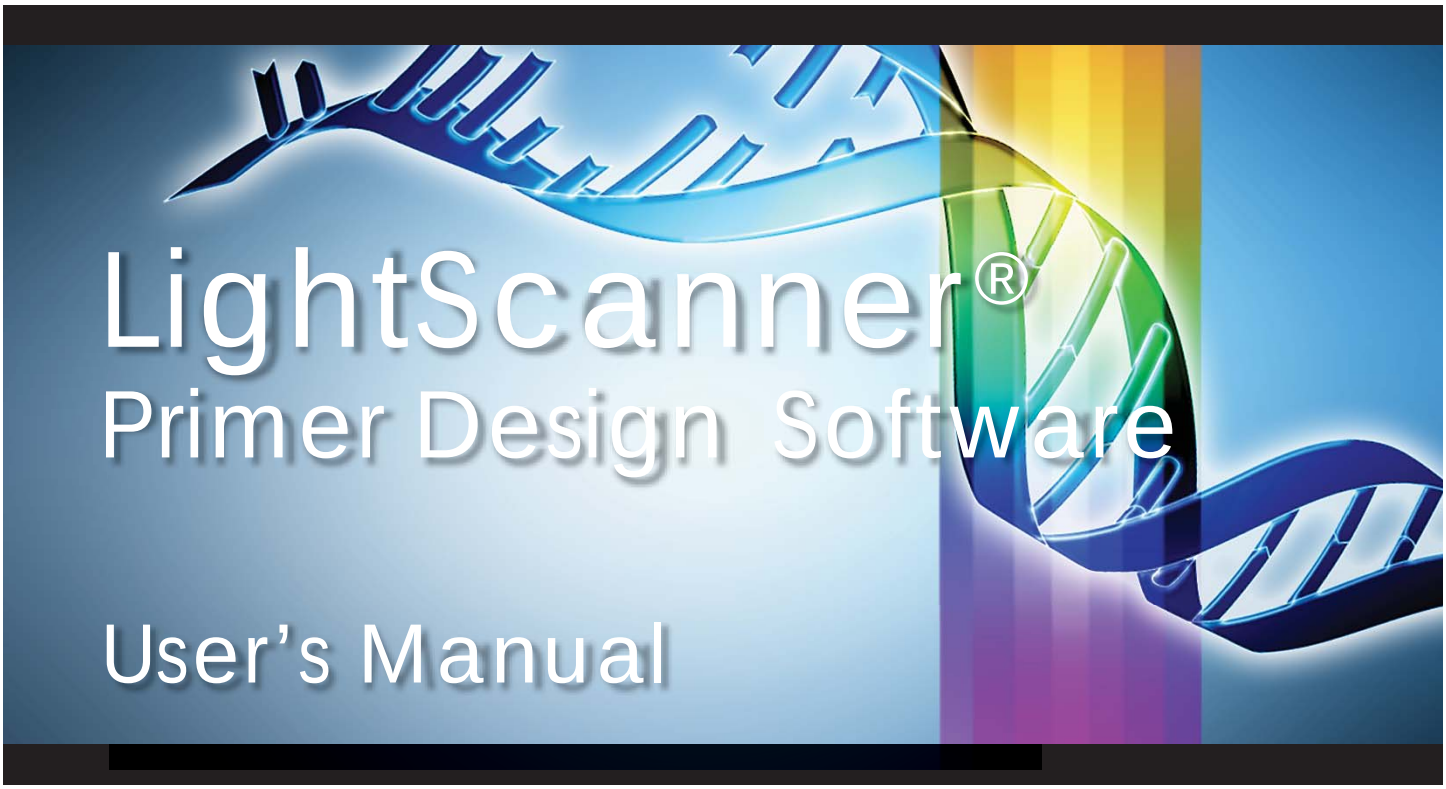

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Part No. LCSN-PRT-0029 Rev 01 11/07

LightScanner® Primer Design Software Manual

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Printed in the United States of America

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We strongly encourage users to visit our Web site for answers to frequently asked questions, updated information and additional insights into operating the LightScanner System.

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CONTENTS

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 functional Tm of an oligonucleotide may be increased by as much as 5–10 ºC 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 ºC (example, 60–65 ºC). 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 maintaining stringency. (We do not recommend using a Tm range of ≥ 5 ºC 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 highest. 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 interactions 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

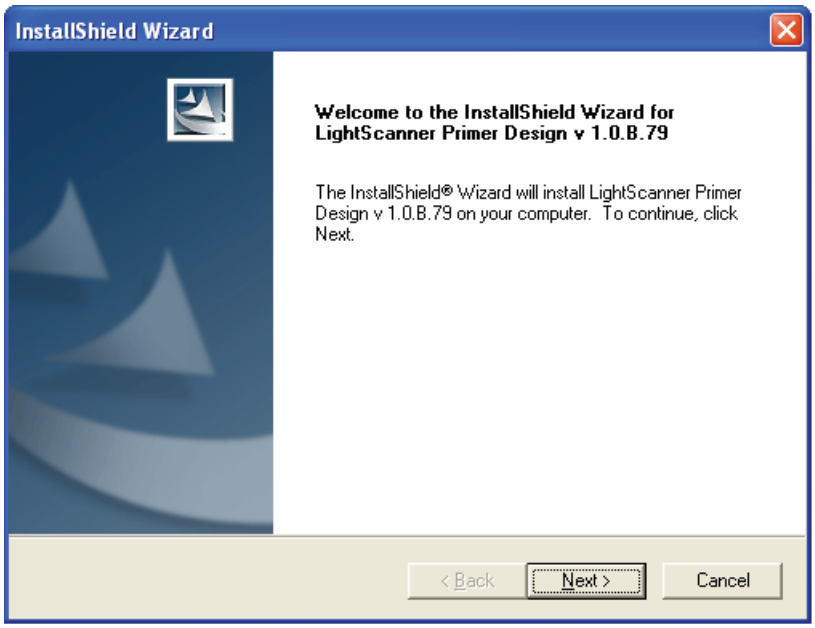
Operating system	Windows XP/ Windows 2000
CPU	80 GB or greater
RAM	512 MB or greater
Display	1024 x 768 or 1280 x 1024
Connections	Network card*

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

Installation Instructions

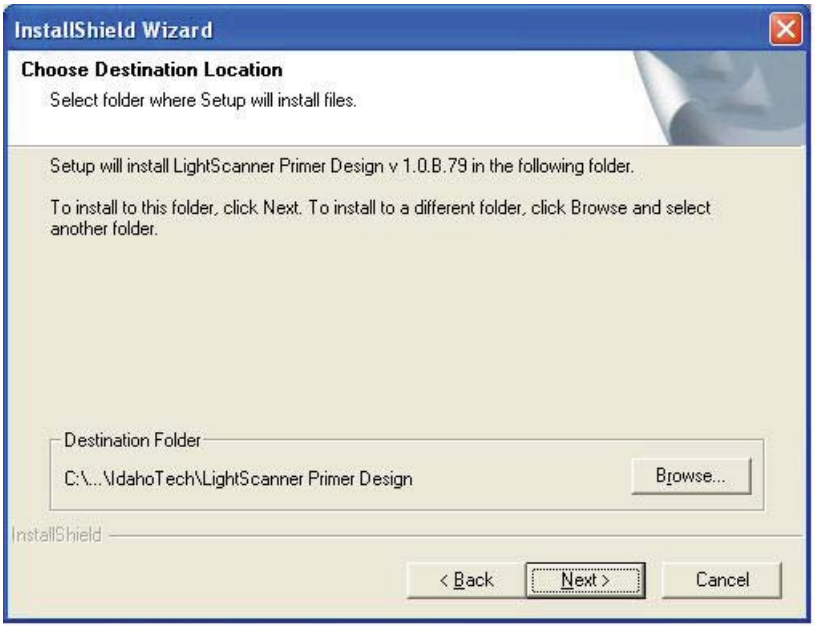
The LightScanner Primer Design (LSPD) software is automatically installed on the computer that is used to run the LightScanner instrument. Users may install the application on independent computers that meet the minimum requirements, if desired, using the installation disk.

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



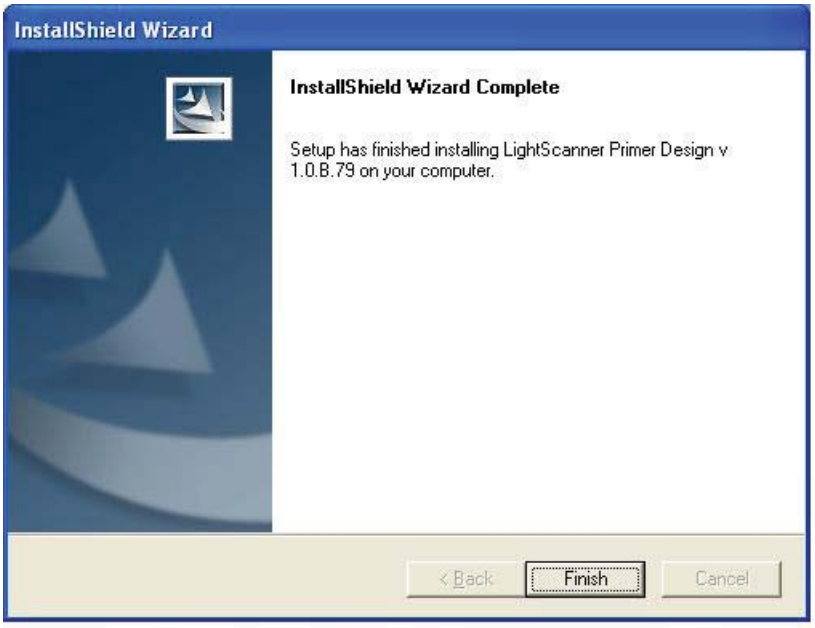
3. The **License Agreement** window is displayed. After reviewing the agreement, select **Yes** to confirm and move to the next window. Selecting **No** will terminate the install process.

4. 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.



5. 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.

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



CHAPTER 2:

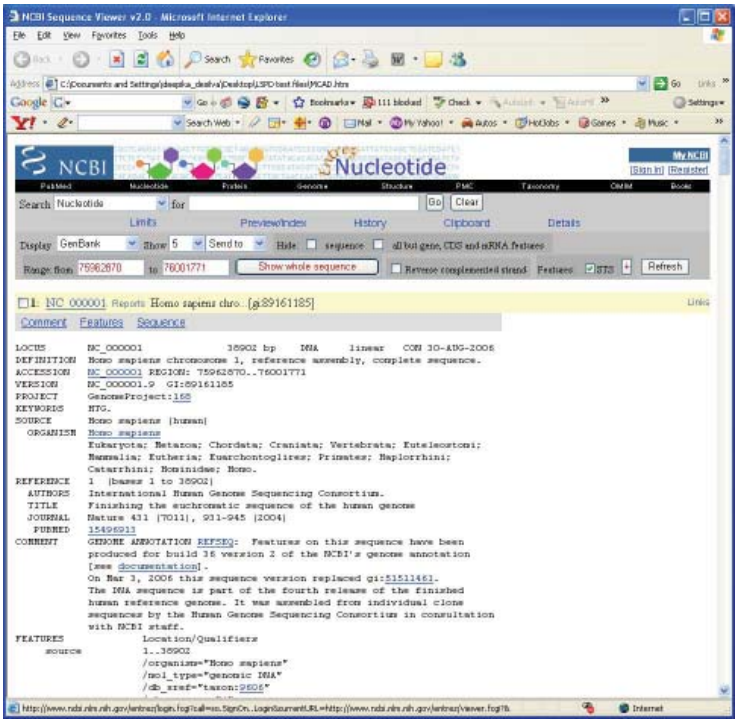
STARTING THE PRIMER DESIGN SOFTWARE

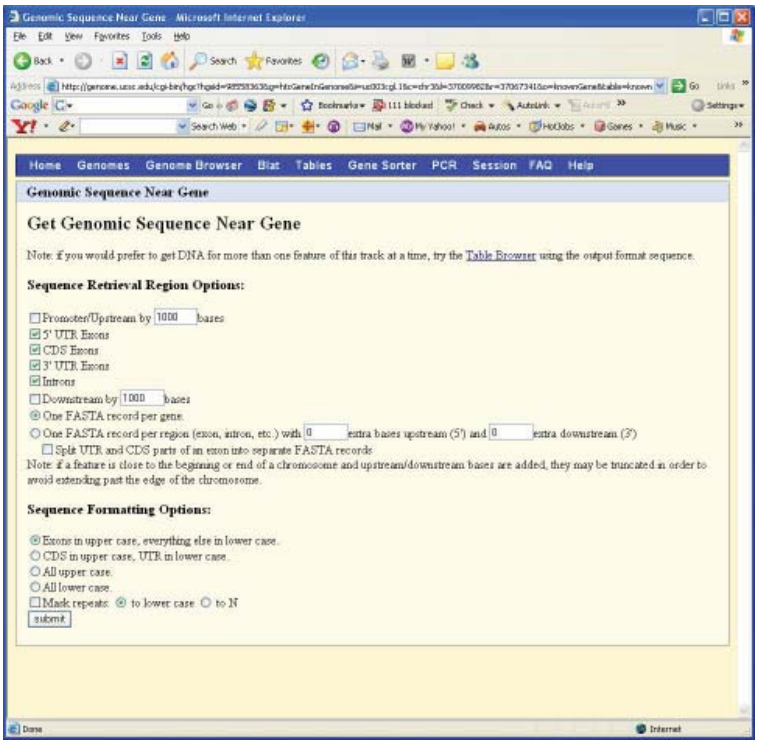
The LSPD software enables users to design primer sets for mutation scanning and discovery using the LightScanner instrument. The primary application is for designing primers that amplify every exon of a given gene specifically for high-resolution melting. A secondary application is the more traditional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

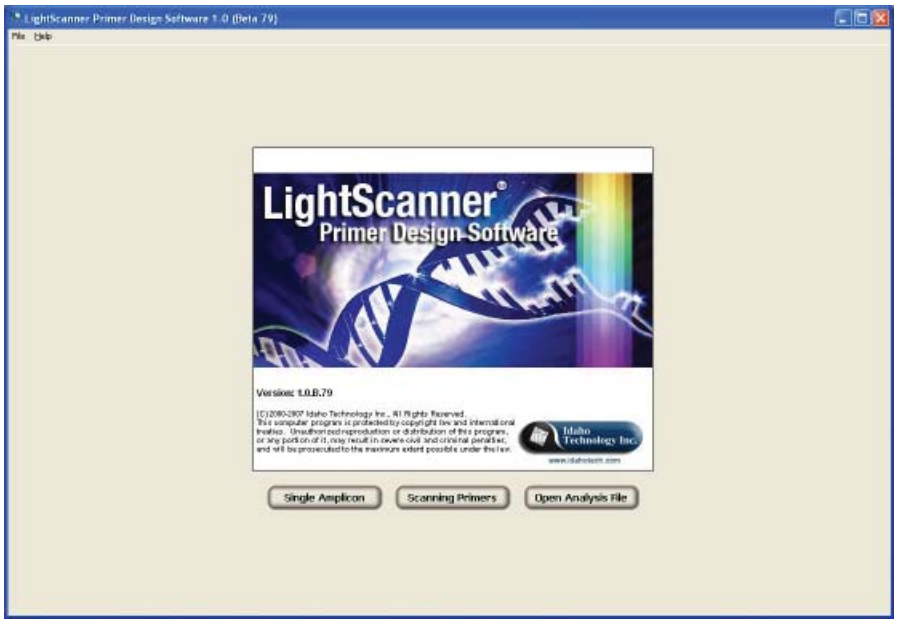
1. Double click on the LightScanner icon on your desktop. The Front Screen displays.

