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Identification of functionally-relevant microRNAs in the regulation of allergic inflammation

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Abstract

Transgenic methods to manipulate CD4 T lymphocytes *in vivo* via forced expression of TCR transgenes and targeted “knock-out” of individual genes by Cre-lox technology are fundamental to modern immunology. However, efforts to scale up functional analysis by modifying expression of larger numbers of genes in T cells *ex vivo* has proven surprisingly difficult. Early RNA interference experiments achieved successful small RNA transfection by using very high concentrations of short-interfering RNA (siRNA) [1], but primary T cells are generally resistant to standard electroporation, cationic liposome-, and calcium phosphate-mediated transfection methods. Moreover, although viral vectors can successfully introduce DNA fragments of varying length, expression of these constructs in primary T cells is low-efficiency and the subcloning process laborious. In this context, the relatively recent discovery of dozens of highly-expressed microRNAs (miRNAs) in the immune system provides both an opportunity and a new challenge [2, 3]. How can we query the miRNAome of a cell to assign particular roles to individual miRNAs? Here we describe an optimized technique for efficient and reproducible transfection of primary mouse CD4 T cells *in vitro* with synthetic miRNA mimics.

Keywords

microRNA; T lymphocyte transfection; Th2; CD4; electroporation; *in vitro* gene expression

1. Introduction

Next generation transfections systems, including the Neon™ Transfection System (ThermoFischer Scientific) and the Amaxa® Nucleofector® (Lonza) are capable of transfecting both cell lines and primary cells with high efficiency (25–99%) and high cell

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viability (60–99%) [4–8]. We have found the Neon system easy to use, economical, and capable of reliably delivering small RNAs into primary mouse T cells to study miRNA function [6, 7, 9, 10]. This technique can be used to test the effect of miRNA overexpression using miRNA mimics without the need to clone into expression or targeting vectors. Electroporation with the Neon Transfection System is amenable to testing a small set of miRNA mimics, and may also be utilized for medium-throughput screens which test the function of >100 distinct miRNAs. After electroporation, CD4 T cells remain capable of proliferation, polarization, and cytokine production, allowing assessment of numerous cellular functions. Molecular consequences of altering miRNA activity in cells can be evaluated through expression studies of predicted, known and novel mRNA targets through techniques including qPCR and RNA sequencing. In addition, Neon electroporation can be used to query the combined effects of multiple miRNAs (such as members of a miRNA family) by co-transfection [10]. This creates an opportunity to observe the potential effects of multiple miRNAs on a convergent mRNA target or pathway.

The transient nature of miRNA mimic transfections *in vitro* provide specific advantages over stable genetic models:

1) Transfection allows temporal control in the manipulation of individual miRNA expression during T cell activation, proliferation, and differentiation.

2) Transfection facilitates rapid functional assessment of multiple miRNAs individually and in combination with one another. This is a particular challenge with genetic models because related miRNAs are often encoded in polycistrons or distributed across multiple genomic loci.

3) Transfection prevents confounding cellular changes that may be seen in stable genetic models as a result of changes in T cell differentiation or adaptations to long-term alterations in gene expression.

This chapter outlines our standard protocol, which begins on day 0 with isolation, activation and Th2 polarization of primary mouse CD4 T cells. Activated Th2-polarized cell cultures are transfected with miRNA mimics on day 1 and again on day 4 to boost miRNA expression. We include optimized instructions for operating the Neon Transfection System with primary mouse CD4 T cells. On day 5 we harvest transfected T cells for analysis by various methods, such as target gene expression and flow cytometry for cellular phenotyping.

2. Materials

2.1 Equipment (see Note 1)

1. Neon™ transfection system (ThermoFisher)

2. Neon™ transfection pipette

3. Neon™ electroporation tubes (a.k.a. cuvettes)

1. Transfection reagents are stored at 4 °C but must be warmed to room temperature (RT) before use.

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4. Neon™ transfection system 10 μl kit (tips)
5. 10-cm TC-treated sterile plates
6. 96-well flat bottom TC-treated sterile plates
7. 96-well round bottom TC-treated sterile plates

