Recombinant DNA Technology

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By

Siddra Ijaz and Imran Ul Haq

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PREFACE

The progressing scientific revolution is resulting in changes in the established rules of human-biotic world interactions. Researchers are now able to identify and characterize genes for almost all biological functions, modify and incorporate them into living cells, exchange genetic materials between species and produce clones (genetic blueprints) of all living beings through the art of Recombinant DNA Technology. Genetic engineering has emerged as the ultimate solution to most everyday life problems, from food safety/security to improving health. A plethora of life's daily issues are now dependent on genetic engineering, either through the development of genetically modified organisms or of new drugs/vaccines to combat diseases. All this is possible due to emerging knowledge in genetic engineering.

This book details different Recombinant DNA Technology techniques and their applications. It is intended to address the approaches of current genetic engineering and their wizardly applications in laboratories, as well as in the field. The book is aimed at professional biologists, university students and scientists in general. This book will have an evocative impact on the minds of its readers and will provide comprehensive knowledge on the further potential of Recombinant DNA Technology. In this book, we have tried to leave no stone unturned to present the complex field of genetic engineering in a fluid way, by using simple and reader-friendly language without compromising its scientific value.

The book is comprised of seven chapters. The first chapter is on Gene Cloning, while the second is about the Construction of Gene Libraries. The third chapter concerns principles and methods for the genetic Transformation of Plants, and light has been shed on Marker Genes and their excision in the fourth chapter. Techniques in Molecular Biology and Approaches in Gene Editing are discussed in the fifth and sixth chapters respectively. The last chapter is about the Applications of Recombinant DNA Technology. We are hopeful that by the end, this effort will lead to the creation of some opportunities to add a little to the already existing ocean of genetic engineering.

Siddra Ijaz, PhD
Imran Ul Haq, PhD

ABBREVATIONS

A	Adenine
AdoMet	S-adenosyl methionine
Amp	Ampicillin
ATP	Adenosine Tri Phosphate
attB	Bacterial attachment Site
attP	Phage attachment Site
BAC	Bacterial Artificial Chromosome
bar	A gene, confers resistance to bialaphos herbicide
BCIP-T	5-bromo-4-chloro-3-indolyl phosphate, p-toluidine salt
BT	Bacillus thuringiensis
C	Cytosine
C2H2	Cys2His2
CaMV35S	Cauliflower mosaic virus 35S
Cas 9	CRISR-associated protein 9
cDNA	Complementary DNA
Cre	Cyclic recombinase
CRISR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
Ct	Cycle threshold
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphate
DSBs	Double stranded Breaks
EDTA	Ethylenediamine tetra acetic acid
FLP	Flippase
G	Guanine
GDS	Gel Documentation System
GFP	Green Fluorescent Protein
GMO	Genetically Modified Organisms
HART	Hybrid Arrest Translation
HCl	Hydrochloric acid
HDR	Homology Directed Repair
He	Helium

HR	Homologous Recombination
HRP	Horseradish peroxidase
HRT	Hybrid Release Translation
Int	Integrase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IR	Illegitimate Recombination
ISSR	Inter Simple Sequence Repeat
KJ	kilojoule
LoxP	Locus of X-over in P1 bacteriophage
LSC	Large single copy
M	Molar
MCSs	Multiple Cloning Sites
$MgCl_2$	Magnesium Chloride
mRNA	Messenger RNA
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NBT	Nitro Blue Tetrazolium
NHEJ	Non Homologous End Joining
NLS	Nuclear localization sequence
NPC	Nuclear pore complex
ORF	Open Reading Frame
ori	Origin of Replication
PAGE	Polyacrylamide Gel Electrophoresis
PAGE	Polyacrylamide Gel Electrophoresis
PAM	Protospacer adjacent motif
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PI domain	PAM interacting domain
qPCR	Quantitative PCR
R gene	Resistance gene
RAMP	Repair associated mysterious protein
RAPD	Radom Amplified Polymorphic DNA
RBS	Recombinase Binding site
Ri plasmid	Root inducing plasmid
RNA	Ribonucleic acid
RNA	Ribonucleic acid
rpm	Revolution per minute
SDS	Sodium dodecyl Sulphate
SSC	Small single copy
SSC	Sodium Chloride-Sodium Citrate
SSCP	Single Strand Conformational Polymorphism

SSR	Simple Sequence Repeat
ssTDNA	single stranded transfer DNA
T	Thymine
TAE	Tris acetate EDTA
TAL	Transcription activator-like
TALEN	Transcription activator-like effector nucleases
Taq polymerase	DNA polymerase from *Thermus aquaticus*
T-DNA	Transfer DNA
Ti plasmid	Tumor inducing plasmid
tracrRNA	transactivating CRISPR RNA
UV	Ultraviolet
Vir gene	Virulence gene
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YAC	Yeast Artificial Chromosome
ZFNs	Zinc Finger Nucleases

CHAPTER ONE

GENE CLONING: AN OVERVIEW

In gene cloning, a set of experiments to assemble DNA fragments is used to generate a recombinant DNA molecule. Gene cloning is the core aspect of Recombinant DNA Technology. In principle, it involves the joining of DNA fragments from different sources. In this technique, the DNA fragment is ligated with a vector to develop a recombinant DNA molecule. Then this recombinant DNA molecule is delivered into the host cell for multiplication, so as to produce multiple identical copies of this molecule.

