DIBE™ coverage analysis

Instructions for Use

1 Safety

This protocol is for use by trained personnel under laboratory conditions only.

All chemicals must be considered potentially hazardous. When handling hazardous chemicals or biological agents, take all appropriate protective measures, such as wearing protective clothing, glasses and gloves.

See material safety data sheets and/or safety statements for specific advice.

The DIBE™ coverage analysis is a two-dimensional electrophoresis and Western blotting-based assay. For more safety information refer to the Cytiva reference materials: *2-D Electrophoresis, Principles and Methods (CY14825)* and *Western Blotting Principle and Methods Handbook*, available on the Cytiva website.

2 Introduction

Description

The Instructions for Use describe the coverage analysis based on two-dimensional differential in blot electrophoresis (2D-DIBE) designed to validate the Amersham™ HCPQuant ELISA kits. The coverage analysis determines the proportion of host cell proteins (HCP) that the ELISA kit recognizes (coverage) in a process specific sample. The coverage analysis should use the same purified antibodies as those present in Amersham HCPQuant ELISA kits.

CyDye™ Cy™3 labelled HCP are separated by 2D electrophoresis, transferred to a membrane, and probed with anti-HCP antibodies that are detected with a CyDye Cy5 secondary detection reagent. The fluorophores on the membrane are detected in two distinct channels to create an overlay of the 2D spot patterns into a multiplexed image. The presence or absence of individual protein spots detected by the anti-HCP antibodies (Cy5) is used to calculate coverage, which is expressed as a percentage of the total number of spots from the labelled HCP channel (Cy3).

The Instructions for Use provide the user with the information needed to prepare the protein sample and perform the coverage assay in two different formats: mini gels and large gels. To obtain the most accurate results, Cytiva recommends using the large gel format to increase the number of protein spots resolved.

Advantages of DIBE coverage analysis

Conventional ECL™ 2D coverage requires the use of two gels for each analysis. The HCPs in one gel are detected with a total protein stain, while the second gel is transferred to a membrane for western blotting with anti-HCP antibodies and ECL detection. The resulting images are then overlaid to determine coverage. Gel to gel variation can produce 2D patterns that are not identical, making overlay challenging. Additionally, the overlay of two different types of signal (total protein stain and ECL) is problematic and requires multiple exposures for the best results.

DIBE coverage analysis uses a single gel/membrane with prelabelled HCP. Detection of the fluorophores is performed in two channels on the same membrane, removing the overlay step. This reduces the risk of misinterpretation caused by poor alignment, simplifying the analysis and resulting in more accurate data.

The following illustration shows an example of the results obtained with DIBE coverage analysis.

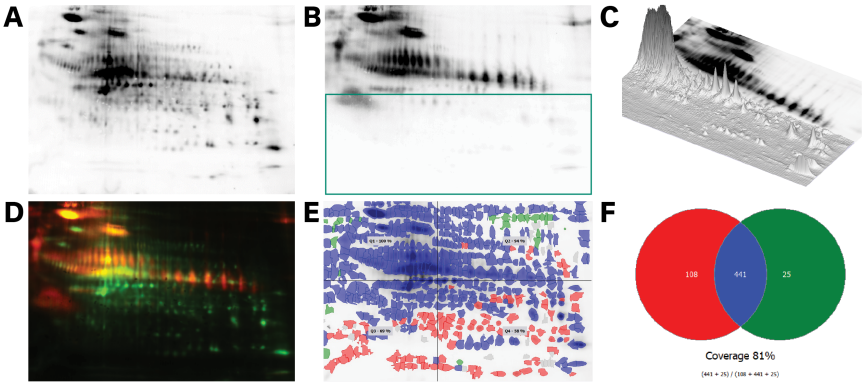

Figure 2.1: DIBE coverage analysis of a mock supernatant sample from a CHO-K1 cell line using the Amersham anti-CHO HCP (Supernatant) antibody. ***A****. 50 µg Cy3 labelled CHO supernatant proteins.* ***B****. Cy5 detected antibody signal.* ***C****. Enlarged 3D view of low molecular weight antibody signal.* ***D****. Two color overlay of Cy3 total protein and Cy5 antibody detected signals.* ***E****. Coverage analysis overlay in Melanie™.* ***F****. Coverage summary. Red spots: Only present in protein channel. Green spots: Only present in antibody channel. Blue spots: present in both protein and antibody channels.*

General recommendations

For accurate data submission to regulatory authorities, Cytiva recommends using an Amersham TYPHOON™ 5 or RGB imager, along with large format (24 cm) isoelectric focusing (IEF) Immobline™ DryStrips and DIGE gels.

For optimization and development, we recommend using the Amersham ImageQuant™ 800 Fluor system and small format (7 cm) gels, which support quicker analysis and savings on reagents.

Both options deliver high image quality and resolution, enabling precise spot definition with the 3D view in Melanie 9 coverage software.

Number of gels/membrane per product

Refer to the table below to estimate the number of gels/membrane that can be run using the products listed.

Product	Product code	Mini Gel	Large Gel
Primary antibody (100 µg) (1:2000 dilution)	See *Related products, on page 30* for available antibodies.	10	2
Primary antibody (1 mg) (1:2000 dilution)	See *Related products, on page 30* for available antibodies.	100	20
Amersham CyDye DIGE Cy3 Minimal (5 nmol)	25801083	12	4
ECL Plex™ Goat-α-Rabbit IgG-Cy5 (150 µg) (1:2500 dilution)	PA45011	18	3
Amersham ECL Prime Blocking Reagent (40g)	RPN418	13	2

Related documentation

- *Amersham HCP CHO solution (supernatant) Datafile (CY11454)*
- *2-D Electrophoresis Principles and Methods Handbook (80642960)*
- *Western Blotting Principle and Methods Handbook (28999897)*

Abbreviations

Term	Definition
CHO	Chinese hamster ovary
DIBE	Differential In Blot Electrophoresis
DIGE	Differential In Gel Electrophoresis
DMF	Dimethyl formamide
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-Linked ImmunoSorbent Assay
HCP	Host Cell Protein
IAA	Iodoacetamide
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
Mock cell line	Parent host cell line with empty vectors
Null cell line	Parent host cell line without vectors
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulphate
TCA	Trichloroacetic acid

3 Required materials

Materials

Cytiva recommends to use the materials listed below. See *Related products, on page 30* for a list of products.

