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Part No. LCSN-PRT-0029 Rev 01 11/07

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LightScanner® Primer Design Software Manual

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CONTENTS

Customer and Technical Support Definitions	
Intended Use and License Agreement	
Chapter 1: Installing the Software Hardware Requirements Installation Instructions	1
Chapter 2: Starting the Primer Design Software Import Sequence Formats Launch the Software	5
Chapter 3: Annotating Sequences Defining Exons, SNPs and Regions Defining an Exon Defining SNPs Defining Regions Inserting Text in a Sequence Replacing a Single Sequence Character	9 9 10 11 11
Chapter 4: Designing Primer Sets Using Common Design Parameters Customizing Design Parameters for Individual Exons Evaluating Your Existing Primer Sets Using the LSPD Software	13 14
Chapter 5: Results	
View Summary Results View Results for Individual Exons Saving and Exporting Data	. 20
BLAST Searches	. 21 21
Checking Cross-Complementarities Checking Specificity Menu Options	. 22
Sequence Menu Settings Menu Tools Menu	. 23 . 23

Chapter 6: Designing LunaProbes [™] Design Your Primers Design Your Probe Determining Probe and Primer Compatibility	. 27 . 29
Chapter 7: Designing a Small Amplicon for Genotyping	. 35
Appendix A: Quick Guide to Using the LightScanner Primer Design Software	39 40 41
Appendix B: Getting Annotated Text Files from the UCSC Genome Browser	.43
Index	.47

Definitions

Function	Description
Single Amplicon	This is a primer design module that can be used for designing individual primer sets. No assumptions are made about exon regions. This module has all the functionality of the scanning primer design module in terms of sequence definitions (single nucleotide polymorphisms [SNP], repeat regions, etc.), and primer design constraints are identical to the scanning primer design module.
Scanning Primers	This module is tailored to design all of the scanning primers required to cover every exon of a given gene. Exons are defined as regions of upper case letters. Exon boundaries can be read directly from GenBank files or text files with the exons already converted to upper case. Primers can be designed using common amplification conditions for every exon or the design conditions can be adjusted for each exon individually. Primer sets are ranked and scored by individual pairs as well as by their compatibility with the entire group of primer set chosen to cover the entire gene. Primer melting temperatures and reaction conditions are adjusted for the LCGreen [®] family of double strand DNA binding dyes.
5' Exclusion Buffer	This parameter defines the length of the 5' exon-intron boundary that is excluded from the search region. No primers will be placed over this boundary, allowing the scanning amplicon to include splice sites as well as the exonic region. The default length of the 5' exclusion buffer is set to 5 bases; however, this value can be manually increased to suit user preferences.
3' Exclusion Buffer	This parameter defines the length of the 3' exon-intron boundary that is excluded from the search region. No primers will be placed over this boundary, allowing the scanning amplicon to include splice sites as well as the exonic region. The default length of the 5' exclusion buffer is set to 5 bases, however this value can be manually increased to suit user preferences.

Function	Description
Minimum Overlap	For high-resolution mutation scanning it is desirable to keep amplicon sizes below 400 base pairs. The software will automatically divided large exons into multiple amplicons in order to obtain the optimal amplicon length. The minimum overlap parameter defines the length of double coverage from adjacent amplicons when an exon is broken up into multiple fragments. The default value for this parameter is set to 5 bases because empirical evidence has shown that mutations that are 3–5 bases away from the 3' end of a primer can still be detected by high-resolution melting.
Primer Tm	This is the predicted melting temperature (Tm) of the primer. The func- tional Tm of an oligonucleotide may be increased by as much as 5–10 °C in the presence of LCGreen dye. It should be noted, however, that this is a theoretical value, and an annealing temperature gradient PCR experiment is strongly recommended to determine empirically the optimum annealing temperature for each primer set.
Minimum/Maximum Primer Tm	The software allows users to specify a Tm range when selecting primer pairs. The recommended Tm range is usually about 5 °C (example, 60–65 °C). The software will search for the best primer sets within this range, while always matching the Tm of the forward and reverse primers within each set. This allows for maximum flexibility in design while still maintain- ing stringency. (We do not recommend using a Tm range of \geq 5 °C when searching for primers due to the overwhelming number of primers that will be generated.)
Primer Score	The software scores each primer set against a hard-coded set of design filters. Scoring is based on penalties with a perfect score (no penalties) equal to zero. Scores with 2–4 digits are coded green (good), scores with 5 digits are scored orange (moderately good), and scores with more than 5 digits are coded red (avoid if possible).
Primer Rank	Primer sets with the lowest score (fewest penalties) are ranked the high- est. Primer sets are displayed by rank in the summary results table.
Primer Sets	Primer sets consist of a single forward and reverse primer. Set scores and rank reflect the compatibility between the two primers that comprise a single amplicon.
Primer Groups	Primer groups consist of multiple primer sets that are needed to cover a large exon or region. Compatibility scores and rank for primer groups help the user decide what primer sets can be used under the same amplification conditions in order to cover a large exon.
Alignment	The position of a set of primers within the sequence context relative to the region the primers are designed to amplify.

Function	Description
Cross- complementarity	The inter- or intra-molecular interactions of oligos within a given primer set. Additional oligos (e.g., probes) can be added to the analysis if necessary.
Delta G	The thermodynamic stability of the inter- and/or intra-molecular interac- tions between a set of oligonucleotides.
Fixed Oligos	User-defined oligo sequences that can be scored by the software to determine their suitability for PCR.
Additives	Chemical additives such as dimethyl sulfoxide (DMSO) or Betaine that are routinely included in PCR reactions to lower the melting temperature of the double-stranded DNA in the reaction. Additives are recommended for high resolution melting with LCGreen dye when amplicons have a GC content greater than 65%.

Intended Use and License Agreement

Intended Use

The LSPD software enables users to design primer sets for mutation scanning and discovery using the LightScanner instrument. The primary application is for designing primers that amplify every exon of a given gene specifically for high-resolution melting. A secondary application is the more traditional primer design workflow for the design of single amplicons.

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CHAPTER 1:

INSTALLING THE SOFTWARE

Hardware Requirements

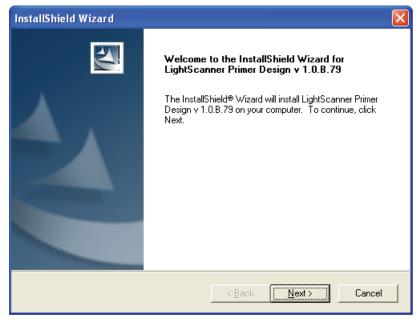
Operating system	Windows XP/ Windows 2000
CPU	80 GB or greater
RAM	512 MB or greater
Display	1024 x 768 or 1280 x 1024
Connections	Network card*

*Optional: Internet access will be required to perform BLAST searches.

Installation Instructions

The LightScanner Primer Design (LSPD) software is automatically installed on the computer that is used to run the LightScanner instrument. Users may install the application on independent computers that meet the minimum requirements, if desired, using the installation disk.

- 1. Insert the LSPD software CD into the CD-ROM drive of the computer.
- The installation wizard will display a welcome window and guide you through the installation process. Select Next to move to the next window.



3. The **License Agreement** window is displayed. After reviewing the agreement, select **Yes** to confirm and move to the next window. Selecting **No** will terminate the install process.

License Agreement		Same /
Please read the following license ag	greement carefully.	(C. 199
Press the PAGE DOWN key to see	the rest of the agreement.	
DAHO TECHNOLOGY, INC. END-USER SOFTWARE LICENSE	E AGREEMENT	<u>^</u>
person, corporation, business, limite operates the computer on which th	(this "License") is a legal agreement betwee dilability company or other entity which owr is Software is installed, run or executed, as v r other personnel using this Software (each a c. ("ITI").	ns or vell as
1. Certain Definitions.		~
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agreement.		

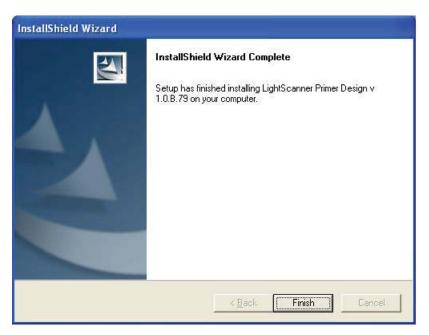
 The Choose Destination Location window opens. Select a location for the software by using the Browse button or leave on the default—C:\Program Files\IdahoTech. Select Next to continue.

hoose Destination Location Select folder where Setup will install files.	
Select folder where Setup will install files.	
Setup will install LightScanner Primer Design v $1.0.B.79$ in the	following folder.
To install to this folder, click Next. To install to a different folde another folder.	r, click Browse and select
Destination Folder	
Destination Folder C:\\IdahoTech\LightScanner Primer Design	Browse
	Biowse

 The Select Program Folder window will open. The Desktop icon allows you to start the application by double-clicking an icon on your desktop and is useful for frequent users. The Program menu icon will appear in the Start > All Programs menu. Click Next to continue.