Methods for the cloning of a particular segment of DNA into a vector are categorized as either (1) ligase dependent or (2) ligase independent methods. However, ligase dependent methods are generally practiced for cloning. Ligation dependent cloning methods are further subdivided into two groups, (1) cohesive ended ligation and (2) blunt ended ligation. These approaches require restriction enzymes to generate cohesive or sticky ends, and multiple enzymatic modifications for blunt ended ligation. PCR development has made this task quite simple and fast.

PCR amplified fragments are cloned through different methods, such as through engineering restriction enzymes sites at the 5' end of primers, ligase independent cloning and TA cloning. Among all these, TA cloning is the more efficient cloning strategy. The TA vector has a 3'-T overhang, while *Taq* polymerase has the ability to generate a 3'-A overhang in amplified PCR products, by introducing a single 3'-A into them. These overhangs are complementary to each other, thus facilitating the direct cloning of a PCR product into the vector. Therefore, in this chapter, an overview of the general cloning strategies is briefly presented.

BASIC FEATURES OF VECTORS

A cloning vector must have the ability to replicate independently in the host cell. Vectors to be used in cloning experiments must have the following features:

- They must be a circular DNA molecule.
- They must have their origin of replication (*ori*) for their autonomous replication within a host.
- They must contain selectable marker gene sequences.
- They must have multiple cloning sites (MCSs) region.

VECTORS FOR CLONING

Different vectors are used in molecular biology for cloning purposes, e.g. plasmid, λ phage, cosmids, BAC (bacterial artificial chromosome) and YAC (yeast artificial chromosome). They accept different sizes of DNA fragments/inserts to carry them as recombinant. A list of cloning vectors and insert sizes which they may carry is given in the table below (Table 1.1).

Table 1.1: Cloning vectors

Vector	Insert size
Plasmid	~ 10 Kb
λ phage	~ 23 Kb
Cosmid	~ 45 Kb
BAC	~ 350 Kb
YAC	~ 1000 Kb

BASIC STEPS OF GENE CLONING

DNA manipulative and DNA modifying enzymes with diverse properties, as well as a series of experimentations involved in gene cloning, are described in detail below. The general steps involved in gene cloning are also illustrated pictorially in Fig. 1-1.

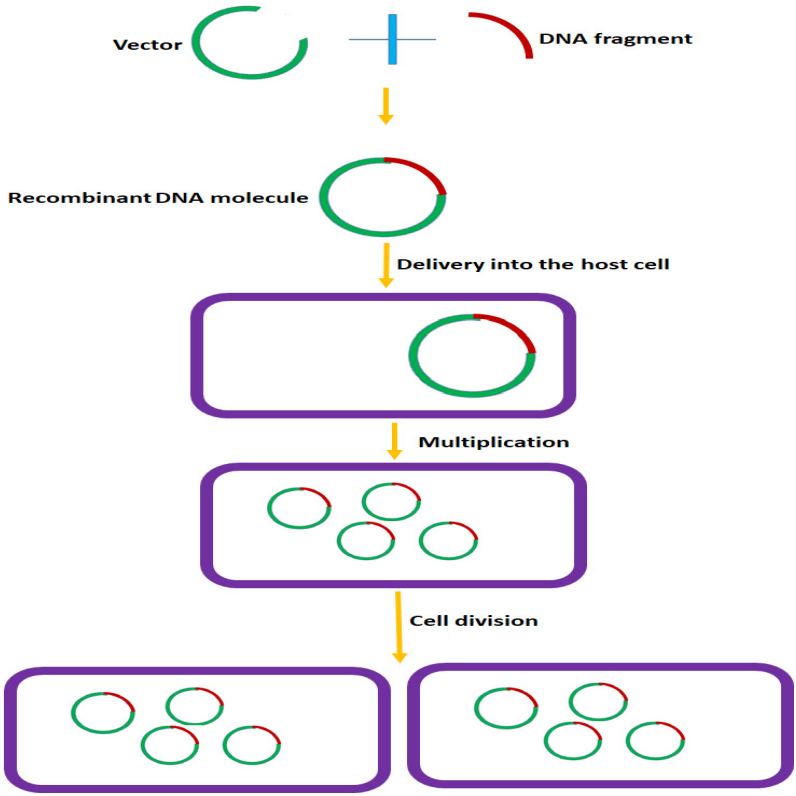

Figure 1.1: A schematic diagram of gene cloning, representing generation of recombinant DNA molecule and its multiplication (idea taken from Brown 2006).

