Human Genomic Vectorette™ Libraries

Product Code HUMAN-VS

INSTRUCTION MANUAL

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1. INTRODUCTION

1.1 FEATURES OF HUMAN GENOMIC LIBRARIES

In most genome walking projects, the distance between known sequence and a restriction cut site is not known. By having several libraries made with different restriction enzymes, amplification of optimized fragments as well as PCR† products of differing sizes containing the region of interest is ensured.

The main feature of a Vectorette library is that the one-day process of DNA digestion/ Vectorette II unit ligation is eliminated. The libraries in the Human Vectorette Libraries have been constructed using four restriction enzymes: Bgl II, EcoR I, Hind III, and Pvu II (blunt end). The gene sequence used for the selection of the positive control primers is from the Human Genome (AL121776) Glyceraldehyde-3-phosphate dehydrogenase (length 143,981 bp). The primers selected are the Control Primer (cut site 110,827, length 23 bp), and the Nested Control Primer (cut site 111,294, length 23 bp), to be used for all four libraries. These primers can be used, along with the Vectorette PCR Primer, to produce amplicons of known size that can act as positive controls when performed alongside the researchers' reactions of interest.

1.2 APPLICATIONS

The Human Vectorette library is useful for researchers who know a specific sequence in the Human Genome and wish to determine the surrounding unknown sequence. Such applications include:

1. Revealing 5' and 3' untranscribed sequence of promoter/enhancer regions that control transcription.
2. Walking from both directions into unknown BAC terminal sequencing, for a greater distance than traditional sequencing allows.
3. Allowing the exon/intron boundary sequence of cDNA to be determined by working off of the exon sequence.
4. Sequencing the flanking regions to microsatellites (small, unique repeats that are not transcribed into RNA).
5. Extension of sequence into gaps between contigs from either side.
6. Providing identification of transgene location in genetically modified organisms.

1.3 KIT CONTENTS

Each Vectorette library is provided in sufficient quantity to perform twenty primary reactions of fifty microliters.

	Amount	Product Code
Bgl II Human Vectorette library	20 rxn	B 0931
EcoR I Human Vectorette library	20 rxn	E 3277
Hind III Human Vectorette library	20 rxn	H 0159
Pvu II Human Vectorette library	20 rxn	P 4612
Control Human Vectorette Primer	1 vial (1 nmol, 50 µl)	P 7987
Control Human Vectorette Nested Primer	1 vial (1 nmol, 50 µl)	P 6737
Vectorette Primer	1 vial (10 nmol, 1 ml)	P 3607
Nested Vectorette Primer	1 vial (10 nmol)	P 3732
Sequencing Primer	1 vial (500 pmol)	P 3857

Materials and Reagents Required but Not Provided

(Sigma Product Codes have been given where appropriate)

- JumpStart™ REDAccuTaq™ LA DNA Polymerase, Product Code D 1313 (or comparable long and accurate product)
- 10 mM dNTP mix, Product Code D 7295
- Dedicated pipettes
- PCR pipette tips
- 0.5 ml thin wall PCR microcentrifuge tubes
- Thermal cycler

2. PROTOCOLS

2.1 PRIMER DESIGN

I. Design of target specific primers to be used with the Human Genomic libraries.

Unlike conventional PCR in which two primers determine specificity, one target specific primer provides the only determinant to ensure faithful amplification in Vectorette PCR. For this reason care must be taken to ensure "uniqueness" of primer design. Sigma recommends the following criteria:

- Primer length should be at least 22 nucleotides with a T_m between 72 °C and 74 °C.
- Specific primers should have at least nine GC bonds, so that the recommended PCR touchdown cycling parameters may be used.
- There should be a GC clamp near the 3' end of the specific primer. Ideally, the last two bases of the 3' end should be a G or C.
- Target specific primers should be designed within the more conserved exon regions of genes if possible, especially when sequence polymorphism among different specimens (e.g. different individuals) is high.
- The 3 ' end of the primers should land on the first or second position of a codon to avoid mismatches caused by synonymous mutations.
- Sigma highly recommends using primer design software [e.g. Oligo, Rychlik 1995a, Hyther (http://jsl1.chem.wayne.edu/Hyther/hytherm1main.html) or Primer3 (www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi)] to avoid hairpin formation, primer-dimers, false priming, etc.

