METHODS BETA

Sanger Sequencing

Sanger sequencing is the process of selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication; it is the most widely used method for the detection of SNVs. Because both alleles of an autosomal locus are sequenced concurrently and are displayed as an analogue electropherograms, Sanger sequencing is unable to detect mosaic alleles below a threshold of 15–20% (Rohlin et al., 2009) and can miss a significant proportion of low-level mosaic mutations (Jamuar et al., 2014). In addition, mosaic mutations at higher allele fractions are miscalled "germ line," which highlights the limitations of Sanger sequencing in detecting mosaicism on both ends of the spectrum (Jamuar et al., 2014).

From: Genomics, Circuits, and Pathways in Clinical Neuropsychiatry, 2016

Related techniques:

Pyrosequencing Exon Indel Mutation DNA Template

Protocols

From: Chapter Sixteen - Sanger Sequencing of Lyssaviruses

Lorraine M. McElhinney, Denise A Marston, Richard J Ellis, Conrad M. Freuling, Thomas F. Müller, Anthony R. Fooks

Current Laboratory Techniques in Rabies Diagnosis, Research and Prevention 2014 • Pages 159-170

The following describes the protocols employed when using the ABI 3130xl, 16 capillary and ABI3730, 48 capillary Genetic Analyzer instruments. Other machines and reagents are available for Sanger sequencing.

16.2.1. Preparing Polymerase Chain Reaction Amplicons for the ABI Genetic Analyzers

After a PCR product is amplified from a lyssavirus-positive sample, it needs to be purified and then sequenced using florescence-based terminator cycle sequencing. The following describes the protocol to be followed when using the ABI Big Dye Terminator ready reaction kit. The kit works by providing a ready mix that contains the enzyme, dNTPs, magnesium chloride, buffer and dye terminators (ddNTPs), all at the appropriate quantities. Purified amplicon DNA and one primer are added to the mix and cycled to obtain dye-terminated products, which can be precipitated and analyzed on the ABI genetic analyzers.

- Sequencing must be performed in both the reverse and forward direction (i.e. using 3′ and 5′ directed primers) so consensus (complementary) sequence can be obtained.
- Sequencing in each direction should be replicated, at least in duplicate, prior to publication of the sequence to reduce the likelihood of errors.
- If cloned PCR products are sequenced, ensure at least five independent clones are sequenced, and

use the consensus sequence for publication to ensure minor variants are not used as the representative sequence.

16.2.2. Purifying the Polymerase Chain Reaction Product

After visualising the PCR product by agarose gel electrophoresis, the remaining PCR product (amplicon) should be purified prior to sequencing. A number of commercial kits are available for the purification of PCR products (e.g. QiaQuick PCR Purification kit; Qiagen). If the PCR product band appears weak on the gel, it can be loaded and extracted directly from the gel using a commercially available kit (e.g. MinElute gel extraction kit (Qiagen)), which will enable the product to be eluted in as little as 10 µL Elution Buffer, thereby increasing the concentration. For large-scale purification (96-well plates), automated purification may be preferable (e.g. Millipure Multiscreen HTS PCR 96-well purification system or Beckman Coulter Agencourt AMPure clean up system).

- Ensure fresh tips are used between samples to avoid cross-contamination.
- Elute purified PCR product in 10–50 µL Elution Buffer depending on amount of product and purification kit used.
- Quantify the DNA concentration using a spectrometer (e.g. the Nanodrop or Polarstar Galaxy plate reader).

16.2.3. Big Dye Terminator Cycle Sequencing Reactions

On ice, add the purified amplicon DNA (approximately 50–100 ng – usually 2–5 µL) to the appropriate wells on a 96-well plate. If plasmid DNA is being used, pre-heat the DNA at 96°C for 1 minute and keep on ice. If many primers are being used for sequencing (forward, reverse, etc.), make a mastermix without the primer and add primers to appropriate wells at this stage. See below as an example for calculating amount of DNA to be added.

- PCR products: (PCR product length/100)×2)/DNA conc. (ng/µL).
- Plasmids: 100/DNA concentration (ng/µL).

Make a mastermix using the volumes shown below. The amount of water can be amended to allow for variation in the amount of DNA product added, ensuring final volume is 20 µL. The primer employed will depend on the template sequence to be analyzed (examples given in Table 16.1, Table 16.2).