Equipment
Amersham ImageQuant 800 Fluor [1]
Amersham TYPHOON 5 (or RGB)
Ettan™ IPGphor™ 3 Isoelectric Focusing Unit
Ettan IPGphor Manifold Complete
Incubation tray for 8 × 7 or 19 × 25 cm membranes
IPGbox
Melanie 9 Coverage
Membrane roller
SDS-PAGE apparatus
Mighty Small Transphor (TE 22 Mini Tank Transfer Unit) (wet transfer)[1]
TE 77 Transfer Unit or TE 77 PWR Transfer Unit (semi-dry transfer)
Thermostatic circulating bath

[1] The product is specific for mini gel protocol. See *Mini gels protocol, on page 20*.

Consumables

2-D Clean-Up Kit

2-D Quant Kit

Blotting paper

DryStrip Cover Fluid

Equilibration tube

Immobiline DryStrip pH 3–11 NL 7 cm[*]/24 cm[†]

Paper electrode wicks

pH indicator papers

Precast 2D mini gel[*]/DIGE Gel[†]

PVDF membrane[‡]

Sample Application Pieces

[*] The product is specific for mini gel protocol, see *Mini gels protocol, on page 20*.
[†] The product is specific for large gel protocol, see *Large gels protocol, on page 24*.
[‡] Use a PVDF membrane compatible with fluorescence applications.

Chemicals

Amersham ECL Prime Blocking Reagent

Bromophenol blue

CyDye DIGE Cy3

DeStreak Rehydration Solution

Dimethylformamide (anhydrous) 99.8% (DMF)

Dimethyl Sulphoxide 99.9% (DMSO) (optional)

Dithiothreitol (DTT)

Ethanol 99.9%

Glycine

Glycerol

IPG Buffer pH 3–11 NL

Iodoacetamide (IAA)

Chemicals

Low melting point agarose

Lysine

Molecular weight marker

Protease inhibitor

Sodium dodecyl sulfate (SDS)

Sodium hydroxide

Trichloroacetic acid (TCA)

Thiourea

Tris

Urea

Antibody reagents

Primary anti-HCP antibody from the same antisera batch as the ELISA kit being validated by this assay.

Secondary Cy5 conjugated anti-IgG antibody, raised against the host species of the primary antibody.

Solutions

The following solutions are required.

Agarose overlay solution

25 mM Tris, 192 mM glycine, 0.5% low melting point agarose, 0.2% SDS, 0.02% bromophenol blue.

Anode transfer buffer (semi-dry transfer only)

48 mM Tris, 39 mM glycine, 20% ethanol.

Blocking solution

5% (w/v) Amersham ECL Prime Blocking Reagent in wash solution (PBS-T).

Bromophenol blue solution

1% Bromophenol Blue, 0.06% Tris.

Cathode transfer buffer (semi-dry transfer only)

48 mM Tris, 39 mM glycine, 1.3 mM SDS.

CyDye DIGE conjugation buffer

30 mM tris, 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, pH 8.5.

Equilibration buffer stock *

75 mM Tris, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, pH 8.8.

Equilibration solution 1 †

10 mg/mL DTT in equilibration buffer stock.

Equilibration solution 2 †

25 mg/mL iodoacetamide in equilibration buffer stock.

DIGE quenching buffer

10 mM lysine.

NaOH solution

100 mM NaOH.

Phosphate buffered saline (PBS)

137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4.

SDS running buffer (10×) — Laemmli buffer

250 mM Tris, 1.92 M glycine, 1% (w/v) SDS.

Transfer buffer (wet transfer only — mini gels protocol)

25 mM Tris, 192 mM glycine, 20% ethanol.

Wash solution (PBS-T)

0.05% Tween-20 in PBS.

* The equilibration buffer stock can be aliquoted and stored at -20°C for several months.
† Prepare fresh solution for each experiment.

4 Preparation of solutions and buffers

Add the quantities listed on the last column to prepare the solutions and buffers.

Agarose overlay solution

25 mM Tris, 192 mM glycine, 0.5% low melting point agarose, 0.2% SDS, 0.02% bromophenol blue, 100 mL) [1]

Low melting point agarose	0.5%	0.5 g
SDS running buffer (10×)		10 mL
Bromophenol blue solution		200 µL
Ultrapure water		to 100 mL

[1] Provided with DIGE Gel.

Anode transfer buffer (semi-dry transfer only)

(48 mM Tris, 39 mM glycine, 20% ethanol, 1 L)

Tris	48 mM	5.8 g
Glycine	39 mM	2.9 g
Ethanol	20%	200 mL
Ultrapure water		to 1 L

Store at room temperature.

Antibody stock solutions

Dilute primary and secondary antibody to 1 mg/mL. Store according manufacturing instructions.

Blocking Solution

(137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween-20, 5% Amersham ECL Prime Blocking Reagent, pH 7.4, 200 mL)

Amersham ECL Prime Blocking Reagent (RPN418)	5%	10 g
Wash solution		to 200 mL

Prepare on the day of use. Store at 2°C to 8°C for maximum one day.

Bromophenol blue solution

(1% Bromophenol Blue, 0.06% Tris, 10 mL)		
Bromophenol Blue	1%	100 mg
Tris	0.06%	60 mg
Ultrapure water		to 10 mL

Store at room temperature.

Cathode transfer buffer (semi-dry transfer only)

(48 mM Tris, 39 mM glycine, 1.3 mM SDS, 1 L)		
Tris	48 mM	5.8 g
Glycine	39 mM	2.9 g
SDS (Sodium Dodecylsulfate)	1.3 mM	0.37 g
Ultrapure water		to 1 L

Store at room temperature.