The key to specific primer design is to balance the binding efficiency and the specificity at the 3' end of the primer. Although this design is slightly different from conventional primer design, it performs well in combination with touchdown PCR cycling protocols, producing good product yield and specificity in the primary Vectorette PCR (Rychlik, 1995).

II. Sigma recommends the use of a second, confirming primer for Vectorette PCR.
While Vectorette PCR does not require a sense and anti-sense primer, Sigma recommends the use of one or more specific primers, preferably designed in a nested fashion, for three reasons:

1. A single primer might not work in Vectorette PCR, and different primers will increase the chance of success.
2. The primary Vectorette PCR might give multiple products and cause confusion as to the identity of the correct product. Two or more primers will allow determination of the desired product by comparing bands in separate PCR reactions generated from different primers.
3. A nested PCR might be needed for some difficult samples to provide the expected specificity and yield.

III. The molar ratio of specific primer to Vectorette primer in primary PCR should be 10 to 1 (1 µM to 0.1 µM).
We have found that this ratio significantly increases product yield and specificity. Since the specific primer is the only primer being extended during the first cycle of PCR, the excess amount of specific primer may help to increase primer-target binding efficiency. Another important aspect of Vectorette PCR is the use of a "touchdown" cycling protocol and the use of a hot start for high specificity. The "touchdown" protocol has seven initial cycles of a high annealing temperature, which is the same as the extension temperature, to increase the specificity of primer to the target sequence. Thirty-two cycles of a lower annealing and extension temperature are then employed for efficient exponential amplification of the targeted PCR amplicon. Hot start can be achieved by using JumpStart REDAccuTaq DNA polymerase.

2.2 PRIMARY VECTORETTE PCR USING HUMAN GENOMIC LIBRARIES

I. Reaction Number: Primary PCR reactions with user-designed primers are usually performed twelve at a time, i.e. for each of the four libraries (Bgl II. EcoR I, Hind III, and Pvu II) three reactions are run of varying primer makeup, containing:

   a. Both the specific and Vectorette primers.
   b. The Vectorette primer alone.
   c. The specific primer alone.

   A set of four control reactions, one for each library, containing control and Vectorette primers will insure the efficacy of the PCR reagents.

II. Primer Dissolution: The Control Human and Control Human Vectorette Primers should be dissolved in 50 µl water or TE, to yield 20 µM final concentration. The Vectorette Primer should be dissolved in 1 ml to give a final concentration of 10 µM. Once dissolved, primers should be stored at –20 °C.

III. Master Mix: Prepare enough master mix for 1.1X the number of reactions you plan to run. This will compensate for loss of solution that does not completely transfer. Use the table below to calculate what you will need and check off components as you add them to the mix.

Component	**For one reaction**	**For _____ reactions**	√	**Final Concentration**
JumpStart REDAccuTaq	2.5 µl			2.5 units (0.05 un/µl)
10X AccuTaq Buffer	5 µl			1X concentration
10 mM dNTP mix	1 µl			0.2 mM
Water	1.5 µl			--------
Final Volume	10 µl			

IV. Reaction Assembly: Prepare reactions as follows using one sterile PCR reaction tube per Human Vectorette Library per condition. Each reaction will need to have primers and library added. Sigma recommends to first add libraries, primers and water, then initiate each reaction with the above master mix. Check off components as you add them to the mix.

Component	Specific Primer + Vectorette Primer	√	Vectorette Primer Alone	√	Specific Primer Alone	√	Comments
Specific Vectorette Library	2 µl		2 µl		2 µl		
10 µM Vectorette Primer	0.5 µl		0.5 µl		----		0.1 µM final concentration
20 µM Control Vectorette Primer **Or** 20 µM Specific Primer (user-designated)	2.5 µl		----		2.5 µl		1 µM final concentration
Water	to 40 µl		to 40 µl		to 40 µl		
Master Mix (see previous page)	10 µl		10 µl		10 µl		See previous page
Final volume	50 µl		50 µl		50 µl		

NOTE: The amount of library to be added to the PCR reaction may need to be empirically determined. Too much DNA from the ligation library will result in non-specific amplification, while too little DNA will give no product. This amount (2 µl) has worked well in our laboratories, and is the amount recommended for the control.