Gene	Forward Primer		Reverse Primer	
Partial Nucleoprotein 1st round	JW12	ATGTAACACCYCTACAATG	JW6UNI	CARTTVGCRCACAT
Partial Nucleoprotein 2nd round	JW12	ATGTAACACCYCTACAATG	JW10UNI	GTCATYARWGTRTG
Glycoprotein	GP01 (GT1)	GGAAAGATGGTTCCTCAGG	GP02 (GT1)	AACTTGAGTTTGCA
Plasmid pCR II	T7	TAATACGACTCACTATAGG	SP6	ATTTAGGAGACTATA

Table 16.1. Examples of Primers used for Sequencing PCR Amplicons

Primer	Orientation	Position EBLV-1	Position EBLV-2	Sequence (5′-3′)	Used for	EBLV
L FOR 1	M	10264–10285 6	10228–10250	GTDGATCAAGAGGTVCGCCATGC	L LD primer	1/2
L REV 2	G	11694–11671	11658–11635	CTTGTAAATCAACCTKATCCAGTG	L LD primer	1/2
JW12 long SacI	M		11658–11635	GCAGAGAGCTCATGTAACACCTCTACAATGGATGC	L LD primer	2
GG2 long XbaI	G		4946–4913	CTAAGTCTAGAGGTATTTGGTCCATTAGACAAGC	L LD primer	2
GG2 FOR	M		4913–4935	GCTTGTCTAATGGACCAAATACC	L LD primer	2
L REV long dist	G		10626–10605	TGAGGCCATGAAGTCATTCACC	L LD primer	2
JW12 EBLV LD	M	52–77	52–77	GAAATGTAACACCCCTACAATGGATG	L LD primer	1/2
EBLV G REV LD	G	4584–4562	4596–4574	GTGGAWGGGTCAGCCAAGGGATG	L LD primer	1/2
EBLV G FOR LD	M	4562–4584	4574–4596	CATCCCTTGGCTGACCCWTCCAC	L LD primer	1/2
EBLV1 L REV LD	G	11221–11197		CTGGAAACCCTCTTACAAGGTCTTG	L LD primer	1
Gen end N E2 REV	G		255–232	GCATTTATTCCGGACAGAATGGAC	5′ end RLM-RACE	2
Gen end N E1 REV	G	254–232		CATTCATCCCTGACAAGATAGAC	5′ end RLM-RACE	1
EBLV-1 L FOR 16	M	10880–10901		CTCTGTTAATGTCAGACTTCTC	3′ end RACE RT	1
EBLV-2 L gen end For 4	M		11417–11438	GACACTTCAATATCTGCTGCAG	3′ end RACE RT	2
EBLV-1 Genomic end L For	M	11555–11576		CCTATTACTTCAAGAAGCAGAC	3′ end RACE PCR	1
EBLV-2 L gen end For 3	M		11532–11552	CCTATTACTTCAAGAAGCAGAC	3′ end RACE PCR	2

Table 16.2. Oligonucleotides used in the Long-Distance PCR and RLM-RACE Strategies for EBLV-1 and -2 Sanger Genome Sequencing

Sequencing Mix volume added per reaction:

- DNA, 5.0 μL
- Molecular grade water, 6.0 μL
- Big Dye Sequencing mix, 4.0 μL
- 5×sequencing buffer, 4.0 μL
- Primer (3.2 pmol/μL), 1.0 μL
- Total volume, 20.0 μL

Examples of primers are shown in Table 16.1. See Table 16.2 for details of primers used for

long-distance PCR to provide overlapping amplicons for European bat lyssaviruses (EBLV) genome sequencing.

Cover the plate with foil, sealing the lid and centrifuge briefly, to collect liquid at the bottom of the wells if necessary.

Transfer the plate to a PCR machine and run on the suitable ABI sequencing program. These can be modified to suit primers if necessary (see below).

Cycling conditions for Big Dye Sequencing reactions:

- 96°C, 10 sec
- 50°C, 5 sec
- 60°C, 4 mins
- 4°C, hold

} ×25

16.2.4. Ethanol Precipitation of Sequencing Reactions

The purification of the sequencing reactions facilitates the removal of unincorporated labeled ddNTPs, which would interfere with base calling. Once the cycling is complete, remove the plate from the PCR machine and clean up the sequencing products. The Ethanol/EDTA/NaOAc clean-up method is recommended. While ethanol/EDTA can be used, the smallest PCR fragments may not be precipitated.

Note: Isopropanol precipitation is NOT recommended.