Gene isolation:

In the first step, a gene, or any DNA fragment to be used in cloning is isolated using different methods, such as a PCR based on sequence-specific primers, to amplify DNA fragments from extracted genomic DNA, RNA, organellar DNA, gene libraries etc. This step in gene cloning is mediated by polymerases.

Restriction digestion

Restriction enzymes (molecular scissors) are involved in the restriction digestion step of gene cloning. These restriction enzymes are categorized into two broad classes based on their mechanism of action; (1) Exonucleases (2) Endonucleases. A diverse range of restriction enzymes is available. The vector and DNA fragment must both be cut with the same kind of restriction enzymes for generating compatible ends in order to facilitate their proper joining.

Multiple cloning sites (MCSs) or polylinkers in a vector are the restriction sites that are specific to restriction enzymes (restriction endonucleases). The sites at which these molecular scissors make the cut are known as recognition sites or restriction sites. These sites may be made up of 4, 5, 6 or 8 base pair-long symmetrical inverted repeats (Palindromic) as well as of asymmetrical sequences; hence, based on the sequence length of recognition sites, endonucleases are referred to as either four cutters, five cutters, six cutters or eight cutters. Among them, four cutters and six cutters are the most commonly used molecular scissors in recombinant DNA technology. The restriction enzymes are cut in different ways and thereby may generate sticky ends or staggered ends (5' protruding ends or 5' phosphate overhangs, and 3' protruding ends or 3' hydroxyl overhangs) as well as blunt ends or flush ends (Fig. 1-2). The ligation efficiency of compatible sticky ends is more than that of blunt ended DNA molecules. Thus, to turn blunt ended DNA into a sticky ended molecule, linkers and adapters are used. Adapters are the short stretches of synthetic oligonucleotides with sticky ends, whereas linkers are blunt ended synthetic oligonucleotides but with restriction enzymes sites, which generate sticky ends.

Classification of restriction endonucleases

Restriction endonucleases are generally classified into four broad categories based on sequence specificity, cleavage position, the composition of their subunits and the requirements of their co-factors etc. These are classified as:-

- Type I restriction endonucleases
- Type II restriction endonucleases
- Type III restriction endonucleases
- Type IV restriction endonucleases

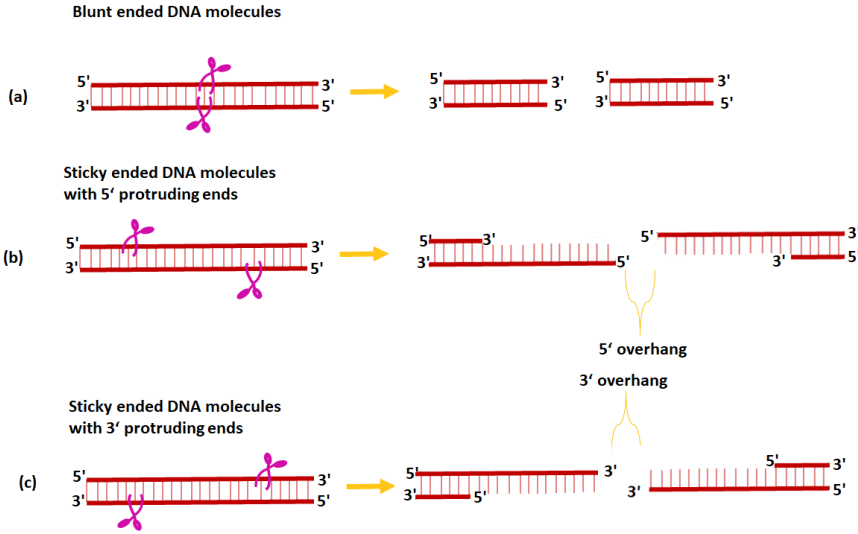

Figure 1.2: Cleavage action of restriction endonucleases (a) blunt ended DNA molecule (b) 5' protruding sticky ended DNA molecule (c) 3' protruding sticky ended DNA molecule (idea taken from Russell 2009).

Type I Restriction Endonucleases

Type I restriction enzymes are made up of multi-subunits and are complex in nature. They have independent subunits for recognition, cleavage of DNA molecules at specific sites and their modifications. The location of these endonucleases' recognition sites is far from their cleavage sites, which make these restriction enzymes no longer suitable for biotechnological applications. These restriction enzymes require AdoMet (S-adenosyl methionine), an Mg^{+} ion and ATP (Adenosine triphosphate) for their activity.

