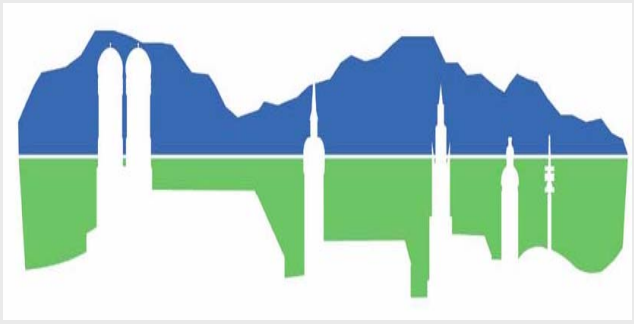


TECHNISCHE UNIVERSITÄT MÜNCHEN

HUPO 2007 Education Program

October 6, 2007

Seoul

Today's 2-D Electrophoresis Technology

Angelika Görg

Andreas Klaus, Carsten Lück, Florian Weiland, Walter Weiss

Technical University of Munich

http://www.wzw.tum.de/proteomik/

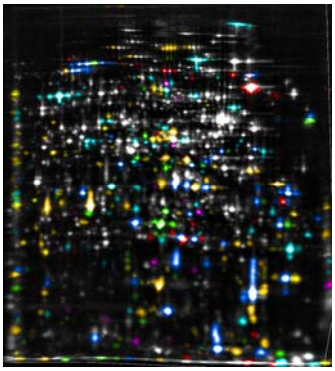
2-D Electrophoresis

Mouse liver

The first proteomic approach

© Görg 2007

Global view at a glance : Stress Signatures



Multiple stress response
in *B. subtilis* -
visualized by color coding
M. Hecker Univ. Greifswald

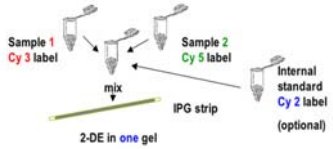
H
P
E

www.decodon.com

© Görg 2007

Differential Display & Quantitation

DIGE



Stress induced proteins

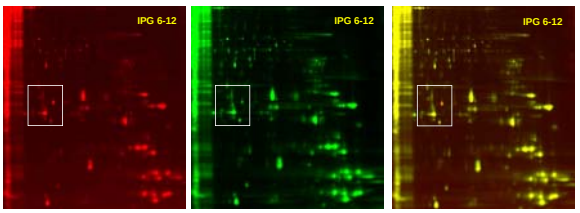


Image Cy 3 (532 nm)
Sample 1

Image Cy 5 (633 nm)
Sample 2

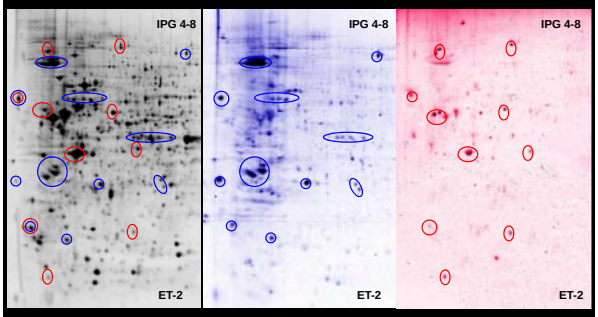
Image overlay

© Görg 2007

Drews,O.,Görg, A. et al.

Global view at a glance: PTM ´s

Multiplexed Fluorescence Stainings: Ewing Tumor Cell Lines



Sypro Ruby
Total Protein Stain

ProQ Diamond
Phosphoprotein Stain

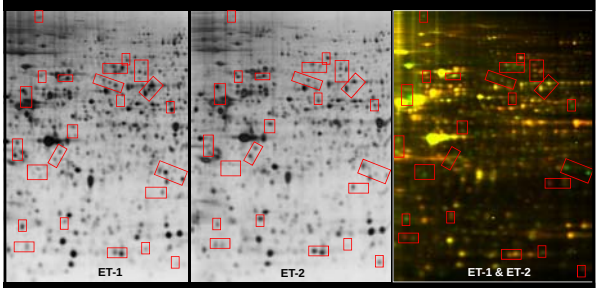
ProQ Emerald 488
Glycoprotein Stain

© Görg 2007

Weiland, F., Görg, A. et al.

Differential Display /Relative Abundance

Differentiation of Ewing Tumor Cell Lines



Silver Stain

DIGE Image Overlay

Weiland, F., Görg, A. et al.

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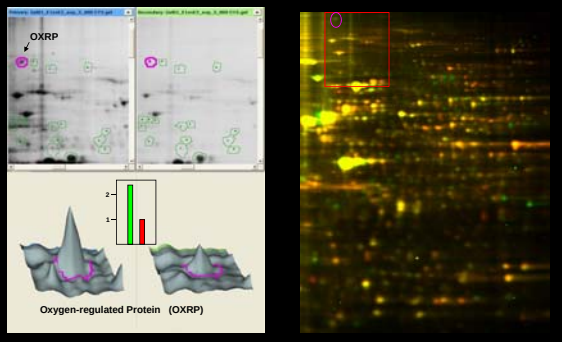
Differential Display & Quantitation :

DIGE of Ewing Tumor Cell Lines ET-1 and ET-2



ET-1 Cy3

ET-2 Cy5

Cy3 & Cy5 Image Overlay

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2-D Electrophoresis

Mouse liver

Limitations :

- Resolution
- Reproducibility
- Automation
- Quantitation
- Analysis of Proteins from 3 - 12
  - extreme acidic or basic pI
  - low abundance
  - integral membrane proteins

?

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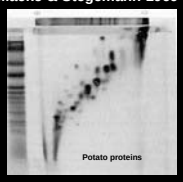
2-D maps

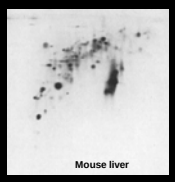
Soluble proteins
Native IEF x PAGE

Macko & Stegemann 1969



Potato proteins

Klose 1975 **275** spots



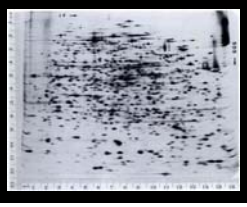
Mouse liver

High Resolution 2-D Electrophoresis

Total protein extracts
Denaturation agents
in both dimensions :
NP-40 x SDS

O'Farrell 1975

1 100 spots/ *E. coli*



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High Resolution 2-D Electrophoresis

Carrier ampholytes
LKB Bromma, Sweden O.Vesterberg 1969

pH gradient instability with time
thousands of amphoteric compounds
batch to batch variability

Reproducibility

Immobilized pH gradients
LKB Bromma, Sweden Bjellqvist, B. et al. 1982

pH gradient stability with time
Selected monomers
pH gradient engineering

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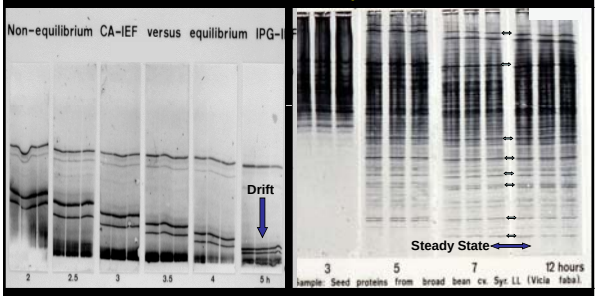
Reproducibility: pH gradient stability with time

Non-equilibrium IEF with CA

Equilibrium IEF with IPGs



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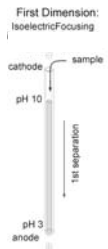
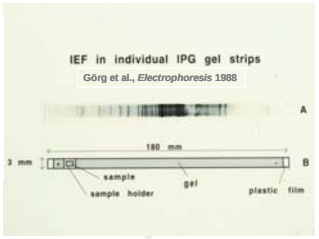
Görg, A. , *Nature 1991*