InstallShield Wizard		×
Select Program Folder Please select a program folder.		N.S.
Setup will add program icons to the Progr name, or select one from the existing fold <u>P</u> rogram Folders:		
LightScanner Primer Design		
Existing Folders: Accessories		~
Administrative Tools Adobe Broadcom CA Registration		
Dell QuickSet Dell Wireless eTrust InoculateIT Games		~
InstallShield		
	< <u>B</u> ack <u>N</u> ex	kt > Cancel

6. The InstallShield Wizard Complete window opens. Select Finish to close the wizard.



CHAPTER 2:

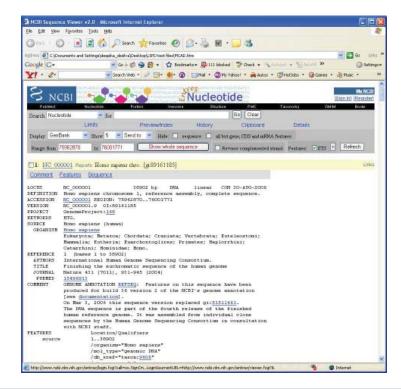
STARTING THE PRIMER DESIGN SOFTWARE

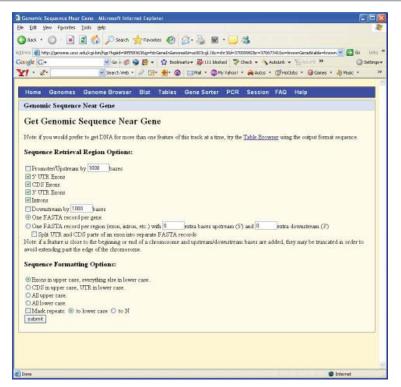
The LSPD software enables users to design primer sets for mutation scanning and discovery using the LightScanner instrument. The primary application is for designing primers that amplify every exon of a given gene specifically for high-resolution melting. A secondary application is the more traditional primer design workflow for the design of single amplicons.

Import Sequence Formats

Before you launch the software, you will want to create sequence files for import. The LSPD software will allow you to import sequence from the following formats: European Molecular Biology Laboratory (EMBL), FASTA, GenBank, or regular text files.

When designing amplicons that cover all exons in a given gene, the most important thing is to correctly identify the boundaries of each exon. Two common ways of obtaining this information is either through annotated GenBank files (http://www.ncbi.nlm.nih.gov/sites/entrez) or through the University of California-Santa Cruz (UCSC) Genome browser (http://genome.ucsc.edu).



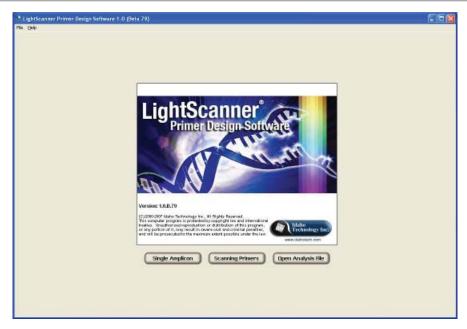


The LSPD software is configured to read files from both sources. Annotated GenBank files opened with the software are displayed with the exon regions highlighted on the screen automatically.

- For EMBL files, go to http://www.ebi.ac.uk/embl
- For FASTA files, go to http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml
- For GenBank files, go to http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html

Launch the Software

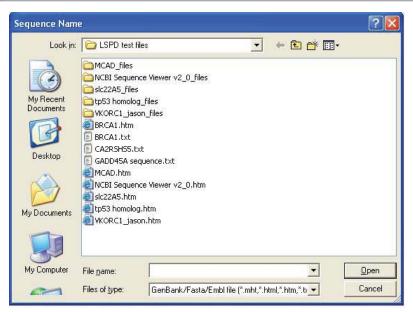
- 1. Double click on the LightScanner icon on your desktop. The Front Screen displays.
- 2. The user has three options to select from:
 - a. Single Amplicon—This is a primer design module that can be used for designing individual primer sets. No assumptions are made about exon regions. This module has all the functionality of the Scanning Primers design module in terms of sequence definitions (single nucleotide polymorphisms [SNP], repeat regions, etc.), and primer design constraints are identical to the Scanning Primers design module.
 - b. Scanning Primers—This module is tailored to design all of the scanning primers required to cover every exon of a given gene. Exons are defined as regions of upper case letters. Exon boundaries can be read directly from GenBank files or text files with the exons already converted to upper case. Primers can be designed using common amplification conditions for every exon or the design conditions can be adjusted for each exon individually. Primer sets are ranked and scored by individual pairs as well as by their compatibility with the entire group of primer set chosen to cover the entire gene. Primer melting temperatures and reaction conditions are adjusted for the LCGreen[®] family of double strand DNA binding dyes.
 - c. Open Analysis File—Open an existing file and work with the data.



3. Select **Open Analysis File** from the Front Screen menus. The **Sequence** screen displays. From the **Sequence** menu, select **Import Sequence**.

LightScanner Primer Design Software 1.0 (Beta 79)	
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	Author deepika_desilva
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	First Position 1 Last 0
	Length %GC Position 1 cDNA
	Comments
	Annotations
	Add Exon Add SNP Add Region
	Import Sequence
	Search All

4. Find and select the desired file, click **Open**.



The sequence will appear in the display window. The software reads the exon annotations imported from GenBank .htm files, finds the exons, automatically converts them to uppercase and highlights the exons on the screen for easy viewing. The software recognizes any uppercase letters as exons, so text files imported from the UCSC Genome Browser with exons annotated in uppercase will also be read correctly.

Note: The sequence must contain at least 200 bases of sequence upstream of the first exon and downstream of the last exon in order for the software to recognize these exons correctly.

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5001		agaaatgatt					
6051		aagataatgt					
	tottttaga						
6151		TTTTTTTT					
6201	agagtgcagt	ggcacastct.	cogttcacca	cagtetcege	ctcccgagtt		

CHAPTER 3:

ANNOTATING SEQUENCES

Defining Exons, SNPs and Regions

It is often useful to manually annotate a sequence with known single nucleotide polymorphisms (SNPs), regions, or exons. The primer design software can be directed to design primers that cover these annotated sites, avoid them, or ignore them. All annotations will be saved with the file once the analysis is complete.

Defining an Exon

If you have imported a file with the exons in uppercase, there is no need to manually define the exons. If you have imported a normal text file and want to define the exons manually, highlight the sequence in the window and select the **Add Exon** button. A pre-filled dialog box will open.

You have the option of renaming the exon and entering a description of the exon for record keeping. An exon is automatically designated as a target to be covered by primers unless the **Target** box is manually unchecked.

Name	Exon 13		🔽 Target	OK
Start	1	c.1 -191		Cancel
End	1	c.1 -191		
Description				

Alternatively

- 1. Select the **Annotations** sub tab, and then select **Add Exon**. The **Edit Exon** dialog box opens.
- 2. Enter the information to define the exon name and location (or leave default name), click OK.

Add Ex	on Add SNP	Add Region		Edit Selected	Delete Se	elected	Delete /		Default Colors
Туре	Name	Start	End	c.Start	c.End	Length		1	Description
Exon	Exon 1	192	221	c.1	c.30	30	Ignore	Target	
Exon	Exon 2	3805	3892	c.31	c.118	88	Ignore	Target	
Exon	Exon 3	8048	8145	c.119	c.216	98	Ignore	Target	
Exon	Exon 4	8257	8326	c.217	c.286	70	Ignore	Target	
Exon	Exon 5	8932	9032	c.287	c.387	101	Ignore	Target	
Exon	Exon 6 Exon 7	10195 15384	10275 15514	c.388 c.469	c.468	81	lgnore Ianore	Target	
Exon	Exon 7 Exon 8	21210	21318	c.469 c.600	c.599 c.708	131 109	Ignore Ignore	Target Target	
Exon	Exon 9	24823	24963	c.709	c.849	103	Ignore	Target	
Exon	Exon 10	25855	24363	c.850	c.945	96	Ignore	Target	
Exon	Exon 11	36526	36774	c.946	c.1194	249	Ignore	Target	
Exon	Exon 12	38096	38167	c.1195	c.1266	72	Ignore	Target	
								2000 — 2008	
1									

Defining SNPs

- 1. Highlight the SNP in the sequence window and select the **Add SNP** button, or select **Add SNP** from the annotations sub tab.
- 2. If the SNP was highlighted in the sequence window, the data is pre-entered in the dialog box. You have the option of renaming the SNP; choosing whether the SNP should be covered, avoided, or ignored; or entering a description of the SNP for record keeping.

Add SNP			
Name	SNP	Target	ОК
Start	1 c.1 -191		Cancel
Status	Avoid 💌		
Descriptio	on 🛛		
Text Colo	r Default 💌	Background Color	Default 💌

Defining Regions

- 1. In the sequence window, highlight the region of interest and select **Add Region** or select the **Add Region** button in the **Annotations** sub-tab.
- The data from the highlighted region is pre-entered in the dialog box. You have the option of renaming the region, or choosing whether the region should be covered, avoided, or ignored. A description of the region can be entered for record keeping.

Name	Region			Target	OK
Start	1	c.1	-191		Cancel
End	1	c.1	-191		
Status	Avoid	•			
Description	n [

3. Repeat steps above as needed to define additional exons, SNPs, and regions.

Inserting Text in a Sequence

- 1. View the sequence of choice. If the **Sequence** locked option is checked, uncheck it and select the **Insert** option.
- 2. Place the cursor at the location where the new sequence information is to be inserted and either type the new characters or paste a copied sequence.

Replacing a Single Sequence Character

- 1. View the sequence of choice. If the **Sequence** locked option is checked, uncheck it and select the **Replace** option.
- 2. Place the cursor to the left of the character to be replaced and type in a new character.

CHAPTER 4: DESIGNING PRIMER SETS

Once the sequence is imported into the software and the exons are coded, each exon will be assigned a tab at the top of the screen. A common set of design parameters will be used to design primers for every exon. Exons that are larger that the recommended size for scanning amplicons will be broken up into two or more fragments for optimum results. The **Search All** button will find scanning primers for all exons with no further user input required.

Using Common Design Parameters

Default settings are used to design primers for every exon. The default settings can be accessed through the Settings Menu.

The user can also define common scanning settings by selecting **Common Scanning Settings** from the **Settings** menu. The user can modify the common settings by typing directly into the fields. These modified settings will be saved and used in all future analyses.

Experiment Settings Reaction C	Conditions			
 Min Amplicon Size Min Primer Tm Min Primer Size 5' Exclusion Buffer 6' Minimum Overlap Forward Primer Conc. (µM) Reverse Primer Conc. (µM) 	74 60.0 17 5 5 0.3 0.3	Max Amplicon Size Max Primer Tm Max Primer Size S' Exclusion Buffer	350 60.0 30 5	

The settings options are as follows:

- Minimum and maximum amplicon size: Recommended amplicon sizes for scanning are between 100 and 400 base pairs.
- Minimum and maximum primer Tm: A primer melting temperature (Tm) range can be used for optimal primer selection. This increases the possibilities for primer selection while still retain-

ing stringency. Individual primers in assigned pairs will always be matched for Tm, but multiple primer pairs will be generated that have Tm that fall within the defined range.

