1st Genomics and Proteomics Workshop

Venue: Macedonian Academy of Sciences and Arts, Skopje, R. Macedonia

Date: November 22-26, 2010

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Organized by: Research Center for Genetic Engineering and Biotechnology, Macedonian Academy of Sciences and Arts

PROGRAMME

November 22, 2010 (Monday)

11.30 –12.00 Registration

12.00 – 12.30 *Welcome address and Introduction to the workshop, Georgi D. Efremov, R. Macedonia*

12.30 – 15.00 Lectures

Niels Tommerup, Denmark: "The use of next-generation sequencing"

Cristina Patuzzo, Italy: "Next-generation sequencing: a revolutionary technology. Our preliminary experience"

Max Chaffanet, France: "Integrated genomic analysis of breast cancers"

November 23, 2010 (Tuesday)

09.00 –12.00 Practical Work (Real-time PCR analysis)

12.00 – 14.30 Lectures

Thilo Dork, Germany:"High throughput technologies for mutation detection"

Jan Gorodkin, Denmark: "Bioinformatics and non-coding RNA"

Rafael Oliva, Spain: "Proteomics: Methods and application in sperm cell research"

November 24, 2010 (Wednesday)

09.00 – 12.00 Practical Work (Capillary electrophoresis: microsatellites, QF-PCR, MLPA, SNaPshot analyses, Detection of allelic imbalances, DNA Sequencing)

12.00 – 13.00 Lunch break

13.00 – 16.00 Practical Work (MicroRNA)

November 25, 2010 (Thursday)

09.00 – 12.00 Practical Work (Microarray analysis; Array CGH analysis for detection of chromosomal anomalies)

12.00 – 13.00 Lunch break

13.00 – 16.00 Practical Work (High throughput technologies: Data analysis)

November 26, 2010 (Friday)

09.00 – 12.00 Practical Work (Introduction to 2D-PAGE/2D-DIGE)

12.00 – 13.00 Lunch break

13.00 – 16.00 Practical Work (2D-DIGE data analysis)

CONTENT

I. REAL-TIME (QUANTITATIVE) PCR

Real-Time PCR is one of the most powerful and sensitive gene analysis techniques available. Real-time PCR is used for a broad range of applications including quantitative gene expression analysis, SNP genotyping, copy number analysis, pathogen detection, drug target validation and for measuring RNA interference. Frequently, real-time polymerase chain reaction is combined with reverse transcription to quantify messenger RNA (mRNA) and microRNA (miRNA) in cells or tissues. RT-PCR or reverse transcription-polymerase chain reaction is a sensitive method for the detection of mRNA expression levels.

In real-time PCR, nucleic acid quantity is measured through the detection of a fluorescent signal proportional to the concentration of double-stranded DNA in the PCR tube. The fluorescent signal is produced by fluorophores added to the reaction mixture. In SYBR Green real-time PCR, the SYBR Green dye forms a complex with double-stranded DNA; it is this complex that fluoresces efficiently. In TaqMan probe assays, a specific fluorophore-labelled oligonucleotide probe, also carrying a quencher residue, hybridises with the target DNA. The probe is subsequently hydrolyzed by the elongating *Taq* polymerase, which results in uncoupling of the fluorophore from its quencher. In both cases, the fluorescent signal produced can be used as a measure of the initial amount of target sequence in the reaction. In the real-time PCR instrument, the fluorescence signal is recorded in "real time" at the end of each PCR cycle. The quantity of the target sequence is then calculated automatically.

In quantification using the Standard Curve Method, one can quantify unknowns based on a known quantity. First a standard curve is created; then unknowns are compared to the standard curve and values are extrapolated.

I.1 QUANTIFICATION OF HBA AND HBB mRNA BY REAL-TIME PCR

β-Thalassaemia mutations lead to abnormally low levels of *HBB* mRNA and HBB polypeptide chains in red blood cells. When blood test results suggest the presence of a *β*-thalassaemia mutation in a patient, it may be necessary to confirm that the quantity of *HBB* mRNA is decreased. The method described below is a real-time PCR assay that can be used for quantification of blood cell *HBB* mRNA against endogenous *HBA* mRNA (used as an internal calibrator). Since some of the common *β*-thalassaemia mutations are associated with only a small decrease in *HBB* levels, standards and samples should be run in triplicates or quadruplicates in order to achieve accurate quantification.

The protocol consists of three separate procedures:

I.1.1 Isolation of total RNA from blood (protocol I.1 or I.2);
I.1.2 Reverse transcription;
I.1.3 Real-time PCR assay.

I.1.1 Isolation of RNA

The basic principle of RNA extraction is very similar to DNA extraction methods, but different in many specific aspects. In general, the cells are lysed, the cell lysate deproteinized and the RNA separated from DNA.

However, RNA is much more labile than DNA and more susceptible to nuclease degradation. Furthermore, RNase are much more resistant to protein denaturants than DNases. These factors make isolation of undegraded RNA relatively difficult.

In order to obtain good quality RNA, it is necessary to lyse the cells and inactive RNase simultaneously. RNase could be inhibited in several ways:

The most effective way to inhibit traces of RNase in solutions is to ethylate proteins with diethylpyrocarbonate (DEPC). All water used should be treated with 0.1% DEPC at 37^{o}C overnight, than autoclaved.

Sterile, disposable plastic ware should preferably be used because it is RNase free. Wherever possible RNase inhibitors should be used in the extraction solutions.

There are number of protocols now available for the isolation of RNA from cells and tissues. The following are two procedures we routinely use to isolate RNA from biological samples.

Extraction of total RNA using guanidinium thiocyanate

Solutions to prepare:

5x Retic solution (5xRe)

140mM NaCl	40.90 g	
4mM KCl	1.85 g	
7mM $MgCl_2x6H_2O$	7.10 g	/ ddH_2O up to 1l / pH 7.1

1x Retic solution (1xRe)

5xRetic solution	200ml
ddH_2O	800ml

Solution D

4 M guanidinium thiocyanate	47.26g	
25mM sodium citrate pH 7.0,	735.25mg	
0.5% sarcosyl	0.5g	
0.1 M 2-mercaptoethanol	0.7ml	/ ddH_2O up to 100ml

Phenol,
US75830, GE Healthcare

Chloroform/Isoamyl alcohol 49:1

Chloroform	49 ml
Isoamylalcohol	1 ml

TE-buffer

10 mM TRIS-HCl pH 7.5
1 mM EDTA

Proteinase K (10mg/ml)

Dissolve 100 mg Proteinase K in 10 ml TE for 30 min at room temperature

Size Marker

φX-174 RF DNA-*Hae* III Digest, 100 µg, 27-4044-02, GE Healthcare

Protocol:

1. Collect 10 ml blood by venepunction using EDTA as an anticoagulant.
2. Centrifuge at 2.500rpm for 10min.
3. Transfer 250µl of reticulocyte rich fraction (the fraction of erythrocytes just below the white blood cell pellet) in a clean 1.5ml Eppendorf tube.
4. Wash twice with physiological saline.
5. Add equal volume (250µl) of solution D and homogenize the solution by pressing three times through a 21 guage needle by a 1-2 ml syringe (by forse).
6. Add 50µl of 2M sodium acetate pH 4.0, 500µl of phenol (saturated with water) and 100µl of chlorophorm-isoamylalcohol mixture (49:1) to the homogenate with through mixing by inversion after the addition of each reagent.
7. The final suspension is shaken vigorously for 10sec. and cooled on ice for 15 minutes.
8. Centrifuge at 13.000rpm for 20 minutes at 4°C. After centrifugation RNA is present in the aqueous phase whereas DNA and proteases are in the interphase and phenol phase.
9. The aqueous phase is transferred in to a fresh tube, mixed with equal volume (600µl) of isopropanol and than placed at -20°C for at least one hour to precipitate RNA.
10. Precipitate RNA by centrifugation at 13.000rpm for 30 minutes at 4°C.
11. Wash the resulting RNA pellet with 70% ethanol.
12. After final wash remove the last traces of ethanol, dry under vacuum for 1min. and dissolve in 20µl of RNase free water.
13. Incubate at 65°C for 10 minutes.
14. Check the integrity of RNA preparation by electrophoresis of an aliquote (2µl) of each sample on 1.5% agarose gel. Two distinct bands corresponding to the 28S and 18S tRNA should be detected. For a good RNA preparation the intensity of the 28S band should be approximately three times that the 18S band on a TBE gel. A low ration of intensity of these two bands indicates degradation (Figure 1).

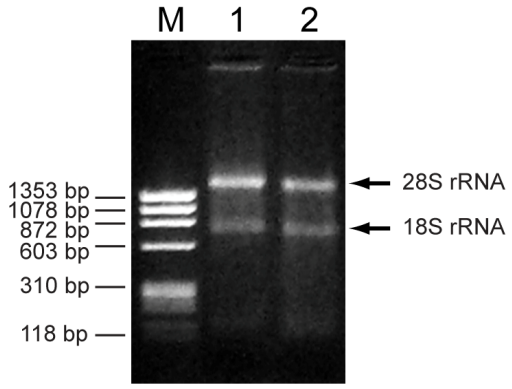


Figure 1. Electrophoretic pattern of isolated RNA samples.

I.1.2 Reverse transcription

Materials:

dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dUTP
Random hexanucleotide mix (Random Hexamers, Promega C1181, 500 ng/µl)
RNasin (Prime RNase Inhibitor, Eppendorf, No.0032 005.357 or rRNasin, Promega N2511)
Superscript II, RNase H- Reverse Transcriptase (Invitrogen. Cat. No. 18064-014 or 18064-022)

1. Protocol:

2. Prepare the Reaction mixture.

Mix the following on ice:

RNase-free water	up to 20 µl	
Total RNA	1 µg	
Random hexanucleotide mix, 100 ng/µl	1.0 µl	(final concentration 5 ng/µl)

Denature at 70°C for 10 minutes; quickly chill on ice; briefly spin down. Add:

5 × Superscript first-strand buffer	4.0 µl	(final 1 ×)
0.1 M DTT	2.0 µl	(final concentration 10 mM)
10 mM (each) dNTP mix	1.0 µl	(final concentration 500 µM each)
RNasin, 40 u/µl	1.0 µl	(final concentration 2 u/µl)

Mix gently and incubate at 42°C for 2 min. Add:

Superscript II	1.0 µl	(200 u)

Incubate at 25°C for 10 minutes and then at 42°C for 60 minutes.

3. Storage. Add 30 µl H_2O, store at -20°C.

I.1.3 Real-Time PCR assay

Materials:

TaqMan® Universal PCR Master Mix (Applied Biosystems, Cat. No. 4304437)

PCR primers: HBA assay: RT-PCR α-gl F/ RT-PCR α-gl R primers and RT-PCR α-gl probe (FAM, ZEN); primers flank and probe spans the exon 2-exon 3 junction

HBB assay: RT-PCR β-gl F/ RT-PCR β-gl R primers and RT-PCR β-gl probe (FAM, ZEN); primers flank and probe spans the exon 2-exon 3 junction

Template: cDNA from unknown samples U 1, U 2 (cDNA to be assayed);
control C 1 (cDNA from an individual who is not anemic)

Standards: Constructs with cloned cDNA amplified by the HBA and HBB assays:

HBA 1: 0.1 pg/µl HBA construct; **HBA 2**: 0.25 pg/µl HBA construct;
HBA 3: 0.62 pg/µl HBA construct; **HBA 4**: 1.6 pg/µl HBA construct;
HBA 5: 3.9 pg/µl HBA construct; **HBA 6**: 9.7 pg/µl HBA construct;

HBB 1: 0.1 pg/µl HBB construct; **HBB 2**: 0.25 pg/µl HBB construct;
HBB 3: 0.62 pg/µl HBB construct; **HBB 4**: 1.6pg/µl HBB construct;
HBB 5: 3.9 pg/µl HBB construct; **HBB 6**: 9.7 pg/µl HBB construct;

Protocol:

1. Design the layout of your plate. Apart from the unknown samples to be quantified, the assay should include standards and at least one control. Purified plasmid DNA containing the HBA and HBB amplicons is used to create a dilutions series of standards. cDNA produced from RNA from a clinically healthy individual is used as a control. An example is shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	HBA1	HBA1	HBA1	HBA1	HBA2	HBA2	HBA2	HBA2	HBA3	HBA3	HBA3	HBA3
B	HBA4	HBA4	HBA4	HBA4	HBA5	HBA5	HBA5	HBA5	HBA6	HBA6	HBA6	HBA6
C	C1	C1	C1	C1	U1	U1	U1	U1	U2	U2	U2	U2
D	HBB1	HBB1	HBB1	HBB1	HBB2	HBB2	HBB2	HBB2	HBB3	HBB3	HBB3	HBB3
E	HBB4	HBB4	HBB4	HBB4	HBB5	HBB5	HBB5	HBB5	HBB6	HBB6	HBB6	HBB6
F	C1	C1	C1	C1	U1	U1	U1	U1	U2	U2	U2	U2

2. Mix the following on ice:

A single reaction: Master mix (example for 40 reactions):

H_2O	4.0 µl	160 µl
2 × TaqMan mix	10.0 µl (final 1 ×)	400 µl
Template	2.0 µl (final variable)	–
Primer, 3 µM working stock	2.0 µl (final 300 nM each)	80 µl
TaqMan probe, 9 µM working stock	2.0 µl (final 900 nM)	80 µl
final volume	20 µl	

Distribute 18 µl master mix into individual wells.
Add 2.0 µl template/product from RT reaction.

