Solicitation S06-182 RNAi Validation

Open Biosystems Inc.

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2 Abstract

RNA interference has become an essential tool to study gene function and the technology has been especially powerful in advancing cancer research. The goal of this proposal is to obtain and functionally validate shRNA constructs in a lentiviral vector system for 136 genes selected by the NCI. Several genome-wide shRNA-based libraries have recently been reported in the literature and our work will focus on the shRNAmir library developed at The Cold Spring Harbor Laboratory. There are unique advantages that make this library stand out: a proprietary design algorithm incorporates the latest knowledge for identifying the best hairpins; all hairpins are embedded in a micro-RNA context that has been shown to increase knockdown efficiency and decrease off-target effects; the lentiviral vector backbone includes a GFP marker for tracking expression and a proven CMV promoter to drive shRNA transcription. By making use of our already existing high throughput laboratory operation, we will test 3 shRNA constructs in two cell lines for all 136 genes for gene knockdown capabilities and report the results. We have chosen two cell lines, T-47D and OVCAR-8 based on the analysis of gene expression data from the NCI database. Given our previous experience with similar types of experiments, we will be able to carry out this work within the 120 day time-frame requested by the NCI. Open Biosystems will be the ideal partner for this project, given our on-going commitment to RNAi technology, the exclusive availability of the shRNAmir library and a strong team of Ph.D. level scientists to carry out this project. Our mission to make cutting-edge technologies openly available to the laboratory researcher will include posting of all validation data obtained as part of this project as well as distribution of the validated constructs in various formats.

3 Definitions and Abbreviations

RT-PCR – Reverse Transcription PCR
QPCR – Quantitative PCR
shRNA – Short Hairpin RNA
siRNA – Short Interfering RNA
RNAi – RNA Interference
dsDNA – Double Stranded DNA
dsRNA - Double Stranded RNA
NCI60 - National Cancer Institute 60

4 Project Description

The SAIC Frederick Inc. is soliciting proposals seeking functionally validated short hairpin RNA (shRNA) constructs for 136 genes selected by the NCI in a lentiviral expression vector system. This contract seeks to cover the acquisition, and/or generation, of specific RNA interference (RNAi) constructs, the biological testing of those constructs, posting of the validation data, and distribution of the material. In response to this solicitation Open Biosystems proposes to produce quantitative PCR data for three shRNAs targeting each of the 136 genes listed in Appendix A in two cell lines. Our reasoning and methodology follows.

4.1 Introduction

The elucidation of gene function is of primary importance in understanding the development and pathology of cancer. RNAi is a valuable tool with which to reveal gene function and thereby identify potential cancer biomarkers and possible therapy targets. RNA interference is an evolutionarily conserved, genetic surveillance mechanism that permits the sequence-specific post-transcriptional down-regulation of target genes. The mechanics of the RNAi pathway have been intensively studied and are outlined in Figure 1. [1-3] [4]

The discovery of microRNA (miRNA) [5] [6] resulted in the development of a new generation of silencing triggers called short hairpin RNA (shRNA), modeled after miRNA hairpin precursors and expressed from DNA vectors. As can be seen in Figure 1 (adapted from Cullen et al. [7]), Drosha in the nucleus cleaves the stem to generate a precursor-miRNA intermediate. [8] This pre-miRNA is transported to the cytoplasm by Exportin-5, [9] [10] where it interacts with Dicer and gets processed into mature miRNAs. The miRNA duplex then interacts with the RNA Induced Silencing Complex (RISC) components, including Argonaute-2, which selectively incorporates the RNA strand whose 5' end is less tightly base-paired. [11] [12] [13] Activated RISC can then down-regulate the expression of homologous mRNAs depending on the level of complementarity between the miRNA and the target mRNA. Since both shRNA and shRNAmir processing are rate limited by enzymatic processing within the cell they are also less susceptible to concentration-dependent off-target effects seen using synthetic siRNA.

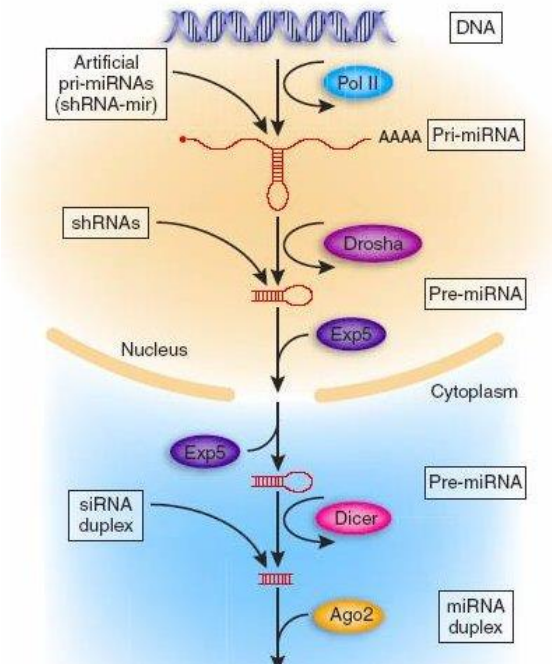


Figure 1. The miRNA biogenesis pathway in vertebrate cells. Artificial siRNAs can enter this pathway as synthetic siRNA duplexes, as shRNAs transcribed by a RNA Polymerase or as artificial pri-miRNAs (shRNA-mir). For simplicity, not all factors involved in miRNA biogenesis are shown. Ago2, Argonaute-2; Exp5, Exportin-5. (adapted from Cullen et al.)

Earlier generation vectors expressing shRNAs were modeled after precursor miRNA and are transcribed under the control of Pol III promoters. [14-16] These shRNAs enter the RNAi pathway earlier than synthetic siRNA, allowing processing by Dicer and active loading into the RISC complex.[17-20] Coincident with advances in our understanding of the miRNA biogenesis pathway shRNAmir adaptations were developed.[14, 15, 19, 20] shRNAmir constructs were designed by replacing the mature miRNA sequence in an endogenous primary-miRNA transcript with gene specific duplexes. The addition of the mir-30 loop and context sequences harnesses endogenous processing by Drosha which increases subsequent Dicer recognition and specificity. shRNAmir constructs thus enter the miRNA biogenesis pathway ahead of either shRNA or siRNA, are processed by both Drosha and Dicer leading to more siRNAs being produced in the cell [16] and subsequently promote active loading into the RISC complex for target mRNA degradation. Rules based design includes destabilizing the 5'end of the antisense strand for strand specific incorporation into RISC allowing for increased and more

specific knockdown. [21, 22] Based on this knowledge and numerous collaborations, Open Biosystems has designed and built-in the most advanced silencing triggers for RNAi studies known to date.

Tangential to the above it was determined that shRNA constructs expressed from plasmid vectors were more versatile than naked siRNAs/shRNA alone, allowing transient transfection, stable integration and *in vivo* RNAi applications. A recent entrant on the gene delivery vector scene, lentiviral vectors overcome deficiencies associated with transient transfection of siRNAs or plasmid-based shRNA expression vectors by integrating directly into the genome of the target cell. Chromosomal integration allows stable, long-lasting expression with little or no evidence for gene silencing as seen with other retroviral-based vectors such as those derived from murine oncoretroviruses. Furthermore, in contrast to other retroviral vectors, lentiviral vectors can efficiently transduce both dividing and non-dividing, post-mitotic cells, including terminally differentiated cells. Lentiviral vectors have been successfully used to deliver cDNAs and shRNAs to cells *in vitro*, to tissues and organs *ex vivo*, and to organs in whole animals. [23] Since no viral proteins are expressed, these vectors cause minimal perturbation to the transduced cells and induce little, if any, immune response in animals. [24] Thus, lentiviral vectors are ideally suited as gene delivery vectors for investigation of cell biology and system biology, and are ideal tools for advancing the development of therapeutics through gene function discovery and target validation.

Open Biosystems has in the last 3.5 years placed itself firmly in the field of RNAi technologies and currently offers the most advanced non-mammalian and mammalian RNAi constructs to researchers. The former includes dsDNA and dsRNA constructs for *Drosophila melangaster*, and a *Caenorhabditis elegans* ORF RNAi feeding library. The mammalian RNAi resources encompass human, mouse and rat libraries which have been developed using the shRNA [14, 15] (http://www.broad.mit.edu/genome_bio/trc/) and the shRNAmir design [15, 16, 20] with Pol III and Pol II promoters respectively. The shRNAmir libraries are available in both retroviral and lentiviral vectors [15, 16, 20, 25] and target a large portion of the genes in the human, mouse and rat genomes. The availability of these ready to use vector-based genome-wide resources for RNAi (see www.OpenBiosystems.com) removes the need to design individual constructs and clone them into expression vectors, a bottleneck that had limited the widespread adoption of RNAi technology.

As stated in the solicitation, there are numerous RNAi systems with disparity among them and a lack of consensus in the scientific community. Variation stemming from hairpin design, the incorporation of epigenetic elements, vector choice, gene sequence target, transfection protocol and cell type all contribute to the need for validation of sequences that reliably down regulate target genes.

4.2 Problem Statement (or statement of need) and significance

To meet the goals outlined by this solicitation in the time allotted the strategy must streamline each critical point in the process including algorithm design, vector design, experimental design and choice of cell lines.