Type II Restriction Endonucleases

Type II restriction enzymes' recognition sites are located within or close to their restriction sites. This feature gives them specificity and defined positions for cutting. They recognize symmetrical palindromic sequences. This class of restriction endonucleases is of practical value for use in gene cloning and other molecular analyses in laboratories. However, they don't have any AdoMet and ATP requirements for their activity. A list of restriction enzymes which are being used in recombinant DNA technology is given in the Table 1.2.

Type III Restriction Endonucleases

These restriction enzymes make a cleavage at DNA molecules outside their recognition sites. They recognize asymmetrical sequences of inverse orientation like the Type I class of restriction enzymes. This group of enzymes also requires AdoMet and ATP for their functioning.

Type IV Restriction Endonucleases

These restriction endonucleases act on modified methylated DNA molecules as recognition sites.

DNA modification

DNA molecules are modified by the addition or removal of their chemical groups according to the cloning strategy's needs. Various types of DNA modifying enzymes, such as alkaline phosphatase (the mediating removal of the phosphate group at the 5' terminal of a DNA molecule), polynucleotide kinase (the mediating addition of the phosphate group at the 5' terminal of a DNA molecule) and terminal deoxynucleotidyl transferase (the mediating addition of more than one nucleotide at the 3' terminal of a DNA molecule) are used in gene cloning experimentation.

Ligation reaction

Restriction digestion as well as modification (in some cases) of a DNA molecule and vector is followed by a ligation reaction. In ligation reactions, the ligase enzyme (molecular scissors) is used to mediate the joining of the DNA fragment and vector molecule together into a recombinant DNA molecule. A ligation mixture contains,

1) Unligated molecules
   a) Unligated vectors
   b) Unligated DNA fragments
2) Self-ligated vector molecules
3) A recombinant DNA molecule

Delivery to host and clone selection

The ligation mixture is delivered into the host cell for the selection of a desired clone (a recombinant DNA molecule), and then to produce multiple copies of this clone. However, the selection of the clone is made using a selective agent (in the culture medium required for the growth of a host cell) against a selectable marker gene present in the vector backbone.

Restriction Enzymes	Ends generated	Restriction Enzymes	Ends generated	Restriction Enzymes	Ends generated	Restriction Enzymes	Ends generated	Restriction Enzymes	Ends generated
AloI	Sticky ends	AccB7I	Sticky ends	BfrI	Sticky ends	BseJI	Blunt ends	BspLU11I	Sticky ends
AluI	Blunt ends	AccBSI	Blunt ends	BfrBI	Blunt ends	BseLI	Sticky ends	BspMI	Sticky ends
AlwI	Sticky ends	AciI	Sticky ends	BfuI	Sticky ends	BseMI	Sticky ends	BspPI	Sticky ends
Alw21I	Sticky ends	AclI	Sticky ends	BfuAI	Sticky ends	BseMII	Sticky ends	BspTI	Sticky ends
Alw26I	Sticky ends	AclWI	Sticky ends	BaeI	Sticky ends	BseNI	Sticky ends	BspT104I	Sticky ends
Alw44I	Sticky ends	AcsI	Sticky ends	BfuCI	Sticky ends	BsePI	Sticky ends	BspT107I	Sticky ends
AlwNI	Sticky ends	AcuI	Sticky ends	BglI	Sticky ends	BseRI	Sticky ends	BspXI	Sticky ends
Ama87I	Sticky ends	AcyI	Sticky ends	BglII	Sticky ends	BseSI	Sticky ends	Eco57I	Sticky ends
Aor13HI	Sticky ends	AdeI	Sticky ends	BisI	Sticky ends	BseXI	Sticky ends	BspLU11I	Sticky ends
Aor51HI	Blunt ends	AfaI	Blunt ends	BlnI	Sticky ends	BseX3I	Sticky ends	BsrI	Sticky ends
ApaI	Sticky ends	AfeI	Blunt ends	BlpI	Sticky ends	BseYI	Sticky ends	BsrBI	Blunt ends
ApaLI	Sticky ends	Afl II	Sticky ends	Bme18I	Sticky ends	BsgI	Sticky ends	BsrDI	Sticky ends
ApeKI	Sticky ends	Afl III	Sticky ends	Bme1390I	Sticky ends	Bsh1236I	Blunt ends	BsrFI	Sticky ends
ApoI	Sticky ends	AgeI	Sticky ends	Bme1580I	Sticky ends	Bsh1285I	Sticky ends	BsrGI	Sticky ends
AscI	Sticky ends	AhdI	Sticky ends	BmgBI	BLUNT	BshNI	Sticky ends	BsrSI	Sticky ends
AseI	Sticky ends	AhlI	Sticky ends	BmrI	Sticky ends	BshTI	Sticky ends	BssECI	Sticky ends
AsiGI	Sticky ends	AjiI	Blunt ends	BmtI	Sticky ends	BsiEI	Sticky ends	BssHII	Sticky ends
AsiSI	Sticky ends	AjnI	Sticky ends	BmyI	Sticky ends	BsiHKAI	Sticky ends	BssKI	Sticky ends
AspI	Sticky ends	AjuI	Sticky ends	BoxI	Blunt ends	BsiWI	Sticky ends	BssNAI	Blunt ends

