REF Select: Expert System Software for Selecting Restriction Endonucleases for Restriction Endonuclease Fingerprinting

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ABSTRACT

REF Select, expert system software, has been developed to assist in the selection of optimal restriction endonucleases for restriction endonuclease fingerprinting (REF), a method for rapid and sensitive mutation screening of long DNA segments (1–2 kb). The REF method typically involves six separate digestions with up to two restriction endonucleases used in each digestion. If done manually, performing a comprehensive review of the large number of possible sets of restriction endonucleases that could be used (over 10^{19} in the example presented here) and making an optimal choice is not feasible. Furthermore, the typical nonoptimal manual selection takes approximately 8 h by someone experienced with REF. REF Select enables a comprehensive review of the possible sets and a consistent, objective and fast selection of an optimal set by using a two-step strategy: the selection of sets that meet specific constraints, which is followed by a ranking of those sets by an optimality score. Based on our experience with REF, we chose default selection and ranking parameters to help the user get started quickly. These parameters form a knowledge base that can be customized and then saved by the user. In conclusion, REF Select facilitates the general application of REF by serving as an expert system for the selection of optimal restriction endonucleases. We demonstrated REF Select using an example segment from the human p53 gene.

INTRODUCTION

Restriction endonuclease fingerprinting (REF) has been demonstrated as a rapid method for screening long segments of DNA for sequence variants (1–4,13,14) with high sensitivity (8,10). REF involves the separate digestions of a PCR-amplified DNA segment, typically with six groups of one or two restriction endonucleases (Figure 1). The test segment could

be one contiguous segment of DNA or could consist of several smaller segments combined for digestion. The fragments from all digestions are combined into one lane of a non-denaturing gel and electrophoresed. Variants may be detected by an altered band pattern that is the result of one or both of two components: an informative restriction component (10) resulting from the loss or gain of a restriction site and/or an informative single-strand conformation polymorphism (SSCP) component (5–7,9,11). Because the test segment is cut at a variety of sites and into a variety of fragment sizes by several groups of restriction endonucleases, the sensitivity of both the restriction component and the SSCP component are increased.

We developed the REF Select software to assist in the selection of restriction endonucleases that maximize the detection sensitivity. REF Select enables the application of consistent and objective criteria to the selection of an optimum set of groups of restriction endonucleases from many possible sets. Before the availability of REF Select, it took approximately eight hours for someone experienced with REF methodology to manually select appropriate but nonoptimal restriction endonucleases. With REF Select, even an inexperienced user without extensive knowledge of restriction endonucleases can make an optimal selection in approximately ten minutes.

MATERIALS AND METHODS

REF Select conforms to the American National Standards Institute (ANSI) standard for the C programming language and is available on the Internet at <http://www.cityofhope.org/molgen/bioinformatics.asp>. This Web site can also be accessed through a link from the **Software Library** on the **BioTechniques** Web site (www.biotechniques.com). Input is provided by a simple interactive text-based user interface and American Standard Code for Information Interchange (ASCII) text files. Default parameter values and online help are provided. The various parameters can be modified and then saved to a parameter file for later reuse. For advanced users, command line options are provided for batch processing.

REF Select is designed to work in concert with the GCG Wisconsin Package (Genetics Computer Group, Madison,

WI, USA; <http://www.gcg.com>); it uses the output of GCG Mapsort and the graphics capabilities of GCG Figure. By using GCG Mapsort to get the set of restriction endonucleases that cut the DNA segment and the corresponding cut sites, REF Select makes indirect use of REBASE (<http://rebase.neb.com>) (12), a restriction endonuclease database. REF Select can also read the restriction sites from two programs that are available from the Internet without cost: TACG (part of the Biology Workbench at <http://biology.ncsa.uiuc.edu>) and Webcutter 2 (<http://www.firstmarket.com/cutter/cut2.html>), which is useful for those who do not have access to GCG.

RESULTS AND DISCUSSION

REF Select uses a two-step strategy of selection that is followed by ranking using an optimality scoring algorithm (Figure 2). The selection step eliminates restriction endonucleases, groups and sets that do not meet the user-specified selection constraints (Table 1) and substantially reduces the burden of the ranking step. The first phase of the selection step is the application of constraints on the individual restriction endonucleases: they must cut the DNA segment at least once, and they must be available and desirable, as defined by the user; typically, it is desired that they be substantially free

of star activity, free of dramatic variation in cleavage efficiency at different sites and not prohibitively expensive, etc. If only this first phase of constraints were applied, the number of possible sets would usually be unmanageable even with a computer, as demonstrated by the *p53* example presented in the Appendix, in which there are over 10^{19} possible sets of six groups of one or two of the 105 available and desirable restriction endonucleases that cut the segment. A dramatic reduction in the number of sets that must be ranked is achieved by applying the group (second phase) and set (third phase) selection constraints. In the *p53* example, only 52 678 sets remain to be ranked after all the selection constraints are applied, a number that can be quickly ranked by a modern desktop computer.