CyDye DIGE conjugation buffer

(7 M urea, 2 M thiourea, 4 % CHAPS, 30 mM Tris, 50 mL)		
Urea	7 M	21 g
Thiourea	2 M	7.6 g
CHAPS	4 %	2 g
Tris (1M, pH 8.8)	30 mM	1.5 mL
Ultrapure water		to 50 mL

Store at room temperature.

DIGE quenching buffer

(10 mM lysine, 10 mL)		
Lysine (M_W 182.6)	10 mM	18 mg
Ultrapure water		to 10 mL

Divide into 1 mL aliquots and store in freezer

Equilibration buffer stock

(6 M urea, 2% SDS, 75 mM Tris , 30% glycerol, pH 8.8, 100 mL)		
Urea	6 M	72 g
SDS	2%	4 g
Tris (1 M, pH 8.8)	75 mM	15 mL
Glycerol	30%	70 mL
Ultrapure water		to 200 mL

Store in freezer.

Equilibration solution 1

(DTT 1% (w/v), 100 mL)		
DTT	1% (w/v)	1 g
Equilibration buffer stock		to 100 mL

Prepare fresh.

Equilibration solution 2

(IAA 2.5% (w/v), 100 mL)		
IAA	2.5% (w/v)	2.5 g
Equilibration buffer stock		to 100 mL

Prepare fresh.

NaOH Solution

(100 mM NaOH, 10 mL)		
NaOH (1 M)	100 mM	1 mL
Ultrapure water		to 10 mL

Store at room temperature.

Phosphate buffered saline (PBS)

(137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 1 L)

NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	10 mM	1.44 g
KH_2PO_4	1.8 mM	0.24 g
Ultrapure water		to 1 L

Adjust pH to 7.4 with HCL.

Store at room temperature.

Phosphate buffered saline (PBS) 10×

(1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, pH 7.4, 1 L)

NaCl	1.37 M	80 g
KCl	27 mM	2 g
Na_2HPO_4	100 mM	14.4 g
KH_2PO_4	18 mM	2.4 g
Ultrapure water		to 1 L

Adjust pH to 7.4 with HCL. Store at room temperature.

SDS running buffer (10×) — Laemmli buffer

(250 mM Tris, 1.92 M glycine, 1% (w/v) SDS, 1 L)

Tris	250 mM	30.4 g
Glycine	1.92 M	144.0 g
SDS	1% (w/v)	10 g
Ultrapure water		to 1 L

Store at room temperature.

100% (w/v) TCA solution

CAUTION
Use Personal Protective Equipment (PPE) when handling TCA.

(100% (w/v) TCA, about 50 mL)

TCA	100% (w/v)	100 g
Ultrapure water		45.4 mL

Store in dark bottle at 4°C.

Transfer buffer (wet transfer only — mini gels protocol)

(25 mM Tris, 192 mM glycine, 20% ethanol, 1 L)

Tris	25 mM	3.0 g
Glycine	192 mM	14.4 g
Ethanol	20%	200 mL
Ultrapure water		to 1 L

Store at room temperature.

Wash solution (PBS-T)

(137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween-20, pH 7.4, 1 L)

Tween-20	0.05%	0.5 mL
PBS		to 1 L

Store at room temperature.

5 Sample Preparation

5.1 Prepare sample for Cy3 labeling and 2D gel electrophoresis

Follow the steps below to remove buffers and impurities from the protein sample that might affect labeling. This protocol also concentrates the proteins in supernatant samples to a volume and concentration that is suitable for separation by 2D gel electrophoresis.

Note: *Grow the cells for the assay according to your own validated procedure.*

Note: *Prepare the HCP sample from a cell line that does not express the product (null/mock cell line) to avoid the product from affecting the assay. To obtain the most accurate results from the assay, grow the null/mock cell line in the same process specific conditions required for the product.*

This protocol is developed for supernatant proteins. For lysate samples, contact your Cytiva representative for more information about sample preparation.

For supernatant protein samples, proceed to *Sample cleanup for samples less than 100 µg, on page 15* or *Sample cleanup for samples greater than 100 µg, on page 17* depending on protein amount.

Sample cleanup for samples less than 100 µg

Follow the steps below to remove buffers and impurities from samples with a total protein amount less than or equal to 100 µg using a 2-D Clean-Up Kit (80648451).

Note: *Keep the tubes on ice in all steps, unless otherwise specified.*

Step	Action
1	If unknown, measure the protein concentration in the sample using 2-D Quant Kit (80648356) or other compatible assay.
2	Transfer the sample into a tube that can be centrifuged at 8 000 × g. ***Note:*** *The tube must have a capacity at least 12× greater than the volume of the sample. Use only polypropylene, polyallomer or glass tubes.*
3	Add three volumes of Precipitant to the sample. Vortex and incubate on ice for 15 min.
4	Add three volumes of the original sample of co-precipitant and vortex to mix.

Step	Action
5	Centrifuge the tubes at 8 000 × g for 10 min. As soon as centrifugation is complete, proceed rapidly to the next step to avoid resuspension or diffusion of the pellet.
6	Remove the supernatant by decanting without disturbing the pellet.
7	Pulse the samples again in the centrifuge (1 min) and then carefully remove the remaining supernatant with a pipette.
8	Add 100 µL co-precipitant to each samples, without disturbing the pellet.
9	Centrifuge the samples again (5 min) and then carefully remove the remaining supernatant with a pipette.
10	Pipette 50 µL ultrapure water onto each pellet and vortex to disperse. ***Note:*** *The pellet should disperse, but not dissolve in the water.*
11	Add 1 mL of pre-chilled (-20°C) wash buffer and 5 µl of wash additive. Vortex until the pellet is fully dispersed.
12	Incubate the tubes at -20°C for 30 min. Vortex the samples 20 to 30 s once every 10 min.
13	Centrifuge the tubes at 8 000 × g for 10 min then carefully remove and discard the supernatant. A white pellet should be visible.
14	Allow the pellet to air dry briefly (no more than 5 min). ***Note:*** *Do not over-dry the pellet as it might become difficult to resuspend.*
15	Resuspend the pellet in CyDye DIGE conjugation buffer.
16	Measure the protein concentration in the sample using 2-D Quant Kit (80648356) or other compatible assay. ***Note:*** *For optimal performance of the coverage kit, the final concentration of protein should be 5 to 10 mg/mL.*

Proceed to label the sample with Cy3 dye.