Asp700I	Blunt ends	AleI	Blunt ends	BpiI	Sticky ends	BsiYI	Sticky ends	BssSI	Sticky ends
Asp718I	Sticky ends	AlfI	Sticky ends	BplI	Sticky ends	BslI	Sticky ends	BssT1I	Sticky ends
AspA2I	Sticky ends	BanII	Sticky ends	BpmI	Sticky ends	BslFI	Sticky ends	Bst6I	Sticky ends
AspEI	Sticky ends	BanIII	Sticky ends	Bpu10I	Sticky ends	BsmI	Sticky ends	Bst98I	Sticky ends
AspHI	Sticky ends	BauI	Sticky ends	Bpu14I	Sticky ends	BsmAI	Sticky ends	Bst1107I	Blunt ends
AspLEI	Sticky ends	BbeI	Sticky ends	Bpu1102I	Sticky ends	BsmBI	Sticky ends	BstACI	Sticky ends
AspS9I	Sticky ends	BbrPI	Blunt ends	BpuAI	Sticky ends	BsmFI	Sticky ends	BstAPI	Sticky ends
AsuC2I	Sticky ends	BbsI	Sticky ends	BpuEI	Sticky ends	Bso31I	Sticky ends	BstAUI	Sticky ends
AsuHPI	Sticky ends	BbuI	Sticky ends	BsaI	Sticky ends	BsoBI	Sticky ends	BstBI	Sticky ends
AsuNHI	Sticky ends	BbvI	Sticky ends	Bsa29I	Sticky ends	Bsp13I	Sticky ends	Bst2BI	Sticky ends
AvaI	Sticky ends	Bbv12I	Sticky ends	BsaAI	Blunt ends	Bsp19I	Sticky ends	BstBAI	Blunt ends
AvaII	Sticky ends	BbvCI	Sticky ends	BsaBI	Blunt ends	Bsp68I	Blunt ends	Bst4CI	Sticky ends
AviII	Blunt ends	BccI	Sticky ends	BsaHI	Sticky ends	Bsp106I	Sticky ends	BstC8I	Blunt ends
AvrII	Sticky ends	BceAI	Sticky ends	BsaJI	Sticky ends	Bsp119I	Sticky ends	BstDEI	Sticky ends
AxyI	Sticky ends	BcgI	Sticky ends	BsaMI	Sticky ends	Bsp120I	Sticky ends	BstDSI	Sticky ends
AarI	Sticky ends	BciVI	Sticky ends	BsaWI	Sticky ends	Bsp143I	Sticky ends	BstEII	Sticky ends
AasI	Sticky ends	BclI	Sticky ends	BsaXI	Sticky ends	Bsp143II	Sticky ends	BstENI	Sticky ends
AatI	Blunt ends	BcnI	Sticky ends	Bsc4I	Sticky ends	Bsp1286I	Sticky ends	BstF5I	Sticky ends
AatII	Sticky ends	BcuI	Sticky ends	Bse1I	Sticky ends	Bsp1407I	Sticky ends	BstFNI	Blunt ends
AccI	Sticky ends	BdaI	Sticky ends	Bse8I	Blunt ends	Bsp1720I	Sticky ends	BstH2I	Sticky ends

