This manual was created by Philip Pellett, PhD while at the CDC in 1995.

Thanks to Alex Greininger for scanning a photocopy of the manual into an editable form.  Users should be on the lookout for typographic errors that might have been introduced by the scanning process.  The manual was created in 1995, so some of the procedures are obsolete or are specific to instruments that are obsolete.

**Herpesvirus Section**

**Procedure Manual**

Cellular Virology and Herpesvirus Laboratories

Centers for Disease Control and Prevention

Version 1

August 1995

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# ****Forward****

This manual is a compilation of procedures used in the Cellular Virology and Herpesvirus Laboratories of the Herpesvirus Section, CDC. Contributors include Jodi Black, Darrell Burns, Marianne Davis, Geraldina Dominguez, Kathleen Kite-Powell, Christine Ko, Phil Pellett, Jeff Rapp, Felicia Stamey, and Dena Tzanetou. No claim is made for originality - many of the protocols are straightforward adaptations from published procedures and from manufacturers' literature. The manual was compiled by Serina Floyd and edited by Phil Pellett.

The procedures described here assume basic familiarity with laboratory procedures, especially as relates to safety. The methods described employ potentially hazardous chemicals, infectious agents, materials that are potentially infected with, among others, human immunodeficiency virus type 1 (HIV-1) and/or hepatitis B virus, and radioactive materials. Detailed description of all of the safety-related aspects of the procedures described here is beyond the scope of this document. Inexperienced laboratorians should not use these protocols without appropriate safety training.

August 1995

This document may be copied freely.

# ****Microscope Photography****

1. Sign in on pad (name, date, time of use)

2. Turn on Xenon power supply [Figure 1]

 -Press silver start button to light Xenon bulb; look for blue light (press for only 1/2 sec. each try; if unsuccessful after 3 attempts, wait for a few minutes then try again)

3. Load film [Figure 2]

 -Use Kodak Ektachrome 400, daylight color film.

 -Open magazine (backing) by sliding catch (left side of camera) in direction of arrow.

 -Make sure lever in the middle is on white dot.

 -Pull out rewind knob (knob on the right).

 -Insert film, push in rewind knob.

 -Pull film to the left, insert into slit in take-up spool, make sure film fits over teeth of transport reel.

 -Turn film transport knob (knob on left) counterclockwise until it stops; depress middle lever again, turn film transport knob counterclockwise again until it stops; this advances the film 2 frames.

 -Close magazine/backing.

 -To set frame counter, push/turn flat disc part of film transport knob until it is set at '1'.

4. Photoautomat [Figure 3]

 -Turn on (switch in back, right side).

 -ASA 400 (adjust with up arrow).

 -RFC (not LIN): Reciprocity failure correction factor.

 -CAL: adjusts exposure factor; keep on 1.0 because others don't make significant difference in pictures.

 -DFC: use DF (dark field) for fluorescence slides; use BF (bright field) when using white light.

 -MODE: keep on 'auto' setting.

 -Use SPOT (not INT); integrates light from limited area in the middle of the field; adjust by moving lever on left side of shutter piece (white section of camera - below main part of camera where film was loaded). [Figure 2]

5. Microscope

 -Silver knob on right side of microscope above the level of the lenses; turning it will change the number in the frame beside it (changes the filters).

 -Use: 1-FITC (blue) 2-Green (rhodamine)

 3-FITC (blue) 4-Bright field

 -FOCUS-Turn on white light source

-Put silver knob on '4'.

-Turn right eyepiece to extreme right; looking through only your right eye, turn eyepiece until circles in the field are focused.

-Turn left eyepiece to extreme left; put random slide in (not a good slide); focus on something; looking through only your left eye, turn eyepiece until field is clearly focused.

6. Take Pictures

 -If using fluorescence, put silver knob on '1'; make sure knob clicks fully into position.

 -Find desired field.

 -Make sure exposure time shown on Photoautomat display is under 30 sec.

 -Make sure no warning lights are blinking.

 - too much light

 - not enough light

 - out of film

 -Press expo button.

 -Don't move to a different field until film advances.

# Preparation and Propagation of Lymphocytes from Cord Blood

## Sample Logging

1. Count the total number of samples received from each hospital and enter into the CORD BLOOD LOG underneath the current date.

2. Using the first letter of the hospital from which it came and a number within the total number of samples received from that particular hospital, name each sample and write name on its tube.

3. Examine samples to see if they are suitable for lymphocyte purification. Discard sample if any of the following apply:

 - greater than 24 hr. old

 - clotted

 - less than 10 ml

 - no date or time

4. For each sample enter the following information into the CORD BLOOD LOG:

 - sample name

 - date sample was taken

 - time sample was taken

 - volume of sample

 - if discarded, write "discarded" and include reason why discarded.

## Lymphocyte Purification

Perform the following operations for each sample:

1. Centrifuge for 10 min at 1500 RPM (Beckman Model TJ-6).

2. Remove and discard most of the top layer (PLASMA).

3. Collect the buffy (WHITE) coat by removing 10 ml from the interface of the red blood cells and the plasma.

4. Transfer the buffy coat to a 50 ml centrifuge tube (ORANGE CAP) containing 10 ml PBS P/S and mix by swirling gently.

5. Underlay with 10 ml of Lymphocyte Separation Medium (LSM).

6. Centrifuge for 20 min at 1500 RPM.

7. Remove and discard most of the top layer (PBS and LSM) and collect the buffy (WHITE) coat by removing 10 ml of liquid starting 5 ml above the buffy coat and stopping 5 ml below.

8. Transfer the buffy coat to a 50 ml centrifuge tube (ORANGE CAP) containing 10 ml PBS P/S and mix by swirling gently.

9. Underlay with 10 ml of LSM.

10. Centrifuge for 20 min at 1500 RPM.

11. Remove and discard most of the top layer (PBS and LSM) and collect the buffy (WHITE) coat by removing 10 ml of liquid, starting 5 ml above the buffy coat and stopping 5 ml below.

12. Transfer the buffy coat to a 50 ml centrifuge tube (BLUE CAP) containing 35 ml of PBS P/S.

13. Centrifuge for 10 min at 1500 RPM.

14. Pour off the supernatant quickly.

15. Resuspend the pellet by rubbing the tube (GENTLY) on the back side of a test tube rack.

16. Add 1.5 ml of PBS P/S to the tube for a total volume of approximately 2 ml.

17. The sample is now ready to be counted and cultured.

## Estimation of Cell Number

Cells are counted in a Coulter Counter

**Counter Preparation**

1. Remove the tubing from the detergent flask (GREEN) and place it into the Isoton flask (CLEAR).

2. MAKE SURE THAT THE DETERGENT AND ISOTON FLASKS ARE FULL AND THAT THE WASTE FLASK IS EMPTY!

3. Lower the sample stand and replace the detergent Accuvette vial with the Isoton Accuvette vial.

4. Slowly return the sample stand to its upper position.

 DO NOT ALLOW THE SAMPLE STAND TO SLAM AGAINST THE APERTURE TUBE!

 MAKE SURE THAT THE APERTURE TUBE IS COMPLETELY IMMERSED IN ISOTON!

5. Pull out the power switch to turn on the counter.

6. Flush the counter by turning both stopcocks to a VERTICAL position.

 DO NOT ALLOW AIR TO ENTER THE COUNTER THROUGH THE APERTURE OR TUBING!

7. When the detergent in the aperture tube has been replaced with Isoton, return both stopcocks to a HORIZONTAL position.

8. At this time the machine is ready to count samples.

**Sample Preparation**

1. Label Accuvette vials with the sample names.

2. Fill vials with 10 ml Isoton each.

3. Add 20 µl of each sample to its corresponding vial.

4. Add 3 drops of Zap-o-Globin to each vial, mix.

5. The samples are now ready to be counted.

**Cell Counting**

1. Lower the counter's sample stand and replace the Isoton vial with a sample vial that has been mixed by inverting slowly a few times.

2. Slowly return the sample stand to its upper position.

 DO NOT ALLOW THE SAMPLE STAND TO SLAM AGAINST THE APERTURE TUBE!

 MAKE SURE THAT THE APERTURE IS COMPLETELY IMMERSED INTO THE SAMPLE!

3. Turn the upper-right stopcock to a VERTICAL position until the counter clicks and the display reads "0" then quickly turn the stopcock back to a HORIZONTAL position.

4. The counter will start counting at this time and will stop on its own.

5. Observe oscilloscope and debris monitor for any disruption in counting.

6. If it is necessary to remove debris from aperture, manually stop counting by lowering the sample stage then lightly brush away debris with the brush provided.

7. Take 2 counts for each sample and average them. Multiply the average by 2000 for the total number of cells.

8. Enter the 2 counts and the total number of cells for each sample into the cord blood log.

**Counter Clean-up**

1. Lower the sample stage and replace the counter sample vial with the detergent vial.

2. Remove the tubing from the Isoton flask and put it into the detergent flask.

3. Flush the counter by turning both of the stopcocks to a VERTICAL position.

4. When the Isoton in the aperture tube has been replaced with detergent, turn both stopcocks to a HORIZONTAL position.

5. Push in the power switch to turn the counter off.

6. EMPTY THE WASTE FLASK AFTER EVERY SESSION!

## Lymphocyte Culture

Label a tissue culture flask for each sample of purified lymphocytes, including the following:

- Sample Name

- Current Date

- Total Number of Cells

Suspend each sample in a stimulation medium to a concentration of one million cells per ml.

If a sample is less than 30 million cells, place it into a T-25 flask; otherwise, place it into a T-75 flask.

If a sample is > or = to 100 million cells, divide it equally into 2 T-75 flasks. Denote that there are duplicate flasks by writing "(2)" immediately to the right of the sample name on both flasks.

Place caps on flasks loosely to allow for gas exchange and store in a 37°C incubator with 5% CO2.

**Materials**

(FBS) Fetal Bovine Serum

Hyclone Laboratories Cat. #A-1111-L

L-Glutamine 200 mM (CDC #CP0038)

Gibco Cat. #25030-024

Hydrocortisone

Sigma Cat. #H0888

Isotonic Detergent (5 liter box)

Baxter Cat. #B3145-16A

(LSM) Lymphocyte Separation Medium

Organon Teknika Corp. Cat. #36427

Penicillin/Streptomycin (CDC #CP0034)

Gibco Cat. #15140-023

Phytohemagglutinin P (mitogen) by Difco Laboratories

Fisher Scientific Cat. #3110-57-3

RPMI 1640 (1x) with L-Glutamine (CDC #CP0069)

Gibco Cat. #11875-036

Zap-o-Globin II Lysing Agent by Coulter Diagnostics

Curtin Matheson Cat. #360-693

**Stimulation Medium**

Combine the following ingredients:

RPMI 1640 500 ml

 Fetal Bovine Serum 50 ml 10% (heat inactivated at 56°C 30 min)

 Penicillin/Streptomycin 5 ml 100 U/ml Penicillin

 100 µg/ml Streptomycin

 L-Glutamine 5 ml 0.29 mg/ml

 Hydrocortisone 1 ml 0.01 mg/ml

 Phytohemagglutinin P 1 ml 0.02 mg/ml or 0.002% w:v

 Filter through a 0.2 µm filter!

**PBS Wash (PBS P/S)**

Combine the following ingredients:

 PBS 500 ml 0.01 M pH 7.40

 Penicillin/Streptomycin 10 ml 100 U/ml Penicillin

 100 µg/ml Streptomycin

 Filter through a 0.2 µm filter.

**Hydrocortisone Preparation**

Hydrocortisone = 1 gram per vial

Prepare a 5 mg/ml stock (1-gram Hydrocortisone in 200 ml)

1. Dissolve 1 gram of Hydrocortisone in 100 ml 95% EtOH.

2. After dissolved, mix with 100 ml sterile Type I water.

# Passage of HHV-6B(Z29) and HHV-7(SB) in Cord Blood Lymphocytes

1. Purify Cord Blood Lymphocytes:

See procedure for the "Preparation and Propagation of Lymphocytes from Cord Blood".

2. After purification of the CBLs, the lymphocytes are placed into a tissue culture flask and suspended in stimulation medium at a concentration of 1x106 cells/ml and placed in a 37°C incubator with 5% CO2. The lymphocytes are allowed to stimulate for 1-3 days (preferably 3 days).

3. After 1-3 days, the stimulation medium is removed from the settled cells by taking off the supernatant with a pipet-aid.

