Novagen®

T7Select® System Manual

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About the System

T7Select®1-1 Cloning Kit		70010-3
T7Select10-3 Cloning Kit		70550-3
T7Select415-1 Cloning Kit		70015-3
T7Select Biopanning Kit		70018-3
T7Select Packaging Kit	6 extracts	70014-3
Uncut T7Select1-1b DNA	10 µg	Discontinued
Uncut T7Select1-2a DNA	10 µg	70042-3
Uncut T7Select1-2b DNA	10 µg	70043-3
Uncut T7Select1-2c DNA	10 µg	70044-3
Uncut T7Select10-3b DNA	10 µg	70548-3
Uncut T7Select 415-1b DNA	10 µg	70040-3

Description

Novagen's T7Select® Phage Display System is a novel phage display system that takes advantage of the properties of bacteriophage T7. This system is easy to use and has the capacity to display peptides up to about 50 amino acids in size in high copy number (415 per phage), and peptides or proteins up to about 1200 amino acids in low copy number (0.1–1 per phage) or mid-copy number (5–15 per phage). Phage assembly takes place in the *E. coli* cytoplasm and mature phage are released by cell lysis. Unlike the filamentous systems, peptides or proteins displayed on the surface of T7 do not need to be capable of secretion through the cell membrane, a necessary step in filamentous phage assembly (1).

T7 has additional properties that make it an attractive display vector. It is very easy to grow and replicates more rapidly than either bacteriophage λ or filamentous phage. Plaques form within
3 h at 37°C and cultures lyse 1–2 h after infection, decreasing the time needed to perform the multiple rounds of growth usually required for selection. The T7 phage particle is extremely robust, and is stable to harsh conditions that inactivate other phage. This stability expands the variety of agents that can be used in biopanning selection procedures, which require that the phage remain infective. T7 is an excellent general cloning vector. Purified DNA is easy to obtain in large amounts, a high-efficiency *in vitro* packaging system has been demonstrated (2), and the DNA is completely sequenced (39,937 bp), so restriction or DNA sequence analysis of clones is quite straightforward.

The T7Select Phage Display System uses the T7 capsid protein to display peptides or proteins on the surface of the phage. The capsid protein is normally made in two forms, 10A (344 aa) and 10B (397 aa). 10B is produced by a translational frameshift at amino acid 341 of 10A, and normally makes up about 10% of the capsid protein (3). However, functional capsids can be composed entirely of either 10A or 10B, or of various ratios of the proteins (4). This finding provided the initial suggestion that the T7 capsid shell could accommodate variation, and that the region of the capsid protein unique to 10B might be on the surface of the phage and could be used for phage display.

There are 3 basic types of T7Select phage display vectors: the T7Select415 vector for high-copy number display of peptides, the T7Select10 vector for mid-copy number display of peptides or larger proteins, and the T7Select1 vectors for low-copy number display of peptides or larger proteins (see Table 1). In all of the vectors, coding sequences for the peptides or proteins to be displayed are cloned within a series of multiple cloning sites following aa 348 of the 10B protein (see *Vector Cloning Regions* on p 4). The natural translational frameshift site within the capsid gene has been removed, so only a single form of capsid protein is made from these vectors. Additional information about this system can be found in *inNovations* No. 6 (5).

Table 1. Phage display vector features

Vector [a]	Use	Copy [b] Number	Vector Size (bp)	Size Limit	Host [c]
T7Select®415-1b	peptides	415	37,314	50 aa	BL21
T7Select10-3b	peptides or proteins	5–15	36,249	1200 aa	BLT5403, BLT5615, BLT5615*rna,* Orgami B 5615, Rosetta 5615, Rosetta-gami 5615
T7Select1-1b	peptides or proteins	0.1–1	36,219	1200 aa	
T7Select1-2a	peptides or proteins	0.1–1	37,245	900 aa	
T7Select1-2b	peptides or proteins	0.1–1	37,244	900 aa	
T7Select1-2c	peptides or proteins	0.1–1	37,243	900 aa	

Notes:

1. The T7Select415-1 capsid gene has wild-type control signals: ϕ10 promoter, Tϕ terminator, and s10 translation initiation signals. The s10 translation initiation signal (ribosome binding site) was modified to produce the mid-copy vector T7Select10-3. ϕ10 and s10 were removed fromT7Select1-1 and 1-2 to further reduce capsid gene expression.
2. The average number of capsid fusion proteins displayed per phage. The T7 capsid shell contains 415 copies of the capsid protein, thus the copy number is 415 for proteins expressed in T7Select 415-1. The copy number for T7Select10-3, T7Select1-1 and T7Select1-2 are calculated values based on measurements from four different protein display trials.
3. Any of the BLT5403 and 5615 strains can be used with T7Select10-3b, 1-1b, 102a, 1-2b, or 1-2c.

Vector Cloning Regions

T7Select415-1b, T7Select1-1b

```
        aa348                                              aa363
...MetLeuGlyAspProAsnSerSerSerValAspLysLeuAlaAlaAlaLeuGlu
...ATGCTCGGGGATCCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA
           BamH I   EcoR I  Sac I    Sal I  Hind III   Not I    Xho I
```

T7Select10-3b

```
        aa348                                                                aa369
...MetLeuGlyAspProAsnSerProAlaGlyIleSerArgGluLeuValAspLysLeuAlaAlaAlaLeuGlu
...ATGCTCGGGGATCCGAATTCTCCTGCAGGGATATCCCGGGAGCTCGTCGACAAGCTTGCGGCCGCACTCGAGTAA
           BamH I   EcoR I  Sse8387 I EcoR V Sma I Sac I  Sal I  Hind III   Not I    Xho I
```

T7Select1-2a

```
        aa348                                                             aa368
...MetLeuGlyGlySerAspIleGluPheGluLeuArgArgGlnAlaCysGlyArgThrArgValThrSer
...ATGCTCGGTGGATCCGATATCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA
             BamH I  EcoR V EcoR I  Sac I    Sal I  Hind III   Not I    Xho I
```

T7Select1-2b

```
        aa348                                                     aa365
...MetLeuGlyAspProIleSerAsnSerSerSerValAspLysLeuAlaAlaAlaLeuGlu
...ATGCTCGGGGATCCGATATCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA
            BamH I EcoR V EcoR I  Sac I    Sal I  Hind III   Not I    Xho I
```

T7Select1-2c

```
        aa348                                                        aa366
...MetLeuGlyIleArgTyrArgIleArgAlaProSerThrSerLeuArgProHisSerSerAsn
...ATGCTCGGGATCCGATATCGAATTCGAGCTCCGTCGACAAGCTTGCGGCCGCACTCGAGTAACTAGTTAA
           BamH I EcoR V EcoR I  Sac I    Sal I  Hind III   Not I    Xho  I
```

Components

T7Select® Cloning Kit

Cloning kits include the following components for construction of up to five libraries:

- 5 µg T7Select415-1, T7Select10-3, or T7Select1-1 *Eco*R I/*Hin*d III Vector Arms
- 0.2 pmol T7Select Control Insert
- 6 T7 Packaging Extracts
- 1 µg T7Select Packaging Control DNA
- 0.2 ml BL21 or BLT5403 and BLT5615 Glycerol Stocks
- 500 pmol T7SelectUP Primer
- 500 pmol T7SelectDOWN primer

T7Select Biopanning Kit

The Biopanning Kit includes the following components for biopanning with target ligands using 96-well microplates or other similar supports:

- 25 g Blocking Reagent
- 2 × 50 ml 10X TBST Wash Buffer
- 10 ml T7 Elution Buffer
- 0.2 ml BL21 Glycerol Stock
- 0.2 ml BLT5403 Glycerol Stock
- 0.2 ml BLT5615 Glycerol Stock
- 10^{10} pfu T7Select Positive Control Lysate
- 10^{10} pfu T7Select Negative Control Lysate
- 50 µl S-protein HRP Conjugate
- 2 × 25 ml SuperSignal® Substrate
- 2 ml T7Select S-protein
- 25 gLOCATOR™ Luminescent Labels
- 25 Development Folders

Storage

*T7Select® Cloning Kits and Storage Conditions		
Catalog Number	**Description**	**Storage Conditions**
70010-3	T7Select 1-1 Cloning Kit	Multi-storage Kit- See table below for T7Select Individual Components and Storage Conditions
70550-3	T7Select 10-3 Cloning Kit	
70015-3	T7Select 415-1 Cloning Kit	

*Avoid repeated freeze-thaw cycles.

