Lab Meeting 10/22/08 (approx.)

Developing a luciferase reporter system for identifying therapeutics in a high-throughput screen…

…and a bunch of other stuff

Drug discovery

Drug discovery has contributed more to the progress of medicine than any other scientific approach.

Biotechs and the larger pharmaceutical industry are interested in academia for its high quality science and also mainly because collaboration increases efficiency and financial return. Academia is interested in big pharma for their support of clinical trials.

Such collaborations can be beneficial for academia but ultimately it is also good for academia to maintain some independence from industry which is why many universities now have established their own compound screening facilities.

UCLA Compound Screening Core

Molecular Screening

E-mail Print

Director: Kenneth Bradley, Ph.D.

Scientific Director: Robert Damoiseaux, Ph.D.

Location:
2805 Molecular Sciences Bldg.
Los Angeles, CA 90095-1489

Telephone: (310) 794-1974

Fax: (310) 206-5231

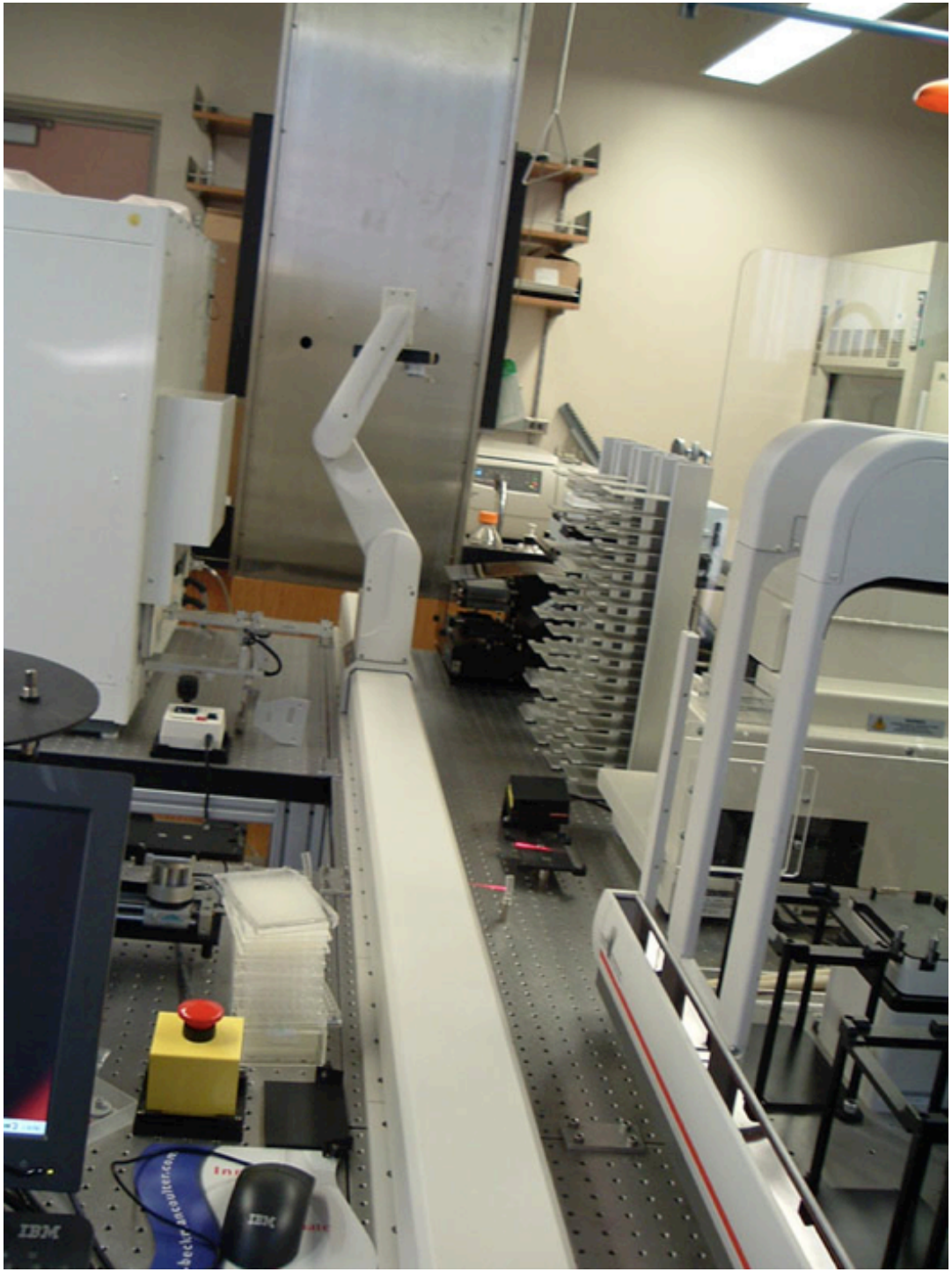
E-mail: rdamoiseaux@mednet.ucla.edu

Website: http://www.mssr.ucla.edu/

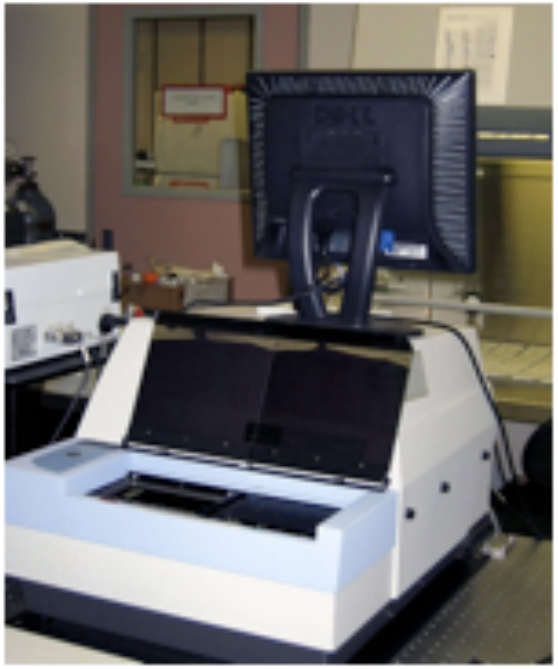
Services:

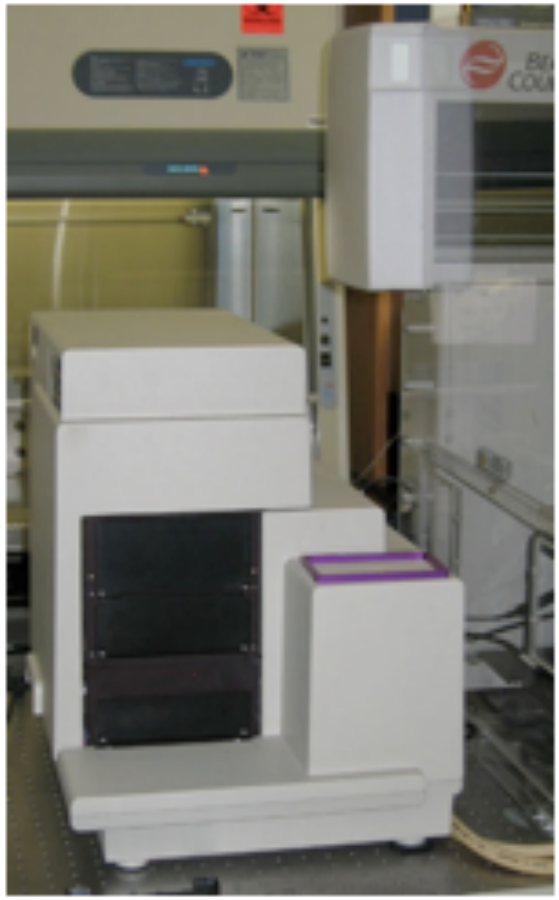
The Molecular Screening Shared Resource (MSSR) provides three types of screens: small molecule, yeast genomic and siRNA. Each screen represents one unit of service, which includes the following:

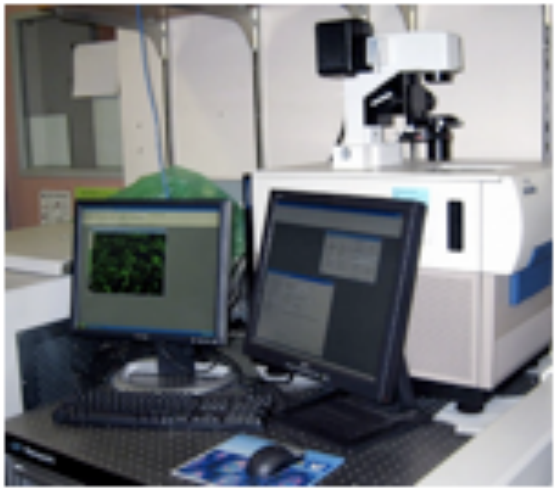
- **Assay development & consultation**: The MSSR provides expert technical assistance for the design and execution of high throughput screening (HTS). Pre-existing assays can be miniaturized and adapted to HTS. Alternatively, MSSR will assist in de novo design of assays. Assay development includes validation using MSSR equipment and pilot runs.
- **High throughput technology & instrumentation**: Initial HTS screens take advantage of automated equipment, much of which is integrated into a single robotics system.
- **Access to libraries**: The total collection of small molecules currently available through the MSSR totals ~70,000 unique compounds. In addition, the MSSR has recently purchased siRNA libraries from Dharmacon representing the druggable genomes (~7000 genes) from both human and mouse (4 duplexes/gene). Yeast knockout collections are also available.
- **Hit validation & follow up**: Following initial screening, compounds that show activity are cherry-picked and re-tested in secondary assays. Based on these results, MSSR staff work with users to identify the best "hits" for follow-up (i.e., dose-response). Additional consultation includes identification of pre-existing compounds for initial structure-activity-relationship (SAR) and/or acting as liaisons with Division of Chemistry faculty for custom synthesis of modified compounds and SAR.


IBM
IBM


BECKMAN
COULTER
Biomek







Chemical Libraries

BioMol Libraries

Bioactive lipids, 204 compounds. *endo-cannabinoid,* 60 compounds, *ion channel,* 72 compounds, *enzyme inhibitors,* 84 compounds and phosphatase and kinase inhibitors, *orphan ligands,* 84 compounds. This library serves primarily for assay validation.

Biomol library compound list

FDA Approved Drug Library

A unique collection of 1120 high-purity chemical compounds (all off patent) carefully selected for:

- Structural diversity
- Broad spectrum covering several therapeutic areas (from neuropsychiatry to cardiology, immunology, anti-inflammatory, analgesia and more)
- Known safety and bioavailability in humans

Microsource Spectrum Collection

2000 biologically active and structurally diverse compounds from our libraries of known drugs, experimental bioactives, and pure natural products.