2.2 Cytokines and Antibodies (Table I; see Note 2)

1. Neutravidin (ThermoFisher; stock 10 mg/mL in PBS)
2. Biotinylated anti-CD3 antibody (clone 2C11; stock 2.8 mg/mL in PBS)
3. Biotinylated anti-CD28 antibody (clone 37.51; stock 2.0 mg/mL in PBS)
4. IL-4 supernatant (10,000 U/mL stock) added as a supernatant from I3L6 cells [11]
5. Anti-IFNγ antibody (3.5 mg/mL stock in PBS)
6. Recombinant human IL-2 (100,000 U/mL in PBS)

2.3 Solutions and reagents

1. “Kool-Aid” media (for 1L): 4.5g glucose, 0.584 g L-glutamine, 3.7 g $NaHCO_3$, 0.116 g L-arginine HCl, 0.036 g L-asparagine, 0.006 g folic acid, 10 mL of 100x NEAA solution, 10 mL of 100x MEM essential vitamin solution
2. Kool-Aid COMPLETE media: Kool-aid media supplemented with 10% FBS, 100 μg/mL Streptomycin, 100 U/mL Penicillin G, 10 mM HEPES, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, and 2 mM L-glutamine
3. siRNA buffer: (5x stock)
4. E buffer: (included in Neon™ transfection 10 μl kit)
5. T buffer: (included in Neon™ transfection 10 μl kit)
6. Dynabuffer 1x PBS supplemented with 2% fetal bovine serum (FBS)
7. Dynabeads™ Mouse CD4 kit (ThermoFisher)
8. DETACHaBEAD® (ThermoFisher)
9. miRIDIAN miRNA mimics and chemistry matched control miRNA mimic (Dharmacon)

[2]. All antibody and cytokine stock solutions are prepared in 1x PBS and stored at 4 °C for up to one month, or −80 °C for long term storage.

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3. Methods

3.1 Isolation of mouse CD4 T cells (day 0)

Primary mouse CD4 T cells may be isolated from peripheral lymph nodes and spleen by standard laboratory protocols. We favor the Dynabead Mouse CD4 kit, following the manufacturer's instructions with slight modification.

1. Havest spleen and peripheral lymph nodes to obtain single cell suspensions (see Note 3).
2. Purify CD4 T cells by positive selection using the Dynabeads™ Mouse CD4 kit using anti-CD4 mAb (clone L3T4)-coated magnetic beads. We use 75 μl of Dynabeads per mouse and elute with an equal volume of the proprietary DETACHAaBEAD reagent.
3. Once cells are attached to beads, all washes should be performed to minimize agitation (see Note 4).

3.2 *In vitro* T cell activation and Th2 polarization (day 0)

1. Prepare tissue culture plates. Make neutravidin working solution by 2000x dilution of 10 mg/mL neutravidin stock in PBS (final 5 μg/mL). Plate 10 mL neutravidin solution onto 10-cm plates and incubate for 2 hours at RT or 1 hour at 37 °C, then gently wash 2x with PBS. Keep neutravidin-coated plates warm at 37 °C in PBS briefly while preparing T cell media (3.2 step 2).
2. Prepare one half the final culture volume of T cell polarizing media. For example, for each 10 cm plate prepare 5 mL of Kool-Aid-COMPLETE media supplemented with T cell polarizing cytokines and blocking antibodies at 2x the desired final concentration. For Th2 culture conditions, supplemented 2x media contains biotinylated anti-CD3 (2x is 0.5 μg/mL), biotinylated anti-CD28 (2x is 2 μg/mL), IL-4 supernatant (2x is 1,000U/mL), and anti-IFNγ antibody (2x is 20 μg/mL). See Table I for working stock and final concentrations of all antibodies and cytokines.
3. Prepare neutravidin-coated plates with T cell media by aspirating PBS and carefully add 2x supplemented Kool-Aid COMPLETE media at half-final volume making sure to fully cover the plate surface. In a 10 cm plate, add 5 mL of 2x cytokine-supplemented media and return plate to 37 °C incubator.
4. Resuspend purified CD4 T cells (from Step 3.1) in Kool-Aid-COMPLETE media (without cytokines or antibodies) at 2×10^6 per mL. Plate cells onto prepared plates such that the cell volume added is equal to the receiving 2x cytokine media, i.e. 5 mL cells in the 10 cm plate. Final cell concentrations will be 1×10^6 per mL. If using other plate sizes, see Table II for appropriate cell numbers and volumes.