Sample cleanup for samples greater than 100 µg

Follow the steps below to remove buffers and impurities from supernatant and cell lysate samples with a total protein amount greater than 100 µg.

Note: *Keep the tubes on ice in all steps, unless otherwise specified.*

Note: *The protocol requires ice cold acetone. Store acetone in the freezer for 1 hour before use.*

Step	Action
1	Measure the protein concentration in the sample using 2-D Quant kit (80648356) or other compatible assay, if unknown.
2	Add a volume of 100% (w/v) TCA solution to the sample to give a final concentration of 13% TCA.
	Note: *Use only polypropylene, polyallomer, or glass tubes.*
3	Incubate the sample at -20°C for 5 min.
4	Incubate the sample at 4°C for an additional 15 min.
5	Centrifuge at 10 000 × g for 10 min.
6	Remove the supernatant by decanting without disturbing the pellet.
7	Pulse the samples again in the centrifuge (1 min) and then carefully remove the remaining supernatant with a pipette.
8	Add 1 mL 100% ice cold acetone.
	Note: *The pellet should disperse, but not dissolve in the acetone.*
9	Incubate at -20°C for 90 min.
	Note: *The sample can be also incubated at -20°C overnight.*
10	Centrifuge at 10 000 × g for 10 min.
11	Remove supernatant and air dry the pellet .
	Note: *Do not overdry the sample as it might become difficult to resuspend.*

Step	Action
12	Resuspend pellet in CyDye DIGE conjugation buffer. ***Note:*** *For optimal performance of the coverage kit, the final concentration of protein should be 5 to 10 mg/mL.*

Proceed to label the sample with Cy3 dye.

5.2 Label sample with Cy3

Follow the steps below to label the protein sample with Cy3 dye. For more information refer to *Appendix A CyDye labeling, on page 40*.

Step	Action
1	Prepare 1 mM stock solution by dissolving the lyophilized Cy3 dye in 5 µL DMF. ***Note:*** *Store the unused stock solution at -20°C in the dark.* ***Note:*** *DMSO can be used instead of DMF.*
2	Prepare a 400 µM Cy3 dye working solution by diluting the stock solution 1:1.5 with DMF. ***Note:*** *Store the working solution at -20°C in the dark, for up to one week.* ***Note:*** *DMSO can be used instead of DMF.*
3	Apply 1 µL of the protein sample to a pH indicator strip and check that the pH is between 8 and 9. If required, adjust the pH using 50 mM NaOH.
4	Transfer a sample volume containing 50 µg protein (for a mini gel) or 150 µg protein (for a large gel) to a new microcentrifuge tube.
5	Add 1 µL Cy3 dye working solution for every 50 µg of protein to be labelled, mix, and centrifuge briefly. Incubate on ice for 30 min in the dark.
6	Add 1 µL DIGE quenching buffer to stop the reaction, mix with a pipette, and incubate on ice for 10 min in the dark.
7	Optional: run 0.5 µl or less of the labelled sample in 1D SDS-PAGE gel.
8	Optional: acquire an image of the gel using the Cy3 excitation and emission settings to verify protein labeling.
9	Proceed to first-dimension isoelectric focusing (IEF). ***Note:*** *Labeled samples can be stored at -70°C for up to 3 months.*

6 Mini gels protocol

Note: *When possible, avoid exposing the labeled samples to direct light during the assay.*

6.1 IEF

Follow the steps below to rehydrate the Immobiline DryStrip and separate the protein sample with IEF.

Note: *Refer to the 2-D Electrophoresis Principles and Methods Handbook (80642960) for alternative loading methods.*

Step	Action
1	Mix 3 mL DeStreak Rehydration Solution with 15 µL IPG Buffer pH 3–11 NL.
2	Bring the volume of the CyDye labelled sample to 125 µL with the solution from step 1. Mix well.
3	Apply the solution into a channel of the Reswell Tray of the IPGbox.
4	Remove the protective cover from a 7 cm Immobiline DryStrip pH 3–11 NL.
5	Place the Immobiline strip into the Reswell Tray channel with the gel side down. Make sure not to trap bubbles under the strip.
6	Incubate overnight at room temperature on a level surface.
7	Follow *Ettan IPGphor 3 Operating Instructions (28963551)* to set up the IEF system.
8	Run the strip at 75 µA and 20°C, using the following protocol steps. a. Step 300 V for 200 Vhr b. Gradient 1000 V for 300 Vhr c. Gradient 5000 V for 4000 Vhr d Step 5000 V for 1250 Vhr
9	Optional: acquire an image of the gel using the Cy3 excitation and emission settings to verify protein IEF separation. ***Note:*** *After completing the IEF, the strip can be stored at -20°C for several months.*

6.2 2D SDS-PAGE

Follow the steps below to separate the protein sample by 2D SDS-PAGE.