3. In the 7500 real-time PCR system software, create an experiment or plate document using the following parameters:

Experiment Properties

Type of experiment:	Quantitation – Relative Standard Curve
Type of reagents:	TaqMan® Reagents
Ramp speed:	Standard

Plate Setup; Define Targets and Samples

Targets:	HBA, HBB
Standards:	Enter quantities following the experimental scheme
Samples:	C 1, U 1, U 2; assign to individual wells

Run Method

Thermal cycling conditions: Enzyme activation and initial denaturation at 95°C for 10 min followed by 40 cycles: 95°C, 15 s; 60°C, 60 s

Notes: For more details refer to the Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Comparative CT/Relative Standard Curve Experiments (Part Number 4387783).

I.2 microRNA DETECTION AND QUANTIFICATION BY REAL-TIME PCR

I.2.1 Introduction to miRNA

MicroRNAs (miRNAs) are small (~21-nucleotide (nt)) noncoding RNAs (ncRNAs) that mediate post-transcriptional gene regulation by pairing with the 3' untranslated region of messenger RNAs, acting as translational repressors, and regulating gene expression post-transcriptionally. After the discovery of the first miRNA in the roundworm *Caenorhabditis elegans*, these short regulatory RNAs have been found to be an abundant class of RNAs in plants, animals, and DNA viruses. About 3% of human genes encode for miRNAs, and up to 30% of human protein coding genes may be regulated by miRNAs. MicroRNAs play a key role in diverse biological processes, including development, cell proliferation, differentiation, and apoptosis.

Thus, potentially all the cellular pathways may be governed by miRNAs, which may contribute to the fine tuning of gene expression on a global level. The importance of miRNAs in gene regulation will be better appreciated when their function or deregulation, or that of the cellular machinery mediating their biosynthesis and function, will be identified among the underlying causes of several genetic disorders. Indeed, it is easy to conceive that protein overexpression resulting from defective miRNA-based mRNA regulation may compromise normal cell function and cause genetic diseases. Accordingly, altered miRNA expression is likely to contribute to human disease, including cancer. The study of miRNA is an emerging and exciting area of research with applications for basic, applied, and therapeutic science. In cancer, miRNAs function as regulatory molecules, acting as oncogenes or tumor suppressors. Amplification or overexpression of miRNAs can downregulate tumor suppressors or other genes involved in cell differentiation, thereby contributing to tumor formation by stimulating proliferation, angiogenesis, and invasion; i.e., they act as oncogenes. Similarly, miRNAs can down-regulate different proteins with oncogenic activity; i.e., they act as tumor suppressors. Several miRNA are associated with breast cancer. It has been shown that there are differences not just between normal and breast cancer tissue, but also between different cancer subtypes. Several methods for global miRNA profiling are currently in common use. These include quantitative RT-PCR (qPCR) involving stem-loop RT primers combined with TaqMan PCR (Applied Biosystems) analysis, qPCR with locked nucleic acid primers (Exiqon), qPCR using poly(A) tailing (QIAGEN, Stratagene), high-throughput sequencing of small RNA libraries, and microarray analysis.

Here we present quantitative RT-PCR (qPCR) involving stem-loop RT primers combined with TaqMan PCR and microarray analysis of the miRNAs.

I.2.1. Protocol for microRNA detection and quantification by real-time PCR

The real-time reverse transcription polymerase chain reaction (RT-qPCR) addresses the evident requirement for quantitative data analysis in molecular medicine, biotechnology, microbiology and diagnostics and has become the method of choice for the quantification of mRNA. RT-qPCR is a combination of three steps:
(i) the reverse transcriptase (RT)-dependent conversion of RNA into cDNA,
(ii) the amplification of the cDNA using the PCR and
(iii) the detection and quantification of amplification products in real time.
The protocol that we use for quantification of microRNA isolated from tissue samples consists of three separate procedures:

- Isolation of total RNA (protocol I.2.2.1);
- Reverse transcription;
- Real-time PCR assay.

The procedures described below are based on the protocols supplied with the following key kits/reagents:
Applied Biosystems TaqMan® MicroRNA Assays;

Applied Biosystems TaqMan® MicroRNA Reverse Transcription Kit.

All relevant details are included below.

I.2.1.1. Isolation of total RNA using RNeasy Mini Kit, Qiagen

Materials:

RNeasy Mini Kit (Qiagen, 74104)

Before starting:

Add 10 µl ß-mercaptoethanol per 1 ml Buffer RLT

Make sure ethanol has been added to Buffer RPE

70% Ethanol	50 ml
96.6% Ethanol	38.8 ml

Follow instructions for isolation of RNA using the RNeasy® procedure (Qiagen RNeasy Mini Kit, catalogue No 74104).

Perform all steps including centrifugations at room temperature. Ensure the centrifuge does not cool below 20°C. During the procedure, work quickly to minimise the risk of RNA degradation.

Protocol:

1. Pour a few millilitres of liquid nitrogen into a mortar and drop a piece of frozen tissue. Wait a few seconds until the nitrogen is just about to evaporate fully and using a pestle crush the tissue into fine powder. If necessary, add more liquid nitrogen and repeat. Do not let thaw. Using a scalpel, quickly transfer powder to a 1.5 ml tube.
2. Immediately add **Buffer RLT** to tissue sample and homogenise further by pipetting up and down several times. Depending on extent of homogenisation, use 300 µl to 4 ml buffer per 100 mg tissue. If using more than 600 µl buffer per sample, distribute homogenate into 600 µl aliquots.
3. Centrifuge the homogenate for 3 min at 12,000 × g (>13,000 rpm).
4. Transfer the supernatant to a fresh tube, add one volume of 70% ethanol and mix well by pipetting.
5. Transfer up to 600 µl of the sample to an RNeasy spin column placed in a 2 ml collection tube. Centrifuge for 15 s at >8,000 × g (>10,000 rpm). Discard the flow-through. Place the column back in the same collection tube. Load the rest of the sample onto the same RNeasy spin column discarding the flow-through after each centrifugation.
6. Add 700 µl **Buffer RW1** to the RNeasy spin column. Close the lid gently and centrifuge for 15 s at >8,000 × g (>10,000 rpm) to wash the spin column membrane.

Discard the flow-through and the collection tube. Transfer column with RNA into a clean 2 ml collection tube.

7. Add 500 µl **Buffer RPE** to the RNeasy spin column. Close the lid gently and centrifuge for 15 s at >8,000 × g (>10,000 rpm) to wash the spin column membrane. Discard the flow-through. Place the column back in the same collection tube.
8. Once again, add 500 µl **Buffer RPE** to the RNeasy spin column. Centrifuge for 2 min at >8,000 × g (>10,000 rpm) to wash and dry the spin column membrane. Discard the flow-through and the collection tube.
9. Place the column in a new 2 ml collection tube. Centrifuge at full speed for 1 min.
10. Transfer the RNeasy spin column to a 1.5 ml collection tube. Add 30-50 µl RNase-free water directly to the spin column membrane. Incubate for 10 min at room temperature. Centrifuge for 1 min at >8,000 × g (>10,000 rpm) to elute the RNA.
11. Optional: Repeat step 10 using another 30-50 µl RNase-free water and collect the eluate in the same collection tube.
12. Analyse RNA using Agilent 2100 Bioanalyzer (see instructions *Agilent Bioanalyzer*). Alternatively, analyse by agarose electrophoresis and quantify on UV spectrophotometer (see instructions *Spectrophotometers*)
13. Store RNA samples at -80°C or proceed with reverse transcription.

I.2.1.2 Reverse transcription

Materials:

TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Cat. No. 4366596)

TaqMan® MicroRNA Assays (Applied Biosystems, Part No. 4427975, Assay Type: mature miRNA, for example Assay ID 002623, Assay name hsa-miR-155)

RNase inhibitor (for example, Prime RNase Inhibitor, Eppendorf, Order No. 0032 005.357 or rRNasin, Promega N2511)

dNTP mix: 25 mM dATP, 25 mM dCTP, 25 mM dGTP, 25 mM dUTP (any supplier of PCR reagents or similar)

Protocol:

1. Mix the following on ice (for a single reaction):

RNase-free water	up to 10 µl final reaction volume
Total RNA	1-10 ng in 3-4 µl
5 × RT Primer	2.0 µl (final 1 ×)
25 mM (each) dNTP mix	1.0 µl (final 250 µM each)
10 × Reverse Transcription Buffer	1.0 µl (final 1 ×)
RNase inhibitor, 20 u/µl	0.2 µl (final 0.4 u/reaction)
MultiScribe Reverse Transcriptase, 50 U/µl	0.75 µl (final 37.5 U/reaction)

final volume 20 µl

Mix gently by pipetting.

2. Incubate at:

16°C for 30 minutes;

42°C for 30 minutes;

85°C for 5 minutes;

If using a thermal cycler include a final hold stage at 4°C; alternatively, transfer tubes on ice.

3. Store the RT reaction at –15 to -25°C. Alternatively, proceed with PCR analysis.

I.2.1.3 Real-Time PCR assay

Materials:

TaqMan® Universal PCR Master Mix (Applied Biosystems, for example Cat. No. 4304437)

TaqMan® MicroRNA Assays (Applied Biosystems, Part No. 4427975, Assay Type: mature miRNA, for example Assay ID 002623, Assay name hsa-miR-155)

Protocol:

1. Design the layout of your plate. Example with 2 assays, miR-155 and RNU6B, and 10 RT reactions from tissues 1 to 5, RT1-155 to RT5-155, RT1-R to RT5-R, is included below:

	1	2	3	4	5	6	7	8	9	10	11
A	miR-155 RT1-155	miR-155 RT1-155	miR-155 RT2-155	miR-155 RT2-155	miR-155 RT3-155	miR-155 RT3-155	miR-155 RT4-155	miR-155 RT4-155	miR-155 RT5-155	miR-155 RT5-155	miR-155 NTC
B	RNU6B RT1-R	RNU6B RT1-R	RNU6B RT2-R	RNU6B RT2-R	RNU6B RT3-R	RNU6B RT3-R	RNU6B RT4-R	RNU6B RT4-R	RNU6B RT5-R	RNU6B RT5-R	RNU6B NTC

2. Mix the following on ice A single reaction: Master mix (example for 11 reactions):

H_2O	7.7 µl	92.4 µl
20 × TaqMan® Small RNA Assay	1.0 µl (final 1 ×)	12.0 µl
2 × TaqMan Universal PCR Master Mix	10.0 µl (final 1 ×)	120.0 µl
Product from RT reaction	1.3 µl (final variable or – in NTCs)	
final volume	20 µl	

Distribute 18.7µl master mix into individual wells. Add 1.3 µl template/product from RT reaction or H_2O in NTCs.

3. In the 7500 real-time PCR system software, create an experiment or plate document using the following parameters:

   Experiment Properties

   Type of experiment: Quantitation – Comparative C_T ($\Delta\Delta C_T$)

   Type of reagents: TaqMan® Reagents

   Ramp speed: Standard

   Plate Setup; Define Targets and Samples

   Targets: miR-155, RNU6B; assign to individual wells; define NTCs

   Samples: RT1, RT2, RT3, RT4, RT5; assign to individual wells

   Run Method

   Thermal cycling conditions: Enzyme activation and initial denaturation at 95°C for 10 min followed by 40 cycles: 95°C, 15 s; 60°C, 60 s

II. CAPILLARY ELECTROPHORESIS

Capillary electrophoresis (CE) is an alternative to conventional slab gel electrophoresis for the separation of DNA fragments. Introduced in the 1960s, the technique of capillary electrophoresis (CE) was designed to separate species based on their size to charge ratio in the interior of a small capillary filled with an electrolyte.

CE offers a number of advantages over slab gel separations in terms of speed, resolution, sensitivity, and data handling. Separation times are generally only a few minutes and the DNA is detected either by UV absorption or by fluorescent labeling. The quantity of DNA required for separation is in the nanogram range. Single-base resolution can be obtained on fragments up to several hundred base pairs. In the presence of appropriate standards, fragments can be accurately sized based on relative electrophoretic mobility. Traditional methods for mutation screening often involve slab-gel electrophoresis analyses which are laborious and difficult to automate. However, recent developments in capillary electrophoresis systems for DNA fragment analysis have made fully automated mutation screening possible and have dramatically increased the possible sample throughput.

II.1 MULTIPLEX LIGATION-DEPENDENT PROBE AMPLIFICATION

Multiplex Ligation-dependent Probe Amplification (MLPA® MRC-Holland) is a high-throughput method developed to determine the copy number of up to 50 genomic DNA sequences in a single multiplex PCR-based reaction. The MLPA reaction results in a mixture of amplification fragments ranging between 100 to 500 nt in length, which can be separated and quantified by capillary electrophoresis. Fundamental for the MLPA technique is that not sample DNA is amplified during the PCR reaction, but the MLPA

probes that are hybridised to the sample DNA. Each MLPA probe consists of two probe oligonucleotides, which should adjacently hybridise to the target DNA for successful ligation. Only ligated probes are exponentially amplified by PCR. MLPA is a method of choice for copy number detection.