- Add 2 µL 125 mM EDTA to each well.
- Add 2 µL 3M Sodium Acetate to each well.
- Add 50 µL of 100% Ethanol to each well.
- Seal plate, vortex briefly and leave for 15 mins at room temperature.
- Centrifuge the plate at 3,000 rpm for 45 mins.
- Invert plate over sink and shake 3 times to remove supernatant.
- Rinse each well with 70 µL 70% ethanol.
- Centrifuge the plate at 3,000 rpm for 15 mins.
- Invert plate over sink and shake 3 times to remove supernatant.
- Keeping the plate inverted, place it on a piece of paper towel and centrifuge upside down for 15–30 seconds at 300 rpm.
- Air dry for 15–10 mins.
- Seal and label the 96-well plate.

16.2.5. Preparing 96-Well Sample Plate(s) for the Genetic Analyzers

Spin samples down if necessary, remove sealing film.

16.2.6. Loading 96-Well Sample Plate(s)

The Genetic Analyzers have a safety feature, which means that no movement will occur inside the machine when the door is open. The machines will not work, and the buttons on the front

of the machines will not function, unless the door is properly closed.

For the 3130xl: Press the button on the front of the machine labeled “TRAY.” Once the tray has stopped moving, the door can be opened. Inside, the machine has two positions for holding plates. Position A is on the left, and Position B is on the right. The plate assembly will only fit in one way with Column 1 furthest away from the door due to a notch in the base plate of the plate assembly. Place the plate assembly into position. Close the door and wait to see the green light appear on the front of the machine, which indicates it is properly loaded.

For the 3730: Open the stacker drawer of the Genetic Analyzer and then open the In Stack door. Place the plate assemblies into the stacker with the plate(s) orientated so that the notched corner of the plate assembly is at the rear right corner of the stacker. Up to 16 plates can be placed in the “In Stack.” Close the “In Stack” door and then the stacker draw.

Note: The In Stack is at the front of the stacker drawer with the “Out Stack” at the rear. Plates to be run are taken from the bottom of the stack and will then be at the top of the “Out Stack” once they have been run.

16.2.7. Checking Run Reagents

Check the polymer and buffers are within expiry dates.

Check there is sufficient volume of polymer in the bottle for the run. Check for bubbles in the tubes (and small tank above) leading from the POP-7. If bubbles are present, an error could occur and your run will stop. To check for bubbles, switch on the light and look inside. Remember to turn off light once checked.

16.2.8. Starting the Run on the 3130xl

In the Data Collection program on the PC, select from the left-hand list 3130xl/Run Scheduler in Plate View. Files which show “Processed” next to them are completed runs. Files which show “Pending” next to them are ready to be run. When you locate your file, it should be “pending.”

You will see on the right of the screen two rectangles representing the two plate positions inside the machine. The color of the rectangles means:

- Grey – no plate(s) in holder.
- Yellow – unlinked plate(s) in holder.
- Green – linked plate(s) in holder.

Click once on the position that relates to your plate, Position A (left) or Position B (right). It should turn green. The Plate Record will now have A or B to the left of the name, indicating linkage to the plate position.

- To start your run, click on the green arrow at the top left hand corner of the window.
- A dialog box will appear telling you that you are about to start processing your plate. Click OK.
- Once the run is finished, check that the run has proceeded correctly and the analyzed sequence data is available.

16.2.9. Starting the Run on the 3730

In the Data collection program on the PC, select from the left-hand list 3730/Run Scheduler in

Plate View. Find the Plate Record file for the plate to be run first and highlight the file.

Click on Add – This will add it to the list in the Input Stack dialog box with Status as Pending. Keep adding plate records in the same order as the order they will be run, until all those to be run are in the In Stack dialog box.

Plates can be added or removed during instrument operation.

Click on Done to close the Add Plates to In Stack dialog box. Note: The Plate Record at the bottom of the list in the In Stack dialog box is marked “1” and will be run first, the next one second, etc.

If the sequencer is running, nothing further needs to be done. If the sequencer is not running, follow these directions: To start your run, click on the green arrow at the top left hand corner of the window. A dialog box will appear, telling you that you are about to start processing your plate. Click OK.

Once the run is finished, check that the run has proceeded correctly and the analysed sequence data are available.

16.2.10. Analyzing Output Data

The raw data will be generated in different formats depending on the sequencer used, but in general you would expect to obtain a chromatogram or trace from which you can derive the sequence (.seq or .fas file). From an ABI sequencer you would expect “.ab1” or “.abi” file formats (Figure 16.1), while from a Beckman sequencer you may expect a “.scf” file format.