2. The user has three options to select from:

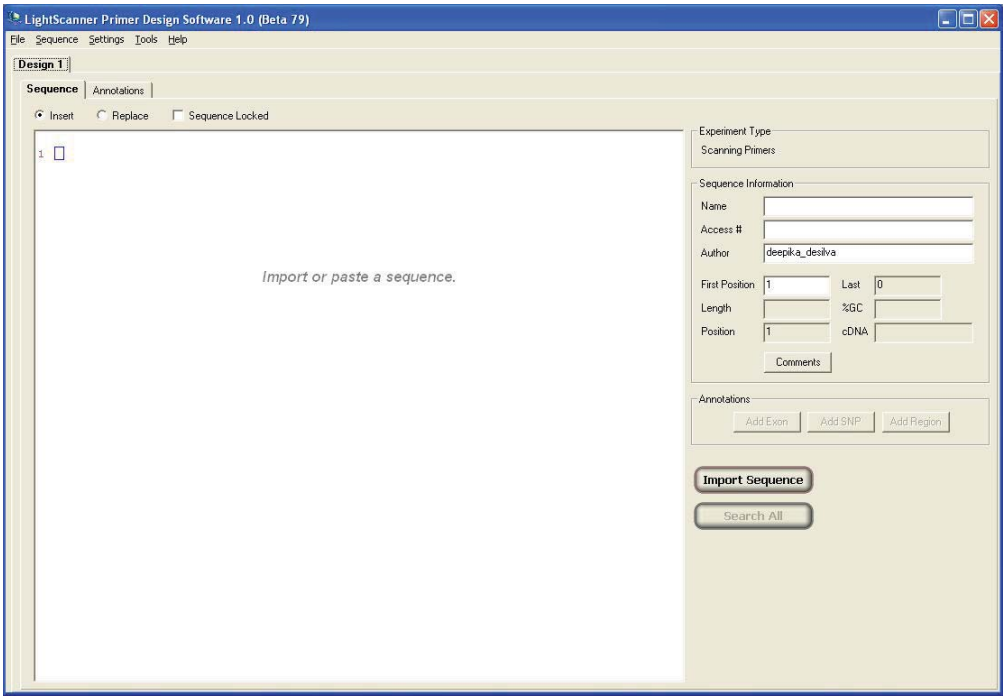
   a. Single Amplicon—This is a primer design module that can be used for designing individual primer sets. No assumptions are made about exon regions. This module has all the functionality of the Scanning Primers design module in terms of sequence definitions (single nucleotide polymorphisms [SNP], repeat 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.

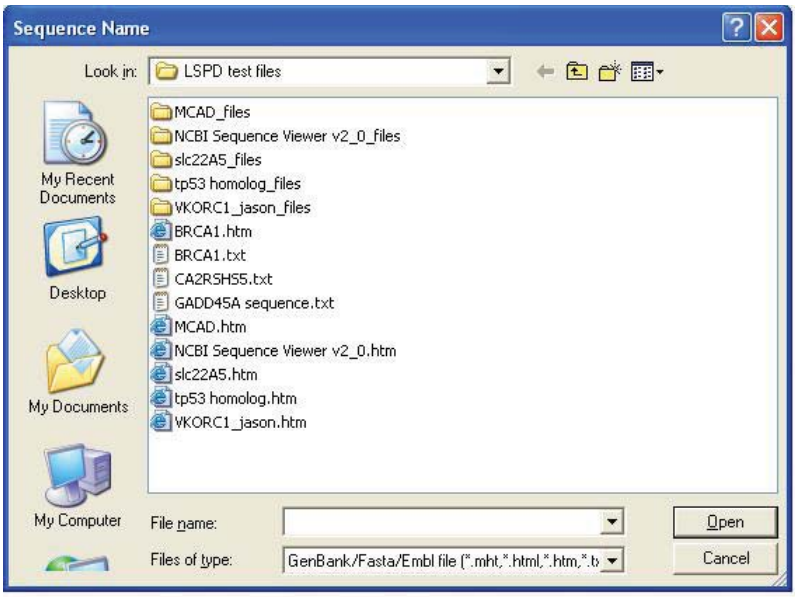
   c. Open Analysis File—Open an existing file and work with the data.



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

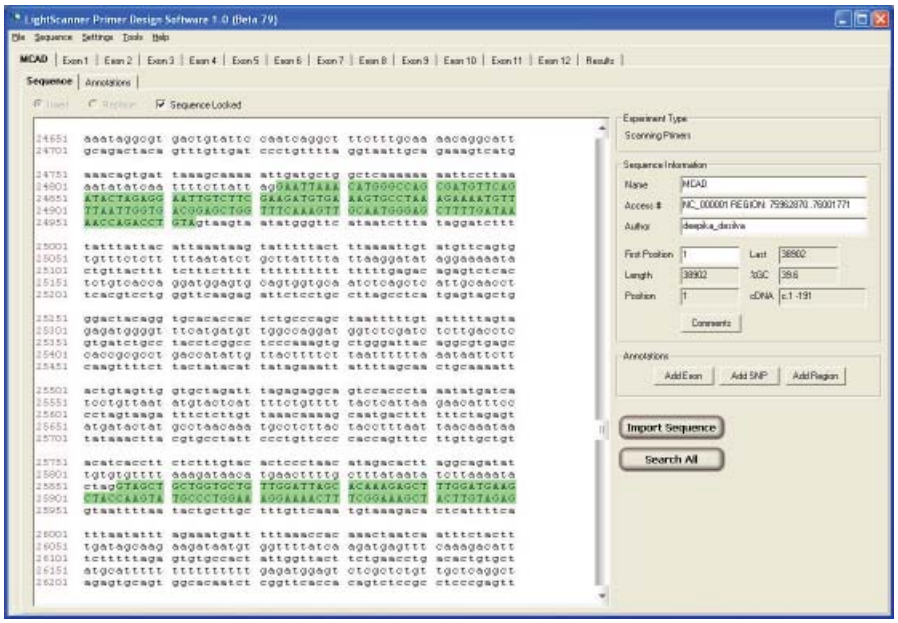


4. Find and select the desired file, click **Open**.



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.



CHAPTER 3:

ANNOTATING SEQUENCES

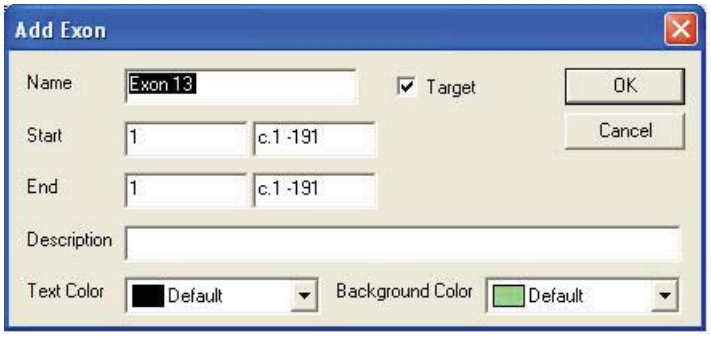
Defining Exons, SNPs and Regions

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

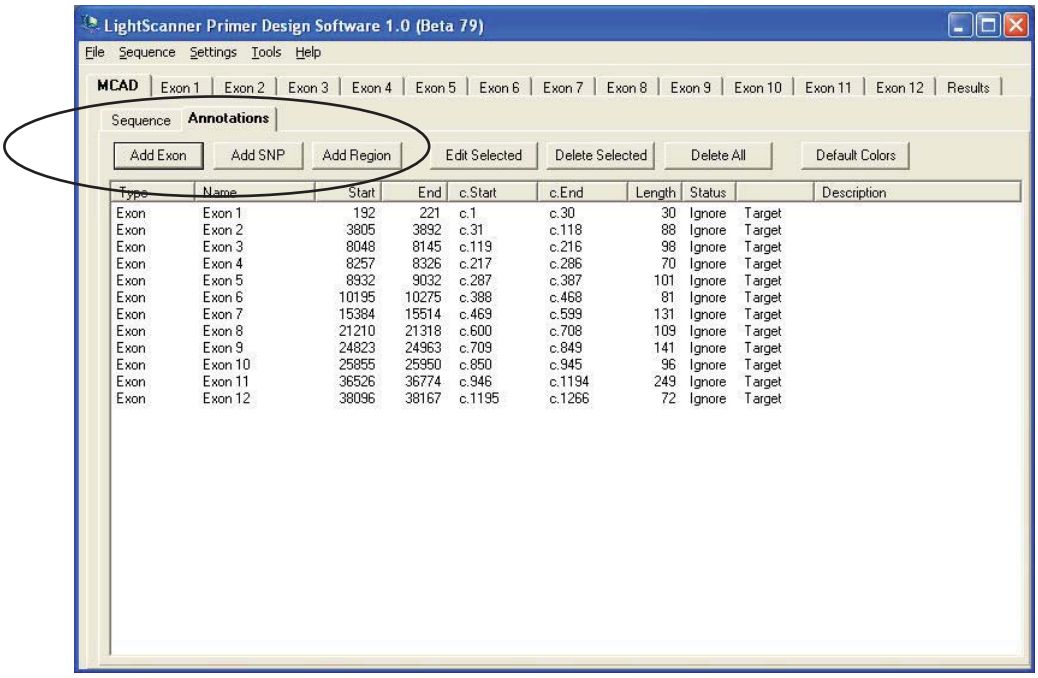
If you have imported a file with the exons in uppercase, there is no need to manually define the exons. If you have imported a normal text file and want to define the exons manually, highlight the sequence in the window and select the **Add Exon** button. A pre-filled dialog box will open.

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.



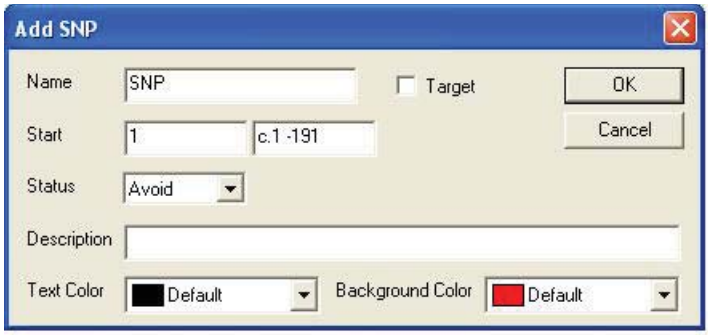
Alternatively

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**.



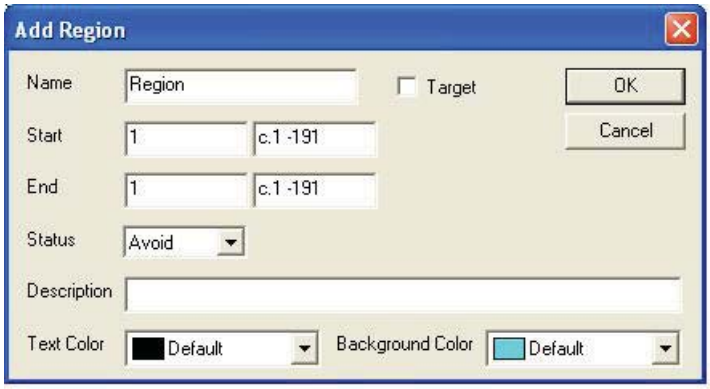
Defining SNPs

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.



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.
2. 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.