Druggable Compound Set

A set of about 8000 compounds which are targeted at kinases, protease, ion channels and GPCR's. These compounds are designed to match our siRNA efforts which are based on sets of siRNA's for the druggable human and mouse genome.

Lead Like Compound Set

This set of 20,000 compounds has been custom tailored for us for lead likeness. The compounds in this set are not to be found in any of the traditional "Russian" collections

Chemically Diverse Library (Combichem Library)

ChemBridge DiverSet, 30,000 chemically diverse small molecules.

siRNA Libraries

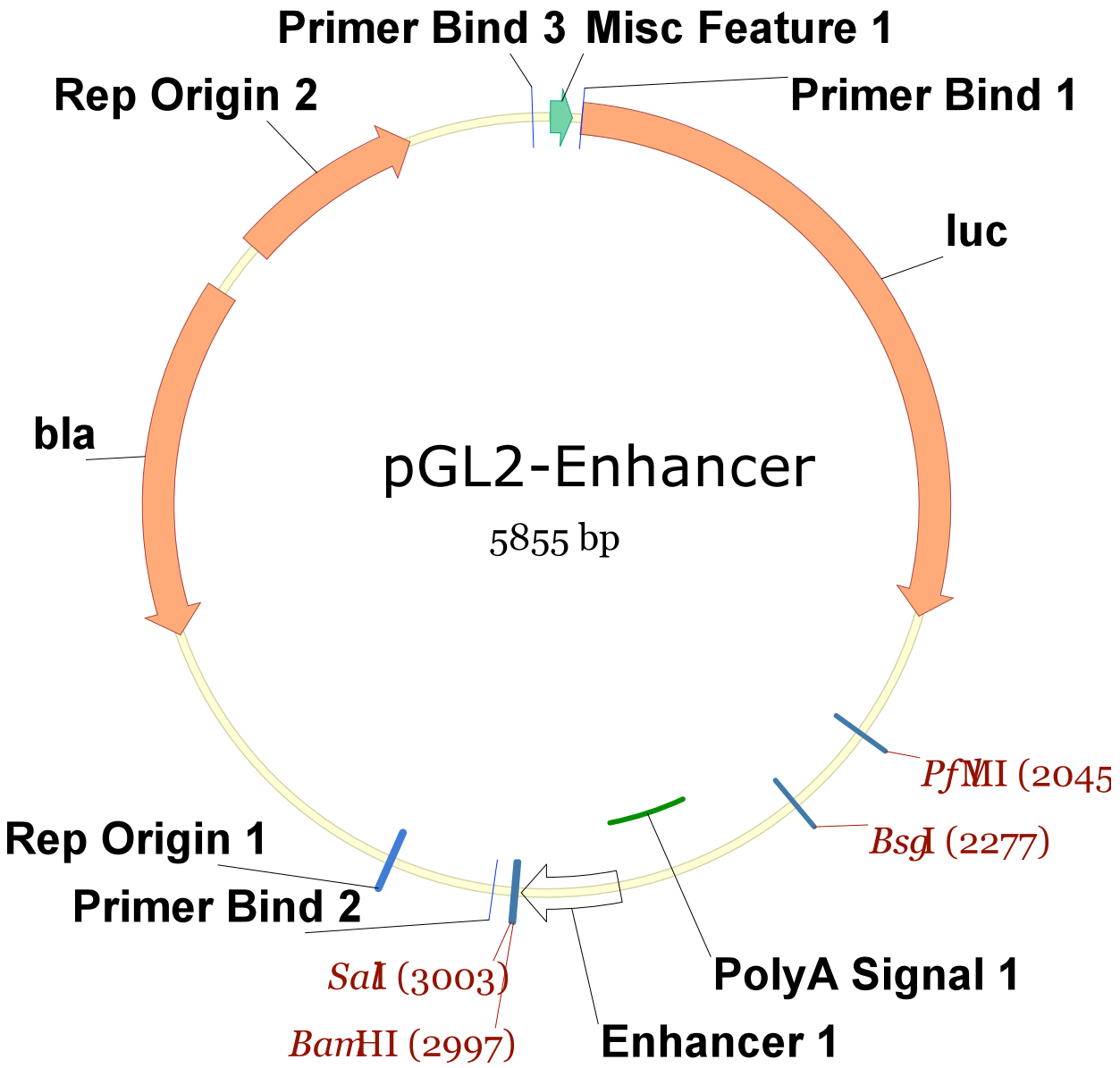
We just acquired sets of siRNA's for the druggable genome of mouse and human. Shortly, we will offer a full range of screens using this technology. We envision to use this technology for the identification of the targets of compounds found in conventional HTS screens as well as for answering basic research questions.

Yeast Knockout Collection

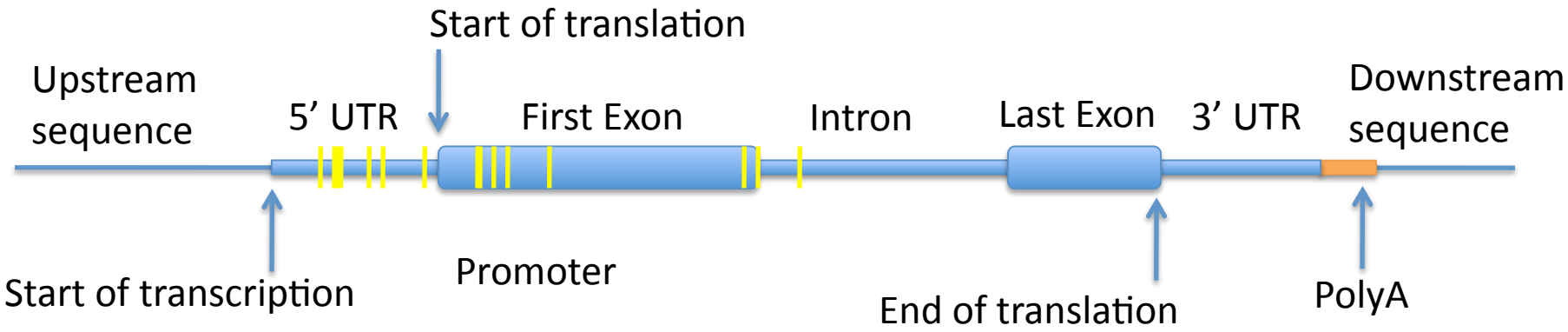
A genome wide set of non-essential knockout mutants of yeast is available for screening in both mating types (alpha and A). The strain collections have been condensed into 384 well plates to facilitate high throughput.

UCLA in-house Collection

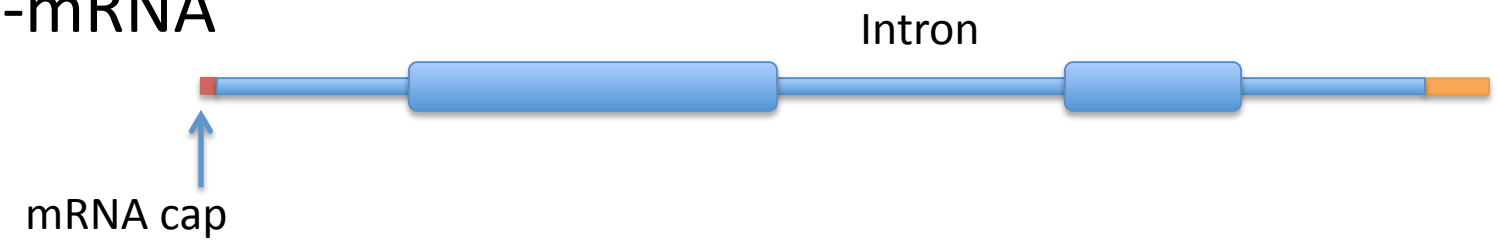
Various professors have donated their compounds to our in-house collection and so far we have collected about 5000 compounds. These libraires are extremely diverse and range from natural products to compounds which have been synthesized using diversity-oriented synthesis.


Primer Bind 3
Misc Feature 1
Rep Origin 2
Primer Bind 1
luc
bla
pGL2-Enhancer
5855 bp
PfMI (2045
BsgI (2277)
Rep Origin 1
Primer Bind 2
SalI (3003)
BamHI (2997)
PolyA Signal 1
Enhancer 1

