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# EAE Induction by Passive Transfer of MOG-specific CD4$^+$ T Cells

Yuki Tanaka[#], Yasunobu Arima[#], Kotaro Higuchi, Takuto Ohki, Mohamed Elfeky, Mitsutoshi Ota, Daisuke Kamimura* and Masaaki Murakami*

Division of Molecular Neuroimmunology, Institute for Genetic Medicine and Graduate School of Medicine, Hokkaido University, Sapporo, Japan

*For correspondence: murakami@igm.hokudai.ac.jp; kamimura@igm.hokudai.ac.jp

#Contributed equally to this work

**[Abstract]** Experimental autoimmune encephalomyelitis (EAE) is an animal model of multiple sclerosis (MS), which is a chronic inflammatory disease of the central nervous system (CNS). It is characterized by focal demyelination and inflammatory responses mediated by myelin-specific autoreactive CD4$^+$ T cells. Using a passive transfer model of EAE in mice, we have demonstrated that regional specific neural signals by sensory-sympathetic communications create gateways for immune cells at specific blood vessels of the CNS, a phenomenon known as the gateway reflex (Arima *et al*., 2012; Tracey, 2012; Arima *et al*., 2013; Sabharwal *et al*., 2014; Arima *et al*., 2015b). Here we describe protocols for passive transfer model of EAE using freshly isolated (MOG)-specific CD4$^+$ T cells or periodically restimulated MOG-specific CD4$^+$ T cell lines, which are suitable for tracking pathogenic CD4$^+$ T cells *in vivo*, particularly in the CNS (Ogura *et al*., 2008; Arima *et al*., 2012 and 2015b).

**Keywords:** Experimental autoimmune encephalomyelitis, Pathogenic CD4$^+$ T cells, Myelin oligodendrocyte glycoprotein, Gateway reflex, Passive transfer

**[Background]** It is widely accepted that autoreactive CD4$^+$ T cells play a significant role in the pathogenesis of MS and EAE (Reboldi, 2009; International Multiple Sclerosis Genetics *et al*., 2011; Steinman, 2014), which are chronic inflammatory diseases of the CNS. The CNS is protected by the blood-brain barrier (BBB), which limits immune cell infiltration from the periphery (Liu *et al*., 2012). Until recently, where and how CD4$^+$ T cells enter the CNS from the peripheral blood was unclear. Although EAE can be induced by immunization of animals with CNS-autoantigens emulsified in complete Freund's adjuvant (CFA) and pertussis toxin (PTx) (Andreasen *et al*., 2009; Lu *et al*., 2016), unwanted systemic inflammation occurs by injection of CFA and PTx, which potentially affects the integrity of the BBB (Schellenberg *et al*., 2012; Marbourg *et al*., 2017). Alternatively, we recommend a passive transfer EAE model, in which activated CD4$^+$ T cells specific for MOG are injected into naïve mice without treatments with CFA or PTx. Using this passive transfer EAE model, we have identified the dorsal vessels of the fifth lumbar (L5) cord as an initial gateway for autoreactive CD4$^+$ T cells to reach the CNS (Arima *et al*., 2012). Mechanistically, gravity-mediated constant activation of sensory neurons in the soleus muscles induces sympathetic nerve activation that connects to the L5 dorsal vessels. The resulting noradrenaline secretion at the vessels enhances NF-κB activity, leading to the production of chemokines that recruit the CNS autoreactive CD4$^+$ T cells (Arima *et al*., 2012). This sensory-sympathetic communication driven

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by anti-gravity responses through the soleus muscles is called 'gravity-gateway reflex' (Arima *et al*., 2012; Tracey, 2012; Sabharwal *et al*., 2014). In addition, this passive transfer EAE model enabled us to discover that other neural activators such as weak electric stimulation or pain sensation create unique gateways for immune cells at different sites (Arima *et al*., 2015a and 2015b). Here, we describe detailed protocols for the passive transfer EAE model using MOG-specific CD4$^+$ T cells, which are suitable for tracking autoreactive CD4$^+$ T cells *in vivo*. Although protocols for EAE have been reported (Racke, 2001), we particularly focus on the passive transfer EAE models and describe the methods in detail. The protocols here induce a transient EAE, in which after adoptive transfer, paralyzed tail (score 1) is expected to appear around 7 days, the clinical signs peak around 10-14 days with score 2 (uneven gait) to 2.5 (one paralyzed rear leg), and then the clinical symptoms will disappear around 20-25 days (Arima *et al*., 2015b). In this remission phase, the mice look healthy. However, activated monocytes remain in the spinal cords, and paralysis returns upon specific neural activation including pain sensation (Arima *et al*., 2015b).