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:

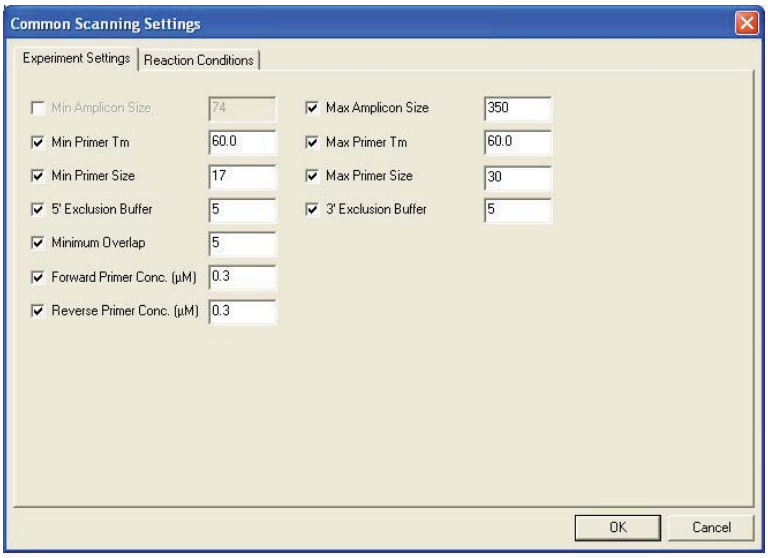
DESIGNING PRIMER SETS

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.



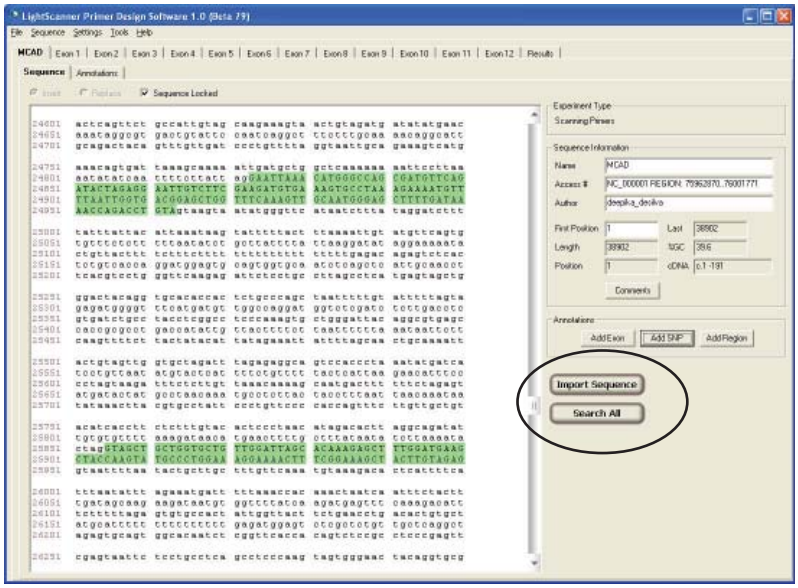
The settings options are as follows:

- 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.

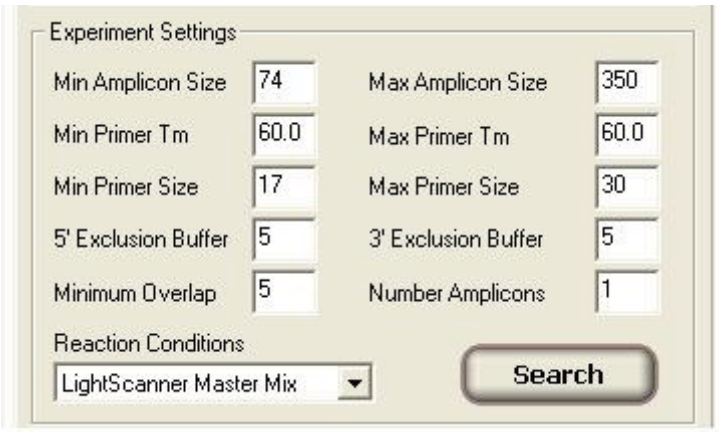
When using common design parameters for all exons, the next step is to select the **Search All** button. This will activate a primer search for every exon, with no further user input required.



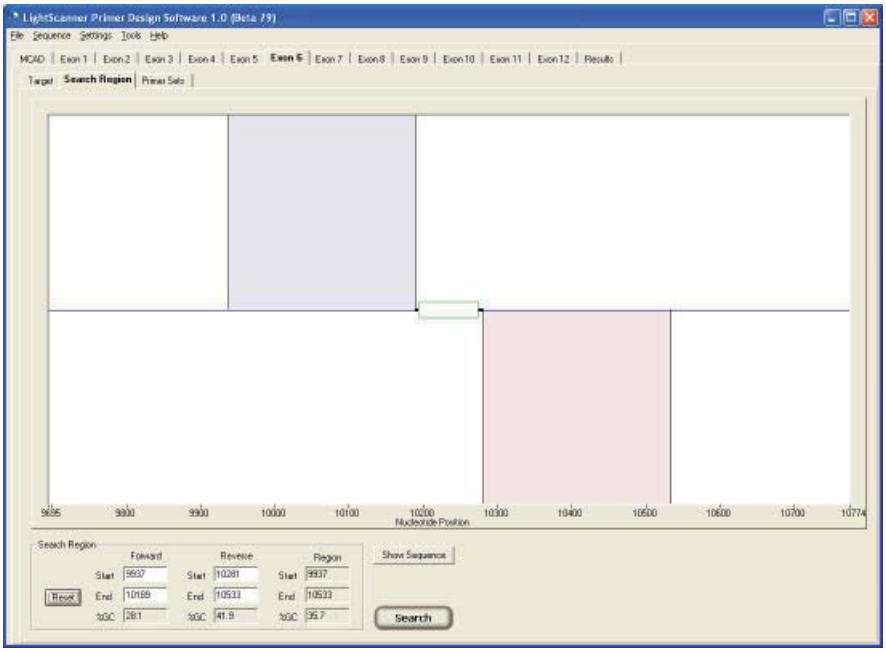
Customizing Design Parameters for Individual Exons

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.

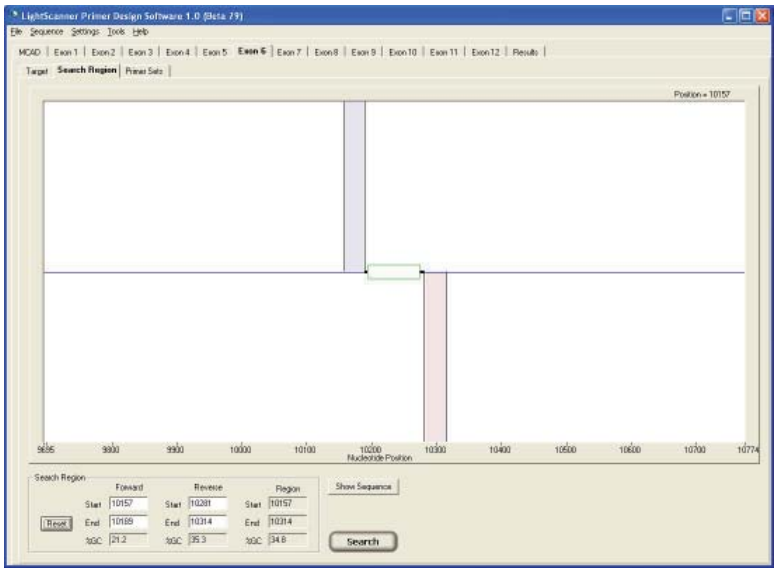
Select the **Search** button to begin the primer search. The software will discover primer sets within the search regions for each amplicon and rank them, displaying them in descending order.



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.



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.

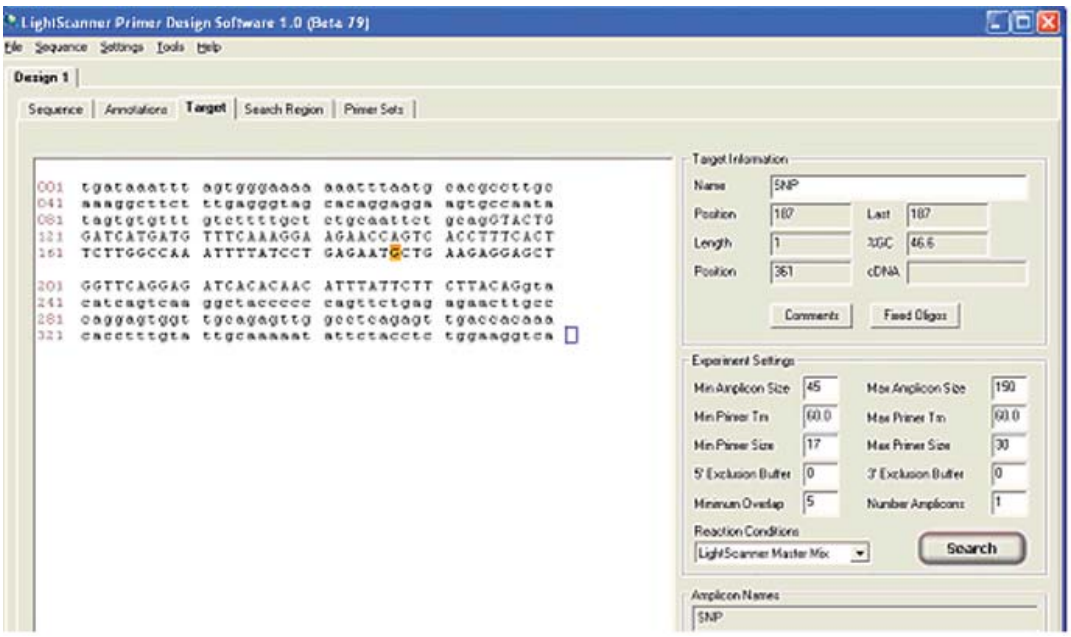
1. Open the Primer Design software and select **Single Amplicon** from the front screen.
2. Paste the sequence into the sequence window.
3. To access the Target, Search Region, and Primer Sets tabs required for analyzing your primers, you must either define the amplicon by selecting the Define Amplicon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

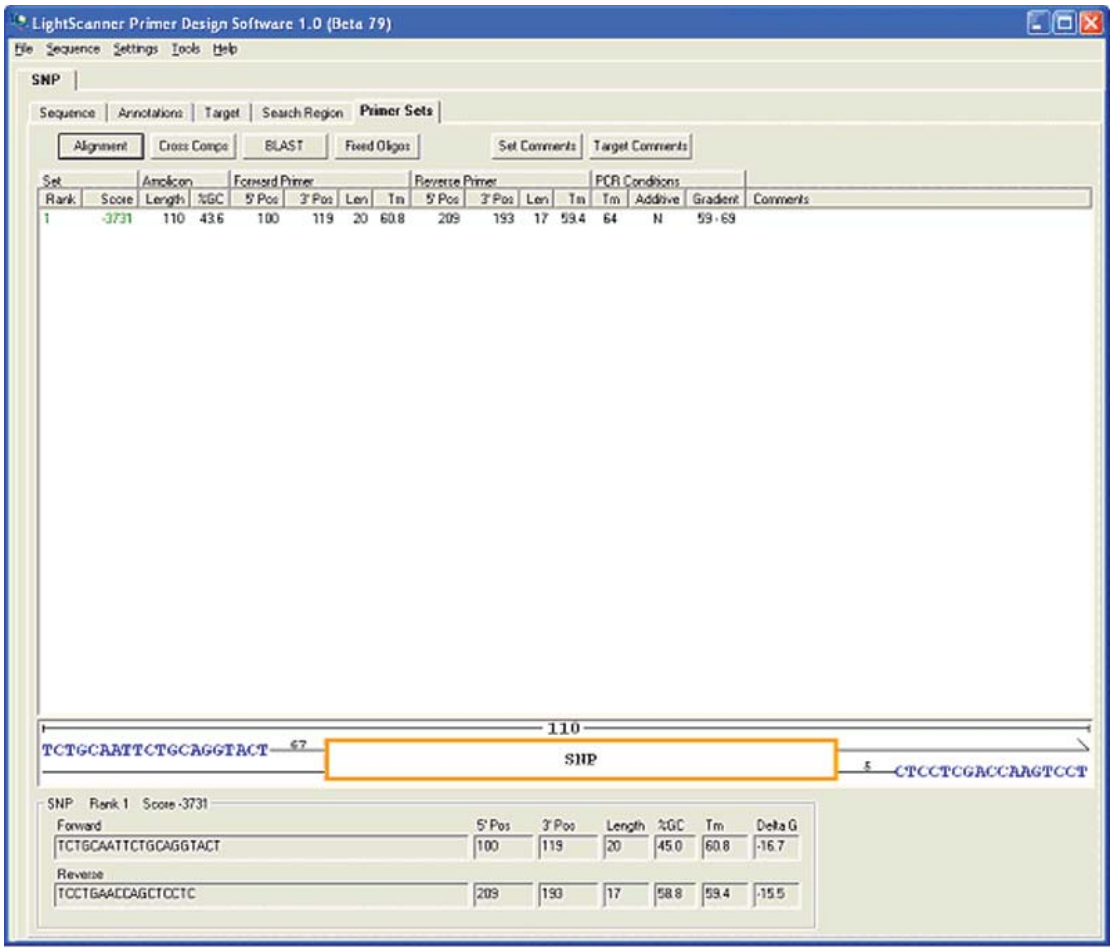
Note: Define an amplicon by selecting the **Define Amplicon** button and highlighting the region of the amplicon in the sequence display box. Define a SNP or a single base by selecting **Add SNP**. Be sure to have the SNP selected as the target. This will enable the Target, Search Region, and Primer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



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

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).