Genomic Sequence

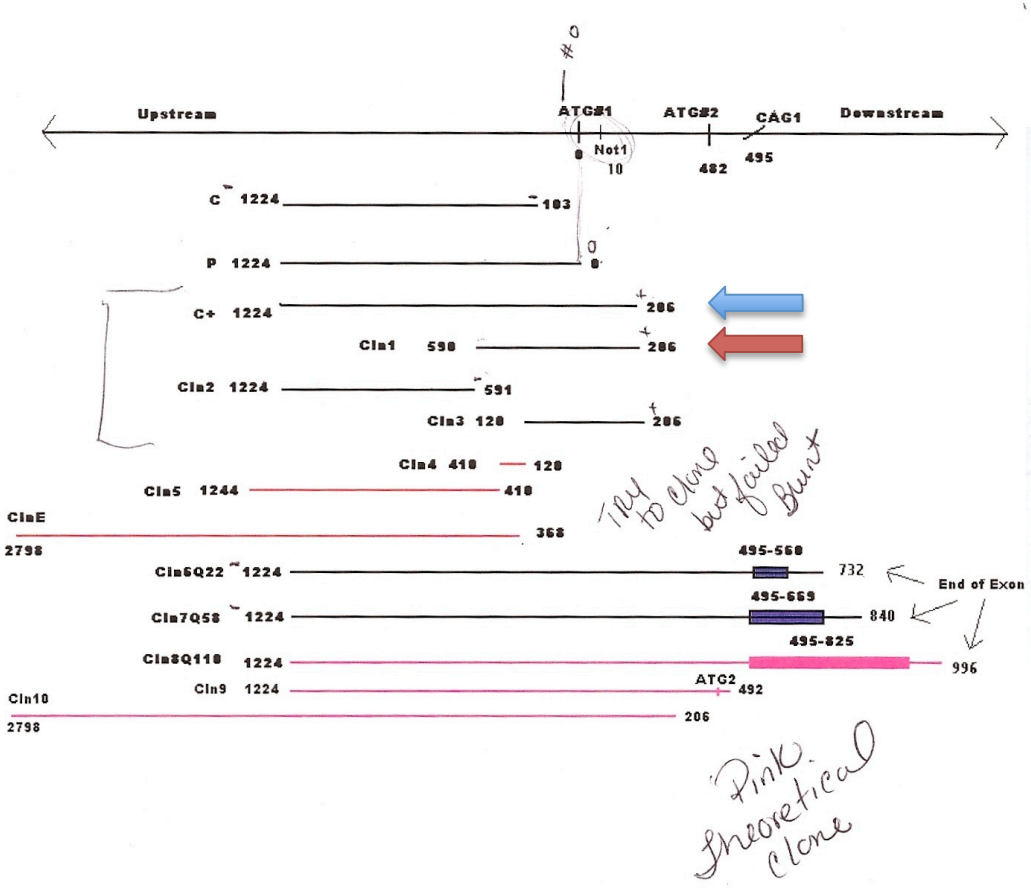


Pre-mRNA



mRNA

Molly's Clones



Normalized

	pGL2	CMV	Clone1	C+	Clone 2	Clone3
Exp. #	**114ng**	**125ng**	**129ng**	**141ng**	**126ng**	**120ng**
6 Cos 24hr	3	10,423	25	15		
6a Cos 48hr	1	3,003	21	25		
6 SHY 48hr	16	262,469	959	201		
6a SHY 72hr	12	335,574	1,121	1,570		
8 PC12 48hr	3	81,802	1,426	923		
9 Neuro 48hr	14	191,180	406	203		
10 Neuro 48hr	38	140,458	472	337		
Exp 11 Hs 72hr	2	11,049	29	34		
12 PC12 48hr	2	58,287	692	521		
13 SHY 72hr	2	16,278	80	-		
14 SHY 72hr	13	360,441	564	1,326		
15 Neuro 48hr	17	43,880	432	543		
16 Neuro 48hr	14	752	220		1	
17 Neuro 48hr	10	48,756	121		1	
18 Neuro 48hr	12		129			55
19 Neuro 48hr	24		189			135
19 PC12 48hr	6		1,450			289

CpG Island?

CAG Island?

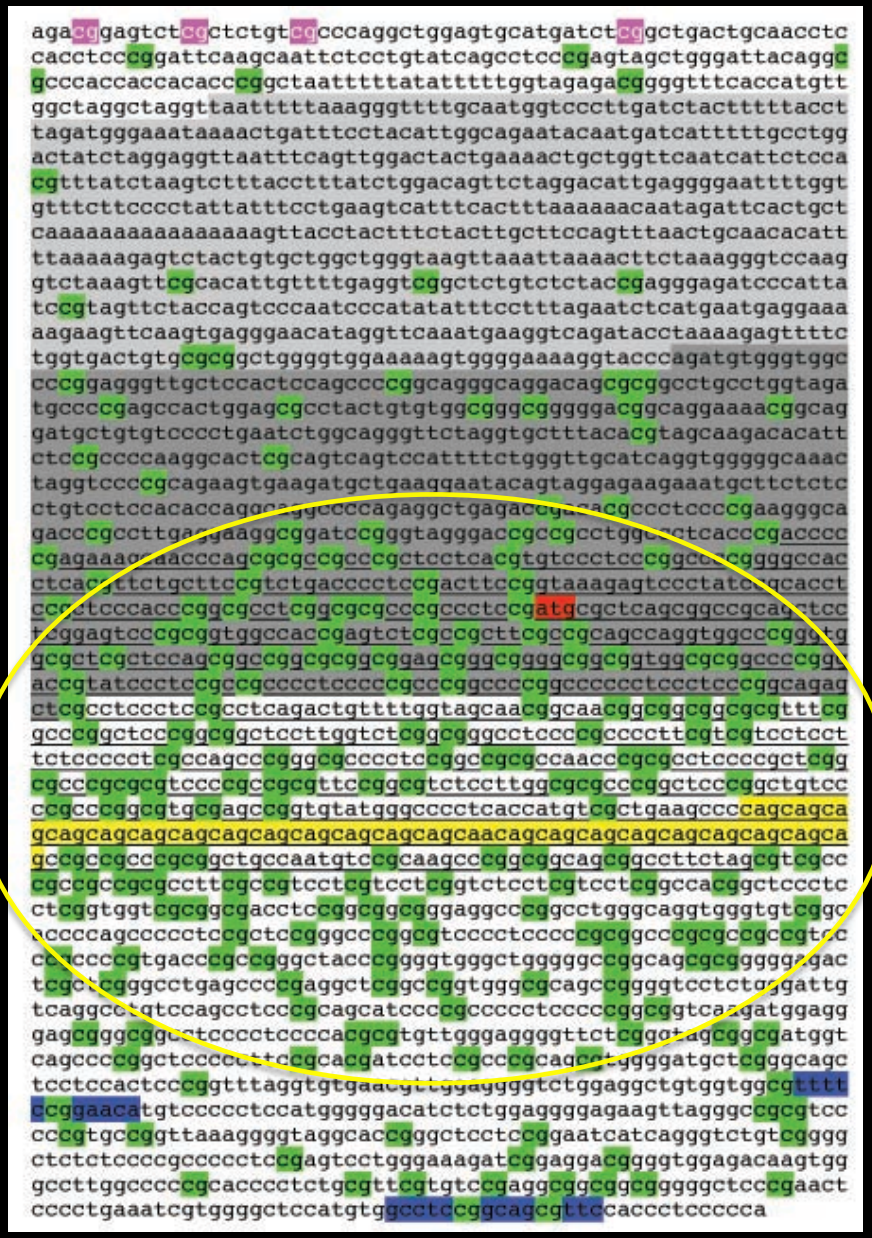
Carlos P. Garcia

Contest… fill in the blank: Castro A. G_____

Image NASA

…other kinds of CpG islands are defined as regions of DNA exceeding 200 bp, having more than 50% C+G content and a CpG frequency at least 0.6 of that expected on the basis of C+G content of the region. Approximately half of the mammalian gene promoters are associated with one or more CpG islands.

Ataxin 2 Upstream sequence-5'UTR-Exon1-Intron1
CpGs and cloned fragments anotated



