructions	IM-2005
Nylon $^{+}$ Membrane Instructions	IM-2003
PowerEase™ Instruction Manual	IM-8634
SeeBlue® Standard Instruction Card	IM-5625
SeeBlue® Plus2 Standard Instruction Card	IM-1003
MultiMark® Standard Instruction Card	IM-5725
Mark12™ Standard Instruction Card	IM-5677

United States Headquarters:
1600 Faraday Avenue
Carlsbad, California 92008
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
Email: tech_service@invitrogen.com
www.invitrogen.com

European Headquarters
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
Email: eurotech@invitrogen.com

IM-9003F 010619

XCell II™ Blot Module

Version F
010430
IM-9051

Novex® Western Transfer Apparatus

Instructions for using the XCell II™ Blot Module

Catalog no. EI9051

United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_service@invitrogen.com
Web: www.invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
E-mail: eurotech@invitrogen.com

Table of Contents

Overview

Introduction

Western or immuno blotting is the transfer of the separated proteins in a gel to the surface of a thin support membrane matrix. The proteins are bound and immobilized on the membrane. Southern and Northern blotting involves the transfer of separated DNA and RNA, respectively, from a gel to a membrane.

To perform a transfer, the gel is layered next to a membrane and placed in a voltage gradient perpendicular to the gel. Negatively charged molecules will migrate out from the gel, move towards the positive electrode, and get deposited on the membrane.

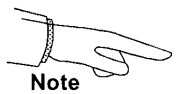
Advantages of Blotting

The advantages of blotting include:

- Removal of gel impurities from the protein or nucleic acid which may hinder further analysis
- Easy access of proteins or nucleic acids on a blot for further analysis
- Faster analysis of proteins on the blots as protein diffusion is minimized
- Shorter staining and destaining time of proteins on the blot as compared to gels
- Multiple successive reprobing of a single blot for analyses after removal of the probe each time
- Convenient way to store separated proteins or nucleic acids for future use

XCell II™ Blot Module

The XCell II™ Blot Module is a simple apparatus which is easily inserted into the XCell *SureLock*™ Mini-Cell in place of the gel/buffer core assembly. The module has rails to guide the unit into the mini-cell. The XCell II™ Blot Module can be used to perform western, Southern, or northern transfer of two mini-gels using only 200 ml of transfer buffer. The XCell II™ Blot Module is a semi-wet transfer unit. An efficient transfer is obtained, as the resistance is constant across the blotting electrodes producing uniform field strength.



Note

The XCell II™ Blot Module can only be used with XCell *SureLock*™ Mini-Cell or XCell II™ Mini-Cell. Please refer to page 24 for ordering information.

Specifications

The specifications of the XCell II™ Blot Module are provided below:

Cell Dimensions	14.5 cm x 14 cm x 11 cm
Blot Module Capacity	200 ml
XCell *SureLock*™ Lower Buffer Chamber Capacity	600 ml
Blot Size	9 cm x 9 cm

Continued on next page

Blotting Membranes

Invitrogen offers three types of blotting membrane and filter paper sandwiches. Please refer to page 24 for ordering information.

- Nitrocellulose for western or Southern blotting
- PVDF (polyvinylidene difluoride) for western blotting
- Nylon for Southern or northern blotting

Please see the table below for more details on each membrane.

Membrane	Properties	Applications	Pore size	Reprobing
Nitrocellulose	Most widely used membrane for western blotting Good binding capacity Proteins bind to the membrane due to hydrophobic interactions Protein binding capacity is 80 µg/cm²	Western transfer Amino acid analysis Solid phase assay systems	0.2 µm 0.45 µm	No
PVDF	Higher binding capacity than nitrocellulose Strong hydrophobic character and solvent resistant Physically stronger than nitrocellulose Compatible with commonly used protein stains and immunodetection methods Protein binding capacity is 50-150 µg/cm²	Protein sequencing Western transfer Amino acid analysis Solid phase assay systems	0.2 µm 0.45 µm	Yes
Nylon	Microporous membrane modified with strongly basic charged groups Ideal for binding negatively charged biomolecules such as DNA and RNA Low background for enhanced resolution Membrane is formed around a non-woven polyester fiber matrix which confers high tensile strength, toughness, and flexibility	Southern, northern and western transfers Solid phase immobilization Dry chemistry test strips Enzyme immobilization Gene probe assays	0.45 µm	Yes

Patent Information

The XCell *SureLock*™ Mini-Cell is covered by US Patent No. 6,001,233. NuPAGE® is covered by US Patent Nos. 5,578,180, 5,922,185; 6,096,182; 6,143,154; 6,162,338; EP0753142, and corresponding patents in other countries.

Preparing for Transfer

Introduction

You need to prepare the transfer buffer, blotting pads, and blotting membranes before performing the transfer. You may prepare the transfer buffer and materials for transfer while electrophoresis of the gel is in progress.

Materials Supplied by the User

- Pre-cut blotting membranes and filter paper sandwiches
- Methanol
- Deionized water
- Transfer buffer (see below)
- Shallow tray for equilibration of membranes, filter paper, and blotting pads

Gloves should be worn at all times during the entire blotting procedure to prevent contamination of gels and membranes, and to avoid exposure to irritants commonly used in electrophoresis and transfer.

Do not touch the membranes or the gel with bare hands. This may contaminate the gel or the membrane and interfere with further analysis.