II.1.1 Spinal Muscular Atrophy analysis by Multiplex Ligation-dependent Probe Amplification (MLPA®)

Materals:

SALSA MLPA KIT P021-A1 SMA (MRC Holland)

Protocol:

Please refer to the manufacturer instruction manuel

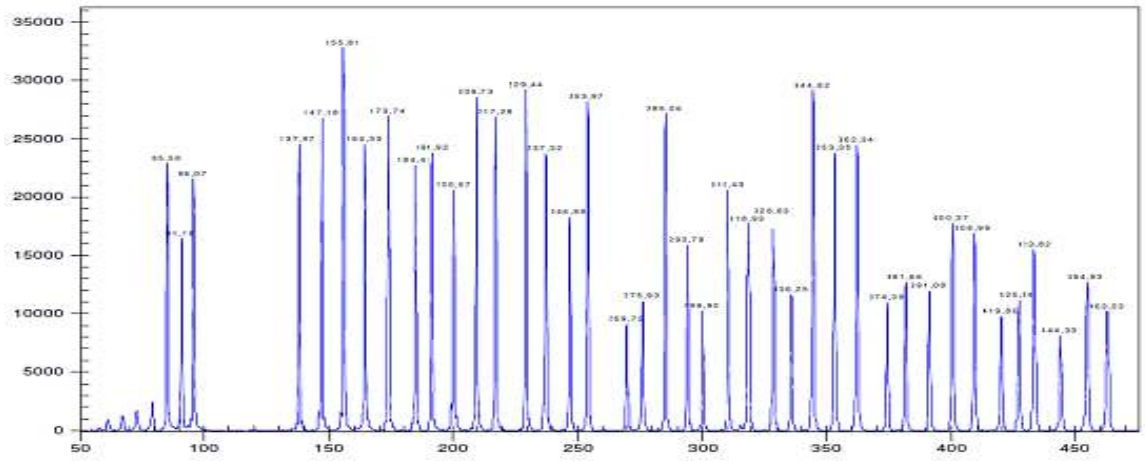


Figure 2. Capillary electrophoresis pattern from a sample of human male control DNA analyzed with SALSA MLPA kit P021-A1 SMA

II.2 MULTIPLEX SNaPshot ANALYSIS

Single-base extension (SBE) or ***minisequencing*** is a method for determining the identity of a nucleotide base at a specific position along a nucleic acid. The method is used to identify a single-nucleotide polymorphism (SNP).

Ten SNP markers can be investigated simultaneously by employing PCR amplification followed by dideoxy single-base extension of unlabeled primers using the ***SNaPshot Multiplex kit*** form ***Applied Biosystems***. The SNaPshot primer is designed to anneal to the sequence adjacent to the SNP sites. Once the primer anneals, the single-base extension occurs by the addition of the complementary dye-labeled ddNTP (dye terminator) to the annealed primer. Each of the four ddNTPs is fluorescently labeled with a different color dye. The result is marker fragments for the different SNP alleles that are all the same length, but vary by color. After electrophoresis and fluorescence detection, the alleles of a single marker appear as different colored peaks at roughly the same size in the electropherogram plot. The size of the different allele peaks will vary slightly due to

differences in molecular weight of the dyes. The GeneMapper Software is used to size and genotype the data.

II.2.1 Detection of the most common CFTR mutations by a multiplex SNaPshot analysis

There are three steps for the detection of the most common CFTR mutations by a multiplex SNaPshot analysis protocol: 1) PCR amplification, 2) Primer extension-(SNaPshot) and 3) Analysis on ABI 3130.

II.2.1.1 PCR amplification (Multiplex PCR reaction)

- Multiplex PCR reaction if performed using 8 primers splited in two separated mixes (mix I and mix II), with concetration of 10pM/ µl for each primer in mix.
- Post PCR, amplicons can be confirmed on 1.5% agarose gel (1.5 g agarose in 100 ml 1xTBE buffer).

II.2.1.2 Primer extension (SNaPshot)

For reason of overlapping of different SNaPshot products, the polymorphisms is analysed with two SNaPShot primer mixes, mix III and mix IV; initial concetration for each primer before dilution is 100pM/ µl (Table 3):

Table 3. List of CFTR SNP primers

	Primer (SNP) ID	Length of SNP primer (nt)	Exon/ Intron	Nucle-otide change	Signal color	
Mix III	G542X	20 (F)	Exon 11	C/A		
	N1303K	22 (R)	Exon 21	G/C		
	Del F508 -1	28 (F)	Exon 10	C/T		
	Intron 8-(5T/7T)	23 (F)	Intron 8	A/T		
	R117H	32 (F)	Exon 4	G/A		
	621+1G->T-1	32 (F)	Exon 4	G/T		
	Intron 8-(7T/9T)	39 (F)	Intron 8	A/T		
Mix IV	R553X-1(D)	22 (R)	Exon 11	G/A		
	G551D-1	20 (F)	Exon 11	G/A		
	W1282X	26 (F)	Exon 20	G/A		
	R1162X-1(D)	27 (R)	Exon 19	G/A		
	1717-1 G->A	29 (F)	Exon 11	G/A		
	2184insA-1	35 (F)	Exon 13	C/A		

- Exo-SAP treatment is required to degrade leftover PCR primers and unincorporated dNTPs. Incubate 0.5 µl Exo-SAP with 1 µl PCR overnight (minimum 90 min.) at 37°C; Inactivate Exo-SAP at 86 °C for 20 min.;
- Assemble SNaPshot reaction and perform PCR:
  In each tube with purified PCR (1.5 µl) ad:

SNaPshot ready mix 1 µl
SNaPshot primers (mix III or mixIV) 1 µl
dH_2O 1 ul
PCR termo-cycler conditions: 25 cycles of 96°C/10s, 50°C/5s and 60°C/30s

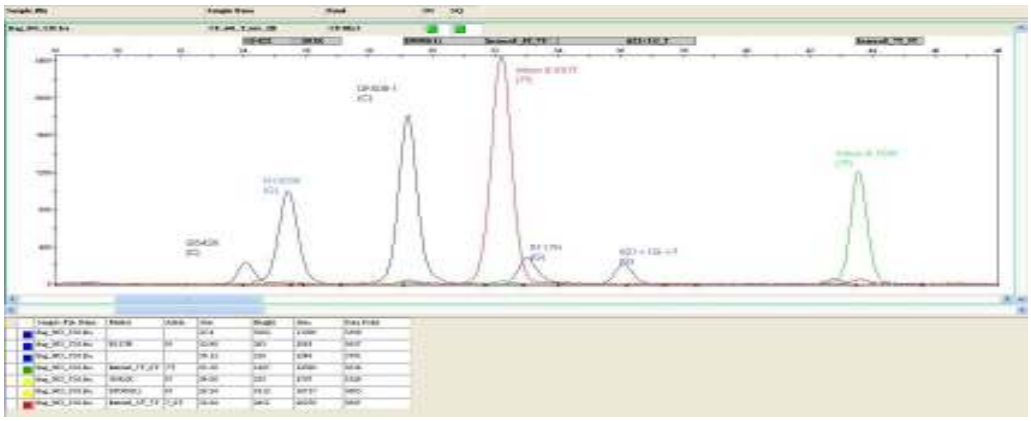
- Post-extension treatment:
  Add 1.5 µL of SAP to each reaction. The SAP is required to inactivate the fluorescently labeled ddNTPs. Incubate 60 min. at 37°C and inactivate at 86 °C for 20 min.;

II.2.1.3 Analysis on ABI 3130

Prepare the sample for ABI Prism 3130 Genetic Analyzer by mixing the following for a single reaction:

Hi-Di Formamide 12 **µl**
Size standard Liz 120 0.3 **µl**
SNaPShot PCR 1 **µl** and denaturate on 95°C/ 5min ,on ice/ 1min

A.



B.

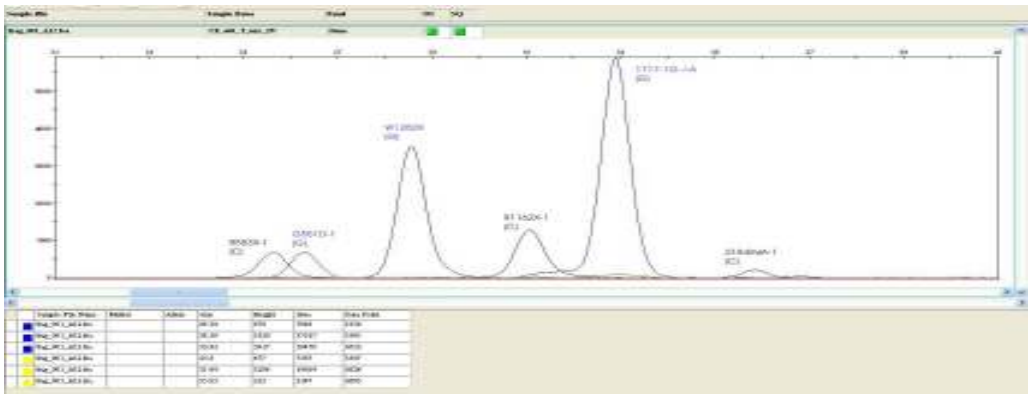


Figure 3. Electrophoreograms from ABI 3130 Genetic Analyzer analysed with GeneMapper 4.0 software. A. CFTR SNaPShot primer mix III; B. CFTR SNaPShot primer mix IV

II.3 DNA SEQUENCING

DNA sequencing is the process of determining the order of nucleotides on a segment of DNA. Cycle sequencing is a method used to increase the sensitivity of the DNA sequencing process and permits the use of very small amounts of DNA starting material. This is accomplished by using a temperature cycling process similar to that employed in the polymerase chain reaction.

The Chain Termination Method

The most popular approach to sequencing is the chain termination method, developed in 1977 by Fred Sanger. This technique makes use of a DNA synthesis reaction and a unique form of a base, called a dideoxynucleotide, that lacks the 3′ hydroxyl chemical group involved in forming the link between nucleotides in a DNA chain. A dideoxynucleotide can be added to a growing chain, but, once incorporated, no further nucleotides can be linked to it. Thus, chain growth is terminated.

In a reaction, the concentration of dideoxynucleotides is optimized so that all possible chain lengths are generated. In automated DNA sequencing, the newly formed chain fragments are marked with four fluorescent dyes, each of which corresponds to one of the four DNA nucleotides (A, C, T, and G). The fragments are then separated by gel electrophoresis or capillary electrophoresis, during which the dyes are excited and detected by the automated DNA sequencing instrument. In this way, the identity of each successive terminating nucleotide is determined, revealing the sequence of the entire chain .

In a sequencing reaction, all the components needed for the synthesis of DNA are present. These include the DNA to be sequenced (the template) and a short (17 to 28 bases in length), single-stranded piece of DNA (the primer), which attaches itself to a specific site on the template and acts as a starting point for the synthesis of a new DNA strand.

II.3.1 Cystinuria SLC3A1 gene analysis - Sequencing protocol

Cystinuria is an autosomal recessive disorder that is characterized by an impaired transport of cystine, lysine, ornithine and arginine in the proximal renal tubule and epithelial cells of the gastrointestinal tract. This condition results in an elevated urine concentration of cystine, lysine, ornithine and arginine. The transport of these amino acids is mediated by the rBAT/$b^{0,+}$AT transporter, the subunits of which are encoded by the genes SLC3A1, located on chromosome 2p16.3-21, and SLC7A9, located on chromosome 19q12-13.1. Based on the urinary cystine excretion patterns of obligate heterozygotes, cystinuria is classified into two types: type I and non-type I. Mutations in SLC3A1 gene cause type I cystinuria, while mutations in SLC7A9 gene, are responsible for non-type I cystinuria. Two novel mutations in SLC3A1 gene (C242R and L573X) determined by direct sequencing as follows:

II.3.1.1. Template preparation

I. PCR amplification of genomic DNA

1. Mix the following for a single reaction:

Reagent	**Quantity**
Deionized H_2O	15.0 µL
10XRb buffer *	2.5 µL
25mM $MgCl_2$	1.5 µL
25mM dNTP	2.0 µL
Primer F+R (10pM)	2.0 µL
Taq Gold	0.2 µL
DNA	2.0 µL
Total Volume	**25µL**

*10XRb buffer no contains $MgCl_2$.

2. Place the tubes in a thermal cycler and set the following conditions:
   a. Initial denaturation at 95˚C for 10 minutes
   b. Repeat the following for 33 cycles: 95 °C for 45 seconds, 59 °C for 45 seconds, 72 °C for 1 minutes
   c. Final extension at 72°C for 10 minutes
   d. Rapid thermal ramp to 4 °C and hold.
   *Analyse the PCR product on 1.5% agarose gel.

II. PCR purification with ExoSAP-IT

The purification of the PCR products is performed with ExoSAP-IT. In general, the method removes unincorporated dNTPs and primers.

1. Remove the ExoSAP-IT from -20 ˚C freezer and keep on ice throughout the procedure.
2. Mix 2 µL PCR reaction with 1 µL of ExoSAP-IT.
3. Incubate at 37 ˚C for 2 hours (or overnight) to degrade remaining primers and nucleotides, and incubate at 86 ˚C for 20 minutes to inactivate ExoSAP-IT.

The PCR product is now ready for DNA sequencing.

II.3.1.2 DNA sequencing

1. Prepare the reaction mixture

For each reaction add the following reagents to each tube containg the purified PCR product:

Reagent	**Quantity**
5XSequencing Buffer	1.0 µL
Ready Reaction Mix*	2.0 µL
Purified PCR product	3.0 µL
Primer F or R (10pM/µl)	0.5 µL
Deionised water	3.5 µL
Total Volume	**10 µL**

Mix well and spin briefly.

2. Place the tubes in a thermal cycler and set the following conditions:

a. Initial denaturation at 96˚C for 5 minutes

b. Repeat the following for 25 cycles:96 °C for 10 seconds, 50 °C for 5 seconds and 60 °C for 4 minutes

c. Rapid thermal ramp to 4 °C and hold.

