**Nojima Culture (Mouse Single B-cell Culture) Protocol**

**Materials**

**Medium**

RPMI-1640, [+] L-Glutamine (Invitrogen, 11875-093)

DMEM, [+] 4.5 g/L D-glucose, [+] L-Glutamine (Invitrogen, 11965-092)

**Supplements**

* Fetal Bovine Serum [FCS HyClone (Thermo Scientific, SH30070.03)]
* Pen Strep (Penicillin (10,000 Units/ml), Streptomycin (10,000 μg/ml); (100x); Invitrogen, 15140-122)
* 2-ME (55mM (1,000X); Invitrogen, 21985)
* HEPES (1M (100X); Invitrogen, 15630-080)
* Sodium Pyruvate (100 mM (100X); Invitrogen, 11360-070)
* MEM NEAA [(100X); Invitrogen, 11140-050]

**Others**

* 0.5% Trypsin-EDTA [(10X); Invitrogen, 15400-054]

Aliquot 5 ml 0.5% Trypsin-EDTA into 15 ml tubes and stored at -20°C. Make 0.05% Trypsin-EDTA in sterile PBS from frozen aliquots for use. Store this diluted 0.05% Trypsin-EDTA at 4 °C.

* PBS pH7.4 (Invitrogen, 10010-023)
* Sodium azide (Sigma): Make 10% (weight/volume) sodium azide solution in sterile, distilled water.

**B cell media (BCM)**

RPMI-1640 supplemented with 10% FCS, 55 μM 2-ME, 1% Pen Strep (100 Units/ml Penicillin, 100 μg/ml Streptomycin), 10 mM HEPES, 1 mM Sodium Pyruvate, and 1% MEM NEAA

\_\_\_ RPMI 1640 500 ml

\_\_\_ FCS 58 ml

\_\_\_ 2-ME 580 μl

\_\_\_ Pen Strep 5.8 ml

\_\_\_ HEPES 5.8 ml

\_\_\_ Sodium Pyruvate 5.8 ml

\_\_\_ MEM NEAA 5.8 ml

**Feeder cell maintenance medium (NB-21 media)**

DMEM supplemented with 10% FCS, 1% Pen Strep (100 Units/ml Penicillin, 100 μg/ml Streptomycin), 1% MEM NEAA

\_\_\_ DMEM 500 ml

\_\_\_ FCS 57 ml

\_\_\_ Pen Strep 5.7 ml

\_\_\_ MEM NEAA 5.7 ml

**Cytokines**

Recombinant mouse IL-4 (Peprotech, 214-14)

Reconstitute lyophilized recombinant mouse IL-4 in 0.22 μm filtered BCM at 20 μg/ml. Aliquot 50 μl or 100 μl of the reconstituted IL-4 into sterile 1.5 ml tube with screw top. Store these aliquots at -80 °C until use.

**Culture plates**

10 cm Tissue Culture Dish (BD Falcon, 353003)

96-well Cell Culture Plate (Denville Scientific, T1096)

**NB-21 Feeder cells**

NB-21 clone 2D9 (manuscript in preparation): NB-21 feeder cells were established from 40LB cells, which express mouse CD154 and mouse BAFF ([Nojima et al., 2011](#_ENREF_1)), by retroviral transduction of mouse IL-21 (mIL-21) cDNA. After transduction, we subcloned NB-21 cells. NB-21.2D9 cells were selected by their capacity to optimally support IgG production by single mature follicular B cells (Kuraoka et al., 2016). The NB21 cells express high levels of MHC class I (H-2Kd) on cell surface, thus NB-21 cells can be isolated from B cells by using anti-H-2Kd antibody, if necessary.

**Handling of NB-21 feeder cells**

It is important to handle NB-21 cells properly, as a function of this feeder cells is crucial for successful single B cell cultures.