CAUTION: DO NOT DISTURB THE CELLS WHEN TAKING OFF THE SUPERNATANT

The cells are then infected with either

 a) a thawed vial of infected CBLs and allowed to absorb for 2 hr. before resuspending them in infection medium to a concentration of 1x106 cells/ml.

or,

 b) 1/10 the volume of the flask to be infected. The inoculum consists of cells and supernatant from a 1-week old infected cord. The cells in the newly infected flask are then suspended in infection medium to a concentration of 1x106 cells/mL.

4. One week post-infection, the flask is passed again, frozen, or used to prepare slides for IFA. Cytopathic effect (CPE) is visible anytime after 5-6 days P.I. The best flask (flask with the highest infection) is always picked and set aside for passage of virus into new cords.

**Materials**

Fetal Bovine Serum (FBS)

Hyclone Laboratories Cat. #A-1111-L

L-Glutamine, 200 mM (CDC #CP0038)

Gibco Cat. #25030-024

Hydrocortisone

Sigma Cat. #H0888

Interleukin-2 (IL-2)

Boehringer Mannheim Cat. #1011456

Penicillin/Streptomycin (CDC #CP0034)

Gibco Cat. #15140-023

RPMI 1640 (1X) with L-Glutamine (CDC #CP0069)

Gibco Cat. #11875-036

**Infection Medium Preparation**

Combine the following ingredients:

 RPMI 1640 500 ml

 Fetal Bovine Serum 50 ml 10% (heat inactivated at 56°C for 30 minutes)

 Penicillin/Streptomycin 5 ml 100 U/ml Penicillin

 100 µg/ml Streptomycin

 L-Glutamine 5 ml 0.29 mg/ml

 Hydrocortisone 1 ml 0.01 mg/ml

 Interleukin-2 (IL-2) 50 µl 0.1 U/ml final concentration

Filter through a 0.2 µm filter.

# Virus and Cell Preservation

## Freezing in 10% DMSO

 To preserve cells and virus, freezing medium containing 10% DMSO is used, along with a slow freezing process. Both of these things will allow the smallest possible ice crystals to be formed and thus less cell damage and death. Remember that DMSO is light sensitive, hazardous to handle, and toxic to cells. Use appropriate protection, sterile technique, and speed.

 1. Pellet cells at 1500 rpm for 10 min.

 2. Resuspend in a 1:1 solution of Freezing Medium A and Freezing Medium D or medium containing 10% serum and 10% DMSO (final concentration), so that the final cell density is 107 cells/ml.

 3. Aliquot 1 ml per vial, labelled with cell and virus name, pass number, and date.

 4. Freeze slowly by adding vials to a box filled with cotton and leaving it in the -70°C freezer overnight.

 5. Transfer vial to liquid nitrogen and write location in the freezer log book.

NOTE: To revive from frozen stock, thaw quickly and add to appropriate medium (if cells) or medium with cells one wishes to infect (if cells plus virus) containing 10% serum. Remove most of media from the culture once it has settled (overnight) and replace with fresh medium in order to reduce DMSO concentration in flask.

## Freezing in Skim Milk

 This procedure is for cell-free virus used for synchronous infections. The skim milk stock is prepared by dissolving a quart size package (90.7 g) of Carnation nonfat dry milk in 1 liter of sterile, endotoxin-free water; autoclaving at 15 lbs. pressure for 15 min; cooling overnight; and repeating the autoclaving/cooling procedure twice more. Use a 250 ml bottle for 100 ml of milk.

 1. Pellet cells at 1500 rpm for 10 min.

 2. Resuspend in a 1:1 solution of skim milk stock and RPMI medium containing 10% fetal calf serum, so that the final cell density is 107 cells/ml.

 3. Aliquot 1 ml per vial, labelled with virus name, the letters SM, pass number, and date.

 4. Store the vials at -70°C.

## Infecting Cells with Skim Milk-frozen Virus

 1. Remove most of the media from uninfected culture without disturbing the cells.

 2. Add the appropriate amount of skim milk-virus stock (quick thawed).

 3. Let virus adsorb to cells at 37°C for 2 hr.

 4. Pellet cells to remove inoculum and resuspend in fresh media so that cells are at a density of 106 cells/ml again.

# Slide Preparation for Immunofluorescence Assay

A. From suspension culture:

 1) Add approximately 13 ml of 0.2 µm-filtered PBS to a 15 ml blue cap tube.

 2) Add 0.3 ml of virus-infected cell suspension (approximately 1 million cells/ml) to the tube.

 3) Centrifuge at approximately 1500 rpm for 10 min.

 4) Pour off supernatant and re-invert tube so that approximately 0.3 ml of PBS remains (will run down from the sides of tube).

 5) Resuspend pellet by rubbing rack or other technique.

 NOTE: If a larger volume is to be needed for making multiple slides, simply add

 that volume of PBS after this step, so that cell density is around 1 million cells/ml.

 6) Apply a "bubble" of the cell suspension per well of a teflon-coated, labelled (date, cells or virus, days post infection) slide (we use 12-well slides).

 7) Allow slide to dry.

 8) Fix slide by putting cold acetone from freezer into a staining dish then adding the dry slide for 5 min.

 9) Remove and allow to dry.

Either use immediately or store for later use in a slide box in the freezer.

B. For monolayer tissue culture there are two slide preparation options. The first is to create a suspension culture by trypsinizing the cells; the second is to grow the cells in a chambered slide, then remove the chamber and fix the slide.

# Indirect Immunofluorescence Assay

1. Prepare a wet chamber (closed container with wet paper towel bottom) and put slide in it.

2. Add antibody, diluted with PBS to a useful dilution (use PBS as negative control).

3. Incubate at 37°C for 30 min.

4. Do two 5 min washes with PBS.

5. Add the FITC conjugate at the appropriate dilution. Use Goat-anti-mouse-FITC; stock vials are stored in freezer; add 2 ml of PBS and filter (1:100 dilution of stock). If primary antibody is not mouse, use the appropriate conjugate.

6. Incubate at 37°C for 30 min.

7. Do two 5 min washes with PBS.

8. Dunk in water, dry in a dark place and mount coverslip by adding 3 drops of buffered glycerol. Slide can be stored in the dark.

9. Read at 40X by picking 3 or 4 fields of 50 to 100 cells and counting infected vs. uninfected cells. Average and express as percent infected cells.

Working dilutions of some frequently used antibodies:

HHV-6 C3108-103 1:200 (ours)

HHV-6 P41 1:500 (Universal Biotechnology)

HHV-6 P82 1:100 " "

HHV-6 P102 1:200 " "

HHV-6 P116 1:100 " "

EBV VCA 1:300

CMV Cocktail 1:2000

HHV-7 KR3 1:5000

HHV-7 KR4 1:5000

# Anti-Complement Immunofluorescence Assay

1. Prepare wet chamber, put slide in it.

2. Add a 1:40 dilution of heat inactivated human serum:

#59 is positive for HHV-6 and HHV-7.

 OSSA is negative for HHV-6 (and lightly positive for HHV-7).

 PBS is the control.

3. Incubate at 37°C for 30 min.

4. Do two 5 min washes with PBS.

5. Add complement (for our vials, add 2.4 ml and resuspend).

6. Incubate at 37°C for 30 min.

7. Do two 5 min washes with PBS.

8. Add fluorescein conjugate. Use a goat anti-human-complement (C3 from Cappel) at 1:16 of original stock dilution (stored made up in the refrigerator; stock is stored in the freezer).

9. Incubate at 37°C for 30 min.

10. Do two 5 min washes with PBS.

11. Dunk in water, dry in dark, and mount coverslip by adding 3 drops of buffered glycerol. Slide can be stored in the dark.

12. Read by picking up 3 or 4 fields of 50 or 100 cells and counting infected vs. uninfected cells. Average and express as percent infected cells.

# Immunoblot Assay

## Materials

Acrylamide Bio-Rad

Bisacrylamide Bio-Rad

Tris-HCl (Trizma-HCl) Sigma

SDS Bio-Rad

Ammonium persulfate Bio-Rad

TEMED Bio-Rad

Tris Base (Trizma Base) Sigma

Glycine Sigma

Methanol Baker Analytical

Skim milk Carnation

Tween 20 Sigma

Nitrocellulose (S&S NC 0.45µm pore) Schleicher & Schuell

Conjugate (Goat Anti-Human IgG H+L,

 Alkaline Phosphate Conjugate) Bio-Rad

BCIP Bio-Rad

NBT Bio-Rad

Dimethylformamide Pierce

NaHCO3 Fisher

NaOH Fisher

**Solutions**

9% Acrylamide Separating Gel:

 30% acrylamide/0.8% bisacrylamide 4.5 ml

 1.5M Tris-HCl containing 0.4% SDS, pH 8.8 3.75 ml

 water 6.75 ml

 10% ammonium persulfate 50 µl

 TEMED 10 µl

Stacking Gel:

 30% acrylamide/0.8% bisacrylamide 1.2 ml

 0.5M Tris-HCl containing 0.4% SDS, pH 6.8 3.0 ml

 water 7.8 ml

 10% ammonium persulfate 120 µl

 TEMED 12 µl

10X Tris/glycine:

 30.2 g Tris base, 144 g glycine, water to 1000 ml

Running Buffer:

 10X Tris/glycine 100 ml

 water 890 ml

 10% SDS 10 ml

Transfer Buffer:

 10X Tris/glycine 300 ml

 water 2070 ml

 Methanol 600 ml

 10% SDS 30 ml

Blotto:

 5% skim milk in Tween 0.05%/PBS 0.01 M

Tween/PBS:

 500 µl in 1000 ml PBS

NBT Solution:

 NBT 250 mg

 dimethylformamide 3.5 ml

 water 1.5 ml

BCIP Solution:

 BCIP 125 mg

 dimethylformamide 5 ml

Sodium Bicarbonate Buffer:

 NaHCO3 8.4 g

 MgCl2 0.2033 g

 water to 1000 ml after adjusting pH to 9.8 with NaOH

Developing Solution (prepared just before addition to Nitrocellulose):

 Carbonate Buffer 20 ml

 NBT Solution 120 µl

 BCIP Solution 120 µl

## Immunoblot Antigen

Infected cell supernatant from human cord blood lymphocytes is harvested between 7 and 11 days post infection. Uninfected cell supernatant is collected from CBL prior to their infection.

Remember: infected-cell cell culture supernatants and pellets contain infectious virus.

1. Filter culture supernatant through 0.45 µm filter coated with 0.2% PVP in water. Coat the filter by suctioning 5 ml of the PVP solution through the filter. Discard the filtrate.

2. Add 150 µl 0.3 M PMSF (in isopropyl alcohol) to each 500 ml bottle of culture supernatant.

3. When approximately 1.25 l of supernatant has accumulated, centrifuge at 15000 rpm overnight in a Type 19 rotor at 5°C.

4. Empty supernatant from bottles, add 0.5 ml Tris Salt EDTA Buffer (TNE) per flask, leave this over pellet in an ice bucket overnight.

5. Pool pellets, centrifuge 1 hr, 25000 rpm, Type 30 rotor, 5°C.

6. Pour off supernatant. Add 0.5 to 1 ml of TNE to pellet (dependent upon pellet size). Leave on ice overnight.

7. Sonicate (Cup-horn, setting 4, 50% duty cycle, continuous) until pellet is re-suspended.

8. Aliquot material into freezer vials and store in -70°C freezer.

9. Before running in gel, a 1:1 dilution of virus/cell protein suspension is made in SDS reducing buffer and is heated at 100°C for 2 min, then placed on ice.

 TNE Buffer: 0.01 M Tris pH 7.4

 0.10 M NaCl

 0.01 M EDTA

 0.1 mM TPCK

 0.1 mM TLCK

 0.1 mM PMSF

## Immunoblot Procedure

1. Prepare 9% acrylamide gels in Bio-Rad Mini-Protean II:

 Add 3.7 ml acrylamide/gel (using 0.75 mm spacer).

 Pour stacking gel using a comb with one small well for the standard and one large well for the immunoblot antigen.

2. Load gel with immunoblot antigen obtained from cell culture supernatant (see procedure), 100 µl/gel. Load 10 µl of Rainbow marker (standard) in its well (molecular weights 14,300 to 200,000).

3. Run electrophoresis at 100 v for 1 hr. and 45 min.

4. Transfer protein from gel to nitrocellulose at 100 v for 1 hr. and 30 min

5. Blot nitrocellulose with Blotto by rocking 1 hr.

6. Add serum to nitrocellulose in Bio-Rad Mini Protean Multi-Screen at a 1:11 dilution in Blotto (30 µl serum in 300 µl Blotto). Rock 2 hr.