Preparing the Transfer Buffer

The recommended transfer buffer is a half-strength .5X Towbin buffer (12 mM Tris Base, 96 mM glycine, pH 8.3) with 20% methanol. This buffer provides sufficient ionic strength for a successful transfer in the XCell II™ Blot Module without generating excess heat. Please prepare the appropriate buffer for your gel type from the recipes given below.

For Blotting Novex® Tris Glycine or Tricine Gels:

Prepare 1000 ml of 0.5X Towbin transfer buffer using Novex® Tris-Glycine Transfer Buffer (25X) as follows:

Novex® Tris-Glycine Transfer Buffer (25X)	40 ml
Methanol	200 ml
Deionized Water	760 ml
Total Volume	1000 ml

See page 23 for a recipe of Tris-Glycine Transfer Buffer, if you are preparing your own transfer buffer.

Continued on next page

Preparing the Transfer Buffer, continued

For Blotting NuPAGE® Novex Bis-Tris or Tris-Acetate Gels:

Prepare 1000 ml of 1X NuPAGE® Transfer Buffer using the NuPAGE® Transfer Buffer (20X) as follows:

	Reduced Samples	Non-Reduced Samples
NuPAGE® Transfer Buffer (20X)	50 ml	50 ml
NuPAGE® Antioxidant	1 ml	--
Methanol	100 ml*	100 ml*
Deionized Water	849 ml	850 ml
Total Volume	1000 ml	1000 ml

*NuPAGE® Transfer Buffer with 10% methanol provides optimal transfer of a single gel in the blot module. If you are transferring two gels in the blot module, increase the methanol content to 20% to ensure efficient transfer of both gels.

Please refer to page 23 for a recipe of the NuPAGE® Transfer Buffer, if you are preparing your own transfer buffer.

For Blotting Novex® TBE or DNA Retardation Gels:

Prepare 1000 ml of (0.5X) TBE transfer buffer using the Novex® TBE Running Buffer (5X) as follows:

Novex® TBE Running Buffer (5X)	100 ml
Deionized Water	900 ml
Total Volume	1000 ml

See page 23 for a recipe of the Novex® TBE Running Buffer, if you are preparing your own transfer buffer.

Preparing the Blotting Pads

Use ~ 700 ml of transfer buffer to soak the blotting pads until saturated. Remove the air bubbles by squeezing the blotting pads while they are submerged in buffer. Removing air bubbles is essential as they can block the transfer of biomolecules.

Preparing the Transfer Membrane and Filter Paper

Cut selected transfer membrane and filter paper to the dimensions of the gel or use Novex® pre-cut membrane/filter paper sandwiches (see page 24 for ordering information).

- **PVDF membrane**: Pre-wet the PVDF membrane for 30 seconds in methanol, ethanol, or isopropanol. Briefly rinse in deionized water and then place the membrane in a shallow dish containing 50 to 100 ml of transfer buffer for several minutes.
- **Nitrocellulose/Nylon membrane**: Place the membrane directly into a tray containing the transfer buffer for several minutes.
- **Filter paper**: Soak briefly in transfer buffer immediately before using.
- **Gel**: The gel should be used immediately following the run (see page 9). **Do not soak the gel in transfer buffer.**

Using the XCell II™ Blot Module

Introduction

The blotting protocol provided below is suitable for majority of protein blotting applications using the XCell II™ Blot Module. However, some optimization may be necessary by the user to obtain best results (see page 16).

Materials Supplied by the User

- Previously electrophoresed mini-gels (maximum gel size 9 cm x 9 cm)
- XCell *SureLock*™ Mini-Cell

Removing the Gel After Electrophoresis

Instructions are provided below to remove the gel from the cassette for transfer after completion of electrophoresis. If you are not ready to perform the transfer immediately, you may continue electrophoresis of your gel at a low voltage of 5 V. The gel can be left in the unit for a few hours until you are ready to transfer the gel.

1. After electrophoresis, separate each of the three bonded sides of the gel cassette by inserting the gel knife into the gap between the cassette two plates. The notched (ell side of the cassette should face up.
2. Push up and down on the knife handle to separate the plates. Repeat on each side of the cassette until the plates are completely separated.

   Caution: Please use caution while inserting the gel knife between the two plates to avoid excessive pressure towards the gel.
3. The gel may adhere to either side of the plates upon opening the cassette. Carefully remove and discard the plate without the gel, allowing the gel to remain on the other plate.
4. Remove wells on the gel with the gel knife.
5. Place a piece of pre-soaked filter paper (prepared as described on the previous page) on top of the gel, and lay just above the oot at the bottom of the gel (leaving the oot of the gel uncovered). Keep the filter paper saturated with the transfer buffer and remove all trapped air bubbles by gently rolling over the surface using a glass pipette as a roller.
6. Turn the plate over so the gel and filter paper are facing downwards over a gloved hand or clean flat surface covered with a piece of Parafilm®.
7. Remove the gel from the plate using the following methods:
   - If the gel rests on the longer (slotted) plate, use the gel knife to push the foot out of the slot in the plate and the gel will fall off easily.
   - If the gel rests on the shorter (notched) plate, use the gel knife to carefully loosen the bottom of the gel and allow the gel to peel away from the plate.
8. When the gel is on a flat surface, cut the oot off the gel with the gel knife. Proceed to **Transferring One Gel**, next page.

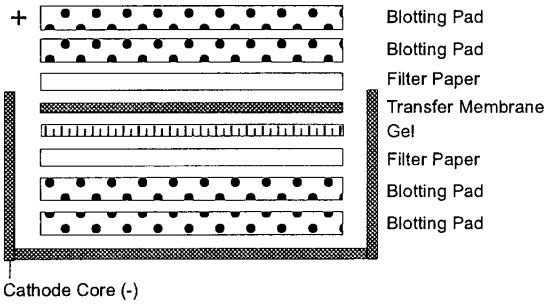
Note: Once you have removed the gel from the unit and the cassette, the transfer should be performed immediately.