1. Thaw frozen vial of NB-21 cells (1 × 106 cells/vial) stored in a liquid nitrogen tank.
   1. Warm up 30 ml of NB-21 media in a 50 ml tube (per one frozen vial) at 37°C.
   2. Thaw frozen vial of NB-21 cells at 37°C in a water bath without shaking a tube.
   3. Transfer thawed NB-21 cells to a 15 ml tube that contain 10 ml warmed NB-21 media in a drop-wise-manner (use 1 ml auto-pipet and 1 drop/second with gently shaking 15 ml tube).
   4. Spin down cells at 400 × *g* for 5 min at 4°C or room temperature (25°C).
   5. Remove supernatants, loose cell pellets by tapping tubes, and then add 15 ml warmed media. After gentle pipetting up and down, transfer cell suspension to a 10 cm dish.
   6. Incubate cells in a CO2 incubator (37°C, 5% CO2).
2. Two days later, passage NB-21 cells.
   1. Warm up NB-21 media and sterile PBS at 37°C.
   2. Remove culture supernatants.
   3. Carefully add 10 ml of warmed PBS to rinse out residual media/cell debris (Edge of the pipet tip should be attached to the side of the dish).
   4. Remove PBS and add 3 ml 0.05% Trypsin-EDTA same way as above. Transfer this 10 cm dish to CO2 incubator and incubate for 3 min.
   5. After incubation, tap side of the dish to completely detach NB-21 cells from the dish, and then add 10 ml of warmed NB-21 media to dilute Trypsin-EDTA.
   6. Mix gently and transfer cell suspension to a 15 ml tube.
   7. Spin down cells at 400 × *g* for 5 min at 4°C or room temperature (25°C).
   8. Remove supernatants, add warmed NB-21 media, and cell count.
   9. Adjust cell concentration to 5 × 105 cells/15 ml/dish, and dispense NB-21 cells into 10 cm dish. \*NB-21 cells should be evenly dispersed in a dish.
   10. Passage numbers should be recorded and labeled.
3. Culture NB-21.2D9 cells for 3 days. These NB-21.2D9 cells are ready for the use of single B-cell cultures.
4. Passage NB-21.2D9 cells every 3 days for near-future-use. **DO NOT** let NB-21.2D9 cells become too confluent. Confluence can result in the loss of their supportive function.
5. **DO NOT** passage NB-21.2D9 cells over and over. Expand your initial cell stock and freeze aliquots for reactivation and future use. We **DO NOT** routinely maintain NB-21.2D9 cells to minimize possible outgrowth of bad clones. If you do not have immediate plan, then freeze down NB-21.2D9 cells and stored at liquid nitrogen for future use.

**Nojima culture**

**Day -6**

1. Thaw and start to culture NB-21.2D9 cells (see Handling of NB-21.2D9 cells).

**Day -4**

1. Expand NB-21.2D9 cells (see Handling of NB-21.2D9 cells).

**Day -1**

1. Harvest NB-21 cells, which had been cultured at least for 5 days from thawing. We typically use NB-21 cells after one additional passage and expansion from the thawing (see Handling of NB-21 cells).
2. After spin down, resuspend NB-21 cells in warmed B-cell media (BCM; 1 ml/dish), count cells, and adjust cell concentrations to 2 × 104 cells/ml in BCM.
3. Add 100 μl of the NB-21 cell suspension to each well of 96-well plates (2 × 103 cells/well).
4. Incubate culture plates in a CO2 incubator for overnight (37°C, 5% CO2).

**Day 0**

1. Warm up BCM at 37°C.
2. Dilute recombinant mouse IL-4 in warmed BCM (at 4 ng/ml).
3. Add 100 μl/well of the diluted IL-4 to each well of 96-well plates, in which NB-21 cells were seeded on day -1. Final concentration of IL-4 is 2 ng/ml.
4. Now plates are ready and sort single B cells directly into each well of the 96-well plates.
5. After cell sorting, initiate Nojima culture in a CO2 incubator (37°C, 5% CO2)

**Day 2**

1. Warm up BCM at 37°C.
2. Remove 100 μl culture supernatants, and then add 200 μl warmed BCM (culture volume: 300 μl, thereafter). \* DO NOT touch bottom of the well with tips. \* Add media carefully and gently so that you do not disturb feeder cell layers.
3. Place culture plates back to a CO2 incubator.

**Days 3-6**

1. Warm up BCM at 37°C.
2. Replace 200 μl culture media with fresh warmed BCM every day.
3. Place culture plates back to a CO2 incubator.

**Days 7-8**

1. Wash tips with sterile 0.9% NaCl between every plate during removal and adding medium, washing by pipetting up and down at full range (set at 200 μL) for 3 times. Avoid introducing bubbles in tips. Replace 200 μl culture media with fresh warmed BCM every day.
2. Place culture plates back to a CO2 incubator.

**Day 10**

We initially harvested culture supernatants on day 9 (24 hours after last media change), however; we found that 12-24 hours of culture extension significantly increased total IgG concentrations in the culture supernatants. Thus we harvest culture supernatants on day 10 (36-48 hours after the last media change on day 8).

1. Harvest all culture supernatants (typically 250-280 μl) into new 96-well plates. To avoid cross-contamination of samples, it is very important not to share tips among samples.
2. Add 2.5 μl of 10% sodium azide (final approximately 0.1%) into each culture supernatant.
3. Store culture supernatants at 4°C for immediate use (within 1 week), or otherwise store them at -20°C or lower temperature.
4. Store tissue culture plates (no culture supernatants but B cells are still there!) at -80°C for subsequent amplification of V(D)J rearrangements and the reconstitution of antibody.

**References**

Nojima, T., Haniuda, K., Moutai, T., Matsudaira, M., Mizokawa, S., Shiratori, I., Azuma, T., and Kitamura, D. (2011). In-vitro derived germinal centre B cells differentially generate memory B or plasma cells in vivo. Nat Commun *2*, 465.

Kuraoka M., Schmidt A.G., Nojima T., Feng F., Watanabe A., Kitamura D., Harrison S.C., Kepler T.B., and Kelsoe G. (2016). Complex Antigens Drive Permissive Clonal Selection in Germinal Centers. Immunity 44(3): 542-552.