3. Ethanol/Sodium Acetate Precipitation

1. In a new 0.5 µL tube mix 50 µL 100% ethanol and 2 µL 3M Sodium Acetate pH 5.2.
2. Add the sequencing reaction in each tube and mix gently by pipetting..
3. Incubate at room temperature for 15 min.
4. Centrifuge at 14000 rpm for 30 min.
5. Carefully remove the ethanol with a pipette.
6. Add 150 µL 70% ethanol to each tube.
7. Centrifuge at 14000 rpm for 10 min.
8. Carefully remove the ethanol with a pipette.
9. Leave for 5 minutes to dry.
10. Dissolve the samples in 20 µL Hi-Di™ Formamide.
11. Before running the samples incubate in thermal cycler at 95˚C for 5 minutes and leave on ice for 5 minutes.

The sample is now ready to run on ABI Prism 3130 Genetic Analyzer .
To store, cover with aluminium foil, and store at 4 °C or -20°C for a longer period.

II.3.1.3 ABI Prism 3130 Genetic Analyzer

Load the samples into MicroAmp™-optical 96 well plate (Applied Biosystems), and run the Sequencing programe on ABI Prism 3130 Genetic Analyzer

Analyse the data with **Sequencing Analysis 5.3.1 softhware**

The results of two identified mutations in CSL3A1 gene leading to cystinuria type 1 patient are presented in Figure 5.

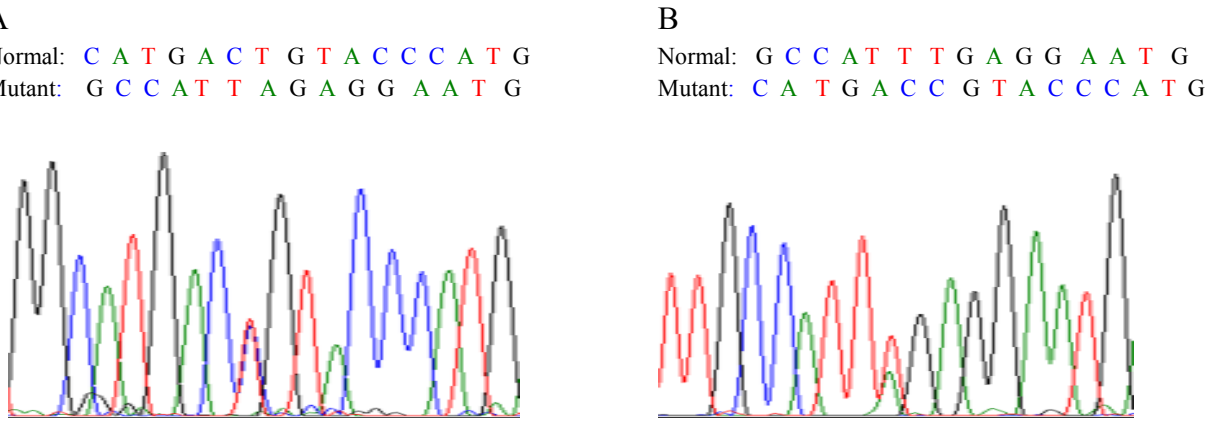


Figure 5. DNA sequencing analysis showing novel mutations in SLC3A1 gene A)T to C nucleotide change leading to missence mutation Cys242Arg and B) T-A nucleotide substitution leading to nonsense mutation Leu573Stop

III. MICROARRAY TECHNOLOGY

A **microarray** is a multiplex lab-on-a-chip. It is a 2D array on a solid substrate (usually a glass slide or silicon thin-film cell) that assays large amounts of biological material using high-throughput screening methods. Types of microarrays include:

- DNA microarrays, such as cDNA microarrays, oligonucleotide microarrays and SNP microarrays
- RNA microarrays, for gene expression and for surveillance of microRNA populations
- Protein microarrays
- Tissue microarrays
- Cellular microarrays (also called transfection microarrays)
- Chemical compound microarrays
- Antibody microarrays
- Carbohydrate arrays (glycoarrays)

A **DNA microarray** is a multiplex technology used in molecular biology. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, called features, each containing picomoles (10^{-12} moles) of a specific DNA sequence, known as *probes* (or *reporters*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel. Therefore arrays have dramatically accelerated many types of investigation.

Microarray-based comparative genomic hybridization (array CGH) is a technique to scan the genome for gains and losses of chromosomal material. This method has significantly high resolution and clinical yield. The limitation of traditional karyotyping is that even high resolution karytoypes are unreliable for detecting many known microdeletion syndromes which range from 3-5 Mb in size.

Array CGH (aCGH) differentially labels DNA from a test sample and a reference sample. Fluorescently labeled reactions are combined and hybridized to DNA substrates from chromosomally normal individuals. Gains and losses of the genome in the test sample relative to the control are measured.

The strengths of this method are that it allows for investigation of the whole genome and has a very high resolution - allowing for the determination of origin of unknown genetic material such as unbalanced rearrangements. The main weakness of aCGH is that it does not detect balanced rearrangements. An uncertainty of aCGH is copy number variation (CNV). CNVs have been found in phenotypically normal people and can occur at a high

frequency in the general population. They are usually inherited and can take up a significant portion of the genome. It is estimated that there are many more CNVs than we expect and they are more common than previously thought. It is not yet clear how CNVs contribute to genetic variability.

III.1. Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis

III.1.1. DNA Isolation

Step 1. gDNA Extraction

gDNA Extraction if performed with standard phenol/chloroform extraction procedure.

Step 2. gDNA Quantitation and Quality Analysis

Accurate assessment of gDNA quantity and quality are crucial to the success of an Agilent Oligo aCGH experiment.

- Use the NanoVue Spectrophotometer to assess gDNA concentration and purity.
- Use agarose gel electrophoresis to assess gDNA intactness and the average molecular weight for each sample.

III.1.2. Sample Preparation

Step 1. Restriction Digestion

1. Thaw 10X Buffer C and Acetylated BSA (supplied with Rsa I). Briefly mix on a vortex mixer and spin in a microcentrifuge. Store all reagents on ice while in use.

2. For each reaction, add the required amount of genomic DNA (0.5 to 1.5 µg) to the appropriate nuclease- free tube or well in the PCR plate and add enough nuclease-free water to bring to the final volume of 20.2 µL (for 4x microarray format).
3. Prepare the Digestion Master Mix by mixing the components in Table 1 based on the microarray format used, on ice in the order indicated.
4. Add 5.8 µL (for 1x, 2x or 4x microarrays) of Digestion Master Mix to each reaction tube containing the genomic DNA to make a total volume of 26 µL (for 1x, 2x or 4x microarrays). Mix well by pipetting up and down.

Table 1. *Preparation of Digestion Master Mix (for 1x, 2x and 4x microarrays)*

Component	**per reaction (µL)**
Nuclease free water	2.0
10X Buffer C*	2.6
Acetilated BSA(10 µg/ µL)	0.2
Alu I(10U/ µL)	0.5
RsaI (10U/ µL)	0.5
Final Volume of Digestion Master Mix	**5.8**

* Buffer C supplied with Rsa I

5. Incubate the samples 2 hours at 37C, then 20 minutes at 65C and hold at 4C.

6. *Optional*. Take 2 µL of the digested genomic DNA and run on a 0.8% agarose gel stained with ethidium bromide to assess the completeness of the digestion. The majority of the digested products should be between 200 bp and 500 bp in length.

7. Proceed directly to "Sample Labeling", or store digested genomic DNA for up to a month at
- 20°C.

III.1.3. Sample Labeling

Step 1. Fluorescent Labeling of Genomic DNA

Cyanine 3-dUTP and cyanine 5-dUTP are light sensitive and are subject to degradation by multiple freeze thaw cycles. Minimize light exposure throughout the labeling procedure.

1. Spin the samples in a centrifuge for 1 minute at 6,000 x g to drive the contents off the walls and lid.
2. Add Random Primers (supplied with the Agilent Genomic DNA Enzymatic Labeling Kit):
   For 1x, 2x and 4x microarrays, add 5 µL of Random Primers to each reaction tube containing 26 µL of gDNA to make a total volume of 31 µL (or 24 µL of gDNA to make a total volume of 29 µL if the optional agarose gel step was done). Mix well by pipetting up and down gently.
3. Transfer sample tubes to a thermocycler. Set the following conditions: 3 minutes at 95C and hold at 4C.
4. Spin the samples in a centrifuge for 1 minute at 6,000 x g to drive the contents off the walls and lid.

5. For 1x, 2x and 4x microarrays prepare one Cy3 and one Cy5 Labeling Master Mix by mixing the components in Table 2 on ice in the order indicated.

Table 2. *Preparation of Labeling Master Mix (for 1x, 2x and 4x microarrays)*

Component	**per reaction (µL)**
Nuclease free water	2.0*
5X Buffer	10.0
10X dNTP	5.0
Cyanine 3-dUTP(1.0mM) or Cyanine 5-dUTP(1.0mM)	3.0
Exo-Klenow fragment	1.0
Final volume of Labeling Master Mix	**19.0 or 21.0***

* Do *not* add nuclease-free water if you skipped the optional agarose gel step

a. Add 19 µL (or 21 µL) of Labeling Master Mix to each reaction tube containing the gDNA to make a total volume of 50 µL. Mix well by gently pipetting up and down.
b. Transfer sample tubes to a thermocycler. Set the following conditions: 2 hours at 37C, then 10 minutes at 65C and hold at 4C.

Reactions can be stored up to a month at - 20°C in the dark.

Step 2. Clean-up of Labeled Genomic DNA

Clean-up of Labeled Genomic DNA is performed using Amicon 30kDa filters following this procedure.

1. Spin the labeled genomic DNA samples in a centrifuge for 1 minute at 6,000 x g to drive the contents off the walls and lid.
2. Add 430 µL of 1X TE (pH 8.0) to each reaction tube.
3. Place an Amicon 30kDa filter into a 1.5- mL microfuge tube (supplied) and load each labeled gDNA into the filter. Spin 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow- through.
4. Add 480 µL of 1X TE (pH 8.0) to each filter. Spin for 10 minutes at 14,000 × g in a microcentrifuge at room temperature. Discard the flow- through.
5. Invert the filter into a fresh 1.5-mL microfuge tube (supplied). Spin for 1 minute at 1,000 × g in a microcentrifuge at room temperature to collect purified sample. The volume per sample will be approximately 21 µL.

6. Add 1X TE or use a concentrator to bring the sample volume to 21 µL.
7. Take 1.5 µL of each sample to determine yield and specific activity. Refer to Table 3 for expected yield of labeled genomic DNA and specific activity after labeling and clean- up, when starting with high quality genomic DNA.
8. Combine test and reference sample using the appropriate cyanine 5- labeled sample and cyanine 3- labeled sample for a Total Mixture Volume of 39 µL.

Labeled DNA can be stored up to one month at - 20°C in the dark.

Determination of yield , degree of labeling or specific activity

Use the Nano Vue 4.0 Spectrophotometer to measure the yield, degree of labeling or specific activity.

$$\text{Degree of Labeling} = \frac{340 \times \text{pmol per µL dye}}{\text{ng per µL genomic DNA} \times 1000} \times 100\%$$

$$\text{Specific Activity*} = \frac{\text{pmol per µL dye}}{\text{µg per µL genomic DNA}}$$

*pmol dyes per µg genomic DNA

Note that the Specific Activity is Degree of Labeling divided by 0.034.
Record the gDNA concentration (ng/µL) for each sample. Calculate the yield as

$$\text{Yield (µg)} = \frac{\text{DNAconcentration (ng/µL)} \cdot \text{Sample Volume (µL)}}{1000 \text{ ng/µg}}$$

Refer to Table 3 for expected yield of labeled genomic DNA and specific activity after labeling and clean- up, when starting with high quality genomic DNA.

Table 3. *Expected Yield and Specific Activity after Labeling and Clean-up*

Input gDNA (µg)	**Yield (µg)**	**Specific Activity of Cyanine-3 Labeled Sample(pmol/ µg)**	**Specific Activity of Cyanine-5Labeled Sample(pmol/ µg)**
0.2	2.5 to 3	15 to 25	15 to 20
0.5	**5 to 7**	**25 to 40**	**20 to 35**
3.0	7 to 10	35 to 55	25 to 40

Step 3. Preparation of Labeled Genomic DNA for Hybridization

1. Prepare the 10X Blocking Agent:

a. Add 1,350 µL of nuclease- free water to the vial containing lyophilized 10X Blocking Agent (supplied with Agilent Oligo aCGH Hybridization Kit).

b. Leave at room temperature for 60 minutes and mix on a vortex mixer to reconstitute sample before use or storage.

2. Prepare the samples for hybridization:

a. Mix the components according to the microarray format in Table 4 below to prepare the Hybridization Master Mix.

Table 4. *Preparation of Hybridization Master Mix for 4x microarray*

Component	**Volume () per hybridization**
Cot-1 DNA (1.0mg/ml)*	5
Agilent 10X Blocking Agent	11
Agilent 2X Hi-RPM Buffer	55
Final Volume of Hybridization Master Mix	**71**

**Use Cot-1 DNA from the appropriate species.*

b. Add the appropriate volume of the Hybridization Master Mix to the 1.5 mL microfuge tube, tall chimney plate well or PCR plate well containing the labeled gDNA to make the total volume 110 µL.

c. Mix the sample by pipetting up and down, then quickly spin in a centrifuge to drive contents to the bottom of the reaction tube.

d. Transfer sample tubes to a thermocycler. Set the following conditions: 3 minutes at 95C, then 30 minutes at 37C.

e. Remove sample tubes from the thermocycler. Spin 1 minute at 6000 × g in a centrifuge to collect the sample at the bottom of the tube.
The samples are ready to be hybridized.