4.2.1 Algorithm and shRNA design justification

Algorithms used to select the hairpin sequences were derived based on the following general guidelines in conjunction with Tom Tuschl's rules [26] and a proprietary algorithm from Rosetta Inpharmatics:

General guidelines:

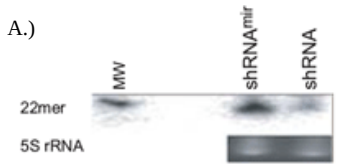
1. siRNA targeted sequence is usually 21nt in length.
2. Avoid regions within 50-100 bp of the start codon and the termination codon
3. Avoid intron regions
4. Avoid stretches of 4 or more bases such as AAAA, CCCC
5. Avoid regions with GC content <30% or > 60%.
6. Avoid repeats and low complex sequence
7. Avoid single nucleotide polymorphism (SNP) sites
8. Perform BLAST homology search to avoid off-target effects on other genes or sequences

Tom Tuschl's rules

1. Select targeted region from a given cDNA sequence beginning 50-100nt downstream of start condon
2. First search for 23nt sequence motif AA(N_{19}). If no suitable sequence is found, then,
3. Search for 23nt sequence motif NA(N_{21}) and convert the 3' end of the sense siRNA to TT
4. Or search for NAR(N_{17})YNN
5. Target sequence should have a GC content of around 50%

A = Adenine; T = Thymine; R = Adenine or Guanine (Purines); Y = Thymine or Cytosine (Pyrimidines); N = Any.

These guidelines and rules have been used in conjunction with the destabilization of the 5'end of the antisense strand for strand specific incorporation into RISC allowing for increased and more specific knockdown. [21, 22] In addition to solid algorithms for hairpin sequence selection our new generation shRNAmir design adds increased performance over the earlier generation shRNA. To validate the performance of the new generation shRNAmir design relative to the earlier generation shRNA, shRNA and shRNAmir constructs were designed to express sequence targeting firefly luciferase. [13] The sequence was inserted such that an identical mature small RNA would be generated from each construct after endogenous processing. Dose-response experiments for shRNA have indicated that repression correlates very well with the amount of siRNA delivered. [18] HEK293 cells were transfected with shRNA-luc and shRNAmir-luc, and assayed for resulting cellular siRNA by northern blotting. Cells transfected with shRNAmir-luc contained ~12 times more siRNA than did cells transfected with shRNA-luc. This shows that the shRNAmir design was much more efficient than shRNA at being processed into siRNA (Figure 2 A). In a separate experiment using a fluorescent reporter assay measuring knockdown, multiple shRNA and shRNAmir constructs directed against proteasomal genes were compared for their gene silencing ability. Multiple shRNA and shRNAmir constructs directed against proteasomal genes were compared for their gene silencing ability [16] See Figure 2 B for results. Overall, shRNAmir constructs produced increased and more consistent knockdown compared to first-generation shRNA constructs.



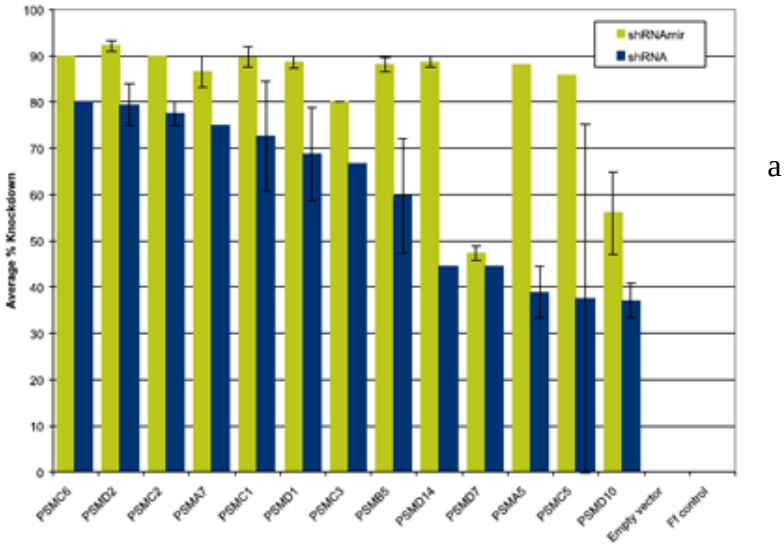
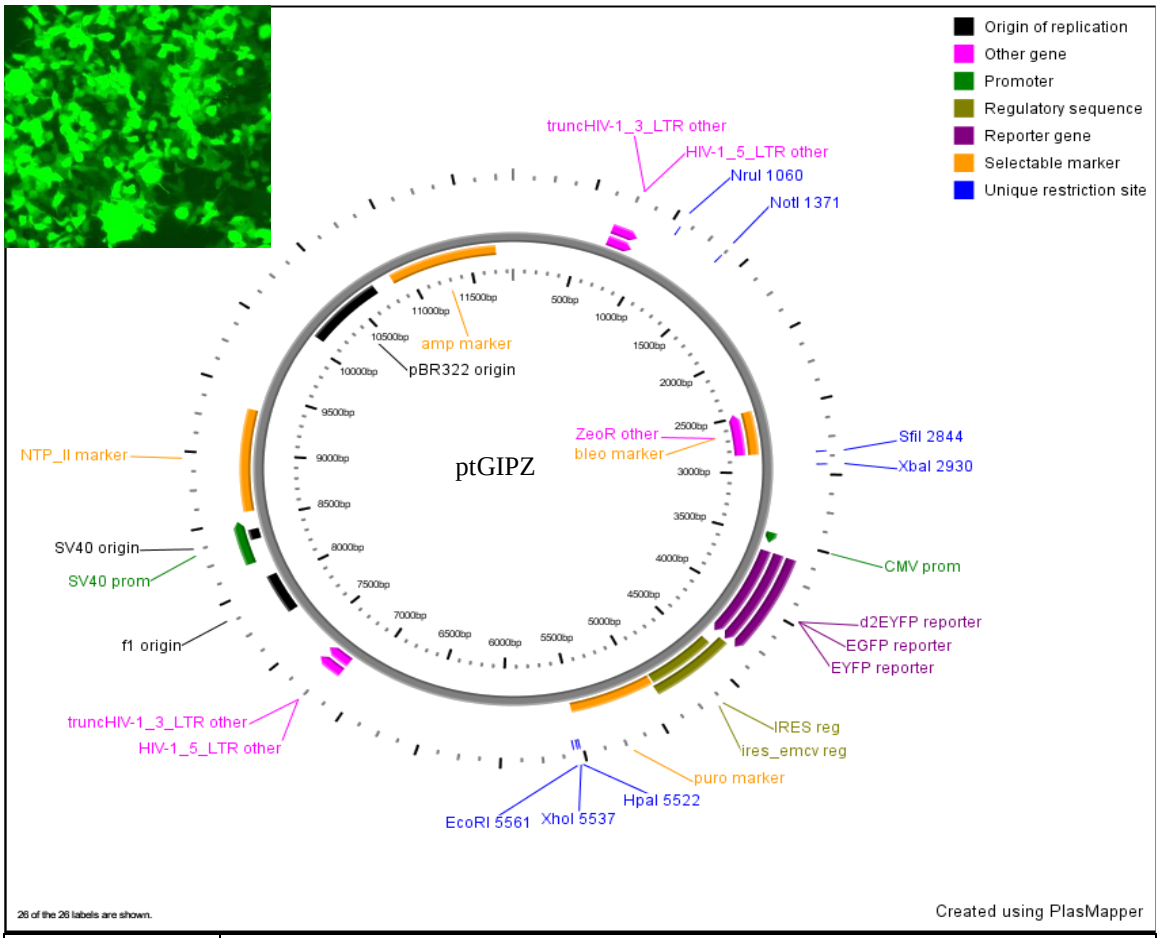


Figure 2. A.)Northern blotting was used to detect the mature siRNA produced after transfection of HEK 293 cells with shRNA-luc or shRNAmir-luc. Transfection was normalized using a co-delivered dsRed expression plasmid. A 12-fold increase in the siRNA produced after shRNAmir-luc processing was detected relative to shRNA-luc (shRNA) processing. B.) Comparison of gene silencing with shRNA vs shRNAmir. Green bars indicate % knockdown using shRNA-mir constructs and blue bars indicate shRNA constructs. Data is plotted as average % knockdown from multiple constructs (26 from shRNA and 29 from shRNAmir).

4.2.2 Vector justification

The retroviral shRNAmir library has been transferred into the ptGIPZ lentiviral vector developed and validated at Open Biosystems. The ptGIPZ vector has been modified from the pPRIME-CMV-GIN vector [25] using the latest in lentiviral technology. The shRNAmir sequence and the tGFP reporter are co-expressed by a CMV promoter. Co-expression of the shRNAmir and tGFP from the same promoter allows us to easily identify which cells in the culture are expressing the shRNA. Addition of an IRES-puro in the vector further allows selection and expansion of only shRNA-expressing cells (see Figure 3 for a summary of additional vector elements).



Vector Element	Utility
CMV Promoter	RNA Polymerase II promoter
cPPT	Central Polypurine tract helps translocation into the nucleus of non-dividing cells
WRE	Enhances the stability and translation of transcripts
GFP	Marker to track shRNAmir expression
IRES-Puro	Mammalian selectable marker
AMPr	Ampicillin bacterial selectable marker.
5'LTR	5' long terminal repeat
pUC ori	High copy replication and maintenance of plasmid in E.coli
SIN-LTR	3' Self inactivating long terminal repeat
RRE	Rev response element

Figure 3. Lentiviral vector components are represented schematically and are listed in the table. The insert depicts the tGFP fluorescence seen in all transduced cells.

Lentiviral vectors are ideal tools for advancing therapeutics through gene function discovery and target validation. One of the major safety concerns with the use of lentiviral vectors is the possibility of producing replication competent lentiviruses (RCL), through recombination of the gene transfer vector with the helper segments co-expressed *in trans* during packaging. At Open Biosystems, we use TranzVector™, a lentiviral vector system distinguished for its efficiency and safety relative to alternative methods. Through additional splitting of helper components (packaging) onto separate genetic elements [27], we have virtually eliminated the possibility of generating RCL. This design ensures a gene delivery vehicle that is orders of magnitude safer than all other lentiviral vectors currently in use. Extended discussion of the vector design and safety aspects can be found in Wu *et al*. [27, 28, 29] In addition to the safety features, the gene transfer vector (ptGIPZ) contains elements that are essential for high efficiency transduction (polypurine tract/central termination sequence or DNA flap); [30] and for high level, sustained expression of the transgene. [31] Protocols are well established for generating TranzVector™ viral stocks expressing shRNAs with titers (unconcentrated) generally ranging from 1 to 3 x10^7 transducing units per milliliter. These methods have also been adapted at Open Biosystems to produce viral stocks in high throughput using a 96-well plate format. As a consequence of the superior algorithm design, the mir30 adaptation, the GFP reporter, and our extensive experience with this lentiviral vector, we are convinced that this design will provide the highest degree of performance and holds the most promise for achieving the goals set forth in the solicitation.