Continued on next page

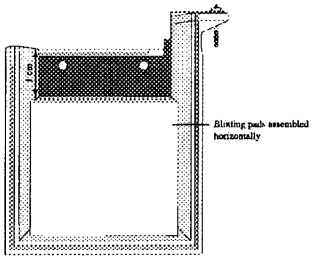
Transferring One Gel

Instructions are provided below for transferring one gel. If you need to transfer two gels at a time, please refer to page 12.

1. Wet the surface of the gel (from step 8, previous page) with the transfer buffer and position the pre-soaked transfer membrane (prepared as described on page 8) on the gel. Remove all air bubbles by gently rolling a glass pipette over the membrane surface.
2. Place the pre-soaked filter paper on top of the transfer membrane. Remove any trapped air bubbles.
3. Place two soaked blotting pads into the cathode (-) core of the blot module. The cathode core is the deeper of the two cores and the corresponding electrode plate is a darker shade of gray. Carefully pick up the gel membrane assembly with your gloved hand and place on the pad in the same sequence, so that the gel is closest to the cathode plate (see figure below).



4. Add enough pre-soaked blotting pads to rise 0.5 cm over the rim of the cathode core. Place the anode (+) core on top of the pads. The gel/membrane sandwich should be held securely between the two halves of the blot module ensuring complete contact of all components. **Note**: The blotting pads lose their resiliency after many uses. To ensure a snug fit between both sides of the blot module, use an additional pad. Pads should be replaced when they begin to lose resiliency and are discolored.
5. The gel membrane sandwich and blotting pads should be positioned in the cathode core of the XCell II™ Blot Module to fit horizontally across the bottom of the unit. There should be a gap of approximately 1 cm at the top of the electrodes when the pads and assembly are in place (see figure below).



Continued on next page

Transferring One Gel, continued

6. Hold the blot module together firmly and slide it into the guide rails on the lower buffer chamber. The blot module will only fit into the unit one way, so the (+) sign can be seen in the upper left hand corner of the blot module. The inverted gold post on the right hand side of the blot module should fit into the hole next to the upright gold post on the right side of the lower buffer chamber.
7. Depending on the mini-cell that you are using, follow the appropriate instructions for positioning the wedge:
   - For the XCell *SureLock*™ Mini-Cell, place the gel tension wedge so that the vertical face of the wedge is against the blot module. Push the lever forward to lock it into place.
   - For the XCell II™ Mini-Cell, place the front wedge (without the screw hole) so that the vertical face of the wedge is against the blot module. Slide in the rear wedge and push it down firmly.

     Note: When properly placed, the rear wedge will not be flush with the top of the lower buffer chamber. There will be a gap between the rear wedge and the lower buffer chamber.
8. Fill the blot module with transfer buffer until the gel/membrane sandwich is covered in transfer buffer. Do not fill all the way to the top as this will generate extra conductivity and heat.
9. Fill the outer buffer chamber with deionized water by pouring approximately 650 ml in the gap between the front of the blot module and the front of the lower buffer chamber. The water level should reach approximately 2 cm from the top of the lower buffer chamber. This serves to dissipate heat produced during the run.

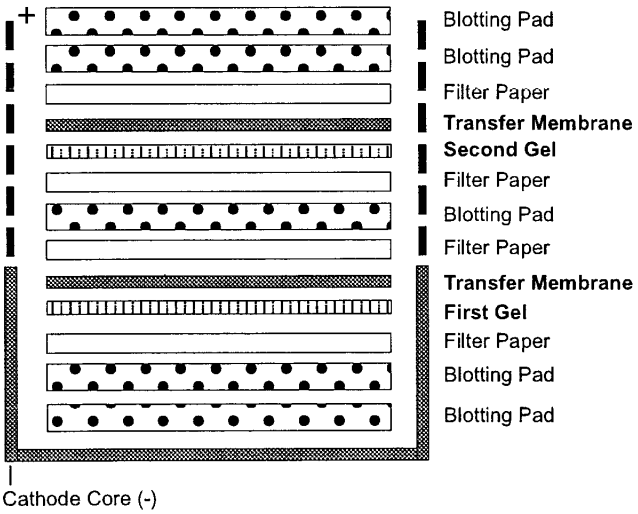
   Note: If you accidentally fill the outer buffer chamber with the transfer buffer, it will not adversely affect the transfer. The liquid in the outer buffer chamber serves as a coolant. We recommend adding deionized water to the outer buffer chamber. The mini-cell is susceptible to methanol and any exposure to methanol should be avoided.
10. Place the lid on top of the unit.
11. With the power turned off, plug the red and black leads into the power supply. Refer to **Recommended Transfer Conditions** on page 13 for transfer conditions.

Continued on next page

Transferring Two Gels

Instructions are provided below for transferring two gels.

1. Remove the gels after electrophoresis as described on page 9.
2. Assemble the gel/membrane sandwich (as described on page 10) twice to make two gel/membrane sandwiches.
3. Place two pre-soaked pads on cathode core of the blot module. Place the first gel/membrane sandwich on pads in the correct orientation, so the gel is closest to the cathode plate (see figure below).



4. Add another pre-soaked blotting pad on top of the first membrane assembly.
5. Position the second gel/membrane sandwich on top of blotting pad in the correct orientation so that the gel is closest to the cathode side.
6. Proceed with steps 5-11 as described in **Transferring One Gel** (pages 10-11).