AccII	Blunt ends	BfaI	Sticky ends	Bse21I	Sticky ends	BspCI	Sticky ends	BstHHI	Sticky ends
AccIII	Sticky ends	BfiI	Sticky ends	Bse118I	Sticky ends	BspCNI	Sticky ends	BstKTI	Sticky ends
Acc16I	Blunt ends	BfmI	Sticky ends	BseAI	Sticky ends	BspDI	Sticky ends	BstMAI	Sticky ends
Acc36I	Sticky ends	BalI	Blunt ends	BseDI	Sticky ends	BspEI	Sticky ends	BstMBI	Sticky ends
BaeGI	Sticky ends	BtsCI	Sticky ends	BcoDI	Sticky ends	BspQI	Sticky ends	CviQI	Sticky ends
Acc65I	Sticky ends	BamHI	Sticky ends	Bse3DI	Sticky ends	BspHI	Sticky ends	BstMCI	Sticky ends
AccB1I	Sticky ends	BanI	Sticky ends	BseGI	Sticky ends	BspLI	Blunt ends	BstMWI	Sticky ends
BstNI	Sticky ends	DseDI	Sticky ends	FspBI	Sticky ends	MfeI	Sticky ends	PauI	Sticky ends
BstNSI	Sticky ends	EaeI	Sticky ends	Fsp4HI	Sticky ends	Mfl I	Sticky ends	PceI	Blunt ends
BstOI	Sticky ends	EagI	Sticky ends	GsuI	Sticky ends	MhlI	Sticky ends	PciI	Sticky ends
BstPI	Sticky ends	Eam1104I	Sticky ends	HaeII	Sticky ends	MlsI	Blunt ends	PctI	Sticky ends
BstPAI	Blunt ends	Eam1105I	Sticky ends	HaeIII	Blunt ends	MluI	Sticky ends	PdiI	Blunt ends
BstSCI	Sticky ends	EarI	Sticky ends	HapII	Sticky ends	MluNI	Blunt ends	PdmI	Blunt ends
BstSFI	Sticky ends	EciI	Sticky ends	HgaI	Sticky ends	MlyI	Blunt ends	PfeI	Sticky ends
BstSNI	Blunt ends	Ecl136II	Blunt ends	HhaI	Sticky ends	Mly113I	Sticky ends	Pfl23II	Sticky ends
BstUI	Blunt ends	EclHKI	Sticky ends	Hin1I	Sticky ends	MmeI	Sticky ends	Pfl FI	Sticky ends
Bst2UI	Sticky ends	EclXI	Sticky ends	Hin1II	Sticky ends	MnlI	Sticky ends	Pfl MI	Sticky ends
BstV1I	Sticky ends	Eco24I	Sticky ends	Hin4I	Sticky ends	Mph1103I	Sticky ends	PfoI	Sticky ends
BstV2I	Sticky ends	Eco31I	Sticky ends	Hin6I	Sticky ends	MroI	Sticky ends	PhoI	Blunt ends
BstXI	Sticky ends	Eco32I	Blunt ends	HinP1I	Sticky ends	MroNI	Sticky ends	PinAI	Sticky ends
BstX2I	Sticky ends	Eco47I	Sticky ends	HincII	Blunt ends	MroXI	Blunt ends	PleI	Sticky ends
BstYI	Sticky ends	Eco47III	Blunt ends	HindII	Blunt ends	MscI	Blunt ends	Ple19I	Sticky ends
BstZI	Sticky ends	Eco52I	Sticky ends	HindIII	Sticky ends	MseI	Sticky ends	PmaCI	Blunt ends

BstZ17I	Blunt ends	Eco72I	Blunt ends	HinfI	Sticky ends	MslI	Blunt ends	PmeI	Blunt ends
Bsu15I	Sticky ends	Eco81I	Sticky ends	HpaI	Blunt ends	MspI	Sticky ends	PmlI	Blunt ends
Bsu36I	Sticky ends	Eco88I	Sticky ends	HpaII	Sticky ends	Msp20I	Blunt ends	PpiI	Sticky ends
BsuRI	Blunt ends	Eco91I	Sticky ends	HphI	Sticky ends	MspA1I	Blunt ends	PpsI	Sticky ends
BtgI	Sticky ends	Eco105I	Blunt ends	Hpy8I	Blunt ends	MspR9I	Sticky ends	Ppu21I	Blunt ends
BtgZI	Sticky ends	Eco130I	Sticky ends	Hpy99I	Sticky ends	MssI	Blunt ends	PpuMI	Sticky ends
BtrI	Blunt ends	Eco147I	Blunt ends	Hpy188I	Sticky ends	MunI	Sticky ends	PscI	Sticky ends
BtsI	Sticky ends	EcoICRI	Blunt ends	Hpy188III	Sticky ends	MvaI	Sticky ends	PshAI	Blunt ends
BveI	Sticky ends	Eco57MI	Sticky ends	HpyCH4III	Sticky ends	Mva1269I	Sticky ends	PshBI	Sticky ends
Cac8I	Blunt ends	EcoNI	Sticky ends	HpyCH4IV	Sticky ends	MvnI	Blunt ends	PsiI	Blunt ends
CaiI	Sticky ends	EcoO65I	Sticky ends	HpyCH4V	Blunt ends	MwoI	Sticky ends	Psp5II	Sticky ends
CciNI	Sticky ends	EcoO109I	Sticky ends	HpyF3I	Sticky ends	NaeI	Blunt ends	Psp6I	Sticky ends
CelII	Sticky ends	EcoP15I	Sticky ends	HpyF10VI	Sticky ends	NarI	Sticky ends	Psp1406I	Sticky ends
CfoI	Sticky ends	EcoRI	Sticky ends	Hsp92I	Sticky ends	NciI	Sticky ends	PspAI	Sticky ends
CfrI	Sticky ends	EcoRII	Sticky ends	Hsp92II	Sticky ends	NcoI	Sticky ends	Psp124BI	Sticky ends
Cfr9I	Sticky ends	EcoRV	Blunt ends	HspAI	Sticky ends	NdeI	Sticky ends	PspCI	Blunt ends
Cfr10I	Sticky ends	EcoT14I	Sticky ends	ItaI	Sticky ends	NdeII	Sticky ends	PspEI	Sticky ends
Cfr13I	Sticky ends	EcoT22I	Sticky ends	KasI	Sticky ends	NgoMIV	Sticky ends	PspGI	Sticky ends
Cfr42I	Sticky ends	EcoT38I	Sticky ends	KpnI	Sticky ends	NheI	Sticky ends	PspLI	Sticky ends
ClaI	Sticky ends	EgeI	Blunt ends	Kpn2I	Sticky ends	NlaIII	Sticky ends	PspN4I	Blunt ends
CpoI	Sticky ends	EheI	Blunt ends	KspI	Sticky ends	NlaIV	Blunt ends	PspOMI	Sticky ends
CseI	Sticky ends	ErhI	Sticky ends	Ksp22I	Sticky ends	NmuCI	Sticky ends	PspPPI	Sticky ends
CspI	Sticky ends	Esp3I	Sticky ends	Ksp632I	Sticky ends	NotI	Sticky ends	PspXI	Sticky ends
Csp6I	Sticky ends	FalI	Sticky ends	KspAI	Blunt ends	NruI	Blunt ends	PsrI	Sticky ends