* = CpG

C+

Clone1

Start Codon

Exon1 underlined

CAG repeat

Blue=reverse primer options for intron 1

60 bp / line

Ataxin 2 Intron24-Exon25-3'UTR-PolyA-Downstream sequence

Blue=Forward primer options

Exon25 underlined

Stop Codon

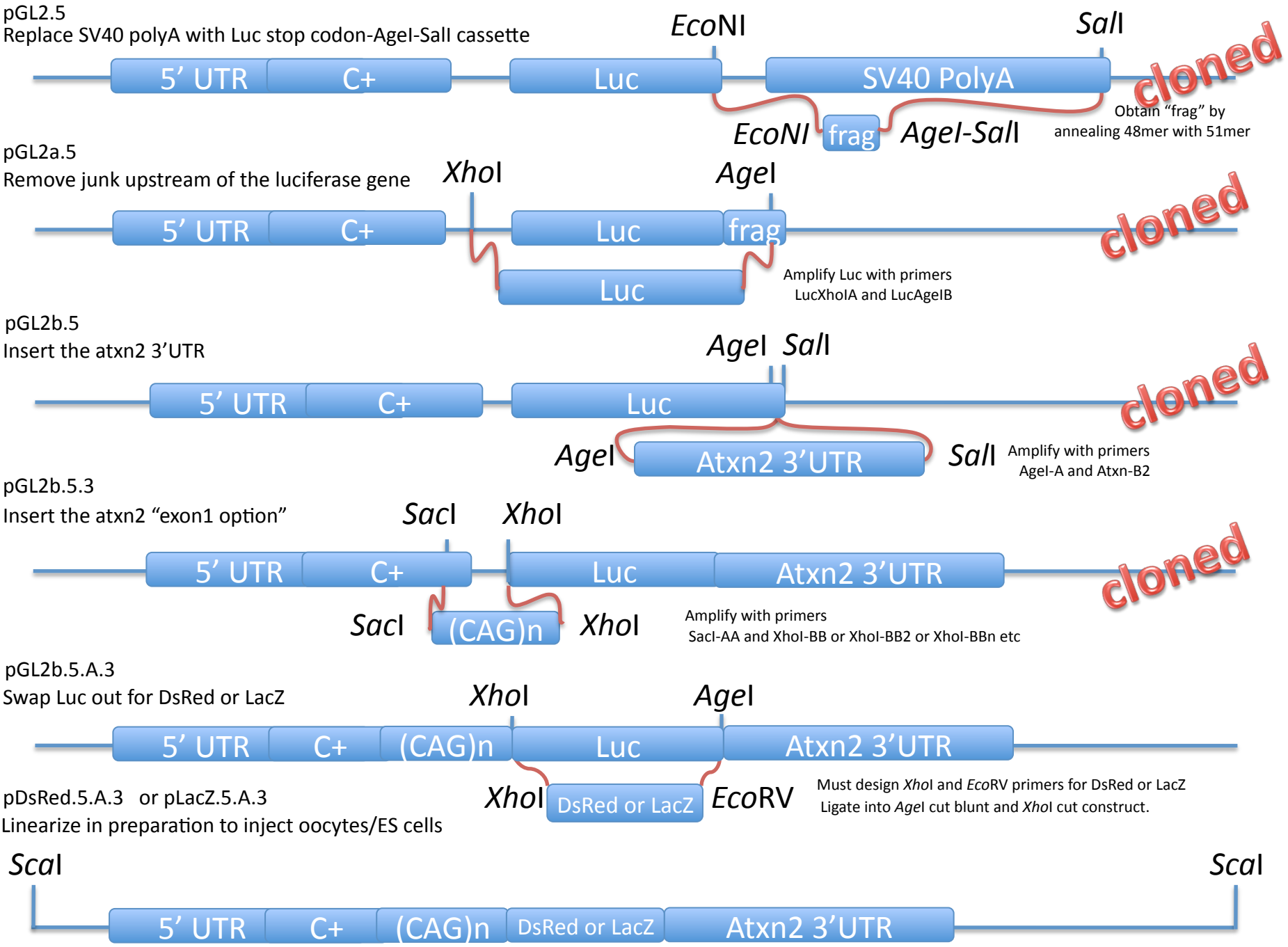
PolyA elements

Green=Reverse primer options

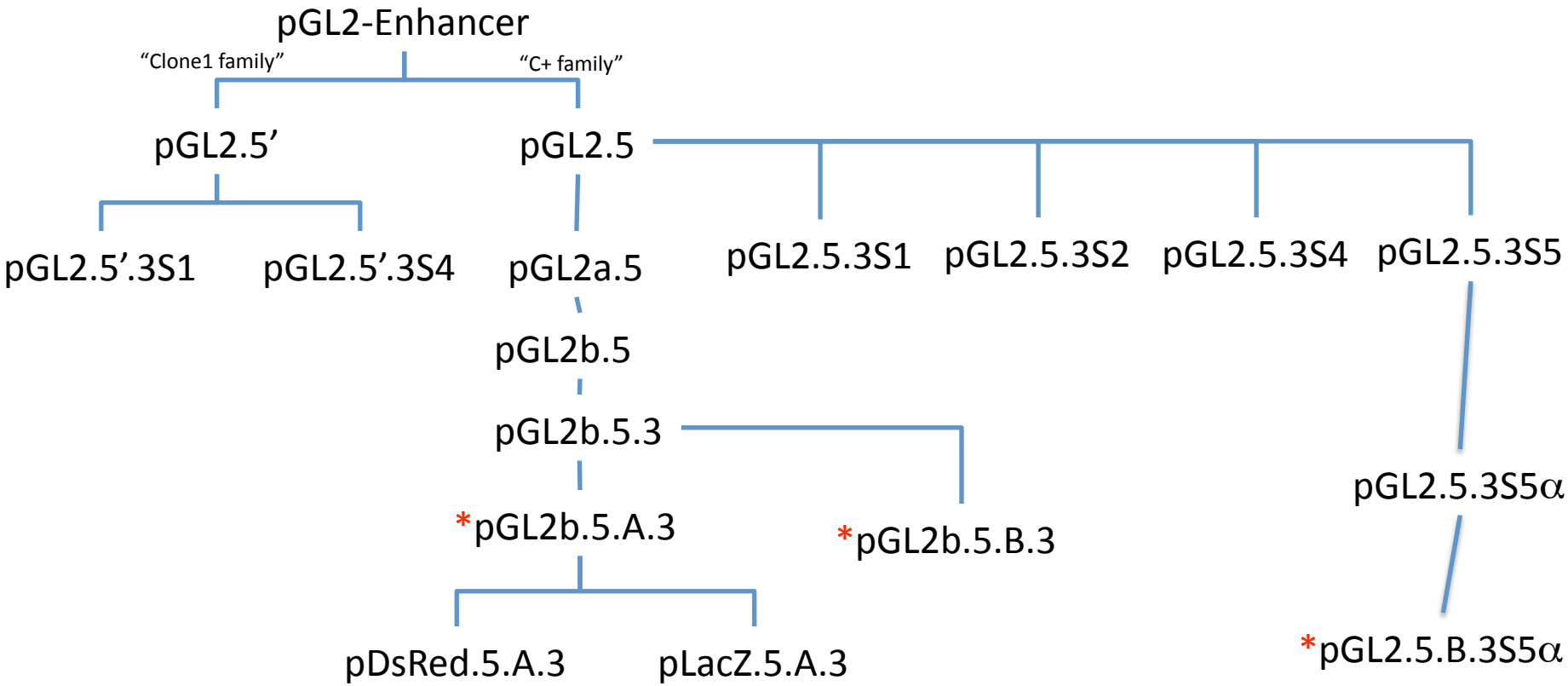
Extent of fragment after AarI deletion

60 bp / line

Cloning pGL2-5.A.3 and pDsRed-5.A.3 (or pLacZ-5.A.3)



Summary of all clones



Key:

pGL2.5	5 denotes "C+" and 5' denotes "clone1"
pGL2a	lowercase letters designate vector modifications, changes to up or downstream sequence of the luc gene
pGL2b.5.3	3 denotes atxn2 3'-UTR
pGL2.5.3S1	S number (S1..S5) denotes the primer set used to obtain different atxn2 3'-UTRs
pGL2b.5.A.3	uppercase letters designate the form of the completion of the atxn2 Exon1 (with or without CAGn) A=includes 2nd ATG before the polyglutamine; B=CAG22
pGL2.5.3S5α	symbols indicate subsequent modification to atxn2 3'UTRs
pDsRed.5.A.3	pDsRed indicates that luciferase was swapped out of the pGL2 construct and replaced with DsRed from pDsRed
pLacZ.5.A.3	pLacZ indicates that luciferase was swapped out of the pGL2 construct and replaced with LacZ from pLacZ
*	A red asetricks indicates that the construct and everything below it in the family has not yet been made

Sequence boundaries in pGL2-5

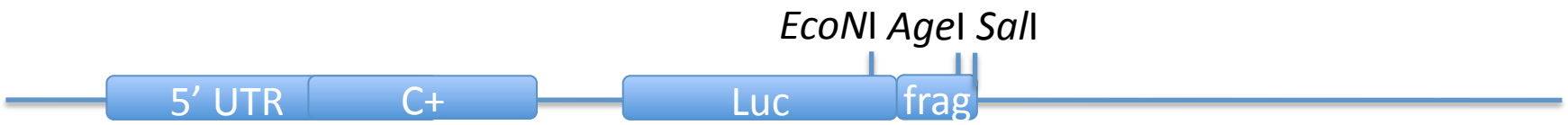
pGL2.5 (known also as C+)

This is pGL2-Enhancer modified by Molly by the addition of a 5'UTR-Exon1 fragment of the Ataxin 2 (Atxn2) gene. The insert consisted of a fragment amplified by PCR with a forward primer containing an additional A to finish an *Ase*I restriction site that was cut and filled with T4 polymerase and ligated to the vector MCS *Sma*I site to result in the vector insert transition sequence (vector...ACCC/TAATTTTTAAAGGG...insert). The reverse primer primed across the *Sac*I site native in Atxn2 which was cut and ligated into the vector MCS *Sac*I site (insert…CCCGGCAGAGCTCTTACGCG…vector, *Sac*I underlined). The resulting fragment was named C+ by Molly.

Boundaries confirmed by sequencing in September 2008

Sequence boundaries in pGL2a.5

pGL2a.5



pGL2a.5 is a modification of pGL2.5 done with the purpose of eliminating unwanted SV40 polyA sequence while at the same time incorporating a convenient *Age*I useful for cloning the 3’UTR later on. The resulting clone has the luciferase gene followed by its regular stop codon then immediately followed by *Age*I then a six bp spacer then *Sal*I. *EcoN*I occurs upstream of the Luciferase stop codon so the fragment replaces this portion of luciferase.

To do this we made two oligos and annealed them. The annealed oligo contains sticky ends ready for ligation into *Eco*NI *Sal*I digested pGL2-5. The oligos are as follows:

As purchased:

```
5’-taaaggccaagaagggcggaaagtccaaattgtaaaccggtactgtag     48mer
5’-tcgactacagtaccggttacaatttggactttccgcccttcttggcctttt     51mer
```

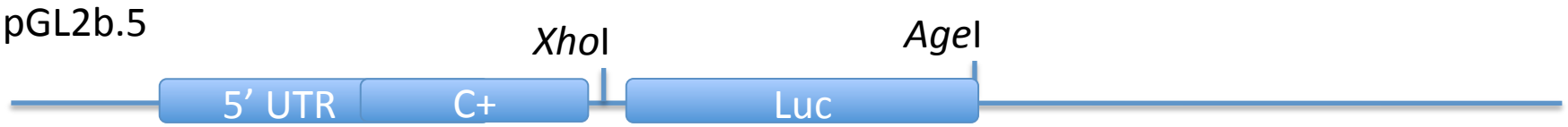
After annealing:

```
5’-taaaggccaagaagggcggaaagtccaaattgtaaaccggtactgtag
3’--tttccggttcttcccgcctttcaggtttaacatttggccatgacatcagct
```

Final sequence through and around *Eco*NI---*Sal*I

```
5’-gagagatcctcataaaggccaagaagggcggaaagtccaaattgtaaaccggtactgtagtcgaccgatgccct
          EcoNI                                  Stop AgeI       SalI
```

Sequence boundaries in pGL2b.5



pGL2b.5 is a modification of pGL2a.5 done with the purpose of eliminating unwanted sequence upstream of the luciferase gene. This was done by amplifying luciferase with a forward primer with a *Xho*I site and reverse primer with *Age*I site. The resulting 5' end of the luciferase gene is altered by having its start codon immediately after *Xho*I while the remaining sequence is unaltered.

The primers ordered to accomplish this were as follows:

gcatctcgagatggaagacgccaaaaacata LucXhoIA
gcataccggtttacaatttggactttccgcccttct LucAgeIB

The resulting alteration is shown here:

Before (*Xho*I underlined and start codon in red):

5'-gctagctcgagatctaagtaagcttggcattccggtactgttggtaaaatggaagacgc

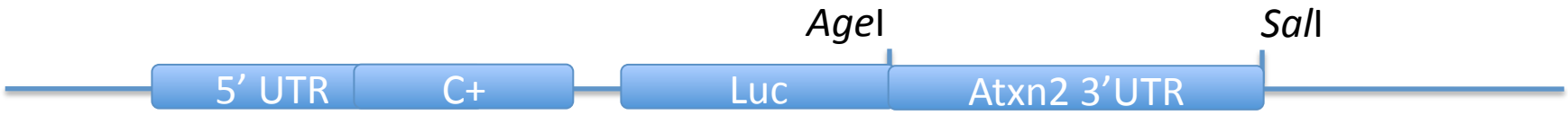
After:

5'-gctagctcgagatggaagacgc

*Note we could have gone from pGL2.5 to pGL2b.5 in one PCR step of the luciferase gene but the reverse *Age*I-*Sal*I primer would have to have been very long

Sequence boundaries in pGL2.5.3

pGL2b.5.3



This a modification of pGL2b.5 whereby the atxn2 3'UTR was amplified by AgeI and SalI primers and ligated between the AgeI and SalI sites of pGL2b-5. The primers used to amplify the 3'UTR are as follows:

cgctaaccggtgttgtaaggctgccctggagga	AgeIA
gctagtcgacagccttcatgccctctggattt	Atxn-B2

The flanking sequences are described here:

Luciferase gene — *Age*I — *Sal*I

ccaaattgtaaaccggtgttgtaaggctgccctggagga---3'UTR---aaatccagagggcatgaaggctgtcgaccgatgccct

Luciferase stop codon

Sequence boundaries in pGL2b.5.A.3

pGL2b.5.A.3 (different clones with exon 1 modifications designated by B, C, D, and so on.)

This a modification of pGL2b.5.3 to include additional portion of the exon 1 including just past the second ATG in Atxn2 but before the polyglutamine (actually includes the first CAG). The primers to make this modification were the following:

ggcagagctcgcctccctccgcctcagac SacI-AA
cacactcgagctggggcttcagcgacat XhoI-BB2

The SacI-AA primer annals across the SacI site native in Atxn2 exon 1 and so digesting the amplicon and annealing with the pGL2b.5.3 SacI site reconstitutes the native sequence shown here with the SacI site underlined:

5'-ccccctccctcccggcagagctcgcctccctccgcctcag-3'

The resulting insert-to-luciferase boundary is as follows, with *Xho*I underlined:

5'-atgtcgctgaagccccagctcgagatggaagacgc

And again with the frame shown:

5'-atg-tcg-ctg-aag-ccc-cag-ctc-gag-atg-gaa-gac-gc

Luciferase start codon shown in red

Sequence boundaries in pGL2b.5.B.3

pGL2b.5.B.3 (different clones with exon 1 modifications designated by B, C, D, and so on.)