Input

The required file input to REF Select is a Mapsort file generated by GCG Mapsort (see the example command sequence in the Appendix). This file lists all the restriction endonucleases that cut the DNA segment and the locations of the cut sites (two alternative restriction site input file formats are also supported; see Materials and Methods and the online help). An optional input file, called the restriction endonuclease property file, serves as a user database of restriction endonucleases (see the example in the Appendix). In addition to limiting the restriction endonucleases to those marked as available and desirable, the property file provides information (optimum digestion temperature and buffer activity levels) that REF Select uses to ensure compatibility between restriction endonucleases used together in a digestion group.

The input parameters are categorized as group selection constraints, set selection constraints, scoring algorithm parameters, output parameters and program mode. REF Select prompts the user for all needed input parameters in a logical sequence (see the example in the Appendix). The default values for the selection constraints are based on experience with REF and form a knowledge base (Table 1) that helps the user get started quickly.

Features

Automatic parameter adjustment. Adjustments to the default selection constraints may be needed to get optimal output for a particular DNA segment. REF Select includes an optional automatic parameter adjustment mode that aids the user when the initial input parameters result in no selected sets or too many selected sets (i.e., too many to process on a reasonable computer in a reasonable amount of time). Because of boundary effects, the number of selected sets may

Figure 1. Schematic of REF. A hypothetical 1.5 kb test segment with a point mutation is digested separately by 6 groups of 1 or 2 restriction endonucleases as shown. A total of 10 hypothetical restriction endonucleases (A–J) are used to generate 72 fragments (36 sense and 36 antisense) ranging in size from 50–450 bp. Group 1 fragment sizes in bases from left to right are: 50, 170, 230, 290, 350 and 410; Group 2: 120, 240, 300, 360, 420 and 60; Group 3: 190, 310, 370, 430, 70 and 130; Group 4: 260, 380, 440, 80, 140 and 200; Group 5: 330, 450, 90, 150, 210 and 270; and Group 6: 400, 100, 160, 220, 280 and 340.

Table 1. Selection Constraints

No.	Constraint	Default	Automatic Adjustment
Group Selection Constraints	1 Maximum Digestion Temperature Difference (compatibility constraint for groups of two enzymes)	5°C	No
	2 Minimum Buffer Activity Levels (compatibility constraint for groups of two enzymes)	Both enzymes must have activities of 100 in at least one available buffer	Adjusted in increments of 25 down to a minimum of 50 if too few groups selected or up to a maximum of 100 if too many groups selected
	3 Fragment Size Range	50–500 bp	Maximum increased in increments of 10% if too few groups selected and minimum buffer activity levels at 50 or lower
	4 Range of No. of Groups to Select	25–125	Specifies when adjustment of other constraints is needed
Set Selection Constraints	5 No. of Groups in a Set	6	No
	6 Total No. of Fragments (sense and antisense fragments from all groups)	100 (+10%, -20%)	No
	7 5' Maximum End Fragment Size	Size over 200 bp limited to no more than 3 groups	Oversize limit increased in increments of one if no valid sets found
	8 3' Maximum End Fragment Size	Size over 200 bp limited to no more than 3 groups	Oversize limit increased in increments of one if no valid sets found
	9 Maximum No. of Common Cuts (between any two groups)	No more than 2 cuts separated by 4 bp or less	No

Figure 2. REF Select two-step strategy of selection followed by ranking. Application of the selection constraints is subdivided into three sequential selection phases: select restriction endonucleases, select groups and select sets. A group consists of either one or two restriction endonucleases. REF Select first considers all possible groups with one restriction endonuclease and then all with two restriction endonucleases. When groups with two restriction endonucleases are considered, compatibility constraints are applied. A set is a combination of the specified number of groups (six by default). The selected sets are ranked by a theoretical optimality score (see Table 1 for the selection constraints and Equation 1 for the scoring formula).

change dramatically with small parameter changes. For example, most available restriction endonucleases may be excluded by a particular maximum fragment-size selection constraint that results in too few selected sets. If this size constraint is relaxed by only a small percentage, however, more restriction endonucleases may become acceptable, which results in a dramatic combinatorial increase in set possibilities. Automatic parameter adjustment helps avoid the tedium of manually homing in on the desired side of such a

boundary by automatically making incremental parameter adjustments (Table 1) and iterating until the number of selected sets is in the desired range. Such adjustments may not always result in convergence, so the number of adjustments is limited.