7. Wash nitrocellulose in PBS/Tween 3X for 5 min each.

8. Add conjugate (anti-human AP reagent) at 1:1000 in PBS/Tween. Rock 2 hr.

9. Wash nitrocellulose in PBS/Tween 2X and PBS 1X for 5 min each.

10. Add color developing AP substrate of BCIP/NBT in carbonate buffer. Rock 1 hr.

11. Rock with water to stop reaction, dry in air.

# DNA Slot Blots

## Sample Preparation

>>>>> Perform sample preparation in a biological safety cabinet.

1. Label a screw-cap eppendorf centrifuge tube for each sample to be taken.

2. Suspend settled cells evenly in each tissue culture flask before the samples are taken.

3. Using a pipet-aid, remove 1 ml of medium (approx. 1x106 cells) from each flask and place it into its corresponding screw-cap eppendorf centrifuge tube.

4. Spin samples at 12000 RPM for 5 min in an eppendorf centrifuge.

5. Pour off supernatant and remove residual medium with a pipet-man.

 (BE CAREFUL NOT TO DISTURB THE CELL PELLET!)

6. Make sure the cap on each tube is replaced and tightened.

At this time, one can either freeze the samples at -70°C or start the procedure for the isolation of DNA.

**Cell Lysis Buffer**

 Final Stock For 100 ml

 Concentration Final Volume

 Stock 0.4 M Tris-HCl pH 8 1 M 40 ml

 Solutions 100 mM EDTA 0.5 M 20 ml

 1% SDS 10% 10 ml

 200 µg/ml 5 mg/ml 4 ml

 Proteinase K

 DO NOT ADD PROTEINASE K TO STOCK SOLUTION UNTIL READY TO USE.

**Isolation of DNA**

To lyse cells, release DNA, and degrade proteins, add 200 µl of Cell Lysis Buffer to each sample and float them in a water-bath at 60°C (2 hr. to overnight).

**Denaturation of DNA**

To denature double stranded DNA, add 1/10 volume (20 µl) 3 M NaOH to each sample and float them in a water bath at 70°C for 1 hr.

**Dilution and Neutralization of Samples**

1. Let samples cool to room temperature.

2. Add 2 volumes (440 µl) 2M NH4Acetate to each sample. Mix well.

3. Total volume in each tube is 660 µl. (You can adjust for a final volume of 800 µl, if preferred, by adding 140 µl more NH4Acetate).

At this point, samples can be quickly slot blotted or frozen away at -20°C.

Remember Before Running Slot Blot:

Before samples can be slot blotted, they must be heated in a 100°C heat block for 10 min. in order to denature the DNA (thaw first if frozen). Keep on ice until used.

Prepare a code sheet so that location of samples on the blot is known.

Prepare a positive control to ensure that proper hybridization occurs, if possible, separate from other samples.

## Running Slot Blot

1. Add 200 µl from each sample to its assigned well.

 This is DNA from:

 1) 303000 cells if final volume was 660 µl

 2) 250000 cells if final volume was 800 µl

IMPORTANT! - Usually this concentration of cells is excessive and will result in halos forming when DNA binds to the nitrocellulose. It is usually necessary to perform serial dilutions of the samples in order to find the concentration that provides the most DNA with no halo formation. Usually a 1:40 or a 1:80 dilution of the original 660 µl or 800 µl sample is ideal.

a) 1:40 dilution b) 1:80 dilution

 5.0 µl from orig. sample 2.5 µl from orig. sample

 +195.0 µl 6X SSC +197.0 µl 6X SSC

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 200.0 µl total 200.0 µl total

2. Add 200 µl 6X SSC to those wells that received no sample.

3. Apply vacuum gently (using finger to control suction) until liquid is removed.

4. Stop vacuum and then wash wells with 200 µl 6X SSC.

5. After wash buffer is removed via suction, disassemble apparatus and place nitrocellulose into 6X SSC in order to wash off any debris.

6. Remove nitrocellulose and blot excess buffer off using GB002 blotting paper. LABEL BLOT APPROPRIATELY (use abbreviations if possible).

 Information to include:

Experiment

Date Blotted

Dilution (if performed)

Agent

 RECORD THIS INFORMATION ON CODE SHEET AS WELL.

7. Bake blot under vacuum at 80°C for 20 min to 2 hr. depending on the number of blots.

8. Wash apparatus with 70% EtOH, dry, and store.

# Unidirectional Southern Blots

1. Soak gel to near equilibrium in 0.4 M NaOH, 1.0 M NaCl. This may take 2 hr. Denaturing buffer is house media #1017.

Denaturing Buffer

 200 ml 5 M NaCl

 40 ml 10 M NaOH [add last]

 760 ml H2O

2. Remove first solution [denaturing solution] and rinse 3 times with distilled water.

3. Soak gel in 1 M Tris-HCl, 2.0 M NaCl until equilibrium has been obtained. This will also take 2 hr. Transfer buffer is house media #1018.

Transfer Buffer

 400 ml 5 M NaCl

 158 g Tris-HCl

 q.s. to 1 liter with water

4. Cut a piece of nitrocellulose or Nytran to fit gel. Cut 2 pieces of GB002 paper to fit gel. Soak all in 10X SSC.

5. Place 3 cm thick stack of GB004 paper in tray. Pour 1 liter of 10X SSC to absorb. Lay 1 piece of GB002 paper on top of GB004 stack. Lay gel on top of GB002. Lay membrane on top of gel. Lay the other piece of GB002 on top of Nytran. Make sure all air bubbles are gone. Add another 3 cm stack of DRY GB004 paper on top of GB002. Wrap all in Saran wrap for overnight transfer. Place a 1-liter weight on top of the blot in the middle. It is alright if there is excess 10X SSC in the bottom of the tray. This will help the capillary action and the transfer of the DNA.

6. After transfer, unwrap blot and mark or label the orientation of the gel wells. Wash the membrane in 6X SSC. Rub the membrane with gloved hand. Blot dry with GB002.

7. Let the blots air dry then back them in an 80°C oven for 1 to 2 hours. At this point they can be stored until prehybridization/hybridization.

# Bidirectional Southern Blots

Photograph gel and ruler (Polaroid Type 55), save negative. Irradiate each side of gel for 30 sec in DNA Transfer Lamp.

1. Soak gel with gentle shaking to near equilibrium in Denaturing Buffer (0.4 M NaOH, 1.0 M NaCl) (CDC #1017). Near equilibrium has been obtained when the gel no longer floats freely in the solution. This normally takes 1.5 to 3 hr. Use 1 liter of solution for 300 ml agarose.

2. Remove denaturing solution and rinse gel with tap water to remove residual denaturing solution. Aspirate residual liquid with water.

3. Soak gel in Transfer Buffer (1.0 M Tris-HCl, 2.0 M NaCl) (CDC #1018), until near equilibrium has been obtained. This takes 1.5 to 3 hr. Again, you will need about 1 liter of solution for a 300 ml gel. Save this solution; you will need it later for soaking the nitrocellulose paper.

4. Wear gloves, mark blue paper, and cut 2 pieces of nitrocellulose and 2 pieces of filter paper to the size of the gel using a fresh razor blade (filter paper, GB002, slightly longer). Use a gel mold to measure size.

5. Remove the gel to a glass plate.

6. Wet nitrocellulose with distilled water and then with the neutralizing solution about 2 min. Do not put it into the water too fast. Start on one edge or it will not wet properly.

7. Place nitrocellulose on top of the gel and insure that there are no air bubbles between the agarose and the nitrocellulose. Be sure that one end of the nitrocellulose sheet covers the sample wells. Mark the corner; later mark the position of the wells by using a 20 well comb. Also mark the lane positions at the bottom so you can cut straight.

8. Wet a sheet of filter paper (GB002) with the neutralizing solution and place it on top of the nitrocellulose. Again, remove any air that may be trapped between the 2 sheets.

9. Stack a 3 to 4 cm thick pile of filter paper, (GB004), or a 5 to 6 cm pile of paper towels on top of the filter paper.

10. Place a glass plate on top of the whole stack and invert.

11. Repeat steps 6 to 9. Place a second glass plate on top of the entire stack, wrap in Saran wrap, and then apply a mass of 500 g to the top plate.

12. When all of the buffer has eluted from the gel (overnight) remove the nitrocellulose and wash in 6X SSC. Rub the nitrocellulose with a gloved hand.

13. Let the blots air dry on GB002 blotting paper, mark to label at this time. Bake nitrocellulose under vacuum at 80°C for 1 to 2 hours or at 65°C overnight. All sliced pieces of nitrocellulose need to be marked. Remember the orientation of the blot. Notch the lower right corner (if it is upside down, cut the lower left). Remember to number left to right or from right to left.

# Prehybridization-Hybridization

1. Place baked filter in heat sealable bag or hybridization tube with a mesh.

2. Add 30 ml hybridization solution. This can be warmed if it precipitates.

3. Remove as many bubbles and air pockets as possible.

4. Seal bag or close tube.

5. Incubate at bag at 65°C with rocking for 2 to 5 hr. For tubes, make sure that rotation direction is correct. At this point label DNA.

6. Cut corner of bag or open tube, and then pour off hybridization solution.

7. Add 30 ml hybridization solution that contains probe which has been boiled 5 min, then cooled in ice for several min.

8. Seal bag or close tube.

9. Incubate at 65°C overnight.

**Hybridization Solution**

 for 30 ml for 100 ml

 4X SSC 6 ml 20 ml 20X stock

 0.1% sodium pyrophosphate 0.6 ml 2 ml 5% stock in water

 100 µg/ml heparin 120 µl 400 µl 50 mg/ml in water

 0.2% SDS 0.6 ml 2 ml 10% in water

 water 22.75 ml 75.8 ml

# Random Primed DNA Labelling with Digoxigenin-dUTP and Detection of Hybrids by Enzyme Immunoassay

## Principle

 The non-radioactive DIG system uses digoxigenin (a hapten) to label the oligonucleotide probe for hybridization and subsequent color or luminescence detection.

## DNA Labelling Protocol

 DIG-labelled DNA probes are generated enzymatically according to the method of random primed labelling using random oligonucleotides (hexanucleotide), Klenow enzyme, and a mixture of deoxyribonucleosides containing DIG-11-dUTP.

1. Denature template DNA (1 µg to 3 µg) by heating for 10 min in a boiling water bath and quickly chilling on ice.

2. Add on ice:

 - 2 µl hexanucleotide mix

 - 2 µl dNTP mixture

 - add water to a final volume of 19 µl

 - 1 µl Klenow enzyme

3. Centrifuge briefly and incubate overnight at 37°C.

4. Add 2 µl 0.2 M EDTA pH 8.0 to stop the reaction.

5. Precipitate the labelled DNA by adding 2.5 µl, 4 M LiCl, and 75 µl prechilled (-20°C) ethanol. Mix well.

6. Leave for 30 min at -70°C or for 2 hr. at -20°C.

7. Spin in a microcentrifuge for 15 min. Wash the pellet with 50 µl of cold 70% ethanol.

8. Dry the DNA pellet briefly under the vacuum and dissolve in 50 µl TE buffer (T10E1 pH 8.0).

- 1-3 µg of DNA can be labelled per standard reaction.

- Linear DNA is labelled more efficiently than circular DNA; in all cases the template DNA must be thoroughly heat denatured prior to random primed labelling.

- We recommend prolonging the incubation time to overnight in order to increase the yield of DIG-labelled DNA.

- DIG-labelled probes are stable for more than 12 months when stored at -20°C. They can also be stored in the hybridization solution and can be re-used a number of times when freshly denatured before hybridization.

- The amount of newly synthesized labelled DNA depends on the amount and purity of the template DNA in the labelling reaction, and on the length of incubation time.

 Amount of template DNA per Amount of synthesized

 labelling reaction DIG-labelled DNA

 1 hour 20 hours

 10 ng 15 µg 50 µg

 30 ng 30 µg 120 µg

 100 ng 60 µg 260 µg

 300 ng 120 µg 500 µg

 1000 ng 260 µg 780 µg

 3000 ng 530 µg 890 µg

## Labelling of DNA in low melting point agarose

1. Cleanly excise the DNA fragment to be labelled from a low melting point agarose gel and transfer into a 1.5 ml microcentrifuge tube.