IPG Strips
- pH gradient stability with time
- easy handling





IEF in tube gels
- mechanically fragile
- carrier ampholyte IEF
- pH gradient drift/ time-dependent

IPG Strips
- mechanically strong, plastic backing
- immobilized pH gradient
- Ready to use

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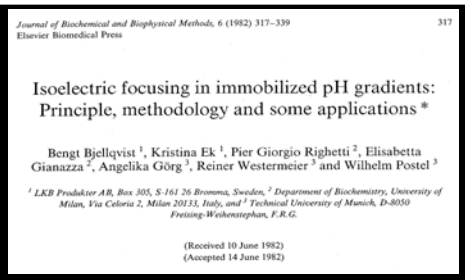


Journal of Biochemical and Biophysical Methods, 6 (1982) 317–339
Elsevier Biomedical Press

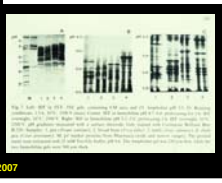
317

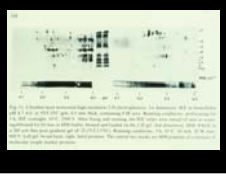
Isoelectric focusing in immobilized pH gradients: Principle, methodology and some applications *

Bengt Bjellqvist [1], Kristina Ek [1], Pier Giorgio Righetti [2], Elisabetta Gianazza [2], Angelika Görg [3], Reiner Westermeier [3] and Wilhelm Postel [3]

[1] LKB Produkter AB, Box 305, S-161 26 Bromma, Sweden, [2] Department of Biochemistry, University of Milan, Via Celoria 2, Milan 20133, Italy, and [3] Technical University of Munich, D-8050 Freising-Weihenstephan, F.R.G.

(Received 10 June 1982)
(Accepted 14 June 1982)





JBBM 1982

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Protocols

1982

1986

Today

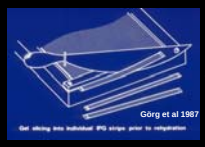
Görg et al., *Electrophoresis* 2000

© Görg 2007

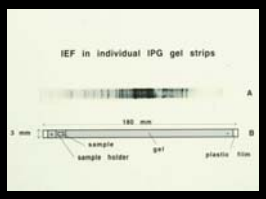
Design of IPG Strips

1982

1985



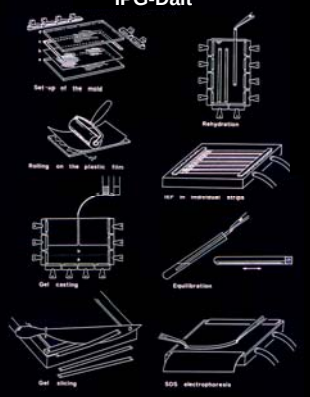
1987



Görg et al., *Electrophoresis* 1988

© Görg 2007

IPG-Dalt



Equilibration of IPG strips

50 mM Tris-HCl pH 8.8 + 2% SDS

+ 6 M urea
+ 30% glycerol
+ 1% DTT
+ 4% iodoacetamide

→ improved protein transfer

→ removes excess DTT (point streaking)
→ alkylation of SH-groups

	DTT	iodoacetamide
1. 15 min (10 min)	+	-
2. 15 min (10 min)	-	+

Preparation of IPG Strips

Görg et al., *Electrophoresis* 1988

© Görg 2007

Equilibration Protocol 1988

50 mM Tris-HCl pH 8.8 + 2% SDS

+ 6 M urea
+ 30% glycerol
+ 1% DTT
+ 4% iodoacetamide

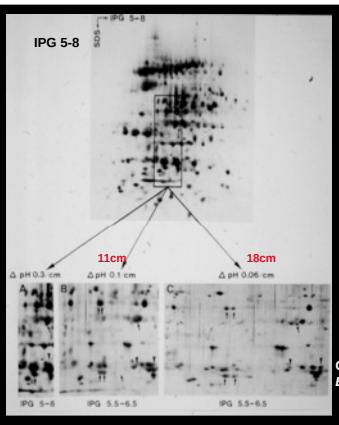
→ improved protein transfer from IPG Strip to SDS gel

→ • removes point streaking
→ • alkylation of SH-groups

	DTT	iodoacetamide
1. 15 min (10 min)	+	-
2. 15 min (10 min)	-	+

Görg et al., *Electrophoresis* 1988

© Görg 2007



IPG 5-8

Narrow IPGs & extended separation distances

Resolution

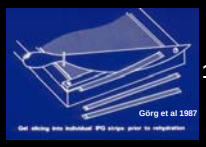
Görg et al., *Electrophoresis* 1988

© Görg 2007

IPG DryStrips

1982

1985



1987

1991

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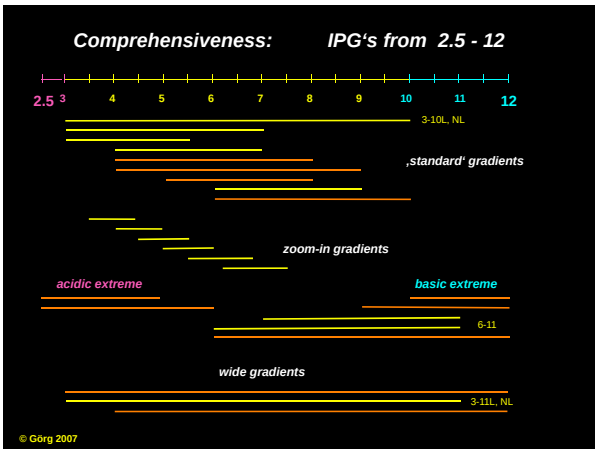
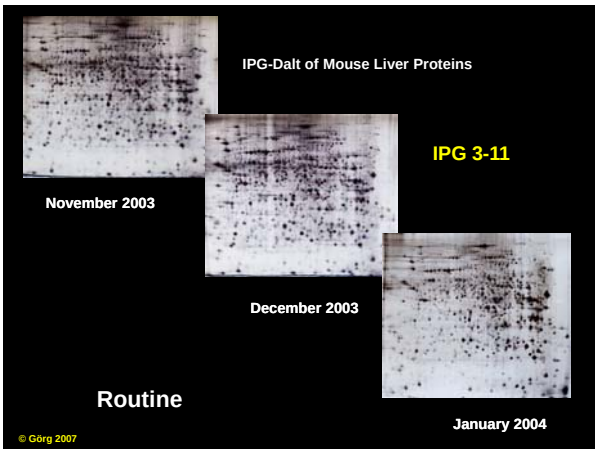
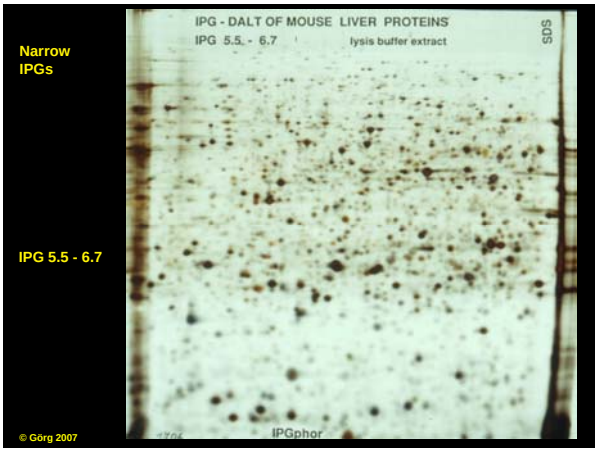
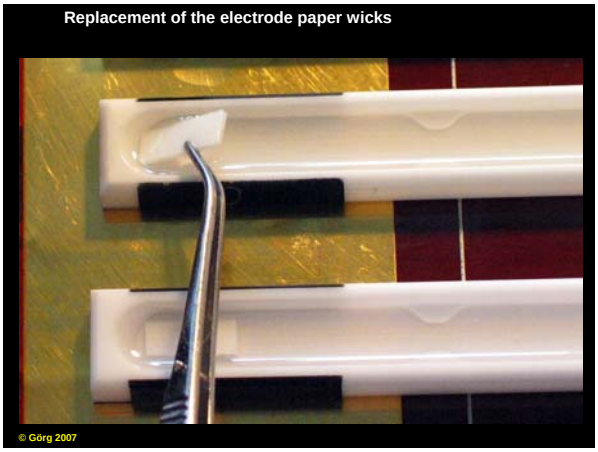
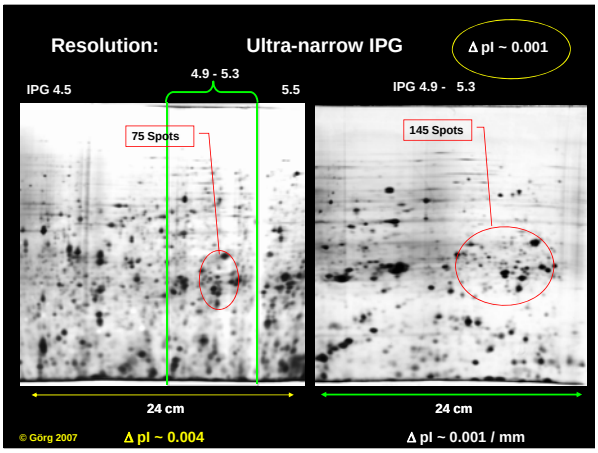
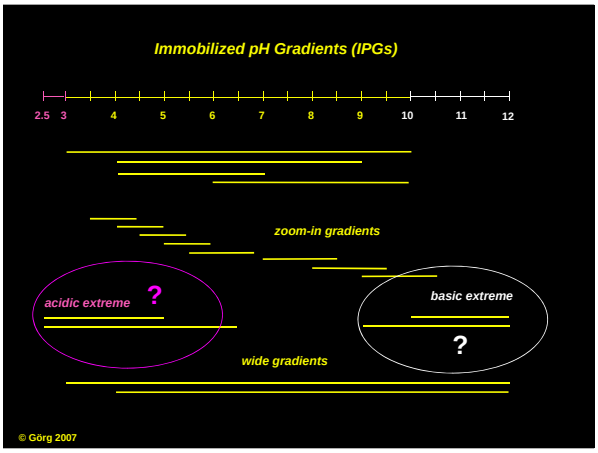