- 5' and 3' exclusion buffer: This is the number of nucleotides on either side of the exon boundary excluded from the analysis. The default value is 5; however, in some cases, users may increase the value to avoid placing primers over exon-intron boundaries of interest.
- Minimum overlap: For large exons, the primer design software will generate more than one amplicon to cover the entire region of interest. The minimum overlap value indicates the number of overlapping bases that will be covered by adjacent amplicons. A minimum of 1 base is required to avoid missing polymorphisms that may be found on the ends of the amplicons.
- Forward and Reverse primer concentrations: The primer Tm calculations are influenced by the concentration of primers used in the reaction. Enter known primer concentrations or use the defaults in the software. Recommended primer concentration ranges are between 0.2 and 0.4 µM.

When using common design parameters for all exons, the next step is to select the **Search All** button. This will activate a primer search for every exon, with no further user input required.

AD EN								
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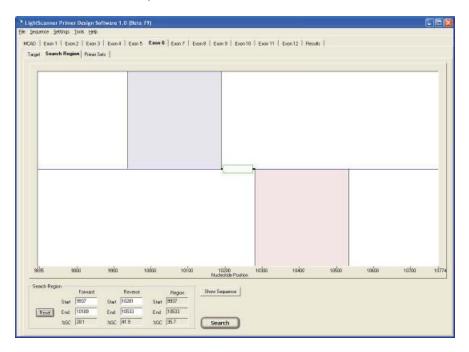
Customizing Design Parameters for Individual Exons

The design parameters for individual exons can be customized by selecting the individual exon tab at the top of the screen. This will bring up a set of Experiment Settings functions that can be modified manually. These include amplicon size, primer melting temperature range, primer size, the size of the region bordering the exon where no primers should be placed (exclusion buffers), the required overlap between amplicons if the exon is broken into multiple amplicons, and the composition of the Master Mix that is used.

Select the **Search** button to begin the primer search. The software will discover primer sets within the search regions for each amplicon and rank them, displaying them in descending order.

Min Amplicon Size	74	Max Amplicon Size	350
Min Primer Tm	60.0	Max Primer Tm	60.0
Min Primer Size	17	Max Primer Size	30
5' Exclusion Buffer	5	3' Exclusion Buffer	5
Minimum Overlap	5	Number Amplicons	1
Reaction Conditions		_	

Alternatively move to the **Search Region** tab. Verify that the region is defined correctly and select the **Search** button or move directly to the **Primer Sets** tab.



(Optional) Select **Show Sequence** to show the entire length of the sequence on the chart with all exons indicated. The button label will change to **Show Near Exon** (or **Target**). Selecting this button again will redisplay the area near the current exon.

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To adjust the search regions for an amplicon, activate the search region tab and then

- 1. Drag the pink and blue bars left or right to move the search regions
- 2. Drag the boundaries of the bars to widen or narrow the regions
- 3. Edit the Start and End fields below the chart to change the region boundaries.

After adjusting the boundaries, select **Search** or select the **Primer Sets** tab. The software will discover primer sets within the search regions for each amplicon and rank them, displaying them in descending order.

Evaluating Your Existing Primer Sets Using the LSPD Software

If you already have a primer set(s) for your target, you may evaluate it for compatibility with LCGreen Plus dye and view the recommended PCR conditions.

Before you begin, obtain the sequence for the amplicon of interest including sequence upstream and downstream, as well as your primer set sequences.

- 1. Open the Primer Design software and select **Single Amplicon** from the front screen.
- 2. Paste the sequence into the sequence window.
- 3. To access the Target, Search Region, and Primer Sets tabs required for analyzing your primers, you must either define the amplicon by selecting the Define Amplicon button and selecting the bases that comprise your amplicon

OR

Select the Add SNP button and define an arbitrary base within the amplicon as a SNP.

Note: Define an amplicon by selecting the **Define Amplicon** button and highlighting the region of the amplicon in the sequence display box. Define a SNP or a single base by selecting **Add SNP**. Be sure to have the SNP selected as the target. This will enable the Target, Search Region, and Primer Sets tabs.

- 4. Select the Target tab then select Fixed Oligos.
- 5. Highlight the forward and reverse primer sequences or type the sequences into the Forward and reverse primer text boxes. The sequence will automatically be highlighted.

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informer 1 carry			() Canacova						
					Target Infor	nation			
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		TTTCAAAGGA			Length	1		2000 46.6	-
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201 GGTT	10010	ATCACACAAC	ATTTATCTT	CTTACICATA	Position	361		cDNA.	
		ggetaccece						101	
		tgeagagttg				Co	merts	Fixed Oligoz	
323 cacct	ttgta	ttgcaaaat	Attetacete	tggaaggtca					
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					Amplicon Na				

6. Select **OK**. This will take you back to the target screen.

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101 ctgcaattctgcagGT&CT6G&TC&TG&TGTTTC&A&AGG&&G&&CC>C&CCTTTC&CTTTTGGCC&&&ATTTT&TCCTG&G&&T gacgttaagacgtcC&TG&CCT>&C&A&AGTTTCCTTCTTGGTC&>G&&A&AGT&A&A&T&AGG&CTCTT&C gacgttaagacgtcC&TG&CCT&GC&CCT&G&
201 GGTTCÅGGÅGÅTCÅCÅCÅTTTÄTTCTTTTÅCÅGgtacatcagtcaaggetacececcagttetgagagaacttgeeeaggagtggttgeagagttg CCÅÅGTCCTCTÅGTGTGTTGTÅÅÅTÅÅGÅÅGÅÅTGTC <mark>eatgtagteegatgeg</mark> ggggteaagactetettgaacggggteeteaecaacgteteaac
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Bases per Line 100 🚊 🔽 Show Positions
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CACAGGAGGAAGTGCCAATAT 61 81 21 61.7
+ · · < > · + Clear Reverse Primer 5' Position 3' Position Length Tm
GGGTAGCCTTGACTGATGTAC 258 238 21 61.8
Allow Mismatched Oligos
Complement Strand Oligos 3' to 5' OK Cancel
Show Fixed Oligos on Sequence

- 7. Select the Primer Sets tab. The software will analyze your primer set.
- 8. Use the color of the score to determine whether you have an acceptable primer set (green = good, yellow = acceptable, and red = poor).

Sequence																					
Sequence	Ann	otations	Target	Searc	ch Regio	n Pr	iner Se	ete													
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9. From the Primer Sets screen you may do a Cross Comps check and BLAST your primers.

CHAPTER 5:

RESULTS

View Summary Results

Select the top-level **Results** tab to view a list of the highest scoring primer set for each exon. A summary screen displays the best scoring primer for each amplicon in tabulated format. The table shows the amplicon length, GC content, primer positions, lengths and melting temperatures, the recommended annealing temperature for the PCR (adjusted for the presence of LCGreen dye), recommendations for the use of additives (usually dimethyl sulfoxide [DMSO]) based on amplicon GC content, and the recommended temperature gradient that should be run for PCR optimization.

- Primers displayed in green are good-scoring primers, orange indicates primers with moderately good scores, and primers in red are considered poor-scoring primers.
- Highlight a single primer set to view the set details. The primer placements and amplicon size are displayed graphically on the lower part of the screen. Details on primer sequence, position, and length, GC content, melting temperature, and stability (Δ G) are displayed numerically below.

				Software 1.0 (Be	ta 79)												
e Sedne	ince Se	ttings <u>I</u>	jools He	alp													
MCAD	Exon 1	Exon	2 Exc	on 3 Exon 4 Exo	n 5 Exon	6 E	xon 7 E	Exon 8	Exon 9 E	Exon 10	Exon 11	Exon 12	Re	sults			
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Group Rank	Score	Set Rank	Com	Seguence Name	Amplicon Length		Forward F 5' Pos	'rimer 3' Pos	Len Tm	Reverse 5' Pos	Primer 3' Pos	Len Tr		Conditions	Gradient	Comments	
ndrik j 1	-713	ndrik	20016	Exon 1	121	64.5	152	171	20 59.4	272	252	21 60.		T Additive Y	58 · 68	Comments	
i i	-2628			Exon 2	205	31.2	3739	3768	30 59.9	3943	3914	30 58	9 63	Ň	58 - 68		
	-6002 -3905			Exon 3 Exon 4	220 182	32.3 31.9	8002 8194	8031 8223	30 60.0 30 59.9		8192 8348	30 60. 28 59.		N	59 - 69 58 - 68		
	-602			Exon 5	195	35.9	8880	8909	30 60.5	9074	9051	24 59.		N	59.69		
	-4779			Exon 6	148	35.1	10158	10187	30 59.8		10281	25 59.		N	58 - 68		
	-3012 -1490			Exon 7 Exon 8	250 188	31.2 37.8	15338 21170	15367 21192	30 59.3 23 60.1	15587 21357	15559 21334	29 59. 24 60.		N	58 - 68 59 - 69		
	-4858			Exon 9	256	33.6	24744	24767	24 60.1	24999	24970	30 59.	6 63	N	58 - 68		
	-2338	31	7000	Exon 10 Exon 11 Amplicon 1	187 252	34.8 36.1	25799 36421	25826 36438	28 60.1 18 59.5	25985 36672	25963 36652	23 60. 21 60.		N	59 - 69 58 - 68		
	-0974	154	-7383 -4365		252	35.2	36609	36631	23 59.5		36831	28 59.		N	58-68		
	-5163			Exon 12		27.3	38020	38049	30 59.5		38196	22 59.		N	58 - 68		
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View Results for Individual Exons

Go to the individual exon tab and choose the Primer Sets sub-tab.