2. Add water to a ratio of 3 ml/g gel and heat the tube for 7 min at 100°C to melt the gel and denature the DNA.

3. After cooling to 37°C the DNA/agarose mixture can be used directly for labelling as described in standard protocol.

## Hybridization

 In addition to the nitrocellulose and nylon membranes generally suitable for use with the DIG system, we especially recommend positively charged nylon membranes from Boehringer Mannheim used according to the following protocol:

## Fixation of nucleic acid to the membrane

1. Bake for 15 to 30 min at 120°C instead of 80°C.

2. Crosslink for 30 min with UV 254 nm (e.g., with a Stratagene Stratalinker). UV-crosslinking is not recommended after alkaline transfer because it is less effective.

Hybridization solution

 Stock Final Concentration

 25 ml 20X SSC 5X SSC

 10 ml 10% Blocking Stock Solution (10X) 1% Blocking Reagent

 1 ml 10% N-Laurylsarcosine 0.1% N-Laurylsarcosine

 0.2 ml 10% SDS 0.02% SDS

 63.8 ml water

## Hybridization protocols for Southern blots

1. Prehybridize membrane in a sealed plastic bag or tube with hybridization solution (20 ml/100 cm2 membrane) at 65°C for at least 1 hr. with gentle agitation.

2. Denature DIG-labelled DNA probe by boiling for 5 min and rapidly cooling on ice.

3. Add the denatured labelled DNA probe (10 ng to 100 ng) to prewarmed hybridization solution (5 ml/100 cm2 membrane) and mix well but avoid foaming.

4. Pour off prehybridization solution and add probe/hybridization solution mixture to membrane.

5. Incubate with gentle agitation for 16 hr. at 65°C.

Post hybridization washes

 Wash membranes 2 x 5 min at room temperature with at least 50 ml of 2X SSC, SDS 0.1% (w/v) per 100 cm2 and 2 x 15 min at 65°C with 0.1X SSC, 0.1% SDS.

## Immunological Detection of Hybrids

**Reagents and solutions**

Maleic Acid Buffer (CDC #0177):

0.1 M maleic acid, 0.15 M NaCl; adjust to pH 7.5 with solid NaOH

Washing Buffer:

Maleic acid buffer plus Tween 20 0.3% (v/v)

Blocking Stock Solution (10X conc.):

Blocking reagent 10% (w/v) in Buffer 1: dissolve blocking reagent by constantly stirring on a heating block (65°C). Store at 4°C.

Buffer 2 Blocking Solution:

The 1X concentration working solution is prepared by diluting the stock solution 1:10 in maleic acid buffer.

Buffer 3 Detection Buffer:

0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2 pH 9.5.

Buffer 4 TE-Buffer:

10 µM Tris-HCl, 1 mM EDTA; pH 8.0

Color Substrate Solution:

Always prepare fresh: add 45 µl NBT-solution (nitro blue tetrazolium salt) and 35 µl x-phosphate solution (5-bromo-4-chloro-3-indolyl phosphate) to 10 ml of buffer 3.

**Detection procedure**

1. After hybridization and stringency washes, rinse membrane briefly (1 to 5 min) in washing buffer.

2. Incubate for 30 min in 100 ml/100 cm2 membrane blocking solution (1X concentration).

3. Dilute anti-DIG-AP conjugate to 150 mU/ml (1:5000) - 75 mU/ml (1:10,000) in blocking solution.

4. Incubate membrane for 60 min with diluted anti-DIG-AP conjugate solution (20 ml/100 cm2 membrane).

5. Remove unbound antibody-conjugate by washing 2 x 15 min with 100 ml/100 cm2 membrane washing buffer.

6. Equilibrate membrane for 2 to 5 min with 20 ml/100 cm2 of buffer 3 (detection buffer).

7. Incubate membrane for 5 min in freshly prepared color solution (10 ml/100 cm2 membrane) in a box in the dark. Do not shake during color development.

8. When desired band intensities are achieved, stop the reaction by washing the membrane for 5 min with buffer 4 (50 ml/100 cm2 membrane).

9. Results are documented by photocopying the wet filter or by photography.

# Radiation-Free Probe Labelling and Detection System

## Principle

 The labelling system employs a photoreactive reagent- psoralen biotin- which intercalates into double or single-stranded nucleic acids and when irradiated with long-wave UV light (365 nm) forms a covalent bond.

 Chemiluminescent detection is performed by addition of streptavidium alkaline phosphatase conjugate and application of the blot to Lumi-Phos 530 substrate sheets. This allows for the detection of nucleic acid blots on nylon membranes.

## Procedure for Photoderivitization with Psoralen Biotin in Solution

1. Nucleic acid to be labelled should be at a concentration of 100 µg/ml in 1X TE buffer with less than 20 mM salt.

2. DNA must be single-stranded prior to labelling reaction. Heat denaturation is the easiest and fastest method. Boil sample for 10 min, then quick-chill on ice.

3. Under reduced lighting add 1.1 µl of psoralen biotin per 10 µl DNA. The psoralen biotin reagent contains methanol which is volatile. The vial should be placed in an ice water bath prior to dispensing and the reagent should be pipetted on ice up and down before withdrawing.

4. Mix sample by tapping.

5. Place up to 150 µl of sample into a well in the microtiter plate on the ice-water bath.

6. Place lamp (RF UV-365 long wave) directly on the plate with bulb centered over wells to be irradiated.

7. Turn on the lamp and irradiate the sample for 1 hr. Remove the sample from the well and place in a clean microcentrifuge tube.

8. Perform two n-butanol extractions. Add 2 volumes of dH2O-saturated n-butanol to the tube. Vortex briefly and centrifuge for 30 seconds in a microcentrifuge at approximately 7000 x g to separate the phases. Discard the top n-butanol layer and repeat one more time.

NOTE: n-butanol extraction removes unincorporated biotin molecules from the probe solution. To prepare dH2O-saturated n-butanol combine equal volumes of dH2O and n-butanol in a bottle. Mix well and let 2 layers separate, n-butanol is the top layer.

9. Probes prepared from heat-denatured material need to be boiled for 10 min prior to use in hybridization. The labelled probe can be stored at -20°C for 6 months to 1 year.

## Transfer and Hybridization

1. Perform Southern using neutral nylon membrane (S&S Maximum Strength Nytran).

2. Immobilize nucleic acids by baking the membrane or UV crosslinking.

3. Remove Southern transfers from storage and wet briefly in 5X SSC. Place membrane in hybridization bag and add 10 ml of prehybridization solution for every 100 cm2 of membrane. Incubate at 65°C for at least 1 hr. with gentle agitation.

NOTE: Use the following prehybridization/ hybridization buffer at 65°C for good results: 0.25 M sodium phosphate pH 7.2, 20% SDS, 0.5% blocking reagent, 1.0 mM EDTA. For preparation of 0.25 M sodium phosphate pH 7.2 combine the following volumes: 171 ml 1 M NaH2PO4 and 79 ml 1 M NaH2PO4. The combined volumes of 1 M stock solutions are diluted to 1000 ml with distilled water.

4. Withdraw sufficient probe to achieve a final concentration of 65 mg/ml in hybridization solution (10 ml/100 cm2 of membrane). Place the probe in a clean microcentrifuge tube and bring volume to 100 µl with 1X TE. If using a probe prepared from heat denatured material, boil the probe for 10 min and immediately place in an ice water bath.

5. Add the probe to a volume of hybridization solution (10 ml/100 cm2 of membrane). Discard the prehybridization solution from the bag and filter the hybridization and probe solution directly into the bag containing the blots through a 0.45 µm cellulose acetate syringe filter with fiberglass pre-filter. Incubate for 16 hr. or more with gentle agitation at 65°C.

## Detection of Hybrids

Make up the following solutions before proceeding with the detection protocol:

Solution Final Concentration

 10X TBS (1 liter) pH 7.5 1X

 63.58 g Tris-HCl (0.5 M) 0.05 M

 11.8 g Tris-base (0.5 M) 0.05 M

 87.66 g NaCl (1.5 M) 0.15 M

 10% SDS (500 ml)

 50 g SDS

Blocking Solution

 25 ml 10X TBS pH 7.5 1X

 222.5 ml dH2O

 7.5 g Rad-Free blocking powder 3%

 2.5 ml 10% SDS 0.1%

(Allow blocking powder to dissolve on stir plate at 60°C for at least 2 hr., add SDS to this stock immediately before use. Store stock at 0°C-4°C for up to 1 week.)

Wash Solution I (1 liter)

 100 ml 20X SSC 2X

 890 ml dH2O

 10 ml 10% SDS 0.1%

(Add in the order indicated to prevent precipitation of the SDS. Prepare immediately before use.)

Wash Solution II (1 liter)

 5 ml 20X SSC 0.1X

 985 ml dH2O

 10 ml 10% SDS 0.1%

Wash Solution III (1 liter)

 100 ml 10X TBS 1X

 890 dH2O

 10 ml 10% SDS 0.1%

1. Remove blot from bag and shake off excess hybridization solution. Save hybridization solution as it can be reused. Add blot to a box containing 500 ml of wash solution I and incubate at room temperature for 5 min with rocking; repeat once.

2. Transfer the blot to wash solution II in a box that has been pre-warmed to 65°C. Incubate at this temperature for 5 min and repeat the wash 1 time.

3. Transfer the blot to blocking solution (30 ml/100 cm2 of membrane) and incubate at room temperature for 3 hr. with gentle rocking.

4. Add streptovidium-alkaline phosphatase at the dilution suggested on the vial (1:650) and incubate for 1 hr. at room temperature with gentle rocking.

5. Transfer the blot into 500 ml of wash solution III and incubate at room temperature for 10 min on a rocking platform. Repeat wash 2 more times.

6. Transfer the blot into 500 ml of 1X TBS for 10 min on a rocking platform at room temperature (TBS removes SDS).

7. Pre-incubate chemiluminescent substrate sheet at 37°C. Place the desired piece of cut substrate sheet (the substrate sheet remains within the sleeve throughout the entire incubation and exposure procedure) into a side-seal reaction bag. Place this assembly into a side-seal reaction bag. Place this assembly into an imaging cassette and incubate at 37°C until use.

8. Remove the membrane from the washing solution and allow excess buffer to drain from the membrane. Place the membrane NUCLEIC SIDE UP on the top of the substrate sheet and replace the top layer of plastic. Assure good contact between the membrane and the substrate sheet. Place the substrate sheet/membrane sandwich back into the side-seal reaction bag. Place into the imaging cassette and continue incubation. After 1 to 2 hr. of incubation, expose to X-ray film at 37°C . The rate of light production continues to increase for the first 10 to 15 hr. Vary the exposure time to obtain the best signal. Colorimetric substrate tablets for detection of nucleic acids on nitrocellulose or neutral nylon transfer membranes can be used. These colorimetric tablets are based on the BCIP/NBT-alkaline phosphatase reaction.

# Human Body Fluid Preparation for PCR

Lysis Buffer: 1% 10-lauryl ether (Sigma Chemical Co), 10 mM Tris pH 8.5, 200 µg/ml Proteinase K in water.

Lymphocytes: Centrifuge 10 ml of lymphocyte cell suspension (1x106 cells/ml) at 800 x g for 15 min. Suspend the pelleted cells in 300 µl of lysis buffer.

Plasma: 50 µl of plasma to 250 µl of lysis buffer.

Saliva:

 Neat Saliva: 150 µl of saliva to 150 µl of lysis buffer.

 800 x g Saliva: Centrifuge 1 ml of neat saliva at 800 x g for 15 minutes. Suspend pellet in 300 µl of lysis buffer.

 Supernatant: Centrifuge supernatant from 800 x g spin at 75000 x g for 90 min. Suspend pellet in 300 µl of lysis buffer.

Throat Swabs:

 Neat Swab: Take swab and swirl in 1 ml water. Mix 150 µl of this and 150 µl of lysis buffer.

 800 x g Swab: Centrifuge remaining swab material at 800 x g for 15 min. Suspend pellet in 1 ml of water. Use 150 µl of this to 150 µl of lysis buffer.

 75000 x g Swab: Centrifuge supernatant from 800 x g spin at 75000 x g and suspend pellet in 300 µl of lysis buffer.

Urine:

 Neat Urine: 150 µl to 150 µl of lysis buffer.

 800 x g Urine: Centrifuge 10 ml of urine at 800 x g and suspend pellet in 1 ml of water. Add 150 µl to this to 150 µl of lysis buffer.

