Program of Genomic and Nutrition Laboratory
UCLA Department of Epidemiology
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5/22/08

Mg Trial Gene-expression SNP Procedures (TRPM6/7)

STEP 1. Converting Total RNA to cDNA (5/22/08)

1. Throw RNA on ice for about 30 minutes

2. Combine the following kit components in a tube on ice. For multiple reactions, a master mix without RNA may be prepared:

2X RT Reaction Mix	20 µl
RT Enzyme Mix	4 µl
RNA (up to 1 µg)	16 µl (12 for 1978 and 1674)
DEPC-treated water	4µl (1978 and 1674 only)
Total	40ul

3. Gently mix tube contents and incubate at 25°C for 10 minutes.

4. Incubate tube at 55°C for 30 minutes.

5. Terminate the reaction at 85°C at 5 minutes, and then chill on ice.

6. Add 2 µl (2 U) of *E. coli* **RNase H** and incubate at 37°C for 20 minutes.

7. Use diluted or undiluted cDNA in qPCR, or store at –20°C until use.

Note: 1ul (1/40) of the final product will be used for RT-PCR per reaction. To avoid the pipetting error with such a low volume, dilute the final to 5x (then use 5ul per reaction based on the protocol).

RNA used in this step:

ID	1978	1748	1532	1784	1075	1491	1482	1674	1172
VISIT	v3	v3	v3	v3	v3	v3	v3	v3	v3
Original volume(ul)	73	73	73	78	78	78	78	78	78
conc.(ng/ul)	147.76	90.28	46.59	81.67	94.57	63.68	81.15	130.69	44.49
ul	12	16	16+12	16	16	16	16	12+8	16

Reactions left in the Invitrogen SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (received 5/20/08): **28 reactions** (4 extra reactions were used due to a broken tube for 1674 and 1532 during the centrifuge)

Step 2: Validate GADPH gene as the endogenous control

1. Quantified CDNA, aliquot and diluted to uniformed concentration (25ng/ul). Randomly select 3 subjects from each group to perform the validation run.

(based on the cDNA before dilution from 5/22/08)

ID	1978	1748	1532	1784	1075	1491	1482	1674	1172
VISIT	v3	v3	v3	v3	v3	v3	v3	v3	v3
conc.(ng/ul)	126.99	128.66	145.35	106.98	162.34	131.87	127.48	147.89	110.63
Overall volume	180ul	180ul	180ul	180ul	180ul	180ul	180ul	180ul	180ul

2. Prob preparation
   - Use the "Human GAPD (GAPDH) Endogenous Control" assay

3. Prepare for assay mix
   - Prepare a clean sterilized 2000ul microcentrifuge tube
   - Prepare the gene expression assay mix for 20 uL total reaction volume each sample (make 5% more overall)

	1X	20X (for 6 samples in Mg Trial)
Master Mix	10	200
H2O	6	120
Assay	1	20
Total	17	340

4. Aliquot 17ul Assay mix for each assigned well

	1	2	3
M1	A1	A2	A3
M2	B1	B2	B3
M3	C1	C2	C3
P1	D1	D2	D3
P2	E1	E2	E3
P3	F1	F2	F3
	NTC	NTC	

(M=Mg, P=placebo, samples were randomly selected)

5. Add 3ul cDNA from the previous step, 3 reactions (triplicate) for each subject

6. Seal plate with the Optical Adhesive Cover

7. Spin plate for 1000rpm, 1 minute. (Important!!)

Step 3: Perform amplification run

1. Add Fam dye to the plate template
2. Assign the plate layout
3. Perform amplification run by "Quantitative"

Step	UNG Incubation	AmpliTaq Gold Activation	PCR	
	HOLD[a]	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

a. If using TaqMan Universal Master Mix without UNG AmpErase, this step is not necessary.

IMPORTANT The 2-min, 50 °C step is required for optimal UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold enzyme.

Note: Over all volume is 20ul.