9. From the **Primer Sets** screen you may do a Cross Comps check and BLAST your primers.

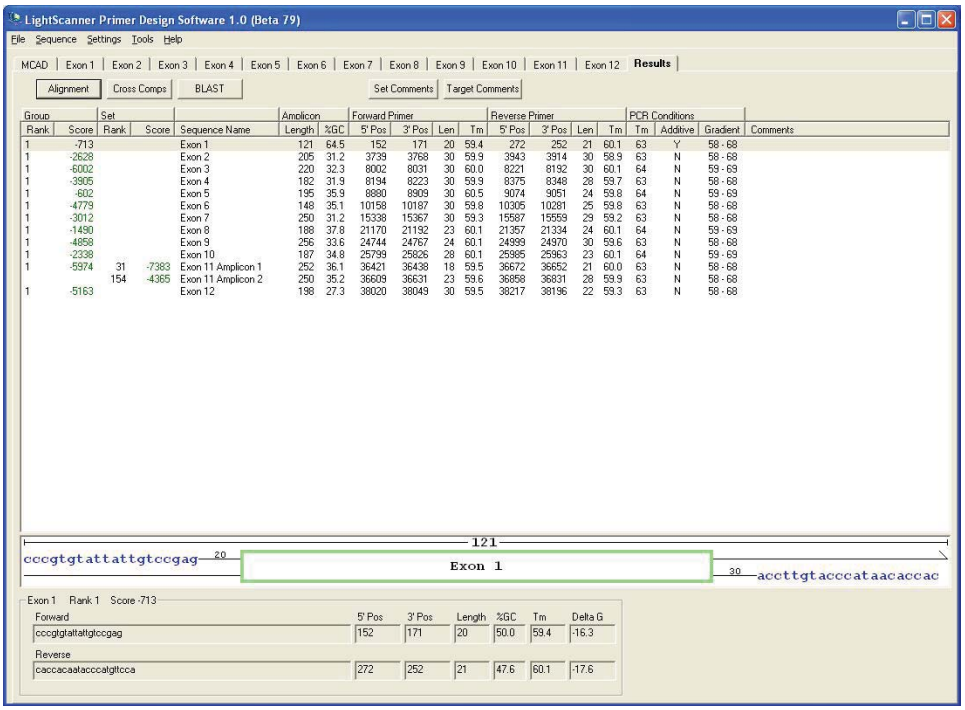
CHAPTER 5:

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.

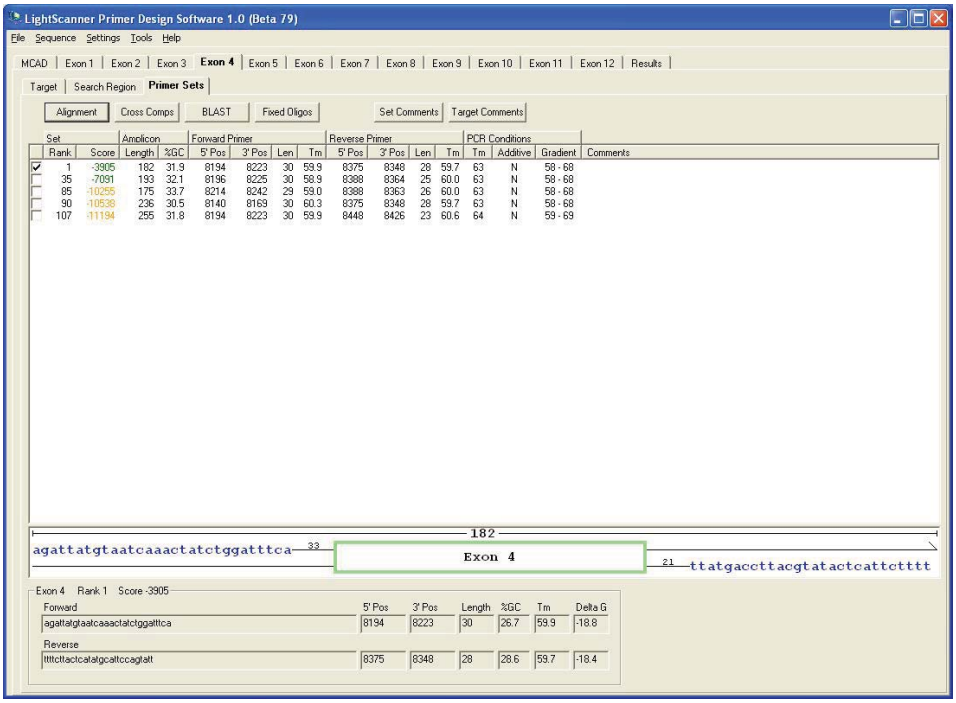


View Results for Individual Exons

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).

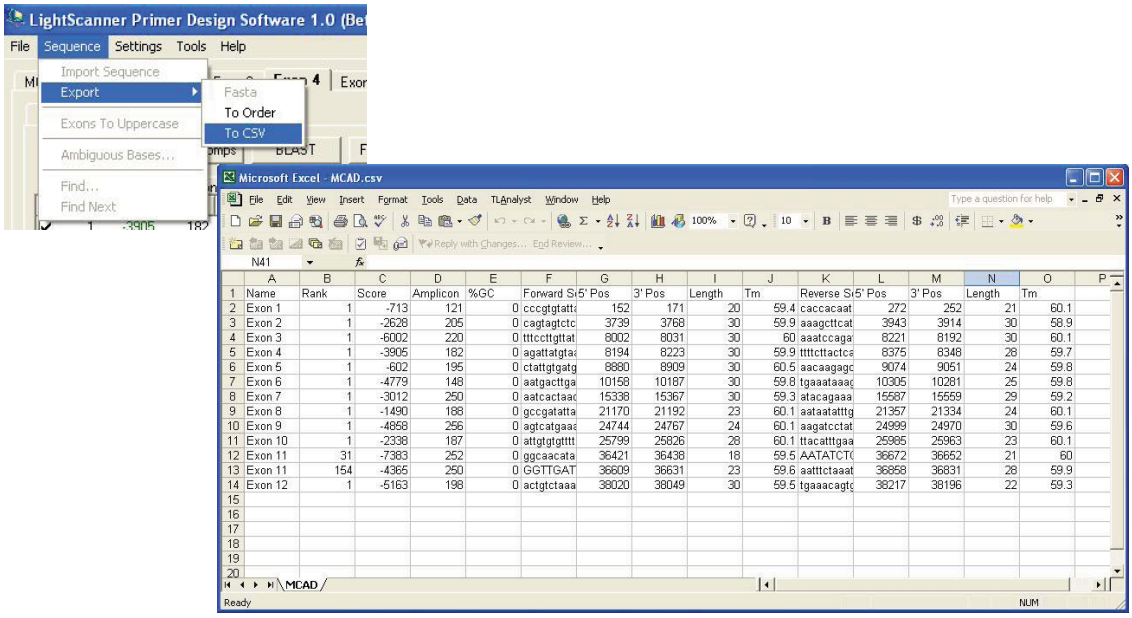
Select the check box next to a primer set to include that set in the final results. Only one check box can be selected per exon.



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.

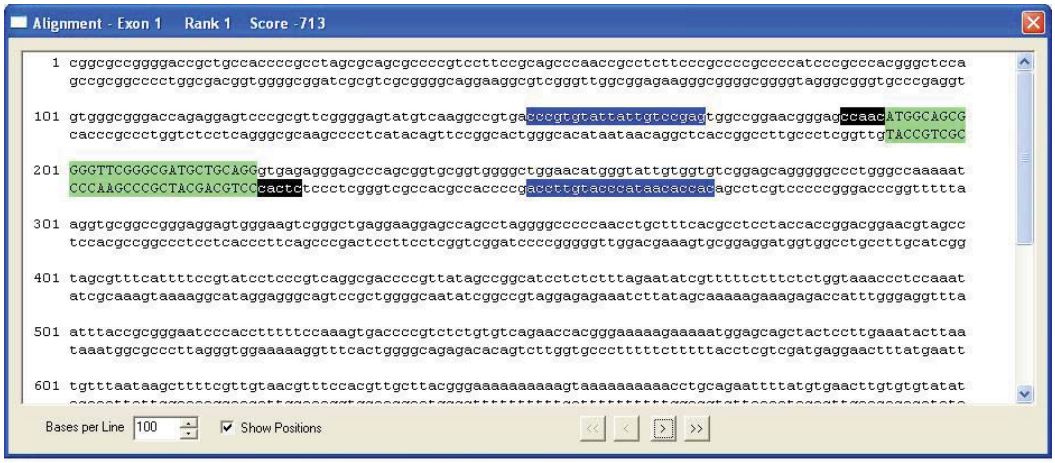
To export data, use the “Export to CSV” option from the Sequence menu and give the saved file a name. The file can then be opened in MS Excel.



BLAST Searches

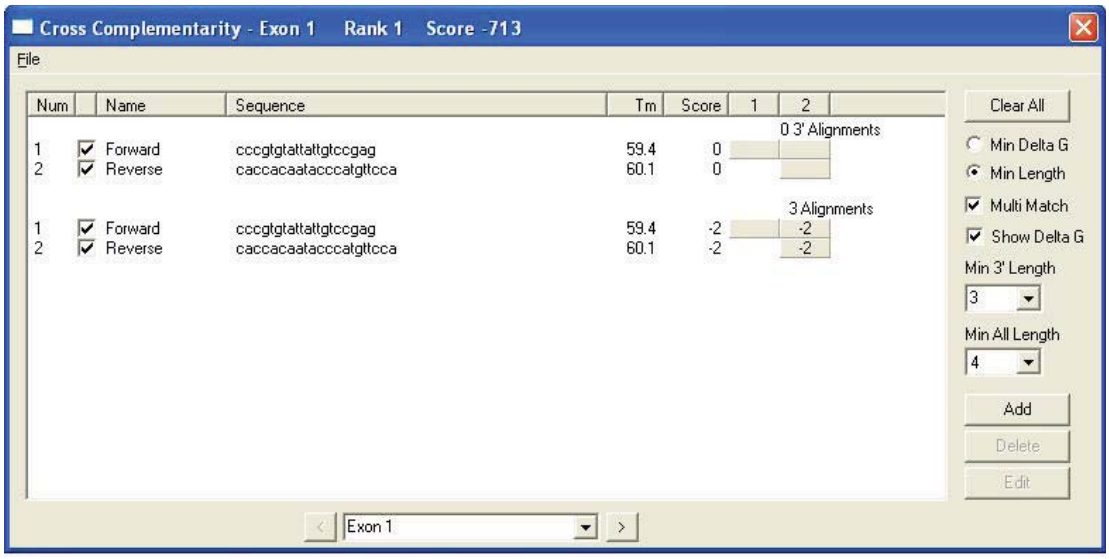
Checking Sequence Alignment

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.



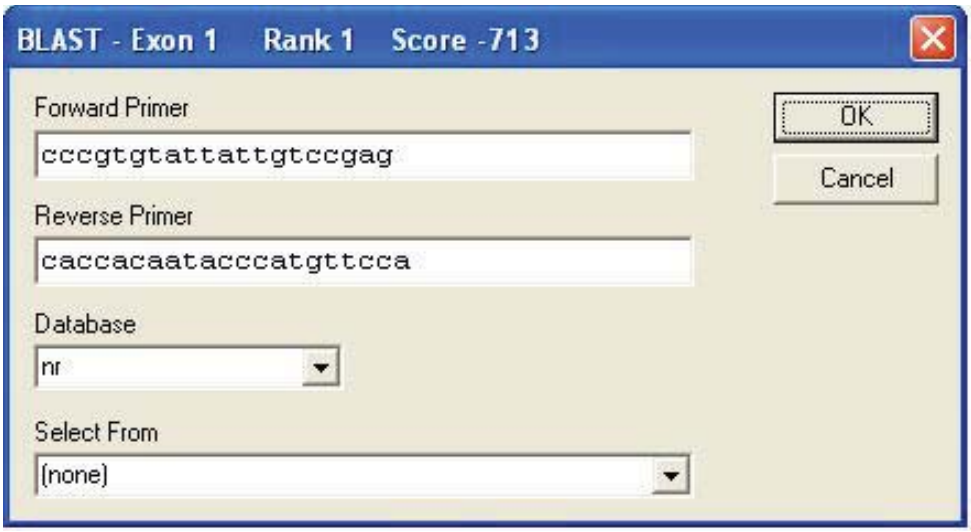
Checking Cross-Complementarities

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



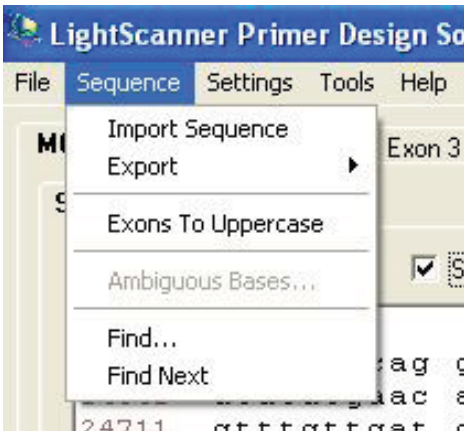
Checking Specificity

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.