This a modification of pGL2b.5.3 to include additional portion of the exon 1. This modification can be done over and over to prepare clones with different CAG lengths or other exon 1 types ending upstream of the CAG. To do so only a different reverse primer need be used. The primers to make this modification were the following:

ggcagagctcgcctccctccgcctcagac SacI-AA
gcatctcgagggccgaggacgaggagac XhoI-BB

The SacI-AA primer annals across the SacI site native in Atxn2 exon 1 and so digesting the amplicon and annealing with the pGL2b.5.3 SacI site reconstitutes the native sequence shown here with the SacI site underlined:

5'-ccccctccctcccggcagagctcgcctccctccgcctcag-3'

The resulting insert-to-luciferase boundary is as follows, with *Xho*I underlined:

5'-gtctcctcgtcctcggccctcgagatggaagacgc

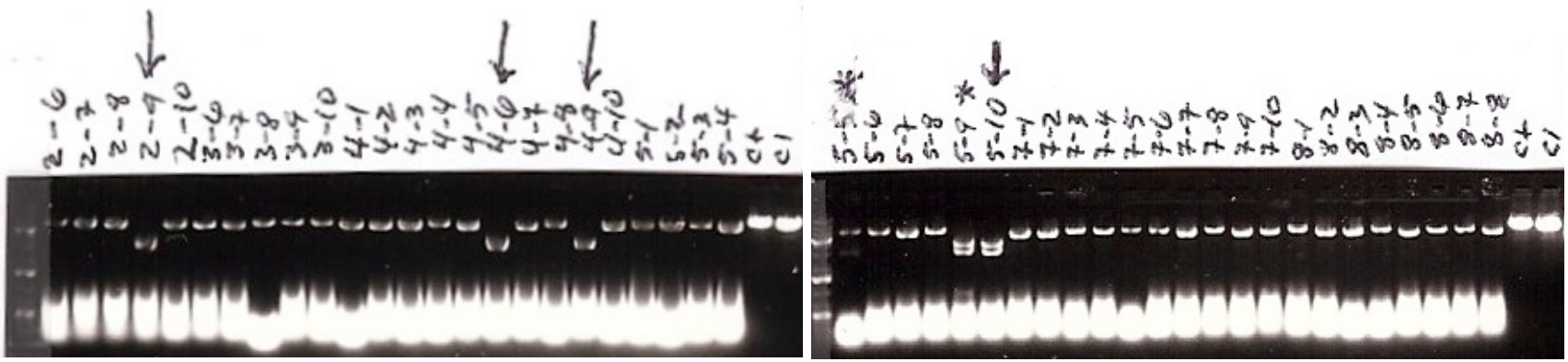
Luciferase start codon shown in red

And again with the frame shown:

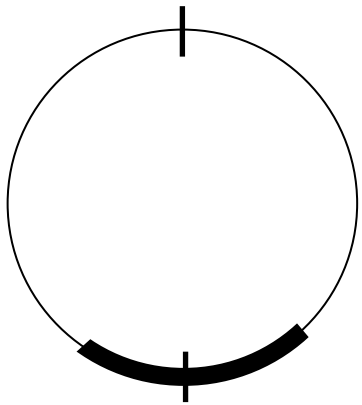
5'-gtc-tcc-tcg-tcc-tcg-gcc-ctc-gag-atg-gaa-gac-gc

Update on cloning progress…

Just some examples of our miniprep screening…

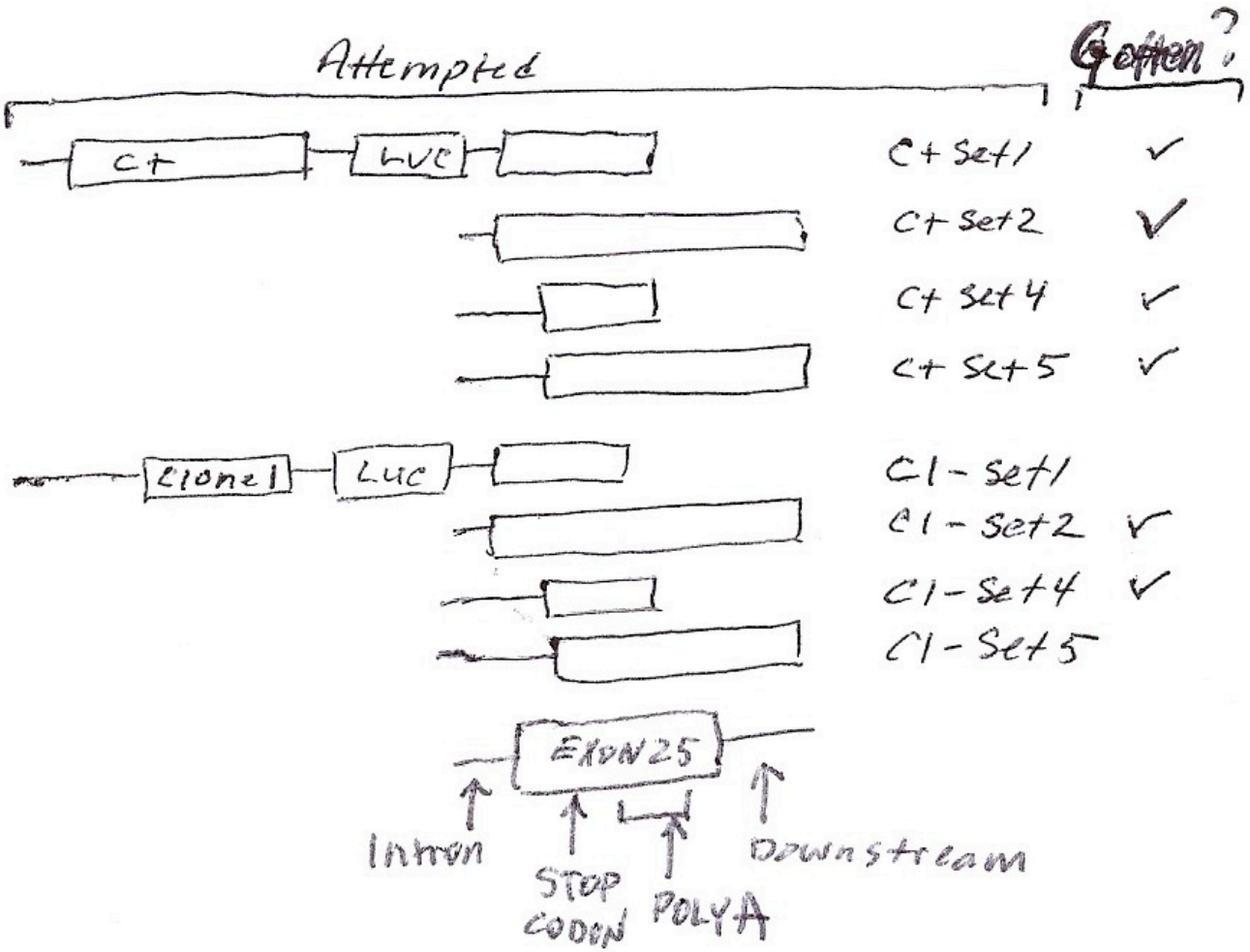


*Kpn*I in original plasmid



*Kpn*I only present in insert

Summary of what we have cloned so far



Real time PCR for Ataxin 2

“Real Time PCR” only means detection of PCR products as they accumulate.

Higuchi et al., 1992 originally described the real time PCR method whereby intercalated ethidium bromide was detected by UV excited fluorescence by a CCD camera.

Higuchi, R., Dollinger, G., Walsh, P. S., and Griffith, R. 1992. Simultaneous amplification and detection of specific DNA sequences. Biotechnology 10:413–417

Three primer pairs designed

5’-agtcctgcatcgaacagagctgtta AtxnEx12-A
5’-cctctgatcttgaagcctggaatct AtxnEx13-B
455bp genomic
386 bp intron
69 bp amplicon from cDNA

5’-aatcagcccttgttcagcatgt AtxnEx19-A “old”
5’-aggactatagacatgaggatgctgaga AtxnEx20-B “old”
380 bp amplicon from genomic (actually last 6 bp in exon 19)
317 bp intron
63 bp amplicon from cDNA

5’-caacagcgcatttcccctatatga AtxnEx24-A
5’-cagggcagccttacaactgctgtt AtxnEx25-B
910 bp amplicon from genomic
836 bp intron
74 bp amplicon from cDNA

Three new primer pairs designed

Each of these amplify only human cDNA and not mouse
Both forward and reverse 3' ends can not anneal mouse cDNA; bases that are different in the mouse are underlined.

aagatatggactccagttatgcaaa Exon5-A
gctccaggtccttctctttgtgt Exon6-B
Annealing T = 50
Product Length = 96 bp
Product length with intron 5 = 27 kb

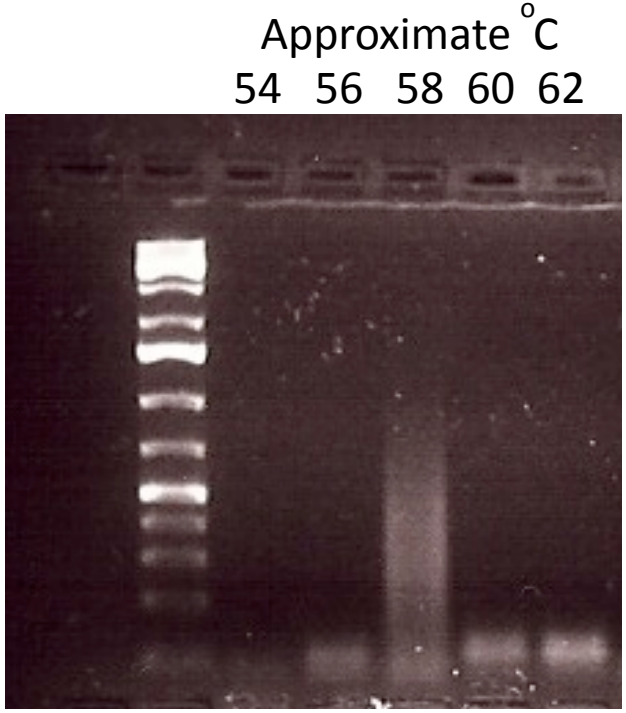
gaacgtgaggggcacagcata Exon8-A
ctggcccatacgcggtgaat Exon9-B
Annealing T = 53
Product Length = 117 bp
Product length with intron 8 = 1,568 bp

gttcccaaatcagcccttgtt Exon19-A
gtgtgttggtggtgccatcatt Exon20-B
Annealing T = 53
Product Length=112 bp
Product length with intron 19 = 429 bp