Step 4: Analysis

1. Highlighted the wells has been used.
2. Press "Analyse" with Fam Dye and "automatic ct"
3. Export the result as excel format and analyse
4. Analyse fold change between Mg group and Placebo group

Sample Name	Detector Name	Ct	Mean	Group mean	delta CT	FOLD
M1	FAM	24.97106	23.65348	23.49139	-0.34561	1.270692
M1	FAM	22.80035				
M1	FAM	23.18904				
M2	FAM	23.54002	23.55674			
M2	FAM	23.56599				
M2	FAM	23.56419				
M3	FAM	23.49914	23.26395			
M3	FAM	23.14487				
M3	FAM	23.14785				
P1	FAM	23.28584	23.02481	23.14578		
P1	FAM	22.8982				

P1	FAM	22.89038				
P2	FAM	23.26695	23.29945			
P2	FAM	23.31506				
P2	FAM	23.31634				
P3	FAM	23.09581	23.11307			
P3	FAM	23.11985				
P3	FAM	23.12355				

Calculation:

- Mean=mean of the triplicate CT within one subject. Formula example: =Average (A1:A3)
- Group Mean=Mean CT of the 3 subjects in the same group (Mg or placebo).
- Delta CT=Δ CT = placebo ct – mg ct
- FOLD = fold change in expression compare with Mg vs placebo = 2^-[Δ CT]

5. Analyze the CT variance between Mg and placebo group using t-test.

Result:

t-Test: Two-Sample Assuming Equal Variances

	Mg	*Placebo*
Mean	23.49139	23.14578
Variance	0.041136	0.01966
Observations	3	3
Pooled Variance	0.030398	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.427813	
P(T<=t) one-tail	0.036076	
t Critical one-tail	2.131847	
P(T<=t) two-tail	**0.072151**	<- Non-significant difference, so GADPH can be used as endogenous gene
t Critical two-tail	2.776445	

Step 5: Gene-expression for TRPM6 and TRPM7 using GAPDH gene as the endogenous control

1. Non-uniformed concentration (25ng/ul)cDNA is needed, just arrange the final cDNA amount per reaction between 10ng to 100ng.
2. Prob preparation for GAPDH, TRPM6 and TRPM7

3. Prepare for assay mix

- Prepare a clean sterilized 2000ul microcentrifuge tube
- Prepare the gene expression assay mix for 20 uL total reaction volume each sample, triplicate (make 5% more overall)

	1X	30X (for 9 samples in Mg Trial, triplicate)
Master Mix	10	300
H2O	6	180
Assay	1	30
Total	17	510

4. Aliquot 17ul Assay mix for each assigned well

	GAPDH			TRPM6			TRPM7		
M1	A1	A2	A3	A4	A5	A6	A7	A8	A9
M2	B1	B2	B3	B4	B5	B6	B7	B8	B8
M3	C1	C2	C3						
M4	D1	D2	D3						
P1	E1	E2	E3						
P2	F1	F2	F3						
P3	:	:							
P4	:	:							
P5	:	:							
	NTC	NTC							

(M=Mg, P=placebo, samples were randomly selected)

5. Add 3ul cDNA from the previous step, 3 reactions (triplicate) for each subject

6. Seal plate with the Optical Adhesive Cover

7. Spin plate for 1000rpm, 1 minute. (Important!!)

Step 5: Perform amplification run

1. Add Fam dye to the plate template
2. Assign the plate layout
3. Perform amplification run by “Quantitative”

Step	UNG Incubation	AmpliTaq Gold Activation	PCR	
	HOLD[a]	HOLD	CYCLE (40 cycles)	
			Denature	Anneal/ Extend
Temperature	50 °C	95 °C	95 °C	60 °C
Time	2 min	10 min	15 sec	1 min
Volume	50 µL			

a. If using TaqMan Universal Master Mix without UNG AmpErase, this step is not necessary.

IMPORTANT The 2-min, 50 °C step is required for optimal UNG enzyme activity. The 10-min, 95 °C step is required to activate AmpliTaq Gold enzyme.

Note: Over all volume is 20ul.