III.1.4. Microarray Processing and Feature Extraction

Microarray processing consists of **hybridization, washing,** and **scanning.**
Feature Extraction is the process by which data is extracted from the scanned microarray image (.tif) and translated into log ratios, allowing researchers to measure DNA copy number changes in their experiments in conjunction with Agilent Genomic Workbench Software.

Step 1. Microarray Hybridization

Each microarray is printed on the side of the glass slide containing the "Agilent"- labeled barcode. This side is called the "active side". The numeric barcode is on the "inactive side" of the glass slide. The hybridization sample mixture is applied directly to the gasket slide and not to the microarray slide. Then the active side of the microarray slide is put on top of the gasket slide to form a "sandwich slide pair".

Hybridization Assembly

1. Load a clean gasket slide into the Agilent SureHyb chamber base with the gasket label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
2. Slowly dispense 100 µL (for 4x microarray) of hybridization sample mixture onto the gasket well in a "drag and dispense" manner.
3. Put a microarray slide "active side" down onto the gasket slide, so the numeric barcode side is facing up and the "Agilent"- labeled barcode is facing down. Assess that the sandwich- pair is properly aligned.
4. Put the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
5. Hand- tighten the clamp onto the chamber.
6. Vertically rotate the assembled chamber to wet the slides and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.
7. Put assembled slide chamber in the rotator rack in a hybridization oven set to 65°C. Set your hybridization rotator to rotate at 20 rpm.
8. Hybridize at 65°C for 24 hours (for 4x or 8x microarrays).

Step 2. Wash Preparation

Prewarming Oligo aCGH Wash Buffer 2 (Overnight)

The temperature of Oligo aCGH Wash Buffer 2 must be at 37°C for optimal performance.

1. Add the volume of buffer required to a disposable plastic bottle and warm overnight in an incubator or circulating water bath set to 37°C.
2. Put a slide- staining dish into a 1.5 L glass dish three- fourths filled with water and warm to 37°C by storing overnight in an incubator set to 37°C.

Step 3. Microarray Washing

Wash Procedure A (without Stabilization and Drying Solution)

1. Completely fill slide- staining dish #1 with Oligo aCGH Wash Buffer 1 at room temperature.
2. Put a slide rack into slide- staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH Wash Buffer 1 at room temperature to cover the slide rack. Put this dish on a magnetic stir plate.
3. Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three- fourths full with Oligo aCGH Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH Wash Buffer 2 at 37°C; monitor using a thermometer.
4. Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.
5. Prepare the hybridization chamber disassembly.

a. Put the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counter- clockwise.

b. Slide off the clamp assembly and remove the chamber cover.

c. With gloved fingers, remove the array- gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide- staining dish #1.

d. Without letting go of the slides, submerge the array- gasket sandwich into slide-staining dish #1 containing Oligo aCGH Wash Buffer 1.

6. With the sandwich completely submerged in Oligo aCGH Wash Buffer 1, pry the sandwich open from the barcode end only. Do this by slipping one of the blunt ends of the forceps between the slides and then gently turn the forceps upwards or downwards to separate the slides. Let the gasket slide drop to the bottom of the staining dish. Remove the microarray slide and put into slide rack in the slide-staining dish #2 containing Oligo aCGH Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*
7. When all slides in the group are put into the slide rack in slide- staining dish #2, stir using setting 4 for 5 minutes. Adjust the setting to get good but not vigorous mixing.
8. Transfer slide rack to slide- staining dish #3 containing Oligo aCGH Wash Buffer 2 at 37°C, and stir using setting 4 for 1 minute.

9. Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

10. Discard used Oligo aCGH Wash Buffer 1 and Oligo aCGH Wash Buffer 2.

11. Put the slides in a slide holder:

- In environments in which the ozone level exceeds 5 ppb, immediately put the slides with Agilent barcode facing up in a slide holder with an ozone- barrier slide cover on top of the array as shown in Figure 1.

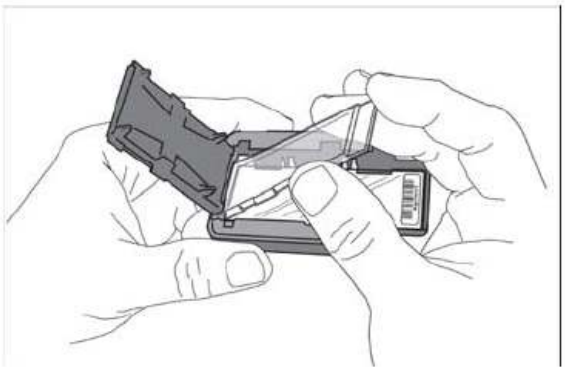


Figure 1. Inserting the ozone-barrier slide cover

12. Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in the original slide boxes in a N2 purge box, in the dark.

Step 4. Microarray Scanning using Agilent C Scanner

An Agilent C- scanner and Agilent Scanner Control software v8.3 or higher is required for 1x1M, 2x400K, 4x180K and 8x60K density microarrays and is optional for 1x244K, 2x105K, 4x44K and 8x15K density microarrays.

Step 5. Data Extraction using Feature Extraction Software

The Feature Extraction (FE) software v10.5 or higher supports extraction of microarray TIFF images (.tif) of Agilent CGH microarrays scanned on the Agilent C Scanner but does *not* support extraction of Agilent CGH microarrays on the GenePix 4000B scanner.

Figure 2 shows an example of Agilent 1M CGH microarray image opened in Feature Extraction software v10.5 in both full and zoomed view.

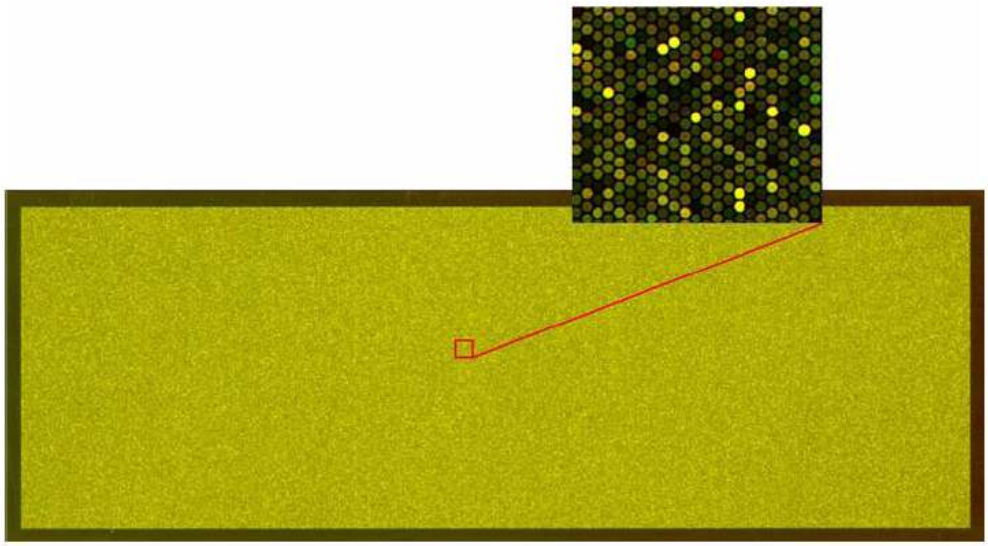


Figure 2. *Agilent SurePrint G3 1x1M CGH microarray shown in red and green channels: full and zoomed view*

Microarray QC Metrics for high DNA quality samples

These metrics are only appropriate for high- quality DNA samples analyzed with Agilent CGH microarrays by following the standard operational procedures provided in this user guide. These metrics are exported to a table in the Feature Extraction QC report and in Genomic Workbench. They can be used to assess the relative data quality from a set of microarrays in an experiment. In some cases, they can indicate potential processing errors that have occurred or suggest that the data from particular microarrays might be compromised. Many factors can influence the range of these metrics including the microarray format (1x, 2x, 4x or 8x) biological sample source, quality of starting gDNA, experimental processing, scanner sensitivity and image processing. The value guidelines presented below represent the thresholds that Agilent has observed when analyzing samples using this protocol.

Table 5. *QC metric thresholds for Enzymatic labeling*

Metric	**Excellent**	**Good**	**Poor**
BGNoise	<5	5 to 10	>10
Signal Intensity	>150	50 to 150	<50
Signal to Noise	>100	30 to 100	<30
Reproducibility	<0.05	0.05 to 0.2	>0.2
DLRSpread	<0.2	0.2 to 0.3	>0.3

Analysis of the data with Genomic Workbench software

Feature Extraction data are imported into the programme for analyzing.

III.2 miRNA microarray system with miRNA labeling and hybridization

RNA extraction recommendation:

1. Absolutely RNA miRNA Kit – Agilent p/n 400814
2. miRNeasy Mini Kit – Qiagen p/n 217004
3. mirVana RNA Isolation Kit – Applied Biosystem p/n AM 1560
4. TRIZOL Reagent (100ml) – Invitrogen p/n 15596-026
5. RNeasy Mini Kit – Qiagen p/n 74104

Procedure:

Agilent's miRNA Microarray System uses cyanine 3-labeled targets to measure miRNA in experimental and control samples. Figure 11 is a standard workflow for sample preparation and array hybridization.

Total RNA (100ng) + Labeling Spike In (optional)
↓
Dephosphorylated RNA *
↓ Add DMSO
↓ Heat, Ice
↓ Assemble Labeling reaction, incubate 2 hours, 16º C
Labeled RNA*
↓ Desalt with Spin column (optional)
Desalted Labeled RNA*
↓ Dry sample with vacuum concentration, approximately 2-3 hours (1 hour with columns), 45º C to 55º C
↓ Assemble Hybridization mixture + Hyb Spike In (optional)
↓ Heat, Ice
↓ Hybridize 20 hours 55º C 20 rpm
↓ Wash, Scan
miRNA Profile

* The sample can be stored frozen at – 80º C if needed.

Figure 11 Workflow for sample preparation and array processing with the optional Bio-Rad columns and spike-ins.

II.2.1 Sample labelling and hybridization

Agilent's miRNA Complete Labelling and Hyb Kit (p/n 5190- 0456) generates fluorescent miRNA with a sample input of 100ng of total RNA. This method involves the ligation

of one Cyanine 3- pCp molecule to the 3' end of a RNA molecule with greater than 90% efficiency. The Agilent miRNA Complete Labelling and Hyb Kit provides all the needed components for sample labelling and hybridization preparation.

Step 1. Prepare the labelling reaction

Dephosphorylation

1. Dilute total RNA sample to 50 ng/µL in 1X TE pH 7.5 or DNase/RNase-free water.
2. Add 2 µL (100ng) of the diluted total RNA to a 1.5 mL microcentrifuge tube and maintain on ice.
3. Immediately prior to use, prepare the Calf Intestinal Alkaline Phosphatase (CIP) Master Mix from:
Add the components in the order indicated in table 14 and maintain on ice.

Table 14 CIP Master Mix without Labelling Spike-In solution

Components	**Volume (µl) per reaction**	**Volume (µl) per 9 reactions**
10x Calf intestinal Phosphatase Buffer	0.4	3.6
Nuclease-free water	1.1	9.9
Calf Intestinal Phosphatase	0.5	4.5
Total Volume	2.0	18.0

4. Add 2 µL of the CIP Master Mix to each sample tube for a total reaction volume of 4 µL. Gently mix by pipetting.
5. Dephosphorylate the sample by incubating the reaction at 37°C in a circulating water bath or heat block for 30 minutes.
If needed, the samples can be stored at -80°C after incubation.

Denaturation
1. Add 2.8 µL of 100% DMSO to each sample.
2. Incubate samples at 100°C in a circulating water bath or heat block for 5 to 10 minutes.
CAUTION: *Incubate the sample for no less than 5 minutes and no more than 10 minutes, or the labelling efficiency of the sample will be affected.*
3. Immediately transfer to ice-water bath.
4. Continue to the next step immediately.

Ligation

1. Warm the 10X T4 RNA Ligase Buffer at 37°C and vortex until all precipitate is dissolved.
2. Immediately prior to use, prepare the Ligation Master Mix by gently mixing the components listed in Table 15 and maintain on ice.

Table 15 Ligation Master Mix for T4 RNA Ligase

Components	Volume (µl) per reaction	Volume (µl) per 9 reactions
10x T4 RNA Ligase Buffer	1.0	9.0
Cyanine3-pCp	3.0	27.0
T4 RNA Ligase	0.5	4.5
Total Volume	4.5	40.5

3. Immediately add 4.5 µL of the Ligation Master Mix to each sample tube for a total reaction volume of 11.3 µL.
4. Gently mix by pipetting and gently spin down.
5. Incubate at 16°C in a circulating water bath or cool block for 2 hours.

Step 2. Purify the labelled RNA (optional)
You may use the Micro Bio- spin 6 columns for purification of the labelled miRNA.

Micro Bio-Spin 6 column preparation
1. Invert the column sharply several times to resuspend the settled gel and to remove any air bubbles.
2. Snap off the tip and place into a 2 mL microcentrifuge tube supplied with the columns from Bio-Rad.
3. Remove the green cap from the column. If the buffer does not drip into the 2 mL microcentrifuge tube, press the green cap back onto the column and remove it again. Let the buffer drain for about 2 minutes.
4. Discard the drained buffer from the 2 mL microcentrifuge tube and then place the column back into the tube.
5. Spin the microcentrifuge tube containing the column for 2 minutes at 1000 x g in a centrifuge.
6. Remove the column from the 2 mL microcentrifuge tube and place it into a clean 1.5 mL microcentrifuge tube. Discard the 2 mL microcentrifuge tube.