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3. All cell culture is performed in a 37 °C incubator with 10% CO_2.
4. Isolation from spleen and peripheral lymph nodes yields approximately $10–20\times10^6$ cells per 6 week old C57BL/6 mouse, purity >90% CD4 T cells.

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5. Activate cells overnight in a 37 °C incubator with 10% CO_2 (see Note 5, 6).

3.3 Preparation for first miRNA mimic transfection (day 1)

1. Prepare a 96-well flat bottom post-transfection culture plate ("receiving plate") by coating with 100 μl/well of neutravidin solution for 2h at RT or 1h at 37 °C in PBS. Wash the neutravidin-coated receiving plate twice with PBS, then keep plate warm at 37 °C in PBS while preparing media (3.3 step 2).
2. Prepare full final culture volume of T cell polarizing media. For 96-well plates, 200 μl/per well of Kool-Aid-COMPLETE media is supplemented with 1x polarizing cytokines and antibodies and will contain biotinylated anti-CD3 (1x is 0.25 μg/mL) and biotinylated anti-CD28 (1x is 1.0 μg/mL), IL-4 supernatant (1x is 500 U/mL), and anti-IFNγ antibody (1x is 10 μg/mL) for Th2 culture conditions. Remove PBS from neutravidin-coated plate and add 200ul per well of this 1x media.
3. Label the receiving plate (*see* Note 7), then place in incubator to equilibrate.
4. Turn on Neon instrument and choose appropriate electroporation settings. For day 1 T cell transfection: 1550 volts, 10 milliseconds, 3 pulses.
5. Add 3 mL of Buffer E into the cuvette's transfection chamber and allow to equilibrate to RT. Carefully transfer the cuvette into Neon pipette stand without spilling any buffer, *see* Note 8.
6. Deposit miRNA mimics in individual Eppendorf tubes for transfection. We typically use 500 nM mimic solution in 0.5–1 μl of siRNA transfection buffer. If combining multiple miRNAs, we use a total mimic concentration of 500 nM (i.e. two miRNA mimics at 250 nM each). Set aside on ice. *See* Note 9.

3.4 Transfecting Th2-polarized T cells with miRNA mimics (day 1)

1. Harvest polarized Th2 cells into 15 mL conical tubes (*see* Note 10), take an aliquot for cell counting, and centrifuge at 400 g-force, 5 min at RT. (Count cells during the spin.) Aspirate supernatant and resuspend cells in 1.5 mL PBS. Transfer the needed number of each group of cells to an Eppendorf tube, then centrifuge at 500g for 5 minutes. Aspirate supernatant, carefully removing as much as possible without disturbing pellet.

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5. This transfection method does not work on naïve unmanipulated mouse T cells. It is necessary to activate the cells for at least 12 hours prior to transfection.
6. If the cell-bead aggregate is fully disrupted during washing, there will be excessive cell loss.
7. When planning and labeling your receiving plate, be sure to account for the number of miRNA mimics multiplied by the number of target cell populations (WT, KO, etc.). Include additional wells for control mimic in each target cell population. Transfections may be carried out in technical singlets for large screens, or in duplicate or triplicate to account for technical variation.
8. If you get an error message during transfection, one possible cause is a bad contact between the metal plate on the cuvette and the pipette stand. This can occur because of bad cuvette positioning, or because of liquid between the contacts. If any liquid spills out of the cuvette, thoroughly dry it immediately. To minimize this risk, never pipette liquid into a cuvette already positioned in the stand.
9. When aliquoting miRNA mimics into Eppendorf tubes to prepare for transfection, you must have a separate tube for each transfection. Each combination of miRNA mimic and cells is considered one transfection.
10. Activated T cells, particularly Th2 cells, can be surprisingly adherent to anti-CD3 and anti-CD28 coated plates. Take care to vigorously and completely blow them off the bottom of the plate. We favor using a P1000 pipetman for this process.

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2. Resuspend cell pellet in 11 μl of T buffer per transfection of 400,000 cells. For example, 10×10^6 cells should be resuspended in 275 μl (*see* Note 11). Take care not to generate any bubbles when resuspending cells (*see* Note 12).