 75000 x g Urine: Centrifuge supernatant from 800 x g spin at 75000 x g for 90 min. Suspend pellet in 300 µl of lysis buffer.

 Neat 75000 x g Urine: Centrifuge 10-20 ml of urine at 75000 x g for 90 min. Suspend pellet in 300 µl of lysis buffer.

 GUSCN Lysis:

 1. Add 200 µl urine to eppendorf.

 2. Add 1 ml GUSCN lysis buffer.

 3. Add 50 µl glass milk beads (Bio 101, GeneClean).

 4. Rotate 30 min.

 5. Centrifuge pulse to pellet beads.

 6. Remove buffer and discard.

 7. Wash beads 2 times with 70% EtOH.

 8. Mix-centrifuge and remove wash.

 9. Wash 1 time with acetone.

 10. Remove all acetone and air dry.

 11. Suspend beads in 200 µl sterile water.

 12. Resuspend beads and heat at 70°C for 15 min.

 13. Pellet beads.

 14. Use 10 µl of resulting supernatant per PCR reaction.

GUSCN Lysis Buffer:

 120 g GUSCN (Guanidine Thiocyanate)

 100 ml 0.1 M Tris-HCl pH 6.4

 8.8 ml 0.5 M EDTA pH 8.0

 2.6 ml Triton X-100

This takes a while to dissolve; wrap bottle in foil.

Heat samples at 65°C for 2 hr. then transfer to a 98°C heat block for 10 min. Sample is now ready for PCR.

**PCR Reactions for fragments less than 1 kb**

Add appropriate volume of lysate to standard 100 µl PCR reactions [50 mM KCl, 10 mM Tris pH 8.5, 1.5 mM MgCl2, 200 µM each of dATP, dGTP, dTTP, dCTP, and 2.5 U of thermostable DNA polymerase (Amplitaq, Perkins-Elmer Cetus)]. Perform 35 amplification cycles, each consisting of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 1 min. Primer concentration is 1.0 µM each.

**Detection of amplified DNA**

Electrophorese 25 µl of amplified DNA for 3 hr. at 120 volts in composite agarose gels consisting of 3% low melting agarose (Nu-Sieve) and 1% low molecular weight agarose (Sea Kem). The running buffer consists of 90 mM boric acid, 90 mM Tris HCl, and 3 mM EDTA. Stain gels with ethidium bromide (0.5 µg/ml) and observe DNA by UV fluorescence. Transfer DNA to nylon membranes (Nytran, Schleicher and Schuell) and hybridize overnight at 65°C with the appropriate probe labelled with 32P using a 5' end labelling kit (KinAce It, Stratagene). Hybridization solution consists of 6X SSC, 0.1% sodium pyrophosphate, 0.2% sodium dodecyl sulfate, and 200 µg/ml Heparin. After overnight incubation at 65°C, wash the blots four times in 2X SSC, 0.1% SDS for 15 min. each at room temperature, and then once in the same buffer at 65°C for 2 hr. Expose membranes to Kodak X-Omat AR film at -70°C for 24 hr. with intensifying screens.

# Quantitative Competitive RT-PCR

## Extraction of infected-cell mRNA

 Total RNA is first extracted with RNAzol B (Tel-Test, Inc. 800-631-0600). Then use a QuickPrep Micro mRNA Purification Kit (Pharmacia, 800-526-3593) to purify poly(A)+ RNA. The capacity of the QuickPrep columns equals the nucleic acid from 107 cells.

## Production of in vitro transcripts (mutant competitor RNA)

 Linearize the mutated plasmid of choice with the appropriate restriction enzyme and purify (e.g. phenol extract, ethanol precipitate) 1 µg for use as a template. Bring the 1 µg of DNA to a volume of 8 µl in diethylpyrocarbonate (depc)-treated water. Add the components of the correct (T7 or T3) MEGAscript kit (Ambion, 800-888-8804) and incubate the reaction at room temperature for 5 hours. I have found that incubation at room temperature (25°C) rather than 37°C results in a higher yield of full-length transcripts. Yield is usually 80-100 µg in vitro RNA per µg template. Treat with DNAse as described in the kit protocol, followed by addition of depc-treated water and NH4Acetate. Phenol/CHCl3 extract, CHCl3 extract, and isopropanol precipitate. Rinse pellet with 70% ethanol, and dry briefly in a vacuum centrifuge. Resuspend in 100 µl of depc-treated water. Remove 5 µl and add to 95 µl depc-treated water for absorbance reading. Save 1 µg of mutant RNA to run on a 0.8% agarose gel under denaturing conditions. Freeze remainder of in vitro RNA at -80°C.

## Serial dilutions of mutant competitor RNA

 Make a 7.2 µg/ml stock of yeast tRNA (Sigma Chemical Co., prepared as per Sambrook et al., 1989, page 6.26) resuspend in depc-treated water. Serial dilutions of the mutant competitor are then made using this solution in order to maintain a relatively constant amount of total RNA in each dilution tube. I make dilutions so that the appropriate amount of competitor RNA is contained in at least a 5 µl volume for pipetting accuracy. A ten-fold dilution series with 10 fg at the low end is perhaps a good starting point if the amount of viral RNA is totally unknown.

## Addition of infected-cell mRNA

 Pipette the 5 µl of each competitor RNA dilution into separate 0.5 ml microfuge tubes on ice, then pipette a constant amount of HHV-6 infected-cell mRNA into each of these tubes. I use mRNA from 3x105 cells/tube which is usually in a volume of 15 to 20 µl. Also include a sample tube containing only infected-cell mRNA and no mutant competitor RNA to be used as the "no reverse transcriptase" control. Desiccate all of the contents of the tubes to dryness in a vacuum centrifuge.

## RQ-1 DNase treatment

 Resuspend each tube in 8 µl of DNase solution:

 2 mM MgCl2 in depc-treated water

 2.5 units/µl RNase inhibitor (Promega)

 0.125 units/µl RQ-1 DNase (Promega)

Spin tubes briefly in microfuge, incubate at 37°C for 50 minutes. Add a drop of mineral oil to each tube and microfuge briefly again. Incubate tubes in a 90°C heat block for 6 minutes, and immediately place on ice.

## Reverse Transcriptase reaction

 I originally used the Perkin-Elmer RT-PCR kit for the remaining steps, but I now use reaction components from different sources. The reasons are that Super-Script II (Gibco BRL) is a better reverse transcriptase than that provided in the kit, and I go through large quantities of reagents (e.g., nucleotides and random hexamers) so I order these in more concentrated forms. I can also adjust the concentrations of the reagents, so the volumes of the reactions are correct.

 To the "no reverse transcriptase" control tubes, add 12 µl of the following solution (RT mix):

 per tube stock concentrations

MgCl2 (depc-treated water) 3.36 µl 25 mM

10X PCR buffer II\* 2.0 µl

dG, dA, dT, dC 4.89 µl 4.09 mM each (Pharmacia)

random hexamers 1.0 µl 50 µM

RNase inhibitor 0.5 µl (20 units) 40 units/µl (Promega)

\*10X PCR buffer II recipe is from Perkin-Elmer RT-PCR kit (500 mM KCl, 100 mM Tris-HCL pH 8.3)

I usually make an amount of RT mix equal to the amount of sample tubes plus 2 or 3 to account for pipetting error.

Then add 0.25 µl SuperScript II reverse transcriptase per remaining sample tubes to the RT mix. Add 12 µl of this mix to each tube and incubate at room temperature for 10 minutes. Place tubes in thermal cycler for RT reaction:

 42°C, 15 minutes

 99°C, 5 minutes

 5°C, 5 minutes.

I use an MJ Research thermal cycler, but I have used a Perkin-Elmer 480 thermal cycler with similar results. Tubes can be frozen at -20°C at this point if time does not permit PCR reactions.

## PCR reaction

 I perform a "hot start" PCR with the following PCR mix:

 per tube stock concentration

MgCl2 4.0 µl 25 mM

10X PCR buffer II 7.5 µl

water 60.5 µl

primer 1 1.0 µl 20 µM

primer 2 1.0 µl 20 µM

32P-dCTP 1.0 µl 3000 Ci/mmol

 75.0 µl

Add 75 µl of this PCR mix to each tube. Heat the tubes in the thermal cycler to 93°C for 3 minutes, then hold at 72°C. Next, add 5 µl of the following Taq mix:

 per tube

water 4.0 µl

10X PCR buffer II 0.5 µl

Taq DNA polymerase 0.5 µl

 5.0 µl

 Once Taq mix has been added, begin thermal cycling program:

 2 minutes at 95°C for 1 cycle

 1 minute at 95°C and 1 minute at 60°C for 28 cycles

 7 minutes at 60°C for 1 cycle

 4°C soak

Cycle numbers for the 95°C to 60°C cycling should be the minimal number which gives distinct product bands (rather than smears) when samples are electrophoresed. This is to avoid the PCR "plateau effect" and to avoid heteroduplex formation between mutant competitor products and viral wild-type products.

## Restriction Digest

Remove 15 µl from each sample and place in a new tube which contains 1.5 µl of 10X restriction digest buffer. Then add 1 µl (containing 5 units) of the appropriate restriction enzyme which will cleave only the mutant competitor PCR products. There is no need to add restriction enzyme to the "no reverse transcriptase" control tubes. Incubate overnight at 37°C. Pour polyacrylamide gels (described below) and allow to polymerize overnight.

## Electrophoresis

The following recipe makes four 8% polyacrylamide gels:

 40% acrylamide/bis (29:1) 45.0 ml

 (BioRad #161-0146)

 water 152.8 ml

 3% ammonium persulfate 4.725 ml

 10X TBE 22.5 ml

 225 ml

Filter this solution, and then add 101 µl TEMED. Pour gel into plates designed for the BioRad Protean II apparatus (20 cm X 16 cm gel size) with a 20-well comb and 1.5 mm thickness spacers. The next day, add 1.5 µl of DNA loading dye (a.k.a. blue juice) to each RT-PCR restriction digest sample tube. Load samples in wells but skip a well between each sample to account for the inability of the Betascope Blot Analyzer to distinguish lanes which are close together. Electrophorese at 180 v, dry gels for 1 hour (4 gels). Count bands of radioactivity on Betascope.

## Data analysis and determination of equivalence point

Enter the Betascope cpms from the bands corresponding to the wild-type, viral RT-PCR (undigested) products into a spreadsheet program which can calculate linear regression. I use SigmaPlot 5.0 (Jandel Scientific, San Rafael, CA). From these cpms, subtract the counts derived from the "no reverse transcriptase" sample lane at a distance of migration equal to the other undigested bands of the gel. This data is now "viral cpm-background". Likewise, enter the Betascope cpms corresponding to the larger band of the two mutant competitor RT-PCR fragments and subtract the background from the "no reverse transcriptase" lane at an equal distance of migration as the mutant competitor bands. This data is now "mutant competitor cpm-background". Multiply the "mutant competitor cpm-background" values by the correction factor which accounts for the length of the mutant competitor fragment. This factor is determined by dividing the total number of cytosine residues (both strands) in the intact RT-PCR fragment by the number of cytosine residues in the larger of the two mutant competitor fragments. This is now "corrected mutant competitor cpm-background". To calculate the y-axis for the equivalence point plot, divide "corrected mutant competitor cpm-background" by "viral cpm-background". In the next column of the spreadsheet, enter the fg (or copies) of the mutant competitor originally added to each sample tube. This is the x-axis of the plot. Plot the data using first order regression. The equivalence point is the amount of mutant competitor present (x-value) when y=1.

# ABI Automated Sequencing Protocol

 The following protocol is for automated sequencing using the Applied Biosystem (ABI) model 373A/373 Sequencer and the ABI TaqDye Deoxy Terminator Cycle Sequencing Kit.

## Set-up of TaqDye Deoxy Terminator Cycle Sequencing Reactions

1. Take ABI kit from -20°C and set up cycle sequencing reactions. Cycle sequencing reactions can be performed in a Perkin Elmer Cetus Model 9600 machine or Perkin Elmer Cetus Model 480 machine. Read the TaqDye Deoxy Terminator Cycle Sequencing Kit Protocol book. Prepare the reaction premix per reaction as follows:

4 µl 5X TACS Buffer, 1 µl dNTP mix, 1 µl ddA, 1 µl ddCTP, 1 µl ddGTP, 1 µl ddTTP and 0.5 µl AmpliTaq DNA Polymerase.