4.2.3 Cell Line Justification

As the solicitation states, "Each construct must be validated in at least one breast cancer cell line and one other cell line." To maximize our chances of success we utilized our ability to further analyze the most recent NCI60 gene expression database. The construction of this database was a tripartite collaboration between the Brown/Botstein laboratories, John Weinstein's group at the Laboratory of Molecular Pharmacology, and the Developmental Therapeutics program both at the National Cancer Institute, Bethesda MD. Initially 60 cell lines were analyzed for gene expression on a 9,000 cDNA microarray by researchers at Stanford University. [32] Subsequently, these same cell lines were re-evaluated for gene expression levels on a 20,000 cDNA microarray. The later findings have been communicated to us via our collaboration with Dr. Douglas Ross and Applied Genomics Incorporated (AGI, http://www.applied-genomics.com/). Our purpose in this analysis was to eliminate probable knockdown failures due to absent or extremely low expression of the target gene in a particular cell line. Focusing on the 136 target genes our analysis is summarized in Appendix A. Our investigation brings at least two points to light. First, to have the greatest probability of successful validation while developing a strategy of attack that realistically fits the time allotment, it is apparent that the T-47D (breast cancer) and OVCAR-8 (ovarian cancer) cell lines are the most suitable choices for initial analysis as they express the highest percentage of the target genes (see Table 1). These two lines also possess other favorable properties such as adherence, and ease of growth. Second, there are a fair number of genes whose expression falls below a GFP intensity cutoff of 1000 in any of the cell lines or are sporadically expressed in isolated cell lines, thus not qualifying for a high throughput primary screen. GFP intensity is the standard unit of measurement used in the NCI60 microarray analysis. This cutoff was decided based on the knowledge (personal communications AGI and Rosetta Inpharmatics) that a cutoff of 1000 GFP intensity will provide us with the most direct and reliable route by which to capture the largest sum of data in a high throughput

Table 1. Summation of targets and chosen cell lines.

# Targets	T-47D	OVCAR-8
73	√	√
10	√	NED$^{\delta}$
16	NED$^{\delta}$	√
19	NED$^{\delta}$	NED$^{\delta}$
3*	NED$^{\delta}$	NED$^{\delta}$
14	No data	No data

* = These genes are present in the database but fail to show gene expression in any of the NCI60 cell lines.
$^{\delta}$ NED = no expression detected.

manner. However, it should be noted that it is possible that these genes will express to a degree amenable to quantitation in the two chosen cell lines.

4.3 Goals and Objectives

Open Biosystems proposes to test 3 shRNAs targeting each of the 136 genes listed in Appendix A by QPCR in two cell lines. The knockdown will be measured as a percent of gene expression as compared to a non-silencing control. This non-silencing control has been designed by Open Biosystems to be identical to vectors used to knockdown target genes with the exception that the shRNA itself has no homology to any sequence in the human, mouse or rat genomes. Therefore, the non-silencing control mimics the process of transduction and stimulation of the RNAi pathway without inducing direct knockdown of any gene (see figure 8c). Each shRNA will be measured for its ability to knockdown the target gene in two cancer cell lines when compared to a non-silencing control using the average of triplicate QPCR reactions of duplicate wells for viral transduction.

4.4 Procedures and Methods

4.4.1 Summary of procedure

Our strategy for validating shRNAs targeting the 136 genes in Appendix A can be briefly summarized as follows. Open Biosystems already has 3 or more shRNAs available for 89 of the 136 genes. Additional shRNAs are also available for the remaining 47 genes leaving only 68 additional shRNAs to be constructed to provide full coverage of the 136 target genes at 3 shRNAs per gene. All shRNAs for this study are or will be sequence validated prior to virus production. Two cell lines, T-47D and OVCAR-8 will then be transduced with viruses expressing each of the 408 (3 shRNA*136 target genes) shRNA using an established robotics protocol. After 48 hours in culture all transduced cells will be lyzed, the total RNA extracted and cDNA synthesized. We are convinced this strategy, as outlined in further detail below, will lead to the most rapid, cost effective validation of shRNA clones.

4.4.2 Clone acquisition/construction

Open Biosystems has a well-established long-term relationship with the Cold Spring Harbor Laboratory (Gregory Hannon, Ph.D., Scott Lowe, Ph.D.), Harvard Medical School (Stephen Elledge, Ph.D.), and the Rosetta Inpharmatics Scientific Foundation. These organizations have been instrumental in developing large mir-adapted shRNA libraries using rules based algorithms. Credence to the design and efficacy of the shRNAs, as well as the vector, is lent by data from independent investigators as well as internally, showing validation of library clones. [25, 33] The Rosetta Inpharmatics Scientific Foundation has provided Open Biosystems with a choice of 6 predesigned shRNA sequences intended to knockdown the 136 target genes listed in Appendix A (Figure 4). As stated above many of these shRNA are already available to be cloned into the ptGIPZ vector at a cost of $5,500. However, there are 68 additional clones needed to meet the 3 shRNA per

A.) A single strand 97nt “mir30-like” DNA oligo (Note that this oligo is not inclusive of the *Bam*HI/*Xho*I cloning tails)

GCTGTTGACAGTGAGCG AGCTGCTGGTGCCAACCCTATT
TAGTGAAGCCACAGATGTA AATAGGGTTGGCACCAGCAGCG
TGCCTACTGCCTCGGA

- Sense target sequence (black)
- Antisense target sequence (blue)
- Common mir30 contexts regions (red)
- mir-30 loop (green)

B.)

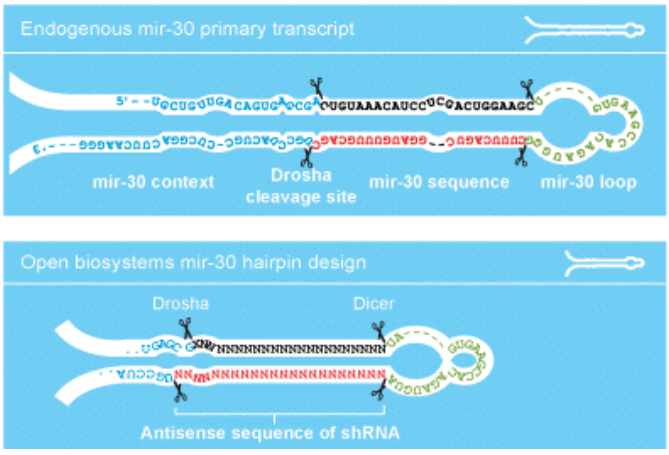


Figure 4. A.) Example shRNA oligo. B.) Example shRNA oligo folded into a hairpin.

target requirement each requiring two oligos to be constructed (making them double stranded). Therefore, the total number of oligos to be synthesized equals 136 (68 shRNA*2 oligos = 136 oligos). Open Biosystems has a current price quote from Operon Inc. for the total oligo production of $3,754 (136 Oligos*115bp*$0.24per bp = $3,754) (see Appendix C). This cost is minimal in light of the ability to maintain consistency throughout the project. The oligo synthesis will be done such that each oligo of a pair is represented in coordinating wells in 96-well format at a normalized concentration. Each oligo will carry the mir adaptation, a unique shRNA sequence, and *Bam*HI/*Xho*I complementary overhangs to allow for efficient ligation into an already prepared and confirmed ptGIPZ vector cut with *Bam*HI and *Xho*I. Oligo pairs will be amalgamated using a Qiagen Rapid Plate robot into a PCR plate and annealed via a melting step followed by a slow cool down in a PCR machine. Annealed oligos will then be pooled in a normalized fashion and ligated to the ptGIPZ vector cut with *Bam*HI and *Xho*I in a single ligation reaction. Pooling allows for a more efficient and reliable means of cloning as it reduces the need for and cost of many individual ligation reactions, and does not add to the sequencing burden. The ligation mix will then be transformed into Prime+™ competent cells that Open Biosystems has found to limit the inherent tendency of viral vectors to recombine. Cells will be plated onto agar with appropriate antibiotic selection and colonies picked. Putative clones will then be screened and identified via sequencing. Our informatics team has developed software allowing rapid alignment of hairpin sequences to given putative clones. Open Biosystems researchers have extensive experience with this cloning strategy, encountering a high degree of success. Our data currently indicates an 82% retrieval rate for non-normalized pools. We anticipate an increase in this percentage when using a normalized oligo pool proposed here and sequencing putative clones to the point of 3-fold representation. We anticipate the need to sequence 3 96-well plates of putative clones to obtain the desired number of shRNAs for validation efforts. Open Biosystems has obtained a quote from Lark Inc. outlining the sequencing costs anticipated as $1,152 for 3 plates (see Appendix D). After sequencing, newly verified clones as well as those clones already in hand will be condensed using a Tecan Genesis robot into 5 96-well plates. The non-silencing control will be added at this time to the last column of the plate. Backup copies of the rearrayed clones will be made, and DNA isolated in 96-well format using Qiagen turbo 96-well DNA isolation kits and a Qiagen Rapid Plate robot. If dropout in the above collection is encountered, the TRC collection will be used.