The software searches for up to 1000 primer pairs for each exon and displays the top 5 unique selections under the individual exon tabs. All primer pairs can be viewed by changing the display option under the Settings menu (Top 50 Sets, Show All Sets, Limit Sets).

Select the check box next to a primer set to include that set in the final results. Only one check box can be selected per exon.

LightScanner Primer Design Software 1.0 (Beta 79)		
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MCAD Exon 1 Exon 2 Exon 3 Exon 4 Exon 5 Exon 6 Ex	ion 7 Exon 8 Exon 9 Exon 10 Exon 11 Exon 12 F	Results
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Alignment Cross Comps BLAST Fixed Oligos	Set Comments Target Comments	
Set Amolicon Forward Primer Rev	rse Primer PCR Conditions	
Rank Score Length %GC 5'Pos 3'Pos Len Tm 5'	Pos 3'Pos Len Tm Tm Additive Gradient Comments	
	975 8348 28 59.7 63 N 58-68 988 8364 25 60.0 63 N 58-68	
	388 8363 26 60.0 63 N 58-68	
	375 8348 28 59.7 63 N 58-68 148 8426 23 60.6 64 N 59-69	
	- 182	
agattatgtaatcaaactatctggatttca-33	Exon 4	1
	Exoli 4	21_ttatgacettacgtatactcattetttt
Exon 4 Bank 1 Score -3905		
Forward	5'Pos 3'Pos Length %GC Tm DeltaG	
agattatgtaatcaaactatctggatttca	8194 8223 30 26.7 59.9 -18.8	
Reverse		
Itttcttactcatatgcattccagtatt	8375 8348 28 28.6 59.7 -18.4	

Saving and Exporting Data

The summary results data generated after a search can be exported in a spreadsheet format for data archiving or can be stored as a primer design file (*.spd file) that can be accessed through the software.

To export data, use the "Export to CSV" option from the Sequence menu and give the saved file a name. The file can then be opened in MS Excel.

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BLAST Searches

Checking Sequence Alignment

To locate the position of the primers in the context of the entire sequence, highlight the amplicon in the screen and click on the Alignment button.

Alignment - Exon 1 Rank 1 Score -713	
1 cggcgccggggaccgctgccaccccgcctagcgcagcgc	~
101 gtgggegggaccagaggagtecegegtteggggagtatgteaaggeegtga <mark>eegytgtattattgteegag</mark> tggeeggaaegggag <mark>eeaae</mark> AT66CA6C6 eaceegeeetggteteeteagggegeaageeeeteataeagtteeggeaetgggeaetaataaeaggeteaeeggeettgeeeteggttg <mark>TAECGTC6C</mark>	
201 GGGTTCGGGCGATGCTGCAGGgtgagagggagcccagcggtgcggtggggctggaacatgggtattgtggtgtcggagcagggggccctgggccaaaaat CCCAAGCCCGGTACGACGTCC <mark>cactc</mark> tccccgggtcgccacgccaccccg <mark>acettgtacccataacaccac</mark> agcctcgtcccccgggacccggttttta	
301 aggtgcggccgggaggagtgggagtgggagtcgggctgaggaagga	
401 tagegttteatttteegtateeteeegteaggegaeeeegttatageeggeateetetetttagaatategttttettte	
501 atttaccgcgggaatcccacctttttccaaagtgaccccgtctctgtgtcagaaccacgggaaaaagaaaaatggagcagctactccttgaaatacttaa taaatggcgcccttagggtggaaaaaggtttcactggggcagagacacagtcttggtgccctttttctttttacctcgtcgatgaggaactttatgaatt	
601 tgtttaataagettttegttgtaacgttteeacgttgettaegggaaaaaaaaaa	~
Bases per Line 100 - V Show Positions	

Checking Cross-Complementarities

To view primer cross-complementarities click on the Cross Comps button.

CHAPTER 5: Results

Num Name	Sequence	Tm Score 1 2	Clear All
Forward Forward Forward Reverse	cccgtgtattattgtccgag caccacaatacccatgttcca cccgtgtattattgtccgag caccacaatacccatgttcca	0 3' Alignme 59.4 0 60.1 0 3 Alignme 59.4 -2 60.1 -2 -2	C Min Delta G Min Length

Checking Specificity

If you are connected to the Internet, BLAST the primer sequences directly by clicking on the BLAST button to check the specificity of the design.

BLAST - Exon 1 Rank 1 Score -713	
Forward Primer	OK
cccgtgtattattgtccgag	Cancel
Reverse Primer	
caccacaatacccatgttcca	
Database	
nr	
Select From	
(none)	

Menu Options

Sequence Menu



- Import Sequence—allows users to open a file for analysis.
- **Export**—If you are in the sequence input screen, you can export the sequence in FASTA format. Alternatively, if you are in the Results tab, you can export all the primer selection information in CSV format that can be opened in a spreadsheet program such as Excel.
- **Exons to Uppercase**—if you are in the sequence input screen and the sequence is annotated with the exons in uppercase, this function will automatically highlight the uppercase characters in green and mark them as exons, opening exon tabs for each selection.
- **Find**—opens a dialog box where a signature sequence can be entered. The software will search the input sequence for this signature sequence.

ile Sequence Settings Tools Help											
MCAD Exo Alignmer Alignmer Alignmer				5 Exon	6 E	xon 7 E	xon 8	Exon	9		
				T Set Comments Target Co							
Group		Set Comr	nents			Amplicon		Forward Primer			
Rank	Sco	Common	Settinas.		e Name	Length	%GC	5' Pos	3' Pos	Len	Tm
1	-7	Target Settings Default Settings			121	64.5	152	171	20	59.4	
1	-26 -60					205	31.2	3739	3768	30	59.9
1	-60	Deraultu	eccings	•2		220	32.3	8002	8031	30	60.0
1	-39 -6	Show Olic	tos Uppe	rcase	1	182	31.9	8194	8223	30	59.9
1	-6		,			195	35.9	8880	8909	30	60.5
1	-47 -30	Top 50 Se	ets			148	35.1	10158	10187	30	59.8
1		 Limit Sets 				250	31.2	15338	15367	30	59.3
1	-14					188	37.8	21170	21192	23	60.1
1	-48	Show All:	Sets			256	33.6	24744	24767	24	60.1
1	-233	8		Exon 10		187	34.8	25799	25826	28	60.1
1	-597	4 31	-7383	Exon 11	Amplicon 1	252	36.1	36421	36438	18	59.5
		154	-4365	Exon 11	Amplicon 2	250	35.2	36609	36631	23	59.6
- C	-516	3		Exon 12		198	27.3	38020	38049	30	59.5

Settings Menu

- Single Amplicon Primers—Used to switch between design applications
- Scanning Primers—Used to switch between design applications
- **Sequence/Target Comments**—This calls up a field that can be used to insert comments about the sequence or the analysis. Comments will be saved with the file for future reference.

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- **Common/Target Settings**—These are the default settings used for primer design in either the Scanning Primer mode (Common settings) or the Single Amplicon mode (Target settings). The settings can be modified by the user and saved as the defaults.
- Show Oligos Uppercase—Normally the bases within an exon are displayed in upper case and the bases in introns are displayed in lower case letters. All of the oligonucleotide primers can be displayed in upper case regardless of location by choosing this option.
- **Top 50 Sets**—Shows the top 50 primers selected by the software. Some of these primer sets may have the forward or reverse primers in common and are thus considered to not be unique; all primers are ranked by score.
- Limit Sets—Shows the top 5 unique primer sets found by the software. These sets should have no primers in common and will be ranked by score.
- Show All Sets—Shows up to 1000 sets of primers selected by the software, including the lowest ranked sets.

tSo	canner	Prime	er Desigr	Software 1.0 (Beta	79)					
que	nce Se	ttings	Tools Help							
1	Exon 1	Exc	Fixed (Fixed (Oligos Oligos - Import Current Se	ection		xon 7 E	×c		
Alignment Cro		Cross	Comp Tool		_	Set C	0			
)		Set		Comp Tool - Import Curre	nt Selectio	n	Forward Pr	rin		
	Score	Rank	Show	Alignment of Current Sele	ection		5' Pos	100		
-713 -2628 -6002 -3905 -602 -602 -4779 -3012		Search Search	- Import Current Selectio All Current Selected	n		152 3739 8002 8194 8880 10158 15338				
-1490 - -4858 -2338			Annota	21170 24744 25799	0.000000					
	-5974 -5163	31 154	-7383 -4365		252 250 198	36.1 35.2 27.3	36421	ACCOUNTS OF A		

Tools Menu

- **Fixed Oligos**—The user has the option of manually entering primer sequences and allowing the design program to score the results. This can be useful when deciding whether to use available primers or embark on a redesign. The fixed oligo feature can also be accessed from every Exon tab at the top right-hand side of the Target subtab.
- Cross-Comp Tool—When a primer set is highlighted in the Results tab, the Cross-comp tool can be used to examine the complementarities between the oligos, including self-complementarity. Cross-comp scores are part of the overall scoring of the primer set and can be a useful clue as to why a primer set has scored poorly. Users have the option of modifying the default parameters used for scoring, including the minimum acceptable length of any complementarities at the 3' end and also across the length of the primer; as well as the acceptable Δ G. A useful feature of the Cross-comp tool is the ability to include an additional oligo, for example a probe, to check for interference. The software will return any complementarity between the additional oligo and the primers as well as the stability (Δ G) of the interactions, if any.
- **BLAST**—This will open a window that can be used to manually enter sequences for a Forward and Reverse Primer. The various NCBI Databases against which the sequences will be queried can be selected using the pull down menu. Use the "Select From" pull down menu to choose the organism against which the sequence is queried. Selecting "Okay" sends the request to the NCBI BLAST web site. When the request has been received, your internet browser opens to the NCBI BLAST home page. The page displays results when the search is complete.

