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U.S. patent no. 5,629,179 has been issued to Novagen, Inc. for the directional random priming method and kit.

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About the System

OrientExpress™ Oligo(dT) cDNA Synthesis Kit	69993-3
OrientExpress Random Primer cDNA Synthesis Kit	69992-3
OrientExpress Oligo(dT) Primer cDNA Library Construction System (λSCREEN™)	69991-3
OrientExpress Random Primer cDNA Library Construction System (λSCREEN)	69990-3
T7Select™1-1 OrientExpress cDNA Cloning System, Oligo(dT)	70200-3
T7Select1-1 OrientExpress cDNA Cloning System, Random Primer	70202-3
T7Select10-3 OrientExpress cDNA Cloning System, Oligo(dT)	70581-3
T7Select10-3 OrientExpress cDNA Cloning System, Random Primer	70580-3
<i>EcoR I/Hind III</i> End Modification Kit	69994-3

Description

Novagen's OrientExpress cDNA Synthesis and Cloning Systems are complete sets of reagents designed for rapid, efficient construction of cDNA libraries having inserts in a defined orientation. Two types of systems are available that differ only in the primer used for first-strand cDNA synthesis. The OrientExpress Random Primer System is based on a patented random primer strategy, whereas the OrientExpress Oligo(dT) Primer System uses a modification of conventional oligo(dT) priming to achieve orientation-specific cloning between *EcoR I* and *Hind III* sites. Starting with high-quality poly(A)⁺ RNA, sufficient reagents are provided in the cDNA Synthesis and Cloning Systems for construction of up to 5 cDNA libraries in λSCREEN-1 or T7Select vectors with either primer approach. The OrientExpress cDNA Synthesis Kits include components for the cDNA synthesis step only.

With this system, cDNA synthesis using either oligo(dT) or *Hind III* Random Primers produces cDNA having an *EcoR I* site on one end and a *Hind III* site on the other, corresponding to the 5' and 3' ends of the mRNA, respectively. Ligation into either λSCREEN-1 or T7Select *EcoR I/Hind III* arms produces inserts having a "sense" orientation relative to upstream T7 expression signals. Both methods produce large libraries; however, random primed libraries provide more even sequence representation and offer the ability to modify average insert size by adjusting the ratio of primers to mRNA in the first strand reaction (see Fig. 2, p. 8). This feature is useful for creating libraries of protein functional domains to be screened by ligand binding assays. It should be noted that unlike oligo(dT), random primers will efficiently prime any RNA present in the sample. Therefore, we recommend using highly purified poly(A)⁺ RNA for random primed library construction to minimize the occurrence of rRNA and other "junk" sequences in the library. The Straight A's™ mRNA Isolation System is designed for the preparation of poly(A)⁺ RNA suitable for random priming.

The strategy for directional random primed cDNA synthesis is diagrammed in Fig. 1 on p. 3. First and second strand cDNA syntheses are simple reactions sequentially carried out in the presence of 5-methyl dCTP, which protects any internal *EcoR I* and *Hind III* restriction sites from digestion. After second strand synthesis, the cDNA is treated with T4 DNA polymerase to blunt the ends. Addition of the *EcoR I/Hind III* Directional Linker d(GCTTGAATTC AAGC) at the d(A)_n:d(T)_n end creates a *Hind III* site d(AAGCTT) in which the two underlined bases are derived from the cDNA. The two dT's are provided on the 5' end of each first strand by the primer [either oligo(dT) or the *Hind III* random primer d(TTNNNNNN)]. Digestion with both *Hind III* and *EcoR I* thus yields cDNA molecules ready for directional insertion into *EcoR I/Hind III* vector arms. For cDNAs having the sequence d(TT) at their 5' ends (statistically 1 in 16 molecules), linker addition will yield *Hind III* sites at both ends. However, because the 5' ends are heterogeneous, cDNA products from every gene will be represented in the library. Excess linkers and small cDNAs (< 300 bp) are removed by a gel filtration step. After vector ligation and packaging in either PhageMaker® λ packaging extracts or T7 packaging extracts (depending on the chosen vector), the phage are plated to determine the library titer.

Following amplification of the library, it can be used for screening by conventional plaque lifts (λSCREEN or T7Select) or biopanning (T7Select) to isolate recombinants containing sequences of interest.

