Single Cell Isolation and Global Amplification

Dissociate Retinas into Single Cell Suspensions

1. Euthanized aniumals with Halothane.
2. Dissect retinas free from retinal pigment epithelium (RPE) in Ca^{++}-free and Mg^{++} free HBSS (**CMF**), pH of 7.4 and a temperature of 37º.
3. Cut the tissue up into 8-10 pieces with sharp tungsten needles.
4. Place retina into CMF containing **papain (5 U/ml)**, pre-activated for 30min @ 37º C with **2.7mM L-cysteine** (reducing agent), and **1mM EDTA**.

Papain solution	
storage	Volume to add for a 1 ml Papain Solution
Frozen stock	• **200µl papain** (25U/ml in CMF)
Refrigerated stock	• **763 µl prewarmed CMF** (1X soln.)
made fresh	• **27 µl L-cysteine** in CMF(100mM) – (176mg/10mlCMF)
Refrigerated stock	• **10 µl of 0.1M EDTA** dissolved in sterile water
	*Activate this cocktail for 30 minutes at 37ºC

5. Incubate tissue for 12 minutes at room temperature.
6. Allow cell clumps to settle to the bottom, remove the majority of the solution and replace 3X with fresh CMF. Repeat again with DMEM containing 1.0 ml of 10% heat inactivated FBS (note- stock in refrigerator are already inactivated). Transfer into a 10ml white capped tube containing 6 ml's of DMEM/10%FBS. Triturate 3X (up and down) with a wide bore glass pipette followed by 3X with a smaller flame treated pipette (Avoid over trituration as this will drastically affect the morphology). The tissue should be forcefully expelled onto the side of the tube, using caution not to create bubbles.
7. Plate out a small portion of cells into 60mm TC dishes containing 10%FCS in DMEM.

Freshly prepare 100µl of Lysis Buffer

Lysis Buffer	
H_2O (Rnase and Dnase Free)	76µl
NP40 ** dissolve in H2O heated to 65°C until dissolved.	0.5µl
5XMMLV buffer (Gibco/BRL) "1st strand buffer"	20µl
PrimeRNase inhibitor (3'-5' Inc.)	1.0µl
RNAsin (Promega)	1.0µl
1/24 dilution of Stock Primer mix	2.0µl
Stock Primer Mix* New aliquot of primer mix	
100mM dATP, dCTP, dGTP, dTTP (Pharmacia)	10µl each
poly-(dt)12-18 (0.5 mg/ml)	16µl
H_2O	14µl

Cell Picking

1. Pick individual rod photoreceptors placed into a separate dish (wash dish) containing DMEM w/ a small amount of serum. The old needle is discarded and a fresh one is used to re-pick the cell and transfer it into a PCR tube containing lysis buffer. (**pre-coat the needle with 10% FBS/DMEM).
2. Spin each cell in the cold-room for 15 sec at 11,000 rpm immediately after it has been picked. Total cells picked are two photoreceptors – PhR1 and PhR2.
3. Proceed to next step.

Single Cell cDNA Amplification

Enzymes- AMV 25 U/µl , MMLV 200 U/µl

1. Lyse the cells by incubating the tubes in a 65°C water bath for 1 min.
2. Remove the tubes from the water bath and place at room temperature for 2 minutes to allow poly-d(T) primer to anneal to the mRNA.
3. Spin down the reaction mixture at 4°C for 1 minute at 11,000 rpm.
4. Keep the tubes on ice and add 0.5 µl of 1:1 (vol:vol) mixture of AMV and MMLV reverse transcriptase and then incubate the tubes at 37° C for 15 minutes (no longer!).
5. Place the tubes on ice to stop the reaction. Incubate the tubes at 65° C for 10 minutes to inactivate the reverse transcriptase.
6. Place the tubes on ice. Spin down the reaction mixture @ 4°C for 1 min.
7. Place the tubes on ice and add 4.5 µl of Terminal Transferase Reaction mix containing 100U of Terminal Transferase. Incubate @ 37°C for 15 minutes.

