Perform Bronchoalveolar Lavage (BAL) and cytospin Protocol

1. Euthanize mice with CO2
2. Position each mouse on its back on the surgical pad. Soak the area with 70% EtOH.
3. Beginning at the lower abdomen, cut open the abdominal cavity and remove skin/upper muscle, moving upwards toward the ribs.
4. Once the ribs are visible, use scissors to carefully puncture the diaphragm. Lungs should collapse away from the diaphragm. Be especially careful not to nick the lungs or heart.
5. Cut away the ribcage to fully expose the lungs/heart (avoid cutting any major blood vessels to keep blood from filling the site).
6. Using a 1 ml syringe with a 27 gauge needle (BD syringes, Franklin Lakes, NJ), puncture the heart ventricles and slowly and carefully pull back the heparinized syringe to collect the blood. Take care to avoid collapsing the heart.
7. Collect the serum from this blood using standard protocol. Store at -70 °C until use.
8. Cut away skin and tissue from the throat until the trachea is revealed. Clear away sufficient tissue to work easily within the field (again, avoid cutting any major blood vessels).
9. Using curved scissors, cut under the trachea to clear a path.
10. Pass the point of a curved forceps under the trachea and grasp the end of a piece of suture. Draw the suture thread (black silk 4-0) under the trachea.
11. Tie a loose half-knot about the trachea, low in the throat.
12. Carefully cut a notch, sufficient in size for the cannula, above the suture thread.
13. Carefully insert the cannula into the hole and down the trachea past the point of the suture thread. Gently press forward until the cannula emerges just at the entrance to the lungs (too far: puncture lungs; too short: collapse trachea when attempting to recover BAL).
14. Tighten suture thread and complete knot to seal trachea around cannula.
15. The mouse lungs are lavaged three times with 0.8 ml of PBS containing 2% BSA (PBS-2%)* - 2% BSA +PBS = 250 ml of PBS + 5 g BSA (final 2%)
16. The BAL fluid was centrifuged at 1500 rpm for 5 minutes and supernatants collected and frozen until ELISA was performed.
17. Gently press the fluid into the lung. Lung lobes should individually inflate slowly. Do not over-fill. For a full grown mouse 0.9-1.0 ml is the absolute maximum. 0.8 ml may be safer.
18. Withdraw fluid from lungs. If resistance is encountered (tissue sucked into cannula), press cannula slowly further into the lung and resume removing. Also try rotating the cannula in place. If all else fails, withdraw the cannula part way; the trachea is much more likely to collapse in this case.
19. Detach syringe from cannula, deposit BAL fluid in container
20. Keep the BAL solution on ice until spun down.
21. Use the BAL fluid and serum to measure the OVA specific IgE using commercially available mouse IgE ELISA kits (MD Bioproducts, St. Paul, MN).
22. The recovered supernatants are collected and stored at -70°C until assessed for cytokine concentration, and the cell pellet are resuspended in 500 μl of Complete media-nutrition, antibiotics, 10% FBS (if few samples, Could use PBS-2%FBS).
23. For histology, after BAL, the mouse lungs are filled with 0.5 ml of 10% formaldehyde solution (Fischer Scientific Cat # 245-684) and rapidly remove the catheter and tie the black silk thread ASAP
24. Then carefully detach the whole trachea and both lungs from thoracic cavity
25. Put the removed lungs into the 10% formaldehyde contained tube.