Comprehensiveness: IPG's from 2.5 - 12
2.5 3 4 5 6 7 8 9 10 11 12
3-10L, NL
‚standard' gradients
zoom-in gradients
acidic extreme
basic extreme
6-11
wide gradients
3-11L, NL
© Görg 2007


IPG-Dalt of Mouse Liver Proteins
IPG 3-11
November 2003
December 2003
Routine
January 2004
© Görg 2007

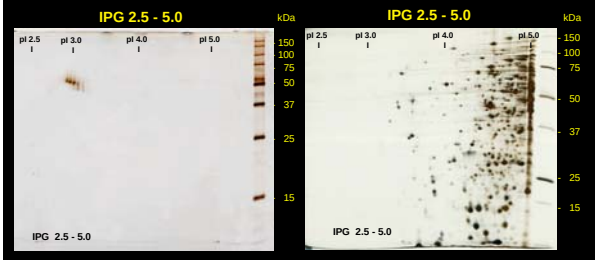

Narrow IPGs
IPG 5.5 - 6.7
IPG - DALT OF MOUSE LIVER PROTEINS
IPG 5.5 - 6.7
lysis buffer extract
SDS
IPGphor
© Görg 2007


Replacement of the electrode paper wicks
© Görg 2007


Resolution: Ultra-narrow IPG
Δ pI ~ 0.001
4.9 - 5.3
IPG 4.5
5.5
IPG 4.9 - 5.3
75 Spots
145 Spots
24 cm
24 cm
© Görg 2007
Δ pI ~ 0.004
Δ pI ~ 0.001 / mm


Immobilized pH Gradients (IPGs)
2.5 3 4 5 6 7 8 9 10 11 12
zoom-in gradients
acidic extreme ?
basic extreme
?
wide gradients
© Görg 2007

Very Acidic Proteins



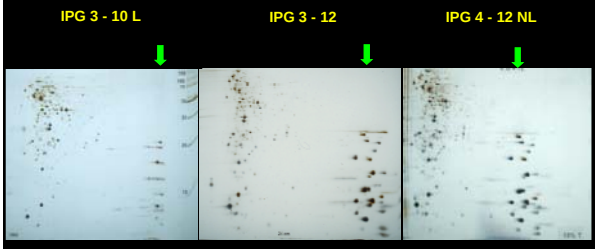
Hyperglycosylated recombinant Erythropoietin (EPO) analog (Aranesp®)

Corynebacterium glutamicum proteins

© Görg 2007

Very Alkaline Proteins > pH 10

Lactococcus lactis NCDO712



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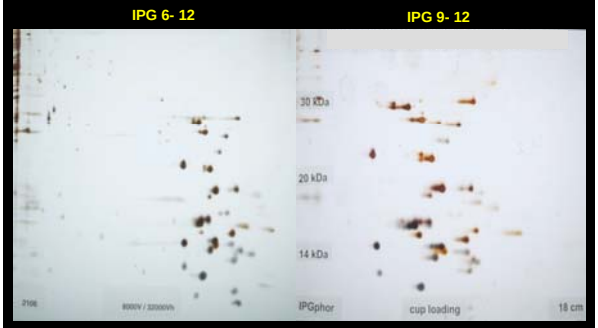
Very Alkaline Proteins > pH 10



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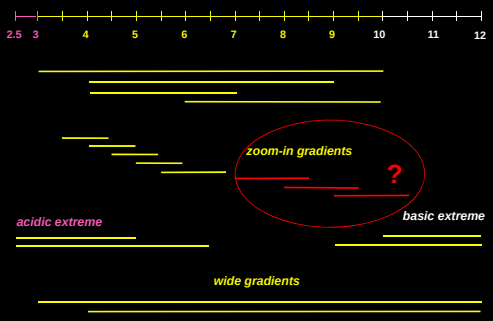
18 cm *Lactococcus lactis* 18 cm

Immobilized pH Gradients (IPGs)



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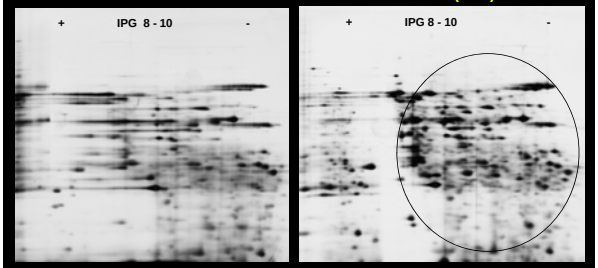
Narrow gradients between pH 7-10

DTT mouse liver DeStreak™ (HED)



Protein-S$^-$ + HO-CH_2-CH_2-S-S-CH_2-CH_2-OH ⟷ Protein-S-S-CH_2-CH_2-OH + OH-CH_2-CH_2-S$^-$

Olsson et al., Proteomics 2002

Protects thiol groups

© Görg 2007

2-D Electrophoresis

The first proteomic approach

The Actual Protocol

Görg *et al.* Electrophoresis 1988
Görg et al, Electrophoresis 2000
Görg et al., Proteomics 2004