## Materials and Reagents

1. Three-way connector (TERUMO Medical, catalog number: TS-TR1K)
2. 1 ml syringe (TERUMO Medical, catalog number: SS-01T)
3. Needle (25 G x 1) (TERUMO Medical, catalog number: NN-2525R)
4. Needle (27 G x ¾) (TERUMO Medical, catalog number: NN-2719S)
5. Cell strainer (100 µm) (Corning, Falcon®, catalog number: 352360)
6. 50 ml polypropylene conical tube (Corning, Falcon®, catalog number: 352070)
7. 2.5 ml syringe (TERUMO Medical, catalog number: SS-02SZ)
8. 10 cm dish (Corning, catalog number: 430167)
9. Needle (18 G x 1 ½) (TERUMO Medical, catalog number: NN-1838R)
10. 96-well U-bottom plate (Corning, catalog number: 3799)
11. Nylon wool
12. 20 ml syringe
13. MACS LS columns (Miltenyi Biotec, catalog number: 130-042-401)
14. C57BL/6 mouse (Japan SLC)
15. M. Tuberculosis H37 RA (BD, catalog number: 231141)
16. MOG peptide 35-55 (MEVGWYRSPFSRVVHLYRNGK) (Sigma-Aldrich), stock solution = 4 mg/ml
17. Incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, catalog number: F5506)
18. Isoflurane (Pfizer)
19. Pertussis toxin from *Bordetella pertussis* (PTx) (Sigma-Aldrich, catalog number: P7208-50UG)
20. CD4 (L3T4) Microbeads, mouse (Miltenyi Biotec, catalog number: 130-049-201)
21. Saline (Otsuka Pharmaceutical Factory, catalog number: 0815)
22. Mouse IL-1β (BioLegend, catalog number: 575102), stock solution = 10 µg/ml

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23. Mouse IL-23 (BioLegend, catalog number: 589002), stock solution = 10 μg/ml
24. Human IL-6 (Toray, order-made) stock solution = 100 μg/ml (Commercially available mouse IL-6 will work)
25. CellBanker (Takara Bio, Clontech, catalog number: CB021)
26. Ammonium chloride ($NH_4Cl$) (Sigma-Aldrich, catalog number: A4514)
27. DDW
28. EDTA-2Na
29. Fetal bovine serum (FBS) (GE Healthcare, HyClone™, catalog number: SH30910.03)
30. RPMI medium 1640 basic (1x) (Thermo Fisher Scientific, Gibco™, catalog number: C11875500BT)
31. Penicillin/streptomycin (Sigma-Aldrich, catalog number: P4333-100ML)
32. 2-mercaptoethanol (NACALAI TESQUE, catalog number: 21417)
33. Iscove's modified Dulbecco's medium (Sigma-Aldrich, catalog number: I3390-500ML)
34. GlutaMAX-1 (100x) (Thermo Fisher Scientific, Gibco™, catalog number: 35050061)
35. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9625-5KG)
36. Potassium chloride (KCl) (Wako Pure Chemical Industries, catalog number: 163-03545)
37. Sodium hydrogen phosphate ($Na_2HPO_4$) (Wako Pure Chemical Industries, catalog number: 197-02865)
38. Potassium dihydrogen phosphate ($KH_2PO_4$) (Wako Pure Chemical Industries, catalog number: 169-04245)
39. Red blood cell (RBC) lysis buffer (see Recipes)
40. Phosphate buffered saline (PBS) (see Recipes)
41. MACS buffer (see Recipes)
42. RP10 medium (see Recipes)
43. Nylon wool column (see Recipes)
44. IM20 medium (see Recipes)

