# 2D Gel Electrophoresis for DiGE at CIAN

**NOTE: This document is a collection of short protocols for running 2D-DiGE experiments at the CIAN DiGE lab. It is meant as an introduction and overview for the beginner, and a quick reference for more experienced users. It is NOT sufficient to read this alone, you will need to refer to more comprehensive resources and instrument manuals for details of the procedure.**

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**Elke Küster-Schöck**

**Content:**

# 1. Time line, excluding data analysis

**Sample preparation**
time: variable, depending on experimental system
follow-up: samples can be stored frozen in sample buffer

**CyDye labeling**
labeling time: ~1h
follow-up: load immediately on IEF strip

**Sample loading and strip rehydration**
preparation time: ~ 1h
rehydration time: at least 10h, or over night
follow-up: IEF has to be carried out immediately after rehydration

**First dimension separation, IEF**
preparation time: < 30min if doing active rehydration
separation time: variable, depending on strip type, length, sample; always several hours; should be monitored during last step
follow-up: immediately load strip on second dimension gel, or freeze at -80˚C for up to several days/weeks

**Second dimension separation, SDS-PAGE**
preparation time: if self-cast gels, add casting time ~ 1h, polymerization over night (ie casting should be done in parallel to rehydration/IEF);
strip equilibration, loading, starting run ~ 1-2h
separation time: variable, 5-7 hours, can be run over night
follow-up: for DiGE, scan immediately; for staining or storage, fix immediately

**DiGE scan**
preparation time: disassembling gel tank and cleaning plates ~ 1h
scan time: prescan to adjust parameters ~ 30min
actual scan per gel, with three fluorophores: ~ 40min
follow-up: fixing for post-staining or storage; if no picking planned, just clean-up

**Post-staining**
preparation time: ~1h
fixation and staining: min. several hours, fixation or staining can be done over night (except for backed gels: 1.5h maximum)
follow-up: fixed and/or stained gels can be kept several days/weeks in the fridge

**Spot picking**
time varies depending on technique used (hand-picking, robot)
follow-up: picked spots can be kept for several days/weeks in appropriate buffer before submitting to mass spectrometry

## 2. General considerations

- Sign up online for use of the first and second dimension separation equipment. If you don't need a booked time slot, cancel your reservation.
- Let Elke know of your plans to run a large 2D or DiGE gel well in advance, so that all supplies can be ordered and received. Typical order-to-delivery times in business days, without backorder issues:
  DiGE CyDyes – 2-10 days
  IEF strips – 1-3 days if in stock, but can be 1-2 weeks
  pre-cast gels – at least 1 week, depending on supplier
- Note on the IEF strip inventory sheet on the freezer how many strips you have used, and at what date. That way our inventory should always be current.
- The lab is based on a "clean-before-and-after" policy; please clean the equipment you are using to your specifications before use, and clean up after yourself and put equipment into storage condition after you are done.
- We are always trying to improve, so please let us know of any suggestions!

## 3. DiGE CyDye labeling

Please see the DiGE manual for detailed instructions.

Here are a few key points:

- Standard labeling reaction: 50µg protein + 400pmol CyDye; according to several unprinted sources and our own experience, half the amount of dye is also sufficient to give a good signal
- Standard labeling conditions should be re-evaluated for samples of lower complexity (reduce amount of total sample?), or samples containing one or several overabundant proteins (increase amount of sample to see low-abundance proteins?), or if you know that you are dealing with something that is unusual in its lysine content.
- The protocol calls for making a 1nmol/µl concentrated stock, and then a 400pmol/µl working solution of the dyes; the latter can't be stored for more than a week. It is also possible to set up the labeling reactions with the concentrated stock directly.
- A slightly basic pH is crucial for labeling. Check the pH of your sample by spotting one µl on pH paper, at least one time for a typical sample, to make sure your buffer maintains the pH in the presence of your proteins.
- DMF for dye dilution should be purchased fresh for each batch of dye, to minimize exposure to humidity.
- A 10mM lysine solution for quenching is in the fridge. Prepare fresh from powder if desired.