Multiple passes. Although REF Select can rank thousands of sets that meet the selection constraints, the user will typically specify that only a few at the top end of the ranking be output (three by default). The difference between these sets

Figure 3A. REF Select output, p53 example. Example output for a 2033 bp DNA segment that includes exons 5-9 from the human p53 gene. Histogram showing the distribution of scores for all 52 678 sets ranked in the first pass. The maximum score is 143.5, the average is 81 and the standard deviation is 17. The score histogram command line option, -sh=50, was used to generate this score histogram with 50 bins.

Cut Site Map

Score vs. Base Number

Figure 3B. Main output page summarizing the first option of the first pass (i.e., the optimum set). The top half contains a cut site map showing the restriction endonuclease groups and any periclares, buffers and activity levels, cut positions, fragment sizes and a list of the fragments sorted by size and keyed to the cut site map. The bottom half contains a plot of the score vs. base number and score statistics for this set option and for the pass. The score vs. base number plot is useful when the user has a particular interest in specific regions of the segment.

Table 2. Restriction Endonuclease Unit Estimates

Enzyme	Buffer ^a		No. of Cuts in Unit Assay Substrate (λ phage) ^b	No. of Cuts in DNA Segment	Approximate No. of Units Required ^c
	Recommended	Used			
AvaI	4	4	1	8	9
BseRI	2	4	1	19	19
AvrII	2	1	1	2	36
HpaII	1	1	1	328	2
BbvCI	4	4	1	7	21
MboII	2	4	1	130	4
EaeI	1	4	1	39	4
HphI	4	4	1	168	3
EciI	2	2	1	32	3
HinfI	2	2	1	148	4
Eco0109I	4	2	1	3	120
NspI	2	2	1	32	5

^aBecause two restriction endonucleases are combined in each digestion, the best buffer for both enzymes must be selected. The REF Select output shows the buffer(s) with the highest activity for both enzymes (see the Cut Site Map in Figure 3B). The recommended buffer (which was used for the unit assay) and the activities in all available buffers are specified by the supplier (New England Biolabs, Beverly, MA, USA; see http://www.neb.com/neb/tech/tech_resource/restriction/buffers/buffer_chart.html). The ratio of the activity in the buffer used to the activity in the recommended buffer is then computed to be used for the adjustment of the required units of enzyme. In most cases, as in this example, the ratio is one.

^bThe supplier (New England Biolabs) defines 1 U as the amount of enzyme required to completely digest 1 μg of the unit assay substrate DNA in a total reaction volume of 50 μL in 1 h using the specified buffer. For all the enzymes here, the assay substrate DNA was Bacteriophage Lambda, which is 48 502 bp. For the number of cut sites for each enzyme, see http://www.neb.com/neb/tech/tech_resource/restriction/restriction_maps/frequencies.html

^cThe number of units required to digest the DNA segment is estimated as the quantity of DNA in micrograms (1 μg assumed here) multiplied by the ratio of the cuts per the 2033 bp in the DNA segment to the cuts per the 48 502 bp in the unit assay substrate and divided by the ratio of the activity in the buffer used to the activity in the recommended buffer. This result is then multiplied by a factor of three to account for possible variation in cutting efficiency with the different substrates. The result of this calculation is only an estimate and serves as a starting point for digestion experiments to determine the optimum amount of enzyme.

will usually consist of only one different group. The user can force more group differences using the multiple pass feature of REF Select. In the second and subsequent passes, the specified number of groups (parameter 11; see the example in the Appendix) that were most often used in the previous pass are removed from consideration. The scores of the sets in these subsequent passes will be lower, but the restriction endonuclease groups used may be preferable to the user.

Scoring Algorithm

The number of selected sets and possible sequence variations typically precludes an experimental approach to ranking the sets by measuring detection sensitivity. Even if an experimental approach is warranted, for example, to optimize a REF procedure that will be run many times, a theoretical ranking is desirable as a starting point. REF Select uses a theoretical variety-scoring algorithm to rank the selected sets. This algorithm focuses on the sensitivity of the SSCP component rather than the restriction component. Because the majority of the

segment usually lies outside restriction sites, the majority of sequence variants must be detected solely by the SSCP component, for example, 79% in the blinded analysis study of Liu and Sommer (10). Furthermore, assuming that a sequence variant is equally likely anywhere along the test segment, the sensitivity of the restriction component is determined by the number of unique restriction sites, which is already limited to a fixed range by the common cuts, fragment size and number of fragment constraints of REF Select.