T7Select® Individual Components and Storage Conditions		
	Vector Arms	Store at 2-8°C
70002-5UG	T7Select1-1 *EcoR* I/*Hind* III	
70549-5UG	T7Select10-3 *EcoR* I/*Hind* III	
70017-5UG	T7Select415-1 *EcoR* I/*Hind* III	
70038-0.2PM	T7Select Control Insert	*Store at -80°C
69139-25UL	T7 Packaging Extracts	*Store at -80°C
69679-1UG	T7Select Packaging Control DNA	Store at 2-8°C
	Glycerol Stocks	*Store at -80°C
69142-0.2ML	BLT5403	
69905-0.2ML	BLT5615	
69386-0.2ML	BL21	
70005-500PM	T7SelectUP Primer	*Store at -20°C
70006-500PM	T7SelectDOWN Primer	*Store at -20°C

*Avoid repeated freeze-thaw cycles.

About the Protocol

Use of the T7Select® system is divided into three parts, as follows:

1. *Cloning in T7 vectors/library preparation*—A population of DNA fragments encoding either peptide or protein sequences is cloned into the T7Select vector, packaged into phage, and amplified to prepare the library for biopanning.
2. *Selecting and amplifying phage*—Several rounds of biopanning are performed to select and amplify phage that bind to a target molecule.
3. *Characterization*—Phage that bind are analyzed by sequencing across the cloning region of the phage DNA. The DNA can be provided by PCR amplification of individual plaques.

For general procedures for culture and handling of T7 phage, refer to the following section, *Growth and Storage of Bacteriophage T7*.

Growth and Storage of Bacteriophage T7

Growth Media

T7 phage can be grown using common bacteriological media. Recipes for TB and LB media and their derivatives are given below. A less expensive equivalent to TB is ZB, in which N-Z-amine A (Quest International) is substituted for Bacto® Tryptone (DIFCO Laboratories). LB and TB are used interchangeably in these protocols.

LB	TB	Top agarose
Per liter: 10 g Bacto tryptone 5 g Yeast extract 10 g NaCl	Per liter: 900 ml deionized water 12 g Bacto tryptone 24 g Yeast extract 4 ml glycerol	Per 100 ml: 1 g Bacto tryptone 0.5 g Yeast extract 0.5 g NaCl 0.6 g agarose
• Adjust pH to 7.5 with 1N NaOH • Autoclave 20 min For plates, add 15 g/l agar	• Autoclave 20 min, cool to 60°C • Add 100 ml sterile K phosphate	• Add 100 ml deionized water • Autoclave 20 min

M9TB (M9LB)	20X M9 salts	K phosphate
5 ml 20X M9 salts 2 ml 20% glucose 0.1 ml 1 M $MgSO_4$ 100 ml TB (or LB)	Per liter: 20 g NH_4Cl 60 g KH_2PO_4 120 g $Na_2HPO_4 \bullet 7H_2O$ • Autoclave 20 min	Per liter: 23.1 g KH_2PO_4 125.4 g K_2HPO_4 • Autoclave 20 min

Table 2. Media and host specifications for the T7Select® vectors

Vector	Host Strain	Liquid Media[2]	Solid Media[2]
T7Select415-1b	BL21[1]	M9TB	LB agar
T7Select10-3b	BLT5403	M9TB, carb	LB agar, carb
T7Select1-1b	BLT5615	M9TB, carb	LB agar, carb
T7Select1-2a, 2b, 2c	BLT5615*rna*	M9TB, carb, kan	LB agar, carb, kan
	Origami™ B 5615	M9TB, carb, kan, tet	LB agar, carb, kan, tet
	Rosetta™ 5615	M9TB, carb, cam	LB agar, carb, cam
	Rosetta-gami™ B 5615	M9TB, carb, kan, tet, cam	LB agar, carb, kan, tet, cam

1. It is possible to use other hosts such as Rosetta, Rosetta-gami B, and Origami B for T7Select415-1 instead of BL21. The Origami B and Rosetta-gami B may provide benefits if expressing constrained (S-S) libraries.
2. After autoclaving and cooling to < 55°C, add the appropriate antibiotics. Add sterile filtered carbenicillin (carb) to 50 µg/ml from a 50 mg/ml stock solution or kanamycin (kan) to 15 µg/ml from a 30 mg/ml stock solution, or tetracycline (tet) to 12.5 µg/ml from a 5 mg/ml stock solution, or chloramphenicol (cam) to 34 µg/ml from a 34 mg/ml stock solution. Amplicillin may be substituted for carbenicillin.

Bacterial Strains

Keep stocks of bacterial strains at –70°C to –80°C. An initial culture is prepared by streaking the bacteria for single colonies on a LB agar plate supplemented with antibiotics (see above table) and incubating at 37°C overnight. Inoculate 50 ml of TB or LB media (with antibiotic if appropriate) in a 250 ml Erlenmeyer flask with a single colony, and incubate at 37°C with shaking until the OD_{600} reaches 0.6–1. Combine a 1.5 ml sample of the fresh log phase culture with 0.15 ml of sterile (autoclaved) 80% glycerol and place directly in the freezer. Cultures are grown from the frozen stocks by inoculating with a few microliters scraped or melted from the frozen surface, without thawing the rest of the sample. Cultures that have been grown from the freezer stock are stored in the refrigerator and used to inoculate further cultures for as long as a month.

Growth of T7 Lysates

T7 grows rapidly on logarithmically growing cells or on cells freshly diluted from stationary phase cultures (but not on cells approaching stationary phase). Lysates grown in shaking flasks typically yield 10^{10}–10^{11} phage/ml. Phage titer is proportionate to aeration of the culture, and the best titers are achieved when the culture volume is 20% or less of the flask volume.

Strains with the 5403 or 5615 designation carry an ampicillin-resistant plasmid that supplies extra 10A capsid protein. In the 5403 plasmid, 10A expression is driven by a T7 promoter, whereas in 5615 it is driven by the lacUV5 promoter. Therefore, in the BLT5403 plasmid, large amounts of capsid protein are produced in the course of T7 infection. In contrast, 5615 hosts require the addition of IPTG to induce capsid protein production prior to infection. Plaques are smaller on BLT5403, but lysate titers are similar to those obtained using 5615 hosts. Phage displaying larger proteins (>600 amino acids) that gave tiny plaques and accumulated deletions in the capsid fusion gene on BLT5403, formed larger plaques and produced lysates without detectable deletion phage on BLT5615 (unpublished observation). To prepare 5615 hosts for phage production, add IPTG to 1 mM about 30 min before infection. For plating add 100 µl, 100mM IPTG to 2.5ml top agar (25 ml agar plates).

The BLT5615*rna* strain is deficient in the RNase I gene, a nonessential ribonuclease that is the primary source of non-specific RNA-degrading activity in *E. coli* extracts. The RNase I gene (*rna*) of BLT5615 was disrupted by insertion of a kanamycin resistant cassette to generate BLT5615*rna*. Phage lysates from BLT5615*rna* do not cause extensive RNA degradation which allows RNA to be used as the “bait” for biopanning experiments. Several RNA binding proteins have been successfully isolated from T7Select cDNA libraries propagated in BLT5615*rna* (6-7). Since BLT5615*rna* is identical to BLT5615 with the exception of the RNase I deficiency, it should be a suitable host for all other applications for which BLT5615 has been used successfully.

Origami™ B 5615 greatly facilitates the expression of active, soluble proteins in E. coli. Mutations in the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes of this host strain creates a cytoplasmic environment compatible with the formation of disulfide bonds (8).

Rosetta™ 5615 is designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. This strain supplies tRNAs under the control of their native promoters for the codons AUA, AGG, AGA, CGG, CUA, CCC and GGA on a compatible chloramphenicol-resistant plasmid.

Rosetta-gami™ B 5615 combines the *trxB/gor* mutations of Origami for disulfide bond formation with the supply of rare tRNAs of Rosetta.

A convenient way to grow lysates on logarithmically growing cells is to dilute a fresh overnight culture in 200 volumes of M9TB, incubate with shaking for 3.5–4 h at 37°C (usually to an OD_{600} of 0.6–0.8), and then infect with a phage stock or plaque. Single plaques will develop on plates at 37°C within the same interval as the cells are growing and can be inoculated into the culture by transferring an agar plug containing the plaque (or the center of the plaque) with the tip of a sterile Pasteur pipet. Good lysates are also obtained by diluting an overnight culture of cells into three volumes of M9TB, adding phage immediately, and shaking until lysis.

The initial phage concentration is not too critical because T7 grows so rapidly. Adding a single plaque or 0.001 to 0.0001 volume of lysate usually works well. For some slower growing recombinants, phage should be added to a lower density of growing cells (or to a higher dilution of overnight culture) so that the culture does not overgrow the phage. If a culture has not lyses after a few hours, dilution with fresh medium and further shaking will usually produce a lysate.