V. Thermal Cycling: Spin down the tube contents and place in a thermal cycler. Begin PCR using the following "touchdown" cycling parameters.

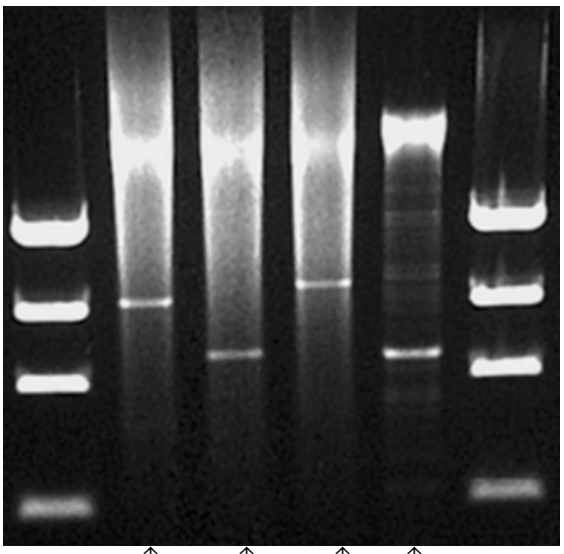
Denaturation	94 °C	5 sec	7 cycles
Annealing/Extension	72 °C	5 min	
Denaturation	94 °C	5 sec	32 cycles
Annealing/Extension	68 °C	5 min	
Final Extension	68 °C	5 min	1 cycle
Soak	4 °C		

Perkin Elmer and MJ Research thermal cyclers are recommended for Vectorette touchdown PCR cycling. The program takes approximately four hours.

NOTE: PCR cycling parameters will need to be determined empirically for each specific primer and template. A "touchdown" PCR cycling protocol is recommended when performing primary Vectorette PCR on genomic DNA. The denaturation temperature in this "touchdown" cycling protocol works well at very small time intervals for genomic DNA. Recommended denaturation time for genomic DNA is two seconds. The recommended positive control PCR denaturation time is 15 seconds but will give positive results at the shorter two-second time interval. Secondary PCR using nested primers may use the same touchdown cycling protocol if the nested specific primer has a high enough T_m. The touchdown cycling PCR protocols were optimized on PE9700 and MJ Research thermal cyclers, and conditions optimal for other thermal cyclers will need to be empirically determined.

VI. Analysis: Analyze PCR products on a 1% - 1.5% agarose gel, depending on the size of the amplified fragment. With complex DNA targets, it is common that faint (or no) bands may be seen after the initial PCR. However, discreet products may appear following a subsequent nested PCR (see Section 2.3).

Figure 1. Bands of expected size for control primer PCR reactions



Products amplified using the Control Human Vectorette Primer, run on a 1% w/v agarose gel with 0.5 mg/ml EtBr.