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CspCI	Sticky ends	FatI	Sticky ends	LguI	Sticky ends	NsiI	Sticky ends	PsuI	Sticky ends
CviAII	Sticky ends	FauI	Sticky ends	LweI	Sticky ends	NspI	Sticky ends	PsyI	Sticky ends
CviJI	Blunt ends	FauNDI	Sticky ends	MabI	Sticky ends	NspV	Sticky ends	PvuI	Sticky ends
DdeI	Sticky ends	FbaI	Sticky ends	MaeI	Sticky ends	OliI	Blunt ends	PvuII	Blunt ends
DpnI	Blunt ends	FblI	Sticky ends	MaeII	Sticky ends	PacI	Sticky ends	RcaI	Sticky ends
DpnII	Sticky ends	Fnu4HI	Sticky ends	MaeIII	Sticky ends	PaeI	Sticky ends	RgaI	Sticky ends
DraI	Sticky ends	FokI	Sticky ends	MalI	Blunt ends	PaeR7I	Sticky ends	RsaI	Blunt ends
DraII	Sticky ends	FriOI	Sticky ends	MamI	Blunt ends	PagI	Sticky ends	RsrII	Sticky ends
DraIII	Sticky ends	FseI	Sticky ends	MbiI	Blunt ends	PalI	Blunt ends	Rsr2I	Sticky ends
DrdI	Sticky ends	FspI	Blunt ends	MboI	Sticky ends	PalAI	Sticky ends	SacI	Sticky ends
DriI	Sticky ends	FspAI	Blunt ends	MboII	Sticky ends	PasI	Sticky ends	SacII	Sticky ends
SalI	Sticky ends	Sfr303I	Sticky ends	SrfI	Blunt ends	TliI	Sticky ends	XagI	Sticky ends
SanDI	Sticky ends	SalI	Sticky ends	Sse9I	Sticky ends	Tru1I	Sticky ends	XapI	Sticky ends
SapI	Sticky ends	SanDI	Sticky ends	Sse8387I	Sticky ends	Tru9I	Sticky ends	XbaI	Sticky ends
SatI	Sticky ends	SapI	Sticky ends	SsiI	Sticky ends	TscI	Sticky ends	XceI	Sticky ends
Sau96I	Sticky ends	SfuI	Sticky ends	SspI	Blunt ends	TseI	Sticky ends	XcmI	Sticky ends
Sau3AI	Sticky ends	SgfI	Sticky ends	SspBI	Sticky ends	TsoI	Sticky ends	XhoI	Sticky ends
SbfI	Sticky ends	SgrAI	Sticky ends	StuI	Blunt ends	Tsp45I	Sticky ends	XhoII	Sticky ends
ScaI	Blunt ends	SgsI	Sticky ends	StyI	Sticky ends	Tsp509I	Sticky ends	XmaI	Sticky ends
SchI	Blunt ends	SinI	Sticky ends	StyD4I	Sticky ends	TspEI	Sticky ends	XmaCI	Sticky ends
ScrFI	Sticky ends	SmaI	Blunt ends	SwaI	Blunt ends	TspGWI	Sticky ends	XmaJI	Sticky ends
SdaI	Sticky ends	SmiI	Blunt ends	TaaI	Sticky ends	TspRI	Sticky ends	XmiI	Sticky ends
SduI	Sticky ends	SmiMI	Blunt ends	TaiI	Sticky ends	TstI	Sticky ends	XmnI	Blunt ends