Continued on next page

Recommended Transfer Conditions

Please choose the transfer conditions from the table below based on type of the gel that you are using.

Note: The expected current listed in the table below is for transferring one gel. If you are transferring two gels in the blot module, the expected current will double.

Type of the Gel	Transfer Buffer (1X)	Membrane	Transfer Conditions	Expected Current
Novex® Tris-Glycine Novex® Tricine	Novex® Tris Glycine Transfer Buffer with 20% methanol. 1X Transfer Buffer should be pH 8.3 before addition of SDS or methanol. Do not use acid or base to adjust the pH.	Nitrocellulose or PVDF	25 V constant for 1-2 hours	Start: 100 mA
NuPAGE® Bis-Tris	NuPAGE® Transfer Buffer with 10% methanol for transfer of one gel. NuPAGE® Antioxidant for reduced samples	Nitrocellulose or PVDF	30 V constant for 1 hour	Start: 170 mA End: 110 mA
NuPAGE® Tris-Acetate	NuPAGE® Transfer Buffer with 10% methanol for transfer of one gel. NuPAGE® Antioxidant for reduced samples	Nitrocellulose or PVDF	30 V constant for 1 hour	Start: 220 mA End: 180 mA
Novex® TBE, TBE-Urea, and DNA Retardation	45 mM Tris 45 mM boric acid 1 mM EDTA	Nylon	30 V constant for 1-2 hours	Start: 390 mA End: 350 mA
Novex® IEF*	0.7% acetic acid, pH 3.0, see page 20	Nitrocellulose or PVDF	10 V constant for 1 hour	Start: 65-85 mA

*Assemble the gel/membrane sandwich in reverse order so that the membrane is on the cathode side (-) of the gel (see page 20)

Continued on next page

Overnight Blotting

For overnight blotting, perform transfer in the cold room to prevent overheating. Since the transfer is performed over a longer period of time, the power should be low. Transfer at constant voltage of 10-15 V overnight. Depending on the transfer efficiency, adjust the transfer conditions accordingly.

Post Transfer Analysis

After completing the transfer, you may proceed directly to immunodetection of proteins, store the membrane for future use, or stain the membrane.

- For immunodetection of proteins, you may use the WesternBreeze® Chromogenic or Chemiluminescent Immunodetection Kits available from Invitrogen (see page 24) or any other immunodetection kit.
- For storing nitrocellulose membranes, air dry the membrane and store the membrane in a Ziploc bag at room temperature or +4°C. Do not store nitrocellulose membranes at -20°C or -80°C, as they will shatter.
- For storing PVDF membranes, air dry the membrane and store the membrane in a Ziploc bag at room temperature, +4°C, or -80°C. When you are ready to use the membrane, rewet the membrane with methanol for a few seconds, followed by thorough rinsing of the membrane with deionized water to remove methanol.
- For staining the membranes after blotting, you may use:
  - 0.1% Coomassie Blue R-250 in 50% methanol. Do not use Novex® Colloidal Blue Staining Kit for staining of membranes, as the background will be very high.
  - 20 ml of SimplyBlue™ SafeStain (see page 24 for ordering information) with dry PVDF membranes and incubate for 1 hour. Wash the membrane with 100 ml of deionized water for 1-3 hours. To avoid high background, do not use SimplyBlue™ SafeStain on nitrocellulose and **wet** PVDF membranes.
  - 0.5% Amido Black (see page 24 for ordering information) in 50% methanol and 10% acetic acid. Remove excess stain with deionized water. Destain with 45% methanol and 10% acetic acid for 30 minutes. Rinse the membrane with deionized water and air dry.
  - 0.1% Ponceau S (see page 24 for ordering information) in 7% trichloroacetic acid (TCA) for 5 minutes. Rinse the membrane in deionized water to obtain transient staining or in 10% acetic acid to obtain permanent staining. Air dry the membrane.

If you do not detect any proteins on the membrane after immunodetection or staining, please refer to the **Troubleshooting** section on page 18. Please refer to the manufacturer recommendations for optimizing immunodetection.

Cleaning the Blot Module

Rinse the blot module with distilled water after use. To clean any residual build-up in the blot module, apply 50% nitric acid in deionized water to areas inside the blot module until residual build-up is removed. Do not submerge the blot module or soak overnight in nitric acid. Use gloves when preparing the nitric acid solution. Once the build-up is removed, rinse the module at least three times in deionized water.

Testing the Efficiency of Blotting

Introduction

Once you have performed the transfer, you may check the efficiency of the transfer by using any one of the methods described below. Testing the blotting efficiency helps you evaluate the transfer and optimize the transfer parameters to obtain an efficient transfer.

We recommend testing the efficiency of blotting when you are performing the western transfer for the first time and if you have changed the buffer system or the gel type.

Materials Supplied By the User

- Pre-stained standards (see page 24 for ordering information)
- Ponceau S or Coomassie stain
- Transfer membrane
- Previously transferred mini-gel (maximum gel size 9 cm x 9 cm)

Procedure

The following methods can be used to test the efficiency of protein transfer.

Using pre-stained standards:

This is the most convenient way to monitor the efficiency of transfer. Pre-stained standards are protein markers that are covalently bound to a synthetic dye. This enables visualization of the protein markers during electrophoresis and after the transfer. Invitrogen offers three types of pre-stained standards (see page 24 for ordering information). The transfer efficiency is good, if most of the standards have transferred to the membrane. Please note that high molecular weight standards do not always transfer completely and this is not indicative of incomplete transfer.