## Equipment

1. X-ray irradiator (Hitachi, model: MBR-1520R) or equivalent
2. Scissors (BONIMED, catalog number: 669-060-72)
3. Micro-dissecting scissors (Karl Hammacher, catalog number: HSB 014-11)
4. Angled serrated tip forceps (Karl Hammacher, catalog number: HSC 187-11)
5. Pipet-aid (Corning, Falcon®, catalog number: 357471)
6. Glass syringe (Tsubasa Industry, 5 ml, lock type), autoclaved
7. Centrifuge (Hitachi, model: CF7D2)
8. Cell culture incubator, 37 °C, 5% $CO_2$ (Panasonic Healthcare, model: MCO-175)
9. Multichannel pipette
10. Water bath

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11. Autoclave

**Part I. Passive transfer method with MOG-specific CD4$^+$ T cells isolated from MOG immunized mice**

**Procedure**

A. MOG immunization in mice (Figure 1)

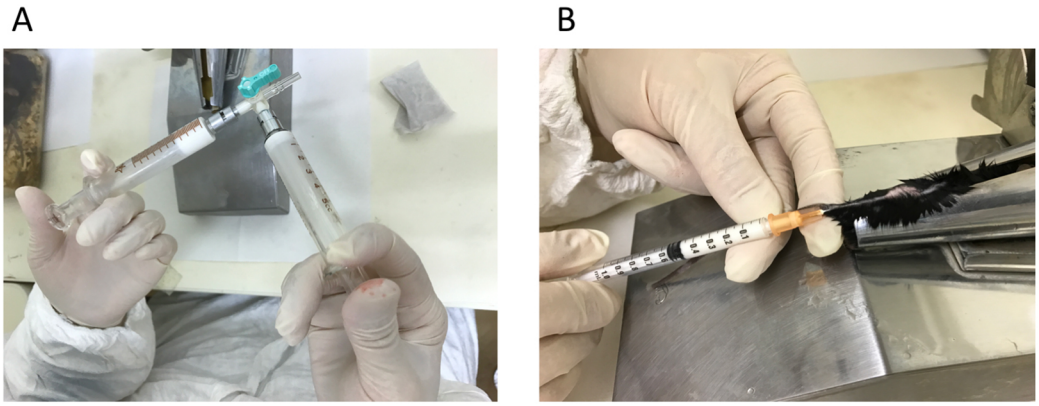


**Figure 1. Making emulsion and immunization in C57BL/6 mice.** A. MOG peptide (35-55) and CFA mixed at 1:1 and emulsified; B. Tail base immunization with 200 µg/100 µl emulsion.

1. Take sufficient volume (2 ml for 30 mice) of 4 mg/ml MOG peptide (35-55) in one glass syringe, take the same volume of CFA in the other glass syringe, and connect two syringes with a two- or three-way connector. Then, emulsify the two solutions by pushing plungers of the two syringes until the MOG/CFA solution becomes white, uniform emulsion (about 50 strokes).
   *Note: CFA is prepared by mixing 10 ml IFA and 2 vials of 10 mg M. Tuberculosis H37 RA.*
2. Move all the emulsion to one-side of the glass syringe, unlock the other empty glass syringe, and attach a new disposable 1-ml syringe to the open side of the connector.
3. Load the emulsion to the 1-ml syringe, and put a 25 G x 1 needle for immunization.
4. Anesthetize a mouse with isoflurane. It is easier for injections if the body of the mouse is fixed in a restrainer (optional). Intravenously administer (i.v.) 200 ng/200 µl PTx to the tail vein of C57BL/6 mice (6-8 weeks old) with 27 G x ¾ needle, followed by immunization by subcutaneous administration (s.c.) of 200 µg/100 µl MOG peptide emulsified in CFA at tail base on day 0. Perform the PTx injection and MOG immunization on the same day.
5. Inject i.v. 200 ng/200 µl PTx on days 2 and 7.

B. Separation of CD4$^+$ T cells from MOG immunized mice by using CD4 Microbeads.
   1. Collect spleens from MOG immunized mice (30 mice) on day 9 or 10.
   2. Homogenize the spleens on a cell strainer attached to a 50-ml tube using a plunger from a 2.5

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ml syringe (7-8 spleens/tube, total four strainers and four 50-ml tubes are used for 30 mice). Add plain RPMI medium up to 50 ml during homogenization.