Step 6: Analysis

1. Highlighted the wells has been used.
2. Press “Analyse” with Fam Dye and “automatic ct”
3. Export the result as excel format and analyze
4. Analyze fold change between Mg group and Placebo group

Ex. TRPM6

	GAPDH	TRPM6	CT	MeanCT	Group mean	Delta CT	FOLD
M1	25.63597	31.43596	5.79999	5.996309	6.710483	-0.24189	1.182541
M1	25.34708	31.27678	5.929705				
M1	25.13606	31.39529	6.259232				
M2	26.37054	33.45231	7.081772	7.126827			
M2	26.29214	33.18905	6.896918				
M2	26.28033	33.68213	7.401792				
M3	26.59281	33.36434	6.771527	7.070762			
M3	26.29869	33.76011	7.461424				
M3	26.41669	33.39602	6.979334				
M4	27.9701	34.37081	6.400711	6.648033			
M4	27.7552	34.3842	6.628996				
M4	27.73299	34.64739	6.914391				
P1	24.42332	31.9873	7.563977	7.39182	6.952372		
P1	24.71703	32.06834	7.351308				
P1	24.67425	31.93442	7.260174				
P2	24.90196	30.97681	6.074855	6.030376			
P2	25.05038	31.22022	6.169844				

P2	25.30311	31.14954	5.846428				
P3	25.54307	32.80062	7.257554	7.209327			
P3	25.56047	32.81279	7.252317				
P3	25.28297	32.40108	7.118111				
P4	25.27516	33.00285	7.727688	7.614733			
P4	25.18701	32.63862	7.451606				
P4	25.21941	32.88431	7.664904				
P5	25.54573	32.08522	6.539485	6.515607			
P5	25.72275	31.96772	6.244966				
P5	25.57759	32.33995	6.762369				

Calculation:

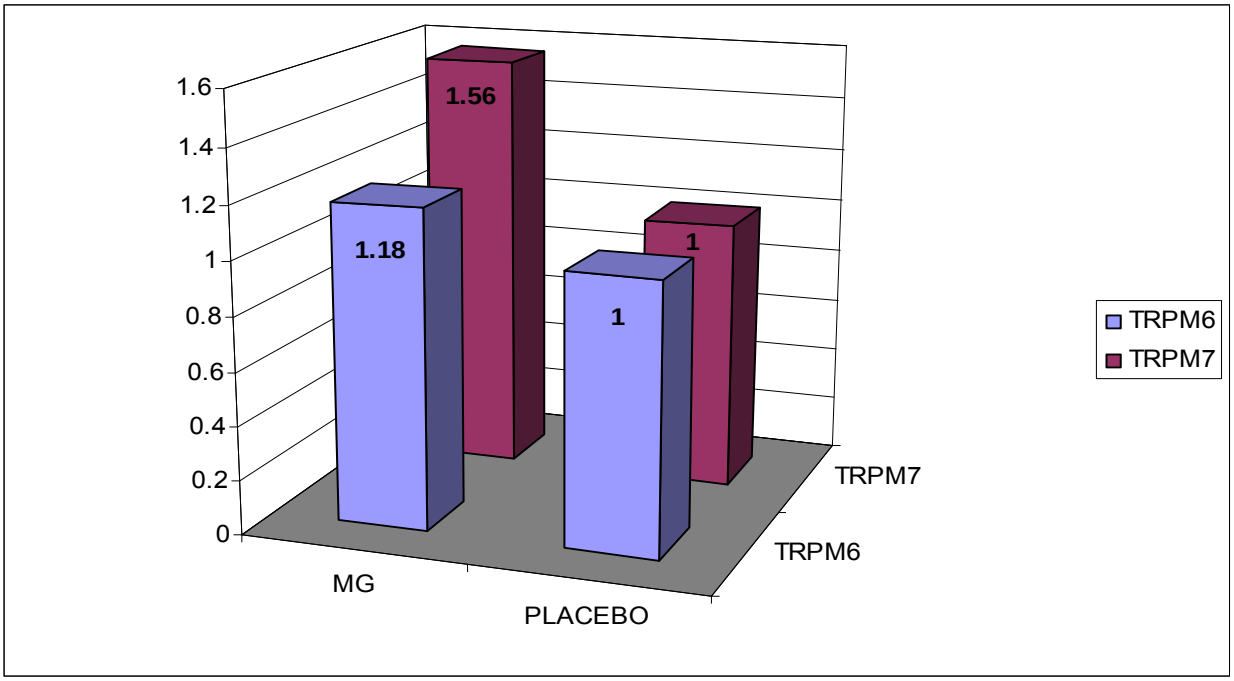
- CT= CT of TRPM6 - CT of GAPDH (or do the difference in means of triplicates)
- Mean ct =mean of the triplicate CT within one subject. Formula example: =Average (A1:A3)
- Group Mean=Mean CT of the 3 subjects in the same group (Mg or placebo).
- Delta CT=Δ CT = placebo ct – mg ct
- FOLD = fold change in expression compare with Mg vs placebo = 2^-[Δ CT]