Each base pair in the test segment resides in one sense and one antisense fragment from each digestion of the test segment. The sensitivity of the SSCP component is the probability that at least one of these fragments will exhibit a detectable band shift, defined as at least half of a band width (10). The premise of the scoring algorithm is that the sensitivity of the SSCP component is maximized for a particular base pair by maximizing the group-to-group variety of the fragments in which the base pair resides. The current scoring algorithm is based on variety in the fragment size across groups. We might consider the degree of fragment overlap

from group to group and the actual sequence differences in the nonoverlapping portions in future algorithms.

The size variety score for a particular base pair is defined in a way that it increases when the range of fragment sizes containing the base pair increases and when the fragments have a more uniform distribution over that range. Formally, the variety score V_i for the i^{th} base pair is:

$$V_i = \min [(s_{i,g} - s_{i,1}), R_s] - \sum_{j=2}^g |(s_{i,j} - s_{i,j-1}) - \Delta_{i,\text{avg}}| \quad [\text{Eq. 1}]$$

where g is the number of groups in a REF set, $s_{i,1}, s_{i,2}, s_{i,3}, \dots, s_{i,g}$ are the sizes of the fragments that contain the i^{th} base pair sorted by ascending size (one size representing both sense and antisense fragments), $\Delta_{i,\text{avg}} = (s_{i,g} - s_{i,1})/(g-1)$ is the average fragment size delta for the i^{th} base pair, $\min[]$ means take the minimum of the terms contained in the brackets, and R_s is the fragment size range to use for scoring. The first term on the right side of Equation 1 adds to the variety score based on the range of fragment sizes. The second term subtracts from the variety score based on the deviation from a uniform distribution of fragment sizes. The overall score for a particular restriction endonuclease set is computed by averaging the individual base pair scores over the entire segment. With the default fragment size range constraint of 50–500 and the default R_s of 75% of this range, the maximum possible score is 337.5. Typical scores are substantially lower than the maximum. Figure 1 shows a hypothetical example with a score of 131.3, and the highest scoring set for the *p53* example in the Appendix has a score of 143.5.

Using a value of R_s that is somewhat less than the maximum possible fragment size range removes some of the bias towards the same range of fragment sizes for all regions of the segment. This bias may result in similar fragment sizes from different regions that may overlap on the gel. Detection sensitivity will not necessarily be compromised because only the fragment with the sequence change will be shifted, but the location of the change will likely be ambiguous. The value of R_s represents a tradeoff between variety across fragments from the same region, which is critical for detection sensitivity and maximized when R_s is 100% of the maximum possible range, and variety across fragments from different regions, which reduces the number of overlapping bands.

Output and *p53* Gene Example

The Appendix shows an interactive run of REF Select for a *p53* example using the default input parameter values. Figure 3A shows the optional page of the figure output that shows the distribution of scores for the 52 678 sets selected and ranked by REF Select in pass 1. The distribution of scores is approximately normal, and the highest score of 143.5 is more than 3 standard deviations greater than the average score of 81. Figure 3B shows the graphical summary page of the figure output for the highest scoring set (i.e., the optimum set). To implement the REF procedure using the optimum set of restriction endonucleases shown in Figure 3B, the amount of each restriction endonuclease to use must first be determined. Table 2 provides estimates that can be used as a starting point for digestion experiments to determine the optimum amounts.

In conclusion, REF Select is expert system software that assists in the selection of the set of groups of restriction endonucleases for restriction endonuclease fingerprinting. It enables a comprehensive review of the possible sets and a consistent, objective and fast selection of an optimal set for use in performing REF on a specific DNA segment. The default selection and ranking parameters used by REF Select were derived from our experience with the REF method and form a knowledge base that a user can customize and save. The user interface is simple and includes online help. The software generates both text and high-quality figure outputs.