3. Remove miRNA mimic aliquots from ice, place at RT and equilibrate for 5 min. Pipette 11 μl of cells into each Eppendorf tube pre-loaded with miRNA mimic. Pipette up and down to mix.

4. Load a Neon pipette tip onto the Neon pipette. Fill a 50 mL conical tube with sterile PBS.

5. Slowly pipette up and down 3 times in PBS to wet the tip, taking care to avoid generating bubbles. Carefully aspirate the 11 μl of cells mixed with miRNA mimic into the Neon pipette tip, again avoiding bubbles.

6. Click the Neon pipette with tip into the cuvette, then press “start” on the screen to electroporate. *See* Notes 13 and 14.

7. Immediately after successful electroporation, transfer transfected cells to the pre-warmed receiving plate pre-loaded with appropriate supplemented media.

8. Repeat steps 5–7 for each cell-miRNA transfection. Between transfections, wash the Neon pipette tip by pipetting up and down 3–5 times in 50 mL conical filled with PBS. The PBS does NOT need be changed between transfections. *See* Note 15.

9. At the end of the transfections, return plate to the incubator then aspirate Buffer E from the cuvette. If the cuvette has been used <50 times and will be saved, wash by filling the cuvette chamber with sufficient 70% ethanol to submerge the metal contact. Aspirate all of the liquid from the chamber (no droplets left) and leave to dry in a biosafety cabinet. Store in a sterile 50 mL conical tube. This will prevent tarnishing of the metal contact.

3.5 Rest activated Th2 cell cultures (day 3)

1. Collect cells by centrifuging the 96-well plate at 400 g-force, 5 minutes.

2. Carefully aspirate 150 μl of supernatant and resuspend plate-bound cells in 200 μl per well of resting media. For Th2-polarized cell cultures, day 3 resting media is Kool-Aid-COMPLETE media supplemented with IL-4 supernatant (1x is 500

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11. Before resuspending the cell pellet, calculate the total number of transfections you plan to perform and confirm that multiplying out 11 ul per 400,000 cells yields sufficient volume for that number of transfections (11 ul × # of transfections + 10% angel’s share). If you have fewer than 400,000 cells/transfection, increase the dilution.

12. If you catch a bubble in the pipette tip when drawing up cells, carefully expel the fluid and re-pipet. If you are unable to re-pipet successfully without bubbles a second time, expel the cells back into the Eppendorf tube, wash the tip with PBS 3–5 times, then try to pipet the cells again. If you still are getting bubbles, check the seal between the pipette tip and change the pipette tip if needed. A bubble within the pipette tip can cause the transfection to “arc” (often with a visible spark and popping sound) and kill the cells.

13. You will hear a click when the pipette is properly positioned within the cuvette station. Listen for this sound before pressing start, or remove and re-position the pipette.

14. The Neon instrument will beep twice if the transfection completes successfully. Wait for these beeps before transferring transfected cells to the receiving plate. See Notes 7 and 20 for additional troubleshooting tips if you do not hear the beeps.

15. In our experience, each Neon pipette tip can be re-used up to 14 times. We recommend changing tips between groups to avoid contamination.

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U/mL), anti-IFNγ antibody (1x is 10 μg/mL) and recombinant IL-2 (1x is 20 U/mL). *See* Note 16. Return cell culture plates to incubator.

3.6 Preparation for miRNA mimic re-transfection (day 4)

1. Prepare a fresh 96-well round bottom 'receiving plate' by plating 200 ul of appropriate media per well. For day 4 transfection, media contains IL-4 supernatant (500 U/mL), anti-IFN-γ antibody (10 μg/mL) and IL-2 (20 U/mL).
2. Label the plate and place in 37 °C incubator to equilibrate.
3. Place miRNA mimics in individual Eppendorf tubes and set aside on ice as before.
4. Turn on Neon instrument. Choose day 4 electroporation settings: 1500 volts, 10 milliseconds, 3 pulses. Prepare cuvette as above.