© Görg 2007

Sample preparation

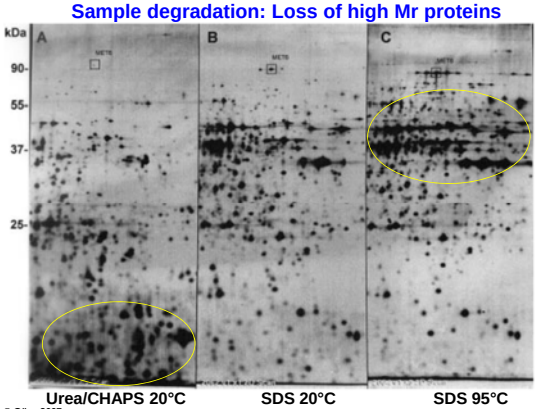
Critically important

- Cells (controlled conditions, synchronous etc.)
- Tissues (heterogeneity in composition)
- Organelles (method of preparation)
- Biological fluids (dynamic range 10^1-10^{10})

- Protein solubilization (urea, thiourea, detergents, reductant)
- Protein precipitation (Clean-up from lipids, polysaccharides, nucleic acids, salts)
- Sample integrity (protein degradation, PTM)

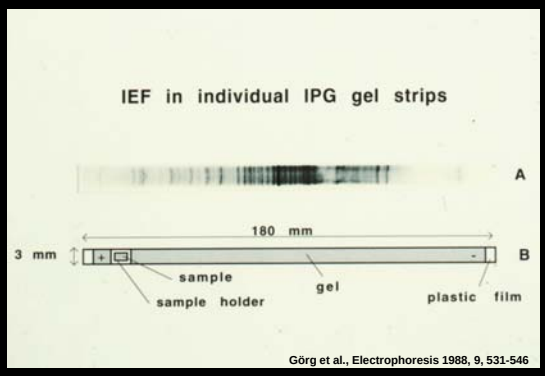
© Görg 2007

Sample degradation: Loss of high Mr proteins



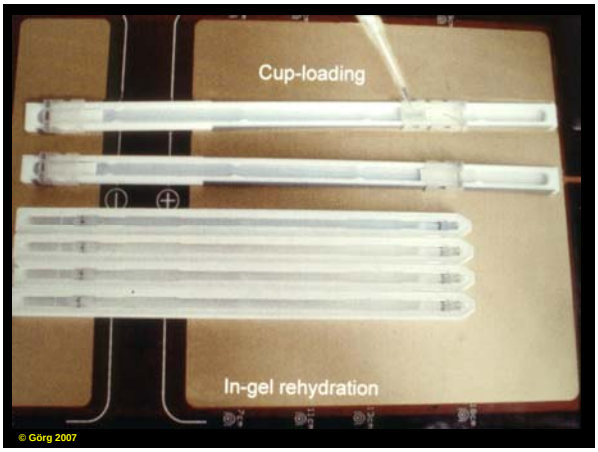
© Görg 2007

Actual Protocol of IPG-Dalt



Görg et al., Electrophoresis 1988, 9, 531-546

© Görg 2007



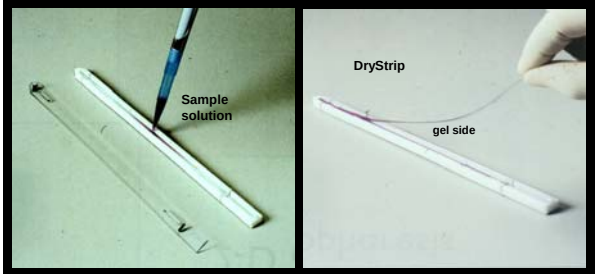
© Görg 2007

Sample application

★ **In-gel rehydration / Cup-Loading**
Analytical & Micropreparative IEF
Wide pH gradients between 3-12

★ **Cup-loading anode**
Narrow pH gradients at the basic extreme
Improved quantification

© Görg 2007

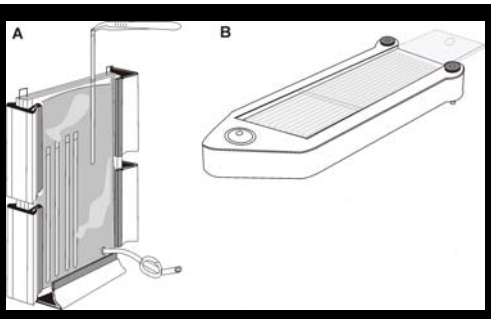


Sample entry by in-gel rehydration
IPG Strip Holder with integrated electrodes

© Görg 2007

Sample Cup Loading

© Görg 2007

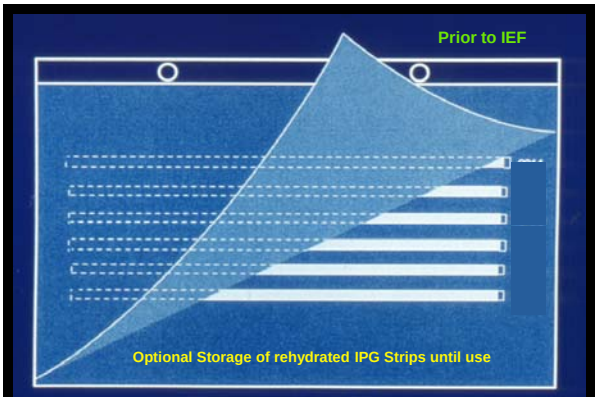


Rehydration of the IPG DryStrips to the original thickness

Rehydration solution: - urea, detergent, 0.5% carrier ampholytes, DTT

- alternatively, DeStreak Solution

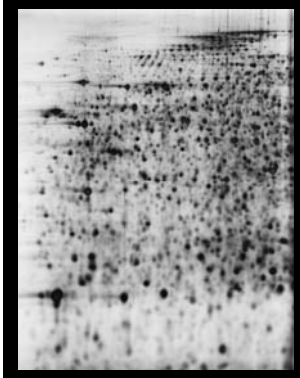
© Görg 2007

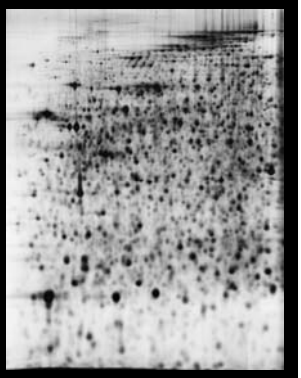


Storage at - 70°C

© Görg 2007

Optional Storage of Rehydrated IPG Strips prior to IEF





Stored at -70°C

No storage

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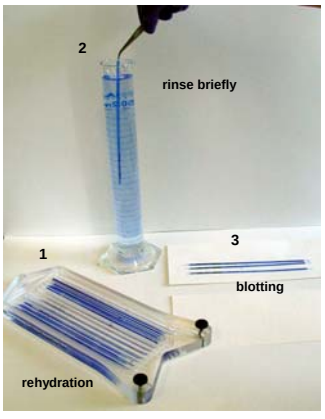
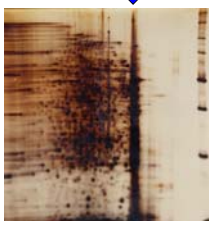


After rehydration, IPG strips are rinsed and blotted to prevent formation of urea crystals during IEF



Urea crystal on IPG strip

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Cup-loading : Minimum Sample Volume 50 µl

Cup-loading Volume 20 µl

Cup-loading Volume 80 µl

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Running conditions: Cup loading

For *all* pH gradients
Mandatory for IPG 6-12 9-12 10-12

Gel length:	180 mm	**Voltage max.**	8000 V
Temperature:	20°C	**Current max.:**	0.07 mA / IPG strip

Sample application	**Anode**
Sample volume	**> 50 µl**
> Initial IEF	**150 V 1 h** **300 V 1 h** **600 V 1 h**
> IEF to the steady-state:	**600 V → 8000 V ≈ 30 min** **8.000 V → steady-state** **32.000 Vh**