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Appendix. REF Select Input Details, *p53* Example

Shown here are the details of REF Select input that demonstrate the process of running the software for a sample 2033 bp DNA segment that includes exons 5–9 from the human *p53* gene. The following log shows how the GCG Mapsort file was generated for the example.

```
bscaring@informatics[1]: fetch hsp53g
hsp53g.gb_pr2
bscaring@informatics[2]: assemble hsp53g.gb_pr2
      Begin (* 1 *) ? 12900
      End (* 20303 *) ? 14932
      Reverse (* No *) ?
That range begins AAAGC and ends TACCA. Is this correct (* Yes *) ?
What should I call the output file (* hsp53g.seg *) ? p53e5to9.seg
bscaring@informatics[3]: mapsort p53e5to9.seg
      Begin (* 1 *) ?
      End (* 2033 *) ?
Is this sequence circular (* No *) ?
Select the enzymes: Type nothing or "*" to get all enzymes. Type "?" for help on which enzymes are available and
how to select them.
      Enzyme(* * *): **
What should I call the output file (* p53e5to9.mapsort *) ?
Mapping .....
```

First, the command, **Fetch**, was used to get the entire human *p53* gene sequence file from the GenBank database. Then, the command, **Assemble**, was used to select the segment of interest and to renumber the segment to start from one. The Assemble step can be skipped if the segment is contiguous and renumbering the original sequence is not desired. Finally, the command, **Mapsort**, was used to find the restriction endonucleases that cut the segment and the corresponding cut sites. When using a restriction endonuclease property file that includes restriction endonucleases with isoschizomers, it is important to ensure that these restriction endonucleases are output by Mapsort, which, by default, lists only one of the isoschizomers. Specifically, the user must instruct Mapsort to output all isoschizomers by using ****** in the enzyme field. The following is an excerpt showing only the first five restriction endonucleases from the restriction endonuclease property file used in the example.

Restriction Endonucleases from the 1998/99 Catalog of New England Biolabs, Beverly, MA
with the 6/10/1999 update of the NEBuffer Activity Chart from NEB's web site at
http://www.neb.com/tech/tech_resource/restriction/buffers/buffer_chart.html

Name	Available	Desirable	Temp	Buf1	Buf2	Buf3	Buf4 ..
AatII	Y	Y	37	0	50	50	100
AccI	Y	Y	37	50	50	10	100
Acc65I	Y	Y	37	10	75	100	25
AciI	Y	Y	37	25	50	100	50
AclI	Y	Y	37	10	10	0	100

(file truncated for brevity)

The restriction endonuclease property file is an ASCII text file that contains a tab- or space-delimited table having columns for availability (Y or N), desirability (Y or N), optimum digestion temperature (Celsius) and buffer activity levels (0–100) for specific buffers. Note that spaces must be removed from restriction endonuclease names when they are entered. The header of the example indicates the supplier from which the optimum digestion temperature and buffer activity level data were obtained, but does not imply that only this supplier can be used. The range of buffer activity levels allowed in the restriction endonuclease property file is 0–100, which is consistent with activity levels normalized to a percentage of a maximum activity level. Any buffer activity levels provided as absolute values must be normalized by the maximum activity to get a percent value. If enzymes from multiple suppliers are used, then the user must devise the appropriate conversion and normalization scheme to get comparable activity levels.

The following is a log (edited for brevity) of the sample interactive run of REF Select using the Mapsort file generated as shown, the restriction endonuclease property file shown excerpted and the default input parameter values.