Note: *Prepare 5 mL equilibration solution 1 and 5 mL equilibration solution 2 for each test sample from the equilibration stock buffer.*

Step	Action
1	Transfer the strip to a 15 mL disposable tube containing 5 mL equilibration solution 1.
2	Incubate for 15 minutes with gentle shaking.
3	Remove the solution from the tube and add 5 mL equilibration solution 2.
4	Incubate for 15 minutes with gentle shaking.
5	Transfer the strip into the well of a precast 2D mini gel. ***Tip:*** *A small amount of SDS running buffer can be applied to the well to help in the placement of the strip.*
6	Seal the strip by applying melted agarose overlay solution into the well. ***Note:*** *Cytiva recommends loading a molecular weight marker to assist with subsequent coverage analysis. If the 2D gel does not have an additional well for loading the marker, apply the marker to a sample application piece and place it adjacent to the IEF strip before sealing with overlay solution.*
7	Follow the manufacturer´s instructions for setting up the electrophoresis apparatus and for running conditions.
8	Run the gel until the dye front reaches the end of the gel. ***Note:*** *Running time can differ between gels from different manufacturers.*
9	Remove the gel from its cassette.
10	Optional: acquire an image of the gel using the Cy3 excitation and emission settings to verify protein separation. ***Note:*** *Make sure the gel stays wet during image acquisition.*

6.3 Transfer

Two transfer protocols are available for mini gels: wet and semi-dry transfer.

Wet transfer

Follow the steps below to transfer the protein sample by wet transfer.

Step	Action
1	Cut the PVDF membrane to the same size as the gel.
2	Pre-wet the PVDF membrane in 99.9% ethanol for 2 minutes.
3	Soak the gel, the pre-wetted PVDF membrane, and two pieces blotting paper in transfer buffer for 10 minutes.
4	Prepare the transfer sandwich according to *MIGHTY SMALL TRANSPHOR Operating Instructions (29277869)*.
5	Transfer at 400 mA for 60 minutes. ***Note:*** *For optimal transfer cool the transfer buffer during the procedure by using a thermostatic circulating bath.*
6	Optional: acquire an image of the membrane using the Cy3 excitation and emission settings to verify protein transfer. ***Note:*** *Make sure the membrane stays wet during image acquisition.*
7	Proceed to *Antibody probing, on page 27*.

Semi-dry transfer

Follow the steps below to transfer the protein sample by semi-dry transfer.

Step	Action
1	Cut the PVDF membrane to the same size as the gel.
2	Pre-wet the PVDF membrane in 99.9% ethanol for 2 minutes.
3	Remove the gel from the glass cassette.
4	Soak the gel in cathode buffer and the pre-wet PVDF membrane in anode buffer for 10 minutes.

Step	Action
5	Soak three pieces of blotting paper in anode buffer and three pieces in cathode buffer.
6	Prepare the transfer sandwich according to the *Amersham ECL Semi-dry Blotters User Manual (28402591)*. ***Note:*** *Make sure that the correct transfer buffer is used on each side of the transfer sandwich.* ***Tip:*** *To minimize disturbances in the transfer, remove bubbles from the sandwich using a membrane roller.*
7	Transfer at 0.8 mA/cm^2 for 60 minutes. ***Tip:*** *Transfer at 45 mA per gel, for standard 8 × 7 cm gels.*
8	Optional: acquire an image of the membrane using the Cy3 excitation and emission settings to verify protein transfer. ***Note:*** *Make sure the membrane stays wet during image acquisition.*
9	Proceed to *Antibody probing, on page 27*.

7 Large gels protocol

Note: *When possible, avoid exposing the labeled samples to direct light during the assay.*

7.1 IEF

Follow the steps below to rehydrate the Immobiline DryStrip and separate the protein sample with IEF.

Note: *Refer to the 2-D Electrophoresis Principles and Methods Handbook (80642960) for alternative loading methods.*

Step	Action
1	Mix 3 mL DeStreak Rehydration Solution with 15 µL IPG Buffer pH 3–11 NL.
2	Bring the volume of the CyDye labelled sample to 450 µL with the solution from step 1. Mix well.
3	Apply the solution into a channel of the Reswell Tray of the IPGbox.
4	Remove the protective cover from a 24 cm Immobiline DryStrip pH 3–11 NL.
5	Place the Immobiline strip into the Reswell Tray channel with the gel side down. Make sure not to trap bubbles under the strip.
6	Incubate overnight at room temperature on a level surface.
7	Follow *Ettan IPGphor 3 Operating Instructions (28963551)* to set up the IEF system.
8	Run the strip with current limit at 75 µA and 20°C, using the following protocol steps. a. Step 500 V for 500 Vhr b. Gradient 1000 V for 800 Vhr c. Gradient 10000 V for 16500 Vhr d Step 10000 V for 22200 Vhr
9	Optional: acquire an image of the gel using the Cy3 excitation and emission settings to verify protein IEF separation. ***Note:*** *After completing the IEF, the strip can be stored at -20°C for several months.*

7.2 2D SDS-PAGE

Follow the steps below to separate the protein sample by 2D SDS-PAGE.

Note: *Prepare 15 mL equilibration solution 1 and 15 mL equilibration solution 2 for each test sample from the equilibration stock buffer.*

Step	Action
1	Transfer the strip into a disposable tube or tray containing 15 mL equilibration solution 1.
2	Incubate for 15 minutes under gentle shaking.
3	Remove the solution from the tube and add 15 mL equilibration solution 2.
4	Incubate for 15 minutes under gentle shaking.
5	Transfer the strip into the well of a precast DIGE gel. ***Tip:*** *A small amount of SDS running buffer can be applied to the well to help in the placement of the strip.*
6	Seal the strip by applying melted agarose overlay solution into the well. ***Note:*** *Cytiva recommends loading a molecular weight marker to assist with subsequent coverage analysis. Apply the marker to a sample application piece and place it adjacent to the IEF strip before sealing with overlay solution.*
7	Follow the manufacturer´s instructions for setting up the electrophoresis apparatus and for running conditions.
8	Run the gel until the dye front reaches the end of the gel. ***Note:*** *Running time can differ between gels from different manufacturers.*
9	Optional: acquire an image of the gel using the Cy3 excitation and emission settings to verify protein separation. DIGE gels from Cytiva can be scanned while still in the cassette. ***Note:*** *Make sure the gel stays wet during image acquisition.*

7.3 Transfer

Follow the steps below to transfer the protein sample by semi-dry transfer.