3. Centrifuge the tubes (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
4. Resuspend the cell pellet in 10 ml/tube (4 tubes if 30 mice are used) of RBC lysis buffer (see Recipe 1) and incubate on ice for about 1 min.
5. Add plain RPMI medium up to 50 ml/tube.
6. Centrifuge the tubes (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
7. Resuspend the pellet in 1 ml/tube of MACS buffer (see Recipe 2).
8. Add 100 µl/tube of CD4 MACS beads and incubate on ice for 30 min.
9. Add plain RPMI medium up to 50 ml/tube.
10. Centrifuge the tubes (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
11. Resuspend the pellet in 5 ml/tube MACS buffer (4 tubes if 30 mice are used). Use the same number of MACS LS columns as that of 50-ml tubes used. Apply the cell suspension to MACS LS columns (5 ml/column). Refer to the manufacturer guide for the use of MACS LS columns.
12. Wash the column with 3 ml/column of MACS buffer twice.
    *Note: Do not discard the flow-through cells, which are used as antigen-presenting cells.*
13. Elute the CD4 positive selected cells with 5 ml/column MACS buffer and pool the eluted fractions to a 50 ml tube.
14. Centrifuge the tubes (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
15. Resuspend the CD4$^+$ T cells in 20 ml RP10 medium (see Recipe 3) and adjust to 8 x $10^6$ cells/ml.

C. Irradiation of splenocytes

1. Collect the flow-through cells obtained in the STEP 12 of the previous section.
2. Centrifuge the flow-through cells depleted of CD4$^+$ T cells (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
3. Resuspend the cells in 10 ml of RP10 medium.
4. Irradiate the cells at 35 Gy, centrifuge (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
5. Resuspend the cells in 20 ml of RP10 medium and adjust to 2 x $10^7$ cells/ml.

D. *In vitro* restimulation and adoptive transfer

1. Co-culture 4 x $10^7$ cells CD4$^+$ T cells and 1 x $10^8$ irradiated splenocytes in 10 ml RP10 medium in a 10-cm dish containing 2 ng/ml IL-23 and 25 µg/ml MOG peptide for 2 days.
2. Add 10 ml RP10 medium in each dish on day 1.
3. Collect the cells by pipetting up and down on day 2, centrifuge (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
4. Resuspend the cells in 10 ml RP10 medium pre-warmed at 37 °C and add the suspension to a nylon wool column (see Recipe 4) (Video 1).
   *Note: Put an 18 G x 1 ½ inches needle to a nylon wool column, and stand the column in a 50 ml tube without a cap. To equilibrate the column, add 20 ml RP10 medium pre-warmed at 37 °C*

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*until the medium spreads uniformly in nylon wool. Incubate the nylon wool column with the co-cultured cells in a $CO_2$ incubator (37 °C, 20 min). After 20 min, elute the cells with 30 ml RP10 medium.*

**Video 1. Making a nylon wool column.** 20 ml of RP10 medium are added to nylon wool and stirred until the medium uniformly spreads in the wool.


5. Centrifuge the eluted cells (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
6. Resuspend the pellets in 1 ml MACS buffer.
7. Add 100 µl/tube of CD4 MACS beads and incubate on ice for 30 min.
8. Add plain RPMI medium up to 50 ml/tube.
9. Centrifuge the tubes (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
10. Resuspend the pellet in 10 ml/tube MACS buffer and apply the suspensions to two MACS columns (5 ml/column).
11. Wash with 3 ml/column MACS buffer twice.
12. Elute the CD4 positive selected cells with 5 ml/column MACS buffer and pool the eluted fractions to a 50 ml tube.
13. Centrifuge the tube (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
14. Resuspend the eluted CD4$^+$ T cells in sterile saline.
    *Note: Resuspended volume should not exceed the maximal volume of i.v. injection allowed in the ethics guideline of your institute.*
15. Inject 1.5 x $10^7$ cells/mouse, i.v.
    *Note: Usually, 6-8 mice can be injected using 30 spleens.*
16. Measure clinical scores as described previously (Ogura *et al*., 2008; Huseby *et al*., 2001; Arima *et al*., 2012 and 2015b).