One control primer was included, designed previously:

5’-CGCCGTGCCTACCATGGTGAC 18S-rRNA-A
5’-CTTGGATGTGGTAGCCGTTTCTCA 18S-rRNA-B

Previously used with annealing temp of 58C

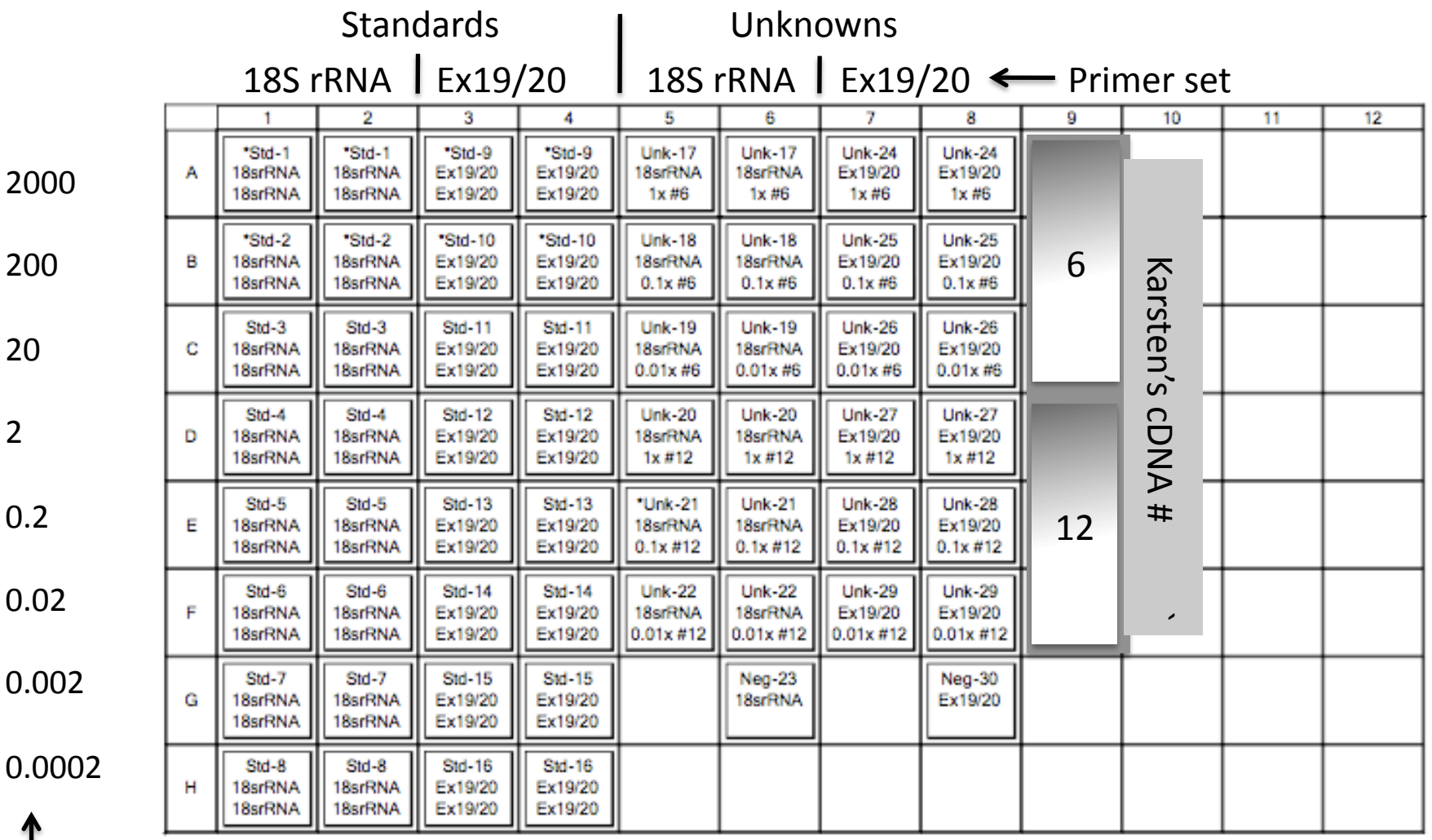


88 bp amplicon
18S-rRNA

No template control

Template=Karsten’s cDNA #6

Plate setup and reaction conditions

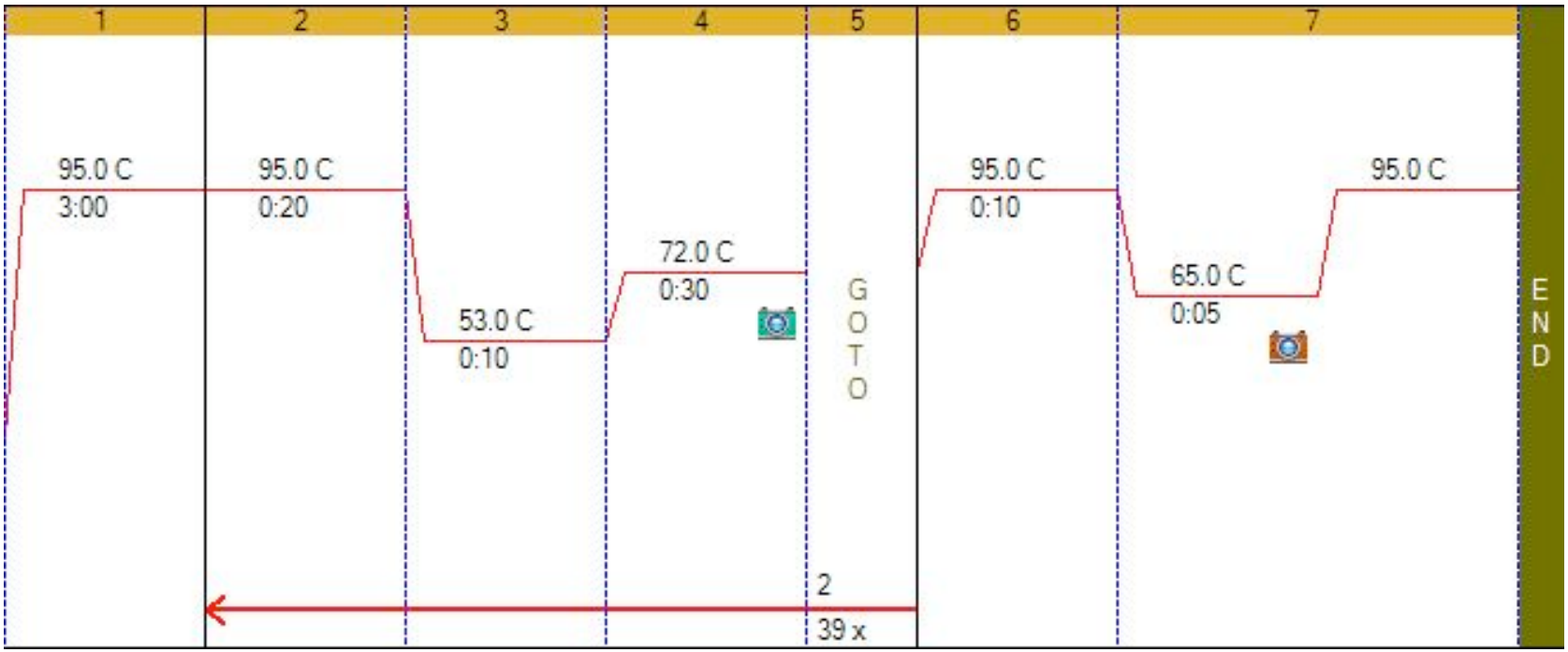


	1	2	3	4	5	6	7	8	9	10	11	12
A	*Std-1 18srRNA 18srRNA	*Std-1 18srRNA 18srRNA	*Std-9 Ex19/20 Ex19/20	*Std-9 Ex19/20 Ex19/20	Unk-17 18srRNA 1x #6	Unk-17 18srRNA 1x #6	Unk-24 Ex19/20 1x #6	Unk-24 Ex19/20 1x #6				
B	*Std-2 18srRNA 18srRNA	*Std-2 18srRNA 18srRNA	*Std-10 Ex19/20 Ex19/20	*Std-10 Ex19/20 Ex19/20	Unk-18 18srRNA 0.1x #6	Unk-18 18srRNA 0.1x #6	Unk-25 Ex19/20 0.1x #6	Unk-25 Ex19/20 0.1x #6				
C	Std-3 18srRNA 18srRNA	Std-3 18srRNA 18srRNA	Std-11 Ex19/20 Ex19/20	Std-11 Ex19/20 Ex19/20	Unk-19 18srRNA 0.01x #6	Unk-19 18srRNA 0.01x #6	Unk-26 Ex19/20 0.01x #6	Unk-26 Ex19/20 0.01x #6				
D	Std-4 18srRNA 18srRNA	Std-4 18srRNA 18srRNA	Std-12 Ex19/20 Ex19/20	Std-12 Ex19/20 Ex19/20	Unk-20 18srRNA 1x #12	Unk-20 18srRNA 1x #12	Unk-27 Ex19/20 1x #12	Unk-27 Ex19/20 1x #12				
E	Std-5 18srRNA 18srRNA	Std-5 18srRNA 18srRNA	Std-13 Ex19/20 Ex19/20	Std-13 Ex19/20 Ex19/20	*Unk-21 18srRNA 0.1x #12	Unk-21 18srRNA 0.1x #12	Unk-28 Ex19/20 0.1x #12	Unk-28 Ex19/20 0.1x #12				
F	Std-6 18srRNA 18srRNA	Std-6 18srRNA 18srRNA	Std-14 Ex19/20 Ex19/20	Std-14 Ex19/20 Ex19/20	Unk-22 18srRNA 0.01x #12	Unk-22 18srRNA 0.01x #12	Unk-29 Ex19/20 0.01x #12	Unk-29 Ex19/20 0.01x #12				
G	Std-7 18srRNA 18srRNA	Std-7 18srRNA 18srRNA	Std-15 Ex19/20 Ex19/20	Std-15 Ex19/20 Ex19/20		Neg-23 18srRNA		Neg-30 Ex19/20				
H	Std-8 18srRNA 18srRNA	Std-8 18srRNA 18srRNA	Std-16 Ex19/20 Ex19/20	Std-16 Ex19/20 Ex19/20								