TERMINAL TRANSFERASE REACTION MIXTURE (STORE @ -80°C)	
5X terminal transferase buffer (Gibco/BRL)	800µl
100mM dATP (Pharmacia)	30µl
H2O (Rnase/Dnase Free)	1.17ml

**Terminal Transferase 400U / µl (Boehringr Mannheim). This buffer contains $CoCl_2$ while the buffer from Invitrogen does not. NOTE: The optimal pH for terminal transferase reaction is 7.2. This was different than the pH of 8.3 which is required for 1st strand synthesis.

8. Incubate @ 65° C for 10 minutes to inactivate the terminal tranferase.
9. Place on ice. Spin down the reaction mixture at 4° C for 2 minutes and then return the tube on ice.
10. Add 90µl of freshly prepared ice-cold PCR Reaction Mixture.

PCR reaction Mixture I	Per rxn.
10X PCR buffer II (Perkin Elmer)	10µl
25mM MgCl2 *(Perkin Elmer)	10µl
20mg / ml BSA (Boehrenger Mannheim)	0.5µl
100 mM dNTP (Pharmacia)	1.0µl each
5.0 % TX-100	1.0µl
5µg/µl Primer † (AL-1)	1.0µl
H2O	63.5 µl
Amplitaq Taq polymerase 5.0 U/µl	2.0µl

† AL-1 Primer: 5' – ATT GGA TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC (T)24- 3'

NOTE: We are changing the pH from 7.2 to 8.3 again for PCR to work. Some batches of terminal transferase buffer have a higher buffering capacity at 7.2 which makes this PCR reaction pH still around 7.8 even after adding 90ul of the PCR mixture.

11. Amplify cDNA in a DNA Thermal Cycler using the following program parameters:

    Cycle parameters – 1st run
    - 94°C for 1 minutes
    - 42°C for 2 minutes
    - 72°C for 6 minutes
    - 25 cycles with 10 sec auto extension per cycle

    Soak file – keep at 4°C ** It is recommended that the 2nd PCR amplification be started as soon as the 1st PCR has finished.

1. After the first round of PCR has finished, reheat the thermocycler block to 94°C, then add 1μl of Amplitaq Taq polymerase (5U/ml) to each tube and restart the PCR with the following program:

   Cycle parameters – 2nd run
   - 94°C for 1 minutes
   - 42°C for 2 minutes
   - 72°C for 6 minutes
   - 25 cycles without extension

   Soak file – keep at 4°C

ORDERING INFO

Catalog #	Company	Description	Size	Contact
27-0815-01	AMERSHAM-PHARMACIA	RNAguard Rnase Inhibitor		
955 15 430-4	EPPENDORF	Prime RNase Inhibitor	7,500 U	
28025-013	INVITROGEN	10% heat inactivated FCS		
	INVITROGEN	M-MLV Reverse transcriptase		
	INVITROGEN	Oligo(dT)12-18 Primer		
	INVITROGEN	AL-1 Primer: 5' – ATT GGA TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC (T)24- 3'		
16314-015	INVITROGEN	Terminal Transferase Buffer 5X		
N808-0172	PE Applied Biosystems	Amplitaq w/ Buffer II	1,000 U	
N8010611	PE Applied Biosystems	Thin walled PCR tubes		
1495062	ROCHE	AMV Reverse Transcriptase		
220 582	ROCHE	Terminal Transferase		
711454	ROCHE	BSA Molecular Biology Grade	20mg/m	
1277049	ROCHE	100mM dNTP set		
	SIGMA	TritonX-100, RNase and DNase free		
	SIGMA	NP-40 (** I believe this is now called IGEPAL)		
C-7880	SIGMA	L-cysteine		
P-5306	SIGMA	Papain		
	QIAGEN	Qia-quick PCR cleanup kit		