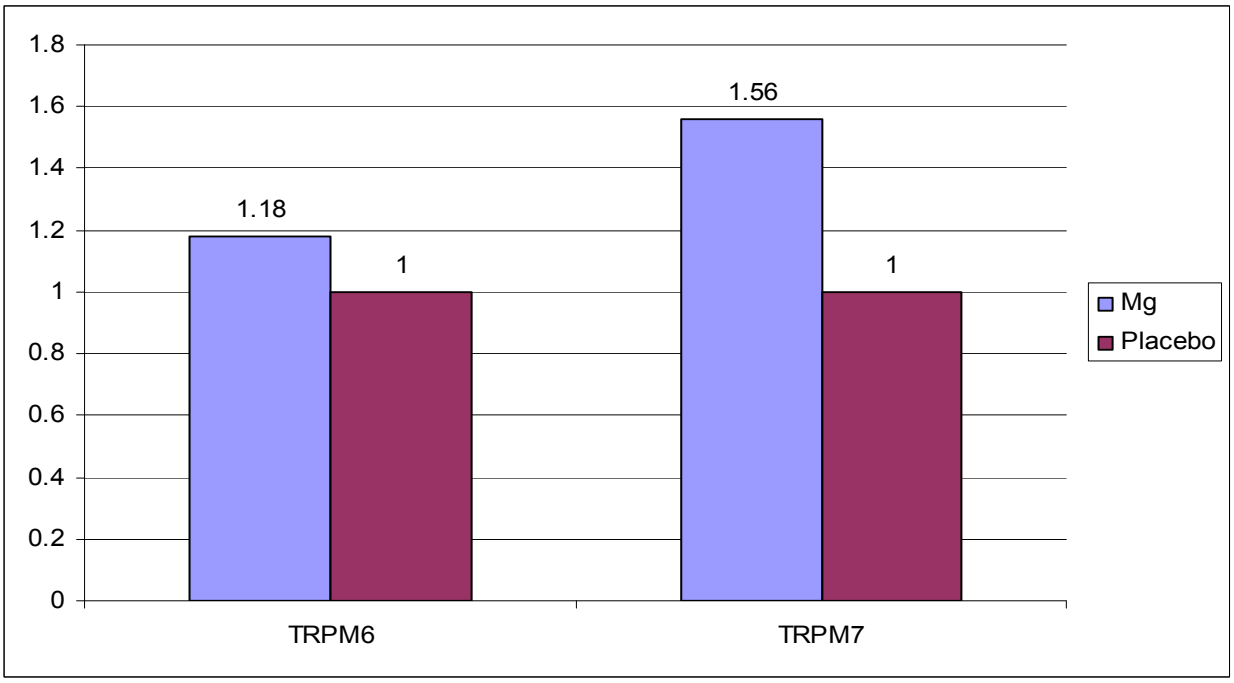
5. Analyze the CT variance between Mg and placebo group using t-test. Result:

t-Test: Two-Sample Assuming Equal Variances
TRPM6

	mg	*placebo*
Mean	6.710483	6.952372
Variance	0.272363	0.434676
Observations	4	5
Pooled Variance	0.365113	
Hypothesized Mean Difference	0	
df	7	
t Stat	-0.59676	
P(T<=t) one-tail	0.284729	
t Critical one-tail	1.894579	
P(T<=t) two-tail	**0.569459**	
t Critical two-tail	2.364624	