If none of the standards or only a few have transferred to the membrane, you may have to optimize the transfer conditions. Refer to **Optimizing Blotting Parameters** on the next page and the **Troubleshooting** section on page 18.

Staining the gel:

The gel can be stained with Coomassie Blue staining or a staining method of choice after the transfer to determine the transfer efficiency. If significant amount of proteins are still present on the gel after staining, this indicates poor transfer. You may need to optimize the transfer as described on the next page.

Staining the membrane:

The membrane can be stained after the transfer to evaluate the transfer efficiency. If you are using the membrane for peptide sequencing, you may stain the membrane with Coomassie Blue stain.

If you are using the membrane for immunodetection, you can stain the membrane with a temporary stain such as Ponceau S (see previous page for Ponceau S staining). After visualizing the transferred protein bands on the membrane, you can rinse the membrane with deionized water to completely remove the staining or incubate the membrane directly in the blocking solution. Ponceau S stain does not interfere with most immunodetection methods.

Optimizing Blotting Parameters

Introduction

Each parameter of the blotting protocol plays a role during the transfer of proteins. An ideal blotting protocol balances each parameter to provide efficient transfer of proteins. When using the XCell II™ Blot module, most proteins will transfer efficiently using the protocol provided on page 10.

Based on the specific properties of a protein or a set of proteins, some optimization of the blotting protocol may be necessary. Please consider the points described below to optimize the blotting protocol.

Gel Percentage

Choose the lowest percentage acrylamide appropriate for the molecular weight of your protein or proteins of interest. Gradient gels are excellent for blotting a range of protein sizes, as the porosity of the gel matrix is well matched with the different sizes of the proteins.

Some proteins can be equally well resolved on a Tris-Glycine gel or a Tricine gel. In general, a Tricine gel will resolve the same range of proteins as a higher percentage Tris-Glycine gel and can be a better choice for some transfers.

Gel Thickness

The 1.0 mm thick gels are better for blotting. Be sure to scale your sample load appropriately for the sensitivity of your antibody detection method.

Field Strength

A higher field strength (volts/cm) may help larger proteins to transfer, but may also cause smaller proteins to pass through the membrane without binding. Our recommended condition for most proteins is 25 Volts for 90 minutes. You may make minor adjustments (5-10V) accordingly, when necessary. Refer to page 13 for **Recommended Transfer Conditions**.

Transfer Time

Increasing the transfer time to two hours will improve transfer of most proteins, but may cause the smaller proteins to pass through the membrane. Transfer time usually has little influence on the detection of proteins that remain bound to the membrane. Please note that transfer times longer than 2 hours at the recommended power settings do not greatly improve transfer of proteins that have failed to transfer completely in 2 hours. This may be due to the exhaustion of the buffer or partial fixation of the protein in the gel as a result of the removal of SDS or a conformational change in the proteins during the transfer interval.

Alcohol in Transfer Buffer

Decreasing or eliminating alcohol may improve the transfer of some proteins, especially large proteins.

For blotting Novex® Tris-Glycine Gels and two NuPAGE® Novex Gels, 20% methanol is added to the transfer buffer (see page 7). This is balanced by the residual SDS in the gel from the running buffer. Please keep this in mind when adjusting the methanol content in transfer buffer.

Continued on next page

Optimizing Blotting Parameters, Continued

SDS in Transfer Buffer

Adding 0.01% to 0.02% SDS to the transfer buffer will facilitate the transfer of proteins, especially large proteins, but may reduce binding of proteins to the membrane, especially nitrocellulose membranes.

In the blotting protocol on page 10, the gel is not incubated in the transfer buffer, leaving residual SDS in the gel. This is balanced by the 20% methanol added to the transfer buffer. Please keep this in mind when adjusting the SDS content in the transfer buffer.

Charge of Protein

For more basic proteins, a carbonate buffer can be used. The high pH of the buffer will confer a higher negative charge on the more basic proteins and cause them to migrate faster. Carbonate buffers improve binding detection for some systems. However, excessive heat may be generated during electroblotting due to the high ionic strength of the carbonate buffer.

Hint

We recommend using two membranes in tandem during initial blotting to closely monitor how much protein is actually transferred and then performing the same visualization technique on both of these membranes. Monitor whether the primary membrane located next to the gel retains the majority of the sample. If the sample is detected on the membrane placed closer to the anode (further away from the gel), you may reduce the rate of transfer by lowering the field strength, allowing more time for the protein to be captured on the primary membrane. Adjust the blotting protocol accordingly using the guidelines provided on this page and on the previous page. Please refer to the **Troubleshooting** section on the next page for more tips on improving the transfer.

Troubleshooting

Introduction Review the information provided below to troubleshoot your experiments.