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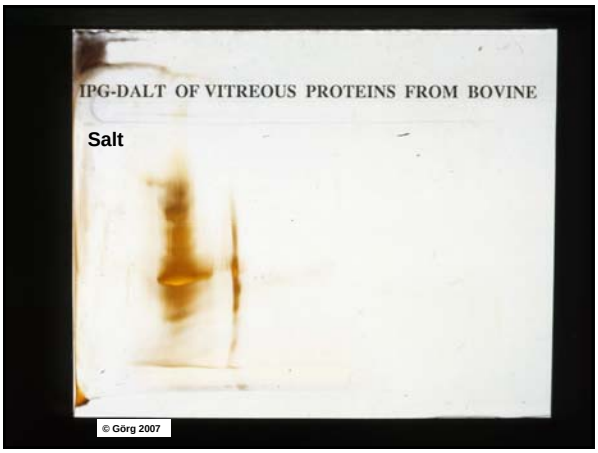
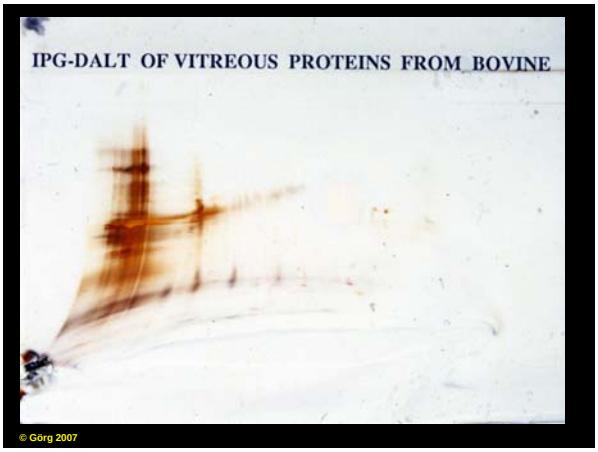
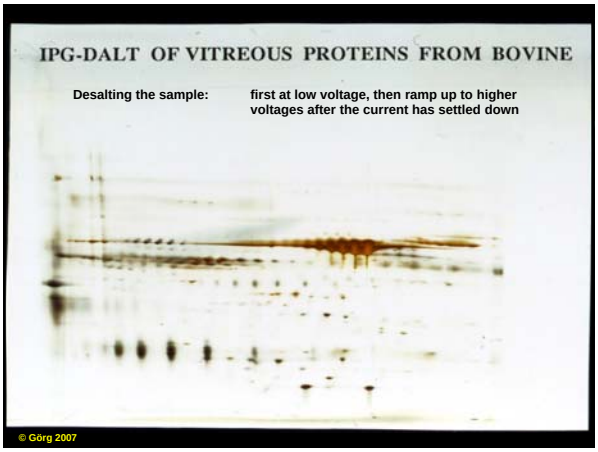
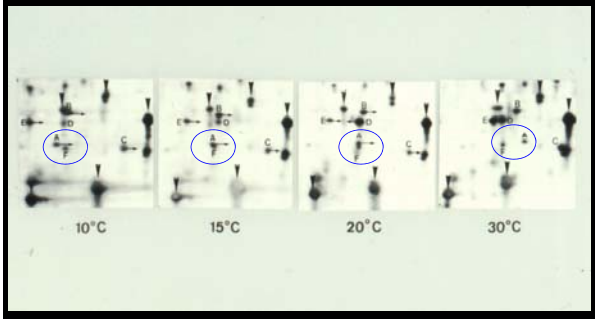






Spot position variabilities due to temperature



Görg et al, *Electrophoresis* 1991,12, 653-58

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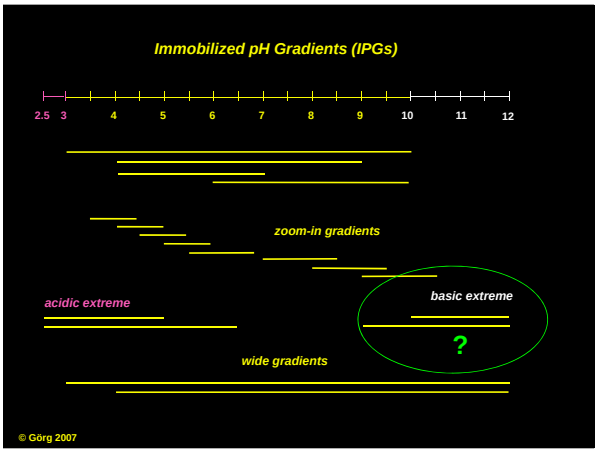

Optimized Protocol for Very Alkaline Proteins

IPG 6 - 12

In-gel rehydration	Cup-loading Anode	Cup-loading Anode
3 500 V max	3 500 V max	8 000 V max

© Görg 2007

IPG-Dalt versus NEPHGE

IPG 6-12 IEF to the Steady-State

8000 V 18 000 Vh 8000 V 32 000 Vh

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Optimized Protocol for IEF of Alkaline Proteins up to pH 12

1.	Sample loading	:	Cup/Anode
2.	Sample entry	:	with limited voltage ~ 3 h not over night
3.	Final Voltage	:	up to 8 000 V
4.	Current max	:	0.05 to 0.07 mA per strip
5.	IEF time (Vh)	:	to the steady-state, no overfocusing

Wildgruber *et al.*, Proteomics 2002; Drews *et al.*, Proteomics 2004

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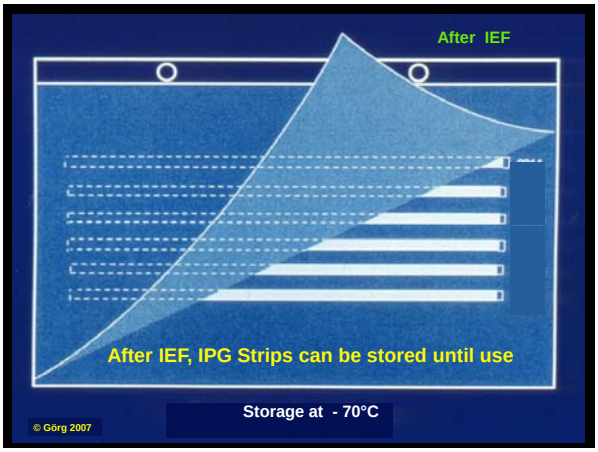
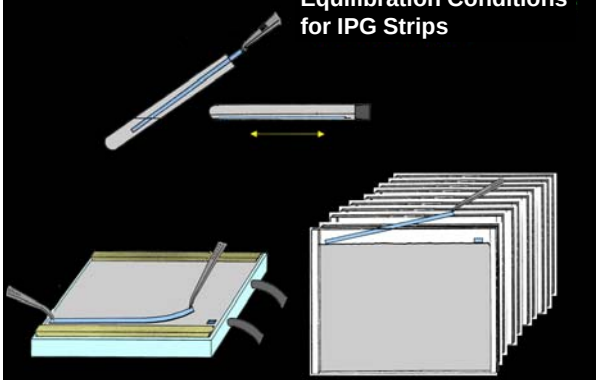


Equilibration Conditions for IPG Strips



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Equilibration Protocol 1988

50 mM Tris-HCl pH 8.8 + 2% SDS

+ 6 M urea
+ 30% glycerol
+ 1% DTT
+ 4% iodoacetamide

→ improved protein transfer from IPG Strip to SDS gel

→ • removes point streaking
• alkylation of SH-groups

	DTT	iodoacetamide
1. 15 min (10 min)	+	-
2. 15 min (10 min)	-	+

Görg et al., Electrophoresis 9, 531-546 (1988)