Refer to *Appendix A* for details regarding the purification of T7 phage and extraction of T7 DNA.

Storage of T7 Lysates

As soon as practical after lysis, the lysate is made 0.5 M in NaCl, centrifuged 10 min at 10,000 rpm, and the supernatant removed from the pelleted debris into a clean tube. Continued shaking after lysis can lead to a decrease in adsorption efficiency, and ultimately to loss of titer. Clarified lysates are usually stable for periods of months to years in the refrigerator. However, different lysates lose titer at greatly different rates, for unknown reasons, and some are totally inactive within a few years.

For long term storage or as a backup, samples of the clarified lysate can be combined with 0.1 volume of sterile 80% glycerol and then frozen at –70°C to –80 °C. These stocks can be frozen and thawed with little loss of titer. However, a few microliters can be scraped from the surface of the frozen stock for inoculation purposes and the remainder placed back into the freezer without the need for thawing the rest of the sample.

Titering T7

A plating assay is used to determine the number of phage present in samples at various points in the T7Select® System protocol. The technique involves combining diluted samples of phage with host cells and molten agarose and plating on TB or LB agar plates. The cells will grow to form a lawn of bacteria. When phage are present, clear areas (plaques) will be observed in the lawn of bacteria which correspond to individual phage infection events produced by single phage in the test sample. When using 100 mm Petri plates, it is convenient to plate dilutions of sample such that 50 to several hundred plaques are produced. The plating assay is used in the T7Select System to determine the efficiency of packaging reactions, to determine the number of primary recombinants in a newly constructed library, to determine the titer of an amplified library and to monitor the enrichment of phage during biopanning. The level of dilution necessary for plating will depend on the source of the sample. The dilution ranges for specific samples are listed in the table below.

Note: When plating a large number of samples, be aware that phage will continue to replicate when combined with host cells. Samples should be plated shortly after phage and host cells have been combined.

Sample Source	Estimated Titer (pfu/ml)	Recommended Dilution Range
Biopanning Eluate	10^3 to 10^6	$1/10^2$ to $1/10^5$
Packaging Reaction	10^7 to 10^9	$1/10^4$ to $1/10^7$
Amplified Lysate	10^{10} to 10^{11}	$1/10^7$ to $1/10^{10}$

TB or LB can be used for all dilutions and in plates for titering T7. Phage remain infective at high dilution in TB or LB but inactivate fairly rapidly in defined media or buffers without protectants such as gelatin.

To count the phage titer, 0.25 ml aliquots of a fresh overnight culture of bacteria are added to a series of 13 × 100 mm tubes at room temperature. Appropriate dilutions of phage in 100 µl buffer are added to the cells, followed by the addition of 2.5–3 ml of melted top agarose at 45–50°C. The contents of the tube are immediately mixed and then poured in a standard 100 × 15 mm Petri dish containing 20 ml of hardened TB or LB agar. Wild-type plaques will appear within 2–3 h at 37°C, as soon as the lawn becomes turbid enough to see them; some recombinants may take longer to develop. In most cases, plates should not be incubated overnight at 37°C because the plaques become too large; however, plaques will usually remain a manageable size if plates are left overnight at room temperature. For additional details regarding phage titering by plaque assay, refer to *Cloning in T7Select Vectors* below.

Cloning in T7Select® Vectors

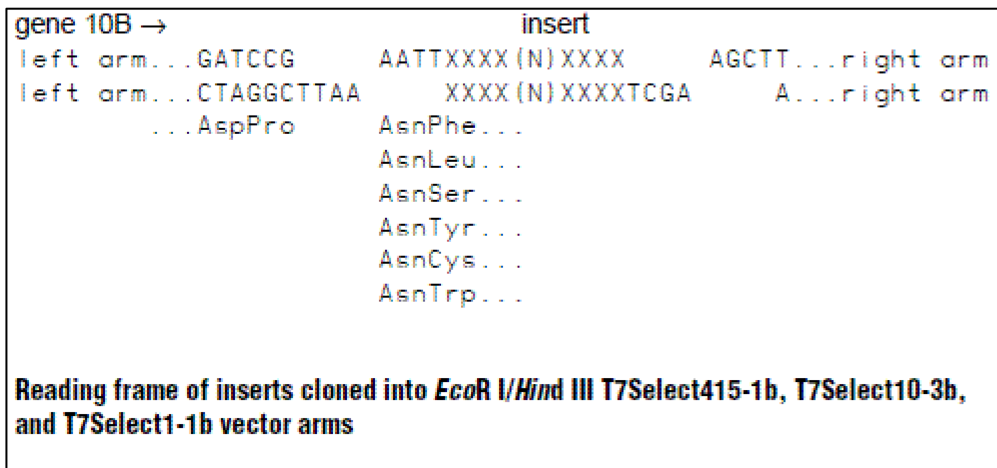
Procedures for cloning in T7Select vectors are similar to those for cloning in bacteriophage λ vectors. Vector arms are prepared and ligated with target inserts, incubated with an *in vitro* packaging extract, and the phage products used for infection of a suitable host. The multiple cloning sites (MCS) in the T7Select vectors are compatible with many existing vectors, including the pET vectors.

Cloning DNA fragments into the MCS of the T7Select vectors requires restriction digestion of the vector DNA, and dephosphorylation of the ends with alkaline phosphatase using standard protocols (9). Confirm restriction digestion by running 100 ng of the digest on a 0.4–0.6% agarose gel at low voltage for several hours alongside uncut vector. The left and right vector arms can be separated with extended electrophoresis at low voltage (0.75 volt/cm, overnight). These steps are not necessary when using prepared T7Select *Eco*R I/*Hin*d III arms.

T7Select *Eco*R I/*Hin*d III Vector Arms

The T7Select Cloning Kits include T7Select415-1b, T7Select10-3b, or T7Select1-1b vectors as prepared *Eco*R I/*Hin*d III arms, ready for directional cloning of appropriately prepared inserts. To provide compatibility with the vector arms and obtain expression in-frame with the 10B protein such that recombinant fusion proteins are displayed, inserts must contain correctly designed termini.

As shown in the diagram below, the inserts require a 5'-AATT "sticky end" on the top strand (amino-terminal side) and a 5'-AGCT "sticky end" on the bottom strand, either created with oligonucleotides or by *Eco*R I/*Hin*d III digestion. The reading frame requires the AAT (Asn) initial codon, followed by a TXX codon (possible second amino acids are shown). Use of *Eco*R I cleavage products places C in the second position, resulting in a TCX codon (Ser). Because the arms are dephosphorylated, inserts should be phosphorylated prior to ligation.



Reading frame of inserts cloned into *Eco*R I/*Hin*d III T7Select415-1b, T7Select10-3b, and T7Select1-1b vector arms

cDNA libraries are easily constructed in T7Select10-3b and T7Select1-1b vector *Eco*R I/*Hin*d III arms using cDNA made with Novagen's OrientExpress™ cDNA Synthesis Kits. Directional random primed or oligo(dT) primed cDNA is prepared from mRNA (supplied by the user) with either the OrientExpress Random Primer or Oligo(dT) Primer cDNA Synthesis Kit. The cDNA is then prepared for cloning using reagents supplied in the *Eco*R I/*Hin*d III End Modification Kit, DNA Ligation Kit, and Mini Column Fractionation Kit. The use of a random primer strategy allows control over cDNA size ranges, thus providing the option to analyze display of various protein domains with the T7Select System. Please refer to the OrientExpress cDNA Manual, TB247, for additional information.

Ligation of Inserts and Vector Arms

For maximal cloning efficiency, it is useful to optimize insert:vector ratios for each insert preparation. The optimal ratio will vary depending on the nature and quality of the insert, but the highest cloning efficiencies are typically obtained with molar ratios between 1:1 and 3:1 (insert:vector).

Insert preparations should be free of interfering substances (salts, primers, nucleotides, etc.). Inserts prepared by annealing purified oligonucleotides are suitable for use without further treatment. Size-fractionated cDNA inserts can be prepared by common techniques, such as gel filtration on a mini column of Sepharose® 4B (supplied in the Mini Column Fractionation Kit). The insert preparation should be at a concentration of about 0.02–0.06 pmol/µl. If necessary, the insert can be concentrated by precipitation with 2 volumes of ethanol after addition of 0.1 volume of 3 M sodium acetate, using 10 µg/ml glycogen as a carrier. After centrifugation, the pellet should be rinsed with 70% ethanol, dried, and resuspended in an appropriate volume of TE buffer or water. Note that Novagen's Pellet Paint® Co-Precipitant is *not* recommended for this application because it is incompatible with the T7 packaging reaction.