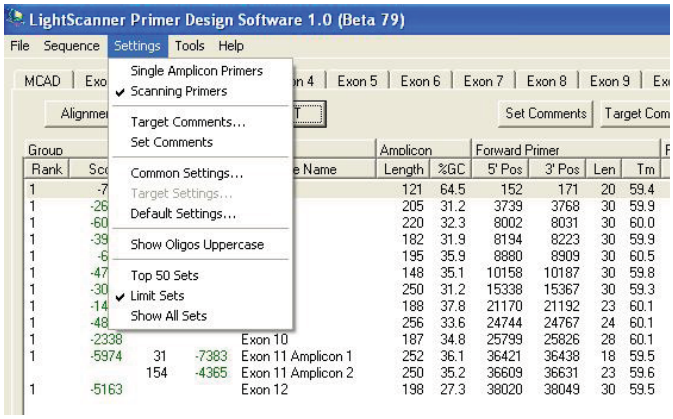


Menu Options

Sequence Menu



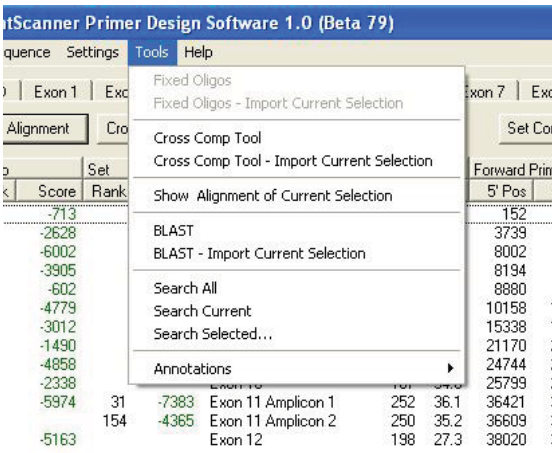
- **Import Sequence**—allows users to open a file for analysis.
- **Export**—If you are in the sequence input screen, you can export the sequence in FASTA format. Alternatively, if you are in the Results tab, you can export all the primer selection information in CSV format that can be opened in a spreadsheet 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.



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.
-

- **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.



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:

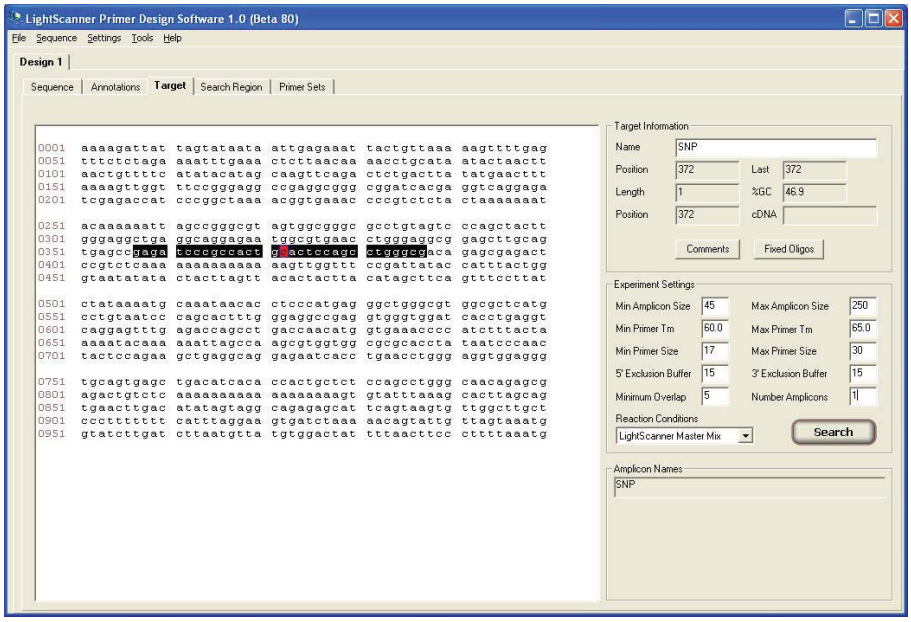
DESIGNING LUNAPROBES™

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.

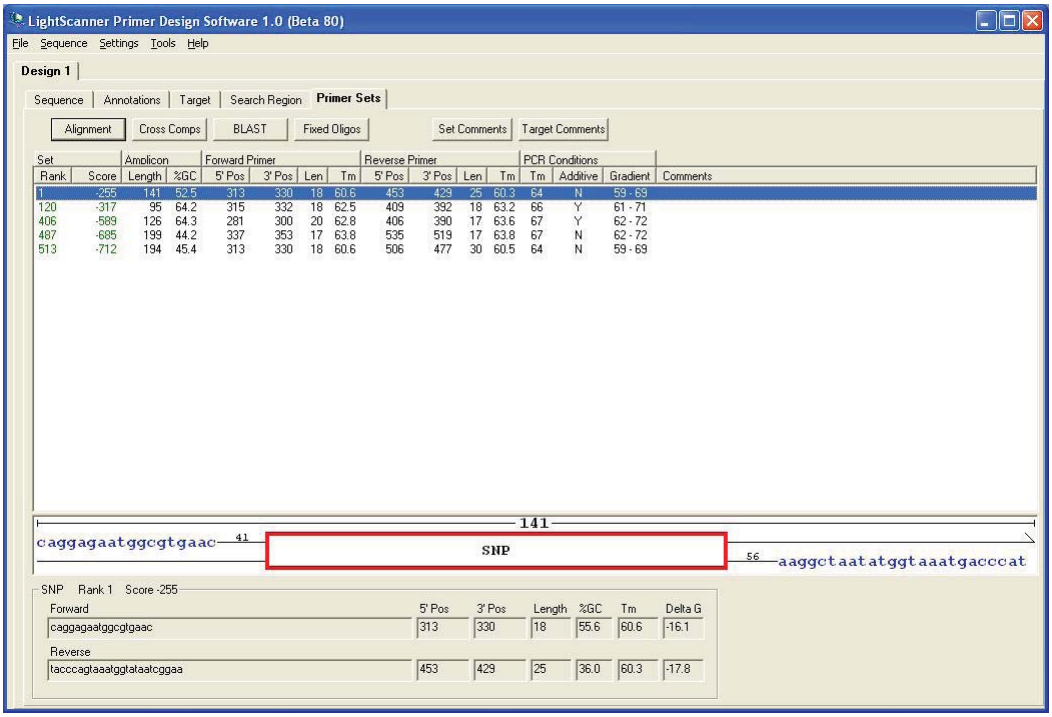
Design Your Primers

Before you begin, design your primers for your target.

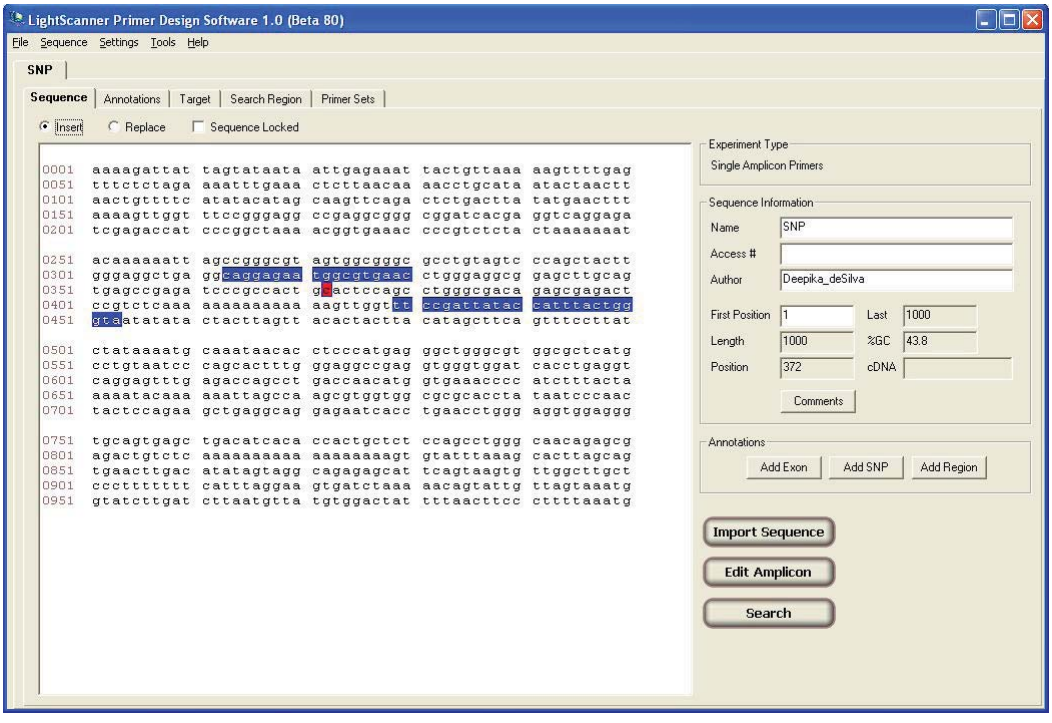
1. Open the Primer Design software and select **Single Amplicon** from the front screen.
2. Paste the sequence into the sequence window.
3. Define the SNP of interest by selecting **Add SNP**. Be sure to have the SNP selected as the target.
4. Move to the **Target** tab.
5. Use the following experimental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



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**.



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

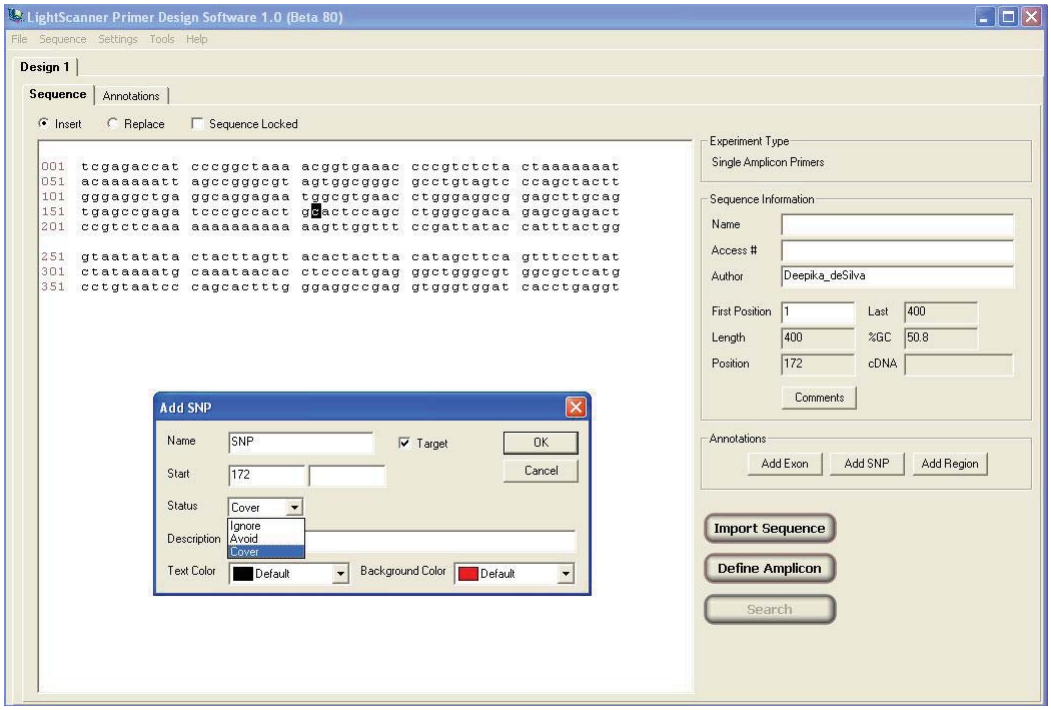


- Select the amplicon sequence as well as some sequence surrounding the amplicon and copy the sequence.

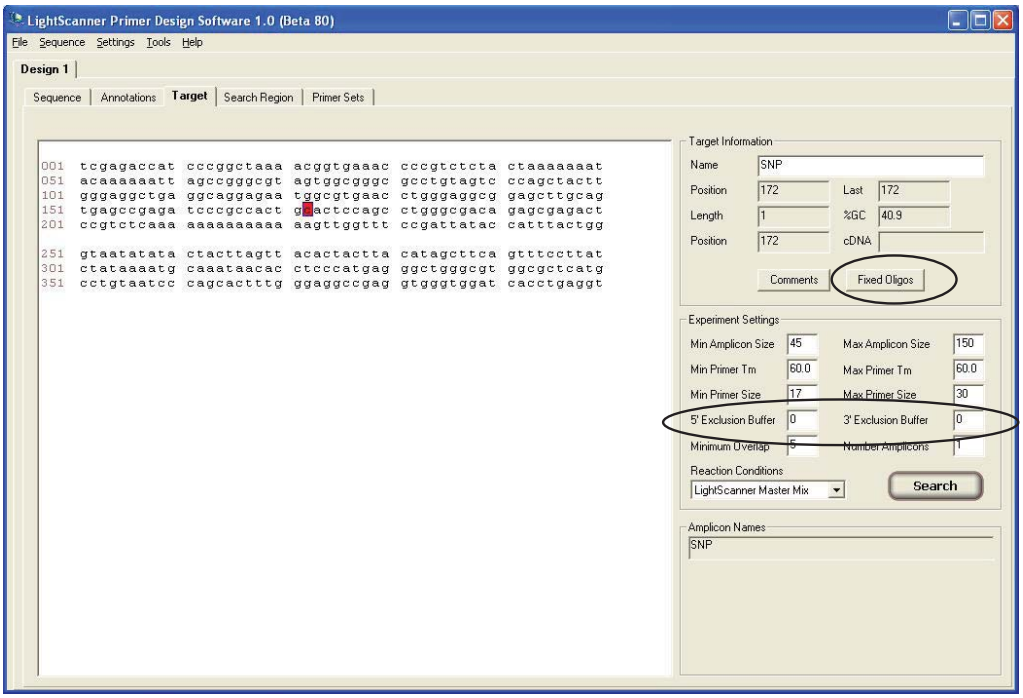
9. Save the file by going to the **File** menu and selecting **Save** or **Save As**. We will call this document the ‘amplicon document.’
10. Minimize the amplicon document.