4.4.3 Viral production/transduction of cells and cDNA synthesis

Open Biosystems has a laboratory facility dedicated to the high throughput production of transgenic lentiviral vectors and is staffed with members experienced in lentiviral production. The facility meets safety specifications for lentiviral production and houses all of the cell culture equipment, and robotics necessary under BSL-2 conditions. To produce lentiviral stocks, each gene transfer vector expressing an shRNA will be transfected into HEK293T cells using a $CaPO_4$ method along with packaging and envelope components, (pTRE-Gag-Pro, pCMV-Vpr-RT-IN, pCMV-VSV-G-poly A, pTRE-Tat/Rev, and pCMV-tetoff). Transfections will be carried out using the Qiagen Rapid Plate robot in 96-well format. The titers (transducing units per ml) of viruses generated using the transfection procedure described above generally have fallen within a tight range between 1 to 3 x 10^7 TU/ml. Each of the shRNA vectors co-express GFP, which is used to quantitate titers using flouorescence microscopy. We will confirm the titers on a number of viral stocks by transducing (infecting) both T-47D and OVCAR-8 cells with serial dilutions of the virus. 48 hours post-transduction, the titers will be scored by counting GFP-positive cells under a flourescent microscope. To obtain a rough estimate of the titers and assess well-to-well variability on all of the viral stocks produced, supernatants from each well will be used to transduce (infect) both T-47D and OVCAR-8 cells seeded in a 96-well plate. The Qiagen Rapid Plate robot will again be used to transfer virus into the cultures. Two days following infection the 96-well plates will be scanned in a plate reader to measure GFP intensity from each well.

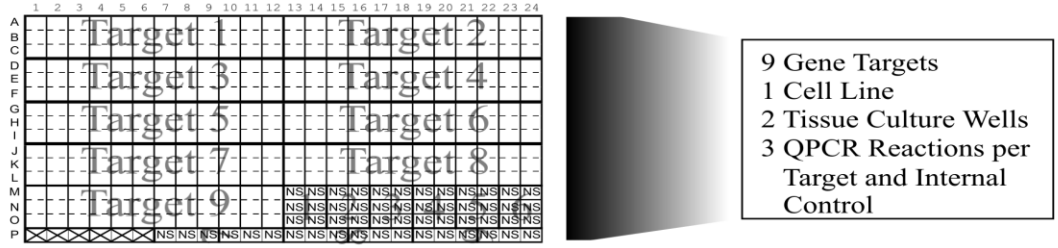
The titer determinations will allow us to establish the amount of virus need to transduce T-47D and OVCAR-8 so that every cell in the culture is expressing the shRNA. All 3 shRNAs to each target will be transduced into both T-47D and OVCAR-8 (in duplicate) in one high throughput round of transductions.

Transduced cells will be lyzed and stabilized in SideStep Lysis and Stabilization Buffer (Stratagene). All, the samples will then undergo cDNA synthesis using the random primer scheme (ABI High Capacity cDNA Archive Kit) in high throughput using the Qiagen Rapid Plate robot along with multiple PCR machines. The total number of 96-well plates to convert to cDNA will be 17. The cost of the ABI Archive kits will total $4,165 (see Appendix E).

4.4.4 **Quantitative Real-time PCR (QPCR) validation of clones**

All QPCR will be conducted using ABI's pre-designed, pre-optimized Taq-man® Gene Expression Assays available for all 136 target genes ($25,651 see Appendix E). QPCR studies will be conducted at Vanderbilt's core facility (http://www.vmsr.org/) see Appendix E. We will use our Tecan Genesis robot to strategically rearray clones and primer/probe sets for QPCR in 384-well format in order to minimize the number of QPCR runs (Figure 5). Using this format, a single 384-well plate can be used to test 3 shRNAs to 9 targets using duplicate wells for the transduction as well as triplicate QPCR reactions. Combining this with a non-silencing control as a reference standard and 18S rRNA as an internal QPCR control ensures the most accurate results possible. The Vanderbilt Microarray Shared Resource has the capacity to run 20 384-well plates in a 24-hour period. Using this facility's machine will allow us to screen 3 shRNA to all 136 target genes in a single cell line in one 24 hour cycle of the machine and the entirety of the project in just two 24 hour runs. Our setup is designed to make the most efficient use of space in the 384-well plate. All data analysis will be done at Open Biosystems. We will be using our ABI Sequence Detection Software in conjunction with the ΔΔCt method to calculate gene expression levels ($\Delta\Delta Ct = \Delta Ct_{,\ NS} - \Delta Ct_{,\ \text{gene of interest}}$). [34] The standard deviation for gene expression measured in the described pipeline will be determined and this will be used to calculate our confidence level for percent knockdown. All knockdown percentages will be given as a percentage of the non-silencing control. The ABI analysis software will highlight outliers within the triplicate QPCR reactions indicating a possible need to repeat the sample. For a workflow of the process see Figure 6.

A.)



B.)

Row	Column	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	shRNA	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3
	Cell culture repetition	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
	QPCR repetition	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	QPCR primer/probe set	T	T	T	T	T	T	IC	IC	IC	IC	IC	IC	T	T	T	T	T	T	IC	IC	IC	IC	IC	IC
B	shRNA	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3
	Cell culture repetition	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
	QPCR repetition	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	QPCR primer/probe set	T	T	T	T	T	T	IC	IC	IC	IC	IC	IC	T	T	T	T	T	T	IC	IC	IC	IC	IC	IC
C	shRNA	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3
	Cell culture repetition	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
	QPCR repetition	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	QPCR primer/probe set	T	T	T	T	T	T	IC	IC	IC	IC	IC	IC	T	T	T	T	T	T	IC	IC	IC	IC	IC	IC

T- Target of shRNA
IC- Internal control

Figure 5. Optimized QPCR 384-well set up. Nine gene targets for one cell line can be assayed in one 384-well QPCR plate. The RNA from two tissue culture transductions per short hairpin will be assayed. The targeted mRNA, internal RNA control, and non-silenceing (NS) will be measured in three QPCR reactions each. A.) One possible layout of a 384-well QPCR plate.

The NS treated samples will be used to normalize the measurements to 100%. B.) Detailed view of each well for two gene targets.

4.5 Figure 6. Workflow and validation of the shRNAs by QPCR in the cell lines.Evaluation

Progress on the project will be gauged according to the timeline in Figure 7. We will consider the project "on track" should we remain on this schedule.

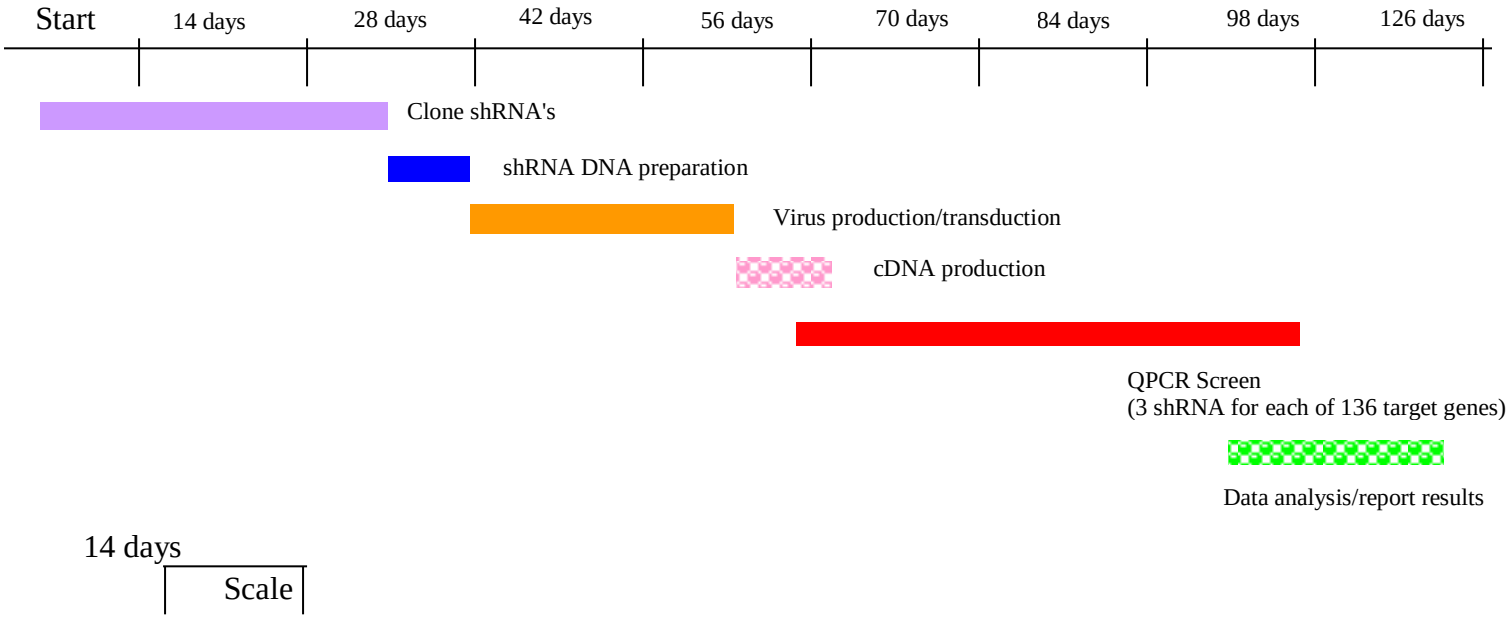


Figure 7. Timeline depicting workflow.

Based on the NCI60 gene expression data we have chosen to conduct our screens in two cell lines, T-47D and OVCAR-8, these two lines provide us the highest probability of success. It should be noted that there are many target genes for which there is no obvious cell line choice for meeting the criteria. While we are reasonably confident in our gene expression analysis results, there remains the possibility that testing these problematic genes in T-47D and OVCAR-8 will yield good results as this prediction is based solely on the gene expression profiles and the cutoff points we set (GFP intensity =1000). This cutoff was decided based on the knowledge (personal communications AGI and Rosetta Inpharmatics) that a cutoff of 1000 GFP intensity will provide us with the most direct and reliable route by which to capture the largest sum of data in a high throughput manner.