Problem	Cause	Solution
No proteins transfer to the membrane	Gel/membrane sandwich assembled in a reverse direction so the proteins have migrated out into the solution	Assemble the sandwich in the correct order using instructions provided on page 10.
Significant amount of protein is passing through the membrane indicated by the presence of proteins on the second membrane	Longer transfer time, inappropriate SDS or methanol content, or sample overloaded	Re-evaluate the percentage of the gel used. Shorten the transfer time by 15 minute increments. Remove any SDS which may have been added to the transfer buffer. If using nitrocellulose membrane, switch to PVDF which has a higher binding capacity. Add additional methanol to increase the binding capacity of the membrane. Decrease the sample load.
Significant amount of protein remains in the gel indicated by staining of the gel after transfer	Shorter transfer time, inappropriate gel type, SDS or methanol content Higher molecular weight proteins usually do not transfer completely as compared to mid to low molecular weight proteins	Switch to a more appropriate lower percentage gel. Increase the blot time by 15 minute increments. Add 0.01% to 0.02% SDS to the transfer buffer to facilitate migration of the protein out of the gel. Decrease the amount of methanol in the transfer buffer.
The pH of the transfer buffer deviates from the required value by 0.2 pH units	Buffer not made up properly	Remake the buffer after checking the reagents and water quality. Do not adjust the pH with acid or base as this will increase the conductivity of the buffer and result in higher current during transfer.
Current is much higher than the expected start current	Concentrated buffer used	Prepare the buffer carefully using the recipe provided on pages 7-8.
	Used Tris HCl instead of Tris Base	Check the reagents used to make the buffer and remake the buffer with correct reagents.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
Current is much lower than the expected start current	Very dilute buffer used resulting in increased resistance and low current	Remake the transfer buffer correctly.
	The circuit is broken (broken electrode)	Check the blot module to ensure that the electrodes are intact.
	Leak in the blot module indicated by a decrease in the buffer volume in the module	Be sure to assemble the blot module correctly to prevent any leaking.
Power supply shuts off using recommended blotting conditions	High ionic strength of the transfer buffer	Prepare the buffer carefully using the recipe provided on pages 7-8.
	Power supply is operating at a current close to the current limit of the power supply	Use a power supply with higher limits.
Diffuse bands and swirling pattern on the membrane	Poor contact between the gel and the membrane	Roll over the surface of each layer of the gel/membrane sandwich with a glass pipette to ensure good contact between the gel and the membrane. The blotting pads need to be saturated with transfer buffer to remove all air bubbles.
	Under or overcompression of the gel	Add or remove blotting pads to prevent any type of compression of the gel.
Empty spots on the membrane	Presence of air bubbles between the gel and the membrane preventing the transfer of proteins	Be sure to remove all the air bubbles between the gel and the membrane using a glass pipette to roll out the bubbles.
	Expired or creased membranes used	Use fresh, undamaged membranes.
Poor transfer efficiency with PVDF	Membrane not treated properly before use	Be sure that the membrane is pre-wetted with methanol or ethanol.
	Poor contact between the membrane and the gel	Use more blotting pads or replace the old blotting pads with new ones.
	Overcompression of the gel indicated by a flattened gel	Remove enough blotting pads so that the unit can be closed without exerting pressure on the gel and the membrane.
High background on western blots	Insufficient blocking of non-specific sites	Increase the blocker concentration or the incubation time.

Special Applications

Introduction

Additional protocols are provided below for certain special transfer applications such as blotting of IEF gels (see below) and native gels (see next page), and semi-dry blotting (see page 22) using the XCell II™ Blot Module. These protocols are slightly different than the standard blotting protocol described on page 10.

Materials Supplied By the User

- Previously electrophoresed mini-gels (maximum gel size 9 cm x 9 cm)
- 0.7% acetic acid in deionized water (for blotting IEF gels)
- Native transfer buffer for blotting native gels (25 mM Tris Base, 25 mM glycine, pH 9.2)

For semi-dry blotting

- Methanol
- Semi-dry transfer unit
- Filter papers
- NuPAGE® Transfer Buffer
- NuPAGE® Antioxidant

Blotting IEF Gels

Novex® pre-cast IEF Gels are composed of 5% polyacrylamide and are more susceptible to hydrolysis due to the heat generated with the recommended blotting protocol. The following protocol has been optimized to prevent hydrolysis and effective transfer of basic proteins due to the low pH of the transfer buffer.

1. Chill the 0.7% acetic acid, which will be used later for transfer.
2. After electrophoresis of the gel, equilibrate the gel in 0.7% acetic acid for 10 minutes.

   Tip: The 5% polyacrylamide gels are more sticky and difficult to handle than higher percentage polyacrylamide gels. To lift the gel from the equilibration solution, submerge the filter paper under the gel while the gel is floating in the equilibration solution. When the gel is in the correct position, lift up the filter paper to attach the gel to the filter paper. This prevents the gel from sticking to the filter paper before it is in the proper position and avoids handling of the gel.
3. Assemble the gel/membrane sandwich as described on page 10, except in a **reverse order** so that the membrane is on the cathode (-) side of the gel. This is the opposite of a typical western blotting protocol, where the negatively charged protein will migrate toward the anode (+) during the transfer.
4. Transfer for 1 hour at 10 V constant.

Continued on next page

Blotting Native Gels

During SDS-PAGE all proteins have a net negative charge due to the SDS in the sample buffer and the running buffer. Proteins separated during native gel electrophoresis do not have a net charge which may cause problems during the transfer. It is possible that some native proteins may have a higher pI than the pH of the Tris-Glycine Transfer Buffer used for standard western transfer protocols. Guidelines are provided below to increase the transfer efficiency of native proteins.

- Increasing the pH of the transfer buffer to 9.2 (25 mM Tris Base, 25 mM glycine, pH 9.2), allows proteins with pI below 9.2 to transfer towards the anode electrode
- Place a membrane on both sides of the gel if you are using the regular Tris-Glycine Transfer Buffer, pH 8.3. If there are any proteins that are more basic than the pH of the transfer buffer, they will be captured on the membrane placed on the cathode side of the gel
- Incubate the gel in 0.1% SDS for 15 minutes before blotting with Tris-Glycine Transfer Buffer. The small amount of SDS will render enough charge to the proteins so they can move unidirectionally towards the anode and in most cases will not denature the protein

It is more likely for native proteins to diffuse out of the membrane into the solution during the blocking or antibody incubation steps, as the native proteins tend to be more soluble. We recommend fixing the proteins to the membrane to prevent diffusion of the proteins. The proteins can be fixed by air drying the membrane or incubating the membrane in 5-10% acetic acid for 15 minutes followed by rinsing the membrane with deionized water and then air drying.

