Love them or Hate them …

Harnessing the power of antibodies is not always easy

Antibodies are extremely powerful reagents in a scientist's toolbox. To make it easier to create novel antibodies for and use existing antibodies in your unique experiments, the Antibody Technology Research Group (ARG) within the ABRF (http://www.abrf.org/ARG) is compiling a dynamic intellectual repository focused on antibodies technologies enabling all of us to address our own antibody challenges by continuously learning from each other.

We invite you to spend **5 to 15 minutes** sharing your knowledge from which the initial installment will be created. In return, you and your institutions will be cited for each contribution in the antibody repository and you will have access to it's complied knowledge *before* it goes public. Submission topics include, but are by no means limited to:

- Generating novel antibodies, i.e.:
  - Immunization approach (Immunogen design, choosing a host, adjuvant)
  - B Cell Fusion
  - Recombinant antibody technologies
  - Screening approaches for monoclonal antibodies possessing your desired specificity
- Producing and purifying antibodies
- Labeling antibodies
- Using antibodies (i.e. Flow Cytometry, Microscopy, ChIp-Seq, In vivo applications)

Your contribution can take whatever form you like. For example, you could submit:

- Reference citations accompanied by 1 to 2 sentences highlighting their value
- Advice
  - "Pearls of wisdom" you would pass down to those you are mentoring / training
  - Things you wish you had known before learning them hard way
- Protocols – highlighting the critical parameters (example protocol follows)

Please send all contributions as separate attachments to weisgarf@mskcc.org. Those received by October 1, 2011 are guaranteed to be part of the first version.

We look forward to hearing from each of you and sharing our collective knowledge thus facilitating all our work.

ARG 2011 Study Sub-Committee,

Frances Weis-Garcia

Memorial Sloan Kettering Cancer Center

Head, Monoclonal Antibody Core Facility

Greg Halverson

New York Blood Center

Chief Immunohematologist

Linda Green

University of Florida

Interdisciplinary Center for Biotechnology Research

Director, Hybridoma Core Laboratory

Kathleen Brundage

West Virginia University

WVU Flow Cytometry Core Facility

Technical Director

Immortalizing Rodent B cells
Via a
PEG based Somatic Cell Fusion Protocol

Memorial Sloan Kettering Cancer Center
Frances Weis-Garcia
646-888-2354
weisgarf@mskcc.org

Purpose:

1. To generate a library of hybridoma lines from mouse, rat or hamsters B cells from which monoclonal antibodies with specific functionality can be isolated
2. To create a naïve rodent B cell library – an immortal representation of the B cells present in the spleen or lymphnodes from and unimmunized rodent
    a. Caveat – Assumes all B cells fuse equally well

Expected Results:

- Average efficiency = 1 hybridoma for every 100,000 pre-fusion viable nucleated splenocytes
- Range of efficiency = 1,500 – 15,000 hybridomas / spleen
    - Failed fusions are those < 1,000 hybridoma / spleen - which is almost always the result of primary adherent outgrowths taking over the culture before the hybridomas can become well established in the original well

Equipment:

- CO_2 Incubators (7% CO_2 / 37°C / humidified with water tray)
- Tissue Culture Hoods
- Clinical Centrifuge
    - Fisher Centrific™, Model # 225, Fisher Cat # 04-978-50
- Microscope appropriate for viewing myelomas and splenocytes

- Hemocytometer
- Pipetaid
- Multi and single channel pipetors

Reagents / Cell Lines / Consumables:

Myelomas:

- Sp2/0-Ag14 For mice (ATCC / Cat# CRL-1581)
  (*NOT ATCC / CRL-8287*)
- P3x63Ag8.653 For rats/hamsters (ATCC / Cat# CRL-1580)

Media / Supplements:

- Hybridoma Serum Free Media (HSFM)
  - Invitrogen – Hybridoma-SFM (1x Liquid – Cat# 12045-076)
  - We purchase powder and prep it here – just to save money
- Fetal Bovine Serum (FBS)
  - Pre-screened by facility for fusion/cloning quality
- Growth Factor Supplement
  - Pre-screened by facility for fusion/cloning quality
  - For mouse fusions –
    - Hybridoma Fusion and Cloning Supplement (HFCS)
    - Roche Applied Sciences / Cat.# 11363735001
  - For rat/hamster fusions –
    - Hybridoma Cloning Factor (HCF)
      (Although it is NOT listed on their US website - PAA Laboratories does sell HCF – contact them directly)
- Gentamycin
  - Invitrogen - 10mg/ml – Cat# 15710-072
- Hypoxanthine, Thymidine (HT)
  - Invitrogen – 100x – Cat# 11067030
- Hypoxanthine, Aminopterin, Thymidine (HAT)
  - Invitrogen - 50x – Cat# 21060017