4.6 Dissemination

This solicitation from SAIC Frederick Inc., requests: "any deliverables required under this subcontract, shall be packaged, marked, and shipped in accordance with commercial standards, or as specified herein. At a minimum, all deliverables shall be marked with the subcontract number and subcontractor name. The subcontractor shall guarantee that all required materials be delivered in usable and acceptable condition." Open Biosystems undertakes to disseminate the results and products from this project both physically and electronically within the guidelines stipulated by the solicitation. The deliverables generated from this validation study can be summarized under the following headings:

4.6.1 Data

The data generated will be annotated with regard to their ability to knockdown a particular gene as well as the cell lines in which they were tested. This annotated data regarding target sites and validation results will then be posted publicly in the following databases: a) the CGAP RNAi web site (http://cgap.nci.nih.gov/RNAi), in a format provided in consultation with the Scientific and Contract Officer b) http://www.ncbi.nlm.nih.gov/genome/RNAi/ the RNAi database at NCBI and c) the searchable database located at http://www.openbiosystems.com. The data for the 136 genes will be made available on the NCI CGAP website and submitted to the NCBI RNAi resource within two weeks of validation. As Open Biosystems already posts their data on the NCI CGAP website, facilitation should be straightforward. Refer to Table 2 for an example of the data that will be made available.

Table 2. Representative sample of annotated validation data.

										% Knockdown in Cell Line	
Oligo ID	Current Accession	Plate	Row	Col	Species	Antibiotic	Vector	Sequence	Gene	T-47D	OVCAR-8
v2HS_58743	XM_296897	2001	a	1	Human	CAM/KAN/TET	pSM2c	TGCTGTTGAC AGTGAGCGAG CAGTGGACCT etc.	LOC342479 (LOC342479) mRNA.	87%	82%
v2HS_64003	NM_013390	2001	a	2	Human	CAM/KAN/TET	pSM2c	TGCTGTTGAC AGTGAGCGAC GAGATAAAGT etc.	Trans membrane protein 2 (TMEM2), mRNA.	84%	86%

4.6.2 Lentiviral clones

Due to Open Biosystems' experience in high throughput manufacturing for researchers, the in-house quality control procedures, established shipping protocols and vendors, as well as superior delivery times and competitive pricing; we are well placed to provide hassle-free and expedient distribution to researchers wishing to acquire the validated shRNA clones. These lentiviral clones will be available in 3 formats: glycerol stocks, vector DNA and the actual viral particles. In addition, as Open Biosystems is at present the designated distributor, 3 shRNA constructs for each of the 136 genes will be available to everyone in the scientific community with immediate effect and no later than two weeks after completion of the work, and will be distributed readily as determined by the NCI using established NCI distribution procedures. All deliverables will be packaged, marked, and shipped in accordance with commercial standards in useable and acceptable condition. For an illustration of Open Biosystems' packaging format see Appendix B. Due to our unique position in this field Open Biosystems already has a trained technical support staff familiar with viral vectors and RNAi ready to assist with any questions the researchers may have.

4.6.3 Reports

As requested in the solicitation, electronic monthly technical reports will be provided to the subcontract specialist Jeanne Lewis (jlewis@ncifcrf.gov) on the 2nd day of every month, and will be generated for each month of contract performance detailing the status and progress of the project. A final report will be generated and emailed likewise within 14 days of the completion of the project.

5 Information regarding vendor, employees and previous work relevant to this RFP.

As highlighted in the introduction, Open Biosystems has placed itself firmly in the field of RNAi technologies (see Table 4 for referees as requested by solicitation). We have on the premises the entire retroviral RNAi libraries of human, mouse and rat, as well the TRC and Hannon mir-adapted lentiviral collections. This speaks for our familiarity and ability to design, generate, clone, sequence-verify and propagate stocks of these collections. As this validation project constitutes a number of labor intensive steps that require robotics for accurate and efficient handling; our manufacturing laboratories are well equipped with resources and experienced staff to effectively handle the load, in both 96-well and 384-well formats; at the level of cloning, shRNA vector preparation, virus production, cell line transduction, cDNA synthesis and QPCR setup (Table 3). We have provided shRNA collections and sub-collections in glycerol stocks, DNA and virus for academic and commercial customers as well as in-house studies. In the latter we have shown consistently high transfection and transduction efficiencies as well as conclusive knockdown of a number of genes (viz. *GAPDH, EG5, Tes* and others) at the phenotypic, mRNA (QPCR) and/or the protein level (see Figure 8).

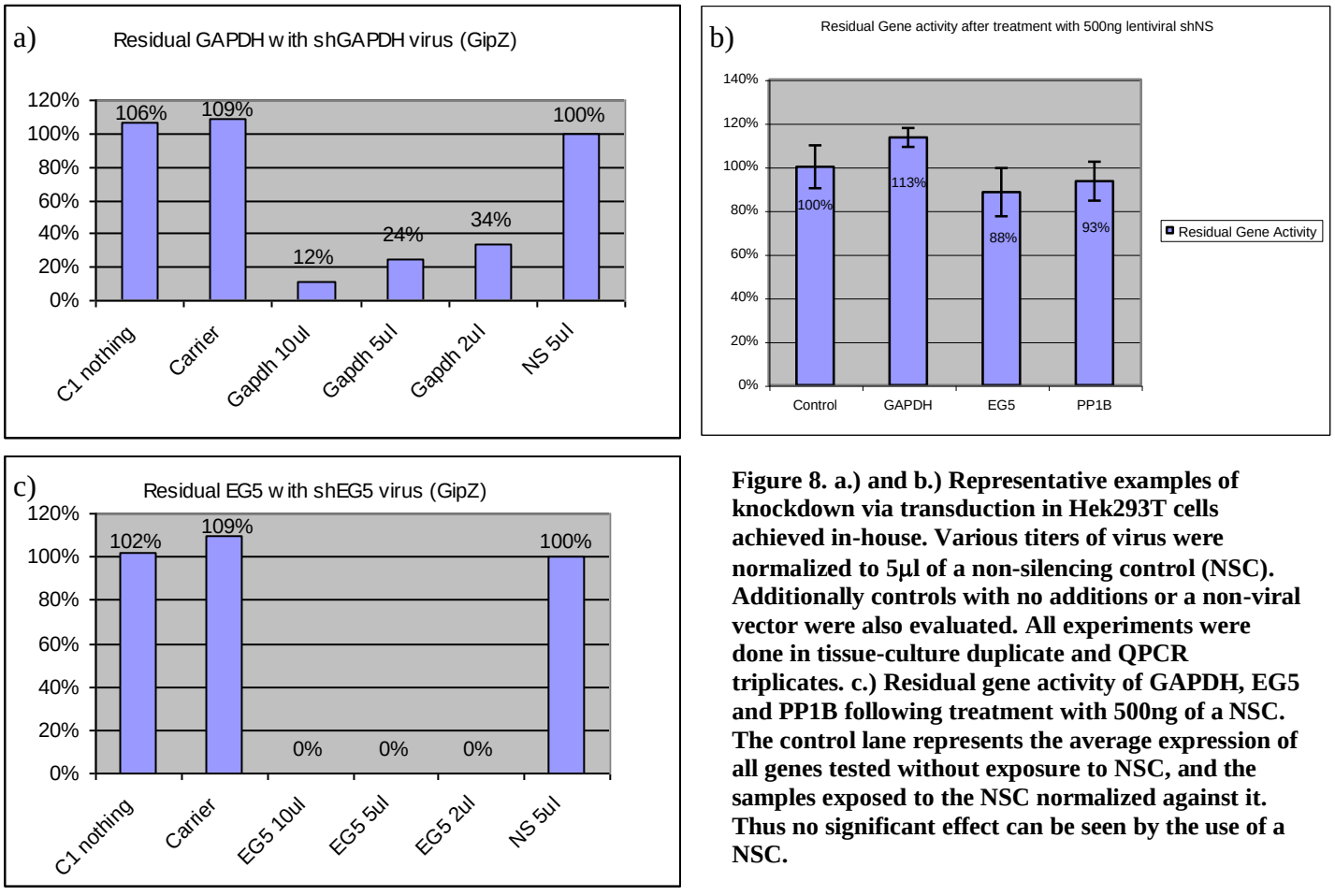


Figure 8. a.) and b.) Representative examples of knockdown via transduction in Hek293T cells achieved in-house. Various titers of virus were normalized to 5µl of a non-silencing control (NSC). Additionally controls with no additions or a non-viral vector were also evaluated. All experiments were done in tissue-culture duplicate and QPCR triplicates. c.) Residual gene activity of GAPDH, EG5 and PP1B following treatment with 500ng of a NSC. The control lane represents the average expression of all genes tested without exposure to NSC, and the samples exposed to the NSC normalized against it. Thus no significant effect can be seen by the use of a NSC.

Our choice of The Vanderbilt Microarray Shared Resource to carryout our 384-well QPCR analysis, is self evident in their ability to do high throughput QPCR analysis, not only as a core facility for their own institution, but also their capability to handle large contract jobs. Finally, we have an expert team of bioinformatic staff that deals with data retrieval, analysis and annotation of databases on a scale of some 10,493,020 distinct cDNA clones and 153,148 distinct shRNA clones just to name two examples.

Table 3. Relevant Information regarding vendor, employees and previous work relevant to this RFP.

Name	Relevant Experience
Rusla Du Breuil, Ph.D. (See attached Biographical sketch)	Over 16 years supervisory experience in clinical and research laboratory settings. Experienced in Quantitative PCR, Mammalian RNAi, gene expression monitoring and molecular biology.
Jamie Cearley, Ph.D. (See attached Biographical sketch)	3 years of experience in mammalian RNAi. Over 13 years of laboratory experience in molecular biology, cloning and transgenic mouse development.
John Wakefield, Ph.D.	16 years experience with HIV and development of lentiviral vectors. Over 7 years of supervisory experience of research staff developing and producing lentiviral vectors.
Andrew Crouse, Ph.D.	3 years of Quantitative PCR experience in monitoring effects of inhibitory small molecules on endogenous gene expression. Over 9 years of laboratory experience in molecular biology, cloning and transgenic mouse development.
Nikolay Korokhov, Ph.D.	Over 10 years experience with molecular biology and gene expression utilizing adenovirus vectors.
Karin Schmitt, Ph.D.	Over 10 years of supervision of genome scale projects. Experienced in molecular biology, mammalian & non-mammalian RNAi and cloning.
Irina Vasenkova, Ph.D.	3 years of experience in Drosophila RNAi, fluorescent microscopy and cell culture. Over 10 years of molecular biology laboratory experience.
Miroslav Novak, Ph.D.	Over 23 years of virology experience.
Roy C. Paul III, MS Comp. Sc.	Over 12 years experience in software development on PC, Unix, Macintosh. 10 years experience in Bioinformatics. Experienced in clone data management, BLAST integration and analysis, data mining, and Microarray systems.
David Kloske, MS Comp. Sc.	16 years experience designing, developing and deploying commercial and enterprise software solutions.