Step	Action
1	Cut the PVDF membrane to the same size as the gel.
2	Pre-wet the PVDF membrane in 99.9% ethanol for 2 minutes.
3	Remove the gel from the glass cassette.
4	Soak the gel in cathode buffer and the pre-wet PVDF membrane in anode buffer for 10 minutes.
5	Soak three pieces of blotting paper in anode buffer and three pieces in cathode buffer.
6	Prepare the transfer sandwich according to the *Amersham ECL Semi-dry Blotters User Manual (28402591)*. ***Note:*** *Make sure that the correct transfer buffer is used on each side of the transfer sandwich.* ***Tip:*** *To minimize disturbances in the transfer, remove bubbles from the sandwich using a membrane roller.*
7	Transfer at 0.8 mA/cm^2 for 60 minutes. ***Tip:*** *Transfer at 400 mA for DIGE gels.*
8	Optional: acquire an image of the membrane using the Cy3 excitation and emission settings to verify protein transfer. ***Note:*** *Make sure the membrane stays wet during image acquisition.*
9	Proceed to *Antibody probing, on page 27*.

8 Antibody probing

Follow the steps below to probe the membrane with the anti-HCP antibody.

Step	Action
1	Prepare for each membrane the following volumes of blocking buffer, wash buffer, and PBS.

	Mini gel	Large gel
Blocking buffer[1]	60 mL	300 mL
Wash buffer	140 mL	700 mL
PBS	20 mL	100 mL

[1] Store unused blocking buffer at 2°C to 8°C for the secondary antibody probing on day 2.

2 Incubate the membrane in 20 mL (mini gel) / 100 mL (large gel) of blocking buffer for 1 hour, under constant shaking.

3 Discard the blocking buffer and add the primary antibody stock solution (1 mg/mL) diluted 1:2000 in 20 mL (mini gel) / 100 mL (large gel) of the blocking buffer.

Note:
Optimization of the primary antibody dilution might be needed.

4 Incubate the membrane overnight at 4°C under constant shaking.

Note:
Alternatively, the membrane can be incubated for 1 hour at room temperature under constant shaking.

5 Remove the antibody solution and wash the membrane 4 × 5 minutes with 20 mL (mini gel) / 100 mL (large gel) wash buffer.

6 Add Cy5 labeled secondary antibody stock solution (1 mg/mL) diluted 1:2500 in 20 mL (mini gel) / 100 mL (large gel) blocking buffer.

Note:
Optimization of the secondary antibody dilution might be needed.

7 Incubate the membrane 1 hour at room temperature under constant shaking.

8 Remove the detection reagent solution and wash the membrane 3 × 5 minutes with 20 mL (mini gel) / 100 mL (large gel) wash buffer.

Step	Action
9	Wash the membrane with 20 mL (mini gel) / 100 mL (large gel) PBS for 5 minutes.
10	Air-dry the membrane.
11	Proceed to the next chapter.
	Note: *The membrane can be stored in the dark at room temperature for several months.*

9 Detection and analysis

Cytiva recommends to use Amersham TYPHOON RGB/5 or Amersham ImageQuant 800 Fluor for image collection and Melanie 9 coverage software for image analysis.

Scan the membrane using the Cy3 and Cy5 excitation and emission settings to detect total HCP and antibody bound HCP respectively. For more information on coverage analysis using Melanie 9, see *http://www.2d-gel-analysis.com*.

10 Related products

ELISA, antibodies and DIBE reagents	Product code
Amersham HCPQuant CHO kit (Supernatant)	29496737
Amersham anti-CHO HCP Antibody (Supernatant), 100 µg	29496739
Amersham anti-CHO HCP Antibody (Supernatant), 1 mg	29496740
Amersham anti-CHO HCP Antibody (Lysate), 100 µg	29411337
Amersham anti-CHO HCP Antibody (Lysate), 1 mg	29411368
ECL Plex Goat-α-Rabbit IgG-Cy5 (150 µg)	PA45011
Amersham DIBE HCP Detection Kit	29613962
Amersham CyDye DIGE Fluor Cy3 minimal dye, 5 nmol	25801083
Amersham ECL Prime Blocking Reagent	RPN418

Equipment	Product code
Amersham ImageQuant 800 Fluor	29399484
Amersham TYPHOON 5	29187191
Amersham TYPHOON RGB	29187193
EPS 3501XL Power Supply Unit	18113005
Ettan IPGphor 3 Isoelectric Focusing Unit	11003364
Ettan IPGphor Manifold Complete	80649838
IPGbox	28933465
MiniVE Electrophoresis Unit	80641877
TE 22 Mini Tank Transfer Unit	80620426
TE 77 PWR Transfer Unit	11001342
TE 77 Semi-Dry Transfer Unit	TE77

Consumable	Product code
2-D Clean-Up Kit	80648451

Consumable	Product code
2-D Quant Kit	80648356
Amersham Hybond P 0.45 PVDF (300mm x 4m)	10600023
Amersham Hybond P 0.45 PVDF (80x90mm)	10600100
Blotting paper (10×10cm)	10426880
Blotting Paper (21×26cm)	TE76
Cleaning Solution IPGPhor Strip Holder	29011927
DIGE Buffer Kit	28937452
DryStrip Cover Fluid	17133501
DIGE Gel	28937451
ECL Plex Rainbow Ladder	RPN850E
Equilibration tube	80646779
Immobiline DryStrip pH 3–11 NL 7 cm	17600373
Immobiline DryStrip pH 3–11 NL 24 cm	17600377
pH Indicator Papers pH 8.0 – 10.0	2600-104A
Paper electrode wicks	80649914
Sample Application Pieces	80112946

Chemical	Product code
Agarose NA	17055401
PlusOne Bromophenol Blue	17132901
DeStreak Rehydration Solution	17600319
Glycerol	17132501
Glycine	17132301
Iodoacetamide	RPN6302
IPG Buffer pH 3–11 NL	17600440
PlusOne Dithiothreitol	17131802