The following estimates can be applied for cloning cDNA inserts. A yield of 400 ng of vector-ready cDNA (20 ng/µl in 20 µl) with an average size of 1.5 kbp corresponds to about 0.02 pmol/µl. The 37 kbp vectors correspond to approximately 0.04 pmol/µg. Therefore, ligation of 3 µl cDNA with 0.5 µg vector corresponds to an insert:vector ratio of 3:1.

A positive control insert is provided with the arms kits (see p 14 for details). We recommend setting up a positive control reaction in parallel with sample insert to test the efficiency of ligation, packaging and plating. To determine the background of non-recombinant phage, another reaction can be set up in which the insert is omitted.

1. Set up ligation reactions by assembling the following components in a sterile 0.5 ml or 1.5 ml tube (add ligase last):

Volume	Component
0–1.5 µl	insert (0.02–0.06 pmol), 1 µl Positive Control Insert, or no insert negative control)
1 µl	T7Select® Vector Arms (0.5 µg; 0.02 pmol)
0.5 µl	10X Ligase Buffer
0.5 µl	10 mM ATP (note final concentration is 1 mM, which is recommended for sticky end ligation)
0.5 µl	100 mM DTT
0 - 1.5 µl	Sterile water
1 µl	(0.4–0.6 Weiss units) T4 DNA Ligase (if necessary dilute enzyme 1:10 in Ligase Dilution Buffer provided with DNA Ligation Kit)
5 µl	Total volume

2. Gently pipet up and down and then incubate 3–16 h at 16°C. Store at 4°C until use.

Note: Ligation components are also available in the DNA Ligation Kit.

In Vitro Packaging

Ligation reactions can be added directly to T7 Packaging Extracts for *in vitro* packaging. The best T7 packaging results are obtained when the extent of dilution of the extract is minimized. The extracts are supplied in 25 µl single reaction volumes, and the volume of the ligation reaction added to the packaging extract should not exceed 5 µl. Large libraries will require scaling up.

1. Allow the T7Select® Packaging Extract to thaw on ice. The volume of the extract is 25 µl and will package up to 1 µg of vector DNA without a loss in efficiency. The extract can be subdivided into several prechilled tubes for testing several DNA samples at once. If performing smaller scale packaging tests, the amount of ligation reaction added must be reduced proportionately.
2. Add 5 µl ligation reaction per 25 µl extract. Mix gently by stirring with a pipet tip; do not vortex. A vial of T7Select Packaging Control DNA is provided with the system. To test the packaging efficiency independently, add 0.5 µg of the control DNA to 25 µl extract.
3. Incubate the reaction at room temperature (22°C) for 2 h.
4. Stop the reaction by adding 270 µl sterile LB or TB medium. If the packaging reaction will be stored for more than 24 h prior to amplification, add 20 µl chloroform and mix gently by inversion. The packaging reaction can be stored for up to one week at 4°C without significant losses in titer. For longer term storage, the packaged phage must be amplified by plate or liquid culture methods.
5. Perform a plaque assay as described below to determine the number of recombinants generated.

When using the T7Select System, *packaged libraries must be amplified by either the plate or liquid culture methods prior to biopanning*. Amplification is necessary for the expression of cloned sequences and their display on the surface of phage particles. Methods for the amplification of libraries are described on p 13. This also applies when using the Positive Control Insert to analyze the display of S•Tag™ peptide on the phage surface.

Plaque Assay

1. Inoculate the appropriate host strain in M9TB medium (see table 2 on p 5) and incubate with shaking at 37°C to an OD_{600} = 1.0.
2. Store the host cells at 4°C until needed. Do not use host cells that have been stored for longer than 48 h.
3. Melt a sufficient volume of top agarose to provide 5 ml for each dilution being plated. Transfer the molten agarose to a 45–50°C water bath.
4. Prepare a series of dilutions of the sample using sterile LB or TB medium as the diluent. Generally, the appropriate dilution for recombinant phage is 10^3–10^6 . When the T7Select® Packaging Control DNA is used, dilutions should be made to 1:10^7. The initial 1:100 dilution can be prepared by adding 10 µl of sample to 990 µl of medium. Serial dilutions can be made by adding 100 µl of the 1:100 dilution to 900 µl medium (10^3 dilution), 100 µl of the 10^3 dilution to 900 µl medium (10^4 dilution), and so on.
5. Prepare a series of 4 ml sterile tubes by pipetting 250 µl of host cells into each tube. Starting with the highest dilution, add 100 µl of the phage dilution to each tube. Be sure to replace the pipet tip between samples to avoid cross contamination.
6. Add 3 ml top agarose to the tube and pour the contents onto a *prewarmed* (37°C) LB or LB/carbenicillin or LB/carbenicillin/kanamycin agar plate. Immediately swirl the plate (gently) to spread the agarose evenly.
7. Allow the plate to sit undisturbed for several min until the top agarose hardens, then invert and incubate for 3–4 h at 37°C or overnight at room temperature.
8. Count the plaques and calculate the phage titer. The phage titer, described in plaque forming units (pfu) per unit volume is the number of plaques on the plate times the dilution times 10 (to account for the 0.1 ml of dilution plated). For example, if there are 200 plaques on a plate from a 1/10^6 dilution, then the titer of the sample is $200 \times 10^6 \times 10 = 2 \times 10^9$ pfu/ml. The total number of phage in a sample is determined by multiplying the titer by the total sample volume. For example, if the sample were a packaging reaction where 1 µg of vector DNA was used, the final volume of the packaging reaction is 0.3 ml (25 µl packaging extract + 5 µl ligation + 270 µl sterile media). The total number of pfu in the packaging reaction is 2×10^9 pfu/ml × 0.3 ml = 6×10^8 pfu. In addition, since 1 µg of vector was used the efficiency is 6×10^8 pfu/µg.

Amplifying Libraries

A single round of amplification is necessary prior to biopanning. There are two basic methods for library amplification depending on the size and complexity of the library. For a cDNA or other library where the total number of primary recombinants is less than 5×10^6, the plate lysate is the preferred method. When amplifying larger libraries the liquid lysate method is required for logistical reasons.

Plate Lysate Amplification

1. Inoculate 50 ml TB with 1 ml overnight culture of the appropriate host (see second table on p 6). Shake at 37°C until the OD_{600} reaches 0.6–1.0. If using a 5615 host strain, add IPTG to 1 mM when the OD_{600} reaches 0.5 and continue shaking for 30 min.
2. If the packaging reaction was stored with chloroform, spin the tube briefly in a microcentrifuge to separate out the chloroform. Leave the chloroform behind when pipetting the phage.
3. Calculate the amount of cells and phage needed. A ratio of 1×10^6 phage per 10 ml cells should be used. Mix the phage (packaging reaction) with the cells in a sterile 50 ml tube. This mixture must be plated within 20 min.
4. Transfer 1 ml aliquots of the phage/host mixture into sterile 15 ml tubes.
5. Add 10 ml molten top agarose at 45–50°C to each tube. Immediately pour the contents of the tube onto a 150 mm LB or LB/carbenicillin or LB/carbenicllin/kanamycin plate. Spread the top agarose evenly by gently swirling the plate.
6. Allow the plates to sit undisturbed on a level surface so the top agarose can solidify. Invert the plates and incubate until the plaques are nearly confluent (3–4 h at 37°C or overnight at room temperature).
7. To elute the phage, cover each plate with 10 ml of Phage Extraction Buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 6 mM $MgSO_4$) and place on a level surface at 4°C from 2 h to overnight.
8. Harvest the phage by tipping the plate slightly and pipetting the liquid into a sterile container. Combine the extract buffer from all the plates in a single tube or bottle. Add 0.5 ml chloroform and gently mix. Centrifuge at 3,000 × g for 5 min to clarify the lysate and transfer the supernatant to a sterile tube or bottle. Determine the titer of the amplified library by plaque assay. Amplified library lysates can be stored at 4°C for several months without a loss of titer. For longer term storage, add 0.1 volume sterile 80% glycerol and store at –70°C.