Sample purification

1. Add 38.7 µL of RNase-Free Water or 1X TE pH 7.5 to the labelled sample for a total volume of 50 µL.
2. Without disturbing the gel bed, pipette the 50 µL sample onto the gel bed from step 6 above.
3. Elute the purified sample by spinning the microcentrifuge tubes containing the columns for 4 minutes at 1000 x g in a centrifuge. You may use the Micro Bio- spin 6 columns for purification of the labelled miRNA.
4. Discard the columns and keep the miRNA sample-containing flow-through on ice.
5. Check that the final flow-through is translucent and slightly pink. The flow-through volume needs to be uniform across the samples and close to 50 µL.

Step 3. Dry the sample

6. After the 16°C labelling reaction or sample purification, completely dry the samples. Use a vacuum concentrator at 45 to 55°C or on the medium-high heat setting. This step may take up to 1 hour after column purification and up to 3 hours without column purification.
7. Flick hard on the tube to check for sample dryness. Check that the pellets do not move or spread.

Step 4. Prepare the 10X Blocking Agent

1. Add 125 µL of nuclease-free water to the vial containing lyophilized 10X GE Blocking Agent supplied with the Agilent miRNA Complete Labeling and Hyb Kit (p/n 5190-0456).
2. Mix by gently vortexing. If the pellet does not go into solution completely, heat the mix for 4 to 5 minutes at 37°C.
3. Drive down any material adhering to the tube walls or cap by centrifuging for 5 to 10 seconds at 17,000 x g.

Step 5. Prepare hybridization samples

1. Equilibrate water bath or heat block to 100°C.
2. Resuspend the dried sample in 17 µL of nuclease-free water when the Spike-In solution is used and 18 µL when the Spike-In solution is not used.
3. *(Optional*) Add 1.0 µL of the Hyb Spike-In solution (3rd Dilution) to each sample.
4. Add 4.5 µL of the 10X GE Blocking Agent to each sample.
5. Add 22.5 µL of 2X Hi-RPM Hybridization Buffer to each sample for a total of 45µL.
6. Mix well but gently on a vortex mixer.

Table 16 Hybridization mix for miRNA microarray without Hyb Spike-In solution

Components	**Volume (µl)**
Labelled miRNA sample	18
10xGE Blocking Agent	4.5
2x Hi-RPM Hybridization Buffer	22.5
Total Volume	45

7. Incubate at 100°C for 5 minutes.
8. Immediately transfer to an ice water bath for 5 minutes.
9. Quickly spin in a centrifuge to collect any condensation at the bottom of the tube.
10. Immediately proceed to "Step 6. Prepare the hybridization assembly".

Step 6. Prepare the hybridization assembly
Refer to the *Agilent Microarray Hybridization Chamber User Guide* (G2534- 90001) for in-depth instructions on how to load slides, assembly and disassembly of chambers, as well as other

helpful tips. This user guide is available with the Agilent Microarray Hybridization Chamber Kit (G2534A) and can also be downloaded from the Agilent Web site at *www.agilent.com/chem/dnamanuals*- protocols.
You can view the general microarray processing procedures for hybridization assembly, microarray washing and scanning at *www.opengenomics.com/arrayvideo*. Note the video is not specific to Agilent's miRNA microarray system.

To process the miRNA system microarray:
1. Load a clean gasket slide into the Agilent SureHyb chamber base with the label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not ajar.
2. Slowly dispense all of the volume of the hybridization sample onto the gasket well in a "drag and dispense" manner.
3. Slowly place an array "active side" down onto the SureHyb gasket slide, so that the "Agilent"-labeled barcode is facing down and the numeric barcode is facing up. Verify that the sandwich-pair is properly aligned.
4. Place the SureHyb chamber cover onto the sandwiched slides and slide the clamp assembly onto both pieces.
5. Hand-tighten the clamp onto the chamber.
6. Vertically rotate the assembled chamber to wet the gasket and assess the mobility of the bubbles. If necessary, tap the assembly on a hard surface to move stationary bubbles.
7. Place assembled slide chamber in rotisserie in a hybridization oven set to 55°C. Set your hybridization rotator to rotate at 20 rpm.
8. Hybridize at 55°C for 20 hours.

II.2.2 Microarray Wash

Step 1. Add Triton X-102 to Gene Expression wash buffers
1. Open the cardboard box with the cubitainer of wash buffer and carefully remove the outer and inner caps from the cubitainer.
2. Use a pipette to add 2 mL of the provided 10% Triton X-102 into the wash buffer in the cubitainer.
3. Replace the original inner and outer caps and mix the buffer carefully but thoroughly by inverting the container 5 to 6 times.
4. Carefully remove the outer and inner caps and install the spigot provided with the wash buffer.
5. Prominently label the wash buffer box to indicate that Triton X-102 has been added and indicate the date of addition. Add the Triton X- 102 to Gene Expression wash buffer 1 and 2 when the cubitainer of wash buffer is first opened. Do this step to *both* Gene Expression wash buffer 1 and 2 before use.

Step 2. Prewarm Gene Expression Wash Buffer 2
The temperature of the Gene Expression Wash Buffer must be at 37°C for optimal performance.
Warm the **Gene Expression Wash Buffer 2** to 37°C as follows:

1. Dispense 1000 mL of Gene Expression Wash Buffer 2 directly into a sterile 1000-mL bottle. Repeat until you have enough prewarmed Wash Buffer 2 solution for your experiment.
2. Tightly cap the 1000-mL bottle and place in a 37°C water bath the night before washing arrays. Alternatively, remove the plastic cubitainer from the box and place it in a 37°C water bath the night before washing the arrays.
3. Place slide-staining dish #3 (see Table 8 on page 13 for numbering of the wash dishes) into a 1.5 L glass dish three-fourths filled with water. Warm to 37°C by storing overnight in an incubator set to 37°C.

Step 3. Prepare the equipment
1. Run copious amounts of Milli-Q water through the staining dish.
2. Empty out the water collected in the dish.
3. Repeat steps 1 and 2 at least 5 times, as it is necessary to remove any traces of contaminating material.
4. Discard the Milli-Q water. Always use designated clean equipment when doing the hybridization and wash steps. Designate and dedicate dishes to miRNA experiments. Wash all dishes, racks, and stir bars with Milli- Q water.

Step 4. Wash the microarray slides

Table 17 Wash conditions

	Dish	**Wash Buffer**	**Temperature**	**Time**
Disassembly	1	GE Wash Buffer 1	Room temperature	
1st wash	2	GE Wash Buffer 1	Room temperature	5 minutes
2nd wash	3	GE Wash Buffer 2	37 ºC	5 minutes

1. Completely fill slide-staining dish #1 with Gene Expression Wash Buffer 1 at room temperature.
2. Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Gene Expression Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.
3. Put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Gene Expression Wash Buffer 2 (warmed to 37°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Gene Expression Wash Buffer 2 at 37°C. Use a thermometer to check the temperature.
4. Remove one hybridization chamber from the incubator and record the time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely. Make note of any significant loss of hybridization volume.
5. Prepare the hybridization chamber disassembly.

a) Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counterclockwise.

b) Slide off the clamp assembly and remove the chamber cover.

c) With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.

d) Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Gene Expression Wash Buffer 1.

6. With the sandwich completely submerged in Gene Expression Wash Buffer 1, pry the sandwich open from the barcode end only:

a) Slip one of the blunt ends of the forceps between the slides.

b) Gently turn the forceps upwards or downwards to separate the slides.

c) Let the gasket slide drop to the bottom of the staining dish.

d) Remove the microarray slide and place into the slide rack in slide-staining dish #2 containing Gene Expression Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. *Touch only the barcode portion of the microarray slide or its edges!*

7. Repeat steps step 4 through step 6 for up to five additional slides in the group. For uniform washing, do up to a maximum of six disassembly procedures yielding six microarray slides.

8. When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4, or a moderate speed setting, for 5 minutes.

9. Transfer slide rack to slide-staining dish #3 containing Gene Expression Wash Buffer 2 at 37°C. Stir using setting 4, or a moderate speed setting, for 5 minutes.

10. Slowly remove the slide rack minimizing droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.

11. Discard the used Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2.

12. Repeat step 1 through step 11 for the next group of six slides using fresh Gene Expression Wash Buffer 1 and Gene Expression Wash Buffer 2 pre-warmed to 37°C.

13. Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a N2 purge box, in the dark.

II.2.3 Scanning and Feature Extraction

This section describes how to scan and extract data from miRNA microarrays. Refer to the Agilent Microarray Scanner *User Guide* for more information on how to use the scanner.

Extract data using Agilent Feature Extraction Software

Feature Extraction (FE) is the process by which information from probe features is extracted from microarray scan data, allowing researchers to measure miRNA expression in their experiments. To get the most recent Feature Extraction software for miRNA expression, go to the Agilent Web site at www.agilent.com/chem/fe. Refer to the Agilent Feature Extraction Software *User Guide* or *Reference Guide* for more information to use the software.

II.2.4. Analysis of the data with GeneSpring software

Refer to the Software Instruction manuel

IV. 2D PAGE and 2D-DIGE electrophoresis

IV.1 2-D electrophoresis

IV.1.1 Introduction to 2-D electrophoresis

2-dimensional (2-D) electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells, tissues, or other biological samples. This technique separates proteins according to two independent properties in two discrete steps. The first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI); the second-dimension step, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according to their molecular weights (Mr, relative molecular mass). Thousands of different proteins can thus be separated, and information such as the protein pI, the apparent molecular weight, and the amount of each protein can be obtained.

A large and growing application of 2-D electrophoresis is within the field of proteomics. The technique is also unique in its ability to detect post- and co translational modifications, which cannot be predicted from the genome sequence. Applications of 2-D electrophoresis include proteome analysis, cell differentiation, detection of disease markers, therapy monitoring, drug discovery, cancer research, purity checks and microscale protein purification.

IV.1.2 Sample preparation

Appropriate sample preparation is absolutely essential for good 2-D electrophoresis results. Due to the great diversity of protein sample types and origins, the optimal sample preparation procedure for any given sample must be determined empirically. Ideally, the process will result in the complete solubilization, disaggregation, denaturation, and reduction of the proteins in the sample.

2-D Clean-Up Kit from GE Healthcare can be used to remove contaminating substances and improve the 2-D electrophoresis pattern. Proteins are precipitated with a combination of precipitation reagents while the interfering substances, such as nucleic acids, salts, lipids, or detergents, remain in solution. Preparation of protein samples with the kit reduces horizontal streaking, improves spot resolution, and increases the number of spots detected compared with samples treated by other means.

IV.1.2.1 Sample preparation from tissue (total extract)

Materials

Mortar, 21G Needles and syringes, spoon, Millipore filters 0.45µm and Microcentrifuge.

Products
(1) Urea, (2) Thiourea, (3) DTT, (4) Amberlite IRN-150, (5) CHAPS

Solutions to prepare
Solution ETU : ***8 M Urea, 2 M Thiourea, 4 % CHAPS, 50 mM DTT***

- ✓ 5 g ***Urea*** + 1.6 g ***Thiourea*** in 10 ml water ***Millipore*** (in 15 ml tube)
- ✓ dissolve without heating
- ✓ Add 0.1 g of Amberlite and shake 10 min
- ✓ Filter the solution on filter 0.45 µm
- ✓ A 9.6 ml of filtrate add 0.4 g ***CHAPS,*** 80 mg ***DTT***

Protocol

- Pre-cooling in liquid nitrogen, mortar, pestle and a spatula
- Crush the pieces of tissue in liquid nitrogen
- Retrieve the powder in a 5 ml tube and weight
- Resume in 15 volumes of Solution ETU
- Thoroughly mix and allow 30 min. at 4 ° C in ice [If necessary make a few times in the syringe (21G needle)]
- Transfer the mixture to a microtube
- Centrifuge at 14,000 rpm for 1 h at 4 ° C
- Withdraw the supernatant and
- Achieve 100 µl aliquots and store at - 80 ° C.

IV.1.2.2. 2-D Clean-Up Kit

Materials
2-D Clean Up Kit Ettan™ (GE Healthcare)

Protocol
According to the manufacturer instructions.

IV.1.2.3. Determination of protein quantity in samples

Electrophoresis of protein samples requires accurate quantitation of the sample to be analyzed to ensure that an appropriate amount of protein is loaded. Bradford is a simple and accurate procedure for determining concentration of solubilized protein. For details on Bradford quantification see protocol *Bio Rad Protein Assay.*

IV. 3. First-dimension isoelectric focusing (IEF)

Isoelectric Focusing is an electrophoretic method that separates proteins according to their isoelectric points (pI). The presence of a pH gradient is critical to the IEF technique. In a pH gradient and under the influence of an electric field, a protein will move to the position in the gradient where its net charge is zero. This is the *focusing* effect of IEF, which concentrates proteins at their pIs and allows proteins to be separated on the basis of very small charge differences. IEF is performed at high voltages and under denaturing conditions, resulting with the highest resolution and the sharpest results. Complete denaturation and solubilization is achieved with a mixture of urea, detergent, and reductant, ensuring that each protein is present in only one conformation with no aggregation, therefore minimizing intermolecular interactions.

IEF is performed with the Immobiline DryStrip gels using a flatbed electrophoresis unit such as Ettan IPGphor II. Immobiline DryStrip gels are available with strip lengths of 7, 11, 13, 18, and 24 cm. To overview total protein distribution, pH 3–11 NL (NL refers to nonlinear) strips are used. Narrow-pH-range strips (1 pH unit) are used to closely study proteins in the regions of interest.