Table 4. Three References

Greg Hannon Cold Spring Harbor Labs hannon@cshl.edu (516) 367-8889	Stephen J. Elledge Harvard Medical School selledge@genetics.med.harvard.edu (617) 525-4510	Marc Vidal Dana-Farber Cancer Institute Marc_Vidal@dfci.harvard.edu (617) 632-5180

6 Budget

The vendor will perform the work as specified for the firm fixed price of **$260,486.**

Materials and Supplies

Cloning

Oligos	$3,754	
Competent cells and enzymes	$328	
Sequencing	$6,192	
DNA isolation	$750	
Plastics	$100	
Rearraying	$30	

Transfection/transduction (17 96-well plates)

Cell lines	$500	
DNA isolation	$1,063	
RNA stabilization	$1,700	
Plastics/media	$489	

cDNA synthesis (17 96-well plates)

ABI Archive kits	$4,165	
Plastics	$153	

QPCR (32 384-well plates)

Rearraying	$1,192	
Plastics/ enzymes	$27,016	
ABI Primer/probe kits	$25,651	
Vanderbilt core facility	$9,803	

Salaries and wages

4 Total FTE's	$106,666	

Indirect costs (overhead)

Consumables	$9,615	11.60%
Salaries	$61,319	73.98%

Sum Total

$260,486

7 Appendices

7.1 Appendix A. 136 Target genes.

2 cell line analysis.

#	Gene Symbol	1º T-47D	2 º OVCAR-8
1	**ABL1**	Yes	Yes
2	**AKT1**	Yes	Yes
3	**AKT3**	Yes	Yes
4	**ATR**	Yes	Yes
5	**BUB1**	Yes	Yes
6	**BUB1B**	Yes	Yes
7	**CAPZB**	Yes	Yes
8	**CCNA2**	Yes	Yes
9	**CDC2**	Yes	Yes
10	**CDK2**	Yes	Yes
11	**CDKN1A**	Yes	Yes
12	**CDKN2C**	Yes	Yes
13	**COL4A6**	Yes	Yes
14	**CSK**	Yes	Yes
15	**CSNK1E**	Yes	Yes
16	**CTNNB1**	Yes	Yes
17	**DAPK1**	Yes	Yes
18	**DDR1**	Yes	Yes
19	**DEK**	Yes	Yes
20	**DYRK2**	Yes	Yes
21	**E2F1**	Yes	Yes
22	**EED**	Yes	Yes
23	**EPHA2**	Yes	Yes
24	**EPHA4**	Yes	Yes
25	**ERBB2**	Yes	Yes
26	**ETS1**	Yes	Yes
27	**EZH2**	Yes	Yes
28	**FADD**	Yes	Yes
29	**FGFR1**	Yes	Yes
30	**FGFR3**	Yes	Yes
31	**FYN**	Yes	Yes
32	**FZD1**	Yes	Yes
33	**H2AFZ**	Yes	Yes
34	**ID4**	Yes	Yes
35	**JAK1**	Yes	Yes
36	**KIT**	Yes	Yes
37	**LYN**	Yes	Yes
38	**MAPK1**	Yes	Yes
39	**MAPK8**	Yes	Yes
40	**MAPK9**	Yes	Yes
41	**MAPKAPK2**	Yes	Yes
42	**MLH1**	Yes	Yes
43	**MTCP1**	Yes	Yes
44	**NCOA3**	Yes	Yes
45	**PAK1**	Yes	Yes
46	**PAK4**	Yes	Yes
47	**PCNA**	Yes	Yes
48	**PCTK3**	Yes	Yes
49	**PIK3CA**	Yes	Yes
50	**PMS2**	Yes	Yes
51	**PRDM2**	Yes	Yes
52	**PRKAR2B**	Yes	Yes
53	**PTK2**	Yes	Yes
54	**PTTG1**	Yes	Yes
55	**RAC1**	Yes	Yes
56	**RAF1**	Yes	Yes
57	**RBL2**	Yes	Yes
58	**RRM2**	Yes	Yes

Data not found Gene Name
APAF1
ATM
BCL3
CCNE1
CCNE2
CDKN2A
DNMT3B
IGF1R
ILK
JUNB
NRAS
RB1
RRM1
SCRIB

59	**STK3**	Yes	Yes
60	**STK6**	Yes	Yes
61	**TACC1**	Yes	Yes
62	**TFAP2C**	Yes	Yes
63	**TFE3**	Yes	Yes
64	**TP53**	Yes	Yes
65	**TYRO3**	Yes	Yes
66	**VAV1**	Yes	Yes
67	**ZNF217**	Yes	Yes
68	**BCL2L1**	Yes	Yes
69	**MAX**	Yes	Yes
70	**PBX3**	Yes	Yes
71	**PRKACB**	Yes	Yes
72	**PTEN**	Yes	Yes
73	**TGFB2**	Yes	Yes
74	**AXL**	Yes	No
75	**CDH1**	Yes	No
76	**FGFR2**	Yes	No
77	**MAPK11**	Yes	No
78	**MDM2**	Yes	No
79	**SYK**	Yes	No
80	**BRCA1**	Yes	No
81	**EDG2**	Yes	No
82	**EPHB3**	Yes	No
83	**NR4A3**	Yes	No
84	**CTGF**	No	Yes
85	**CXCR4**	No	Yes
86	**FOS**	No	Yes
87	**FRAT1**	No	Yes
88	**INSR**	No	Yes
89	**MET**	No	Yes
90	**TNFRSF12A**	No	Yes
91	**CDKN1C**	No	Yes
92	**CDKN2B**	No	Yes
93	**EPHB4**	No	Yes
94	**ERBB4**	No	Yes
95	**FLT1**	No	Yes
96	**JAK2**	No	Yes
97	**MMP11**	No	Yes
98	**SOCS1**	No	Yes
99	**TEK**	No	Yes
100	**EPHB2**	No	No
101	**HOXA10**	No	No
102	**PDK1**	No	No
103	**TEC**	No	No
104	**WNT5A**	No	No
105	**ABL2**	No	No
106	**ANXA8**	No	No
107	**BCL2**	No	No
108	**BCL9**	No	No
109	**BLK**	No	No
110	**DDR2**	No	No
111	***FER**	No	No
112	**FES**	No	No
113	**FLT3**	No	No
114	**HOXA9**	No	No
115	**ITK**	No	No
116	**LCK**	No	No
117	**MSH2**	No	No
118	***PTK2B**	No	No
119	**RBL1**	No	No
120	***TXK**	No	No
121	**WNT10B**	No	No

* Indicates genes that did not express in any of the NCI60 lines

Complete 9 cell line analysis.

		Breast Cancer Cell Lines						Top 3 Non-Breast Cancer Lines for Most SOW Genes Expressed		
#	Gene Symbol	T-47D	MCF-7	BT549	HS-578T	MDA-MB-231	NCI-ADR-RES	OVCAR-8	OVCAR-4	U-251-EXP
1	ABL1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
2	AKT1	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
3	AKT3	Yes	No	Yes	Yes	No	Yes	Yes	Yes	No
4	ATR	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
5	BUB1	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
6	BUB1B	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
7	CAPZB	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
8	CCNA2	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
9	CDC2	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
10	CDK2	Yes	No	Yes	No	No	Yes	Yes	Yes	Yes
11	CDKN1A	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
12	CDKN2C	Yes	No	Yes	No	Yes	No	Yes	Yes	No
13	COL4A6	Yes	Yes	No	No	No	No	Yes	Yes	Yes
14	CSK	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes
15	CSNK1E	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
16	CTNNB1	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
17	DAPK1	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
18	DDR1	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes
19	DEK	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
20	DYRK2	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
21	E2F1	Yes	Yes	No	No	Yes	Yes	Yes	Yes	Yes
22	EED	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
23	EPHA2	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
24	EPHA4	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
25	ERBB2	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes
26	ETS1	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes
27	EZH2	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
28	FADD	Yes	Yes	No	No	Yes	No	Yes	Yes	Yes
29	FGFR1	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
30	FGFR3	Yes	Yes	No	No	No	Yes	Yes	Yes	No
31	FYN	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
32	FZD1	Yes	Yes	No	Yes	Yes	No	Yes	Yes	Yes
33	H2AFZ	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
34	ID4	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
35	JAK1	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
36	KIT	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
37	LYN	Yes	Yes	Yes	No	No	Yes	Yes	Yes	Yes
38	MAPK1	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
39	MAPK8	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes

40	**MAPK9**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
41	**MAPKAPK2**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
42	**MLH1**	Yes	Yes	No	No	No	Yes	Yes	Yes	Yes
43	**MTCP1**	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes
44	**NCOA3**	Yes	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes
45	**PAK1**	Yes	No	No	Yes	No	Yes	Yes	Yes	Yes
46	**PAK4**	Yes	Yes	No	No	No	No	Yes	Yes	No
47	**PCNA**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
48	**PCTK3**	Yes	No	No	No	No	No	Yes	Yes	No
49	**PIK3CA**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
50	**PMS2**	Yes	No	No	No	No	No	Yes	Yes	Yes
51	**PRDM2**	Yes	No	Yes	No	No	No	Yes	Yes	Yes
52	**PRKAR2B**	Yes	No	No	No	No	No	Yes	Yes	Yes
53	**PTK2**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
54	**PTTG1**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
55	**RAC1**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
56	**RAF1**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
57	**RBL2**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
58	**RRM2**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
59	**STK3**	Yes	No	Yes	Yes	No	No	Yes	Yes	Yes
60	**STK6**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
61	**TACC1**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
62	**TFAP2C**	Yes	Yes	No	No	No	No	Yes	Yes	Yes
63	**TFE3**	Yes	No	Yes	No	Yes	Yes	Yes	Yes	Yes
64	**TP53**	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes
65	**TYRO3**	Yes	No	No	No	No	No	Yes	Yes	Yes
66	**VAV1**	Yes	No	No	No	No	No	Yes	Yes	No
67	**ZNF217**	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
68	**BCL2L1**	Yes	Yes	No	No	Yes	Yes	Yes	No	No
69	**MAX**	Yes	No	No	No	No	No	Yes	No	No
70	**PBX3**	Yes	No	Yes	No	No	Yes	Yes	No	Yes
71	**PRKACB**	Yes	Yes	No	Yes	Yes	No	Yes	No	Yes
72	**PTEN**	Yes	Yes	No	No	No	No	Yes	No	No
73	**TGFB2**	Yes	No	No	Yes	Yes	No	Yes	No	Yes
74	**AXL**	Yes	Yes	Yes	Yes	Yes	Yes	No	Yes	Yes
75	**CDH1**	Yes	Yes	No	No	No	No	No	Yes	No
76	**FGFR2**	Yes	No	No	No	No	No	No	Yes	No
77	**MAPK11**	Yes	No	No	No	No	No	No	Yes	No
78	**MDM2**	Yes	No	No	No	No	No	No	Yes	No
79	**SYK**	Yes	Yes	No	No	No	No	No	Yes	No
80	**BRCA1**	Yes	Yes	No	No	No	No	No	No	Yes
81	**EDG2**	Yes	No	No	No	No	No	No	No	No
82	**EPHB3**	Yes	No	No	No	No	No	No	No	No
83	**NR4A3**	Yes	No	No	No	No	No	No	No	No
84	**CTGF**	No	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes
85	**CXCR4**	No	No	No	Yes	No	No	Yes	Yes	Yes

86	**FOS**	No	No	No	No	No	No	Yes	Yes	Yes
87	**FRAT1**	No	Yes	No	No	No	No	Yes	Yes	Yes
88	**INSR**	No	No	No	No	No	No	Yes	Yes	No
89	**MET**	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
90	**TNFRSF12A**	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
91	**CDKN1C**	No	No	No	No	Yes	No	Yes	No	No
92	**CDKN2B**	No	No	Yes	Yes	No	No	Yes	No	No
93	**EPHB4**	No	Yes	Yes	No	Yes	No	Yes	No	No
94	**ERBB4**	No	No	No	No	No	No	Yes	No	No
95	**FLT1**	No	No	No	No	No	No	Yes	No	No
96	**JAK2**	No	No	Yes	No	No	No	Yes	No	No
97	**MMP11**	No	No	No	No	No	No	Yes	No	No
98	**SOCS1**	No	No	No	No	No	No	Yes	No	No
99	**TEK**	No	No	No	No	No	No	Yes	No	Yes
100	**EPHB2**	No	No	No	Yes	No	No	No	Yes	Yes
101	**HOXA10**	No	Yes	No	No	No	No	No	Yes	No
102	**PDK1**	No	No	No	No	No	No	No	Yes	No
103	**TEC**	No	No	No	No	No	No	No	Yes	No
104	**WNT5A**	No	No	No	Yes	No	No	No	Yes	Yes
105	**ABL2**	No	No	Yes	No	Yes	No	No	No	Yes
106	**ANXA8**	No	No	No	No	No	No	No	No	No
107	**BCL2**	No	Yes	No	No	No	No	No	No	No
108	**BCL9**	No	No	No	No	No	No	No	No	No
109	**BLK**	No	No	No	No	No	No	No	No	No
110	**DDR2**	No	No	Yes	Yes	No	No	No	No	Yes
111	**FER**	No	No	No	No	No	No	No	No	No
112	**FES**	No	No	No	No	No	No	No	No	No
113	**FLT3**	No	No	No	No	No	No	No	No	No
114	**HOXA9**	No	No	No	No	No	No	No	No	No
115	**ITK**	No	No	No	No	No	No	No	No	No
116	**LCK**	No	No	No	No	No	No	No	No	No
117	**MSH2**	No	No	No	No	No	No	No	No	No
118	**PTK2B**	No	No	No	No	No	No	No	No	No
119	**RBL1**	No	No	No	No	No	No	No	No	Yes
120	**TXK**	No	No	No	No	No	No	No	No	No
121	**WNT10B**	No	No	No	No	No	No	No	No	No

7.2 Appendix B. Packaging Illustration

7.3 Appendix C. Operon Quote

2705 Artie Street
Building 400, Suite 27
Huntsville, AL 35805
www.operon.com

7.3.1.1.1.1.1

April 18, 2006

7.3.1.2
7.3.1.3 Quote No. PW041806_A

Valid until December 31, 2006

Troy Moore
Open Biosystems
6705 ODYSSEY DRIVE
256-319-1462
Troy.Moore@openbiosystems.com

Dear Troy,

Operon Biotechnologies is happy to offer the following pricing:

Custom DNA Synthesis Charges:

Standard Salt-free DNA:

Synthesis Scale – Plates(96 -well)	**0.2 umol**
Price per base	**$0.24/base**
Number of oligos	**~1600**
Maximum	

Length	115mer

- Please reference quotation number **PW041806_A** when placing your order.

Patrick A. Weiss
CEO

Operon Biotechnologies Inc.
2705 Artie Street, Bldg. 400 Ste. 27
Huntsville, AL 35805
USA

p: (256) 704-8180
f: (251) 252-7760
email: patrick.weiss@operon.com
web: www.operon.com

7.4 Appendix D. Sequencing quote

April 12, 2006

Jaime Cearly
Open Biosystems, Inc.
6705 Odyssey Drive
Huntsville, AL 35806
PHONE: 256-319-1418
EMAIL: jcearly@openbiosystems.com

Re: High Throughput Sequencing; Quote [604-12-04]

Dear Dr. Cearly,

On behalf of Lark, I am pleased to present you with the attached quotation for DNA sequencing services. As a leading contract services company, Lark looks forward to the opportunity of serving your needs now and in the future.

Lark Company Overview. Lark, a Genaissance company, provides a broad range of custom molecular biology services to life scientists in the pharmaceutical, biotechnology, agricultural, academic, and government sectors. The Houston, Texas office serves the Americas and the branch office near Cambridge, England serves the European community. With over 15 years of experience in the genomic services business, Lark has developed an extensive portfolio that supports the growing needs of our commercially driven client base.

Flexibility. Lark is committed to meeting our customer's needs. We provide a wide variety of sequencing services, as well as other related offerings, such as DNA extraction and primer design and synthesis. Our sequencing services range from single research grade sequencing to cGMP sequencing of recombinant DNA constructs.

Quality. DNA Sequencing quality is continually monitored using PHRED quality scores. We typically obtain >600 PHRED 20 bases for over 80% of the high quality samples received. Projects designated as regulatory submission and submitted to the Lark US facility will be conducted under the requirements of Good Laboratory Practices (GLP) and Current Good Manufacturing Practices (cGMP) as promulgated by the U.S. Food and Drug Administration (FDA) 21 CFR Part 58 and 21 CFR Parts 210 & 211 and International Conference on Harmonization (ICH) Q7a, where applicable to the laboratory services provided.

Ordering Information. When you are ready to send your samples, go online to www.lark.com and fill out an order form. Refer to the quote number on your order form.

Please attach your expected sequence file and any other technical information for your project by clicking on the "upload file" button at the bottom of the form. In the notes section of the form, please indicate any special instructions for your project. A copy of the order form will be sent to your email address. Please print this emailed copy of your order form and send it in with your samples, along with a signed copy of the quotation. Please be sure to include your billing arrangements on your order form (PO number or credit card number). Dr. Cearly, should you require any further information or clarification on any aspect of this quotation, please do not hesitate to contact me.

Kind regards,

Cal Froberg
Sr. Director of Sales - Regional Accounts
Cogenics®, A Division of Clinical Data®
Comprehensive Pharmacogenomics & Molecular Services™
9441 West Sam Houston Parkway South Suite 103
Houston TX 77099
T: 281-684-8459
Email: cfroberg@clda.com

April 12, 2006

QUOTE NUMBER: 604-12-04
Jaime Cearly
Open Biosystems, Inc.
6705 Odyssey Drive
Huntsville, AL 35806
PHONE: 256-319-1418
EMAIL: jcearly@openbiosystems.com

QUOTATION FOR MOLECULAR BIOLOGY SERVICES:
7.4.1.1 High Throughput Sequencing

Project Description

Lark will provide DNA sequencing on approximately twenty 96 well plates provided by Dr. Cearly, using the 3730*xl* DNA Analyzer. Lark will perform one sequence reaction per clone using automated instrumentation. Text and/or chromatogram data will be delivered through Lark's secure FTP site, as an email attachment, or on CD via FedEx.

Service-Specific Terms

Price:

High Throughput sequencing of 20-96 well plates at $384 per plate
$7,680.00

Shipping and handling, per shipment, if needed
$25.00

Project Total
$7,705.00

Turnaround Time: 4-5 business days **Quote Expiration Date**: One Month

Materials Required:
Dr. Simmons will provide plasmid DNA in 96 well plates.

Sample requirements, per sample per sequencing reaction

- Plasmid DNA should be at a concentration of 20ng/ul with a mass of ≥ 300 ng in water or 10 mM Tris (not TE).
- If sending primers, please send 10 µl of 8 pmol/µl per sequencing reaction. Primers should be about 20 bases long and the melting temperature should be approximately 50-65°C.