Design Your Probe

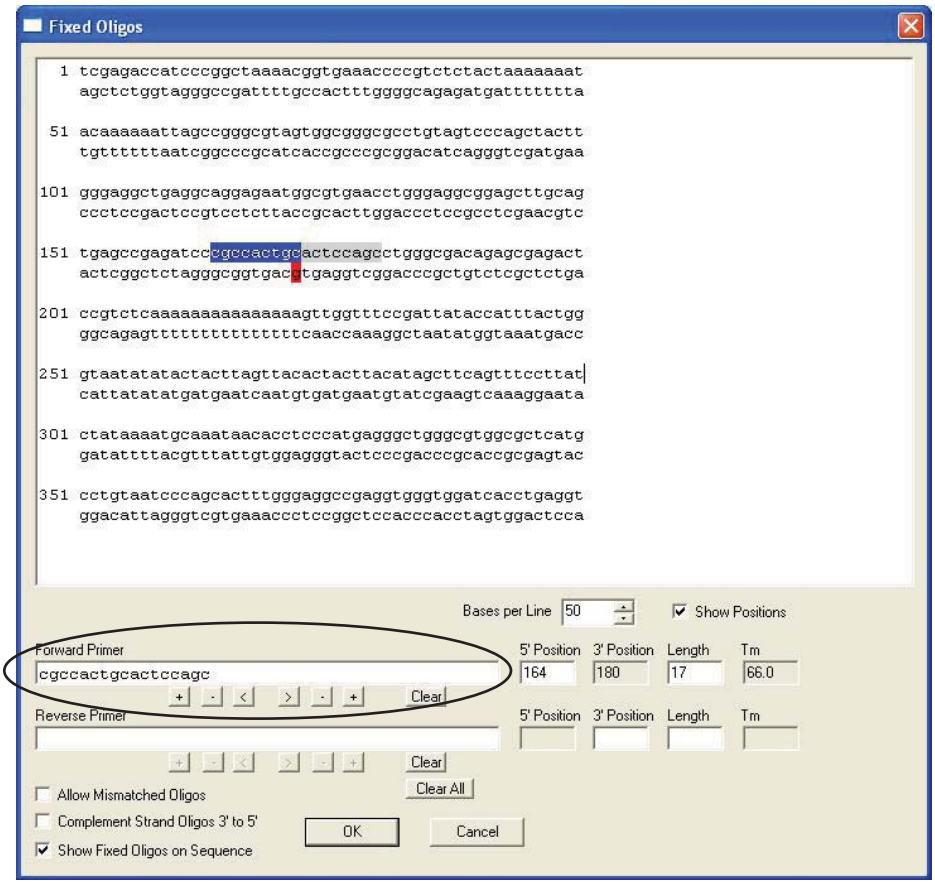
1. Open a new LSPD Software window and choose the **Single Amplicon** module. Paste your fragment into the new document.



2. 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**.



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



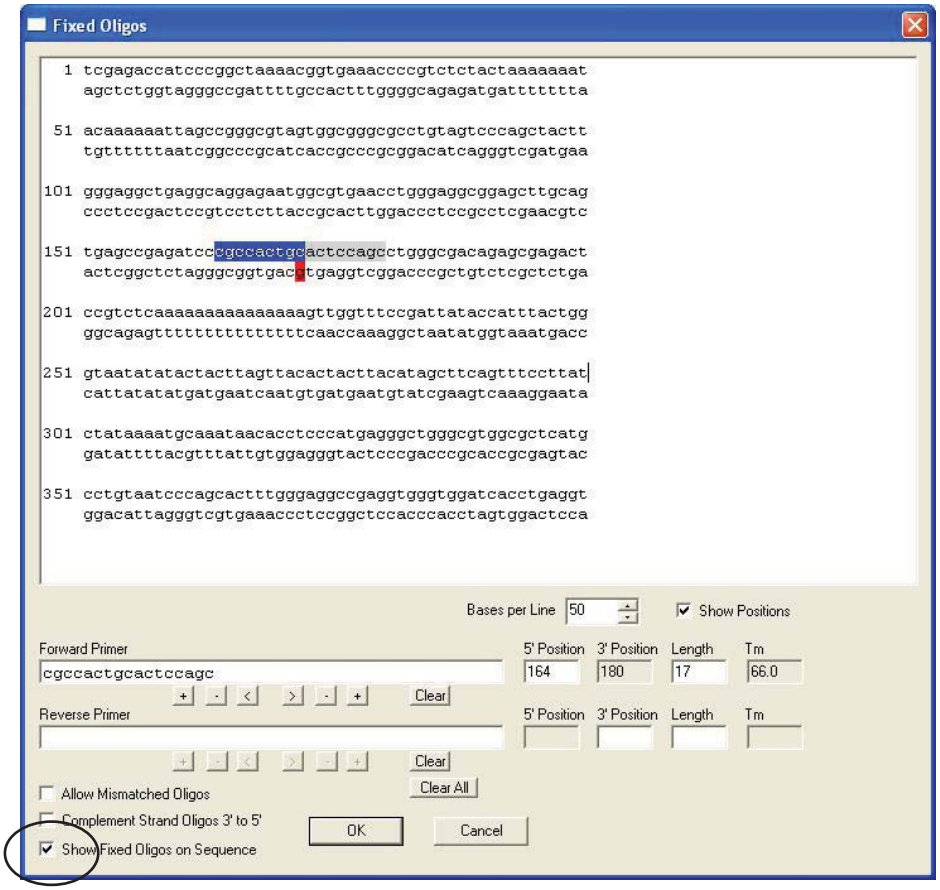
7. Highlight the region over the SNP. We recommend designing the probe to match the variant sequence. For example if a base change is C>T, with the T allele being the variant, design the probe to the sequence containing the T base. This will result in a perfect match between the probe and the variant 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.
8. 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 wild-type 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**.



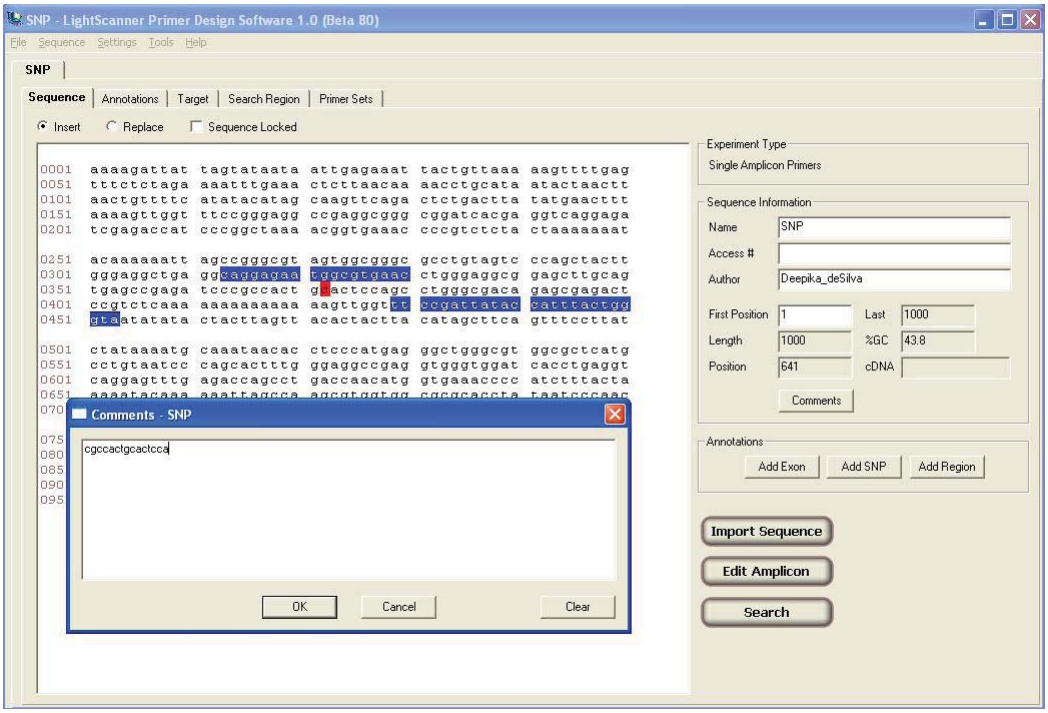
Note: The sense primer was moved and the SNP of interest is in the center of the oligo.

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

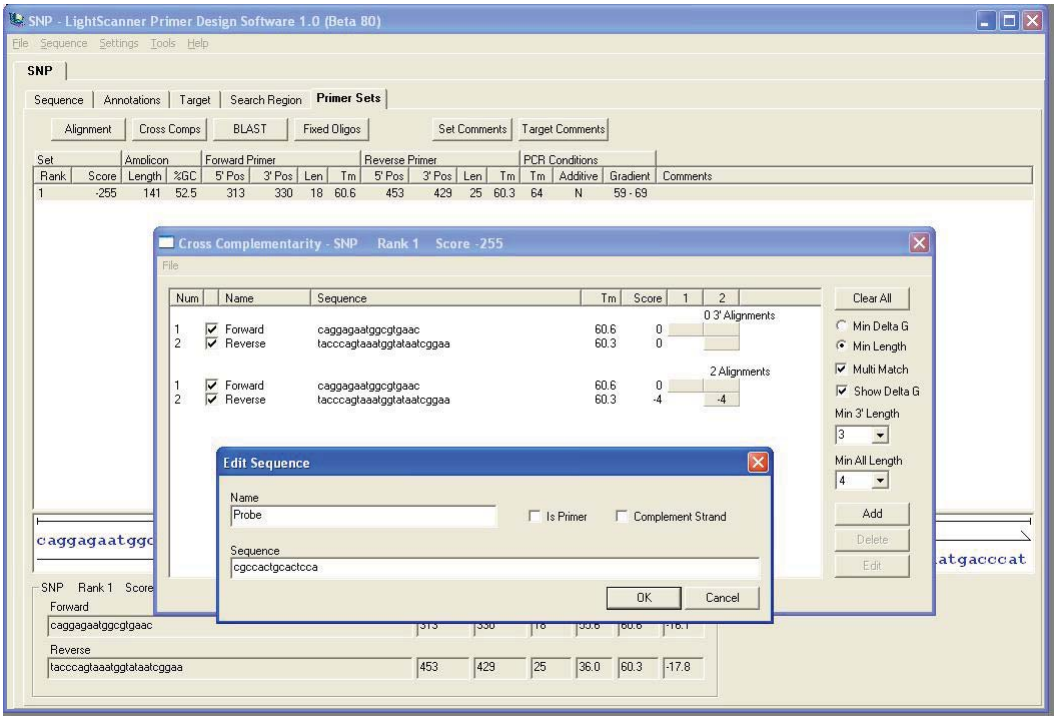
1. Maximize the previously saved amplicon document window that contains the primers that will be used with this probe.
2. Select the **Comments** option located in the sequence information box on the right hand side of the page.

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



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.



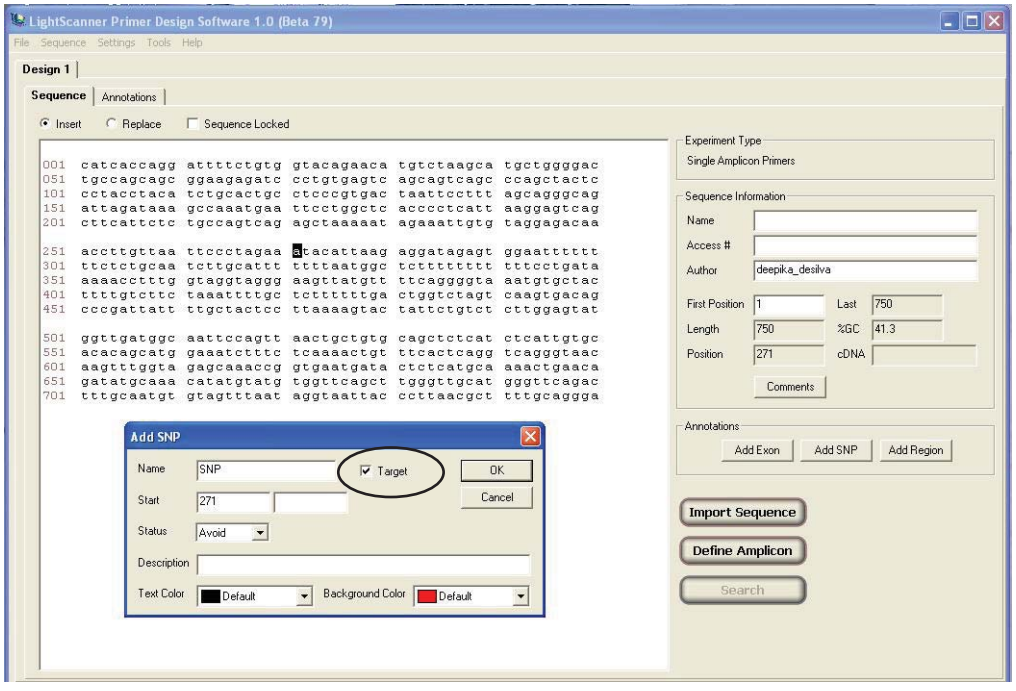
CHAPTER 7:

DESIGNING A SMALL AMPLICON FOR GENOTYPING

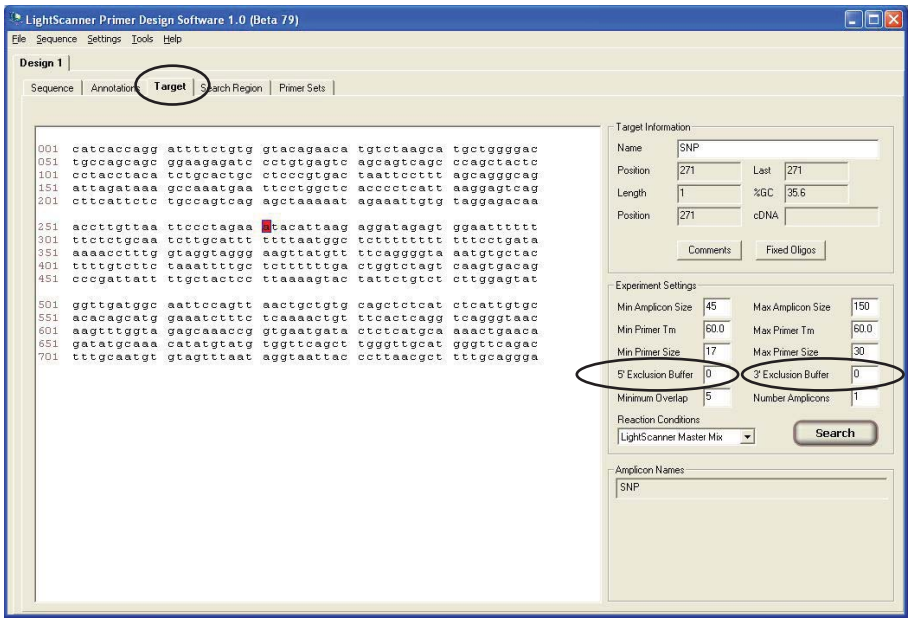
The LSPD Software can be used to design small amplicons for genotyping. A small amplicon is a 50–70 bp region in which the primers are designed adjacent to a known SNP. This section will enable you to define a SNP within your sequence and design primers adjacent to the SNP of interest.