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Insufficient protein transfer from first to second dimension
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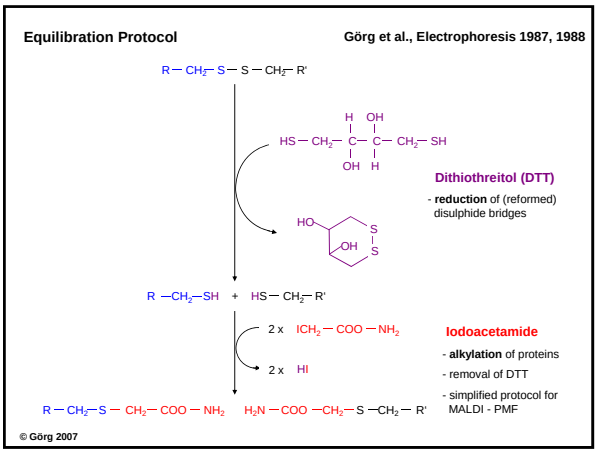
Equilibration Protocol
Görg et al., Electrophoresis 1987, 1988
R — CH2 — S — S — CH2 — R'
HS — CH2 — C — C — CH2 — SH
H OH
OH H
Dithiothreitol (DTT)
- reduction of (reformed) disulphide bridges
HO
OH
S
S
R — CH2 — SH + HS — CH2 — R'
2 x ICH2 — COO — NH2
2 x HI
Iodoacetamide
- alkylation of proteins
- removal of DTT
- simplified protocol for MALDI - PMF
R — CH2 — S — CH2 — COO — NH2
H2N — COO — CH2 — S — CH2 — R'
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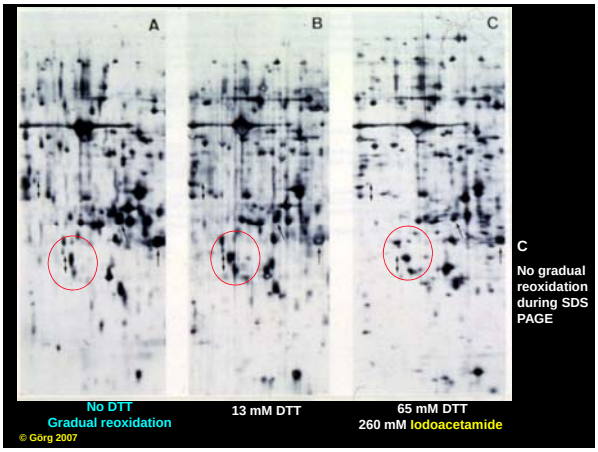
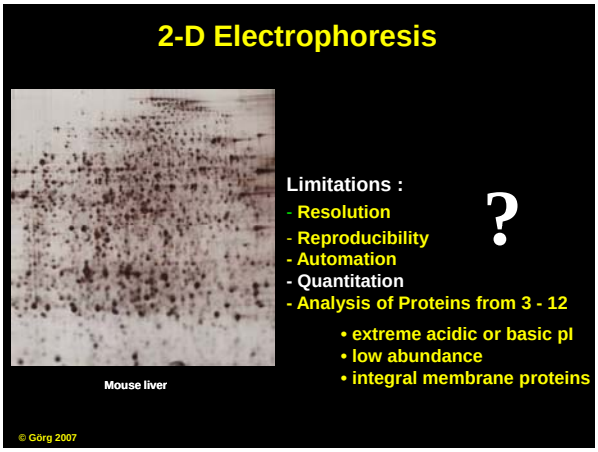

A
B
C
No DTT
Gradual reoxidation
13 mM DTT
65 mM DTT
260 mM Iodoacetamide
C
No gradual reoxidation during SDS PAGE
© Görg 2007


2-D Electrophoresis
Mouse liver
Limitations :
- Resolution
- Reproducibility
- Automation
- Quantitation
- Analysis of Proteins from 3 - 12
• extreme acidic or basic pI
• low abundance
• integral membrane proteins
?
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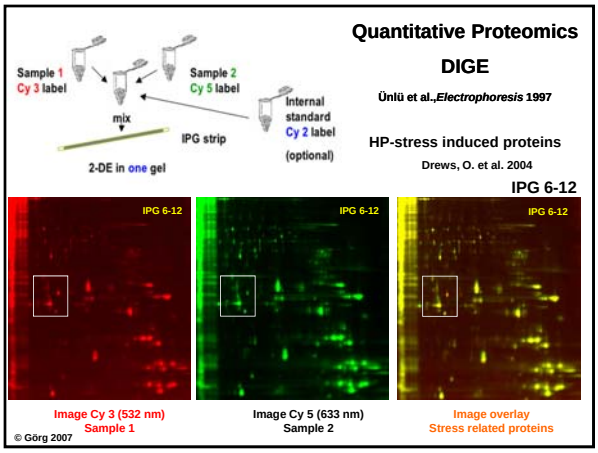
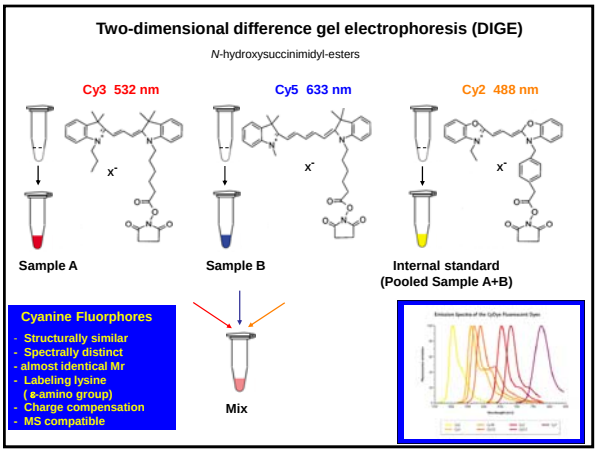

Quantitative Proteomics
DIGE
Ünlü et al.,Electrophoresis 1997
Sample 1
Cy 3 label
Sample 2
Cy 5 label
mix
IPG strip
2-DE in one gel
Internal standard
Cy 2 label
(optional)
HP-stress induced proteins
Drews, O. et al. 2004
IPG 6-12
IPG 6-12
IPG 6-12
IPG 6-12
Image Cy 3 (532 nm)
Sample 1
Image Cy 5 (633 nm)
Sample 2
Image overlay
Stress related proteins
© Görg 2007


Two-dimensional difference gel electrophoresis (DIGE)
N-hydroxysuccinimidyl-esters
Cy3 532 nm
Cy5 633 nm
Cy2 488 nm
Sample A
Sample B
Internal standard
(Pooled Sample A+B)
Cyanine Fluorphores
- Structurally similar
- Spectrally distinct
- almost identical Mr
- Labeling lysine
(ε-amino group)
- Charge compensation
- MS compatible
Mix

Minimum labeling	**< 5% of total protein** so that only proteins containing a single dye molecule are visualized on the gel
Sample solution :	Lysis buffer pH 8.5 without carrier ampholytes, no DTT 30 mM Tris, 7M urea, 2M thiourea, 4% CHAPS
Labeling:	CyDyes are reconstituted in DMF **50 µg protein is labeled with 400 pmoles of dye on ice-water for 30 min** reaction is stopped by adding 1 µL of 10 mM lysine

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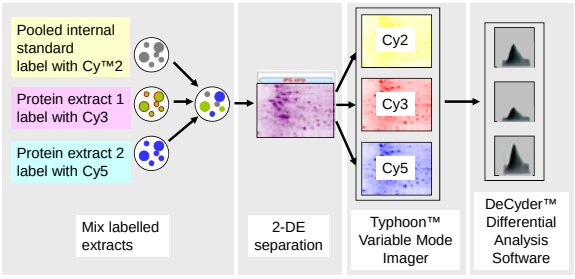
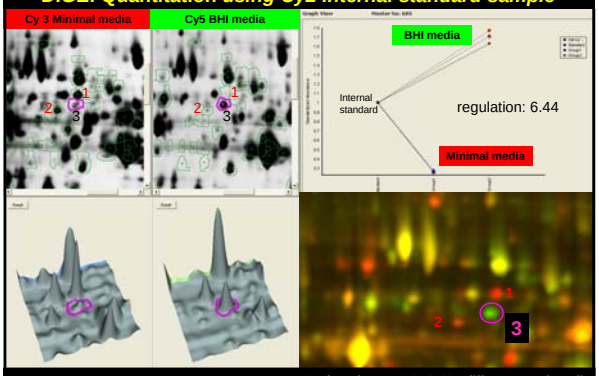
Achieving accurate quantitative data

Ettan™ DIGE system



Minimal labeling : < 5 % of protein labeled

DIGE: Quantitation *using Cy2 internal standard sample*



NAD-dependent aldehyde dehydrogenase

C. glutamicum ATCC 13032 - different growth media
BHI= brain heart infusion

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Lück, et al. in preparation

2-D Electrophoresis

Mouse liver

Limitations :

- Resolution
- Reproducibility
- Automation
- Quantitation
- Analysis of Proteins from 3 - 12
  - extreme acidic or basic pI
  - low abundance
  - integral membrane proteins

?