Import Current Selection will bring up the BLAST dialog box with the selected primers entered into the Forward and Reverse Primer Sequence fields.

- **Search All**—Performs the same function as the button on the Sequence tab. By selecting Search All the software will discover all primers for the annotated exons.
- **Search Current**—is active if a single exon tab is activated. Primers will be selected for the selected exon.
- Search Selected—Will bring up a dialog box with a list of all the exons that were discovered within the sequence with a check box next to each exon. The user has the option of selecting specific exons by using the check boxes next to the name of the exon, limiting the searches to only the selected exons.
- **Annotations**—users can access the same functions as described in Chapter 3, Defining Exons, SNPs and Regions.

CHAPTER 6: DESIGNING LUNAPROBES™

The LSPD Software may be used to design LunaProbes. LunaProbes are unlabeled probes used in genotyping applications. This chapter will explain how to design a probe in the same Tm range (60–65 °C) as your primers with minimal cross-reactivity.

Design Your Primers

Before you begin, design your primers for your target.

- 1. Open the Primer Design software and select **Single Amplicon** from the front screen.
- 2. Paste the sequence into the sequence window.
- 3. Define the SNP of interest by selecting Add SNP. Be sure to have the SNP selected as the target.
- 4. Move to the **Target** tab.
- 5. Use the following experimental settings:
 - Maximum amplicon size 250 bp
 - Primer Tm between 60–65°C
 - 15 bp exclusion buffer on both the 5' and 3' end

0051 ttttictetaga aatttegaa oteitaacaa aactigocta atactatti Poulion 77 0051 taatgttega catteratag cagttega oteigagaga gegaggagagagagagagagagagagagagagagagaga	SNP 45 M& 60.0 Mz 45 Mz	Last 372 %GC 46.9 Fixed Oligos
2051 ttttictictaga aasttitgaaa otoitaacaa aasotigoata atactaatti Poullon 37 2051 tttictictaga aasttitgaa otoitaataataa aagttigg ooggacaaga gotoagagaga 1 2051 acaaaaatti agcoggogt agtgooggo ooggacaaga gotoagagaga 1 2051 acaaaaaatti agcoggoog agtgooggo gotogaagagagagagagagagagagagagagagagagag	372 La 372 cD Comments	2GC 46.9
1101 aactgtttte aattacatag cagtgergg creating creating Poilion 37 1101 aacagttggt terggrag creating gragergg gragergg gragergg Lengh 1 1101 acaattiggt artgordgag growtgag growtgag gragergg gragergg Import and	26 372 cD Comments 28 45 Ma 60.0 Ma	2GC 46.9
1111 aaagttggt tagggagg cgggggggggggggggggggg	26 372 cD Comments 28 45 Ma 60.0 Ma	2GC 46.9
2201 tcgagaccat cccggctaaa acggtgaaac cccgtctta ctaaaaaat Leggin 2251 acaaaaaat gcgaggtgagat 2251 acaaaaaat gcgaggtgagat 2351 acaaaaaat gcgaggtgggggggggggggggggggggggggggggggg	272 cD Comments 28 45 Ma 60.0 Ma	:DNA
2201 togagacat cocggotaa acggtgaaac cocgtotta otaaaaaat 73 221 acaaaaat agocggoggt aggggggg goctggagt coggtaat 73 221 gagagotga aggggggg aggtgggggggggggggggg	272 cD Comments 28 45 Ma 60.0 Ma	:DNA
2251 acaaaaat agcoggggt agtgggggg cetgtagte coagtagt 2351 acaaaaaat agcoggggt agtgggggg 2351 tgagotgagat tgggggtgg 2351 tgagotgagat tggggtgg 2351 tgagotgagat tggggtgg 2351 tgagotgagat tggggtgg 2352 tgagotgagat tggggtgg 2353 tgagotgagat 2354 tgagotgagat 2354 tgagotgagat 2351 tgagotgagat 2361 tgagotgagat 2372 tgagotgagat 2381 tgagotgagat 2392 tgagotgagat 231 ttataaaat 2329 tatatata 2331 ttataaaat 2332 tatatata 2333 tatatata 2334 tatatata 2335 tatatata 2331 tatatata 2332 tatatata 2333 tatatata 2334 tatatata 2335 tatatata 2335 tatatata 2332 tatatata 2333 tatatata <td>Comments</td> <td></td>	Comments	
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V451 gtaatatata ctacttagtt acactactta catagettea gttteettä ExperimentSeting V501 tataaaatg caataaac cteccatgag ggtggggg gggeteatg Mn Ampleon See V501 tatacaacaagette gaggteggag gtggggggggggggggggggg	ze 45 Ma 60.0 Ma	
1501 dtataaaatg caaataacac dtocoatgag ggdggggg gggdtocatg Min Ampicon Siz 1551 octgtaatco cagcactttg ggaggocgag gtgggtggat cacotgaggt Min Ampicon Siz 1661 ocaggagtttg agaccagott gaccaacatg gtggaacoco atotttacta 1651 aaaataaaaaatatagoca agottggtgg gggggggggggggg 1701 tactocagaa gotgaggcag gagaatcaco tgaacotggg aggtggaggg 5 Febamin Buff	ze 45 Ma 60.0 Ma	
1551 octytaatoc cagoactty ggaggocgag gtgggtggat caetygaggt 1601 caggagtty agaccagoot gaccaacat gtgaaacooc atottacta 1651 aaatacaa aaatagoca agotyggtgg ogogacota taatoccaac 1701 tactocaga gotyaggoag gagaatoaco tgaacotgg aggtggaggg 5 Fedamo Ruff	60.0 Ma	
1551 octgtaatee eageaettig ggaggeegag gtgggtggat eaetigaggt 1651 ocgggaggttig agaecageet ggeecaacatg gtgsaaceee atettaata 1651 aaaatacaaa aaatageea agegtggtgg egegeaetta taateeeaa 1701 taeteeaga getgaggeag gagaateaee tgaacetggg aggtggaggg 5 Fedamo Ruffe	60.0 Ma	Max Amplicon Size
1651 aaaatacaaa aaattageca agegtggtgg egegeaceta taateecaac 7701 taeteeaga getgaggeag gagaateace tgaacetggg aggtggaggg 5 Fedamon Ruffe		sector and the sector set
1701 tactccagaa gctgaggcag gagaatcacc tgaacctggg aggtggaggg Min Pimer Size	17 Ma	Max Primer Tm
5 Exclusion Buffe		Max Primer Size
5' Exclusion Buffe	1	
	fer 15 31	3' Exclusion Buffer
1751 tgcagtgage tgacateaca ceaetgetet ceageetggg caacagageg 1801 agaetgtete aaaaaaaaaa aaaaaaagt gtatttaaag caettageag Minimum Ovelen	-	
	p 5 Nu	Number Amplicons
1851 tgaacttgac atatagtagg cagagagcat tcagtaagtg ttggcttgct 1901 cccttttttt catttaggaa gtgatctaaa aacagtattg ttagtaaatg ReacionCondhio	ions	
1951 gtatettgat ettaatgtta tgtggaetat tttaaettee etttaaatg		Search
- Amplicon Names		
SNP		

- 6. Search for primers by selecting the **Search** button.
- 7. Choose the desired primer set from the list by highlighting and then select **Fixed Oligos**.

LightScar <u>S</u> equence					e 1.0 (Beta 8	0)										.)6 ×
esign 1	5 <u>2</u> 000	igo <u>1</u> 00	лэ <u>П</u> ол	٢													
100	É		í	. 1. 6		0.											
Sequence				-													
Aligr	nment	Cross	Comps	BLA	ST	Fixed	Oligos		Set	Comm	ents	Target	Comments				
Set		Amplicor		Forward F				Reverse F					onditions		1		
Rank	Score -255	Length 141	%GC	5' Pos 313	3' Pos 330		Tm 60.6	5' Pos 453	3' Pos 429		Tm 60.3	Tm 64	Additive	Gradient 59 - 69	Comments		
120 406 487 513	-317 -589 -685 -712	95 126 199 194	64.2 64.3 44.2 45.4	315 281 337 313	332 300 353 330	18 20 17	62.5	403 406 535 506	392 390 519 477	18 17	63.2 63.6 63.8 60.5	66 67	YYNN	61 - 71 62 - 72 62 - 72 59 - 69			
Forward caggag Reverse	}ank 1 d gaatggc⊆ e	Score -2	55	ac 41	C				5' Pos 313 453		Pos IO	141- Leng 	th %GC 55.6 36.0	Tm 60.6	Delta G [-16.1	56—aaggetaatatggtaaatgae	ccat

8. Return to the sequence tab, the primers will be highlighted in blue in the sequence window.

uenc	e Annotations Ta	rget Search Region	Primer Sets				
Insert		Sequence Locked					
						Experiment Ty	pe
001				*********		Single Amplic	on Primers
051			attgagaaat ctcttaacaa				
101			caagttcaga			- Sequence Info	are sting
151			ccgaggcggg			Sequence min	
201			acggtgaaac			Name	SNP
						A	
251			agtggcgggc			Access #	1
301			tggcgtgaac			Author	Deepika_deSilva
351			geactccage				1
401			aagttggt <mark>tt</mark>		catttactgg	First Position	1 Last 1000
451	gregatatata	ctacttagtt	acactactta	catagettea	gttteettat		
501	cteteeesta	reseteerer	ctcccatgag	aactaaacat	aacactceta	Length	1000 %GC 43.8
551			ggaggccgag			Position	372 cDNA
601			gaccaacatg			rosidon	DIA CDINA
651			agcgtggtgg				Comments
701	tactccagaa	gctgaggcag	gagaatcacc	tgaacctggg	aggtggaggg		Comments
751	tgcagtgagc	tgacatcaca	ccactgetet	ccaqcctqqq	caacagagcg	- Annotations -	
301	agactgtctc	aaaaaaaaaa	aaaaaaaagt	gtatttaaag	cacttagcag		
851	tgaacttgac	atatagtagg	cagagagcat	tcagtaagtg	ttggcttgct	Ad	d Exon Add SNP Add Region
901			gtgatctaaa				
951	gtatcttgat	cttaatgtta	tgtggactat	tttaacttcc	cttttaaatg		
						Import Se	quence
						Edit Am	plicon
						Sear	ch
						-	

• Select the amplicon sequence as well as some sequence surrounding the amplicon and copy the sequence.