6. Can also present in the graph like

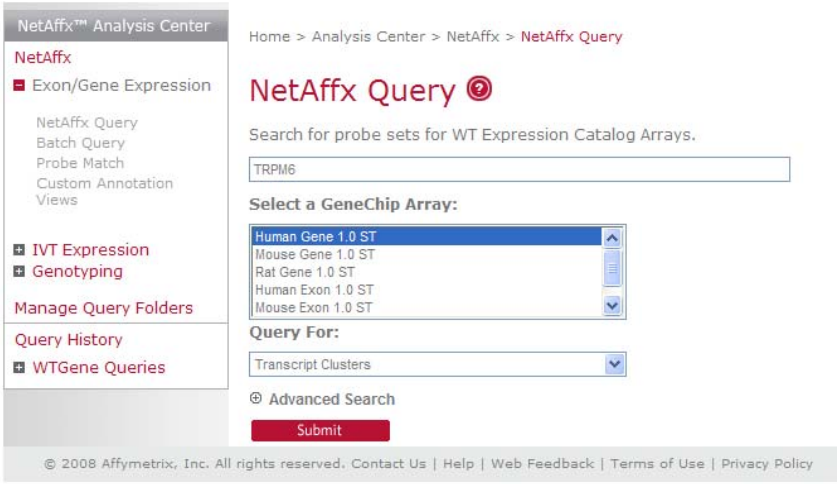

1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.56
1.18
1
1
MG
PLACEBO
TRPM7
TRPM6
TRPM6
TRPM7


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.18
1
1.56
1
TRPM6
TRPM7
Mg
Placebo

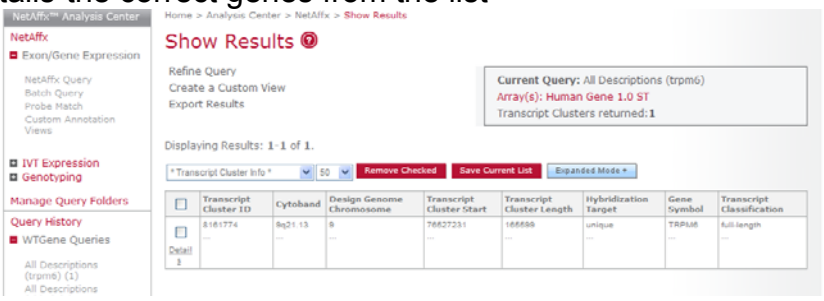
Appendix I: How to select gene expression assay validating Affixchip result?

1. Go to Affymetrix.com -> Net Affy. Log in, and select "**Exon/Gene Expression**" on the right, then choose "**Human Gene 1.0ST**"

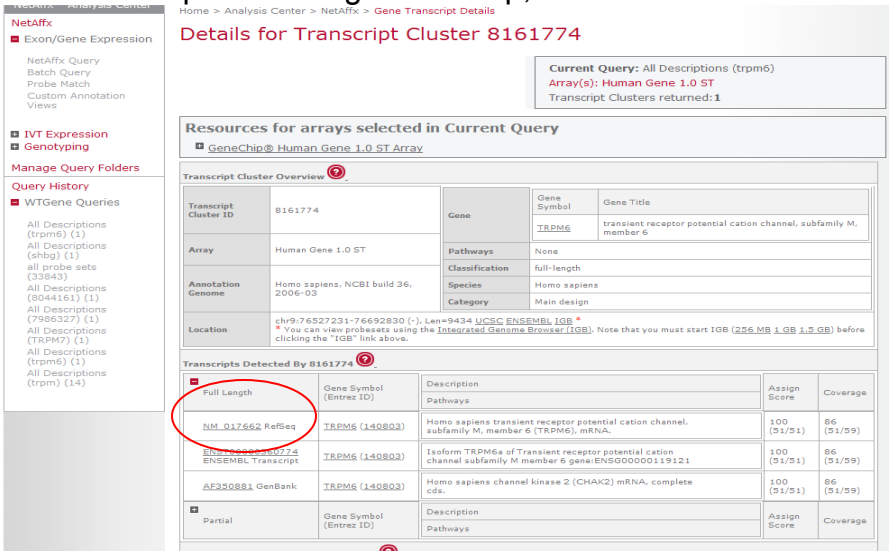
2. Key in the gene name of interest, ex. TRPM6, and submit