One can prepare for twenty reactions and store unused portion at 4°C for one month.

2. Mix the following reagents in a labeled 0.6 ml or 0.2 ml MicroAmp reaction tube in the following order:

1. dH2O q.s. to 20 µl.

2. Primer for dsDNA or purified PCR fragments 3.2 pmol

3. Template DNA (dsDNA or PCR fragment) between 0.5 µg to 1 µg.

4. Reaction Premix 9.5 µl.

Final reaction volume should be 20 µl. Overlay the reaction mixture with one drop of mineral oil if using the Perkin Elmer Cetus Model 480 machine. No oil is required for the 9600 PCR machine.

3. Place the tubes in a thermal cycler preheated to 96°C

4. Immediately after placing the tubes in the thermal cycler, begin thermal cycling as follows:

 Rapid thermal ramp to 96°C

 96°C for 30 seconds (Model 480) or 15 seconds (Model 9600)

 Rapid thermal ramp to 50°C

 50°C for 15 seconds (Model 480) 1 second (Model 9600)

 Rapid thermal ramp to 60°C

 60°C for 4 minutes

Perform 25 cycles total (approximately 3.0 hr. for 480 Model 2.5 hr. for 9600 Model) and then rapid thermal ramp to 4°C and hold.

Detailed procedures for preparation of cycle sequencing reactions along with other information regarding purification of template DNA is found in the Taq DyeDeoxy Terminator Cycle Sequencing Kit protocol book which can be obtained by request from the company free of charge with the purchase of the kit (not automatically included).

## Plate Assembly

 The first time the plates are used, mark the notched plate on one side, the plain plate is already marked. Clean plates with Liqui-Nox detergent and rinse well with ddH20. Place plates on a large test tube rack and rinse with 100% ethanol. Airdry plates, never wipe with any type of tissue. Plates must be free dust and lint (very important).

 Assemble plates by inserting the spacers between the plates. Make sure the orientation of the plates is correct, marked sides should go on the outside. Check for gasket marking on the notched plate, it also should be on the outside. Tape the bottom of the plate using Teflon tape and clamp the remainder of the plate using large binder clips.

## Gel Preparation

 Prepare gel. Final volume for one gel is 50 ml. Gel composition is 50% (w/v) urea (8.3 M), 1X TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA).

For 75 ml gel:

- 37.5 g urea

- 11.25 ml of 40% acrylamide stock (19:1). Acrylamide stocks should be less than 4 weeks old.

- 21 ml dH2O

- Mix together in a 150 ml beaker

- Stir over low heat until urea is dissolved

- Deionize with ~1 g of BIO-REX MSZ 501(D) mixed bed ion exchange resin

- Continue to stir for ~5 min with no heat

- Filter through disposable 115 ml filter (0.45 or 0.2 µm)

- Degas for 10 to 15 min.

- Pour into 100 ml graduated cylinder

- Add 7.5 ml of 10X TBE

- Adjust final volume to 75 ml

- Pour solution into clean 125 ml flask

- Immediately before pouring gel add: 0.375 ml 10% ammonium persulfate and 37.5 µl TEMED. Gently mix and pour into the assembled glass plates using a 25 ml disposable pipet. Air bubbles can be gently tapped out of the solution during the pouring process. Insert either the 16 well comb or the flat end of the casting comb. Clamp over comb with large "pony" clamps and level the gel. Let the gel polymerize for 2 to 4 hr.

## Column Preparation

 Take an appropriate number of Centri Sep columns (one per reaction) from box, tap contents to the bottom and snap bottom of column tightly. Always do batches of even number of columns and hydrate a few extra ones. Add 0.75 to 0.80 ml of ddH2O to the columns and resuspend by vigorous vortexing. Make sure no air bubbles are trapped. Allow columns to hydrate for at least 30 minutes at room temperature. Extra hydrated columns can be stored at 4°C for 3 to 4 months.

## Plate Check

 Turn on the 373A/373 sequencer; it will take a few minutes to warm up. Remove "pony" clamps and then the comb very slowly, allowing air to enter into the wells. The casting comb should also be removed slowly in order to keep the top of the gel as a flat edge. Next, remove side clamps and tape. Rinse the plates well, make sure they are free of precipitated urea and debris and let them air dry (do a 100% ethanol rinse to speed up drying). Place the plates in the 373A/373 sequencer resting on top of the bottom buffer chamber and clamp down the laser safety bar. Close the door to the 373A/373 sequencer and look at the Main Menu which appears on the sequencer. Use the Configure Menu to choose the correct filter wheel: Set A (dye primers regardless of enzyme and dye terminators using TAQ enzyme) or Set B (dye terminators using T7 Sequenase enzyme). Go back to Main Menu and select Start Pre-run then Plate Check and then select Full Scan.

 Turn on Apple computer and click on the 373A/373 Application folder. Open up the Data Collection program. On the Apple in the Data Collection window, click on "Scan" and "Map"; wait 30 sec. A flat line should appear on the "Scan" window, peaks indicate dirty plates or something in the gel. Clean plates as necessary. Look at the "Map" window. Four independent lines should be present (each a different color); the y-value is indicative of the PMT of the laser, it should be between 800 and 1000. The x-value is used as a guide to bad lanes (a sheet of lane assignments is kept on top of the 373/373A sequencers). To adjust the y-value go to the Main Menu on the 373A/373 sequencer and choose Configure Menu and adjust PMT value.

## Pre-running Gel and Setting Run Parameters

 Set the upper buffer chamber on the top platform, put the well aligner bar in front of the plate and tighten the clamps to secure the gel. Insert the sharks-tooth comb (24 wells) approximately 1 mm into the gel (the number on the aligner bar should coincide with the numbers on the comb). Load the upper buffer chamber with 1X TBE followed by the lower buffer chamber (make 2 liters of 1X TBE); the electrodes in both buffer chambers should be covered with buffer. Use a syringe fitted with bent needle to flush the wells with 1X TBE. On the 373A/373 sequencer go to Start Pre-run and choose Pre-run gel. The timer will automatically set itself for 1 hr. Pre-electrophorese the gel for a minimum of 10 to 20 minutes.

 While pre-electrophoresing, go to the menu bar and choose Edit and select "Settings" and select the following parameters:

- Run length 12 hr. (can be changed on the sequencer using the Main Menu and then choosing Set-up Run).

- Gel file name; name it according to established protocol, i.e., spx408; if you do not name it the last file will be overwritten.

- Sample file name; name it as above.

- Folder name; name it as above.

- Comb size, choose either 16, 24 (used most often), or 36 well comb.

- Dye set used, choose type of sequencing chemistry used; i.e. Dye Deoxy Terminator.

- Analyze all samples; choose YES.

- Plot raw data; choose NO.

- Plot analyzed data; choose YES.

## Column Purification of Cycle Sequencing Reactions

 When cycle sequencing reactions are finished excess nucleotides are removed using the hydrated Centri Sep columns. Protocol for removal of Dye Terminators prior to sequencing is included with the columns. Briefly:

- Remove column caps first, then bottoms.

- Let the columns drain by gravity; if no flow, prime them by using a rubber bulb on the top of the column tube.

- Spin for 2 to 4 minutes at 3,000 rpm to finish draining the columns.

- Check columns to make sure they have drained properly; the columns should not be wet looking. The resin inside the columns should not touch the side of the tubes and the top of the resin should be slanted. If columns appear wet, they can be respun.

- Load cycle sequencing reaction on the top center of the resin. Respin (same rpm and time) columns maintaining the same column orientation.

- Dry the sample in a vacuum centrifuge with no heat for approximately 15 minutes (do not over dry).

## Preparing and Loading Samples

 Prepare the following reagents:

- 5 µl of deionized formamide and 1 µl of 50 mM EDTA, pH 8.0.

- Add 4 µl of this mixture to each tube and vortex vigorously to dissolve the dry pellet. Spin the solution to the bottom of the tube. Samples must be loaded within several hours.

 After the gel has pre-electrophoresed for 20 minutes, heat the samples at 90°C for 2 min to denature, then put on ice. Flush the wells again before loading and load samples (4 µl) in to the well of the gel (a black piece of paper and top lighting helps view the wells). Load every other well followed by 5 min pre-electrophoresing then load the remaining wells.

 Fill out sample sheet or make any changes for individual samples if necessary. Go to the menu bar and click under File and save the sample sheet by clicking "Save As" and give it a name, i.e., spx408ss. Close the sample sheet and go to the menu bar, click on Collect this enables the computer to receive data from your run. In order to get a hard copy of your analyzed data (chromatograms) choose a printer by clicking on the apple icon and choosing "chooser" and clicking on 2.0 Color Quick. Make sure the AppleTalk is inactive. Turn down the screen brightness on the Apple and wait until the next day.

## Saving Results

 The results are found in a folder with the name given previously i.e., Results40812/23/94. In order to save the chromatograms as well as the sequence data insert a blank disk (3.5"; 1.44MB) into the Apple; format and name the disk. Open up your result folder, Results40812/23/94, and highlight the files you are interested in saving, drag them to the disk which will automatically copy them to your disk.

 If you want to save your sequence data only in a DOS format go to the Apple File Exchange folder and open it up. First choose your result folder, Results40812/23/94, and click on "Open". Insert an unformatted disk and format it choosing DOS. To save your sequence files highlight the file (sequence files have a seq extension) and click on "Translate". To exit Apple File Exchange, go to the menu bar and click on File and then on Quit. After you are done do not forget to take plates down and clean the plates and the buffer chambers.

## Automated Sequencing Supply List

Applied Biosystems

0850 Lincoln Centre Drive

Foster City, California 94404

1-800-874-9868

1-415-572-2743 (FAX)

Glass plate plain: catalog #401068; price $200.00

Glass plate notched: catalog #401069; price $225.00

Gel spacers, pair: catalog #400571; price $50.00

24-well shark's-tooth comb: catalog #401048; price $90.00

Gel casting comb: catalog #401046; price $50.00

16-well assembly: catalog #400880; price $65.00

TaqDye Deoxy Terminator Cycle Sequencing Kit: catalog 401150; price $625.00

Perkin Elmer

761 Main Avenue

Norwalk, Connecticut 06859

1-800-762-4002

MicroAmp reaction tube with cap: catalog #N801-0540; price $99.00

BioRad

2000 Alfred Nobel Drive

Hercules, California 94547

1-800-424-6723

1-800-756-4246 (FAX)

Acrylamide/Bis 19:1 (30g): catalog #161-0120; price $23.00

Ammonium Persulfate (10g): catalog #161-0700; price $9.00

TEMED (5ml): catalog #161-0800; price $10.50

Urea (1kg): catalog #161-0731; price $49.00

Analytical Grade Monosized Mixed Bed Resin (BIO-REX MSZ 501(D)): catalog #142-7425; price $135.00

Princeton Separations

P.O. Box 300

Adelphia, New Jersey 07710

1-908-431-3338

1-908-431-3768

Centri Sep Spin Columns (100): catalog #PSR00105; price $240.00

Available from glassware and the in-house catalog is #95103

PGC Scientifics

P.O. Box 7277

Gaithersburg, Maryland 20898-7277

1-800-424-3300

1-301-990-0740 (FAX)

Liqui-Nox Liquid Detergent (1 gallon): catalog #19-0840-03; price $22.00

## Template Preparation

 Template preparation is of the utmost importance in sequencing. For plasmid DNA purification the QIAwell 8 Plasmid Purification system is the recommended system for fluorescent DNA sequencing. The QIAwell 8 System provides 8 to 48 ultrapure, double-stranded plasmid templates rapidly and with minimal effort. The QIAwell 8 Plus Plasmid Kits eliminate alcohol precipitation by combining the QIAwell 8 Plasmid Kit with the time-saving advantage of in-line desalting on QIAprep 8 strips. Pure DNA is eluted for QIAwell 8 strips directly into QIAprep 8 strips for binding. After washing to remove salts, DNA is eluted at a typical concentration of 150-200 ng/µl in Tris (pH 8.0) or water. The QIAwell system comes with a very detailed handbook including the procedure for plasmid DNA purification.

 The QIAquick purification products allow for purification of DNA from agarose gels and solutions whose quality is high enough to use in a variety of applications including automated sequencing. Each QIAquick kit comes with its own detailed protocol manual.

QIAGEN Inc.