Labeling workflow:

- put measured protein amount in clean Eppendorf tube
- add appropriate amount of dye solution, mix, spin down
- incubate 30min on ice in the dark
- quench by adding 1µl of 10mM lysine, mix, incubate 10min on ice in the dark

# 4. First dimension, IEF

### Rehydration loading

- get out/thaw sample, sample buffer, DTT (MW 154.2) or DeStreak solution
- clean coffin(s) with “Strip Holder Cleaning Solution” and toothbrush, let drain and air-dry or blot dry with Kimwipes; clean two pairs of forceps; prepare some Kimwipes on work surface to put down strip if needed
- combine sample, sample buffer, IPG buffer, and DTT to make the appropriate rehydration volume for the strip used
- [if using DeStreak reagent, omit DTT, add DeStreak to 12µl/ml, i.e. for 24cm strip, use 5.4µl]

| IPG strip length | Total volume per strip | final conc. 10mg/ml DTT | final conc. 1% IPG buffer |
|---|---|---|---|
| 13 cm | 250 µl | 2.5 mg<br>16.2µl of 1M* | 2.5 µl |
| 24 cm | 450 µl | 4.5 mg<br>29.2µl of 1M* | 4.5 µl |

* use of a 1M stock made in water will dilute the other components of the rehydration solution, so adding the solid is preferred

- if more than one sample, note sample ID, (coffin ID,) and strip ID on a piece of paper, DO NOT write on strips or coffins as lab markers contain fluorescent components; the printed number on the strip will be visible on your DiGE scan
- pipet sample into middle of coffin, try to avoid bubbles
- get strip from -20˚C freezer, sign out at freezer door, remove from package, peel off protective foil using forceps, starting at “+”/acidic/anodic end (“-“/basic/cathodic end is softer and stickier)
- lower strip onto sample in coffin, gel side down (writing is right side up), with “+” end towards pointed end of coffin
- remove bubbles and distribute sample by carefully lifting and lowering strip from its ends and sliding it back and forth
- overlay strip with 1-2ml of DryStrip Cover Fluid (mineral oil) using disposable Pasteur pipette or blue tip
- close lid (bubbles in oil are not detrimental), place coffin on IPGphor in the designated orientation and position, space coffins evenly
- [if using only one coffin, place another, empty coffin on IPGphor]
- place lid adaptor onto coffins, close IPGphor lid

- start IPGphor (power button back left), select program; for active rehydration, don't use the "rehydration" section, but program it as a first step using 30-50V at 20˚C for at least 10h

**Isoelectric Focusing after Rehydration Loading**

- end and exit rehydration program (hit stop button 3 times)
- on a clean surface, dampen electrode wicks with 7µl water or 50mM DTT (7.7mg/ml), respectively (or dip in and blot dry)
- using forceps, slip $H_2O$ wick under "+" end, DTT wick under "–" end of strip, so that it is between gel and electrode
- refill oil if needed, replace coffin lid, put coffin(s) back on IPGphor
- [if you want the computer to log your run, start IPGphor control software on Typhoon computer; leave on during run, save logfile after run ends]
- check/edit program, start IEF program (see hints below)
- clean equilibration tubes, allow to dry before use
- at the end of the program, take strips out of coffins, blot oil from back and sides of strip on Kimwipes, and place strips one equilibration tube each; freeze rapidly at -80˚C for storage, or proceed directly to second dimension

**IEF program hints (too many variables to make rules)**

- use the manufacturer's leaflet that came with your strips as a guideline
- check the recommendations in the GE 2DE Handbook, p.65 ff
- the high voltage in the final step(s), e.g. 8000V, might only be reached incrementally, or not at all, depending on the sample conductivity; for consistency among runs, the length of this step should be limited by Vh, not time
- if you might not be around when the program ends, add a step at the end of your program at 500V, for several hours, to avoid the proteins diffusing from their positions; try to keep this to a minimum, and add the total accumulated kVh at the end of the run to your notes

## 5. Second dimension, SDS-PAGE on Ettan DALTsix

**Casting single-percentage gels** with gel-strengthener

- clean gel caster, black wedge, funnel, separator sheets (thin and thick), dummy plates, glass plates; use RO water and lint-free wipes
- assemble gel caster lying flat; start with thin separator sheet, then glass plates or dummy, thin separator, etc. until six gel cassettes or dummies are assembled, finish with the last thin separator, and fill the gap with as many thick separators as needed
- make sure the foam seal on the face plate is lubricated lightly (GelSeal) and sits well in the groove
- turn two screws into threaded holes for two or three full turns, place face plate on caster so that notches are resting on screws, clamp sides with six spring clamps, then hand-tighten screws
- place assembly upright onto bench pad, check that it is level
- prepare 0.1% SDS solution in plant sprayer, at least 50ml per gel
- prepare acrylamide mixture, fresh APS solution (or add as solid)
- after adding catalysts, mix by swirling, start casting rapidly using funnel in filling channel in the back until it is about 1cm below the top of the short plate
- overlay with 0.1% SDS by spraying exhaustively along top from about 30cm away (or water-saturated butanol via pipette, change to 0.1% SDS or Milli-Q water after 3h to avoid corrosion of plastic parts on the caster)
- wrap caster in plastic wrap and allow to polymerize at RT over night
- remove gels and clean off any polymerized acrylamide, clean caster
- use right away, or wrap in foil and store in container at 4˚C, keep moist with running buffer or 0.1% SDS