SexAI	Sticky ends	SmlI	Sticky ends	TaqI	Sticky ends	Tth111I	Sticky ends	XspI	Sticky ends
SfaNI	Sticky ends	SmoI	Sticky ends	TaqII	Sticky ends	Van91I	Sticky ends	ZraI	Blunt ends
SfcI	Sticky ends	SmuI	Sticky ends	TasI	Sticky ends	Vha464I	Sticky ends	ZrmI	Blunt ends
SfiI	Sticky ends	SnaBI	Blunt ends	TatI	Sticky ends	VneI	Sticky ends	Zsp2I	Sticky ends
SfoI	Blunt ends	SpeI	Sticky ends	TauI	Sticky ends	VpaK11BI	Sticky ends	HpyAV	Sticky ends
Sfr274I	Sticky ends	SphI	Sticky ends	Tfi I	Sticky ends	VspI	Sticky ends	TspMI	Sticky ends

Table 1.2: A List of restriction enzymes along with ends generated by these endonucleases, used in recombinant DNA technology. Made after consulting information provided by http://rebase.neb.com/rebase/rebase.html

Protocols in Gene Cloning

DNA molecules generated from different sources may be used in gene cloning for diverse downstream applications. This section will explain the general cloning workflow and protocols involved in gene cloning experimentation, and describe how the DNA fragment of interest (the insert) is amplified through a Polymerase Chain Reaction (PCR). Restriction sites of specific restriction enzymes are engineered at the 5' ends of specific primers to the DNA fragment of interest. PCR analysis is performed to amplify the DNA fragment of interest flanked by specific sites against particular restriction enzymes. The PCR product (amplicon) is resolved on agarose using gel electrophoresis. Agarose gel is prepared in a 0.5X TAE buffer (Appendix A). The gel is visualized and photographed using the gel documentation system (GDS). Subsequent to this procedure, the following methods are employed.

DNA Elution

For DNA elution, agarose gel containing a DNA fragment of the required size is excised with a clean scalpel. Gel extraction is performed by using available gel extraction kits (according to the manufacturer's protocol) for eluting the required fragment.

PCR purification

Instead of DNA elution, PCR purification may also be performed if the PCR product is a lone specified amplicon. For purification, PCR purification kits are available. Therefore, PCR purification is performed according to the manufacturer's protocol which gives instructions on which PCR purification kit to use.

Competent Cell Formation

Competent *Escherichia coli* (E. coli) cells are prepared so as to build up their ability to take DNA up. For cloning purposes, plasmidless strains of E. coli are used. The general protocol for the preparation of bacterial competent cells is given below.

- Take a loop of E. coli fresh culture and transfer into L.B liquid for broth cultures (Appendix B).
- Incubate this culture at 37°C @180rpm overnight and then transfer 2ml from overnight culture into 100ml of LB liquid in a flask.

- Incubate it at 37°C @ 180rpm to get a fresh culture.
- Centrifugate (for 5 minutes, at 3000-4000 rpm, 4°C) to harvest E. coli cells.
- Discard the supernatant.
- Add 0.1M $MgCl_2$ to pellet
- Resuspend it by gentle mixing and then centrifugation (5 minutes, 3000-4000 rpm, 4°C).
- Discard the supernatant.
- Add 0.1M $CaCl_2$ to pellet.
- Resuspend it by gently swirling and incubating on ice for 15-30 minutes followed by centrifugation (for 5 minutes, at 3000-4000 rpm, 4°C).
- Discard the supernatant.
- Add 0.1M $CaCl_2$ to pellet and resuspend it.
- Discard supernatant, add 2ml 0.1M of $CaCl_2$ and resuspend pellet into it.
- Prepare the aliquots by adding glycerol and keep in storage at -80°C.

Restriction digestion and Ligation reaction

The restriction digestion of an eluted DNA or PCR purified product (insert) and vector is performed with the same restriction enzymes. Subsequently the ligation reaction is performed using T4 DNA ligase.

Heat Shock Method and Blue White Selection

The delivery of a ligation product into a bacterial cell (a competent E. coli cell) is generally and commonly executed by the heat shock method. Heat shock treatment is given to the competent cell culture after the addition of a ligated product to it at 42°C, for 2 minutes. After heat shock treatment, transformed bacterial cells are incubated in a small quantity of liquid medium without antibiotics, for a short time period. Because of this incubation, the expression of the resistance gene (an enzyme) reaches the level at which this enzyme may detoxify the antibiotic in the culture regime and thereby allows the growth of the transformed bacterial cell on culture plates. The bacterial culture is spread onto an L.B. solid medium of ampicillin along with X-Gal and IPTG. This is done for blue white selection to select a recombinant clone. Cultured plates are incubated at 37°C overnight, and bacterial colonies appear on the culture plate if bacterial transformation is achieved. Keeping the culture plates for a long period of time results in the formation of satellite colonies.