Media Formulations:

- Fusion Base Media:
  - HSFM
  - 15% FBS
  - 1 x Appropriate Growth Factor Supplement (see above)
  - 10 mg/ml Gentamycin
- HT Media (Fusion Growth Media):
  - Fusion Base Media
  - 1x HT
- Fusion Recovery Media:
  - HT media
  - 2x Growth Factor Supplement (see above) ***rather*** than 1x
- 1.3x HAT Media (Fusion Selection Media = 1x):
  - Fusion Base Media
  - 2x Growth Factor Supplement (see above) ***rather*** than 1x

Misc:

- 50% PEG 1500 in 75 mM Hepes (w/v)
  - Roche Applied Sciences (Cat# 10783641001)
- 500 ml FRESH 70% ethanol made in bottle
- 1% Eosin in PBS
- 60 mm petri dishes
- Conical tubes (cell culture grade) – 50 and 15 ml
- Serological pipets (cell culture grade) – 25 – 10 – 5 and 2 ml
- 1 ml syringe
- 10 ml syringe
- 26g needle
- Sterile frosted glass slides for disrupting spleen – Prepared by scraping the excess frosting off with the edge of another slide, washing glass dust off the slides with ultra pure water and autoclaving the slides in sets of 2/bag.
- Sterile 40 μm single cell filter
  - Fisher Cell Strainers (Cat# 22-363-547)
- T75 cell culture flasks
- 30 sterile flat bottom 96 well tissue culture plates
- Sterile basin/trough from which to pipet media/cells into 96 well plates with multichannel pipettor

Protocol:

Day -3/-4 (Monday):

1. Administer one final challenge with the immunogen
    - This is an acute dose – thus there is no adjuvant
    - Routes:
        - IV (Tail vein or retro-orbital) is preferred
        - If the buffer system toxic to the animals (i.e. 8M Urea) – then perform an interperitoneal injection
    - 5 – 15 μg if that much antigen is available
    - Best and second best animals are injected
        - Second best is only used if best dies
        - "Best" means the animals with the strongest sera antibody response in all target assays
    - See "Words of Wisdom" / Trouble shooting – Point #1 for more information on adverse side effects

Day -1 (Wed/Thur):

1. Passage the myeloma cells TWICE:
    - Early in the morning with HSFM + 10% FBS – for:
        - SP2/0-Ag14 to 0.4×10^6 viable cells/ml
        - P3x63Ag8.653 to 0.5×10^6 viable cells/ml
    - End of the day to with HT media – for:
        - SP2/0-Ag14 to 0.20×10^6 viable cells/ml
        - P3x63Ag8.653 to 0.25×10^6 viable cells/ml

Day 0 (Thur/Fri):

1. Before 10 am, passage the myeloma cells cells/ml with HT media:
    - SP2/0-Ag14 0.4×10^6 viable cells/ml
    - P3x63Ag8.653 0.5×10^6 viable cells/ml
2. Harvest spleen and place it in a conical tube containing HT media.
3. Collect blood from the animal and prepare the sera by:
    - Incubating it at 37°C for 30 min to facilitate clotting

- Incubating overnight at 4°C to shrink the clot
- Remove clot
- Microfuge at max for 10 min
- Sera is liquid supernatant

4. Pour the spleen and HT media into a 60 mm petri dish. Tweeze/cut away as much other tissue from the spleen as possible **without** disrupting the capsule of the spleen. Transfer spleen to a new 60 mm petri dish.
5. Obtain a single cell suspension of splenocytes by employing BOTH the "syringe" and "slide" methods in this order.
    - Syringe Method – Take 10 ml of HT in a syringe with a 26 gauge needle and inject it into the spleen (once) allowing it fill with HT (swell) and allowing the splenocytes to spew out – This method recovers the most viable splenocytes
    - Slide Method – Obtain the remaining splenocytes by pressing the spleen between the frosted area of 2 sterile slides that have been scrapped and washed to a more fine level of frosting (See Equipment – Misc for more details) –
    - For more details on each of these – refer below to "Words of Wisdom" #4
6. Filter out large aggregates with a single cell filter into a 50 ml conical tube to remove aggregates – This seems to decrease the frequency which a primary adherent outgrowth overtake the wells in which the new hybridomas grow up -
7. Rinse petri dish with ~ 2 mls of HT media and pass this through the same single cell filter into the 50 ml conical tube -
8. Mix cells in the 50 ml conical tube with the pipet you transferred them to the tube with and take ~ 100 μl for counting -
9. Determine total cell number and viability of the nucleated splenocytes using eosin - Depending upon how well the animal responded to the final boost, you could get anywhere from 150 to 450 x 10^6 total nucleated splenocyes –
10. Count the myeloma cells to determine cell number and viability -