Chemical	Product code
Sodium Dodecyl Sulfate	17131301
Tris	17132101
Thiourea	RPN6301
Urea	17131901

Software	Product code
Melanie 9 Coverage Floating Licence	29455821
Melanie 9 Coverage Node-locked Licence	29455822

11 Troubleshooting

IEF

Possible cause	Corrective action
Problem: voltage below the set limit	
High ionic strength of the sample	Reduce the ionic strength of the sample to less than 10 mM by performing buffer exchange.
High carrier ampholytes concentration	Reduce the concentration of IPG buffer to 0.5%.
Incorrect carrier ampholytes formulation	Use the IPG buffer with the corresponding pI range of the Immobiline DryStrip (e.g. 3-11 NL).
Problem: current too low or zero	
Poor contact of electrodes with the Immobiline DryStrip	Make sure that the electrodes are in contact with the paper wicks and that the paper wicks are wetted with distilled water. Make sure that the paper wicks sufficiently overlap the gel of the Immobiline DryStrip. Consult the equipment instructions for specific guidance.
Insufficient rehydration of the Immobiline DryStrip	Rehydrate the Immobiline DryStrip with the correct volume of solution according to the manufacturer's instructions.
Problem: sparking or burning of the Immobiline DryStrip	
High current setting	Do not exceed the maximum current setting of 50 µA per Immobiline DryStrip (75 µA for 24 cm strips).
Problem:Immobiline DryStrip remains blue	
Poor contact of electrodes with Immobiline DryStrip	Make sure that the electrodes are in contact with the paper wicks and that the paper wicks are wetted with distilled water. Make sure that the paper wicks sufficiently overlap the gel of the Immobiline DryStrip. Consult the equipment instructions for specific guidance.

Possible cause	Corrective action
Problem: Immobiline DryStrip turns white	
Insufficient rehydration volume	Rehydrate the Immobiline DryStrip with the correct volume of solution according to the manufacturer's instructions.
Immobiline DryStrip dried out during focusing	Make sure that the Immobiline DryStrip is covered with Immobiline DryStrip Cover Fluid to prevent dehydration.
Immobiline DryStrip dried out due to prolonged air exposure	Avoid leaving the Immobiline DryStrip exposed to air. For extended storage, store the strips at -20°C.

2D SDS-PAGE

Possible cause	Corrective action
Problem: vertical streaks	
Insufficient equilibration of strips	Equilibrate the Immobiline DryStrip with each equilibration solution for 15 minutes.
Insufficient concentration of SDS in the running buffer	Adjust the concentration of SDS in the running buffer to 0.1%.
Problem: horizontal streaks	
Sample under-focused	Increase the focusing time.
Impurities in the sample during focusing	Increase the focusing time. Remove impurities using the 2D Clean Up kit or buffer exchange.
Problem: individual proteins appear as multiple spots or are missing, unclear or in the wrong position	
Oxidation of the sample	Use DeStreak Rehydration buffer or DeStreak reagent if using an alternative rehydration buffer. During equilibration, add DTT in the first step to reduce the disulfide bonds. Add iodoacetamide in the second step to alkylate the thiol groups to prevent proteins from reoxidizing.

Possible cause	Corrective action
Carbamylation of the sample	Do not heat any solutions containing urea above 30°C. Cyanate (a urea degradation product) carbamylates proteins and changes their pI.
Poor contact of the strip with the 2D gel	Avoid bubbles when placing the strip into the gel and applying the sealing solution.
Problem: no distinct spots are visible in the gel	
Insufficient sample amount	Increase the amount of protein used.
Impurities in the sample during focusing	Increase the focusing time. Remove impurities using the 2D Clean Up kit or buffer exchange.
Incorrect alignment of the pH gradient	Make sure the immobiline DryStrip is placed in the correct orientation according to polarity.
Poor CyDye labeling of the sample	Make sure that the pH of the sample is between 8 and 9 before labeling. Use the correct ratio of 400 nmol CyDye to 50 µg protein.
Problem: intense, dominating spot present in the 2D spot pattern	
Biologic product in the sample	Perform 2D DIBE with mock cell lines that do not express biologic product.
Problem: slow gel run	
Incorrect concentration of running buffer	Use the correct running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.
Leaking current	Make sure that there is a watertight seal between the anode and cathode chambers of the tank.
Expired acrylamide	Make sure the acrylamide or precast gels have not expired.
Problem: gel smile	
High temperature during focusing	Use active cooling.
High current or voltage	Lower the current or the voltage limits.

Semi-dry transfer

Possible cause	Corrective action
Problem: a significant proportion of proteins remains in the gel after transfer	
Ethanol in the buffer used to equilibrate the gel	Equilibrate gel with transfer buffer containing SDS (cathode transfer buffer) before transfer.
Incorrect orientation of the gel stack	Make sure that the filter papers soaked in anode transfer buffer (containing ethanol) are on the membrane side of the stack, and the blotting papers soaked in cathode transfer buffer (containing SDS) are on the membrane side of the stack.
Problem: the proteins spots on the membrane appear smeared, smudged, or diffuse	
SDS in the buffer used to equilibrate the membrane	Equilibrate membrane with transfer buffer containing ethanol (anode transfer buffer) before transfer.
Incorrect orientation of the gel stack	Make sure that the blotting papers soaked in anode transfer buffer (containing ethanol) are on the membrane side of the stack, and the blotting papers soaked in cathode transfer buffer (containing SDS) are on the gel side of the stack.
Degraded ethanol in the transfer buffer	Prepare fresh transfer buffer for each experiment.
Gel not equilibrated	Equilibrate gel with transfer buffer containing SDS (cathode transfer buffer) for 10 minutes before transfer.
Problem: no protein spots on membrane	
Incorrect orientation of the gel stack	Make sure that the membrane is on the anodal side of the stack and the gel is on the cathodal side of the stack. Consult the transfer equipment instructions for the correct orientation.
Problem: fewer spots than expected appear on the membrane	
Current bypasses the gel	Make sure that the filter papers and membrane are cut to the same size as the gel.
Dried areas of the membrane	Assemble the transfer stack quickly to prevent the membrane from drying. Do not leave wetted membranes exposed to the air.