1. 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.

2. 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**.

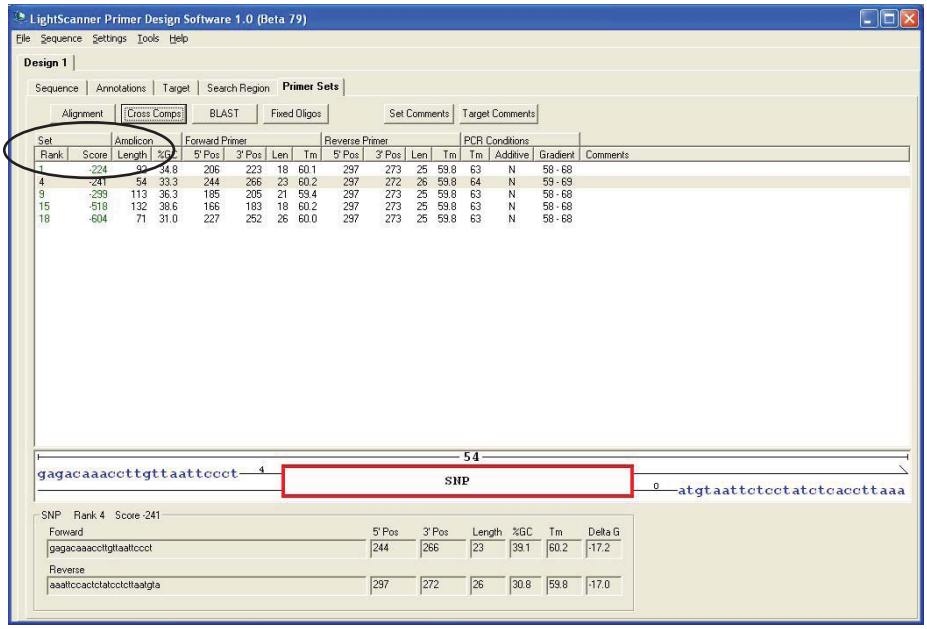


3. Select the **Target** tab that appears and refer to the **Experiment Settings** at the right hand side of the screen. Verify that the **Min** and **Max Amplicon Sizes** range from 45–150 and change both the 5' and 3' exclusion buffers to 0.

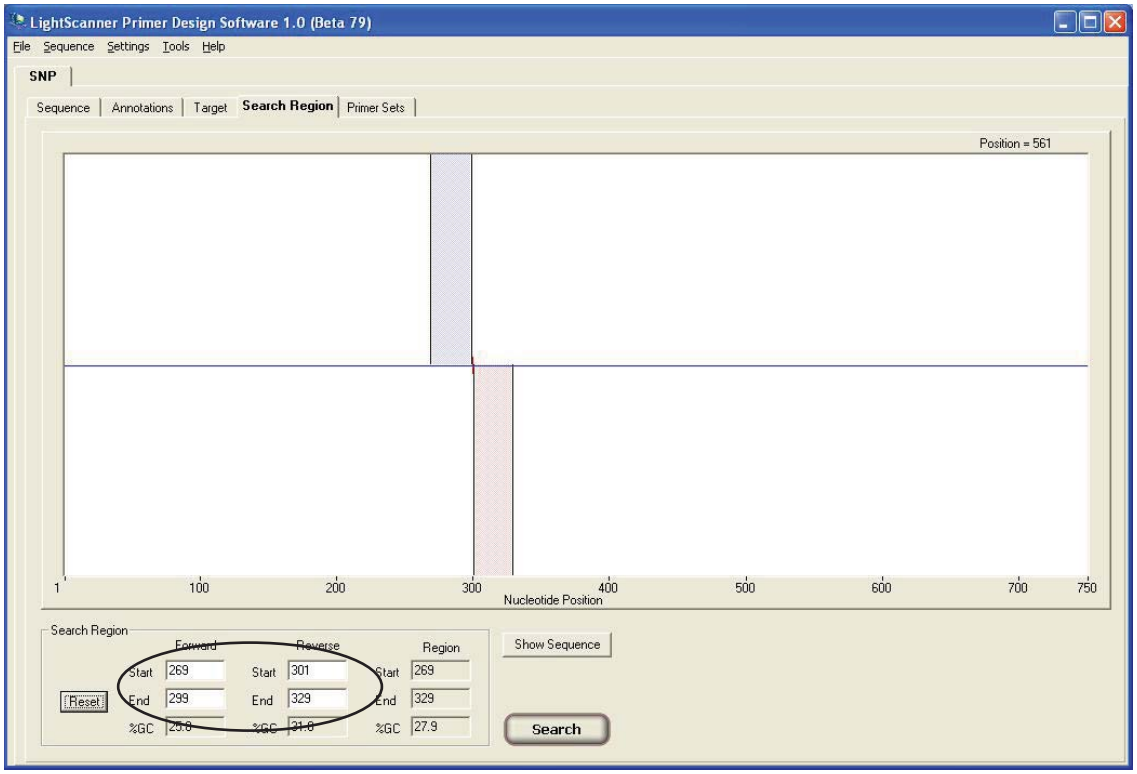


4. Select **Search** for primer sets.

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.



6. 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**.



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.

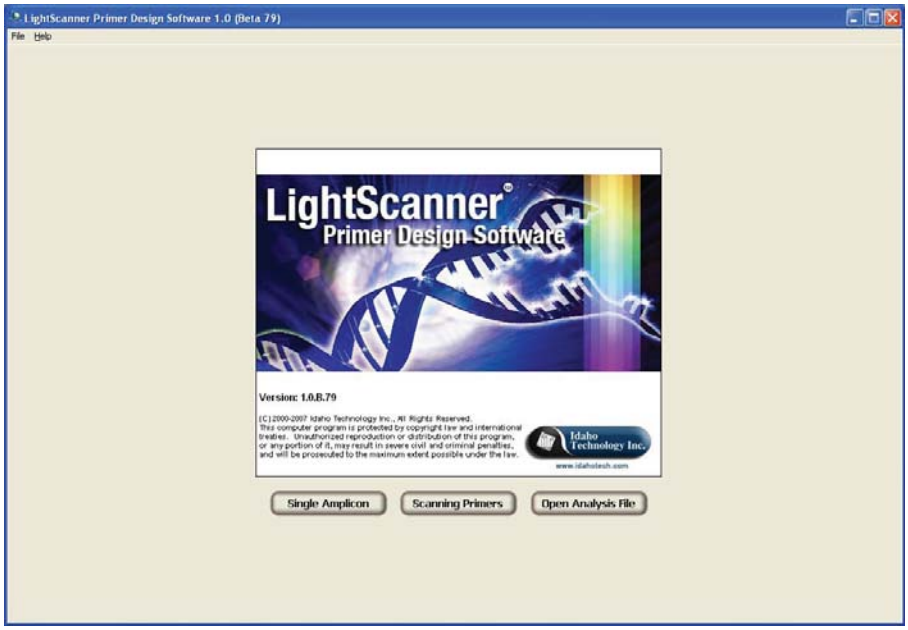
8. If primers do not display any significant cross-complementarities and are specific to the target region, primers can be ordered.

APPENDIX A:

QUICK GUIDE TO USING THE LIGHTSCANNER PRIMER DESIGN SOFTWARE

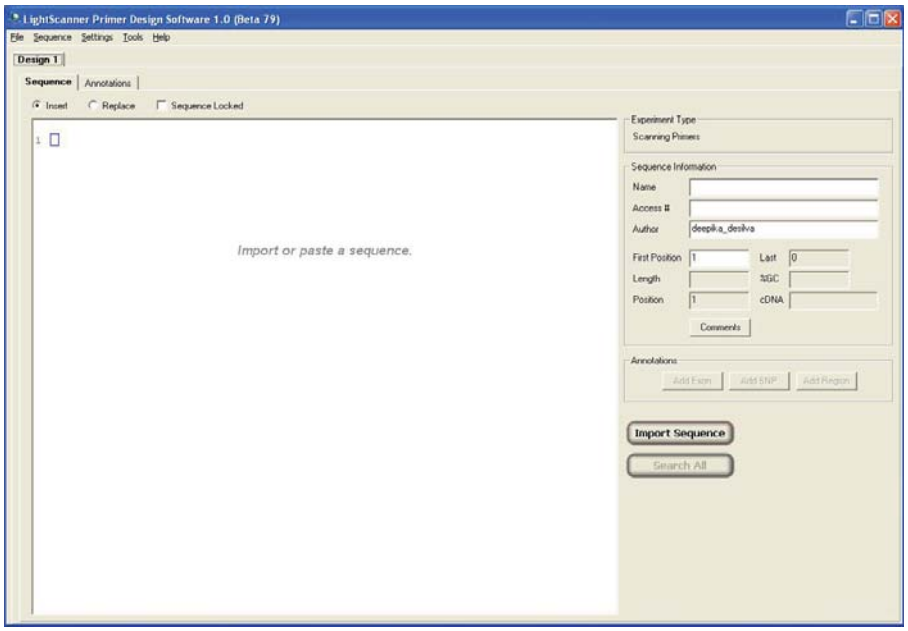
The LSPD software enables users to design primer sets that amplify small segments of large regions of interest. These primer sets can be added to the users' gene files. The primers designed will only be used for scanning analysis.

Step 1. Launch the Software

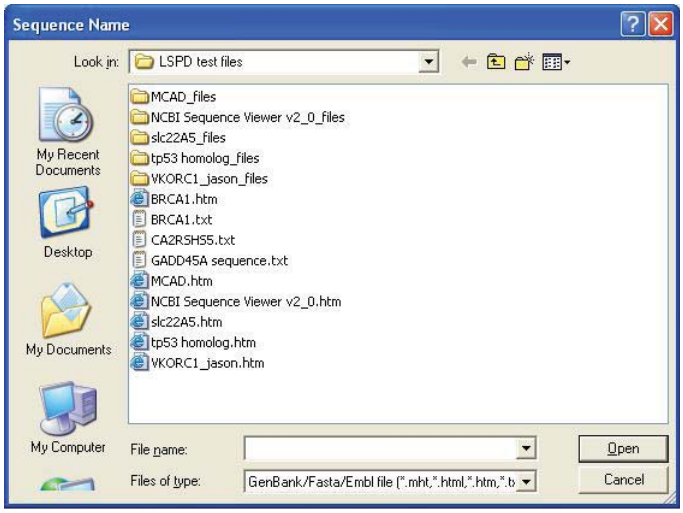


Step 2. Import a Gene Sequence

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.



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

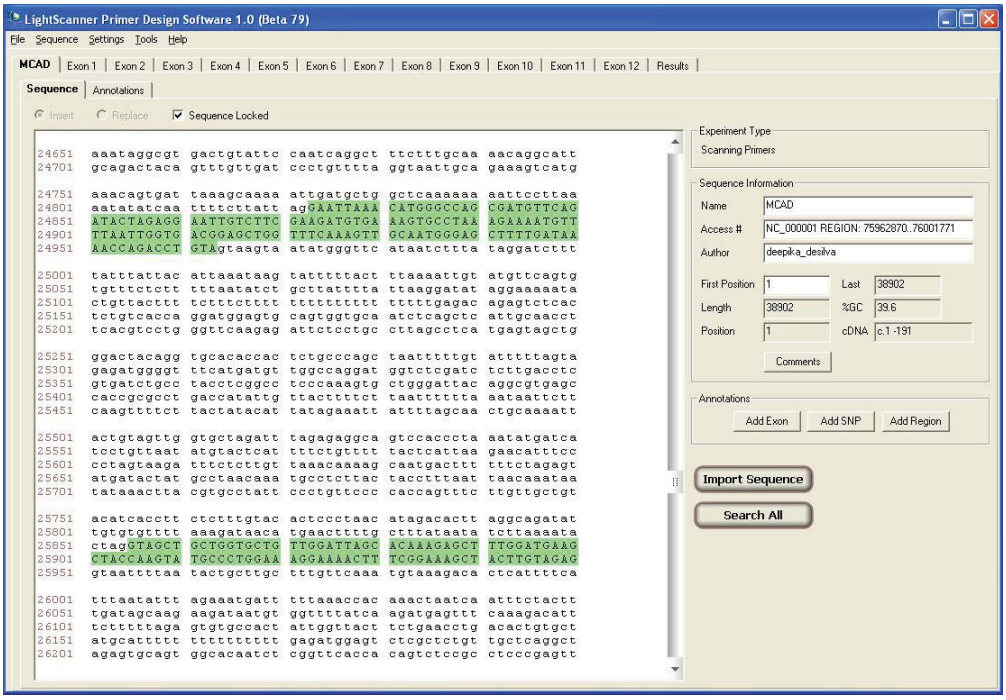


Exons will be highlighted and bases in exons will be converted to uppercase.