2.3 NESTED PCR

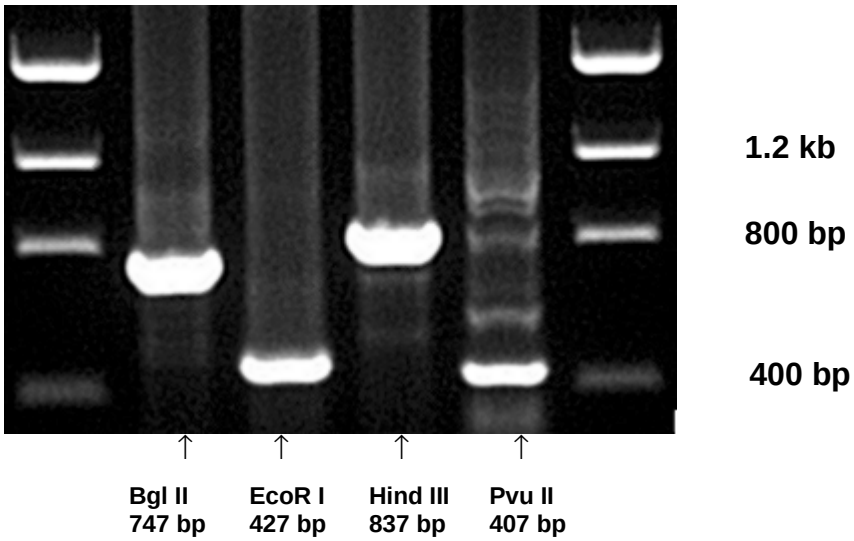
A second (nested) PCR reaction may be needed to obtain the fragment of interest or to eliminate nonspecific PCR amplicons. The nested specific primer will ideally be directed to a region not covered by the original primer, and be 3' of the initially targeted region. At the very least, the nested primer should be extended at the 3' end by 3–5 bases, relative to the initial specific primer. The nested Vectorette PCR primer is included in the kit. Reactions should be set-up as delineated in the table below.

NOTE: Before first use, the Vectorette Nested Primer should be dissolved in 500 µl to give a final concentration of 20 µM. Once dissolved, primers should be stored at –20 °C.

Component	Amount	Comments
Primary PCR product	1 µl	Of a 1:10 to 1:1,000 dilution
10X AccuTaq buffer	5 µl	Final concentration of 1X.
10 mM dNTP mix	1 µl	Final concentration of 0.2 mM
20 µM Nested Vectorette Primer	1 µl	Final concentration of 0.4 µM
20 µM Nested custom primer	1 µl	Final concentration of 0.4 µM
JumpStart REDAccuTaq LA DNA Polymerase	2.5 µl	Final amount 2.5 units
Sterile water	X µl	Final volume of 50 µl

NOTE: If the nested specific primer has a high enough T_m, use the same PCR cycling parameters that were used in the primary PCR reaction. Analyze the nested PCR product on a 1.5–4% agarose gel, depending on the size of the fragment.

Figure 2. Bands of expected size for control nested primer secondary PCR reactions

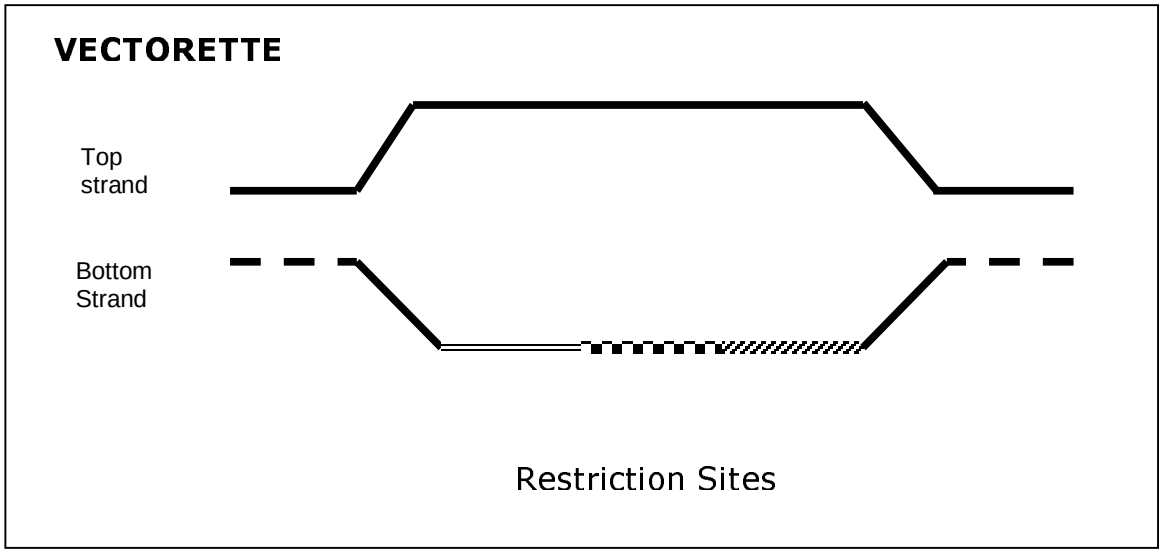


Products amplified using the Control Human Nested Vectorette Primer, run on a 1% w/v agarose gel containing 0.5 mg/ml EtBr.