Appendix (cont.)

```
bscaring@informatics[4]: refsel p53e5to9.mapsort -sh=50
A score histogram will be computed with 50 bins.
Enter the format of the restriction site file p53e5to9.mapsort or enter "?" for help.
1. GCG Mapsort file.
2. TACG file with "Display Cut Sites by Enzyme" option. saved as a text file.
3. Webcutter 2 file with "Table of sites, sorted by enzyme name" option saved as a text file and edited to delete
   everything but this table.
(* 1 *):
Reading Mapsort file p53e5to9.mapsort...
1. AatI cuts at AGG'CCT 2x: 392 1032
2. AccI cuts at GT'mk_AC 1x: 16
3. AccII cuts at CG'CG 2x: 247 253
(output of restriction endonuclease list truncated for brevity)
306 restriction endonucleases that cut segment read from file p53e5to9.mapsort
Use default restriction endonuclease property file named re_prop.txt ?
Hit return to use or
enter "c" to change to a different property file or
enter "?" for help or
enter "n" to not use a property file:
194 restriction endonucleases read from property file re_prop.txt
193 were marked as available and desirable.
105 also cut the segment and will be used.
Use a previously saved parameter file?
Hit return for default parameter file refsel.par or
enter "c" to change to a different parameter file or
enter "n" to use default values:
n
Current Input Parameters
-----
GROUP SELECTION CONSTRAINTS
1. Max optimal digestion temp difference within a group(C): 5
2. Min buffer activity levels: 100, 100
3. Fragment size range: 50 - 500
4. Desired range of #groups to select: 25 - 125
SET SELECTION CONSTRAINTS
5. #restriction endonuclease groups in a set: 6
6. Desired total #fragments for all groups: 100 (+10%,-20%)
7. 5' end frag max size(#bp), limit(#groups): 200, 3
8. 3' end frag max size(#bp), limit(#groups): 200, 3
9. Common cuts tolerance(#bp), limit(#cuts): 4, 2
SCORING ALGORITHM PARAMETERS
10. Scoring Algorithm Options: % fragment size range to use for scoring: 75
OUTPUT PARAMETERS
11. #passes, #groups to not use in next pass: 3, 2
12. #options to show for each pass: 3
13. Base pair range size for score plot: 25
14. Fragment size bin size: 50
15. Output text file line length, lines per page: 80, 60
PROGRAM MODE
16. Auto parameter adjustment: ENABLED
-----
Use these parameter values?
Hit return for yes or
enter item number for help or to change or
enter "d" to use default values:
The output text file name will be p53e5to9.ref
Hit return to use or enter "c" to change:
The output GCG figure file name will be p53e5to9.figure
Hit return to use or enter "c" to change:
Selecting from 5.565000e+03 possible restriction endonuclease groups
which would form 4.114220e+19 possible REF sets...
71 restriction endonuclease groups selected...
14 groups had pericleavers (all cut sites were identical within +/-4 bp).
Best pericleaver will be used and others will be listed as alternatives.
```

Appendix (cont.)

```
Best pericleaver is that with
1. Fewest number of restriction endonucleases.
2. Highest total buffer activity.
15 total pericleavers found.
New group count is 56.
Pass 1
56 restriction endonuclease groups selected...
Selecting from 3.246844e+07 possible REF sets...
Pass 1 Statistics:
56 restriction endonuclease groups selected,
3.246844e+07 possible REF sets.
52678 REF sets selected for scoring,
3 best scoring sets saved.
  Min Score = 18.6
  Avg Score = 80.8, sdev = 16.7
  Max Score = 143.5
  Score Range = 124.9
Pass 2
54 restriction endonuclease groups selected...
Selecting from 2.582716e+07 possible REF sets...
Pass 2 Statistics:
54 restriction endonuclease groups selected,
2.582716e+07 possible REF sets.
25473 REF sets selected for scoring,
3 best scoring sets saved.
  Min Score = 22.3
  Avg Score = 80.0, sdev = 15.9
  Max Score = 139.6
  Score Range = 117.3
Pass 3
52 restriction endonuclease groups selected...
Selecting from 2.035852e+07 possible REF sets...
Pass 3 Statistics:
52 restriction endonuclease groups selected,
2.035852e+07 possible REF sets.
8241 REF sets selected for scoring,
3 best scoring sets saved.
  Min Score = 26.0
  Avg Score = 72.2, sdev = 13.5
  Max Score = 118.9
  Score Range = 93.0
Text output written to file p53e5to9.ref
GCG Figure output written to file p53e5to9.figure
If you have GCG set up for figures, to print the output, type figure p53e5to9.figure
bscaring@informatics[5]: figure p53e5to9.figure
```

After reading the Mapsort file, REF Select indicated that it found 306 restriction endonucleases that cut the segment. Next, REF Select read the restriction endonuclease property file and found that it contained 193 restriction endonucleases marked as available and desirable, 105 of which cut the segment. From these 105 restriction endonucleases, 5565 groups of one or two can be formed and from those groups, 4×10^{19} sets of six groups can be formed.

Proceeding with the selection step using the default parameter values, REF Select determined that 71 of the 5565 possible groups satisfied the group selection constraints. Of these 71 groups, 15 were found to be pericleavers of (to have the same cut sites as) other groups within a tolerance of 4 bp. Thus, 56 groups were selected in the first pass. Of the 32 468 436 possible sets of six groups that can be formed from these 56 groups, 52 678 were found to satisfy the set selection constraints. In the ranking step, REF Select scored these 52 678 sets and output the 3 best scoring sets and other run information. Figure 3B shows the output for the highest scoring set. The multiple pass feature was used in this example so that, in passes 2 and 3, the two groups most often used in the previous pass were removed from consideration and that set selection proceeded by using the remaining 54 and 52 groups, respectively.