By performing any of these two fixing methods the proteins will be sufficiently unfolded to expose hydrophobic sites and will bind more efficiently to the membrane.

Continued on next page

Semi-Dry Blotting of NuPAGE® Novex Bis-Tris Gels

The NuPAGE® Novex Bis-Tris Gels do not transfer efficiently using a semi-dry transfer cell as compared to blotting with XCell II™ Blot Module. If you decide to use semi-dry blotting for NuPAGE® Novex Bis-Tris Gels, use the protocol provided below to ensure efficient transfer of proteins.

1. Prepare 100 ml of 2X NuPAGE® Transfer Buffer from 20X NuPAGE® Transfer Buffer as follows:

NuPAGE® Transfer Buffer (20X)	10.0 ml
NuPAGE® Antioxidant (for reduced sample)	0.1 ml
Methanol	10.0 ml
Deionized Water	79.9 ml
Total Volume	100 ml

   If you are blotting large proteins, please see the **Note** below.

2. Soak the filter paper and transfer membrane in the transfer buffer.
   - If you are using Novex® pre-cut membrane/filter sandwiches, use three filter papers (0.4 mm/filter in thickness) on each side of the gel or membrane.
   - If you are not using the Novex® pre-cut membrane/filter sandwiches, use two thick filter papers.
3. Assemble the gel/membrane/filter paper sandwich on top of the anode plate as follows:

   Filter Paper
   Filter Paper
   Filter Paper
   Membrane
   Gel
   Filter Paper
   Filter Paper
   Filter Paper
4. Perform the transfer at 15 V constant for 15 minutes if you are using the Bio-Rad Trans-Blot Semi-Dry Transfer Cell. For any other semi-dry transfer cell, follow the manufacturer recommendations.

   Note: For transfer of large proteins (>100 kDa), pre-equilibrate the gel in 2X NuPAGE® Transfer Buffer (without methanol) containing 0.02-0.04% SDS for 10 minutes before assembling the sandwich.

Buffer Recipes

20X NuPAGE® Transfer Buffer

20X NuPAGE® Transfer Buffer is available from Invitrogen (see next page).

1. To prepare 20X NuPAGE® Transfer Buffer, dissolve the following reagents in 100 ml of deionized water:

		Concentration (1X)
Bicine	10.2 g	25 mM
Bis-Tris (free base)	13.1 g	25 mM
EDTA	0.75 g	1 mM
Chlorobutanol*	0.025 g	0.05 mM

2. Mix well and adjust the volume to 125 ml with deionized water. The pH of the buffer is 7.2.
3. Store at room temperature. The buffer is stable for 6 months at room temperature.
4. For transfer, dilute the 20X NuPAGE® Transfer Buffer (see page 8).

* Chlorobutanol is used as a preservative in the transfer buffer and is not necessary for efficient transfer of proteins. If you do not have chlorobutanol, you may prepare the buffer without chlorobutanol but the buffer will not be stable for long periods. Use the buffer within 2 weeks.

25X Novex® Tris-Glycine Transfer Buffer

25X Novex® Tris-Glycine Transfer Buffer is available from Invitrogen (see next page).

1. To prepare 25X Novex® Tris-Glycine Transfer Buffer, dissolve the following reagents in 450 ml of deionized water:

		Concentration (1X)
Tris Base	18.2 g	12 mM
Glycine	90.0 g	96 mM

2. Mix well and adjust the volume to 500 ml with deionized water. The pH of the buffer is 8.3. Do not adjust with acid or base.
3. Store the buffer at room temperature. The buffer is stable for 6 months at 25°C.

5X Novex® TBE Running Buffer

5X Novex® TBE Running Buffer is available from Invitrogen (see next page).

1. To prepare 5X Novex® TBE Running Buffer, dissolve the following reagents in 950 ml of deionized water:

		Concentration (1X)
Tris Base	54.0 g	89 mM
Boric Acid	27.5 g	89 mM
EDTA (free acid)	2.9 g	2 mM

2. Mix well and adjust the volume to 1000 ml with deionized water. The pH of the buffer is 8.3.
3. Store the buffer at room temperature. The buffer is stable for 6 months at 25°C.

Accessory Products

Additional Products

A large variety of pre-cast polyacrylamide gels, pre-mixed buffers, blotting membranes, and molecular weight markers are available separately from Invitrogen for the separation and analysis of proteins. Ordering information for most widely used products is provided below. For more detailed information, please visit the web site at www.invitrogen.com or call Technical Service (see next page).