9259 Eton Avenue

Chatsworth, California 91311

1-800-426-8157

1-818-718-2056 (FAX)

QIAwell 8 Plasmid Kit (50): catalog #17124; price $740.00

QIAwell 8 Plus Plasmid Kit (50): catalog #16144; price $1180.00

QIAvac Manifold 6S: catalog #19503; price $295.00

QIAquick Gel Extraction Kit (50): catalog #28704; price $60.00

QIAquick PCR Purification Kit (50): catalog #28104; price $60.00

# Super Quickie Plasmid DNA Prep

Adapted from Holmes and Quigley

All centrifugation steps are at max rpms in Eppendorf centrifuge (~15,000 x g) at room temperature or 4°C.

1. Starting from 3 to 5 ml overnight cultures, spin down Eppendorf tube of bugs (1 to 2 min).

2. Remove all medium - there should be <3 µl remaining.

3. Suspend bug pellet in 0.4 ml STET/lysozyme mixture (see below) by vigorous vortexing for 15 sec.

4. Place in boiling water bath. Timing is critical!

 40 sec for JM103 55 sec for JM83

 50 sec for DH5-‡ and DH10B 75 sec for DH1

5. Spin 10-15 min.

6. Without disturbing the pellet, remove the supernatant to new tube containing 0.4 ml isopropanol. Pasteur pipettes work well. Mix by inversion or brief vortexing. Discard the tube containing the pellet.

7. Chill in -20°C freezer for 5 min. A longer time only increases the amount of crud precipitated.

8. Spin 5 min. A longer time only increases the amount of crud precipitated. Orient the tubes in the centrifuge so you know where the pellet should be.

9. Remove all liquid. There should be <0.5 µl liquid in the tube.

10. Suspend in 1 ml T10E1, pH 7.5-8.0.

 If the vector is pUC or a relative, 25 µl should give a good digest. If the vector is pBR322, suspend in 100 µl TE.

 For 500 ml

 STET: 8% sucrose 40 g

 5% Triton-X-100 25 ml

 50 mM EDTA 50 ml 0.5 M stock (CDC #6932)

 50 mM Tris, pH 8.0 25 ml 1 M stock

 q.s. to 500 ml with H2O

 Stable > 1 year

 Lysozyme: 10 mg/ml in T10, pH 8.3. Make fresh daily.

 STET/Lysozyme (this makes a little extra): STET 10 mg/ml lysozyme

 For 12 superquickies 5.25 ml 0.375 ml

 " 24 " 10.5 0.75

 " 36 " 15.75 1.125

 " 48 " 21.0 1.5

# 96 Well Super Quickie DNA Prep

Adapted from Current Protocols in Molecular Biology

Use an adjustable multichannel pipettor.

1. Aerate Super Broth (CDC #8441) containing 25-50 µg/ml ampicillin in a 2 l flask by shaking in bacterial shaker, 250 rpm, room temperature for 1 to 3 hr.

2. Aliquot 200 µl of the aerated media to each well of a 96 well dish (Costar 3799).

3. Toothpick colonies to wells of the dish.

4. Incubate overnight at 37°C without shaking.

5. Remove 10 µl from each well to another 96 well dish containing 100 µl of Super Broth and antibiotic. This dish will serve as a stock of bacteria. Store at 4°C.

6. Centrifuge dish 5 min, 3000 rpm, 4°C.

7. Remove supernatant media.

8. Add 80 µl STET/lysozyme to each well, mixing by pipetting up and down 3-5 times vigorously.

9. Wrap securely in plastic wrap, float on boiling water for 1.5 min.

10. Add 40 µl 7.5 M ammonium acetate to each well, mixing by pipetting up and down. Incubate at 4°C for at least 10 min.

11. Centrifuge 30 min, 3000 rpm, 4°C.

12. Remove 90 µl/well, being careful to avoid the lysate pellet, to wells containing 70 µl isopropanol. Mix well by pipetting up and down. Incubate 10 min, room temperature.

13. Centrifuge 15 min, 3000 rpm, 15°C.

14. Remove all supernatant. Air dry pellets.

15. Resuspend in 100 µl/well T10E1 pH 8.0 by vibrating plate gently on vortexer.

16. Use 20 µl/digest. Do digests in 96 well dish.

 ml STET ml lysozyme

 For 1 Dish 10.5 0.75

 2 21.0 1.50

 3 31.5 2.25

 4 42.0 3.00

 STET: for 500 ml

 8% sucrose 40 g

 5% Triton X-100 25 ml

 50 mM EDTA 50 ml 0.5 M

 50 mM Tris, pH 8.0 25 ml 1 M

 Lysozyme: 10 mg/ml in 10 mM Tris, pH 8.3, make fresh daily.

# Purification of Plasmid DNA in CsCl Gradients

1. Weigh 29.70 g CsCl, put in capped 50 ml blue cap tube for each DNA sample.

2. Add 20.0 ml T10E1 pH 8.0. Shake. Put in 37°C water bath for 20 min to dissolve almost all the salt.

3. Add 8.0 ml of DNA solution or DNA solution + T10E1 to make up to 8.0 ml total.

4. Mix gently but well. Remaining salt crystals should dissolve quickly. They may need to be squashed with a pipette.

5. Add 4.0 ml 5 mg/ml ethidium bromide.

6. Mix gently but thoroughly.

7. Load into self-sealing tube for Vti50 rotor.

8. Spin overnight at 44,000 rpm at 20°C.

9. Remove lower band with a 16-gauge needle. Make "breather" hole in the top of the tube first.

10. Extract DNA with CsCl and H2O saturated isopropanol or 1-Butanol, 6 or 7

 times

 \*\* In 1-liter brown bottle, mix 600 ml alcohol, 300 ml good water, and 300 g CsCl.

11. Dialyse DNA vs. T10E1. Change at least 3 times.

12. Dialyse once vs. T5E.1 pH 7.6.

SECOND GRADIENT (not always necessary)

1. CsCl stock solution:

 Add 100.00 g CsCl to 100.0 ml H20.

 Dissolve completely.

 Store tightly sealed at room temperature.

2. Add DNA from first gradient (without doing the extractions or dialysis) to Vti65 tube - use judgment based of thickness of first gradient band to determine whether to use 1 or 2 Vti65 tubes.

3. Fill tubes nearly to top with CsCl solution.

4. Add 0.2 ml ethidium bromide solution (5 mg/ml stock).

5. Top off with CsCl solution.

6. Seal tubes.

7. Centrifuge at 44,000 rpm, 20°C, overnight, resume at step 9 of first procedure.

# Whole Cell DNA Prep

For ~20 x 106 mammalian cells.

1. Pellet cells at ~800 x g for 10 min.

2. Suspend cells in 0.4 ml Solution 1, transfer to 1.5 ml eppendorf tube. Incubate at room temperature for 15 min. AFTER INITIAL RESUSPENSION, HANDLE GENTLY TO AVOID SHEARING DNA.

3. Add 0.13 ml Solution 2. Mix gently by inversion. 60°C to 65°C 2 hr. to overnight.

4. Add 0.5 ml phenol/chloroform (isoamyl alcohol is harmless but not necessary). Use a glass pipette. Rotate gently for 10 min.

5. Centrifuge at ~1000 x g 10 min at 15°C in tabletop centrifuge.

6. Remove phenol/chloroform from beneath the aqueous layer using Pasteur pipette.

7. Repeat steps 4, 5, and 6 until aqueous phase is clean (usually 3 to 4 extractions).

11. Extract twice with 0.5 ml chloroform.

12. Divide sample into 2 eppendorf tubes, add 1 ml EtOH to each. Mix gently. Incubate at 4°C 1 to 24 hr. until only one phase is present - do not mix hard to accomplish this.

13. Centrifuge in eppendorf centrifuge 12,000 to 15,000 x g 15 min.

14. Remove supernatant. Air dry pellet. Suspend in a total of 0.5 ml T10E1 per preparation by letting it sit at 4°C overnight.

15. Somewhere between 5 and 20 µl of this DNA should give a decent restriction profile.

DNA BUFFER

 for 100 ml

 500 mM NaCl 10 ml 5 M stock

 10 mM EDTA 2 ml 0.5 M stock

 50 mM Tris, pH 8.0 5 ml 1 M stock

 0.5 % SDS 5 ml 10% stock

 2 % Sarkosyl 2 g

 q.s. to 100 ml with H20 (stable > 1 year at RT)

Make Solutions 1 and 2 as needed.

SOLUTION 1

DNA buffer + 0.25 mg/ml RNAse A (from boiled 10 mg/ml stock)

SOLUTION 2

DNA buffer + 8 mg/ml Proteinase K (as powder)

# Cytoplasmic DNA From Roller Bottles

1. At 100% CPE (but before cells are blown apart), shake and/or scrape cells off glass/plastic.

2. Pellet at 1000 rpm for 5 min in JA-10.

3. For each large roller bottle (RB): Resuspend cells by vortexing relatively gently in 5 ml T10E1, pH 7.5 + 0.5% NP 40 (resuspend in 3 ml for small RB).

4. Swell 15 min on ice.

5. Add 25 ul (15 ul for sm RB) RNase A\*\*, wait 5 min. Spin 2000 rpm in conical bottom tube. Supernatant may be frozen at this point (-80°C).

6. Collect supernatant. Add 0.25 ml/RB Proteinase K\*\*\* (0.15 per small RB) and 0.25 ml/ RB 20% SDS (0.15 ml per small RB).

7. Incubate 45°C to 55°C for 2 to 6 hrs.

8. Especially with CMV, treat gently from here on; use blunt tip pipettes, etc.

9. Make volume to exactly 16.0 ml with T10E1 pH 7.5 and add 24 ml saturated sodium iodide in T10E1 pH 7.5 (keep in dark). Up to 3 RB can go in a gradient.

10. Add 0.3 ml 5 mg/ml ethidium bromide. The refractive index should be 1.435.

11. With a 50 ml syringe and a 16-gauge needle load Vti50 quick-seal tube (cloudy ones are best), balance, heat seal, and spin 44,000 rpm for 18 hr.

12. Stop centrifuge. Carefully take the tubes out without disturbing them. Keep them in the dark as much as possible. Using a UV light and a ring stand, clamp setup, slowly collect the band with a 10 ml syringe and a 16-gauge needle. Do not poke your hand. Collect the discarded tubes in a large beaker.

13. Gently extract with isoamyl alcohol saturated with water.

14. Dialyse vs T10E1 pH 8.0, changing at least 3 times. The first dialysis should be in the dark; cover the flask with aluminum foil.

 \*\*RNAse A: Make 10 mg/ml in T10N15 pH 7.5, Incubate at 100°C for 5 min, aliquot for freezer.

\*\*\*Proteinase K: 10 mg/ml in T10E1, pH 7.5. Incubate at 37°C to 65°C for 30 min, aliquot for -80°C freezer.

# HHV-6 Nucleocapsid DNA Preparation

Adapted from Martin et al., J. Med. Virol. 9:69,1982, which was adapted from Straus et al. J.V. 40:516,1981

For ~109 pelleted lymphocytes (no supernatant).

Should be done in an externally exhausted biosafety cabinet through step 9. Phenol and Chloroform extractions should be done in a chemical hood.

Lysis buffer solution needs to be made fresh - it crystalizes.

1. Suspend cells in 6 ml lysis buffer in 16 ml polyallomer or polypropylene screw cap Nalgene tube.

2. Sonicate for 30 pulses at 50% duty cycle, power level 4.

3. Add 6 µl 10 mg/ml DNAse I and 6 µl 10 mg/ml boiled RNAse A. Mix. Incubate at 30°C for 30 min. Turn water bath to 55°C to 60°C when done.

4. Using a glass pipette, add 6 ml 1,1,2-trichloro-trifluroethane (TCTFE). Mix vigorously 2 min. (Do in virus hood with external exhaust.) Centrifuge at 800 x g (2000 rpm in Sorvall tabletop centrifuge) for 10 min at 4°C.

5. Prepare step gradients as follows:

 To a 13.2 ml tube (ultraclear) for SW41 rotor, add 3.5 ml 40% glycerol in lysis buffer, then gently layer 3.5 ml 5% glycerol in VZV lysis buffer on the 40% layer. Layer 4.2 ml of sample gently on top of cushion. Fill tube to within 1 to 2 mm of the top of the tube with lysis buffer.

 Similarly, into tubes for an SW50 rotor, layer 1.5 ml of the 40% glycerol solution, 1.5 ml of the 5% solution, and then 2 ml of the virus suspension.