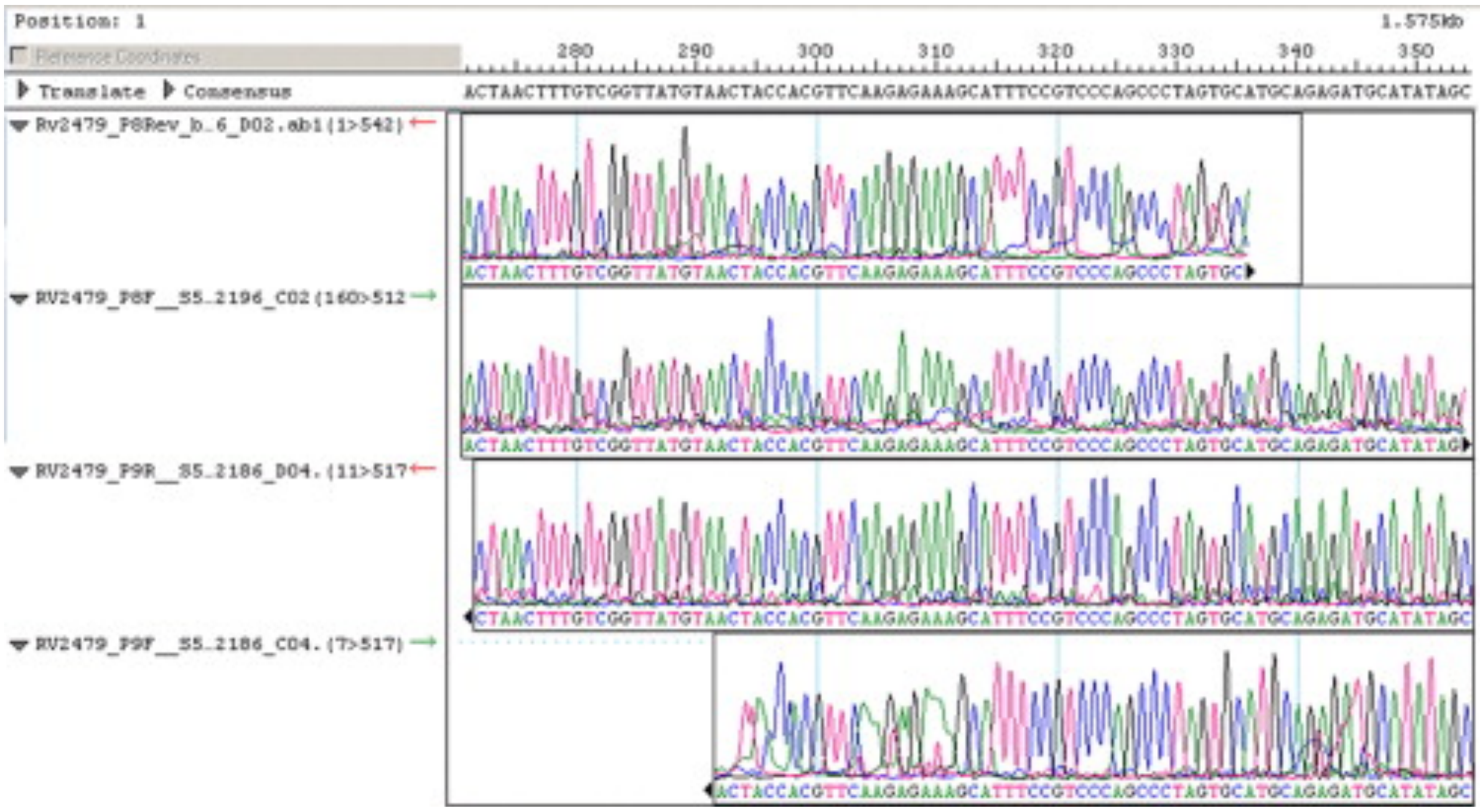

Figure 16.1. Sanger sequencing of overlapping long distance PCR amplicons (forward and reverse) facilitate genome sequencing via “Walking the Genome.” The four ABI traces of four overlapping PCR products (RV2479, 1993 EBLV-2 M. daubentonii from Switzerland) yield contiguous sequences that are superimposed (aligned) to generate a consensus sequence (top frame) determined using the Seqman Programme (DNASTAR Lasergene 10).

A number of software packages exist for analyzing the data and generating consensus sequences from the replicate forward and reverse outputs (e.g. Seqman in Lasergene). If the sequences are good quality, they will form a contiguous sequence (called a contig). The forward and reverse contiguous sequences are aligned to generate consensus sequences (Figure 16.1).

If the consensus sequence saved is more than the required length, it can be trimmed to the correct length by opening in a sequence editing program (e.g. Seqman or Editseq in Lasergene 10 or MEGA13).

Key researchers

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70 h-index | 128 Citations | 245 Documents

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Relevant methods

An inducible nitric oxide synthase (NOS) is expressed in hemocytes of the spiny lobster Panulirus argus: Cloning, characterization and expression analysis

Kozsik, T.Tóth, M.Bozó, I.