**Recipe** for 12.5% homogeneous gels

| | **2 gels** | **4 gels** | **6 gels** |
|---|---|---|---|
| 40% acrylamide/bis 37.5:1 | 77.8 ml | 108.9 ml | 140 ml |
| Rhinohide | 20.8 ml | 29.2 ml | 37.5 ml |
| 1.5M Tris pH8.8 | 62.8 ml | 87.9 ml | 113 ml |
| Milli-Q $H_2O$ | 85.3 ml | 119.4 ml | 153.5 ml |
| 10% SDS | 2.5 ml | 3.5 ml | 4.5 ml |
| 10% APS, fresh | 1.0 ml | 1.4 ml | 1.8 ml |
| TEMED | 125 µl | 175 µl | 225 µl |
| Total | 250 ml | 350 ml | 450 ml |

**Preparation of DALTsix apparatus**

- get out and clean all parts of the DALTsix
- prepare 5l of 1x SDS-PAGE running buffer for lower tank (= anode), and 1.2l of 2x SDS-PAGE running buffer (or 1x buffer containing 2x the amount of SDS by adding 1/100 volume of 10% SDS stock) for upper tank (= cathode)
- assemble tank, fill with 4l lower tank buffer, switch on buffer circulation pump by plugging in black cable
- switch on MultitempIII water bath, adjust temperature (see below)
- prepare or melt agarose sealing solution (1% LMP agarose in upper tank buffer), 1-2ml per gel, let cool to ~60˚C in heat block
- get out and clean outsides of gels, rinse sample well with running buffer

**Equilibration of IPG strips**

- thaw equilibration buffer stock at room temperature
- for each 24cm strip, prepare 10ml of equilibration buffer 1 (+ 5mg/ml DTT, 50mg/10ml), and 10ml of equilibration buffer 2 (+ 45mg/ml iodoacetamide, 450mg/10ml), disolve by shaking at RT
- equilibrate strip for 15min each in buffer 1 and 2, at RT with gentle shaking

**Loading of strips on gels**

- place gel on bench, long plate on bottom
- wet strip with running buffer, place strip on long edge of plate, gel side up, acidic/+ end to the left of long plate
- place gel upright into rack, push backing of strip down onto gel surface with a thin spacer or ruler, avoid trapping bubbles
- seal strip into place by slowly pipetting agarose onto both ends of the strip

**Running gels**

- place gels into slots in anode assembly, fill empty slots with dummies
- slide upper buffer chamber over gels, lubricate with upper buffer if needed, fill upper buffer chamber to between marks, top up lower buffer to same level using funnel, close lid, [cover gel tank with black plastic bag for DiGE]
- start run; standard conditions: 25˚C, step1 - 1W/gel, 60min, step2 – 13W/gel, until blue front leaves gel, about 5-6 hours
  overnight: 30˚C, step1 - 1W/gel, 60min, step2 – 2W/gel, ~16h

## 6. DiGE gel scan

Gels have to be scanned right after the second dimension separation. If running three or more gels, scan two at a time and leave the rest in the gel tank under a very low voltage, or wrap in plastic foil and store at 4˚C in the dark. Allow to warm to room temperature before scanning.

- make sure that scanner is on, start Typhoon control software
- clean scanner platen with 70% ethanol, 10% $H_2O_2$, last with water
- take gels out of the tank
- rinse glass plates clean with distilled water, dry with Kimwipes
- place long gel alignment guide onto Typhoon scanner
- put gel “gripper” on short end of gel sandwich, metal parts will be on the bottom
- carefully lower gel sandwich onto scanner platen, avoid water or buffer getting under glass sandwich, note orientation
- use “DiGE” template in control software; important settings are:
  acquisition mode: fluorescence
  tray: DiGE Ettan DALT, select 1 or 2 gels
  focal plane: +3mm, press sample “on”
  DiGE naming format

| Dye | Emission filter | Laser |
|---|---|---|
| Cy2 | 520 BP 40 | Blue (488) |
| Cy3 | 580 BP 30 | Green (532) |
| Cy5 | 670 BP 30 | Red (633) |

- prescan at 1000µm resolution, to adjust PMT voltage, start out around 500V
- final scan at 100µm resolution

Note on adjustment of PMT voltage:

Intensity values can maximally be 100K. The values from pre-scans tend to be ~7K lower than in the high-resolution scan. Aim for a maximum of ~75K (check in ImageQuant) in the pre-scan to avoid oversaturation in the final scan.