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2-D Electrophoresis

Mouse liver

Limitations :

- low abundance proteins

Detection and Identification:

- high resolution technology
- with micropreparative quantities of samples

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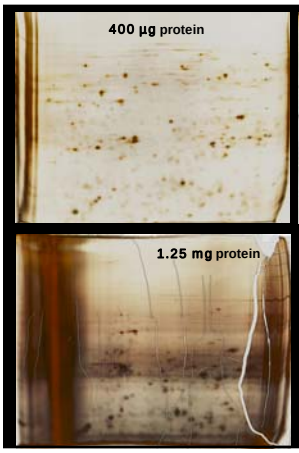

Micropreparative Sample Load

IPG 4.9 - 5.3 (24 cm)

Mouse liver proteins

Görg et al. Proteomics 2002, 2, 1652-1657

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2-D Electrophoresis

Mouse liver

Enrichment of low abundance proteins

- Subcellular fractionation
- Affinity depletion / enrichment
- Prefractionation - *solubility* - *pI*

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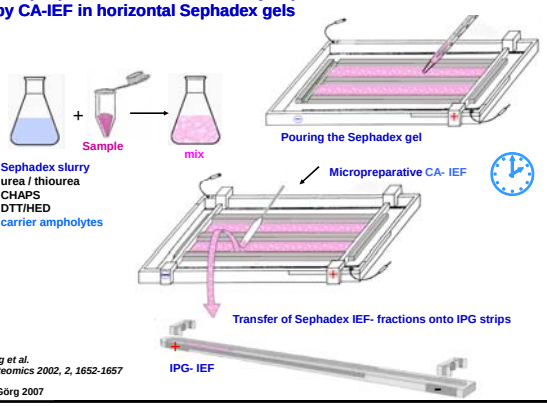
Sample prefractionation according to pI by CA-IEF in horizontal Sephadex gels



Görg et al. Proteomics 2002, 2, 1652-1657

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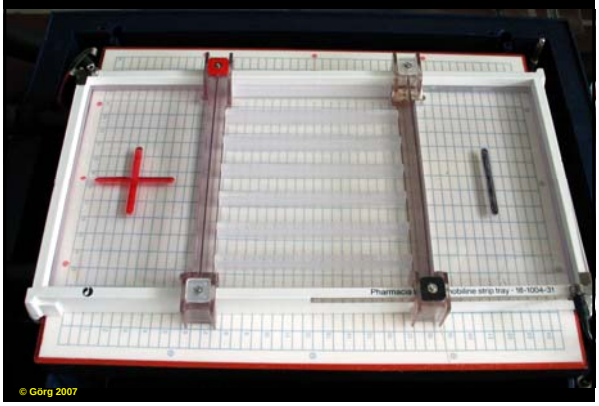
Sephadex IEF tray on the Multiphor® instrument



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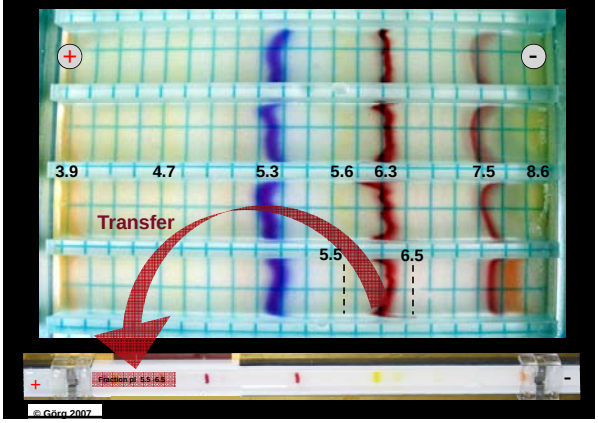
Sample fractionation by Sephadex-IEF, 10 mg protein-load per lane



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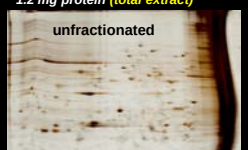
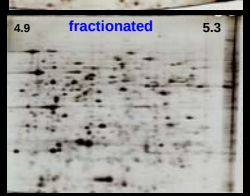
1.2 mg protein (total extract)



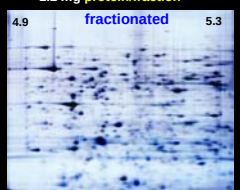


Ultra-narrow IPG 4.9 - 5.3

Mouse liver proteins

Sample load

1.2 mg protein/fraction



After Sephadex-IEF Fractionation

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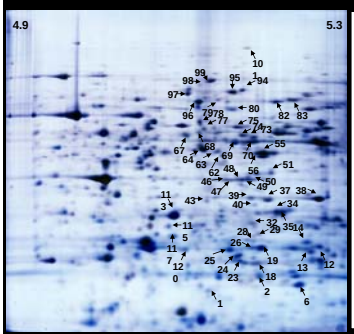
Identified mouse liver proteins: ultra-narrow IPG 4.9-5.3 after Sephadex-IEF fractionation



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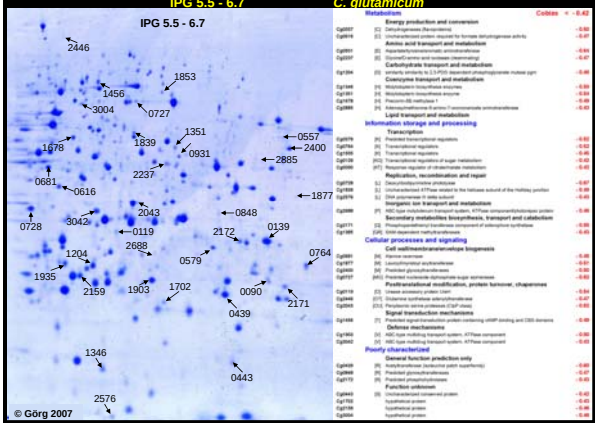
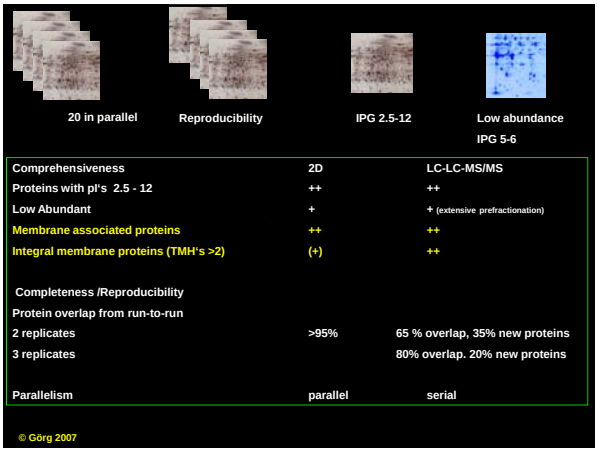
Putative Low Abundance Proteins after Sephadex-IEF Prefractionation