*=outlier removed

<u>Reaction Conditions</u>
Template
250 pmol each primer
H20 to 10 ul
10 ul SYBR green supermix

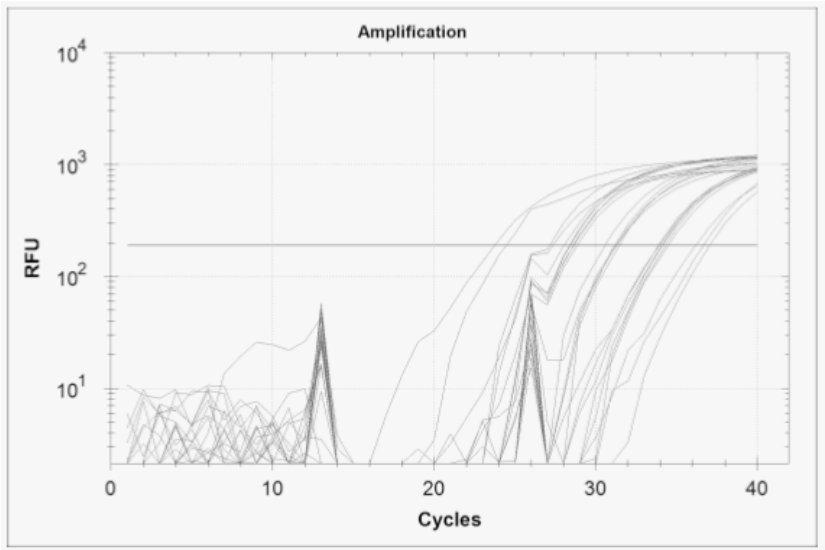
“Run Information”



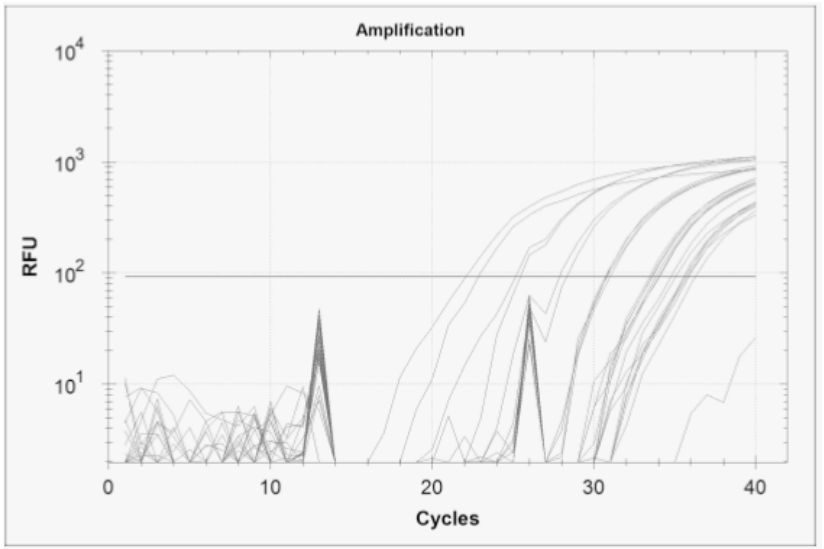
1: 95.0°C for 3:00
2: 95.0°C for 0:20
3: 53.0°C for 0:10
4: 72.0°C for 0:30
Plate Read
5: GOTO 2, 39 more times
6: 95.0°C for 0:10
7: Melt Curve 65°C to 95°C : Increment 0.5°C for 0:05
Plate Read

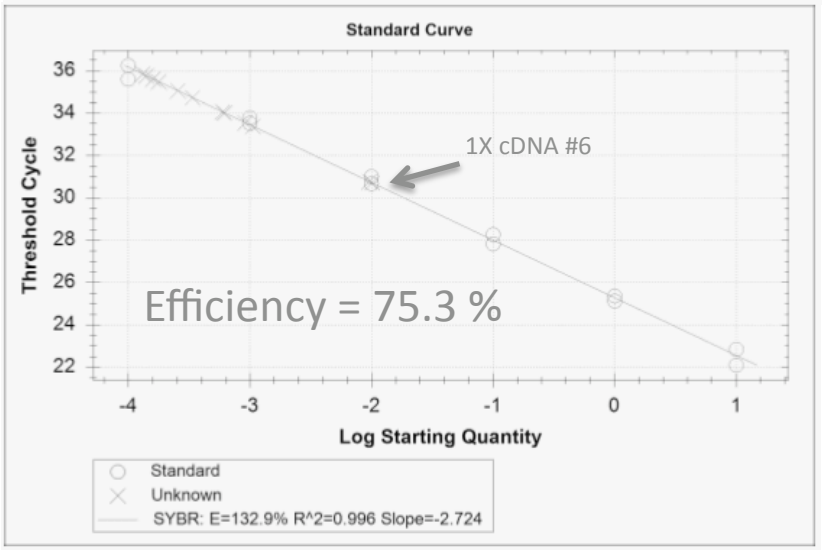
Standard Curves

18S rRNA

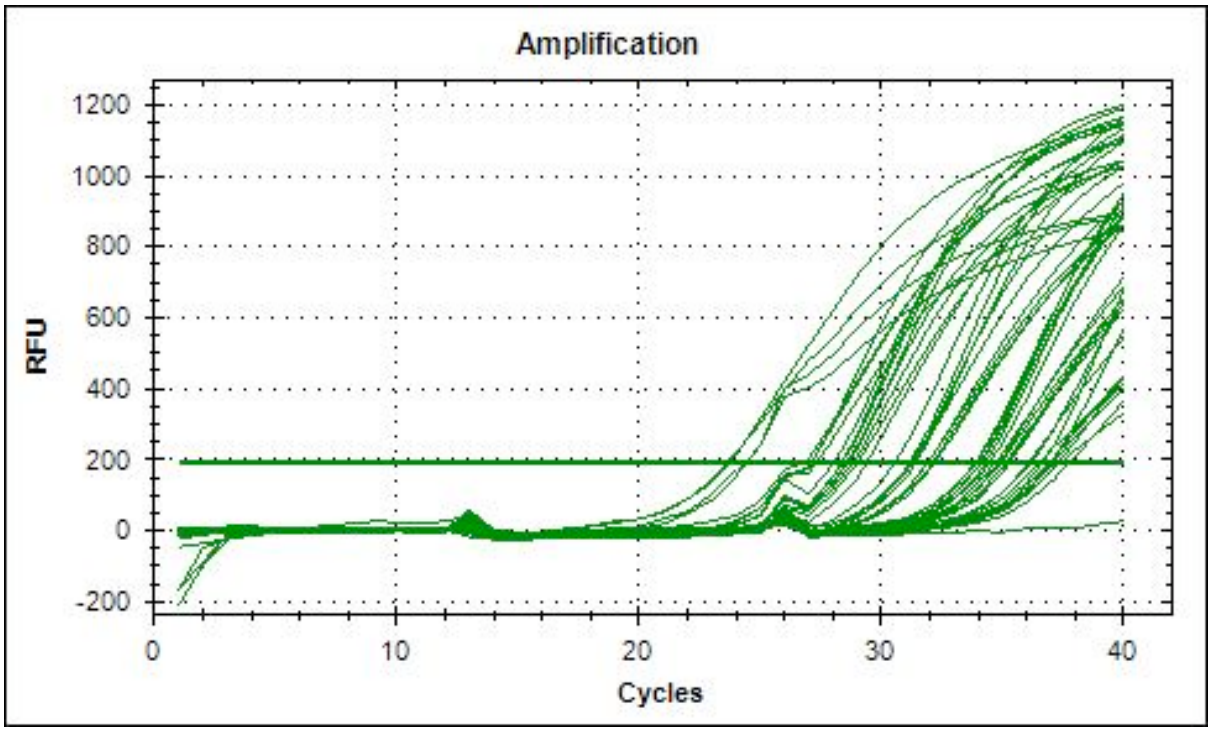


Ex19/20



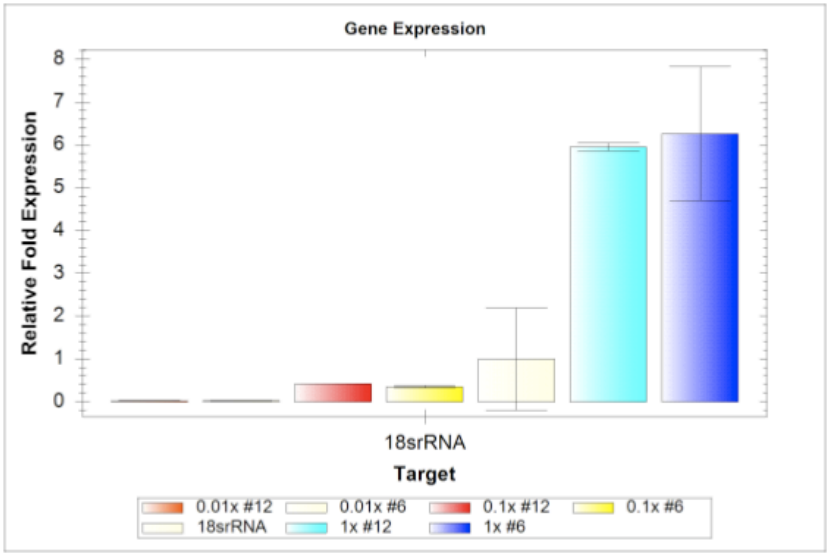


Regular View (not log)