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**Part II. Passive transfer model with MOG-specific T cells lines**

Passive transfer EAE can also be induced using a MOG-specific T cell line generated by periodical antigen stimulations *in vitro*. Once the T cell line is established, this method is useful to reduce the number of mice used to induce the transfer EAE.

## Procedure

A. MOG immunization
   1. Perform MOG immunization in the same way as Part I. A. MOG immunization in mice, except for the number of mice to be immunized. Usually, 3-4 mice are sufficient to obtain MOG-specific T cells lines.

B. Culture and passage of T cell lines
   1. Collect inguinal lymph nodes (iLNs) from MOG immunized mice (3-4 mice) on day 9 or 10 (Figure 2).

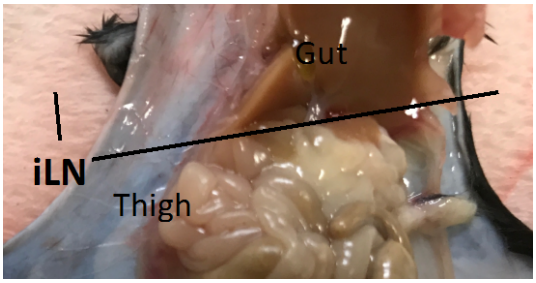


**Figure 2. The location of the inguinal lymph nodes (iLN).** iLN of the immunized C57BL/6 mouse.

   2. Homogenize the iLNs on a cell strainer in a 50 ml tube using the plunger from a 2.5 ml syringe.
   3. Centrifuge the tubes (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
   4. Resuspend the cell pellet in 10 ml/tube RBC lysis buffer (see Recipe 1) and incubate on ice for 1 min.
   5. Add plain RPMI medium up to 50 ml/tube.
   6. Centrifuge the tubes (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
   7. Resuspend the cells in 2 ml IM20 medium (see Recipe 5).
   8. Filter the cells using a cell strainer.
   9. Count the cells.
   10. Seed 2.5 x $10^5$ cells/well/200 µl iLN cells in the presence of 4 µg/ml MOG peptide, 0.5 ng/ml IL-1β, 5 ng/ml IL-6 and 0.5 ng/ml IL-23 in 96-well U-bottom plates for 10 to 14 days.
   11. Proceed to step C1.

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C. T cell preparation

1. Collect CD4$^+$ T-cell-rich iLN cells from 96U plates in a 10-cm dish by gently pipetting 2 to 3 times using a multichannel pipette.
2. Collect the cells into 50 ml tubes and centrifuge (600 *x g*, 5 min, 4 °C).
3. Aspirate the supernatant and resuspend the T cells in 5 ml IM20 medium.
4. Count the cells and adjust the concentration to 2.5 x $10^5$ cells/ml.

D. Irradiation of splenocytes

1. Collect the spleens of naive C57BL/6 mice and homogenize using the plunger from a 2.5 ml syringe.
2. Add plain RPMI medium up to 50 ml/tube and centrifuge (600 *x g*, 5 min, 4 °C).
3. Aspirate the supernatant and add 1 ml/spleen RBC lysis buffer.
4. Add plain RPMI medium up to 50 ml and centrifuge (600 *x g*, 5 min, 4 °C).
5. Irradiate the cells at 35 Gy, centrifuge (600 *x g*, 5 min, 4 °C) and aspirate the supernatant.
6. Suspend the irradiated splenocytes in 10 ml IM20 medium, count and adjust the concentration to 2.5 x $10^6$ cells/ml.

E. *In vitro* stimulation

1. Mix 2.5 x $10^5$ cells/ml CD4$^+$ T-cell-rich iLN cells and 2.5 x $10^6$ cells/ml irradiated splenocytes at 1:1.
2. Seed the cells at 200 µl/well containing 4 µg/ml MOG peptide, 0.5 ng/ml IL-1β, 5 ng/ml IL-6 and 0.5 ng/ml IL-23 in 96-well U-bottom plates.
3. Incubate the co-cultured cells at 37 °C, 5% $CO_2$ for 10-14 days.
4. Repeat steps C1 to E3 to enrich the percentage of MOG-specific CD4$^+$ T cells from total iLN cells (Figure 3). Use fresh irradiated splenocytes in each repeating cycle.