For more details see ‘CIAN Typhoon mini-manual’.

## 7. Post-staining

Before starting a staining protocol, clean containers, locate reagents. Read detailed protocols for specific instructions, this is only an overview.

### Deep Purple total protein stain

All steps at RT, with gentle shaking; protect stain/stained gel from light; required volume per gel min. 300-500ml, 1l for fix, wash

| | | |
|---|---|---|
| Fixation | A: 1% citric acid<br>15% ethanol | 1.5h or over night |
| Stain | B: 1:200 thawed concentrate, diluted in<br>100mM sodium borate, pH10.5 | 1.5h |
| Wash | C: 15% ethanol | 30min |
| Acidification | A: 1% citric acid<br>15% ethanol | 30min (up to over night to reduce background) |
| Rinse | C: 15% ethanol | 5min, before imaging |
| Storage | D: 1% citric acid | |

Scan on Typhoon, excitation: green laser (532nm), emission: 560LP or 610BP; pre-scan 1000µm resolution, scan 100µm resolution

**Silver stain (compatible with mass spectrometry)** see separate protocol

**ProQ Diamond Phosphoprotein stain** see manufacturer's instructions; use *before* total protein stain if desired

### Sypro Ruby total protein stain

All steps at RT, with gentle shaking; protect stain/stained gel from light; required volume per gel ~300-500ml (?)

| | | |
|---|---|---|
| Fixation | 7% acetic acid<br>50% methanol | 30min, repeat |
| Stain | Ready-to-use stain | over night |
| Wash | 7% acetic acid<br>10% methanol | 30min |
| Rinse | Water | 5min, repeat |

Scan on Typhoon, excitation: blue laser (488nm), emission: 610BP; pre-scan 1000µm resolution, scan 100µm resolution

## 8. Data analysis with DeCyder

See below for introduction; use manual for details.

**DeCyder 6.5 overview and (very) short instructions**

**Gel Loader** – loading the image data from the Typhoon scanner into the Oracle database for use in DeCyder; only done once, images can be used for multiple analyses by creating new projects/workspaces that point to the same raw data

**DIA** – spot detection and first in-gel analysis for the images from each single gel

- *create workspace* (a DIA workspace is a folder that contains the DIA analysis data for one gel; it can be filed in the same project folder as the image data, or a new project can be generated)
- select gel images to be analyzed (remember, only the images of a single gel can be analyzed at one time)
- *process – process gel image* ; you may change estimated number of spots to match expectations, then look at the results in the scatter plot, adjust contrast and brightness of the image, but no further steps are necessary to proceed to BVA
- *save workspace* for use in BVA

**BVA** – analysis of DIA spot maps of all gels in one DIGE experiment, merges gel spot maps, normalizes intensities, statistical analysis (t-test, ANOVA)

- *create workspace* (a BVA workspace is a folder that contains the BVA analysis data for a DIGE experiment; it can be filed in the same project folder as the image and DIA data, or a new project can be generated)
- from the menu, select DIA workspaces to be included in this analysis
- assign images to groups in *Experimental Design View* in the upper corner of the ST, spot map table screen; the images of the internal standard should already be assigned to a group named “Standard”
- (define landmarks: in MT, match table, display standard images of all gels, check *landmark mode*, click on landmark spots in master image and then all the other images)
- Match gels
- Check matches in MT image view, display all Standard images and look at match vectors in zoom, if needed, break or add matches, rematch
- In PT, protein table view, perform protein statistics calculations
- Check potential candidate spots individually

**EDA** – extended data analysis of one or several BVA workspaces, unsupervised and supervised clustering algorithms (heat maps, SOM, k-means, Principle Component Analysis); short instructions not available