Shipping Information:

Your *96 or 384* well plates and/or primer samples should be shipped frozen on dry ice. Ship the following to Lark, 9441 West Sam Houston Parkway South, Suite 103, Houston, TX 77099:

(a) Sample(s)
(b) Completed order form with payment information (P.O. Number or credit card information)
(c) Signed and dated copy of this Quotation.

Lark will not process samples that are received without items (b) and (c).

Additional Terms and Conditions. **See Appendix A.**

The terms and proposed herein are accepted by Dr. Simmons

_____ _____
Signature Date

7.5 Appendix E. QPCR quote

Applied Biosystems
an Applera Corporation Business

Quotation

North American
Sales and Service
850 Lincoln Centre Drive
Foster City, CA 94404 U.S.A.
(800)874-9868; F(650)638-5875

To: Andy Crouse
Open Biosystems
HUNTSVILLE AL 35806

Quote No.: **20337085**
Quote Valid To: 06/30/2006
Quote Date: 03/14/2006
Pay Terms: Net 30 Days
Freight Terms: FOB FACTORY - FRT PPD & ADD

Please reference Quote No. when placing your orders.

Telephone No.
Fax No.
Reference andy.crouse@openbiosystems.com

Item	Part Number	Description	QTY	Unit List Price	Unit Net Price	Total Extended Price
0001	4319413E	FG,18S r RNA MGB	7.00	450.00	405.00	2,835.00
0002	4322171	FG,HIGH CAP CDNA ARCHIVE KIT^	5.00	560.00	392.00	1,960.00
0003	4316813	Bulkpack 96-well Rxn Plates, 50xN8010560	1.00	2,200.00	1,320.00	1,320.00
0004	4311971	FG,OPTICAL ADHESIVE COVERS	5.00	174.00	104.40	522.00
0005	4326614	FG,50mL TAQMAN UNIV PCR MSTR MIX,NO UNG Sufficient for 20,000 reactions @ 5ul s cale.	7.00	3,060.00	1,836.00	12,852.00
0006	4331182	FG,OFF THE SHELF GX SET	134.00	150.00	120.00	16,080.00

Sales Representative: Barbara Pepper
Prepared by: MaryLou

ACCEPTANCE OF THIS QUOTATION IS LIMITED TO THE ATTACHED TERMS

Applied Biosystems
an Applera Corporation Business

Quotation

North American
Sales and Service
850 Lincoln Centre Drive
Foster City, CA 94404 U.S.A.
(800)874-9868; F(650)638-5875

QUOTE NO.: 20337085
QUOTE VALID TO: 06/30/2006
QUOTE DATE: 03/14/2006

To: Andy Crouse
Open Biosystems

Please reference Quote No. when placing your orders.

Item	Part Number	Description	QTY	Unit List Price	Unit Net Price	Total Extended Price
0007	4351372	KIT,TQMN GENE EX ASSAYS, SM	2.00	250.00	237.50	475.00

****

Orders for the Custom Assays may be placed online at
www.appliedbiosystems.com
OR by email to: genomics@appliedbiosystems.com

Contact Oligo Order Admin @ x5660 with any questions.

****

To receive the pricing shown above please reference this quotation number when placing your order. Thank you!

To place your order by phone, please contact our Order Administration Call Center at 1-800-327-3002.
To place your order via fax, please send it to our Order Administration Dept. - fax# 650-638-5998.
- O R -
Visit us on the web !! @ appliedbiosystems.com
Available on the web: Reagent Ordering, Product Information, Certificates of Analysis, and more.....

7.6 Appendix F. Insurance Certificates

ACORD™ CERTIFICATE OF LIABILITY INSURANCE Page 1 of 2

DATE: 08/03/2006

PRODUCER: 877-945-7378

Willis North America, Inc.
26 Century Blvd.
P. O. Box 305191
Nashville, TN 372305191

THIS CERTIFICATE IS ISSUED AS A MATTER OF INFORMATION ONLY AND CONFERS NO RIGHTS UPON THE CERTIFICATE HOLDER. THIS CERTIFICATE DOES NOT AMEND, EXTEND OR ALTER THE COVERAGE AFFORDED BY THE POLICIES BELOW.

INSURED: Open Biosystems, Inc.
6705 Odyssey Drive
Huntsville, AL 35806

INSURERS AFFORDING COVERAGE	NAIC#
INSURER A: St. Paul Fire and Marine Insurance Compan	24767-005
INSURER B: Travelers Indemnity Company of Connecticu	25682-002
INSURER C:	
INSURER D:	
INSURER E:	

COVERAGES

THE POLICIES OF INSURANCE LISTED BELOW HAVE BEEN ISSUED TO THE INSURED NAMED ABOVE FOR THE POLICY PERIOD INDICATED. NOTWITHSTANDING ANY REQUIREMENT, TERM OR CONDITION OF ANY CONTRACT OR OTHER DOCUMENT WITH RESPECT TO WHICH THIS CERTIFICATE MAY BE ISSUED OR MAY PERTAIN, THE INSURANCE AFFORDED BY THE POLICIES DESCRIBED HEREIN IS SUBJECT TO ALL THE TERMS, EXCLUSIONS AND CONDITIONS OF SUCH POLICIES. AGGREGATE LIMITS SHOWN MAY HAVE BEEN REDUCED BY PAID CLAIMS.

INSR LTR	ADD'L INSRD	TYPE OF INSURANCE	POLICY NUMBER	POLICY EFFECTIVE DATE (MM/DD/YY)	POLICY EXPIRATION DATE (MM/DD/YY)	LIMITS	
A		GENERAL LIABILITY [X] COMMERCIAL GENERAL LIABILITY [ ] CLAIMS MADE [X] OCCUR GEN'L AGGREGATE LIMIT APPLIES PER: [X] POLICY [ ] PRO-JECT [ ] LOC	TT06800443	8/1/2006	8/1/2007	EACH OCCURRENCE DAMAGE TO RENTED PREMISES (Ea occurence) MED EXP (Any one person) PERSONAL & ADV INJURY GENERAL AGGREGATE PRODUCTS - COMP/OP AGG	$ 1,000,000 $ 250,000 $ 10,000 $ 1,000,000 $ 2,000,000 $ 2,000,000
A		AUTOMOBILE LIABILITY [ ] ANY AUTO [ ] ALL OWNED AUTOS [ ] SCHEDULED AUTOS [X] HIRED AUTOS [X] NON-OWNED AUTOS	TT06800443	8/1/2006	8/1/2007	COMBINED SINGLE LIMIT (Ea accident) BODILY INJURY (Per person) BODILY INJURY (Per accident) PROPERTY DAMAGE (Per accident)	$ 1,000,000 $ $ $
		GARAGE LIABILITY [ ] ANY AUTO				AUTO ONLY - EA ACCIDENT OTHER THAN AUTO ONLY: EA ACC AGG	$ $ $
A		EXCESS LIABILITY [ ] OCCUR [X] CLAIMS MADE [ ] DEDUCTIBLE [X] RETENTION $ 10,000	TT06800443	8/1/2006	8/1/2007	EACH OCCURRENCE AGGREGATE	$ 5,000,000 $ 5,000,000
B		WORKERS COMPENSATION AND EMPLOYERS' LIABILITY ANY PROPRIETOR/PARTNER/EXECUTIVE OFFICER/MEMBER EXCLUDED? If yes, describe under SPECIAL PROVISIONS below	HEUB6835C94106	8/1/2006	8/1/2007	[X] WC STATU-TORY LIMITS [ ] OTH-ER E.L. EACH ACCIDENT E.L. DISEASE - EA EMPLOYEE E.L. DISEASE - POLICY LIMIT	$ 500,000 $ 500,000 $ 500,000
A		OTHER Commercial Property	TT06800443	8/1/2006	8/1/2007	$2,625,000 - Blanket Personal Property Limit incl Computer Prop $4,500,000 Blanket Business Income $5,000 Deductible/24 Ded for BI	

DESCRIPTION OF OPERATIONS/LOCATIONS/VEHICLES/EXCLUSIONS ADDED BY ENDORSEMENT/SPECIAL PROVISIONS

Solicitation #S06-182

It is agreed that National Cancer Institute - FCRDC is included as an additional insured as respects to General Liability.

CERTIFICATE HOLDER

National Cancer Institute - FCRDC
Attn: Jeanne Lewis
NCI-Frederick Cancer Research & Development Center
SAIC-Frederick, Inc.
P.O. Box B, Building 1050, Research Contracts
Frederick, MD 21702-1201

CANCELLATION

SHOULD ANY OF THE ABOVE DESCRIBED POLICIES BE CANCELLED BEFORE THE EXPIRATION DATE THEREOF, THE ISSUING INSURER WILL ENDEAVOR TO MAIL 10 DAYS WRITTEN NOTICE TO THE CERTIFICATE HOLDER NAMED TO THE LEFT, BUT FAILURE TO DO SO SHALL IMPOSE NO OBLIGATION OR LIABILITY OF ANY KIND UPON THE INSURER, ITS AGENTS OR REPRESENTATIVES.

AUTHORIZED REPRESENTATIVE

ACORD 25 (2001/08) Coll:1707064 Tpl:528757 Cert:7585801 © ACORD CORPORATION 1988

IMPORTANT

If the certificate holder is an ADDITIONAL INSURED, the policy(ies) must be endorsed. A statement on this certificate does not confer rights to the certificate holder in lieu of such endorsement(s).

If SUBROGATION IS WAIVED, subject to the terms and conditions of the policy, certain policies may require an endorsement. A statement on this certificate does not confer rights to the certificate holder in lieu of such endorsement(s).

DISCLAIMER

The Certificate of Insurance on the reverse side of this form does not constitute a contract between the issuing insurer(s), authorized representative or producer, and the certificate holder, nor does it affirmatively or negatively amend, extend or alter the coverage afforded by the policies listed thereon.

8 References

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