Pathobiology of Human Disease • Volume 79 • 2018

Material and methods

Lobsters between 0.5 and 0.8 kg of weight were collected by diving in the Golfo de Batabanv≥, which lies in the southwestern part of the Cuban archipelago. They were kept in 100-l tanks (two lobsters per tank) connected to a flow through seawater supply system, using biological, Äìmechanical filtration, seawater UV-sterilization, central aeration system, and controlled photoperiod of 12: 12 h light: obscurity. All experiments were performed after one week in these conditions.

To obtain hemocytes, the same amount of hemolymph from five lobsters was extracted using a modified Citrate-EDTA buffer (400 mM NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 20 mM EDTA, pH 4.6) as anticoagulant [39]. The hemolymph was extracted from the fourth walking leg coxa using a pyrogen-free disposable syringe containing the same volume of pre-cooled anticoagulant solution. The mixture was centrifuged at 700 g for 10 min at 4 °C. The cell pellet was washed twice with anticoagulant and suspended again in anticoagulant solution.

Attempts at producing a hybridised Penaeus mondon cell line by cellular fusion

Kerry Claydon; Katrina G.Roper; Leigh Owens

Fish & Shellfish Immunology • Volume 29, Issue 3 • September 2010 • Pages 539-

Material and methods

P. monodon were collected from wild stocks off the coast of northern Queensland, Australia, or from a commercial farm located at Innisfail, Queensland, Australia. Following collection, P. monodon were maintained at 28¬±3°C at James Cook University in 1t Reln¬© recirculating tanks with biological filtration. Two immortal cell partners,

Epithelioma papulosum cyprinid (EPC) and Spodoptera frugiperda pupal ovarian cells (Sf9) were investigated in cell fusion experiments. Cells were maintained in 25cm2 flasks containing 10ml of Leibovitz 15 (L-15) (Gibco, Brisbane, Australia) or tissue culture medium 100 (TC-100) (Gibco, Brisbane, Australia) for the maintenance of the EPC and Sf9 cells respectively, and supplemented with 10% foetal bovine serum (FBS) (Trace Biosciences, Castle Hill, Australia). The cells were observed daily with an Olympus IMT-2 inverted phase-contrast microscope, and once a monolayer formed, cells were trypsinised, split and re-seeded.

Identification of upregulated immune-related genes in Vibrio harveyi challenged Penaeus monodon postlarvae

S. Nayak, S.K. Singh, N. Ramaiah, R.A. Sreepada

Fish & Shellfish Immunology • Volume 29, Issue 3 • September 2010 • Pages 544-549

Material and methods

Ten-day old postlarvae (PL) of P. monodon (Crustacea, Decapoda) were obtained from a commercial hatchery and, held in the laboratory in a 500 L tank filled with seawater under continuous aeration. The PL were fed twice daily with a commercial artificial feed and, maintained throughout the 3 day experimental period under aeration at 25 °C and 29 psu salinity.

Replicates of two glass containers of 2-L capacity were filled with 1 L filtered autoclaved seawater. PL14 of P. monodon (60 nos/container) were acclimatized for a day under aeration. A strain of V. harveyi D3 (identity confirmed through partial sequencing of its 16 S rRNA gene) found to cause lethargy or mortality in all 3 trial runs was used for challenging the PL. Broth cultures of this strain grown for 24 h in seawater nutrient broth were used for the experimental challenge. Cells were harvested by centrifugation and re-suspended in sterilized seawater and the PL were 'bath' challenged by adding the harvested cells to attain 2.5-3.2 (X 106) cells ml-1 in the experimental containers for 48 h. Two glass containers filled with 1 L filtered autoclaved seawater holding ~60 PL with no added bacterial cells served as experimental controls.

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Lesley-Ann Sutton, Larry Mansouri, Rebeqa Gunnarsson, Adam Ameur, Sean Hooper, Markus Rasmussen, Anders Isaksson, Ulf Gyllensten, Richard Rosenquist

Clinical Lymphoma Myeloma and Leukemia • Volume 11, Supplement 2 • October 2011 • Pages s148-s149

Chapter Fourteen: Sanger Dideoxy Sequencing of DNA

Sarah E. Walker, Jon Lorsch

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Dr Timothy Walker MBBS, Camilla L PhD, Ruth H Harrell PhD, Jason T Evans PhD, Georgia Kapatai PhD, Martin J Dedicoat PhD, ..., Prof Tim E Peto DPhil

The Lancet • Volume 380, Supplement 3 • 23 November 2012 • Page s77