Liquid Lysate Amplification (500 ml scale)

1. Inoculate 50 ml LB with appropriate antibiotics with a single colony from a freshly streaked plate. Shake at 37°C overnight. Host cells can be stored at 4°C for up to two days prior to use.
2. Add 5 ml of the overnight culture to 500 ml LB or LB/carbenicillin or LB/carbenicillin/kanamycin medium and grow to a OD_{600} of 0.5–1.0. If using the 5615 strains, add IPTG to 1 mM when the OD_{600} reaches 0.5 and continue shaking for 30 min. Calculate the number of cells in the culture using the following formula:

   $$OD_{600} \times \frac{(2 \times 10^{8} \text{ cells/ml})}{0.5\ OD_{600}} \times \text{culture volume} = \text{total cells}$$

3. Infect culture with phage library at an MOI of 0.001–0.01 (i.e., 100–1000 cells for each pfu). It is important to infect the culture with cells in substantial excess to prevent rapid lysis of all available host cells. For example, if a 500 ml host culture has an $OD_{600} = 0.75$, the total number of cells is:

   $$OD_{600} \times \frac{(2 \times 10^{8} \text{ cells/ml})}{0.5\ OD_{600}} \times 500 \text{ ml} = 1.5 \times 10^{11} \text{ cells}$$

   This culture can be infected with 1.5×10^{8} to 1.5×10^{9} pfu.
4. Incubate with shaking at 37°C for 1–3 h until lysis is observed. Cell lysis will result in a visible reduction in the OD of the culture and the accumulation of long thin strands of debris in the medium. Stop incubation once lysis is observed. Prolonged incubation will result in a loss of lysate titer.
5. Clarify the lysate by spinning at 8,000 × g for 10 min. Decant the supernatant into a sterile bottle. Titer the lysate by plaque assay on p 12.
6. Amplified library lysates can be stored at 4°C for several months without a loss of titer. For longer term storage, add 0.1 volume sterile 80% glycerol and store at –70°C.

Use of T7Select® Positive Control Insert/Plaque Lift Analysis

The T7Select Control Insert in the kit encodes the 15 aa S•Tag™ sequence that binds with high affinity to S-protein. When cloned into *Eco*R I/*Hin*d III arms of T7Select415-1, T7Select-10-3 or T7Select1-1, an in-frame fusion protein is produced resulting in the display of the S•Tag peptide on the surface of the phage.

When using T7Select415-1 or T7Select10-3, recombinant plaques are easily detected using S-protein HRP conjugate with chemiluminescent substrate. T7Select10-3 plaques expressing the S•Tag peptide require longer exposure times for detection. The assay cannot be used for the detection of insert in T7Select1-1 due to the low copy number displayed. A PCR assay can be used to distinguish recombinant and non-recombinant plaques when using the T7Select Control Insert with the T7Select1-1 vector. The protocol for the detection of T7Select415-1 S•Tag recombinants by plaque lift is described next. The plaque lift protocol can also be modified to detect other ligands expressed in T7Select415-1 or T7Select10-3 using appropriate ligand-specific reagents.

Using this protocol, T7Select415-1 recombinant plaques expressing the S•Tag peptide produce extremely strong signals in a 10-minute exposure, whereas T7Select10-3 recombinants produce a weaker signal corresponding to the lower copy number (average of 10/phage vs. 415/phage). With both vectors, recombinants should be easily distinguished from non-recombinant plaques lacking inserts. By comparing the number of positive plaques on the plaque lift with the total number of plaques on the plate, the background levels of non-recombinant phage can be determined. The non-recombinant background for control insert ligations in T7Select *Eco*R I/*Hin*d III arms is typically 0.15–1.5%.

Plaque lift protocol

1. Chill plates for 1 h at 4°C prior to making plaque lifts to minimize the tendency of the top agarose to stick to the membrane. Using plates containing 500–1500 plaques, perform plaque lifts using nitrocellulose membranes (e.g., Whatman Corporation Protran™, No. 10402579). Plaques are ready for screening even when small (0.5–1 mm in diameter).

   Carefully overlay the plates with the membranes (wear gloves and handle the filters by the edges). Bend the membrane slightly and allow the center to touch the plate first. Number both the filter and the plate. Following placement of the membranes, use pin holes or ink to align the membrane with the plate. After one minute of contact, carefully peel the membrane off the plate. (Store plates at 4°C. Plates may be used for two rounds of duplicate lifts without significant loss of signal.)
2. Invert the membranes on plastic wrap and allow to air dry for 10–20 min.
3. Prepare 200 ml of 1X TBST by combining 20 ml of 10X TBST with 180 ml deionized water.
4. Prepare a solution of 5% Blocking Reagent in 1X TBST (e.g., 5 g/100 ml). Typically 25–50 ml blocking solution is sufficient for processing up to ten 82 mm membranes.
5. Soak the membranes in blocking solution for 30 min with gentle rocking.
6. Dilute S-protein HRP Conjugate 1:5000 in 1X TBST and apply enough solution to cover the membranes. Incubate for 30 min at room temperature.
7. Wash the membranes 3 times for 5 min with 25 ml 1X TBST.
8. Prepare the SuperSignal substrate working solution by combining equal parts 2X Luminol/Enhancer and 2X Stable Peroxide Solution and mixing briefly. Prepare sufficient substrate to apply 0.5 ml per membrane.
9. Remove the membranes from the final TBST wash and transfer to a clean container. Apply the SuperSignal substrate working solution and be sure that the entire membrane has become wet. Incubate at room temperature for 1–2 min.
10. Remove the membrane from the working solution and place in a Development Folder (make sure the membrane is wet, but not dripping). Remove any bubbles between the plastic and the membrane. Remove any excess liquid on the exterior of the envelope.
11. Expose a gLOCATOR™ label to light for 10–20 sec. Place on the exterior of the development folder and record experimental details on the label.
12. Place in a film cassette with autoradiographic film (e.g., Kodak OMAT AR) and expose for
    1–10 min for T7Select415-1 recombinants, or 10– 30 min for T7Select10-3 recombinants. Be careful not to move the film after initial placement or multiple images may result. Light output continues over several hours.

Screening the Library by Biopanning

Procedures for selecting phage from a T7 phage display library are very similar to those used in filamentous phage display. Usually, a target ligand is immobilized on a solid phase and a lysate containing the amplified phage library is allowed to bind to the target. After several washes to remove non-specifically bound phage, the remaining phage are eluted and amplified for the next round of selection. There are some important parameters to consider when designing a biopanning experiment, which are described next.

Choice of Solid Phase and Ligand Immobilization

A variety of solid phases have been used successfully for biopanning with phage display libraries, including plastic ELISA plates or uncoated cell culture dishes, magnetic particles, glass beads, and beaded agarose. The most convenient and commonly used solid phase is plastic and the most commonly used method for coating is non-covalent adsorption. However, because the adsorption of proteins onto plastic surfaces is thought to be a hydrophobic interaction, some ligands, particularly highly hydrophilic proteins or low molecular weight compounds, may bind inefficiently to plastic unless a covalent attachment method is used. The methods used for the preparation of ELISA plates are directly applicable to biopanning, and detailed ligand immobilization protocols can be found in enzyme immunoassay laboratory manuals (10-11). To enhance binding, proteins that adsorb poorly to plastic can be partially denatured with a chaotropic agent such as guanidine, urea, or thiocyanate, or with acid or heat. In addition, target lipids or lipoproteins can be adsorbed to plastic in the presence of deoxycholate.

The solid phase used for immobilization of the target ligand usually depends on the volume of phage lysate screened. For most applications, a plastic 96-well ELISA plate (e.g., Corning, No. 25801) allows up to 10^{10} phage to be screened in a single well. However, when larger volumes (> 0.2 ml) must be screened, uncoated 6 to 24-well plastic cell culture plates can be used. When screening very large lysate volumes (> 2 ml), plastic Petri dishes can be used. Since the titers of T7 phage lysates are typically 10–100-fold lower than M13 lysates, larger volumes may be required in the initial rounds of biopanning to ensure that a sufficiently representative sample has been exposed to the target ligand. The protocol presented next for coating an ELISA plate should be suitable for the majority of target proteins.

Coating an ELISA Plate

1. Wash the wells of a plastic ELISA plate (qualified for protein binding) several times with deionized water and remove excess water by inverting the plate and tapping onto a clean paper towel.
2. Dilute the target protein with deionized water or Tris-buffered saline (TBS) to between 1 and 10 µg/ml.
3. Apply 100 µl of diluted target protein (or S-protein for biopanning control experiments) to the wells and incubate at room temperature for 3–4 h or at 4°C overnight. Cover the plate with Parafilm® or plastic wrap to prevent evaporation of the buffer.
4. Wash wells three times with 300 µl 1X TBS or water to remove unbound target protein.
5. Prepare a 5% solution of Blocking Reagent in water and apply 200 µl to the wells.
6. Incubate for 60 min at room temperature or overnight at 4°C.
7. Wash plate five times with deionized water and pipet 200 µl of water into each well. Cover the plate and store at 4°C until ready for use. The plate can be stored for several weeks.

