Methodology: Development of Prakriti-specific iPSC models from LCLs

Lymphoblastoid Cell Lines (LCLs) to Induced Pluripotent Stem Cells (iPSCs) reprogramming

LCL to iPSC reprogramming is widely used these days for the generation of cellular models for rare genetic disorders. LCLs, as we know, can be developed from peripheral blood using Epstein-Barr Virus transfection. Culturing and maintaining LCLs are comparatively easier than other cell types. These are suspension cell lines with an average doubling time of 24 hours. Several methods are available for LCL to iPSC reprogramming, from commercially available Sendai virus to episomal plasmid-based methods. During initial experiments, we have tested both methods for LCL to iPSC development. In the Sendai virus-based method, we could not achieve success in reprogramming. But in the episomal plasmid-based method, after a few attempts, we were able to see the reprogramming. The episomal plasmid-based method is non-integrative to the host genome and an economically viable option, as we have to reprogram multiple lines into iPSCs. So, we have used the episomal plasmid-based method for our LCL to iPSC development.

LCL culture

Individual-specific Lymphoblastoid Cell Lines (LCLs) derived from extreme Prakriti healthy individuals were utilized for iPSC cellular model development. All eight LCL lines have been used for reprogramming. Before utilizing these LCLs in iPSC development, their cellular morphology was confirmed, and they were screened for any mycoplasmal or bacterial contaminations. LCLs were cultured using RPMI complete media comprising RPMI media with 15% Fetal Bovine Serum (FBS) and 1X Antibiotics (Anti-Anti, Thermo). Before utilizing these LCLs in iPSC development, their cellular morphology was confirmed, and they were screened for any mycoplasmal or bacterial contamination during culturing to ensure their cellular health and growth.

Episomal Plasmids

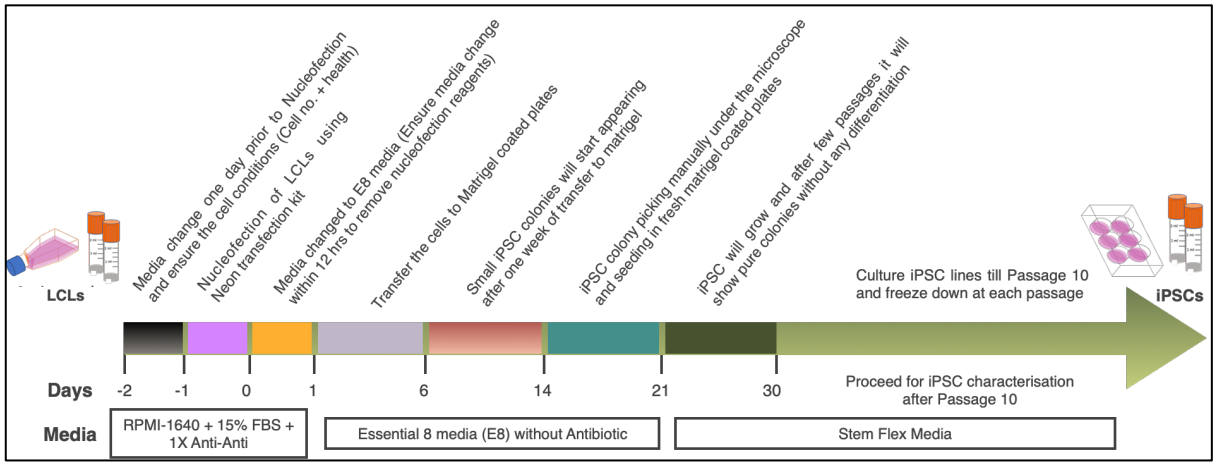
Three episomal plasmids, pCXLE OCT3/4, pCXLE hSK, and pCXLE hUL (Addgene: #27078, 27080 & 27077), have been employed to facilitate reprogramming into iPSCs. These plasmids are non-integrative to the host cell genome and, thus, widely utilized. They contain transcription factors such as *SOX2*, *KLF4*, *OCT4,* and *Nanog*. The plasmids feature ori P and EBNA-1 sequences, which amplify the persisted reprogramming factors.

Before Neon transfection

LCLs were cultured using complete RPMI media (RPMI media with 15% FBS and 1X Anti-Anti). Approximately 1.5 million cells were seeded in a T25 flask for each LCL line two days prior to neon experiments. Spent media has been changed to fresh complete RPMI media 12 hrs. before the neon experiment. On neon transfection day, cells were collected and washed with 1X PBS and approx. 1.5 million cells were taken into 1.5 ml MCT tubes in 1 ml of PBS. Using a neon nucleofection kit (Thermo, MPK1025), we have taken the R buffer, recommended for LCL nucleofection. From all three episomal plasmids, 1-1 ug of plasmid has been taken in 7 ul of buffer R for one sample nucleofection, and volume has been made up to 10 ul using NFW. MCT tubes containing cells were spin again to collect the cell pellet, PBS was completely removed. Nucleofection buffer R and plasmid cocktail of 10 ul have been added to the cell pellet and mixed gently. Immediately after adding cells were subjected to nucleofection using Neon transfection system using 1100 Voltage with 20 ms width and 3 pulses.

After Neon transfection

Cells were seeded in 6 well-untreated plates with complete RPMI media and kept in a CO_2 incubator. Massive cell death due to nucleofection was observed the next day. Half of the spent RPMI media has been changed to fresh reprogramming media, Essential 8 media. Complete media was replaced with fresh Essential 8 nutrition media on the next day, and cells were cultured for the subsequent 5 days with media top-up in between. We have observed the cellular morphology daily to confirm the cell's health and recovery after nucleofection. On the sixth day, we have coated 6 well plates with Matrigel (Corning, cat 354277) with DMEM F-12 media and cells were transferred to the coated plates along with their culture media. On the very next day, initially, we did half media change and from the next day full media was changed with fresh Essential 8 media. From this point onwards cells media was daily changed to fresh Essential 8 media. After transfer, we could see the attachment of cells to the Matrigel-coated plates. Only the reprogrammed cells will attach to the Matrigel, but only those cells that have transfection of all three plasmids will be reprogrammed into iPSCs. We were able to see the attachment of cells with distinguished changes in their morphology after two weeks. We were able to see the proper iPSC-like morphology one week later, where they formed dense colonies and had a high nuclear-to-cytoplasmic ratio.



iPSC Colony picking and sub-culturing

Once we have the dense iPSC colonies with distinct boundaries and high nuclear-to-cytoplasmic ratios, we plan for pure colony picking and subculturing. Matrigel has been coated to 12 well plates with DMEM F-12 media. From the master reprogramming plate, iPSC colonies were marked based on size for colony picking. During this time, iPSC colonies, along with some unreprogrammed cells, were also growing in the master plate. So, to get the pure iPSC colonies we have picked some portion of the growing colonies and streaked it into freshly coated 12 well plates with Matrigel with the help of sterile pippet tips. From this step, iPSCs were culture in Stem Flex media. We have added Revita cell along with Stem Flex media to ensure the proper attachment of picked iPSC conies to the subculturing plates. Then, both the master plate and their colony plates were kept in a CO2 incubator. For every cell line, we have taken 4 iPSC colonies minimum for further culture. Once we ensured the pure iPSC colonies in cultured clones with proper colony formation and undifferentiated cells, half of the Master plate cells were frozen down, and half was used for DNA isolation, which we used further for characterization. Pure iPSC colonies were further cultured till passage 10 and then we followed the characterization methods.