## 9. Spot picking workflow

- If picking from DiGE gels, treat samples and gels with care to **avoid keratin contamination**; keep handling to a minimum, wear protective clothing at all times.
- Consider loading **extra, unlabeled protein** along with CyDye labeled samples to increase the amount of protein in each spot.
- Check with MS facility about their staining preferences. Adjust protocol accordingly.
- After scanning gels, open glass plates carefully with wedge tool, **fix gels** in fixative appropriate to downstream stain.
- **Store gels** either in fixative, or in 1% acetic acid, or in ultrapure water, at 4˚C (cold room) while you do the Decyder analysis.
- When happy with pick list, contact MS facility to **make appointment** for sample or gel drop-off; specify number of spots to process.
- Time a fluorescent post-stain to be fresh for robotic picking; keep in mind that the post-stained gel image has to be loaded, matched, and annotated in Decyder before submitting for picking.
- **Stain gel**: silver or SyproRuby (DeepPurple) for floating gels, SyproRuby or DeepPurple for backed gels. Scan or image gel.
- For loading into **Decyder**, first make copy of your BVA workspace into a new project using the Organizer (from the Decyder main menu screen), to make sure you carry over all matching information, and annotations, but not to destroy the analysis should things not work as planned.
    - Load gels into new project containing copied BVA workspace.
    - Perform spot detection in DIA.
    - Open copy of BVA workspace, add pick gel image (‘edit – add template/DIA workspace’); to match, put master Cy2 image and pick gel image into windows, create a few landmarks, use ‘match primary’ option when matching (that way, all the other matched images stay as they are); check matching, especially of the pick list spots
- **Create images** to use for picking, only of the post-stained gel, with and without spot outlines and annotations. See document ‘CIAN Viewing and Editing of Typhoon Images’, section VII, for details.
- Gel or gel pieces submitted to MS facility should be in ultrapure water or 1% acetic acid. Check with your MS facility contact about the specific requirements.

# 10. Recipes of solutions provided in the CIAN DiGE lab

based on recipes in the GE DiGE manual

## SDS Equilibration Buffer Stock

| Reagent | Quantity | | Final concentration |
|---|---|---|---|
| Tris (1.0M, pH8.0) | 20ml | 40ml | 100mM |
| Urea (MW 60.06) | 72.07g | 144.14g | 6M |
| Glycerol (99.5% [v/v], MW92.09) | 60ml/75.6g | 120ml/151.2g | 30% (v/v) |
| SDS (MW288.33) | 4g | 8g | 2% (w/v) |
| Bromophenol Blue (1% stock) | 400µl | 800µl | 0.002% (w/v) |
| | | | |
| $H_2O$ MilliQ | to 200ml | to 400ml | |

## Rehydration Buffer

| Reagent | Quantity | Final concentration |
|---|---|---|
| Urea (MW 60.06) | 10.5g | 7M |
| Thiourea (MW 76.12) | 3.8g | 2M |
| CHAPS (MW 614.89) | 1g | 4% (w/v) |
| Bromophenol Blue (1% stock) | 50µl | 0.002% (w/v) |
| | | |
| $H_2O$ MilliQ | to 25ml | |

## SDS-PAGE Running Buffer, 10x

| Reagent | Quantity | Final concentration |
|---|---|---|
| Glycine (MW 75.07) | 1152g | 1.92M |
| Tris (MW 121.1) | 242g | 250mM |
| SDS (MW 288.38) | 80g | 1% (w/v) |
| | | |
| $H_2O$, destilled/RO | to 8l | |

## 11. Sources/Bibliography

**personal communication:**

Jackie Vogel, Hong Han, Renee Wang, Frédéric Dallaire, Madeleine Pool, Yovany Moreno, Wissal El-Assaad, all McGill University

David Friedman, Vanderbilt University, Nashville, TN, USA

Tracy Andacht, University of Georgia, Athens, GA, USA

**company manuals/handbooks*:**

Ettan DiGE System User Manual, GE Healthcare, 2005

2-D Electrophoresis – Principles and Methods, GE Healthcare, 2004

manuals for:
IPGphorII, Immobiline DryStrip IPG strips, DALTsix, MultiTempIII, Powersupply EPS 601, Deep Purple stain, Typhoon trio+ scanner, ImageQuant software, Decyder software; all GE Healthcare
Sypro Ruby stain, ProQ Diamond stain, Rhinohide; Invitrogen/Molecular Probes

* *pdf versions of most manuals available from the company websites, or from Elke*

**textbook:**

R Westermeier, T Naven, H-R Höpker (2008) Proteomics in Practice – A Guide to Successful Experimental Design, Wiley-VCH

**papers:**

S Viswanathan, M Ünlü, JS Minden (2006) Two-dimensional difference gel electrophoresis, *Nature Protocols* 1(3):1351-8
-- *protocol-style overview of the technique from the inventors.*

NS Tannu, SE Hemby (2006) Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling, *Nature Protocols* 1:1732-42
-- *another protocol, more focused on GE-products*

**other sources:**

GE Healthcare 2D Electrophoresis Discussion Board:
http://life-sciences-forums.com/
** Anyone can read and post on 2D-related issues; replies provided by forum members, some of whom are experts in the field*