Determining the Number of Phage for Screening

The number of phage that must be screened depends on the complexity of the target sequences and the titer of the amplified library lysate. For example, if a random heptapeptide library is screened, the number of permutations of a seven amino acid sequence is $20^7 = 1.28 \times 10^9$. Assuming a library of sufficient size has been prepared, a minimum of 1.28×10^{11} amplified phage must be screened to represent every possible permutation with a multiplicity of 100. A considerably smaller number of phage are required to screen a displayed cDNA library. Typically about 10^6 phage would allow for sufficient sequence representation to find rare cDNAs, especially in the OrientExpress™ directionally cloned libraries (6). However, with biopanning it is just as convenient to screen up to 10^8 clones, which would provide an increased opportunity to select sequences of interest.

Kinetics

Selection of the highest affinity interactions between displayed phage polypeptide and target molecule requires selection of phage that have rapid on-rates and slow off-rates. For this reason, decreasing the duration of the binding step and increasing the duration of the elution will increase the stringency of selection for high affinity interactions. For any phage that have very slow off-rates, the best method for recovery is to add cells to the plate and allow infection and growth of bound phage to occur *in situ*.

Elution Conditions

The T7Select® Biopanning Kit includes T7 Elution Buffer (1% SDS), which allows for efficient disruption of most expressed peptides/proteins and their ligands. T7 phage are stable to a broad range of alternative treatments including up to 5 M sodium chloride, 4 M urea, 2 M guanidine-HCl, reducing conditions with DTT at up to 100 mM, and treatment with alkali up to pH 10. Unlike filamentous phage, T7 is not stable to acidic conditions below about pH 4.0, so acetic acid cannot be used for elution.

An important consideration is whether the elution reagent will inhibit growth of cells during subsequent amplification or plating steps. As an alternative to elution, it has been reported that bound phage can be directly amplified, *in situ,* by the addition of mid-log host cells (12) to the solid phase. Fusion proteins displayed on capsid do not interfere with the tail assembly or the infectivity of bound phage. This method allows for the amplification of phage with very slow off-rates, and may be useful for recovery of the highest affinity phage.

When eluted phage are transferred to liquid culture for amplification, it is important that the elution buffer does not inhibit cell growth. For example, when using T7 Elution Buffer, the phage must be diluted a minimum of 200-fold when transferred to culture (i.e., 100 µl elution to 20 ml culture).

If some information is available regarding the type of interaction sought, the elution reagent can be modified accordingly. For example, if an ionic interaction was desired, 5 M NaCl would be a sensible choice for elution since it would mask any ionic interactions during elution, in a manner similar to ion-exchange chromatography.

Number of Rounds of Biopanning

The number of rounds of biopanning generally determines the extent of the enrichment for phage that bind to the target ligand. A population of phage with high affinity for the target molecule can usually be selected in three or four rounds of biopanning. To determine whether enrichment is occurring, it is useful to perform biopanning with two identically coated wells using the same input phage. One of the wells can be used to titer the retained phage as a measure of enrichment, and the other can be used for amplification. The number of eluted phage recovered from the first and second rounds will be low, generally from 10^3–10^5 phage before amplification. The enrichment of phage that bind the target molecule during the first two rounds usually results in increases in titer for recovered phage in later rounds. The total number of phage recovered in rounds three and four will often exceed 10^6. When no further increases in recovered phage number are observed, further enrichment is unlikely to occur with additional biopanning.

The T7Select Positive Control Lysate can be used to monitor biopanning procedures. When T7Select Positive Control Lysate is combined with phage without insert (T7Select Negative Control Lysate) at ratios of $1{:}10^6$–$1{:}10^7$, a nearly homogeneous population of positive phage can be isolated in three or four rounds of biopanning on S-protein-coated ELISA plate wells. The saturation point for ELISA wells coated with S-protein is estimated to be 10^8–10^9 pfu, so increases in recovered phage beyond this level would not be expected. Using the T7Select Positive Control Lysate, the percentage of positive plaques can be monitored using the plaque lift protocol described previously. By expressing the ratio of positive plaques to total plaques on the plate and comparing this to the initial ratio of positive to total, an enrichment factor can be calculated. For example, if 89 positive plaques were detected in a field of 1200 total plaques, the ratio of positive to total is (89/1200)=0.074. If the input ratio of positive:total was $1{:}10^7$, then the enrichment factor is $(0.074/10^{-7}) = 7.4 \times 10^5$.

Biopanning Protocol

1. Inoculate a 50 ml culture of LB with the appropriate host strain and antibiotic according to the table on p 7. Incubae with shaking at 37°C until the OD_{600} = 0.5–0.6. This culture will be used as the host for selected phage eluted from the solid phase in step 9 below.
2. Based on the titer of the amplified library and the desired number of phage to be screened (see p 13), calculate the volume of phage lysate necessary for first round screening. For example, if a peptide library contains 1×10^9 primary recombinants, the desired multiplicity of screening is 100, and the amplified titer is 3×10^{11} pfu/ml, then the volume of lysate necessary for first round screening is calculated as follows:

   $$\frac{\text{primary recombinants} \times \text{multiplicity}}{\text{amplified library titer}} = \text{volume required}$$

   $$\frac{[1 \times 10^9 \text{ pfu}] \times 100}{3 \times 10^{11} \text{ pfu/ml}} = 0.33 \text{ ml}$$

   As another example, if 10^7 clones of a cDNA library are to be screened and the amplified library titer is 1×10^{10} pfu/ml, then the volume needed for first round screening is 1 µl.
3. Prepare 100 ml of 1X TBST by combining 10 ml 10X TBST (provided in biopanning kit) with 90 ml deionized water.
4. Apply the calculated amount of phage lysate to ligand coated plate prepared according to the protocol above or other suitable method. If the calculated volume exceeds the well volume, several wells can be processed in parallel, and recovered phage pooled prior to amplification. If the volume of phage lysate is less than 100 µl, use 1X TBST as diluent to bring the volume in the well to 100 µl.
5. Incubate the plate at room temperature for 30 min. Longer incubation times (e.g., overnight at 4°C) may increase the recovery of rare clones. If incubating for longer periods, cover the plate with plastic wrap to prevent evaporation.
6. Wash the plate five times with 1X TBST. This can be done with a vacuum plate washer or by adding >200 µl buffer to each well, incubating briefly, inverting the plate, and shaking out the buffer. The inverted plate can be blotted briefly on a paper towel to remove excess buffer.
7. Elute bound phage by adding 200 µl T7 Elution Buffer (or other elution reagent). Incubate at room temperature for 10–20 min.
8. Transfer eluted phage from the well to a sterile 15 ml tube.
9. Add up to 250 µl of eluted phage to the 50 ml culture prepared in step 1 and incubate, with shaking, at 37°C. Continue incubation until lysis is observed (1–3 h).
10. Transfer the lyses culture to a clean 50 ml centrifuge tube and centrifuge at 8,000 × g for 10 min. Transfer supernatant to a clean tube and store at 4°C until ready for next round of biopanning.

After the desired number of rounds of biopanning have been completed, the final lysate contains a large percentage of positive recombinants. In preparation for further analysis of the selected population of positives, the final lysate must be titered and plated at low density (less than 100 pfu/plate). Individual, well isolated plaques are used for PCR amplification as described in *Sequence Analysis of Selected Phage Recombinants* below. It is useful to save a labeled series of samples derived from plaques used for PCR amplification. To accomplish this, for each "scrape" prepared for PCR amplification, remove a plug from the same plaque using a sterile Pasteur pipet and place the plug in 1ml phage extraction buffer (see *Amplifying Libraries*, p 13). Label the scrape and plug samples identically, and store the plug samples at 4°C as an archive. Each plaque will contain 10^6–10^7 phage. If a long term archive of these samples is desired, phage eluted from plugs can be combined with 0.1 volume sterile 80% glycerol and stored at –70°C.

Sequence Analysis of Selected Phage Recombinants

There are two primary methods of preparing T7Select® phage for sequencing. PCR amplification with the T7SelectUP and T7SelectDOWN primers allows amplification of the region surrounding the multiple cloning site. The PCR reaction products are purified away from excess primer and nucleotides (with Pellet Paint® Co-Precipitant), and sequenced using a cycle sequencing protocol and end-labeled primers. Alternatively, isolated plaques can be grown in liquid culture, the phage isolated and the DNA extracted for direct sequencing. While PCR provides a convenient and rapid means of isolating the region of interest, sequencing of PCR products generally requires the use of a cycle sequencing protocol to generate sufficient signal. Commercial cycle sequencing kits (e.g., Epicentre SequiTherm EXCEL™ DNA Sequencing Kit, Catalog No. SEK6020) allow sequencing of either PCR products or purified T7 phage DNA.