   *Notes:*

   a. *T cells can be frozen after step E3 in CellBanker or equivalent solution at -80 °C. When thawing, use a 37 °C water bath, and wash the cells in plain RPMI medium. Then, the T cell line can be used for culture (start from step E1).*
   b. *Typically, more than 95% of live cells will be CD4$^+$ T cells after four rounds of in vitro stimulation (Figure 3).*

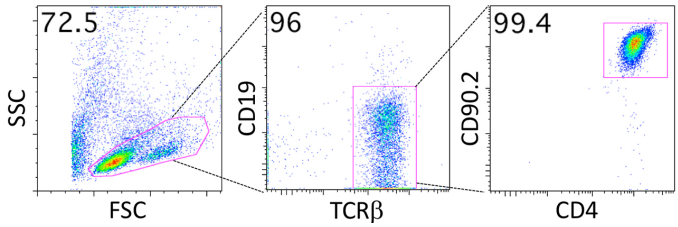


**Figure 3. Representative FACS plots of a T cell line.** Most living cells (> 95%) are CD4$^+$ T cells (CD19$^-$ T cell receptor (TCR)β$^+$CD90.2$^+$CD4$^+$ T cells) after 5 days of the fourth round of *in*

*vitro* stimulation (Procedure E). Irradiated splenocytes can be seen as forward scatter (FSC)$^{low}$, side scatter (SSC)$^{low}$ dead cells.

F. Adoptive transfer of T cell lines
   1. Set up culture the same way as 'E. *In vitro* stimulation'.
      *Note: Do not forget to add 4 μg/ml MOG peptide, 0.5 ng/ml IL-1β, 5 ng/ml IL-6 and 0.5 ng/ml IL-23.*
   2. Seed these cells at 200 μl/well in 96-well U-bottom plates (about 5 plates for 1 mouse).
   3. Incubate the co-cultured cells in 37 °C, 5% $CO_2$ for 2 days.
   4. Collect the cells from the 96-well U-bottom plates in a 10-cm dish by gently pipetting 2 to 3 times with a multichannel pipette.
   5. Collect the cells in 50 ml tubes and centrifuge (600 *x g*, 5 min, 4 °C).
   6. Aspirate the medium and resuspend the cells in 1 ml plain RPMI medium.
   7. Count the cells and adjust the concentration to 2.5-3.75 x $10^7$/ml.
      *Note: Count only living cells.*
   8. Inject 1-1.5 x $10^7$ cells i.v. into C57BL/6 mice.
   9. Measure clinical scores as described previously (Huseby *et al*., 2001; Ogura *et al*., 2008; Arima *et al*., 2012 and 2015b).

## Data analysis

The clinical symptoms of EAE are evaluated as follows: grade 1, paralyzed tail; grade 2, uneven gait; grade 2.5, one paralyzed rear leg; grade 3, rear limb paralysis; grade 4, paralyzed front and rear legs; and grade 5, moribund (Huseby *et al*., 2001; Ogura *et al*., 2008; Arima *et al*., 2012 and 2015b).

## Recipes

1. Red blood cell (RBC) lysis buffer (500 ml)
   4.41 g $NH_4Cl$
   500 ml DDW, then autoclave
2. Phosphate buffered saline (PBS)
   1.46 g NaCl
   1.86 g KCl
   3.5 g $Na_2HPO_4$
   3.4 g $KH_2PO_4$
3. MACS buffer (1,000 ml)
   950 ml PBS
   1.86 g EDTA-2Na

Add 50 ml heat-inactivated FBS after autoclaving

4. RP10 medium (500 ml)
   500 ml RPMI medium 1640 basic
   50 ml heat-inactivated FBS
   5 ml 100x penicillin/streptomycin
   1.86 µl 2-mercaptoethanol
5. Nylon wool column
   Nylon wool (1.2 g) is unraveled with a brush, put into a 20 ml syringe, and sterilized by autoclave
6. IM20 medium (600 ml)
   500 ml Iscove's modified Dulbecco's medium
   100 ml FBS
   5 ml GlutaMAX-1 (100x)
   1.8 µl 2-mercaptoethanol
   5 ml 100x penicillin/streptomycin

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