Product	Quantity	Catalog no.
NuPAGE® Novex 4-12% Bis-Tris Gel	1 gel	NP0321
Novex® 10% Tris-Glycine Gel	1 gel	EC6075
XCell *SureLock*™ Mini-Cell & XCell II™ Blot Module	1 unit	EI0002
XCell II™ Blot Module	1 unit	EI9051
PowerEase® 500 Power Supply	1 unit	EI8600
SeeBlue® Plus2 Pre-Stained Standard	500 µl	LC5925
SeeBlue® Pre-Stained Standard	500 µl	LC5625
MultiMark® Multi-Colored Standard	500 µl	LC5725
SimplyBlue™ SafeStain	1 L (stains 50 mini-gels)	LC6060
Nitrocellulose Membrane (0.2 µm pore size)	20 membrane/filter paper sandwiches	LC2000
PVDF Membrane (0.2 µm pore size)	20 membrane/filter paper sandwiches	LC2002
Nylon Membrane (0.45 µm pore size)	20 membrane/filter paper sandwiches	LC2003
WesternBreeze® Chromogenic Western Blot Detection Kit	1 Kit (αMouse)	WB7103
	1 Kit (αRabbit)	WB7105
	1 Kit (αGoat)	WB7107
WesternBreeze® Chemiluminescent Western Blot Detection Kit	1 Kit (αMouse)	WB7104
	1 Kit (αRabbit)	WB7106
	1 Kit (αGoat)	WB7108
Novex® Tris-Glycine Transfer Buffer (25X)	500 ml	LC3675
NuPAGE® Transfer Buffer (20X)	125 ml	NP0006
Novex® TBE Running Buffer (5X)	1 L	LC6675
NuPAGE® Antioxidant	15 ml	NP0005
Ponceau S Solution	500 ml	33427-01
Amido Black	25 g	12310-01
SERVA® Blue R Stain (tablets)	5 g	35056-01

Technical Service

World Wide Web

Visit the Invitrogen Web Resource using your World Wide Web browser. At the site, you can:

- Get the scoop on our hot new products and special product offers
- View and download vector maps and sequences
- Download manuals in Adobe® Acrobat® (PDF) format
- Explore our catalog with full color graphics
- Obtain citations for Invitrogen products
- Request catalog and product literature

Once connected to the Internet, launch your web browser (Netscape 3.0 or newer), then enter the following location (or URL):

http://www.invitrogen.com

...and the program will connect directly. Click on underlined text or outlined graphics to explore. Don't forget to put a bookmark at our site for easy reference!

Contact us

For more information or technical assistance, please call, write, fax, or email. Additional international offices are listed on our web page (www.invitrogen.com).

United States Headquarters:
Invitrogen Corporation
1600 Faraday Avenue
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail: tech_service@invitrogen.com
Web: www.invitrogen.com

European Headquarters:
Invitrogen Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Tel (Free Phone Orders): 0800 269 210
Tel (General Enquiries): 0800 5345 5345
Fax: +44 (0) 141 814 6287
E-mail: eurotech@invitrogen.com

Limited Warranty

Invitrogen is committed to providing our customers with high-quality goods and services. Our goal is to ensure that every customer is 100% satisfied with our products and our service. If you should have any questions or concerns about an Invitrogen product or service, please contact our Technical Service Representatives.

Invitrogen warrants that all of its products will perform according to the specifications stated on the certificate of analysis. The company will replace, free of charge, any product that does not meet those specifications. This warranty limits Invitrogen Corporation liability only to the cost of the product. No warranty is granted for products beyond their listed expiration date. No warranty is applicable unless all product components are stored in accordance with instructions. Invitrogen reserves the right to select the method(s) used to analyze a product unless Invitrogen agrees to a specified method in writing prior to acceptance of the order.

Invitrogen makes every effort to ensure the accuracy of its publications, but realizes that the occasional typographical or other error is inevitable. Therefore Invitrogen makes no warranty of any kind regarding the contents of any publications or documentation. If you discover an error in any of our publications, please report it to our Technical Service Representatives.

Invitrogen assumes no responsibility or liability for any special, incidental, indirect or consequential loss or damage whatsoever. The above limited warranty is sole and exclusive. No other warranty is made, whether expressed or implied, including any warranty of merchantability or fitness for a particular purpose.

MSDS Information

Introduction

In an effort to provide our customers with the best possible information any time they need it, Material Data Safety Sheets (MSDSs) are now available 24 hours a day, 7 days a week from either our web site or the 3E Company (see below). **Note:** MSDSs will no longer be included with product shipments.

MSDS Information On-line

1. To request an MSDS, please visit our web site (www.invitrogen.com).
2. From the left-hand column, under echnical Resources select SDS Requests
3. Follow instructions on the page and fill out all the required fields.
4. To request additional MSDSs, click the dd Another button.
5. All requests will be faxed unless another method is selected.
6. When you are finished entering information, click the ubmit button. Your MSDS will be sent within 24 hours.

MSDS Information by Phone

Customers of Invitrogen can call the 3E Company, 24 hours a day, 7 days a week for MSDS information. This information can be obtained directly over the phone, faxed, or mailed to the customer. MSDS information can be accessed by compound, product catalog number, or compound part number listed in the upper right hand corner of the label.

3E Company
1905 Aston Avenue
Carlsbad, CA 92008
Voice: 1-800-451-8346 (U.S., Canada, and Guam)
Voice: 1-760-602-8700 (See below for other toll-free numbers)
Fax: 1-760-602-8888

Emergency Information

In the event of an emergency, the 3E Company can help with disposal or spill information. They can also connect the customer with poison control or the University of California at San Diego Medical Center doctors.

Toll-Free Numbers

You can contact the 3E Company using the toll-free numbers listed below.

Country	Toll-Free Number	Country	Toll-Free Number
Belgium	008071178	Mexico	958008342735
China	108001100017		958004518346
France	0800903046		958003603220
Germany	0130829154		958003466737
Indonesia	00180316570620	Netherlands	08000220091
Italy	167870990	Singapore	8001100987
Japan	006633800185	Spain	900931263
Korea	0308116570820	Turkey	00800136570900
Malaysia	1800808714	United Kingdom	0800967491