PCR Amplification of Plaques

A "scrape" from an individual plaque yields a sufficient amount of phage DNA for PCR using the following protocol:

1. Using a sterile loop or pipet tip, scrape up a portion of the top agarose of an individual plaque of interest and disperse it in a tube containing 100 µl of 10 mM EDTA, pH 8.0.
2. Vortex the tube briefly, then heat at 65°C for 10 min.
3. Cool to room temperature and centrifuge at 14,000 × g for 3 min to clarify.
4. Combine the following components in a sterile 0.5 ml PCR tube (see step 5 for "hot start" prior to adding Nova*Taq*™ DNA polymerase):

1–2 µl	Phage lysate
5 µl	10X Nova*Taq* Buffer with $MgCl_2$
1 µl	T7SelectUP Primer (5 pmol/µl)
1 µl	T7SelectDOWN Primer (5 pmol/µl)
1 µl	dNTP mix (10 mM each: dATP, dCTP, dGTP, dTTP)
1.25 U	Nova*Taq* DNA polymerase (Cat. No. 71003-3)
to 50 µl	deionized water

5. Perform a "hot start" by heating the reaction to 80°C prior to the addition of DNA polymerase. Process for 35 cycles in a thermal cycler (Perkin-Elmer):
   94°C for 50 sec
   55°C for 1 min
   72°C for 1 min
   Final extension at 72°C for 6 min.

   Note: Alternative "hot start" options include: NovaTaq™ and Hot Start DNA
6. When the amplification is completed, analyze the reaction products to confirm that the amplification product is the expected size. First, remove the oil overlay by adding 100 µl of chloroform and mixing. Add 5 µl of 10X loading dye to the top aqueous phase and load 10–25 µl per lane on a 1% agarose gel containing 0.5 µl/ml ethidium bromide.

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Notice to Purchasers of the T7Select® Vectors

The T7Select System is covered by U.S. Patents 5,223,409, 5,403,484, 5,571,698, 5,766,905, 6,979,538, European Patent 436,597, and other patents pending. The system is sold for research use only. Any commercial use of the T7Select System, including drug discovery or development of commercial products, requires a license from Dyax and EMD Chemicals Inc. Information on commercial licenses in the field of therapeutic recombinant antibodies can be obtained by contacting Dyax Corporation, 300 Technology Square, Cambridge MA 02139.

Appendix A: Purification of T7 Phage and T7 DNA

T7 is purified from clarified lysates by precipitation with polyethylene glycol (PEG 8000; formerly called PEG 6000) followed by banding in a CsCl step gradient. The technique requires an ultracentrifuge equipped with a swinging bucket rotor. Procedures are described below for purifying phage from different volumes of lysate. In each case, phage are extracted from the PEG pellet in 1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and the concentrated phage solution is layered atop four steps of different density CsCl solutions in a clear ultracentrifuge tube (e.g., Beckman SW50.1 for small scale cultures, Beckman SW41 for larger scale preparations).

Although the SW50.1 rotor is convenient for small scale purification, the larger Beckman SW41 rotor and Beckman 344059 centrifuge tubes (14 × 89 mm) allow gradients to be loaded with 1–5 ml PEG-extracted phage. This volume range is suitable for banding of phage from 50 ml to 500 ml initial culture volume per tube.

The four CsCl layers are made by mixing a stock solution of 62.5% CsCl (25 g CsCl + 15 ml deionized water) with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) in the following ratios (volume in centrifuge tube):

CsCl:TE	SW50.1	SW41
1:2	1 ml	2 ml
1:1	1 ml	2 ml
2:1	1 ml	2 ml
1:0	0.5 ml	1 ml

Successively denser solutions are underlayered in the tube using a Pasteur pipet or automatic pipet. Because of the large differences in density between the layers, this can be done easily with little mixing. Up to 1.5 ml (SW50.1 tubes) and 5 ml (SW41 tubes) of concentrated phage solution is layered on top of the CsCl steps. The tubes are centrifuged at room temperature for 35 min at 40,000 rpm (SW50.1) or for 60 min at 35,000 rpm (in an SW41 rotor).

After centrifugation, the tubes typically contain a thick layer of debris and empty phage heads atop the 1:2 layer, a sharp, turbid band of phage particles above the 2:1 layer, and in many cases a lower turbid band atop the 1:0 layer. The lower band is a carbohydrate fraction that contains no phage particles and is present in variable amounts from one preparation to the next. The turbid bands are seen most easily when the tube is illuminated from above and the room light is dim. The band of purified phage particles can be collected by dripping through a puncture in the bottom of the tube, or by careful removal from above with a Pasteur pipet (after initial removal of the top layers).

Phage particles at this stage are pure enough for most purposes. They may be further purified by adding enough 62.6% CsCl to make the solution denser than the 2:1 CsCl:TE mixture, then floating up to an interface with an upper layer of 1 M NaCl by again centrifuging at the same speeds and times cited above.

Purified T7 particles may be stored in the CsCl solution in the refrigerator, where they are reasonably stable. They typically have a titer around 4×10^{11} pfu/ml per A_{260} unit (i.e. a solution with $A_{260} = 1$ has 4×10^{11} pfu/ml). For convenience, stocks in use are usually diluted to an A_{260} of 6 in 0.2 M NaCl, 2 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, where they also are quite stable. For higher dilutions, autoclaved gelatin (100 µg/ml) will help to protect against inactivation. However, purified stocks, whether diluted or not, gradually lose titer over periods of many months. Purified T7 particles may also be stored at -80°C in solutions containing 8% glycerol, but indications are that a slow loss of titer occurs under these conditions as well.

Two procedures for preparation of T7 DNA from purified phage particles are given on p 24.

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Purification of T7 from 35 ml Lysates

A standard stock of each new recombinant can be prepared by using a single plaque to infect 35 ml of growing cells in a shaking 125 ml flask (or other convenient volume, such as 50 ml in a 250 ml flask). This volume of lysate can be processed in a single 50 ml polypropylene centrifuge tube so that many lysates can be processed at the same time.

1. Inoculate 50 ml of sterile LB broth in a 250 ml Erlenmeyer flask with a single colony of an appropriate T7 host strain. Shake at 250 rpm overnight at 37°C. Use appropriate antibiotics for the chosen host strain.
2. The next morning, add 0.15 ml of the fresh overnight culture to 35 ml M9LB or M9TB. Shake at 37°C until the OD_{600} reaches 0.6–0.8 (about 3.5 h). If using a 5615 host strain, add IPTG to 1 mM when the OD_{600} reaches 0.4 and continue shaking for another 30 min.
3. Using a large-bore pipet tip, transfer a plug containing a single T7 plaque from an agar plate, or add 5 µl of high titer phage lysate to the culture. Continue shaking at 37°C until lysis (usually 1–1.5 h). Lysis will be recognized by "clearing" of the culture; strings of cell debris and a translucent appearance instead of the cloudy appearance of intact cells.
4. Upon lysis, add 3 ml of 5 M NaCl to 35 ml cultures (or 5 ml of 5 M NaCl to the 50 ml cultures, swirl to mix, and transfer to 50 ml Falcon tubes. Centrifuge at 7,000 rpm in a Beckman JA-12 rotor or equivalent for 10 min at 4°C.
5. To prepare phage stocks, transfer 5 ml of the supernatant to a sterile tube (e.g., 15 ml Falcon). If frozen stocks are desired, add 1 ml supernatant to 0.1 ml sterile 80% glycerol in a cryovial, mix thoroughly, and store at -80°C. Store the remainder tightly capped at 4°C as the working lysate.
6. Pour the remaining supernatant from step 4 into a fresh 50 ml Falcon tube. Add 1/6 volume of sterile 50% PEG 8000 (100 g PEG dissolved with stirring in 100 ml deionized water and autoclaved, and then brought to 200 ml with sterile deionized water). Cap the tube and mix thoroughly by vortexing. Thorough mixing is important because the 50% PEG is very viscous, and if a small film were to remain at the bottom of the tube, the high concentration of PEG would prevent subsequent extraction of phage from the pellet.
7. Allow the lysate-PEG mixture to stand on ice for at least 30 min to precipitate the phage particles. The mixture can be left indefinitely at this stage, and it is often convenient to leave tubes overnight in the refrigerator.
8. Centrifuge the lysate-PEG mixture for 10 min at 7,000 rpm and carefully decant the supernatant. Allow excess liquid to drain away from the pellet by leaving the tubes in an inverted position on paper towels for a few min.
9. Add 1.2 ml of 1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA to the drained pellet and vortex vigorously for 30 seconds to extract the phage.
10. At this point, transfer the mixture to a 1.5 ml microcentrifuge tube. Centrifuge at 12,000 × g for 10 min and layer the supernatant on a CsCl step gradient in an SW50.1 ultracentrifuge tube prepared as described above.
11. Centrifuge 35 min at 40,000 rpm at room temperature to band the phage. Collect the phage as described above. If collecting with a pipet from above, first remove the top layer of debris to within approximately 1 cm of the phage band. Use a fresh Pasteur pipet to collect the phage band in as small a volume as possible. Transfer the phage into a sterile polypropylene tube, cap tightly, and store at 4°C. Typically, about 0.2 ml of purified phage at a concentration of a few times 10^{12} per ml is obtained, although the concentration may be lower for recombinants that grow poorly.