B. Count Cells and Determine Differentials

1. Centrifuge the BAL fluid, 1500 rpm for 5 minutes, 4 °C.
2. The cell pellet are resuspended in 500 μl of Complete media (or PBS-2% BSA) and keep on ice.
3. Load a standard Neubauer hemacytometer with the diluted cell suspension (20 μl of sample + 20 μl of dye) and count the cells.
4. Remove aliquots of 4 x 10⁴ cells in 200 μl volume for cytopsins. Dilute cells if necessary.
5. Add entire cell mixture to double cytopsin funnel and centrifuge 10 min at 600 rpm, using double cytoslides for duplicate samples (if it is possible).
6. Allow the slides to dry at room temperature for 30 min -1 h prior to staining.
7. Stain one of the duplicated slides by using Diff-Quick stain (Siemens, Newark, DE).- dip slides 30 sec. each solutions.
   Alcohol Fixation → red → blue → tap water jar washing enough times
8. Another stain the duplicated slides by using Wright-Giemsa stain (Thermo Fisher Scientific Fisher HealthCare™ PROTOCOL™ Wright and Wright-Giemsa Stain Solutions, Cat # 23-264-984), which stain method is better the differentiation of eosinophils from other cells than Diff Quick stain
9. 300-500 cell differential counts were performed to determine the absolute numbers of inflammatory cells.

Diff-Quick (Diff-Quik) Staining Protocol

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NovaUltra Special Stain Kits

Principle
"Diff-Quick" is a proprietary brand of a Romanowski stain. The Romanowski group of stains are defined as being the black precipitate formed from the addition of aqueous solutions of methylene blue and eosin, dissolved in methanol. The variants of the Romanowski group differ in the degree of oxidation (polychroming) of the methylene blue stain prior to the precipitation.

The stain class was originally designed to incorporate cytoplasmic (pink) staining with nuclear (blue) staining and fixation as a single step for smears and thin films of tissue (spread preparations of omentum). Minor modifications of working stain concentration and staining time have been made over the years for fixed tissue sections.

Technical Points

(step 5) - exposure to alcohol should be as brief as possible to prevent excessive decolourisation.
Method

Paraffin Sections

1. Bring sections to distilled water
2. Stain with “Diff Quick” solution II .30 secs
3. Counterstain (optional) with “Diff Quick” solution I for 30 seconds
4. Rinse rapidly in tap water
5. Rapidly dehydrate in absolute alcohol
6. Clear and mount

Smears/Imprints

1. Air-dry the smear
2. Fix in “Diff Quick” Fixative (or methanol) for 30 secs/drain
3. Stain with “Diff Quick” solution II for 30 secs/drain
4. Counterstain (optional) with “Diff Quick” solution I for 30 secs/drain
5. Rinse in tap water to remove excess stain
6. Rapidly dehydrate in absolute alcohol
7. Clear and mount

Results

helicobacter........................................dark blue
background....................................light blue
platelets............................................violet to purple

neutrophils nucleus.............................dark blue
cytoplasm.................................pale pink
eosinophils nucleus...............................blue
cytoplasm.................................blue
granules...............................red to red/orange
basophils
nucleus..............................purple or dark blue
granules..............................dark purple/black
monocytes
nucleus(lobated)..............violet
cytoplasm..............................sky blue

**Wright and Wright-Giemsa Stain, Dip Method (Rapid)**

1. Place approximately 50 ml Wright-Giemsa Stain in a Coplin jar.
2. Fill another Coplin jar with water or phosphate buffer.
3. Place thoroughly dried blood film, feather edge DOWN, in Wright-Giemsa Stain for approximately 1 minute-1 minute 30 seconds. NOTE: Rapid dipping for 5-10 seconds may reduce water artifacts on films that are not thoroughly dried. (6 times dipping)
4. Remove slide from stain and place in deionized water or phosphate buffer, pH 6.8-7.2, feather edge DOWN, for approximately 1-10 minutes. DO NOT AGITATE SLIDE WHILE IT IS IN DEIONIZED WATER.
5. Rinse briefly in running deionized water and air dry thoroughly before evaluation.

References
5. [http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/1/wg.pdf](http://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/General_Information/1/wg.pdf)
Inflammatory Airway Disease

- Increased cellularity
- Increase of:
  - Neutrophils,
  - Lymphocytes,
  - Macrophages,
  - Eosinophils
- Mucus, goblet cells, Curthmann's spirals