3.7 Re-Transfection with miRNA mimics (day 4)

1. Harvest each well of cells into an Eppendorf tube, keeping each well separate, and centrifuge at 500g, 5 min to pellet. Aspirate supernatant. Wash once in PBS followed by 500g, 5 minute centrifugation. Then carefully aspirate as much supernatant as possible without disturbing the cell pellet. Maintain cells at RT throughout this procedure.
2. Resuspend each cell pellet in 11 μl T buffer per transfection (*see* Note 17). Pipet up and down gently to mix.
3. Load a Neon pipette tip onto the Neon pipette. Fill a 50 mL conical tube with sterile PBS.
4. Slowly pipette up and down 3 times in PBS to wet the tip, taking care to avoid generating bubbles. Carefully aspirate the 11 μl of cells mixed with miRNA mimic into the Neon pipette tip, again avoiding bubbles.
5. Click the Neon pipette with tip into the cuvette, then press "start" on the screen to electroporate. *See* Notes 13 and 14.
6. Immediately after successful electroporation, transfer transfected cells to the pre-warmed receiving plate pre-loaded with appropriate supplemented media.
7. Repeat steps 4–6 for each cell-miRNA transfection. Between transfections, wash the Neon pipette tip by pipetting up and down 3–5 times in 50 mL conical filled with PBS. The PBS does NOT need be changed between transfections. *See* Note 15.
8. At the end of the transfections, return plate to the incubator (see Note 18, 19).

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16. By day 3, T cell activation is complete, and the T cells should be "rested" in IL-Therefore, no neutravidin coating step is done, and there is no anti-CD3 or anti-CD28 in the media.
17. We generally do not re-count cells before the day 4 transfection unless the wells look overpopulated. As a result of a low proliferation rate and increased cell death within the first day after initial transfection, the number of cells per well is usually within range. In our experience, successful transfections can be performed with as few as 50,000 cells and as many as 800,000 cells.

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3.8 Alternative applications

This chapter discusses the approach to CD4 T cell transfection with miRNA mimics, which our laboratory used to successfully identify functional roles for miR-29 in Th1 polarization [6] and miR-24, miR-27 and miR-19 in Th2 cell differentiation and cytokine production [9, 10]. Alternatively, T cells can be transfected with miRCURY LNA™ miRNA Power Inhibitors (Exiqon) to block endogenous miRNA function. The Neon may also be used to introduce synthetic siRNAs into mouse and human CD4 T cells [7, 10]. In addition, CD4 T cells may be transfected with Cas9 protein pre-loaded with CRISPR guide RNAs as a method to target gene expression in human CD4 T cells [12]. Larger CRISPR/Cas9 screens successfully identified host CD4 T cell factors influencing HIV infection [13, 14]. The latter experiments employed the AMAXA Nucleofector, which uses capillary-loop electroporation technology similar to Neon in a high-throughput 96-well format.

References

1. McManus MT, Haines BB, Dillon CP, et al. (2002) Small interfering RNA-mediated gene silencing in T lymphocytes. J Immunol 169(10):5754–5760. [PubMed: 12421955]
2. O'Connell RM, Rao DS, Baltimore D (2012) microRNA regulation of inflammatory responses. Annu Rev Immunol 30:295–31doi: 10.1146/annurev-immunol-020711-075013 [PubMed: 22224773]
3. Baumjohann D, Ansel KM (2013) MicroRNA-mediated regulation of T helper cell differentiation and plasticity. Nat Rev Immunol 13(9):666–67doi: 10.1038/nri3494 [PubMed: 23907446]
4. Chicaybam L, Sodre AL, Curzio BA, Bonamino MH (2013) An efficient low cost method for gene transfer to T lymphocytes. PLoS ONE 8(3):e6029doi: 10.1371/journal.pone.0060298
5. Chebel A, Rouault J-P, Urbanowicz I, et al. (2009) Transcriptional activation of hTERT, the human telomerase reverse transcriptase, by nuclear factor of activated T cells. J Biol Chem 284(51):35725–3573doi: 10.1074/jbc.M109.009183 [PubMed: 19843528]
6. Steiner DF, Thomas MF, Hu JK, et al. (2011) MicroRNA-29 Regulates T-Box Transcription Factors and Interferon-γ Production in Helper T Cells. Immunity 35(2):169–18doi:10.1016/j.immuni.2011.07.009 [PubMed: 21820330]
7. Montoya MM, Ansel KM (2017) Small RNA Transfection in Primary Human Th17 Cells by Next Generation Electroporation. J Vis Exp. doi: 10.3791/55546
8. Kim JA, Cho K, Shin MS, et al. (2008) A novel electroporation method using a capillary and wire-type electrode. Biosens Bioelectron 23(9):1353–1360. doi: 10.1016/j.bios.2007.12.009 [PubMed: 18242073]
9. Simpson LJ, Patel S, Bhakta NR, et al. (2014) A microRNA upregulated in asthma airway T cells promotes TH2 cytokine production. Nat Immunol 15(12):1162–1170. doi: 10.1038/ni.3026 [PubMed: 25362490]
10. Pua HH, Steiner DF, Patel S, et al. (2016) MicroRNAs 24 and 27 Suppress Allergic Inflammation and Target a Network of Regulators of T Helper 2 Cell-Associated Cytokine Production. Immunity 44(4):821–83doi: 10.1016/j.immuni.2016.01.003 [PubMed: 26850657]