Purification of T7 from 200 ml Lysates

Larger amounts of phage are conveniently purified from 200 ml of lysate, which can be accommodated in a single 250 ml polypropylene bottle for popular centrifuge/rotor types (e.g., Sorvall GSA), allowing six lysates to be processed at once.

1. Prepare a fresh overnight culture of the appropriate host strain as described in step 1 for the 35 ml scale procedure.
2. Inoculate 200 ml of sterile M9TB or M9LB in a 1 liter Erlenmeyer flask with 1 ml of overnight culture. Shake at 250 rpm at 37°C until the OD_{600} reaches 0.6–0.8 (about 3.5 h). If using a 5615 host strain, add IPTG to 1 mM when the OD_{600} reaches 0.4 and continue shaking for another 30 min.
3. Infect the culture by adding 0.02 ml high titer T7 phage lysate and continue shaking at 37°C until lysis, usually 1–1.5 h.
4. Upon lysis, add 40 µl of DNase I (1 mg/ml, or use 40 µl Novagen's RNase-free DNase I, Cat. No. 69182) and continue shaking for 15 min. Add 5 g solid NaCl, swirl to dissolve, and pour the lysate into a centrifuge bottle. Centrifuge at 8,000 rpm for 10 min at 4°C.
5. Transfer the supernatant into another centrifuge bottle containing a stir bar. Add 20 g PEG 8000 slowly, with stirring. Continue stirring at room temperature until the PEG is dissolved. The lysate-PEG mixture can be further processed after 30 min, but can also be stored overnight at 4°C.
6. Centrifuge the lysate-PEG mixture at 8,000 rpm for 10 min at 4°C. Decant the supernatant and allow the pellet to drain by inverting the bottle on paper towels for a few min. Wipe the inside of the rim of the bottle to remove as much liquid as possible.
7. Resuspend the PEG pellet (which is typically spread along the walls of the bottle, as well as at the bottom) in 1.5 ml of 1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
8. Transfer the suspension to a suitable centrifuge tube (or divide into 2–3 1.5 ml microcentrifuge tubes) and centrifuge for 10 min at 10,000 rpm. Layer the supernatant on a CsCl step gradient in an SW50.1 ultracentrifuge tube and process as described in step 11 for the 35 ml scale procedure.

Purification of T7 from 2 liter Lysates

Rather large amounts of T7 (a few times 10^{13} phage particles) can be purified from 2 liters of lysate, grown as 4×500 ml lysates in 1–2.8 liter flasks. This procedure includes additional steps (7 and 8 below) to wash the PEG pellet prior to extraction of the phage with 1 M NaCl.

1. Prepare a fresh overnight culture of the appropriate host strain as described in step 1 for the 35 ml scale procedure.
2. Inoculate each of four sterile flasks containing 500 ml M9TB or M9LB with 2.5 ml of overnight culture. Shake at 250 rpm at 37°C until the OD_{600} reaches 0.6–0.8 (about 3.5 h). If using a 5615 host strain, add IPTG to 1 mM when the OD_{600} reaches 0.4 and continue shaking for another 30 min.
3. Infect the culture by adding 0.05 ml high titer T7 phage lysate and continue shaking at 37°C until lysis, usually within 1–1.5 h.
4. Upon lysis, add 50 µl of DNase I (2 mg/ml) and continue shaking for 15 min. Add 12.5 g solid NaCl, swirl to dissolve, and pour the lysate into centrifuge bottles. Centrifuge at 8,000 rpm for 10 min at 4°C.
5. Combine the supernatants into a 3–4 liter Erlenmeyer flask or beaker containing a stir bar. Add 200 g PEG 8000 slowly, with stirring. Continue stirring at room temperature until the PEG is dissolved (15–30 min). During this time the phage particles precipitate. The lysate-PEG mixture can be further processed as soon as the PEG is dissolved, but can also be stored overnight at 4°C.
6. Swirl the flask to make sure the precipitate is evenly suspended and transfer into 6×500 ml centrifuge bottles. Centrifuge at 8,000 rpm for 10 min at 4°C. Decant the supernatant and allow the pellet to drain by inverting the bottles on paper towels for a few minutes. Wipe the inside rims of the bottles to remove as much liquid as possible.
7. Resuspend the pellets (which are typically spread along the walls of the bottles, as well as at the bottom) in a combined volume of about 40 ml of sterile 10% PEG (10 g PEG 8000 in 100 ml of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, autoclaved). It is convenient to resuspend the pellet in 10 ml solution per bottle, transferring the suspension to the next bottle, such that pellets are combined. Make sure that the pellets are evenly suspended by repeated pipetting up and down with a 10 ml pipet. Transfer the resuspended mixture into two 50 ml Falcon tubes (approx. 22 ml/tube) or other suitable centrifuge tube.
8. Centrifuge the mixture for 10 min at 7,000 rpm at 4°C in a Beckman JA-12 or equivalent rotor. Decant the supernatant and drain the washed phage pellet as completely as possible.

9. Resuspend each pellet in 5 ml of 1 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA. Vortex thoroughly to extract the phage (there should be no remaining clumps).
10. Centrifuge for 10 min at 7,000 rpm at 4°C. Carefully transfer the supernatants to a fresh 50 ml Falcon tube. If desired, the pellets can be re-extracted with another 5 ml of salt solution as in step 9. Pool the second supernatants with the first.
11. Layer the final phage suspension on CsCl step gradients in SW41 ultracentrifuge tubes. Four gradients should hold slightly more than 20 ml of phage suspension (about 5 ml/gradient). Centrifuge at 35,000 rpm for 60 min at 20°C. Collect the phage bands as described in step 11 for the 35 ml scale procedure. Pool into a 15 ml Falcon tube; the total volume should be about 4 ml.

Preparation of T7 DNA From Phage by Phenol Extraction

1. Dialyze the CsCl-purified phage against 0.1 M NaCl, 0.1 M Tris-HCl, pH 8.0. Use a minimum of 100 volumes of buffer. Change buffer four times over 1 h at room temperature.
2. Transfer the dialysate into a polypropylene tube and extract with an equal volume of phenol equilibrated with 0.1 M Tris (pH > 7 but < 8). Mix by inversion until the phases are thoroughly mixed.
3. Centrifuge at 1500–5000 × g for 5–10 min (phases should be clearly separated). Transfer the top aqueous phase to a fresh tube using a sterile glass pipet (or wide-bore pipet tip for smaller volumes).
4. Repeat the extraction with phenol twice more (steps 2 and 3).
5. Extract twice with an equal volume of chloroform:isoamyl alcohol (CIAA; 24:1) as in steps 2 and 3 to remove residual phenol.
6. Dialyze the final aqueous phase into TE buffer. Store at 4°C.

Preparation of T7 DNA From Phage by Heat Treatment and Ethanol Pecipitation

This procedure is convenient for processing multiple small scale samples.

1. Add 10 volumes of 10 mM EDTA (pH 8.0) to 1 volume of CsCl-banded phage in a sterile tube.
2. Heat at 65°C for 5 min, and then to room temperature.
3. Add 2.5 volumes of 100% ethanol, and place at –20°C for 20 min.
4. Centrifuge at 10,000 × g for 5 min.
5. Remove the supernatant. Rinse the pellet twice with 0.5 ml 70% ethanol. Centrifuge if necessary to collect the precipitate at the bottom of the tube before removing the supernatant.
6. Allow the pellet to air dry or place briefly under vacuum.
7. Resuspend the DNA in the desired volume of TE buffer. The DNA may take some time to dissolve completely; it will go into solution if kept at room temperature overnight or at 37°C for several hours. However, restriction digests for analyzing the DNA can be performed immediately.