- 9. Save the file by going to the **File** menu and selecting **Save** or **Save As**. We will call this document the 'amplicon document.'
- 10. Minimize the amplicon document.

Design Your Probe

1. Open a new LSPD Software window and choose the **Single Amplicon** module. Paste your fragment into the new document.

LightScanner Primer Design Software 1.0 (Beta 80)	
File Sequence Settings Tools Help	
Design 1	
1 1	
Insert C Replace E Sequence Locked	
	Experiment Type
001 tcgagaccat cccggctaaa acggtgaaac cccgtctcta ctaaaaaaat	Single Amplicon Primers
051 acaaaaaatt agccgggcgt agtggcgggc gcctgtagtc ccagctactt 101 gggaggctga ggcaggagaa tggcgtgaac ctgggaggcg gagcttgcag	Sequence Information
151 tgagccgaga tcccgccact geactccagc ctgggcgaca gagcgagact	Sequence Information
201 ccgtctcaaa aaaaaaaaaa aagttggttt ccgattatac catttactgg	Name
251 gtaatatata ctacttagtt acactactta catagettea gttteettat	Access #
301 ctataaaatg caaataacac ctcccatgag ggctgggcgt ggcgctcatg	Author Deepika_deSilva
351 cctgtaatcc cagcactttg ggaggccgag gtgggtggat cacctgaggt	
	First Position 1 Last 400
	Length 400 %GC 50.8
	Position 172 cDNA
	Comments
Add SNP 🛛 🔀	
Name SNP I Target OK	Annotations
Name SNP 🔽 Target OK	
Start 172 Cancel	Add Exon Add SNP Add Region
Status Cover 💌	
Ignore Description Avoid	Import Sequence
Cover	
Text Color Default 👻 Background Color Default 👻	Define Amplicon
	Search

- In the Sequence tab highlight the SNP, or mutation of interest, select Add SNP. In the dialog box, select the Target box and change Description to Cover. Select OK. The LSPD Software window will display a SNP tab.
- 3. Select Target tab to view target information.
- 4. Under Experiment Settings change 5' and 3' exclusion buffers to 0.

CHAPTER 6: Designing LunaProbes

gn 1							
quence Annotations Target	Search Region Primer Sets						
				Target Inform	nation		
151 acaaaaatt ago 101 gggaggctga ggo 151 tgagccgag tco 101 ccgtctcaaa aaa 151 gtaatatata cta 101 ctataaaatg caa	ggctaaa acggtgaaac cgggcgt agtggcggg agggga tggcgtgac cggcoact ggactcoagc aaaaaa aagttggtt icttagtt acactactta icttagtt ctcccatgag icactttg ggaggccgag	gcctgtagtc ctgggaggcg ctgggcgaca ccgattatac catagcttca ggctgggcgt	ccagctactt gagcttgcag gagcgagact catttactgg gtttccttat ggcgctcatg	Name Position Length Position Experiment S Min Amplico Min Primer S S'Exclusion Minimum UV Reaction Co	SNP 172 1 172 1 172 172 172 Comments Settings m Size 17 Buffer 0 Settings m Size 17 Buffer 0 settings er Master Mix	Lest 172 KGC 40.9 DNA Fixed Oligos Aax Amplicon Size Aax Primer Tm Aax Primer Size Y Exclusion Buffer Humber Amplicons Sea) 150 600 30 0 1 1 rch

- 5. Select Fixed Oligos.
- 6. The Fixed Oligos screen will display.

💻 Fib	red Oligos	X
1	tcgagaccatcccggctaaaacggtgaaaccccgtctctactaaaaaaat agetctggtagggeegattttgecaetttggggeagagatgattttttta	_
51	acaaaaaattagccgggcgtagtggcggggcgcctgtagtcccagctactt tgttttttaatcggcccgcatcaccgcccgcgggacatcagggtcgatgaa	
101	gggaggetgaggeaggagaatggegtgaacetgggaggeggagettgeag eceteegaeteegteetettaeegeaettggaeeeteegeetegaaegte	
151	tgagccgagatcc <mark>ogccactge</mark> actccagcctgggcgacagagcgagact actcggetctagggggggggac <mark>,</mark> tgaggtcggaccgetgtetcgetctga	
201	ccgtctcaaaaaaaaaaaaaaagttggtttccgattataccatttactgg ggcagagtttttttttt	
251	gtaatatatactacttagttacactacttacatagcttcagtttccttat cattatatatgatgaatcaatgtgatgaatgtatcgaagtcaaaggaata	
301	ctataaaatgcaaataacacctcccatgagggctgggcgtggcgctcatg gatattttacgtttattgtggagggtactcccgacccgcagcacc	
351	cctgtaatcccagcactttgggaggccgaggtgggtggatcacctgaggt ggacattagggtcgtgaaaccctccggctccacccactagtggactcca	
	Bases per Line 50 😤 🔽 Show Positions	
	ard Primer cactgcactccagc	
Reve	rse Primer 5' Position 3' Position Length Tm	
	low Mismatched Oligos Clear All	
	omplement Strand Oligos 3' to 5' OK Cancel	

- 7. Highlight the region over the SNP. We recommend designing the probe to match the variant sequence. For example if a base change is C>T, with the T allele being the variant, design the probe to the sequence containing the T base. This will result in a perfect match between the probe and the variant template (T:A) giving the highest Tm melt. The advantage of doing this is that the wild-type now behaves like a single base mismatch, and any novel variant under the probe is the equivalent of a double mismatch. This makes it very easy to identify novel variants and differentiate them from the SNP of interest.
- To determine if your probe should be on the sense or antisense strand, choose the mismatch that is the most destabilizing. For base pair neutral mutations, either strand will work. See stability ranking below.

General Ranking of Base Pair Stability

Stable G:C>A:T>G:T>G:A>T:T>G:G> A:A>C:C>C:T>C:A Unstable

- 9. Using the example above, if the probe is designed to the variant sense-strand, the probe wildtype duplex will harbor a T:G mismatch. If the probe is designed to the variant antisense strand, the probe wild-type duplex will harbor a C:A mismatch. Since a T:G mismatch is more stabilizing than C:A (see chart above), you would choose the antisense strand for the probe. This will give you the biggest Tm separation between the genotypes in the actual experiment.
- 10. If you choose the antisense strand for your probe, highlighting this region will display the sequence in the **Reverse Primer** text box. A probe designed to the sense strand will be displayed in the **Forward Primer** text box.
- 11. Use the +, -, <, > keys after the oligo box to get required length and Tm. Recommended length is 20–30 base pairs and recommended Tm is 60–65° C.
- 12. Check the Tm. You may increase or decrease the oligo length to achieve the recommended Tm. Make sure the SNP is at least 3 base pairs from the end of the oligo.
- 13. Select Show Fixed Oligos on Sequence.

14. Copy the probe sequence from the primer text box on the Fixed Oligos page. Select OK.

Fixed Oligos		
1 tcgagaccatcccggctaaaacggtgaaaccccgtctctact agctctggtagggccgattttgccactttggggcagagatga		
51 acaaaaattagccgggcgtagtggcgggggcgcctgtagtccc tgttttttaatcggcccgcatcaccgcccgcggacatcaggg		
101 gggaggetgaggcaggagaatggegtgaacetgggaggegga ceeteegaeteegteetettaeegeaettggaeeeteegeet		
151 tgageegagatee <mark>egeeaetge</mark> aeteeageetgggegaeaga acteggetetagggeggtgae <mark>t</mark> gaggteggaeeegetgtet		
201 ccgtctcaaaaaaaaaaaaaaagttggtttccgattatacca ggcagagtttttttttt		
251 gtaatatatactacttagttacactacttacatagcttcagt cattatatatgatgaatcaatgtgatgaatgtatcgaagtca		
301 ctataaaatgcaaataacacctcccatgagggctgggcgtgg gatattttacgtttattgtggagggtactcccgacccgcacc		
351 cctgtaatcccagcactttgggaggccgaggtgggtggatca ggacattagggtcgtgaaaccctccggctccacccactagt		
Bases	per Line 50 📑 🔽 Show Positions	
Forward Primer	5'Position 3'Position Length Tm	
cgccactgcactccagc + · · · · · + Clear Reverse Primer	164 180 17 66.0 5' Position 3' Position Length Tm	
+ - X > - + Clear		
Allow Mismatched Oligos Clear All Complement Strand Oligos 3' to 5'	- Í	
Show Fixed Digos on Sequence		

Note: The sense primer was moved and the SNP of interest is in the center of the oligo.

15. Go to the Sequence tab on the LSPD Software Screen; your probe will be displayed. Save this search by going to the file menu and selecting save or save as.

Determining Probe and Primer Compatibility

- 1. Maximize the previously saved amplicon document window that contains the primers that will be used with this probe.
- 2. Select the **Comments** option located in the sequence information box on the right hand side of the page.