11. Mix the cells in a ratio of **5 nucleated splenocytes** to **1 myeloma** (Total # not Viable #) –
    - Save some myeloma as a control for the selection media
12. Wash the cells mixture 3x with HSFM – 45 ml each wash – because the FBS will inhibit the fusion -
    - Cell aggregates often develop as the FBS is washed away - Remove them by passing the cell suspension through the another single cell filter
13. The following steps need to be done consecutively without stopping –
    - Resupend the pellet after the final wash
    - While agitating by tapping the tube against the wall of a large beaker containing warm water (37-40°C)
        - Warm the cells for 1 min.
        - Add 0.5 ml PEG 1500 over 60 sec. – washing any cells on the wall down to the bottom of the tube
        - Add 15 ml HSFM – DROPWISE – over 90 sec.
    - Incubate cells with**out** agitation for 8 min at room temperature
    - "Heat-shock" the cells for 2 min in 37 – 40°C water bath
    - Centrifuge fusion mixture for 4 min. setting the Fisher clinical centrifuge (see equipment list above) just before "5" -
    - Stop fusion **immediately** by"
        - Aspirating as much media as possible without disturbing the pellet.
        - Begin re-suspending pellet by **gently** tapping the tube only **3 - 5 times.**
        - Re-suspension with a large bore (i.e. 25 ml) pipet in 30 ml of freshly prepared Fusion Recovery Media. Only pipet up and down a few times without forcing the cells between the pipet and bottom of the tube.
        - Allow any remaining clumps to settle for a few seconds and then transfer all "single" cells which are temporarily suspended in the media into a T75 flask(s).

- Continue to recover more "single" cells with additional aliquots of Fusion Recovery Media and add each to the flask(s) - At this point – if the clumps do not break apart easily - pipet more forcefully with **each wash** volume.

14. Depending upon how long the freshly fused cells are left to recover from the process - the final suspension of cells should be:
    - ~ 4 to 4.5 x 10^6 pre-fusion viable cells/ml for 4 to 8 hours
    - ~ 2.5 to 3 x 10^6 pre-fusion viable cells/ml for 12 - 16 hours (preferred)
15. Incubate 4 to 20 hours in the cell culture incubator –

Day 1 (Fri/Sat): … Assuming the cells recovered overnight …

16. Dispense 150 μl/well of 1.3x HAT media into 96 well plates and number them.
17. In a sterile trough, dilute enough fused cells with Fusion Recovery Media to obtain 100 x 10^6 pre-fusion viable nucleated/lymphoid in 50 ml (0.1 x 10^6/50 μl).
18. Without delay, dispense in 50 μl of the diluted fusion suspension into 10 of the 96 well plates pre-filled in step 16.
19. Repeat steps 17 and 18 until the desired number of plates are made (i.e. 25).
20. Plate 50 μl/well of the myeloma cells set up at the time of the fusion into 1 pre-filled 96 well plate.
21. Incubate plates for 5 - 8 days UNDISTURBED. A plate or 2 can be checked for growth, but care should be taken to not disturb colonies.

Day 5 to 8:

22. Incubate plates for 5 - 8 days UNDISTURBED. A plate or 2 can be checked for growth, but care should be taken to not disturb colonies –
23. To determine fusion efficiency, count the number of clones (cluster of ≥ 10 spherical/glowing cells) using a 4x objective, usually on day 6/7 - Record # colonies /well and % wells with hybridoma growth –

24. Feed the fusion (aspirate and add 150 μl Fusion Recovery Media) on day 7/8 (Fri), unless the clones are particularly:
    - Small (most colonies are < 25 cells) or infrequent (< 0.5 clones/well) - then begin feeding on the Day 5/6 (Wednesday)
    - Large (can be seen by the naked eye on day 5/6) and show noticeable growth by day 7/8 (Fri) - then delay the first feeding until day 11 (Mon)

Day 21 to 28:

25. Count and calculate what % wells from "all" the plates contained hybridomas as part of the Quality Control data - Adjust the # colonies/well with this final count –

Words of Wisdom / Trouble Shooting Tips:

1. Final Challenge with immunogen: When injected intravenously, there is a significant chance of the animal becoming moribund – Our vets best guess is this is cause by an anaphylactic reaction – MOST of the time the animal can be "rescue" before with anti-histamine (0.3 mgs IP per 20-30 g mouse) before it gets too sick – With that said we find that immortalized splenic B cells from the animals who do because sick (and not died) are more likely to be successful – presumably because they tend to:
    - Have larger spleens
    - Generate more hybridomas
    - Have more hybridomas secreting antigen specific antibodies –
    - Have more background signal (antibody coming from unfused antigen specific B cells)

    We have not administered anti-histamine prophylactively – rather we wait for the following clinical presentation and administer as indicated below – This does not always follow this linear progression:

    a. Significant increase in scratching – particularly of the ears
    b. Hunched / scruffy appearance
    c. Increased respiration rate
    d. Lethargy – 3 classes
        a. More sedentary than cage mates who have not received the injection – but still responds to stimuli and moves around to some degree -
        b. Markedly sedentary – stay in one place - but still responsive to cage mate and humans bothering them
        c. Markedly sedentary – NOT significantly responsive to cage mate or human bothering them
            - Best results if administered at or just before this point is reached
    e. Blueness around whiskers (at this point it NEEDS to be administered)
    f. Limpness – shallow breathing
2. A few points about the myeloma/fusion partners:

- They need to be in culture for at least 2 weeks prior to fusion and not more than 6 months.
- A new stock is thawed every 3 months. The old stock is used for "real" fusions until the new batch gives similar efficiencies to the old stock in a "test" fusion.
- They are tested monthly for mycoplasma –
- Maintained without antibiotics to ensure they are not carrying an "undetectable" level of contamination that could bloom up after the fusion/during the screening -
- For 72 hours prior to the fusion, they are to be maintained in log phase growth to ensure > 95% viability and "100%" of the population cycling at the time of the fusion. This is done by counting and splitting the cells daily, keeping them as close to 0.5×10^6 viable cells per ml (lower limit = 0.1 – 0.2 overnight and reaching more than = 0.75 by the next day).
- A maximum of 100×10^6 myeloma cells will be needed at the time of the fusion (~ 200 ml @ 0.5×10^6/ml).

3. Terminally bleed the animal by cutting open the chest in such a way that the blood pools into the thoracic cavity after the heart is cut. Transfer the blood into a microfuge tube using a 1 ml syringe (no needle). Removing the lungs before cutting the heart maximizes recovery:
4. Obtain the splenocytes by:
    - <u>The "syringe" method:</u>

      Fill a 10cc syringe with HT media. Attach a 26 g needle. Slowly inject HT media into the spleen with the bevel side of the needle up. You should see the spleen swell and RBDs leak out. When you feel you have "emptied" a spot of the spleen because it looks rather pale, move to a different part. Some sections will be disrupted easier than others, so move around. You will have gotten most of the splenocytes out when the spleen looks pale/translucent. If the spleen is not pale/translucent, do **not** refill the syringe with the cell/media mixture because you do not want to diminish the integrity of the cell walls by passing them through a needle. The remaining cells will be removed in the slide method.

- The "slide" method:

  Press the spleen between the frosted areas of two slides until the spleen starts breaking into clumps. Then push the bulk of the organ above the frosted area and dissociate the clumps by gently rubbing them between the frosted areas. Rinse the cells off the frosted areas into the HT. Repeat this until the spleen is completely white or the spleen does not become any paler.

5. Adherent cells from the spleens of hamsters can be a significant problem because they can overtake the fusion by growing better than the hybridomas. This can be minimized by replacing the "slide method" with tweezing the spleen into small pieces.
6. The un-fused myeloma cells are a control for HAT selection as well as "revertents" in the myeloma population. They should all die within 3 days of HAT selection .
7. All centrifugations are done with the Fisher clinical centrifuge at "3" unless otherwise noted.
8. Cells should be agitated by continuously tapping the tube against the wall of the beaker.
9. Make every attempt not to touch the cells with the pipet, but rather to coat the walls of the tube with the PEG by allowing it to flow down the sides of the tube starting near the cells. Thus, they flow with the PEG to the bottom of the tube and you do not loose any cells on the pipet.
10. This step allows the cells to recover as well as help clear the fusion of many adherent cells that can over grow the hybridomas later.
11. When handling cell suspension, reuse pipets whenever possible to minimize cell loss to walls of pipet.

References:

- This protocol is a compliation of 4 protocols from the following sources:
  - Harlow and Lane – Antibodies
  - Charles Janeway's lab
  - ATCC
  - Current Protocols in Immunology (from 1999)