3. choose "Details"the correct genes from the list

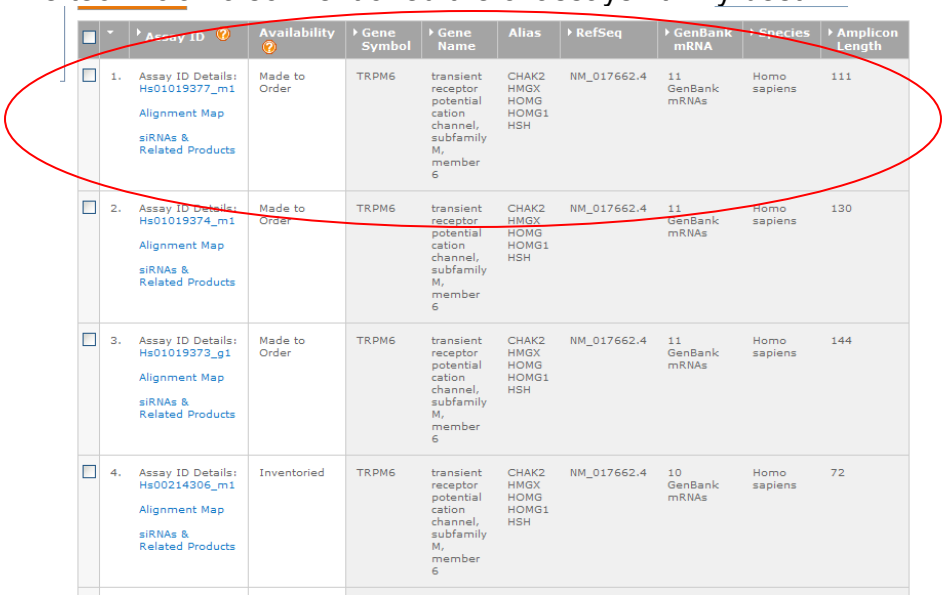


4. Find out the RNA sequence using in this chip, ex. NM017662 for TRPM6



5. Go to Applied Biosystems website, go to **Taqman Gene Expression Assays,** type TRPM6

6. Examine the results with the following hints:
   - Make sure the RefSeq is consistent with AffyMetrix
   - Make sure the "Species" is "Homo Sapiens"
   - For the assay ID, "….._m1" stand for exon to exon while "…._g1" will pick up the genomic DNA and mRNA. For valildating this chip, choose "m1" is better
   - Pick up the most transcript assays under "GenBank mRNA", the larger numbered the better. Ex. 11 for TRPM6
   - If the assay is inventoried, can save shipping time.
   - The technician also mentioned the 3' assays fit Affy best.



	Assay ID	Availability	Gene Symbol	Gene Name	Alias	RefSeq	GenBank mRNA	Species	Amplicon Length
1.	Assay ID Details: Hs01019377_m1 Alignment Map siRNAs & Related Products	Made to Order	TRPM6	transient receptor potential cation channel, subfamily M, member 6	CHAK2 HMGX HOMG HOMG1 HSH	NM_017662.4	11 GenBank mRNAs	Homo sapiens	111
2.	Assay ID Details: Hs01019374_m1 Alignment Map siRNAs & Related Products	Made to Order	TRPM6	transient receptor potential cation channel, subfamily M, member 6	CHAK2 HMGX HOMG HOMG1 HSH	NM_017662.4	11 GenBank mRNAs	Homo sapiens	130
3.	Assay ID Details: Hs01019373_g1 Alignment Map siRNAs & Related Products	Made to Order	TRPM6	transient receptor potential cation channel, subfamily M, member 6	CHAK2 HMGX HOMG HOMG1 HSH	NM_017662.4	11 GenBank mRNAs	Homo sapiens	144
4.	Assay ID Details: Hs00214306_m1 Alignment Map siRNAs & Related Products	Inventoried	TRPM6	transient receptor potential cation channel, subfamily M, member 6	CHAK2 HMGX HOMG HOMG1 HSH	NM_017662.4	10 GenBank mRNAs	Homo sapiens	72

7. Order the assay and do the gene expression assay following the protocol.

Appendix II: How to select endogenous control
The purpose is to find a gene that is not differentially express between the study groups (intervention and placebo).

1. Follow the literatures (for the specific gene of interest)
2. If can't find it in the literatures, need to follow the validation steps as in **STEP 2** to find a good endogenous gene. The suggested most common used endogenous genes: **GAPDH, β-actins, and 18S**.
3. How to buy one? In the **Taqman Gene Expression Assays** search page, check "**Endogenous controls**" then can find the Cat number and product size to order.