3. Paste the probe sequence in the comments section of your amplicon document.

NP Sequence Annotations Target Search Region Primer Sets Insert C Replace Seauence Locked	
• New Nepace i sequencecced 0001 aaaagattat tagtataata attgagaaat tactgttaaa aagttttgag 0001 attgataata tagtataata attgagaaat tactgttaaa aagttttgag 0051 ttctctraga aaattgaaa ctcttaacaa aacctgacta atactaactt 0101 aatgttttc atatacatag caagtcaga ctctgacta tatgaactt 0101 aatgttttc atatacatag caagtcaga ctctgacta tatgaactt 0101 aatgttttc ccgggtagg ccgagggggg cggatcacga ggtcagggg 0201 tcgagaccat cccggctaaa acggtgaaac cccgtctct actaaaaaat 0201 tcgagggctga gggagddagaa 0201 tggggggtga gggagddagaa 0201 tggggggtga gggagddagaa 0201 tgggggggtga dcccgcgcat 0351 tgggggggg a dcccgcaca dggggggg 0351 tggggggg a accttaga 0451 gtagccgaga tcccggcat 0451 gtagcggag tccagcat 0451 gtagcggag tccagcat 0451 gtagcgaga tccctgcatt 051 ctataaaatg caataacac tcccatgag ggtgggggg tggggggg tggggggtgggggtgggggg	Experiment Type Single Amplicon Primers Sequence Information Name SNP Access # Author Deepika_deSilva First Position 1 Last 1000 Length 1000 %GC 43.8 Position 641 cDNA
070 Comments - SNP	Annotations Add Exon Add SNP Add Region Import Sequence Edit Amplicon Search

- 4. Do a cross-complementarity check between your probe and primer set:
 - a. Copy the probe sequence
 - b. Move to the Primer Sets tab, select Cross Comps.
 - c. On the **Cross Comps** screen, select **Add** and paste your sequence into the **Sequence** text box.
 - d. If your probe lies on the anti-sense strand, select the complement strand box in the **Edit Sequence** box.
 - e. Select OK.
- 5. All of the complementarities between the probe and the primers are now displayed in the **Cross Complementarity** window.

6. If your probe does not have any significant complementarities, order your probe from your oligo house of choice. Be sure to add a C3 block or a phosphate on the 3' end.

💺 SNP - LightScanner Primer Design Software 1.0 (Beta 80)
Ele Sequence Settings Tools Help
SNP
Sequence Annotations Target Search Region Primer Sets
Alignment Cross Comps BLAST Fixed Oligos Set Comments Target Comments
Set Amolicon Forward Primer PCR Conditions Rank Score Length %26C 5° Pos 3° Pos Len Tm Tm Additive Gradient Comments
1 - 255 141 525 313 330 18 606 453 423 25 60.3 64 N 59-69
Cross Complementarity - SNP Rank 1 Score -255
File
Num Name Sequence Tm Score 1 2 Clear All
0 3' Alignments
1 v Forward caggagaatggcgtgaac 60.6 0 v Min Delta G 2 v Reverse tacccagtaaatggptaatcggaa 60.3 0 v Min Length
2 Alignments ⊽ Multi Match
1 V Forward caggagaatgggggaac 60.6 0
2
3
Edit Sequence MinAl Length
4
Name Probe Fis Primer Complement Strand Add
caggagaatgc
Sequence Edit atgacocat
- SNP Bank 1 Some
Forward DK Cancel
caggagaatggcgtgaac
Reverse transmission from the transmission of transmis
tacccagtaaatggtataatoggaa 453 429 25 36.0 60.3 17.8

CHAPTER 7:

DESIGNING A SMALL AMPLICON FOR GENOTYPING

The LSPD Software can be used to design small amplicons for genotyping. A small amplicon is a 50–70 bp region in which the primers are designed adjacent to a known SNP. This section will enable you to define a SNP within your sequence and design primers adjacent to the SNP of interest.

- Launch LSPD software and select the Single Amplicon button on the front screen. Go to Import Sequence and open the sequence file containing the SNP of interest, including at least 100 bases upstream and downstream of the target SNP.
- Once the sequence has been imported, highlight the target SNP in the Sequence tab and press Add SNP. In the dialog box, select the Target box and verify that the SNP status is Avoid. Press OK.

gn 1							
quen	ce Annotations						
Inse	ert C Replace	C Sequence Locker	ł				
						Experiment T ₃	pe
001	catcaccagg	attttctgtg	gtacagaaca	tgtctaagca	tgctggggac	Single Amplic	on Primers
051		ggaagagatc					
01	cctacctaca	tctgcactgc	ctcccgtgac	taattccttt	agcagggcag	C Sequence Inf	ormation
151		gccaaatgaa					1
:01	cttcattctc	tgccagtcag	agctaaaaat	agaaattgtg	taggagacaa	Name	1
					territoria de la factoria	Access #	
51		ttccctagaa					
51		tcttgcattt gtaggtaggg				Author	deepika_desilva
101		taaattttgc					
51		ttgctactcc				First Position	1 Last 750
		1.1.8.1.0.1.1.1				1	750 %GC 41.3
501	ggttgatggc	aattccagtt	aactgctgtg	cageteteat	ctcattgtgc	Length	1750 %dC [41.5
551	acacagcatg	gaaatctttc	tcaaaactgt	ttcactcagg	tcagggtaac	Position	271 cDNA
501	aagtttggta	gagcaaaccg	gtgaatgata	ctctcatgca	aaactgaaca		1 1
551		catatgtatg					Comments
101	tttgcaatgt	gtagtttaat	aggtaattac	ccttaacgct	tttgcaggga		
	-					- Annotations -	
	Add SNP					Annotations	
						Ad	d Exon Add SNP Add Region
	Name	SNP	(🖓 Ta	raet) O	K	-	
		1					
	Start	271	\sim	Car	ncel		
		1.0.0		-		Import Se	equence
	Status	Avoid 💌					
						Define A	mplicon
	Descripti	on				-	
	Text Cold	Default	 Background C 	olor Default	•	Sear	ch

3. Select the **Target** tab that appears and refer to the **Experiment Settings** at the right hand side of the screen. Verify that the **Min** and **Max Amplicon Sizes** range from 45–150 and change both the 5' and 3' exclusion buffers to 0.

LightScanner Primer Design Software 1.0 (Beta 79)	
Elle Sequence Settings Tools Help	
Design 1	
Sequence Annotation Target Search Region Primer Sets	
\bigcirc	
Target Information	
001 catcaccagg attttctgtg gtacagaaca tgtctaagca tgctgggggac Name SNP	
101 cctacctaca tctgcactgc ctccgtgggt tagtcagt tagtcagt caggggag Position 271	Last 271
201 ettegatata gebaaatgaa titetggete acceltatt aggagteag Length 1	%GC 35.6
Position 271	cDNA
251 accttgttaa ttccctagaa atacattaag aggatagagt ggaatttttt	
301 ttctctgcaa tcttgcattt ttttaatggc tctttttttt tttcctgata	1
351 aaaacctttg gtaggtaggg aagttatgtt ttcaggggta aatgtgctac Commer	nts Fixed Oligos
401 ttttgtcttc taaattttgc tcttttttga ctggtctagt caagtgacag	
451 cccgattatt ttgctactcc ttaaaagtac tattctgtct cttggagtat	
501 ggttgatggc aattccagtt aactgctgtg cagctctcat ctcattgtgc Min Ampicon Size 45	Max Amplicon Size 150
551 acacageatg gaaatettte teaaaactgt tteacteagg teagggtaac	
601 aagtttggta gagcaaaccg gtgaatgata ctctcatgca aaactgaaca MinPrimerTm 60.0	0 Max Primer Tm 60.0
651 gatatgcaaa catatgtatg tggttcagct tgggttgcat gggttcagac Min Primer Size 17	Max Primer Size 30
701 tttgcaatgt gtagtttaat aggtaattac ccttaacgct tttgcaggga Min Pimer Size 17	Max Primer Size 30
5' Exclusion Buffer 0	3' Exclusion Buffer 0
Minimum Overlap 5	Number Amplicons 1
Reaction Conditions	
	Search
Light/Scanner Master Mix	
- Ampicon Names	
SNP	

- 4. Select **Search** for primer sets.
- 5. Under the **Primer Sets** tab, a list of the calculated primers will be displayed with a corresponding rank and score. Primers displayed in green are high scoring primer sets and most likely to be optimal in the defined range. Small amplicon primers can be successful with a fragment size ranging from 40–150 bases; however, 50–70 base fragments are ideal for this application. Try to select a primer set from the tabulated list with both high rank and score that falls within the 50–70 base fragment size.

	htScanne					e 1.0 (B	leta 1	79)									
	equence ;	jettings	Toop	s <u>H</u> elp	0												
	ign 1																
Se	quence	Annota	tions	Targe	et Searc	ch Regio	n Pi	rimer 9	Sets								
	Alignme	nt	Cross (Comps	BLAS	ST	Fixed	Oligos	5	Set	Comm	ients	Targe	t Comments			
Se			olicon		Forward Pr				Reverse Pr					Conditions		1	
R		ore Le			5' Pos	3' Pos									Gradient	Comments	
4 9 15 18	5 4	24 241 299 318 304	113	34.8 33.3 36.3 38.6 31.0	206 244 185 166 227	223 266 205 183 252	23 21 18	60.2 59.4 60.2	297 297	273 272 273 273 273 273	25	59.8 59.8 59.8 59.8 59.8 59.8	63 64 63 63 63		58 - 68 59 - 69 58 - 68 58 - 68 58 - 68		
S	agacaa NP Rank Forward		ore -24		ttece	t—4	[5' Pos	3	SN Pos		ngth %GC 39.1	Tm 60.2	Delta G	atgtaattotootatotoacottaaa

 If the software does not return primers for amplicons in the desired size range, return to the Search Region window and reduce the search regions to approximately 30–50 bases on either side of the SNP. Select Search.

LightScanner Primer Design Software 1.0 (Beta 79) File Sequence Settings Iools Help					
SNP Sequence Annotations Target Search Region Prime	er Sets				
				Position = 561	_ 1
1 10 200	300 400 Nucleatide Position	500	600	700	750
Search Region Forward Reverse Start 263 Start 301 End 239 End 329 %GC 209 %GC 210	Region Start 269 And 329 %GC 27.9 Search				

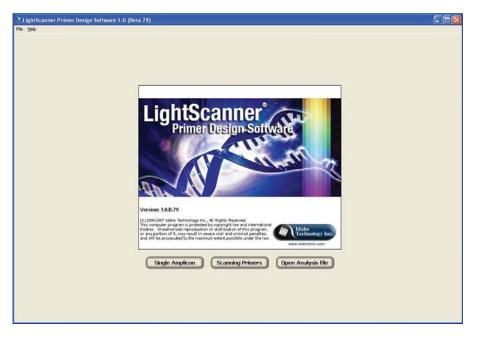
- 7. Highlight optimal primer set and select **Cross Comps** to check cross-complementarities. If connected to the Internet, BLAST primer sequences to check the design specificity.
- 8. If primers do not display any significant cross-complementarities and are specific to the target region, primers can be ordered.