Possible cause	Corrective action
Incomplete hydration of the membrane	Do the following: 1. Pre-wet the PVDF membrane in 99.9% ethanol for 2 minutes. 2. Equilibrate the pre-wetted PVDF membrane in anode buffer for 10 minutes.
Handling issues	Avoid touching the membrane. Always wear gloves and handle the membrane with blunt ended tweezers.

Wet transfer

Possible cause	Corrective action
Problem: a significant proportion of proteins remain in the gel after transfer	
Insufficient electrophoresis conditions	Run the transfer with a current limit of 400 mA. Consult the equipment instructions for instrument specific guidance.
Insufficient transfer time	Transfer for at least 60 minutes.
High concentration of ethanol in the transfer buffer	Reduce the concentration of ethanol in the transfer buffer to 15%.
Problem: the proteins spots on the membrane appear smeared, smudged, or diffuse	
SDS in the buffer used to equilibrate the membrane	Equilibrate the membrane with transfer buffer containing ethanol before transfer.
Degraded ethanol in the transfer buffer	Prepare fresh transfer buffers for each experiment.
Excessive heat during transfer	Use active cooling of the transfer apparatus and pre-chilled transfer buffers.
Problem: no protein spots on membrane	
Incorrect orientation of the gel stack	Make sure that the membrane is on the anodal side of the stack and the gel is on the cathodal side of the stack. Consult the transfer equipment instructions for the correct orientation.

Detection

Possible Cause	Corrective action
Problem: a large number of spots are detected in the negative control (no primary antibody control)	
Insufficient blocking of the membrane	Block the membrane for at least one hour with blocking buffer.
Problem: weak signal	
Low antibody concentration	Increase the primary antibody concentration.
Insufficient incubation time with antibody	Incubate at least one hour at room temperature or overnight at 4°C.
Low detection reagent concentration	Increase the Cy5 conjugated secondary antibody concentration.
Insufficient incubation time with detection reagent	Incubate at least one hour at room temperature with diluted Cy5 conjugated secondary antibody.
Expired or incorrectly stored reagents	Follow manufacturer's instructions.
Short exposure time or low PMT	Increase the exposure time (camera) or increase the PMT setting (scanner).
Insufficient sample amount	Increase the amount of protein used.
Problem: high background	
High antibody concentration	Decrease the primary antibody concentration.
Long incubation time with antibody	Incubate for one hour at room temperature or overnight at 4°C. Incubation at room temperature can increase non-specific signals if the incubation time is too long.
High detection reagent concentration	Decrease the Cy5 conjugated secondary antibody concentration.
Long incubation time with detection reagent	Incubate for one hour at room temperature. Increased incubation times can increase background.
Insufficient blocking of the membrane	Block the membrane for at least one hour with blocking buffer.

Possible Cause	Corrective action
Insufficient washing	Wash membranes for the recommended 4 × 5min after each incubation step. Use an orbital or rocking platform shaker.
Detergent present on membrane	Perform a final wash using PBS without added detergent. Residual detergent on the membrane can give increased background at certain wavelengths.
Problem: saturated spots	
Long exposure or high PMT	Decrease the exposure time (camera) or decrease the PMT setting (scanner). Saturated spots may make subsequent analysis of coverage difficult.
Problem: poor resolution	
Poor gel quality	Make sure that the acrylamide or precast gels have not expired.
Low resolution settings	Increase the resolution of the scanner to at least 50 µm for small membranes and 100 µm for large format membranes.

Appendix A

CyDye labeling

Recommendation

The quality of the DMF/DMSO is critical for a successful protein labeling.

Use 99.8% anhydrous Dimethylformamide (DMF) or Dimethyl sulphoxide (DMSO) less than 3 months old from day of opening. After opening, over a period of time, DMF and DMSO degrade producing amine compounds. Amines will react with the NHS ester CyDye reducing the concentration of dye available for protein labelling.

Make sure DMF or DMSO are anhydrous and not contaminated with water.

Stock dye solutions preparation

Reconstitute CyDye minimal dyes solid compounds DMF to a concentration of 1 mM e.g., 5 µL DMF to 5 nmol of dye.

Note: *The stock dye solutions are stable at -20°C for several months.*

Note: *The stock solution of Cy3 a deep red, and Cy5 a deep blue color.*

Follow the steps below to prepare 5 µL the stock dye solutions.

Step	Action
1	Take a small volume of DMF from its original container and dispense into a microcentrifuge tube.
2	Take the CyDye from the -20°C freezer and leave to warm for 5 minutes at room temperature.
3	Add 5 µL of the DMF to each new vial of CyDye.
4	Replace the cap on the dye microcentrifuge tube and vortex vigorously for 30 seconds.
5	Centrifuge the microcentrifuge tube for 30 seconds at 12000 × g in a benchtop microcentrifuge.

Working dye solutions preparation

Follow the steps below to prepare 5 µL of working dye solution at 400 µM. 1µL of working dye solution contains 400pmol of dye.

Note: *1 µL of working dye solution contains 400 pmol of dye enough for labeling 50 µg of protein.*

Note: *The working dye solutions are only stable for 2 weeks at -20°C.*

Step	Action
1	Spin down the stock dye solution in a microcentrifuge. Make sure that all the liquid is collected at the bottom of the tube.
2	Add 3 µL of the DMF first to the sterile microcentrifuge tube.
3	Add 2 µL of the stock dye and mix.

cytiva

cytiva.com/hcp

For local office contact information, visit cytiva.com/contact

29657336 AB V:2 11/2021