Samples can be applied either by including it in the rehydration solution (rehydration loading) or by applying it directly to the rehydrated Immobiline DryStrip gel via sample cups or a paper bridge. Each specific set of conditions (e.g. sample and rehydration solution composition, Immobiline DryStrip gel length, and pH gradient) requires empirical determination for optimal results.

IV.3.1. IEF of wide range strips (pH 3-10/ 3-11 NL)

Materials

IPGphor and accessories, rehydration cassette, Manifold, strip 18/24 cm, pH3-11NL/pH3-10.

Products

(1) Urea, (2) Thiourea, (3) DeStreak Reagent, (4) Amberlite IRN-150, (5) Bromophenol blue, (6) CHAPS, (7) IPG-Buffer pH 3-11 and (8) Mineral oil.

Solutions to prepare

Rehydration buffer : ***8M Urea, 2M Thiourea, 2% CHAPS, DeStreak Reagent, 0.5% IPG-Buffer pH 3-11NL, Trace of Bromophenol blue***

- ✓ 5 g Urea + 1.6 g Thiourea in 10 ml Millipore water (in tube of 15 ml)
- ✓ Dissolve without heating
- ✓ Add 0.1 g of Amberlite and shake 10 min.

- ✓ Filter the solution with 0.45 µm filter
- ✓ To 4.8 ml of filtrate add 0.1 g CHAPS, 60 µl of Reagent DeStreak, 25 µl of IPG-Buffer pH 3-11NL and a few grains of Bromophenol blue

Protocol

1. Rehydratation

(Strip 24 cm = 450 µl Rehydration buffer + sample)

- ❖ Make sure the rehydration tray is perfectly horizontal.
- ❖ Place 450 µl of rehydration buffer and sample in a channel of the rehydration tray.
- ❖ Evenly distribute throughout the channel.
- ❖ Remove the plastic protector of the strip
- ❖ Position the strip in the channel so that the gel side is in contact with the buffer.
- ❖ Avoid the presence of any air bubbles.
- ❖ Cover with 2 to 3 ml of previously degassed mineral oil (DryStrip Cover).
- ❖ Close the casket and let rehydrate overnight.

2. Focusing

- ❖ Turn the IPGphor to cool (20 ° C).
- ❖ Transfer Manifold in IPGphor.
- ❖ Degas 150 ml of mineral oil (DryStrip Cover Fluid)
- ❖ Place 9 ml of mineral oil per channel (always fill the 12 channels, even when using less than 12 strips).
- ❖ Place the strips in the channels (in mineral oil).
- ❖ Prepare 2 electrodes paper (paper Wicks) per strip.
- ❖ Apply 150 µl of distilled water to each paper electrode.
- ❖ Place the paper electrodes at each end of the strips.
- ❖ Put the electrodes and start focusing.

3. Example of classical program:

20°C; 50 µA/strip

S1	Step-n-hold	300 V	3 h	0.9 kVh
S2	Gradient	1 000 V	8 h	5.2 kVh
S3	Gradient	10 000 V	3 h	16.5 kVh
S4	Step-n-hold	10 000 V	3 h	30.0 kVh
Total:			**17 h**	**52.6 kVh**

The strips are treated directly in the 2nd dimension, or immediately frozen at - 80 ° C in a plastic bag.

Figure 1. IEF of erythrocyte membrane proteins on 24 cm IPG strip pH 3-10.

IV.4. Second-dimension SDS-PAGE

SDS-PAGE is an electrophoretic method for separating polypeptides according to their molecular weights (Mr). The technique is performed in polyacrylamide gels containing sodium dodecyl sulfate (SDS). Besides SDS, a reducing agent such as DTT is also added to break any disulfide bonds present in the proteins. When proteins are treated with both SDS and a reducing agent, the degree of electrophoretic separation within a polyacrylamide gel depends largely on the molecular weight of the protein and there is an approximately linear relationship between the logarithm of the molecular weight and the relative distance of migration of the SDS-polypeptide complex.

SDS-PAGE consists of four steps:

- Preparing the system for second-dimension electrophoresis
- Equilibrating the Immobiline DryStrip gel(s) in SDS equilibration buffer
- Placing the equilibrated Immobiline DryStrip gel on the SDS gel
- Electrophoresis

IV.4.1. Equilibrating the Immobiline DryStrip gels

The equilibration step saturates the Immobiline DryStrip gel with the SDS buffer system required for the second dimension separation. The equilibration solution contains buffer, urea, glycerol, reductant, SDS and dye. An additional equilibration step replaces the reductant with iodoacetamide.

Materials

Equilibration tube, Agitator

Products

(1) Glycerol, (2) DTT, (3) Tris-Base, (4) SDS, (5) Iodoacetamide and (6) Bromophenol blue

Solutions to prepare

Stock Solution Bromophenol Blue: ***50 mM Tris-base, 1% Bromophenol blue***

- Tris-base **60 mg**
- Bromophenol blue **100 mg**
- Millipore water **to 10 ml**

Gel Buffer Stock: ***1.5 M Tris-base pH 8.6 , 0.4% SDS***

- Tris-base **90,85 g**
- SDS **2 g**
- Millipore water **to 500 ml**

(Adjust the pH to 8.6 with HCl)

Equilibration Buffer : ***6 M Urea, 2 % SDS, 30% Glycerol,***

50 mM Tris-HCl, pH 8,6

(This solution may be retained in brown flask at room temperature for 15 days)

- Urea **180 g**
- SDS **10 g**
- Glycerol **150 g**
- Gel Buffer Stock **16,7 ml**
- Millipore water **to 500 ml**

Equilibration buffer 1 (Eqb 1) : 1 % of ***DTT*** in the equilibration buffer.

Equilibration buffer 2 (Eqb 2) : 4,7 % of ***Iodoacétamide*** in the equilibration buffer.

Protocol

- Incubate each strip individually in 10 ml of Eqb 1, with stirring at room temperature for 15 min
- Remove the solution and incubate each strip individually in 10 ml of Eqb 2, with stirring at room temperature for 15 min.
- Before you place the strip on the SDS-PAGE, quickly dipped it into the Migration buffer

IV.4.2. Second dimension gel preparation and migration

Ettan DALT*six* Large Vertical electrophoresis systems combined with 24-cm-long Immobiline DryStrip gels offer the highest possible 2-D resolution. The system is designed for simplified assembly and rapid electrophoresis of the second-dimension gel. Ettan DALT*six* system accepts up to six large, second-dimension gels (26 × 20 cm). When running fewer gels, unused slots are filled with blank cassette inserts. System recirculate the buffer so that even gel temperatures are maintained during electrophoresis.

Material

Ettan-Dalt six and accessories

Products

(1) 40% Acrylamide/Bis Solution 37.5:1, (2) Glycerol, (3) Tris-Base, (4) SDS, (5) Ammonium persulphate, (6) TEMED, (7) iso-propanol and (8) Bromophenol blue

Solutions to prepare

Gel Buffer Stock: **1.5 M Tris-base pH 8.6 , 0.4% SDS**

- Tris-base 90,85 g
- SDS 2 g
- Millipore water to 500 ml

(Adjust the pH to 8.6 with HCl)

10% Ammonium persulfate: Ammonium persulfate 500mg/5ml

(Prepare fresh)

30% Isopropanol: 30 ml isopropanol with 70 ml MilliQ water

12.5% Acrylamide solution:

(450 ml of solution are necessary for preparation of 6 gels in the casting tray of Ettan-Dalt 6)

- 40% Acryl/Bis= 37.5:1 140 ml
- Gel Buffer Stock * 112.5 ml
- H_2O MilliQ 197.5 ml

* or 112.5 ml of 1.5 M Tris-HCl (pH8.8) + 4.5 ml 10% SDS + 193 ml H_2O

Thoroughly mix, degas and at the last moment add:

- TEMED **60 µl**
- 10 %Ammonium persulphate **4.5 ml**

Migration buffer: ***25mM Tris, 192mM Glycine, 0.1% SDS***

- Migration buffer TGS [10x] **100 ml**
- MilliQ water **900 ml**

Agarose sealing solution: ***0,6% Low-melting Agarose, 25mM Tris, 192mM Glycine, 0.1% SDS, trace of Bromophenol blue.***

- Low-melting agarose **0,6 g**
- Migration buffer **100 ml**
- Bromophenol blue **Few grains**

Heat in microwave (do not boil) to melt the agarose, then aliquote in 2 ml eppendorf and store at 4 ° C.

Protocol

Gel preparation

The gels are prepared under the hood. The gels should be casted the day before the run is planed.

- Prepare the casting unit with 7 glass plates (tighten screws)
- Degas and pre-cool the 12.5% gel solution to 4 °C.
- Put the TEMED and ammonium persulfate when you are ready to pour the gels

- Pour approximately 450 ml (it stops at 1 cm from the top edge of the glass plates) of 12.5% gel solution
- Cover quickly (but without damaging the front) ~ each plate with 1 ml of 30% isopropanol
- Allow to polymerise overnight.

Migration

Before equilibration step of the strips, put Agarose sealing solution to heat to 90 °C solution (1 eppendorf /strip).

- Fill the tank bottom of the Ettan-DALT6 with 4.5 l Migration buffer
- Connect the cryostat: 2 buttons, cooling (15 ° C) and traffic
- At the end of the equilibration step, quickly dip the strip in the migration buffer
- Place the strip on the gel
- Quickly add ~ 1.0 ml of Agarose sealing solution
- Remove carefully and quickly air bubbles.
- Allow to cool.
- Cover with migration buffer and place the gel in the tank of the Ettan-DALT6
- After assembly pour the migration buffer at the top of the tank (about 1.4 l)
- Start electrophoresis with the following program:
  - **Step 1 :** 45 min at 2.5 W/gel
  - **Step 2 :** 5.00 h at 16 W/gel

IV.5. Visualizing results

Most detection methods used for SDS gels can be applied to second-dimension gels: ***autoradiography and fluorography, silver staining, coomassie staining, fluorescent labeling*** and ***fluorescent staining.*** Here we give the protocol for silver staining and cooloidal coomassie staining.

Fluorescent labeling is the basis for the 2D-DIGE experiments and detailed information about it is given in section **21.2.**

IV.5. 1. Silver staining

For details on protocol, please refer to the instruction manuel of the *Silver staining Kit* (GE healthcare)

IV.5.2. Coomassie colloidal blue staining

Products

(1) Orthophosphoric acid 85%, (2) Ammonium Sulfate, (3) Coomassie Brillant Blue G250, (4) Ethanol

Solutions to prepare

Stock solution of Coomassie Blue G250:

Coomassie Blue G250	2g
Water (hot)	100 ml

dissolve at 100 °C, cool and add:

sodium azide	0.2 g / l

30% ETOH, 2% Phosphoric acid

Ethanol absolute	300 ml/l
Phosphoric acid	20 ml/l

2% Phosphoric acid

Phosphoric acid	20 ml/l

Pre-Staining Buffer

$(NH_4)_2SO_4$	120 g/l
Millipore water	720 ml
2% Phosphoric acid	20 ml/l
18% ETOH (add last)	180 ml/l

Staining buffer

Pre-staining buffer	950 ml
Stock solution of Coomassie G250	5 ml

Protocol

- ✓ Wash gels in millipore water
- ✓ Fix the gel 2x30' in **30% ETOH, 2% phosphoric acid**
- ✓ Fix the gel O/N in **30% ETOH, 2% phosphoric acid**
- ✓ Rinse 3x10' in **2% phosphoric acid**
- ✓ Balance 30' in **Pre-staining Buffer**
- ✓ Add in the same solution 5% of a **Stock solution of Coomassie G250**
- ✓ Incubate 24 to 72 hours (for a color balance to allow 5 days) in the **Staining buffer**
- ✓ Wash the gels with 1% Acetic Acid or Millipore water
- ✓ To store: Let in the staining buffer or in 25% $(NH_4)_2SO_4$

IV.6. Introduction to 2-D DIGE electrophoresis

2-D Fluorescence Difference Gel Electrophoresis (2-D DIGE) is a method that labels protein samples prior to 2-D electrophoresis, enabling accurate analysis of differences in protein abundance between samples. It is possible to separate up to three different samples within the same 2-D gel. The technology is based on the specific properties of spectrally resolvable dyes, CyDye™ DIGE Fluor dyes, that have been designed to be both mass- and charge-matched. As a consequence, identical proteins labeled with each of the CyDye DIGE Fluor dyes will migrate to the same position on a 2-D gel. 2D analysis experiments commonly address questions like protein level differences caused by a disease state, drug treatment, life-cycle stage etc. Some protein level differences are small and the results are affected by experimental variation originating both from the system and from inherent biological variation.

System related result variation may arise either from gel-to-gel variation, which can result from differences in electrophoretic conditions between first dimension strips or second dimension gels, gel distortions, sample application variation and user-to-user variation, of from variation due to user-specific editing and interpretation when using the data analysis software.

Inherent biological variation arises from intrinsic differences that occur within a population. For example, differences from animal-to-animal, plant-to-plant or culture-to-culture which have been subjected to identical conditions.

System variation cannot be overcome when using conventional 2D electrophoresis but by using DIGE system it is possible to minimize the gel to- gel variation effects on results. Biological variation cannot be removed from any 2D electrophoresis experiments. However, using DIGE system allows the inherent biological variation to be effectively differentiated from induced biological changes using highly accurate measurement of protein abundance changes, an appropriate experimental design and advanced statistical analysis.