[18]Effective miRNA overexpression in cells capable of endogenous miRNA expression may not be possible with the protocol outlined here. Limited availability of Argonaute due to high occupancy by endogenous miRNAs and/or previous saturation of the mRNA targets of highly expressed miRNAs may hamper transfected miRNA mimic activity in a cell. Studies using this transfection protocol can be enhanced by starting with miRNA-deficient T cells (e.g. from *Dgcr8*-deficient animal strains) to study the gain of functional miRNA activity [6, 15]. Complementary miRNA inhibitor transfections can also be used to test loss of function [10].

[19]If performing a rescue screen in miRNA-deficient T cells from *Dgcr8*-deficient strains, as was pioneered in *Dgcr8*-deficient ES cells [16], be aware that CD4 T cells will include both miRNA-deficient cells and "escapees" that fail to delete *Dgcr8* and therefore remain miRNA-sufficient. For best results, include a method to identify true knockout cells (e.g. a Cre-activated reporter gene) to mark the miRNA-deficient cell population prior to assessing the phenotype "rescued" by transfection with individual miRNAs [6].

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11. Tepper RI, Pattengale PK, Leder P (1989) Murine interleukin-4 displays potent anti-tumor activity in vivo. Cell 57(3):503–512. [PubMed: 2785856]
12. Schumann K, Lin S, Boyer E, et al. (2015) Generation of knock-in primary human T cells using Cas9 ribonucleoproteins. Proc Natl Acad Sci USA 112(33):10437–1044doi:10.1073/pnas.1512503112 [PubMed: 26216948]
13. Hultquist JF, Schumann K, Woo JM, et al. (2016) A Cas9 Ribonucleoprotein Platform for Functional Genetic Studies of HIV-Host Interactions in Primary Human T Cells. Cell Rep 17(5):1438–145doi: 10.1016/j.celrep.2016.09.080 [PubMed: 27783955]
14. Park RJ, Wang T, Koundakjian D, et al. (2017) A genome-wide CRISPR screen identifies a restricted set of HIV host dependency factors. Nat Genet 49(2):193–20doi: 10.1038/ng.3741 [PubMed: 27992415]
15. Wang Y, Medvid R, Melton C, et al. (2007) DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. Nat Genet 39(3):380–38doi: 10.1038/ng1969 [PubMed: 17259983]
16. Wang Y, Baskerville S, Shenoy A, et al. (2008) Embryonic stem cell-specific microRNAs regulate the G1-S transition and promote rapid proliferation. Nat Genet 40(12):1478–148doi:10.1038/ng.250 [PubMed: 18978791]

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Table I.

Cytokines and antibodies

Reagent	Stock Concentration	1× Final Dilution Factor	1× Final Concentration
Biotinylated anti-CD3	2.8mg/mL	11,200	0.25ug/mL
Biotinylated anti-CD28	2mg/mL	2,000	1ug/mL
mouse IL-4	10,000U/mL	20	500U/mL
anti-IFNγ mAb	3.5mg/mL	350	10ug/mL
recombinant human IL-2	100,000U/mL	5000	20U/mL

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Table II.

Cell numbers, media volume and tissue culture plate size for starting cultures

Cell number	Media Volume	TC plate size
1.5×10^5	200uL	96 well
3×10^5	450uL	48 well
5×10^5	750uL	24 well
1×10^6	1.5mL	12 well
2×10^6	3mL	6 well
$6-10\times10^6$	10mL	10cm plate