6. Spin the SW41 at 35 krpm, 4°C for 1 hr., and the SW50 at 40 krpm for 40 min.

7. Discard the supernatant.

8. Suspend the nucleocapsid pellet gently in 2 ml 2X STEP, transfer to 16 ml screw cap polyallomer or polypropylene Nalgene tube.

 From this step on, you have exposed DNA which is very sensitive to shear forces. Pipette slowly and use wide bore pipettes whenever possible.

9. Add 1 ml 2X STEP containing 3 mg/ml proteinase K. Mix gently. Incubate at 55°C to 60°C, 60 min to overnight.

10. Add 2 ml phenol (TE saturated), mix gently on rotator 5 min, spin 2500 rpm in Sorvall tabletop centrifuge for 10 min. Remove phenol phase from under the aqueous phase with Pasteur pipette.

11. Extract once with 50% chloroform 50% phenol as in step 10.

 Remove the phenol/chloroform phase from under the aqueous phase.

12. Extract once with chloroform as above.

13. Add 9 ml ethanol, mix gently. Can be stored at 4°C at this point.

14. Spin 9000 rpm in JS13, 4°C, 30 min.

15. Remove all supernatant, air dry. Suspend DNA very gently in a total volume of 0.4 ml T10E1, pH 7.6. If time allows, simply add the buffer, and put the tube in the refrigerator overnight. Agitate gently the next day, then transfer by slowly pipetting the suspension to a screw cap vial.

16. Check 10 µl of the DNA in a restriction digest (BamHI or ClaI).

**LYSIS BUFFER**

 Make fresh before each use.

 Prewarm NP-40 if refrigerated.

 Order of addition is important.

 Add to ~80 ml H20

 0.5% Na Deoxycholate 0.625 g

 (Deoxycholic acid, Sodium salt)

 Dissolve completely with stirring before continuing.

 0.5% NP-40 0.625 ml 100% stock

 30 mM Tris pH 7.5 3.75 ml 1M stock

 5.0 mM Mg Acetate 0.625 ml 1M stock

 125 mM KCl 15.6 ml 1M stock

 0.5 mM EDTA 0.25 ml 0.25M stock

 3.6 mM CaCl2 0.45 ml 1M stock

 6.0 mM 2-Mercaptoethanol 53 µl 100% stock

 q.s. to 125 ml with H20

**2X STEP** **BUFFER**

 for l00ml

 2% SDS 20 ml 10% SDS

 0.1 M Tris pH 7.5 10 ml 1 M Tris

 20 mM EDTA 8 ml 0.25 M EDTA

 q.s. to 100 ml with H20

# CHEF Procedures

Electrophoresis parameters for herpesviruses

Initial A=18

Final A=18

15°C

200 v

22 hr.

This gets l1 1 to 2 cm from the bottom of the gel.

**Gel buffer**

0.5x TBE 10x stock:

 0.9 M Tris base (108.99 g/l)

 0.9 M boric acid (55.65 g/l)

 20 mM EDTA (40 ml 0.5 M/l)

**Gel**

1% GTG SeaKem

l ladder

10 µl Boehringer l DNA

90 µl TE

20 µl blue juice

use 8 µl/lane

**Cell preparation**

 Have cells at 1 x 108/ml in T10E10, pH 8.0.

 Mix equal volume of cell suspension (37°C) and 2% LMP agarose (37°C) made up in T10E10, pH 8.0.

 Pipette into teflon taped cuvette, let harden.

 Push into 15 ml blue capped tube.

 Incubate 1 hr. at 37°C in 0.5 M EDTA, 1% sarkosyl (SE).

 Replace buffer with SE containing 0.5 mg/ml proteinase K (keep this away from areas where protein work is done). Incubate at 37°C overnight with gentle agitation.

 Rinse 3x in SE over 6 hr.

 Store at 4°C in SE.

# Use of the Class II Biological Safety Cabinet

1. Plan the experiment.

2. List the equipment and items needed, and reagents and media required.

3. Accumulate everything needed to do the work and place it on a cart.

4. Turn on lights, blower and electrical receptacle. Check intake and exhaust grille; clear obstructions. Allow 5 min for the airflow to purge the cabinet.

5. Wash hands with germicidal soap.

6. Put on gloves, long sleeve lab coat or gown.

7. Wipe all interior surfaces with 70% ETOH, or in the case of virus work use 1% sodium hypochlorite followed by wiping with water to prevent corrosion.

8. Place absorbent pad on work surface to minimize splatter and to absorb spills and facilitate easy cleanup.

9. Place all needed items in the hood so that you have a clean area and a working area. Avoid blocking the front intake and the rear exhaust grilles. Do not pass over the clean items when working with the contaminated items. Place pan for contaminated items to the side of the dirty area. Do not use toxic, explosive or flammable substances in the cabinet.

10. Use a pipetting device. Mouth pipetting is never permitted.

11. Use syringes and needles, scalpels, or scissors only when absolutely necessary. If you do use them be very cautious. Needles should not be resheathed, bent, or removed from the syringe but dispose into a sharps container where no one can be punctured.

12. With hands inside the cabinet ready to work, allow 2 to 3 minutes for the turbulence to settle down before beginning work.

13. Work as deep inside the cabinet as you can; at least 4 inches or more inside the cabinet.

14. Use of centrifuges, blenders, sonicators, vortexers etc., should be near the rear of the cabinet.

15. Limit room activity, no opening or closing of door to the area.

16. Movement of objects or hands causes turbulence. To minimize contamination, move slowly, keep open mouths of bottles or tubes parallel to downflow of air. Recap bottles between pouring. Don't place caps on work surface. When working with more than one sample or specimen only work with one at a time. Cap and completely clean up before going to a second specimen. Change gloves frequently.

17. To wipe up a spill, salvage material if possible. Discard all contaminated tubes, caps, beakers etc. and the absorbent pad. Wipe down the work surface with hypochlorite, rinse with water.

18. In the event of an uncontained spill of infectious or other hazardous material, in addition to informing the supervisor, inform coworkers in the vicinity. Err on the side of caution in deciding whether to evacuate and seal the area. Biosafety is equipped for serious decontamination problems.

19. If you sustain an injury requiring medical assistance, go directly to the Clinic if you are able. Do not wait for supervisor approval.

20. Cleanup. Wipe down and remove all noncontaminated materials and equipment from the hood. All contaminated materials need to be placed in a discard pan with a lid before removing. Do one final wipe down. After completion allow blower to run for 5 minutes.

21. Turn off blower and lights.

22. Wash hands carefully with germicidal soap.

Additional information is in the biosafety manual, Biosafety in Microbiological and Biomedical Laboratories.

# Radiological Isotope Record Keeping

## Ordering Radioactive Compounds

 Order radioactive compounds through Darrell Burns. He will fill out an order form on LAN e-mail to Ann Gilliam, DVRD purchasing agent (DVRD blanket order, radioactive). Radioisotope suppliers on CDC quotation include Amersham, ICN, and NEN. If you want to know if your order has arrived, call Greg Bridges (x3400) in Radioactive Shipping Arrival.

## Receiving Radioactive Compounds

 When isotope arrives, place box behind plexiglass shield in Room 244. Remove the shipping invoice. Remove isotope vial and place in the plexiglass box in -20°C freezer in Room 244, taking note of the assay date on the vial. Check the shipping box for contamination with survey meter. Discard any "radioactivity" labels from the outside of the box and put the box in the cardboard recycling bin. Discard the styrofoam box in regular garbage container if the radioisotope company does not have a styrofoam recycling program.

 Go to the yellow 3-ring binder labelled "Log in Use" found in Room 244 and find the "Radiation Received" section. Fill out the info including the assay date from the vial of isotope (date received, isotope, etc.). Next, obtain an "Isotope Usage" sheet from the black 3-ringed binder labelled "All Kinds of Blank Forms". Fill out the "Isotope Usage" sheet and put it in the yellow "Log in Use" binder directly behind the "Radiation Received" sheet that you just filled out moments ago. Punch binder holes in the shipping invoice and place it in the very back of the yellow "Log in Use" binder.

## Using Radioactive Compounds

 Before you begin work, check the isotope work area in room 244 with the survey meter. The area should be clean (not radioactive) when you begin to work, and cleaner (less radioactive) when you finish. Verify that the area is clean by filling out and initialing the "Radiation Survey Report (in house)" form located on the hybridization oven. Place sheets of blue diaper paper (found in the cabinet below drawer #21) on the surface of the work area. Obtain a yellow "Radioactive Waste for Disposal" sticker (CDC form 0.999) from drawer #15. Fill out the information on the sticker except "activity", "date of disposal", and "date of assay". Place the sticker on a clear autoclave bag from drawer #19. This bag will contain all of your solid radioactive waste (pipette tips, gloves, eppendorf tubes, etc.). When you begin your experiment, take note of how much radioisotope (in mCi) you are using. As you generate solid radioactive waste, place it in the bag. All liquid radioactive waste is placed in the carboy behind the plexiglass screens.

 When you are finished with radioactive work for the day, place your bag containing solid radioactive waste into the plexiglass box located under the radioactive work area. Clean up the work area and be sure to check again with the survey meter. Verify that you have left the area clean (not radioactive) by filling out and initialing the "Radiation Survey Report (in house)" form located on the hybridization oven.

## Radioactive Waste Disposal

 When you have completed an entire experiment and all of the radioactive waste is either in the autoclave bag (solid) or the carboy (liquid), complete the yellow "Radioactive Waste for Disposal" sticker on the autoclave bag. Seal the bag with either tape or a wire "twisty" tie. Make an estimate of the solid waste radioactivity in mCi (usually 50% solid and 50% liquid for total radioactivity used in an experiment). Record the amounts of solid and liquid radioactive wastes that you are discarding on the "Radioactive Disposal Log" sheet found on top of the plexiglass radioactive waste box located under the hybridization oven bench. Place the autoclave bag full of your solid radioactive waste labelled with the "Radioactive Waste for Disposal" sticker into this plexiglass waste box.

## Duties of the Radiation Safety Representative

**Monthly Tasks for Office of Health and Safety (OHS)**

**Isotope Inventory Report**

 The isotope Inventory Report is a kind of accounting system for how much radioactivity comes into the lab and how much goes out during a four-month period. Blank isotope inventory reports (CDC form 0.1003) are found in the black binder labelled "All Kinds of Blank Forms". Information from the "Radiation Received" sheet in the yellow binder is used to fill out the third and fourth columns of the isotope inventory report. Information from the "Radioactive Waste Disposal Log" is to fill out the sixth column of the isotope inventory report. Complete the form for all isotopes (14C, 35S, etc.) even if none was received during the four-month period. After the report is completed, make a copy and put it in the black binder labelled "Past Inventory Reports". Also, put all forms related to the previous four months such as shipping invoices, isotope usage forms, radiation received forms, etc. into their respective "Past" black binders. Mail the completed isotope inventory report to Paul Simpson (mailstop F5).

**Radiation Survey Report**

 The Radiation Survey Report is a way of assuring that radioactive spills are not left around the lab. Every month, certain areas of the lab are swabbed with a long Q-tip (cotton tip applicator, Glassware #75160) and the tip is broken off and placed in scintillation cocktail. The dpms counted for each swab are then recorded in the Radiation Survey Report (CDC form #0.1002). If the scintillation counts reveal a radioactive spill, clean up the appropriate area. Make a copy of the form and at the designated four-month intervals send these copies from the previous four months to Paul Simpson (mailstop F5).

**Radiation Badges**

 Every three months, new radiation badges are sent to the lab from OHS. Simply trade each laboratory worker a new badge for their old one and mail the old badges back to OHS (Paul Simpson, mailstop F5). New badges for new employees can be obtained from Paul Simpson or Thomasina McFadden at OHS.

**General Lab Tasks**

 Try to keep the radioactive area clean and tidy. This may require a kindly reminder to lab personnel. Stock the cabinet under drawer #21 with a good supply of blue diaper paper (Underpads, Glassware #94790). In addition, keep drawer #13 stocked with aerosol-free pipette tips for P-20s (Glassware #95101) and P-200s (Glassware #99110). Keep a stack of yellow "Radioactive Waste for Disposal" labels (CDC form #0.999) in drawer #15. Keep at least three cans of Rad-Con spray (obtained from Paul Simpson) on hand. Fresh batteries for survey meters can also be obtained from Paul Simpson. Keep functional pens near the Radiation Record black binders and on top of the radioactive waste bin. Keep a cheerful attitude and hope that the lab adopts nonradioactive technologies in the near future.