2.4 SEQUENCING OF VECTORETTE PCR PRODUCTS

The sequence of Vectorette PCR product can be determined after cloning the amplicon into a vector of the user's choice, or from direct sequencing of the PCR product. Cloning can be accomplished by using TA or blunt systems, or after digestion of the EcoR I, Bam HI, or Hind III site in the Vectorette II adaptor (see Figure 3). Sequencing primers are included in the kit. In either case, the Vectorette PCR product should be gel purified using a suitable system (e.g. GenElute™ PCR DNA Purification Kit, Product Code GEN-PCR).

Figure 3. Makeup of the Vectorette Adaptor



A detailed explanation of the Vectorette system can be obtained from our website (http://www.siigma-aldrich.com/vectorette) or can be ordered free of charge by contacting our Technical Service department at 800-325-5832.

3. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
No PCR product is observed	A PCR component is missing or degraded.	A positive control should always be run to insure components are functioning. A checklist is also recommended when assembling reactions.
	The reaction does not have enough enzyme.	1.0 µl (2.5 units) is sufficient for most applications. It is recommended that the cycling parameters be optimized before the enzyme concentration is increased. In rare cases, the yields can be improved by increasing the enzyme concentration. However, if the enzyme amount is above 2 µl (5 units), higher background levels may be seen.
	Lack of specificity	Increase annealing temperature during PCR. Alternately, try the touchdown protocol that has been included in this manual.
		Use thin wall tubes and minimize the incubation time during the annealing and extension steps. This will limit the opportunities for mispriming and extension.
		Ensure the primers and enzyme concentrations are not too high. This will help reduce mispriming.
	DNA fragment too large	Modify PCR conditions by increasing extension time.
Smearing or multiple bands	Lack of specificity (Also see Note below)	Increase annealing temperature during PCR. Alternately, try the touchdown protocol that has been included in this manual.
		Use thin wall tubes and minimize the incubation time during the annealing and extension steps. This will limit the opportunities for mispriming and extension.
		Ensure the primers and enzyme concentrations are not too high. This will help reduce mispriming.
		Magnesium ion concentration plays an important role in specificity. Changing the magnesium ion levels can improve specificity (and perhaps yield) by increasing the stringency of the reaction or by direct effects on the polymerase itself. The magnesium ion:dNTP ratio also plays a role in PCR specificity must be greater than one.

IMPORTANT NOTE: For certain applications (particularly when using complex target DNA) nested PCR is required for complete specificity. Try to use a nested "custom" primer with the nested Vectorette PCR primer.

4. REFERENCES

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Lilleberg, S. and Patel, S. Isolation of DNA flanking retroviral integration sites using Vectorette II. *Genosys* Origins, **II** (1998)

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Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239** (4839), 487-491 (1988)

Sambrook, J., *et al. Molecular Cloning: A Laboratory Manual*, third edition, Cold Spring Harbor Laboratory. Cold Spring Harbor (2000) (Product Code M8265)

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5. RELATED PRODUCTS

Product Name	**Product Codes**	
JumpStart REDAccuTaq LA DNA polymerase	D1313	
Ultrapure dNTPs	**10 mM**	**100 mM**
dATP	D 6920	D 4788
dCTP	D 7045	D 4913
dGTP	D 7170	D 5038
dTTP	T 7791	T 9656
Sets of all four nucleotide solutions	DNTP-10	DNTP-100, DNTP-100A
dNTP Mixture (10 mM each dATP, dCTP, dGTP and dTTP)	D 7295	

† The PCR process is covered by patents owned by Hoffman-LaRoche, Inc.

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