When designing 2D DIGE experiments, the following recommendations should be considered:

1 Inclusion of an internal standard sample on each gel

2 The requirement for biological replicates such as multiple cultures, tissue etc.

3 Randomization of samples to produce unbiased results

4 No gel replicates of the same sample is needed

The differences in methodology between 2-D DIGE and traditional 2-D electrophoresis are outlined in Table 2.

Table 2. Differences in methodology between 2-D DIGE and traditional 2-D electrophoresis.

Step	**Step 2-D DIGE**	**Traditional 2-D electrophoresis**
Sample preparation	Exclude carrier ampholyte and reductant until after labeling	Carrier ampholyte and reductant included
	Concentrate protein to 1–10 mg/ml.	Concentration of sample is optional.
Labeling	Addition of CyDye required	No CyDye required
	Labeling reaction terminated with addition of lysine	No lysine addition require
Electrophoresis	Use low-fluorescence glass plates	Use standard glass plates

The other steps of the 2-D DIGE technique such as first dimension IEF and second dimension PAGE are the same as in the traditional 2-D PAGE analysis. Differences also arise in visualization of the results (no staining is required in DIGE compared to 2-D PAGE) and software analysis.

IV.6.1. 2-D DIGE sample preparation and labeling

The workflow for sample preparation and labeling is the following:

1. Sample preparation: Prepare cell lysates compatible with CyDye DIGE Fluor minimal dye or CyDye DIGE Fluor saturation dye labeling.
2. pH adjustment of the cell lysates.
3. Preparation of an internal standard and labeling of protein samples using CyDye DIGE Fluor minimal dyes or CyDye DIGE Fluor saturation dyes.
4. Preparation of labeled protein samples for first dimension isoelectric focusing.

Sample preparation

The cell lysis solution should not contain any primary amines, such as ampholytes or thiols (e.g. DTT) as these will compete with the proteins for CyDye DIGE Fluor dyes

resulting in fewer labeled proteins. This decrease in labeling efficiency may affect the data after scanning and spot detection.

The protocol for sample preparation is practically the same as for the conventional 2D-PAGE analysis (see protocol **21.1.2.1)**, except that sample proteins should be dispersed into the solution UTC. Also clean-up of samples with 2-D Clean-Up Kit (see section 21.1.2.2.) is recommended. Protein concentration of samples should be in the range from 5 to 10 µg/µl.

Solutions to prepare

Solution UTC : ***8 M Urea, 2 M Thiourea, 4 % CHAPS***

(Prepare fresh)

- ✓ 5 g Urea + 1.6 g Thiourea in 10 ml water Millipore (in 1.5 ml tube)
- ✓ Dissolve without heating
- ✓ Add 0.1 g of Amberlite and shake 10 min.
- ✓ Filter the solution on filter 0.45 µm
- ✓ Add 0.4 g CHAPS, ajust to 10 ml
- ✓

pH adjustment of samples

The labeling reaction with CyDye DIGE Fluor minimal dyes is most efficient at pH 8.5. Therefore, lysis and sample solutions should be buffered using NaOH or Tris to pH 8.5.

Materials

pH indicator strips, pipet from 0.1 to 10 µl

Products

(1) Tris base

Solutions to prepare

1.5 M Tris: ***181,71mg in 1 ml ddH$_2$O***

Protocol

- ✓ Check the pH of the cell lysate (protein sample) by spotting a small volume (1-2 µl) on a pH indicator strip
- ✓ Adjust the pH to 8.5 if the pH of the cell lysate is outside the desired range by careful addition of 1.5 M Tris (0.5 µl Tris solution to 30 µl sample)

Labeling of protein samples using CyDye DIGE Fluor minimal dye

As an example of a DIGE analysis, a comparison of protein abundance between two different types of tissue (normal breast-N and carcinoma breast-C) each with three biological replicates using CyDye DIGE Fluor Cy2, Cy3, and Cy5 minimal dyes will be presented below. For this analysis, 3 gels are needed with the following sample distribution:

N° Gel	Cy2	Cy3	Cy5
1	IS	N1	C2
2	IS	C3	N2
3	IS	N3	C1

Homogenization of sample volumes

Samples need to contain 45-50 µg of protein each, for labeling with 400 pmol minimal dyes. Sample volumes containing 45-50 µg protein need to adjust to the same volume and the most diluted sample volume is taken. The samples volumes are adjusted with UTC buffer (pH 8.5). For example:

9.1 µL *final volume* = **45µg**

- **N1** (45µg) → 8.7 µL **BCT-9** + 0.4 µl UTC (pH 8.5)
- **N2** (45µg) → 6.4 µL **BCT-18** + 2.7 µl UTC (pH 8.5)
- **N3** (45µg) → 5.6 µL **BCT-20** + 3.5 µl UTC (pH 8.5)
- **C1** (45µg) → 9.1 µL **BCT-12**
- **C2** (45µg) → 6.4 µL **BCT-13** + 2.7 µl UTC (pH 8.5)
- **C3** (45µg) → 5.3 µL **BCT-3** + 3.8 µl UTC (pH 8.5)

where: BCT-9, 18 and 20 are normal breast tissues and BCT 3, 12 and 13 are breast cancer tissues.

Preparation of the internal standard

The internal standard is created by pooling an aliquot of all biological samples in the experiment and labeling it with one of the CyDye DIGE Fluor dyes (usually Cy2 when using CyDye DIGE Fluor minimal dyes and Cy3 when using CyDye DIGE Fluor saturation dyes). The internal standard is then run on every single gel along with each individual sample. This means that every protein from all samples will be represented in

the internal standard, which is present on all gels. In this way every protein spot on all gels will have an internal standard.

For 3 gels: 3x45µg=135 µg total

3 x 9.1 µl = 27.3 µl

6 samples by 22.5 µg each

IS = 4.4 µL **N1** + 3.2 µL **N2** + 2.8 µL **N3** + 4.6 µL **C1** + 3.2 µL **C2** + 2.7 µL **C3**+ 6.4 µL UTC (pH 8.5) = 27.3 µl

Preparation of « Dye Stock Solution » at 1 nmol/µl (1 mM)

CyDye DIGE Fluor minimal dyes are supplied as a solid powder and are reconstituted in dimethylformamide (DMF) giving a concentration of 1 nmol/µl. Once reconstituted, the minimal dye stock solution is stable for three months or until the expiry date on the container, whichever is sooner.

Materials
Vortex, microcentrifuge

Products
(1) 5 nmol of CyDye DIGE Fluor minimal dye kit (2) dimethylformamide (DMF) <0.005% H2O, ≥ 99.8% pure

Protocol

- ✓ Remove CyDyes from the freezer and leave 5 min. at room temperature
- ✓ Take a small volume of DMF from its original container and dispense into a microfuge tube

- ✓ Add 5 µL of DMF into each tube and cap the tube
- ✓ Vortex vigorously for 30 sec.
- ✓ Centrifuge at 12 000 g for 30 sec.
- ✓ The fluorophore can then be used

Preparation of « Dye Working Solution » at 400 pmol/µl (400 µM)

It is recommended that 400 pmol of dye is used to label 50 µg of protein. If labeling more than 50 µg of protein then the dye:protein ratio must be maintained for all samples in the same experiment. Prior to labeling, the dye stock solution is diluted with DMF (see above) to a working dye solution. The working dye solution is only stable for one week at –20ºC.

Materials

Microcentrifuge, Pipet from 0.1 to 10 µl

Products

(1) dimethylformamide (DMF) <0.005% H2O, ≥ 99.8% pure

Protocol

- ✓ Briefly spin down CyDye stock solution
- ✓ Add 1 volume of stock solution to 1.5 volume of DMF (1.5 µl stock + 2.25 µl DMF) (*Add first DMF to the tube, than stock !!!)*

Labelling

Materials

Microcentrifuge, Pipet from 0.1 to 10 µl

Products

(1) Minimal Dye Working Solution **(**Cy2, Cy3, Cy5), (2) lysine

Solutions to prepare

10 mM lysine (« DIGE Stop Solution «) ***18 mg lysine in 10 ml H_2O***

Protocol

Labelling must be done on ice. In the eppendorf containing the sample, add CyDye (mix with the cone) according to the scheme:

* ***Cy3*** :

- ➢ 9.1µL **N1**+ 0.9µL **Cy3**
- ➢ 9.1µL **C3**+ 0.9µL **Cy3**
- ➢ 9.1µL **N3**+ 0.9µL **Cy3**

* ***Cy5*** :

- ➢ 9.1µL **C2**+ 0.9µL **Cy5**
- ➢ 9.1µL **N2**+ 0.9µL **Cy5**
- ➢ 9.1µL **C1**+ 0.9µL **Cy5**

* *Cy2 :*

➢ 27.3 µL **SI**$_{FRIM}$ + 2.7 µL **Cy2**

✓ Centrifuge briefly.
✓ Put the tubes on ice and in the dark for 30 min.
✓ Add 0.9 µl of DIGE Stop Solution for Cy3 and Cy5 and 2.7 µl for IS (Cy2)
✓ Mix by pipetting, pulse and leave 10 min. on ice, in the dark.

Labeling is now finished. The labeled samples can be processed immediately or stored for up to 3 months at -70°C in the dark.

IV.6.2. Preparation of labeled protein samples for first dimension isoelectric focusing

The main difference between conventional 2D electrophoresis and Ettan DIGE system is that the latter will enable up to three different protein samples to be run on a single 2D gel. To achieve this you need to mix the differently labeled protein samples *BEFORE* the first dimension run.

Protocol

For the example given above, the labeled samples are mixed according to the scheme:

Strip **01** [Strip code]→ 10 µL **N1**$_{cye\ 3}$ + 10 µL **C2**$_{cye\ 5}$ + 10 µL **IS**$_{cye\ 2}$ + 420 µL Rb

Strip **02** [Strip code]→ 10 µL **C3**$_{cye\ 3}$ + 10 µL **N2**$_{cye\ 5}$ + 10 µL **IS**$_{cye\ 2}$ + 420 µL Rb

Strip **03** [Strip code]→ 10 µL **N3**$_{cye\ 3}$ + 10 µL **C1**$_{cye\ 5}$ + 10 µL **IS**$_{cye\ 2}$ + 420 µL Rb

where: Rb is the rehydration buffer depending on pH range of the strip. The Rb volume calculation is based on 24 cm strip where **V$_{rehidration}$ =450 µl.**

The samples are mixed by pipeting, centrifuged briefly and ready for the rehidration step (see protocols in section IV.3)

IV.6.3. Visualization of results – image acquisition

Typhoon Variable Mode Imager and Ettan DIGE Imager are recommended for scanning of DIGE second dimension SDS PAGE gels. Typhoon Variable Mode Imager is fully optimized as part of Ettan DIGE System. Ettan DIGE Imager is a scanning CCD camera-based instrument designed for high resolution images of 2D DIGE applicationsData produced by Typhoon Variable Mode Imager and Ettan DIGE Imager is

directly compatible with ImageQuant TL, ImageMaster 2D Platinum and DeCyder 2D softwares.

The workflow for scanning gels with above Imagers and cropping gel image files consist of following steps:

- Cleaning the Imager
- Placing the gels in the Imager
- Selecting scan parameters
- Scanning of the gels
- Cropping of image files (with the ImageQuant TL software)

IV.6.4. Evaluations of results – image analysis

Two different software have been specially designed for image analysis of 2-D PAGE/ DIGE System: DeCyder 2D and ImageMaster 2D Platinum software. DeCyder 2D is recommended in complex experiments when more than two groups or conditions are used in an experiment. ImageMaster 2D Platinum software is suitable for basic experiments containing control vs. treated or healthy vs. nonhealthy conditions. A detection of less than 10% difference between samples can be made with more than 95% statistical confidence. At Figure 2, one of the modules that both software's have –a 3D view of a differentially expressed protein in normal vs disease state is presented.

A B

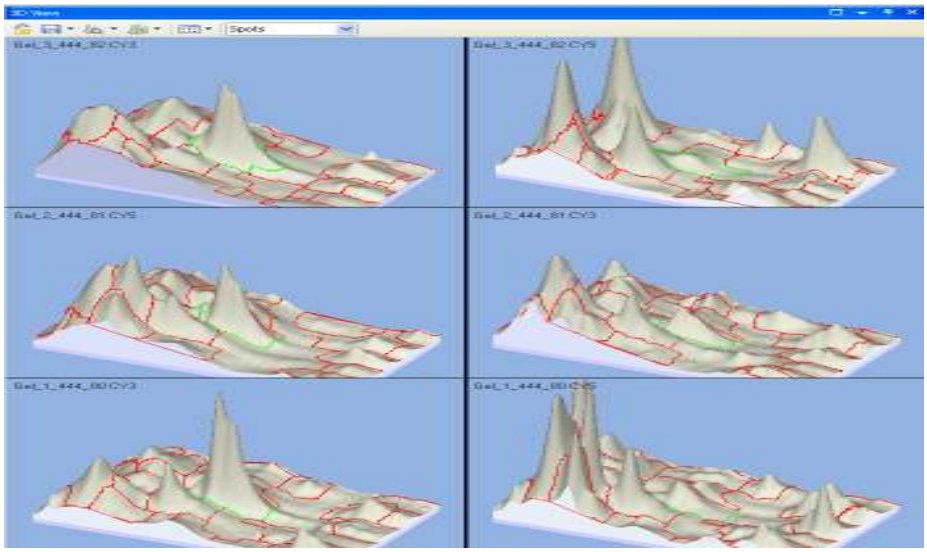


Figure 2. 3D view of a differentially expressed protein in normal (A) and cancer tissue (B) from breast when Annova<0.005 filter was applied, analyzed with ImageMaster 2D Platinum.