IPG 5.5 - 6.7 *C. glutamicum*





Comprehensiveness	2D	LC-LC-MS/MS
Proteins with pI's 2.5 - 12	++	++
Low Abundant	+	+ (extensive prefractionation)
Membrane associated proteins	++	++
Integral membrane proteins (TMH's >2)	(+)	++
Completeness /Reproducibility		
Protein overlap from run-to-run		
2 replicates	>95%	65 % overlap, 35% new proteins
3 replicates		80% overlap. 20% new proteins
Parallelism	parallel	serial

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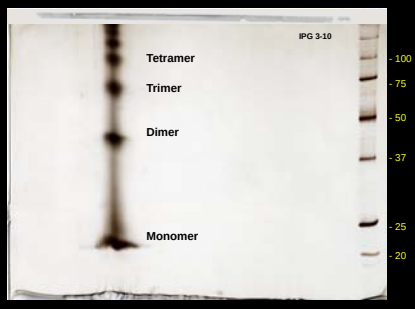
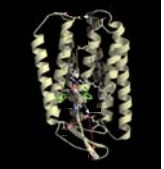
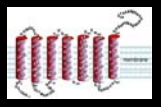
Purified Bacteriorhodopsin







(Source: D. Oesterhelt 1998)

Theoretical pIs = 3.93 & 4.43 / Mr = 27 kDa (Monomer)
7 Transmembrane helices
GRAVY: 0.787

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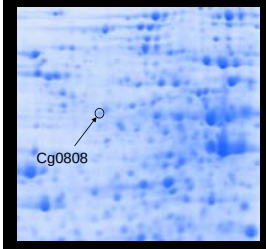
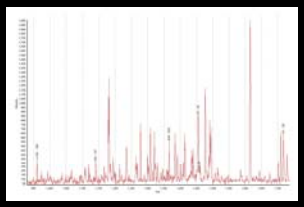
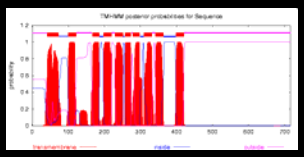
Membrane Protein Cg0808
Lipopolysaccharide Biosynthesis Acyltransferase







- **Theoretical pI: 5.35 Mr: 80 kDa**
- **GRAVY: 0.19**
- **10 Transmembrane helices**
- **6 matched tryptic peptides**

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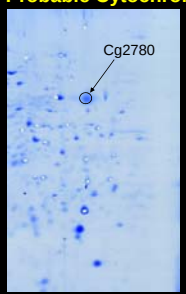
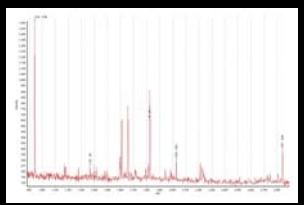
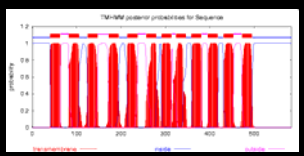
Membrane Protein Cg2780
Probable Cytochrome C Oxidase Polypeptide Subunit







- **Theoretical pI: 6.9 Mr: 65 kDa**
- **GRAVY: 0.4**
- **12 Transmembrane helices**
- **5 matched tryptic peptides**

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IPG-Dalt of *C. glutamicum* Membrane Extracts

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Further Reading

Görg *et al., Electrophoresis* 1988, *9*, 531-546

Görg *et al., Electrophoresis* 2000, *21*, 1037-1053

Görg, Weiss & Dunn, *Proteomics* 2004, *4*, 3665-3685

Görg, Drews & Weiss in: *Purifying Proteins for Proteomics. A Laboratory Manual* (R. Simpson. Ed.), CSHL Press, NY, 2004, pp. 391-430

2-DE Manual: http://www.wzw.tum.de/proteomik

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Sample solubilisation for 2-DE

"Classical" O'Farrell lysis buffer (1975)
9.5 M urea, 4% NP-40, 1% DTT, 2% synthetic carrier ampholytes

Alternative reagents

Chaotropes: increase sample solubility, minimize protein aggregation, proteins are unfold, denatured progressively
2 M thiourea, 7 M urea (Rabilloud et al, 1997)

Detergents: disrupt hydrophobic interactions (protein-lipid and protein-protein interactions)
Linear sulphobetaines (SB 3-10, SB 3-12) (Rabilloud et al, 1997)
CHAPS (Perdew et al, 1983), C6BZ (Rabilloud et al, 1999)
Triton X-114 phase partitioning (Wissing et al, 2000)
SDS pre-solubilisation and detergent exchange

Reducing agents: disulfide bond cleaving agents
DTT
Tributyl phosphine (Herbert et al, 1998)

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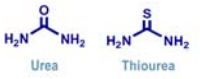
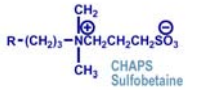
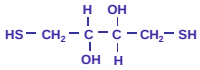
Sample preparation for 2D electrophoresis



Chaotropes: Disrupt hydrogen bonds and hydrophobic interactions
Note: Urea forms an equilibrium with ammonium cyanate and may modify (carbamylate) proteins. Do not heat > 37°C!



Detergents:
Break hydrophobic interactions & solubilize proteins
Note: Must not carry a net charge (-> non-ionic or zwitterionic)



Reductants:
Reduce disulphide (-S-S-) bonds

Carrier Ampholytes:
- Improve sample solubilization
- Act as cyanate scavengers

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Sample application by „*in-gel rehydration*"

Sample volume	350 µl 450 µl	Gel length:	180 mm 240 mm
Protein concentration		5-10 mg/ml Lysis buffer	
Lysis buffer		7 M urea/2M thiourea 4% CHAPS 2 % Carrier Ampholytes 1% DTT	
Rehydration solution		6 M urea/2M thiourea 1-2% CHAPS 1 % Carrier Ampholytes 0.4% DTT	

For in-gel rehydration the sample (dissolved in lysis buffer) is diluted with rehydration solution to the appropriate amount of volume

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Sample application by „*cup loading*"

Protein concentration	5-10 mg/ml Lysis buffer
Sample volume	50 µl min 100 µl max
Lysis buffer	7 M urea/2M thiourea 4% CHAPS 2 % Carrier Ampholytes 1% DTT 10 mM PMSF
Rehydration solution Alternatively:	6 M urea/2M thiourea 1-2% CHAPS 1% Carrier Ampholytes 0.4% DTT DeStreak Solution (HED instead of DTT)

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Sample *Prefractionation* by IEF in Sephadex Gels

Sephadex Slurry	210 mg Sephadex G-100SF reswollen (> 24 h) in 3.0 ml rehydration solution (8M urea, 1%CHAPS, 200mM HED, 2.5% carrier ampholytes) (alternatively:DeStreak Solution)
Sample Solution	5-10 mg protein / ml in 9.5M urea, 2% CHAPS, 1% DTT, 1% carrier ampholytes, 10mM Pefabloc (or PMSF)
Preparation of Sephadex Gel	1.5 ml sample solution + 5 µl coloured pI marker are added to 3.0 ml Sephadex slurry and poured onto the flatbed tray
Sephadex-IEF	Temperature: 20°C Separation distance: 10 cm Settings: 100 V (30 min); 200 V (30 min); 600 V (1h); 1000 V (2 h)
IPG-IEF	Transfer of Sephadex fractions onto rehydrated IPG strips - 1 cm of Sephadex fraction is applied onto surface (near the anode) of the corresponding narrow range IPG strip - protect surface with 2 ml of IPG strip cover fluid - continue with IPG-IEF as described for cup-loading

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Görg et al., *Electrophoresis* 2002