APPENDIX A:

QUICK GUIDE TO USING THE LIGHTSCANNER PRIMER DESIGN SOFTWARE

The LSPD software enables users to design primer sets that amplify small segments of large regions of interest. These primer sets can be added to the users' gene files. The primers designed will only be used for scanning analysis.

Step 1. Launch the Software

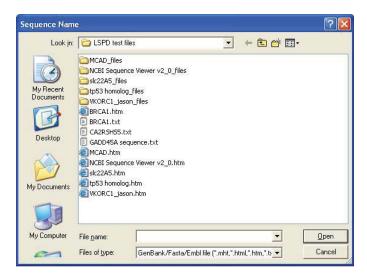


Step 2. Import a Gene Sequence

1. From the **Sequence** menu, select **Import**, and then select the file type. May import from European Molecular Biology Laboratory (EMBL), FASTA, GenBank, or regular text files.

ntScanner Primer Design Software 1.0 (Beta 79) spience Settings Tools Help	
a j	
ence Amotations	
Inum C Replace C Sequence Locked	Espeninen Type Scanning Phines Sequence Information Name Access B Aufor despla_deshva First Postion 1 Last 0 Length 2000. Commerks Accelations Accelations Accelations Commerks Search All

2. Find and select the desired sequence, click Open.

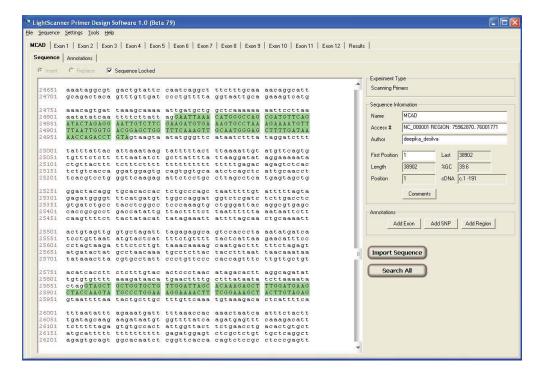


Exons will be highlighted and bases in exons will be converted to uppercase.

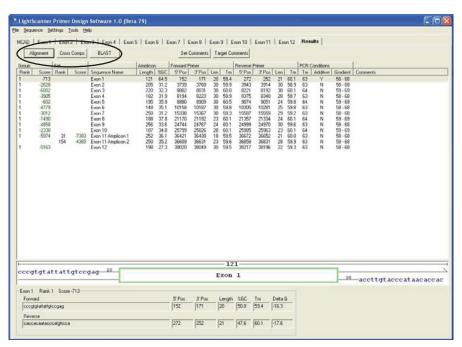
Note: The sequence must contain at least 200 bases of sequence upstream of the first exon and downstream of the last exon for the software to recognize these exons correctly

Step 3. Search for Primer Sets Using Common Design Parameters

Select Search All to find scanning primers for all exons.



Step 4. View and Export Results



1. A results summary screen displays the best scoring primer for each amplicon in tabulated format. All of this data can be exported in a spreadsheet format for data archiving or can be saved as a primer design file (*.spd file) that can be re-opened in the software. 2. To locate the position of the primers in the context of the entire sequence, highlight the amplicon in the screen and select the **Alignment** button.

Alignment - Exon 1 Rank 1 Score -713	D
1 cggcgccggggaccgctgccaccccgcctagcgcagcgc	^
101 gtgggggggaccagaggagtcccgcgttcggggagtatgtcaaggccgtga <mark>ucegtgtatattgtccggag</mark> tggccggaacgggag <mark>ccaac</mark> a cacccgccctggtctcctcaggggcgcaagccctcatacagttccgggcactgggcacataataacaggctcaccggccttggcctcggttg <mark>1ACCGTC6C</mark>	
201 <mark>GGGTTCGGGCGKTGCTGCLGG</mark> gtgagagggagcccagcggtggggtggggctggacatgggtattgtggtgtggggcggggccctgggccaaaaat CCCLLGGCCCCTLCGLCGTCC <mark>cactc</mark> tcctcgggtcgccacgccaccccgacttgtaccacaggcctcgtcccccgggacccggttttta	
301 aggtgcggccgggaggagtgggaagtgggaagtcggggacgaggaggaggacgggacccccaacctgctttcacgcctcctaccaccggacgga	
401 tagegttteatttccgtatcctcccgtcaggegaccccgttatageeggcatccttcttttagaatatcgtttttcttttgtaaaccctccaaat atcgcaaagtaaaaggcataggaggggggggcgtccgctgggggcaatatcggccgtaggagagaaatcttatagcaaaaaggaaccatttgggaggttta	
501 atttaccgcggggaatcccacctttttccaaagtgaccccgtctctgtgtcagaaccacgggaaaaagaaaaatggagcagctactccttgaaatacttaa taaatggcgcccttaggggggaaaaaggtttcactggggcagagacacagtcttggtgccctttttttt	
601 tgtttaataagettttegttgtaacgtttecaegttgettaegggaaaaaaaaaa	~
Bases per Line 100 😴 Show Positions 🥢 🤇 🔊	

3. To view primer cross-complementarities, select the **Cross Comps** button in the results summary screen.

Num Name	Sequence	Tm Score 1 2	Clear All
2 V Forward 2 Reverse 1 V Forward 2 V Reverse	cccglglatlattgtccgag caccacaalacccatgttcca cccgtglattattgtccgag caccacaalacccatgttcca	03'Alignments 59.4 0 60.1 0 3 Alignments 59.4 -2 -2 60.1 -2 -2	C Min Delta G Min Length ✓ Multi Match ✓ Multi Match ✓ Show Delta Min 3' Length 3 ✓ Min All Length 4 ✓ Add Delete Edit

4. If you are connected to the Internet, BLAST the primer sequences directly, by selecting the **BLAST** button to check the specificity of the design.

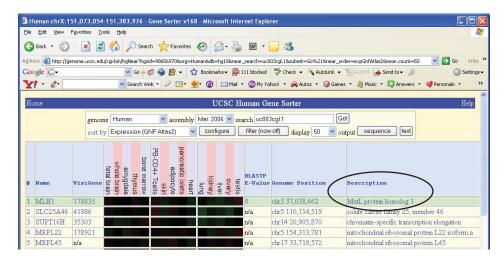
BLAST - Exon 1 Rank 1 Score - 713	
Forward Primer	OK 1
cccgtgtattattgtccgag	Cancel
Reverse Primer	
caccacaatacccatgttcca	
Database	
nr 💌	
Select From	
(none)	•

APPENDIX B:

GETTING ANNOTATED TEXT FILES FROM THE UCSC GENOME BROWSER

The LSPD Software is able to read text files obtained from the UCSC Genome Browser with the exons converted to uppercase. The following section is a brief guide to getting files of interest in this format.

- 1. Go to the UCSC genome browser home page at http://genome.ucsc.edu.
- 2. Go to Gene Sorter and type in the name of the gene of interest.



- 3. Click on the description box.
- 4. Find the Page Index box and select Sequence.

Human Gene MLH1 Description: Mat. protei RefSeq Summary (NM_	Search Web + 22 iencme Browser Blat (uc003 cgl.1) Descript a homolog 1 000249). This gene was id A minimitch repair gene was id A minimitch repair gene was id	Tables C tion and Pag ientfied as a loco £L, consistent w	Ene Sorter Po e Index us frequently mutate	ol • 🚔 Antos • 🕃 Gau R. Session FAD d in hereditary nonpolyp	 Marcer Standber Standber Sandber Wasses Help coist colon cancer (HNPCC) the requences (BEE+ chemoty 	It is a human
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5. From the next page choose genomic (Chr information).

Human Gene C	ADD45A (uc001	ddz.1) Description ar	nd Page Index - W	indows Internet I	xplorer			-6
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6. This will take you to a page where you can select how you export the gene sequence, for example: Exons in upper case, Promoter/Upstream 200 bp, Downstream 200 bp, etc. For the Light-Scanner software to find primers for the last exon, you must have at least 200 base pairs downstream of the exon in your text file. Selecting **submit** will bring up the desired sequence with the exons displayed in upper case.

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7. Save the file as a text (.txt) file that can be opened with the primer design software.

INDEX

Α

Additives	ix
Alignment	
Definition of	viii
Amplicon, adjusting search regions	16
Amplicon size, scanning	13
· · · · · · · · · · · · · · · · · · ·	

В

BLAST	
Cross-complementarities	21
Sequence alignment	22

С

Cross-complementarities	iii,	21
Cross-comp tool		24

D

Data, saving and exporting	20
Delta G	13
Designing a probe	29
Designing primers	27

Ε

End-user License Agreement for Software (EULA) xi
Exclusion buffer, values for
Results for 20
F
Fixed oligos ix, 24
G
Genotyping, small amplicons
Н
Hardware requirements 1
I
Installation 1

L

LunaProbes	27
Μ	
Minimum overlap	14
Ρ	
Primer concentrations Primers	14
Groups	viii
Rank	viii
Score	viii
Primer Tm	
Definition of	viii
Minimum/maximum	vii
Primer Tm, minimum and maximum	13
Probe and primer compatibility	32
Q	

(

Quick guide 39	9
----------------	---

R

Region	
Defining	11
Results tab	19

S

Sequence formats	
Importing	23
Single amplicon	
Definition of	10

Т

Technical Support	iii
Tools menu	

U

UCSC genome browser...... 43



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