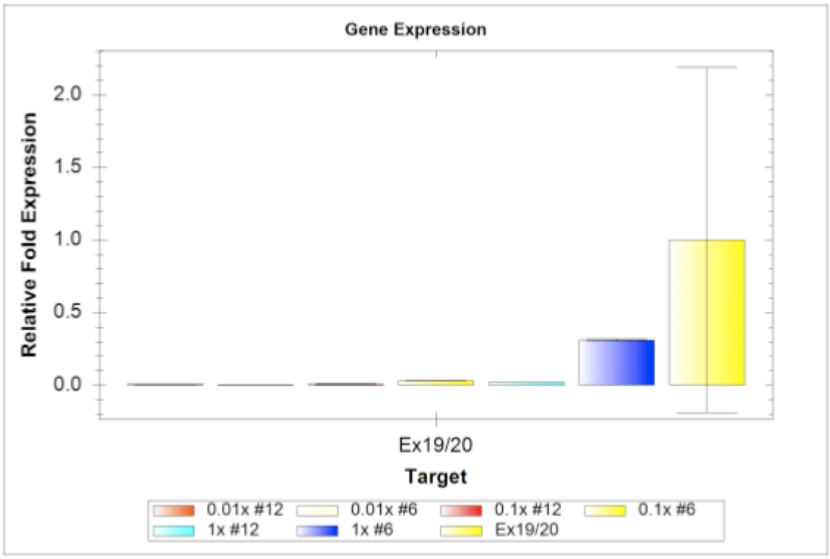


Expression calculations for unknowns

18S rRNA

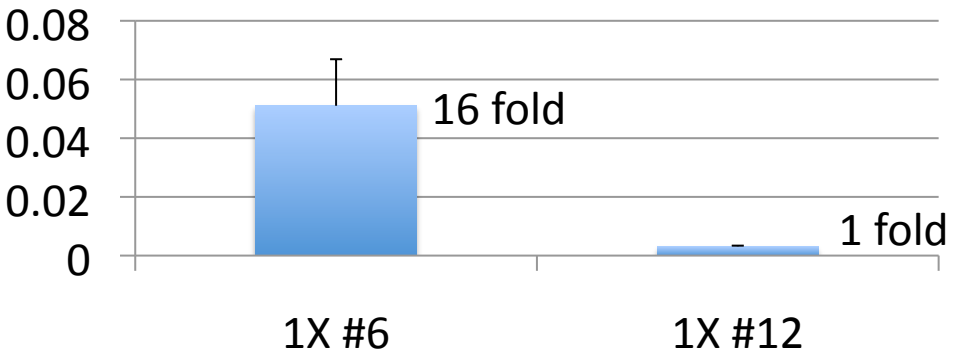


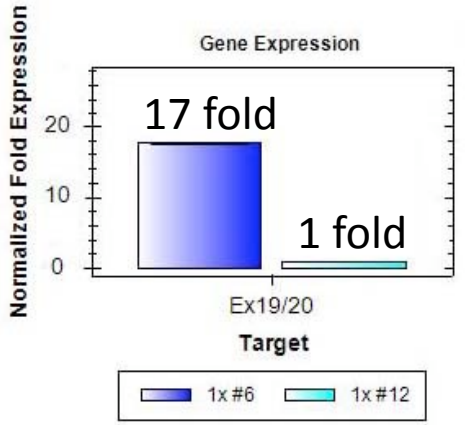
Ex19/20



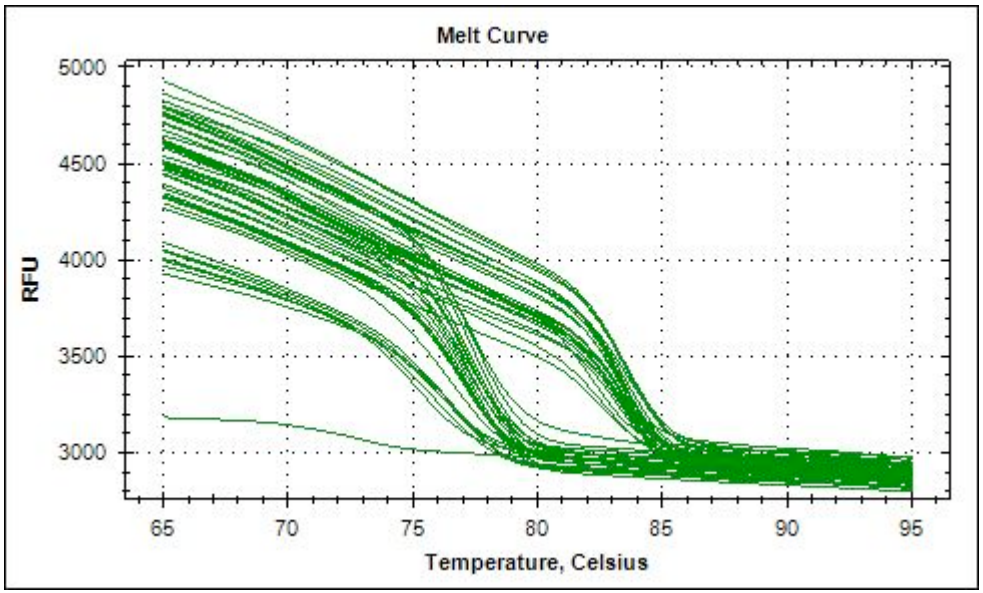
Relative expression between cDNAs #s 6 & 12

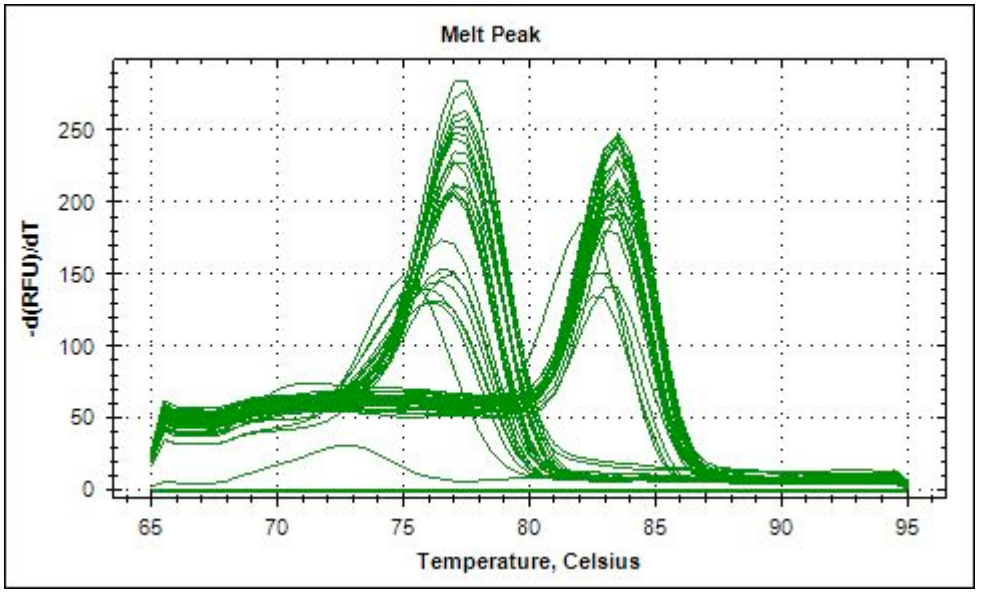
$$\frac{SQ_{Ex19/20}}{SQ_{18S\ rRNA}}$$

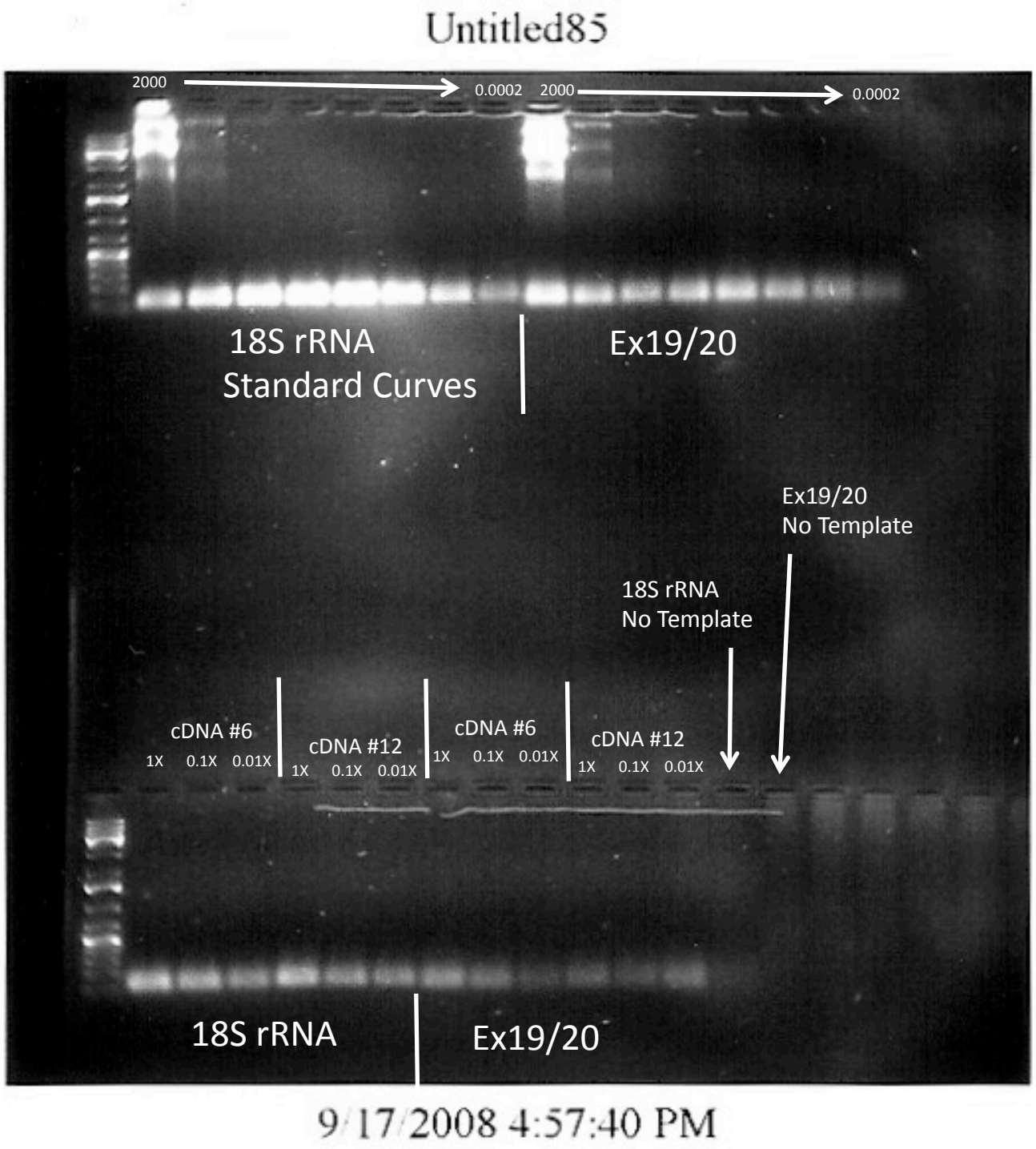




Melt Curve Analysis






Untitled85
2000
0.0002
2000
0.0002
18S rRNA
Standard Curves
Ex19/20
Ex19/20
No Template
18S rRNA
No Template
cDNA #6
1X 0.1X 0.01X
cDNA #12
1X 0.1X 0.01X
cDNA #6
1X 0.1X 0.01X
cDNA #12
1X 0.1X 0.01X
18S rRNA
Ex19/20
9/17/2008 4:57:40 PM