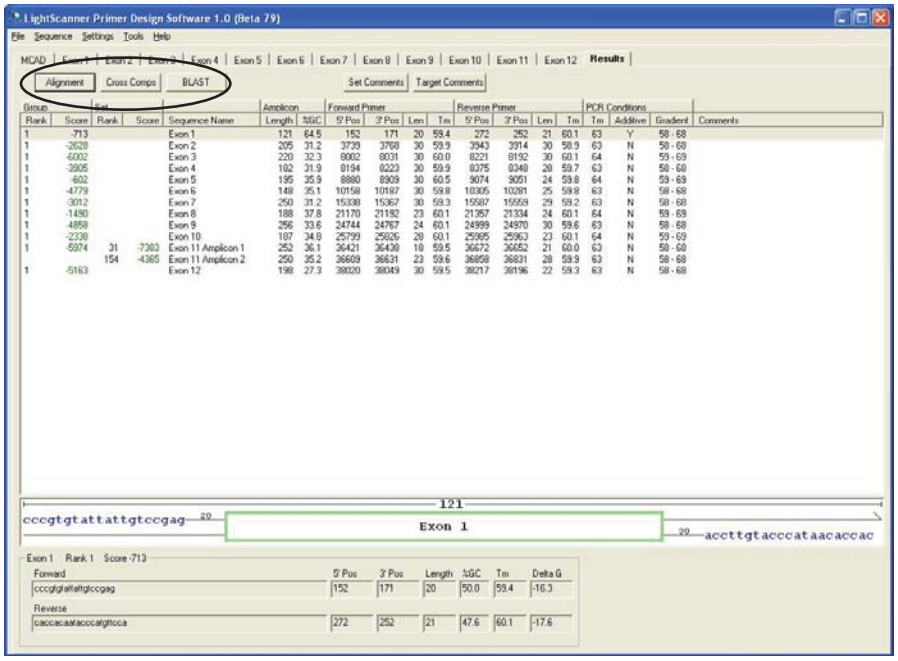
Note: The sequence must contain at least 200 bases of sequence upstream of the first exon and downstream of the last exon for the software to recognize these exons correctly

Step 3. Search for Primer Sets Using Common Design Parameters

Select **Search All** to find scanning primers for all exons.

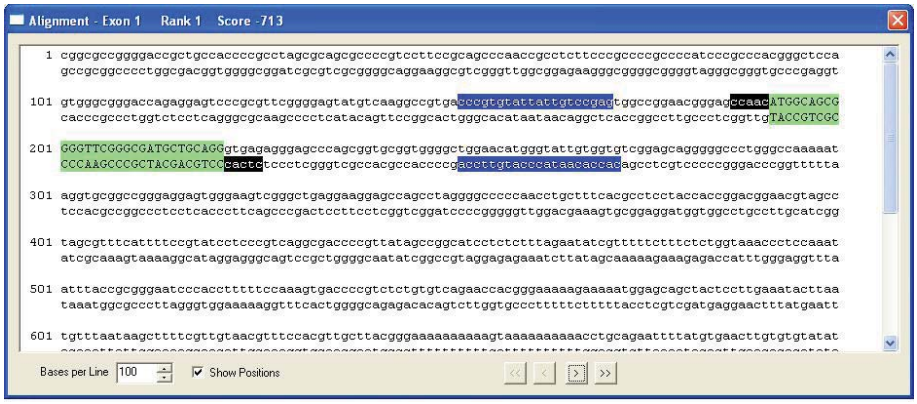


Step 4. View and Export Results

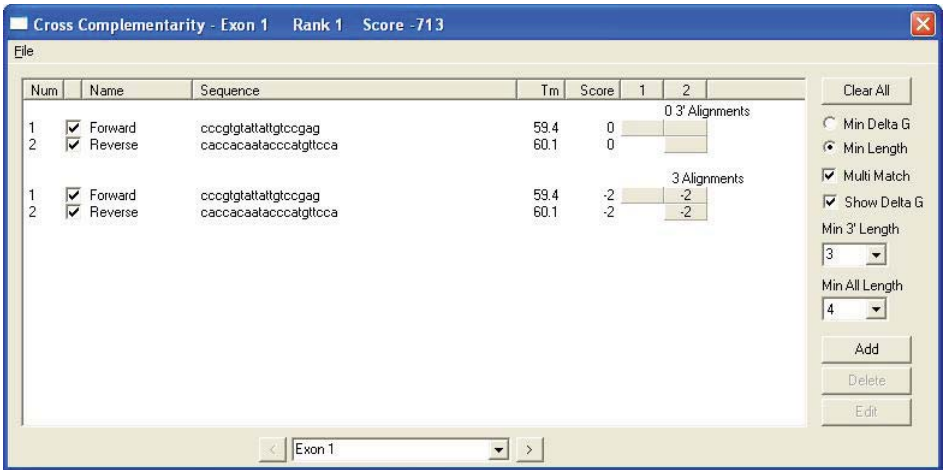


1. A results summary screen displays the best scoring primer for each amplicon in tabulated format. All of this data can be exported in a spreadsheet format for data archiving or can be saved as a primer design file (*.spd file) that can be re-opened in the software.

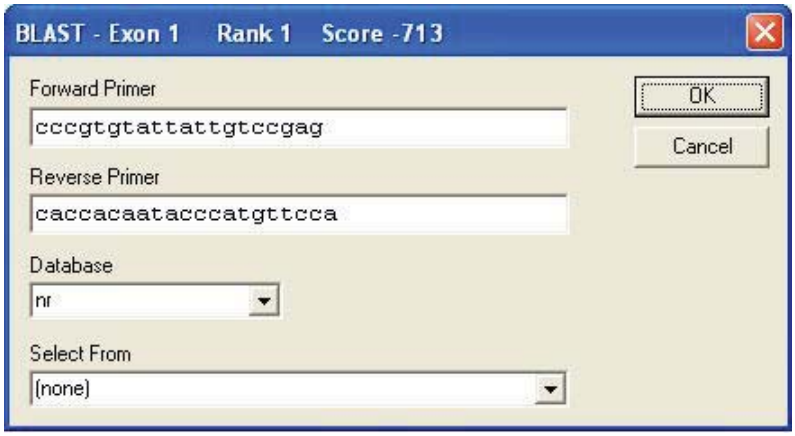
2. To locate the position of the primers in the context of the entire sequence, highlight the amplicon in the screen and select the **Alignment** button.



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



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.

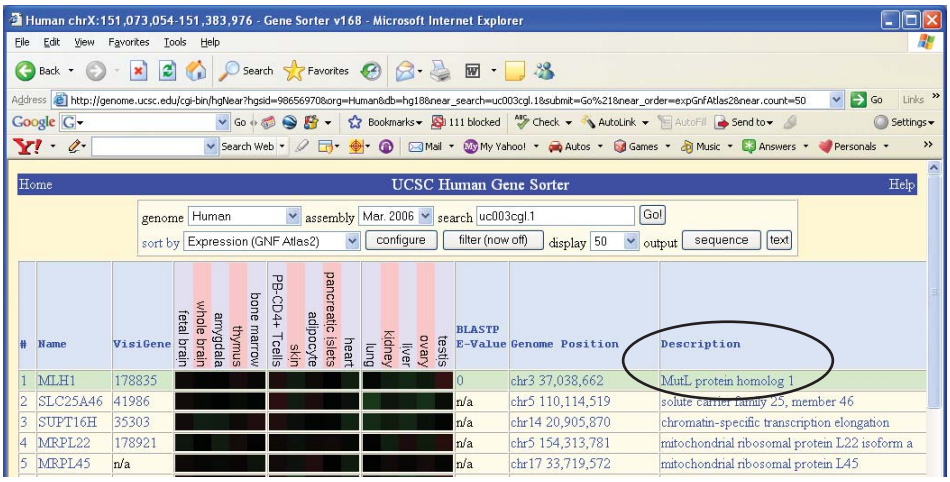


APPENDIX B:

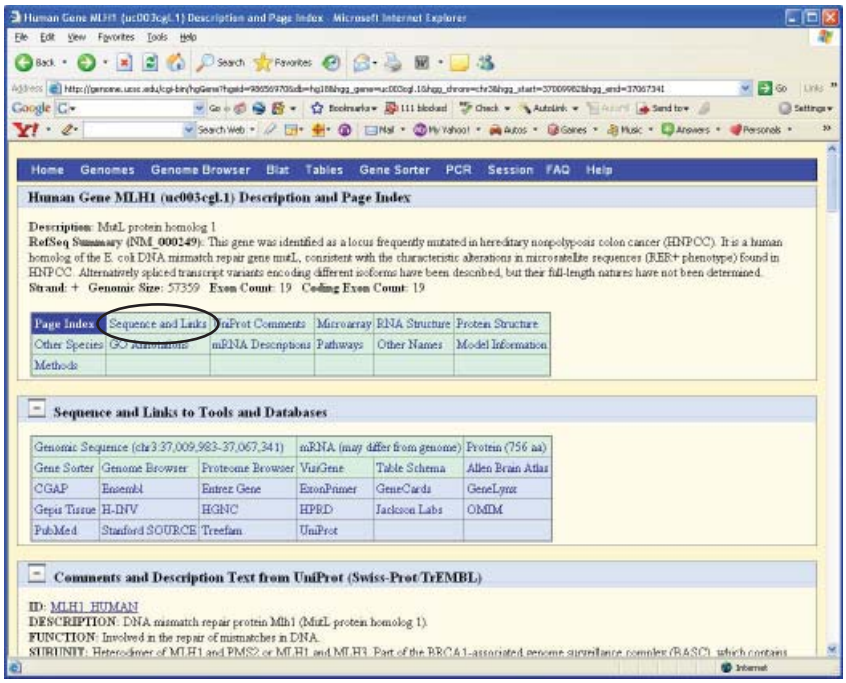
GETTING ANNOTATED TEXT FILES FROM THE UCSC GENOME BROWSER

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.

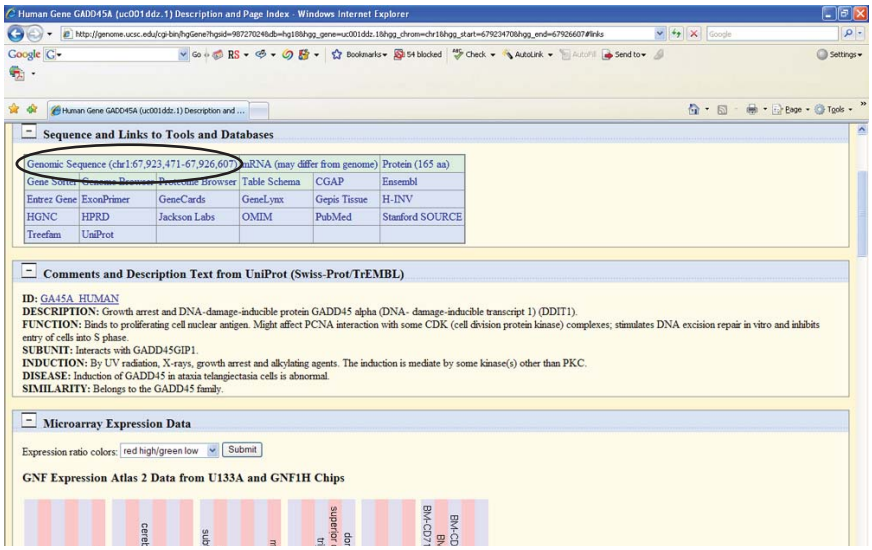
1. Go to the UCSC genome browser home page at **http://genome.ucsc.edu**.
2. Go to **Gene Sorter** and type in the name of the gene of interest.



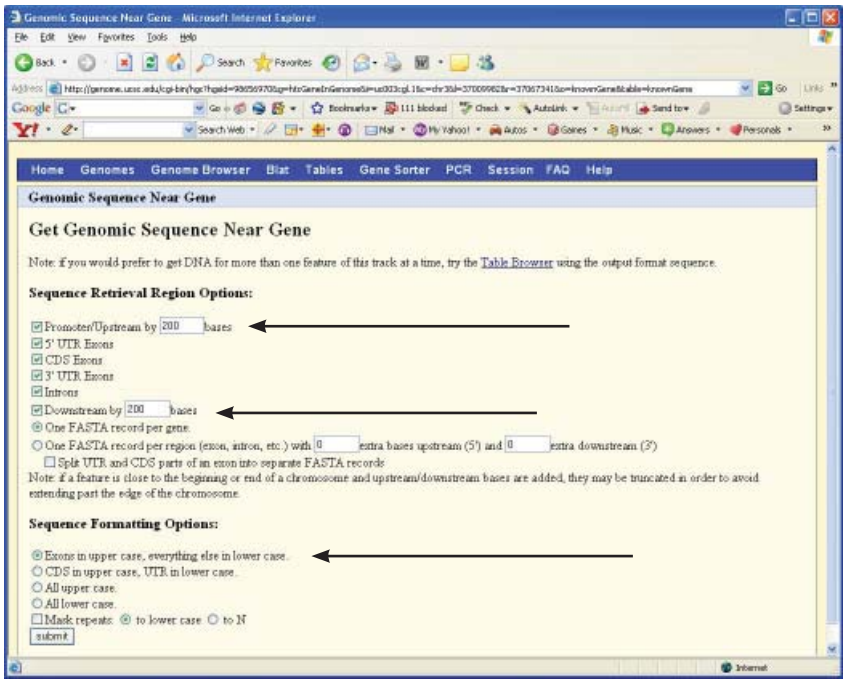
3. Click on the description box.
4. Find the **Page Index** box and select **Sequence**.



5. From the next page choose **genomic (Chr information)**.



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 LightScanner 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.



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

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