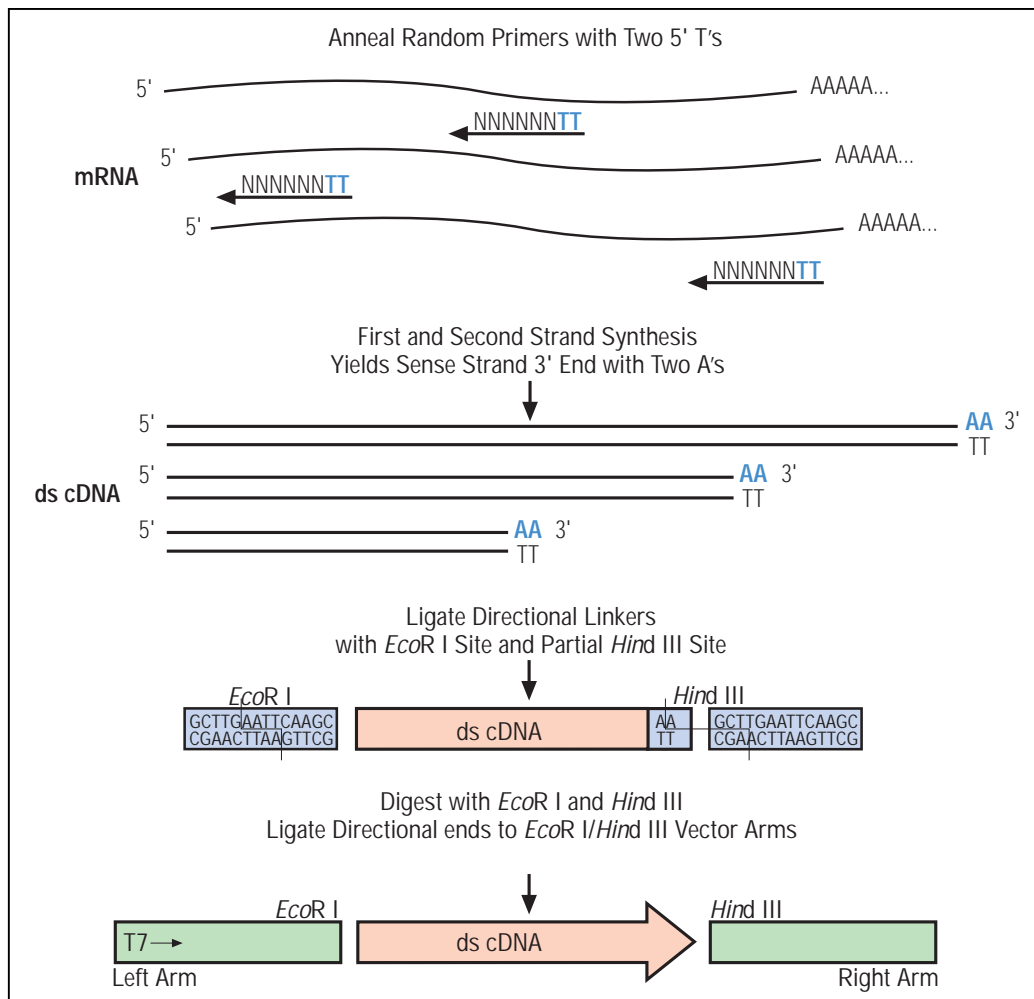


Fig. 1. OrientExpress directional random primer strategy

λSCREEN and T7Select Vectors

The λSCREEN-1 vector accepts cDNA inserts from 0–8 kbp into *EcoR I/Hind III* arms. Inserts are cloned downstream from T7 expression signals and vector-encoded peptides such that cDNAs can be expressed as fusion proteins that are easily detected and purified by several strategies. The amino terminal fusion sequences include, in order, the 260 aa T7•Tag[®], 6 aa His•Tag[®], thrombin cleavage site, 15 aa S•Tag[™], and enterokinase cleavage site. An SP6 promoter is placed before the enterokinase recognition site, which allows synthesis of non-fused RNA for probes and *in vitro* translation (e.g., with the Single Tube Protein[™] System 3). Libraries can thus be screened for expressed polypeptides with antibodies or other ligands, as well as with conventional nucleic acid probes. For non-directional cloning, the vector is also available as *EcoR I* digested, dephosphorylated arms. In this case, the *Hind III* digestion is omitted from the protocol and cDNAs containing an *EcoR I* site at each end are produced.

λSCREEN-1 also features an advanced automatic subcloning system for conversion of phage recombinants to plasmids *in vivo*. A complete plasmid flanked by two 34 bp *loxP* sites derived from bacteriophage P1 is built into the phage. Plasmid subclones are generated simply by infection of hosts expressing the P1 cre recombinase, which recognizes the *loxP* sites and forms the plasmid by site-specific recombination. When plated in the presence of ampicillin (or carbenicillin), colonies appear which are the result of plasmid excision. Inserts are faithfully and efficiently subcloned without the generation of deletions or rearrangements. This is in contrast to systems employing single stranded replication intermediates, such as those based on M13-type helper phage infection. Please refer to the λSCREEN Vector Manual, TB119, for complete protocols for cloning, screening and autosubcloning.



There are two types of T7Select phage display vectors suitable for cDNA library construction. The T7Select10-3 vector displays an average of 10 copies of recombinant fusions, whereas the T7Select1-1 vector displays an average of 0.1–1 copy per phage. In both vectors, coding sequences for the peptides or proteins to be displayed are cloned into the *EcoR I/Hind III* sites following aa 348 of the 10B capsid protein. 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. The cloning capacity of T7Select1-1 and T7Select10-3 vectors is 0–3.6 kbp. Please refer to the T7Select System Manual, TB178, for a complete description of the vectors and associated protocols.

System Components

OrientExpress cDNA Library Construction Systems (λ SCREEN)

- OrientExpress Random Primer cDNA Synthesis Kit or Oligo(dT) Primer cDNA Synthesis Kit
- *EcoR I/Hind III* End Modification Kit
- DNA Ligation Kit
- Mini Column Fractionation Kit
- λ SCREEN-1 *EcoR I/Hind III* Arms Kit plus PhageMaker

T7Select OrientExpress cDNA Cloning Systems

- OrientExpress Random Primer cDNA Synthesis Kit or Oligo(dT) Primer cDNA Synthesis Kit
- *EcoR I/Hind III* End Modification Kit
- DNA Ligation Kit
- Mini Column Fractionation Kit
- T7Select1-1 Cloning Kit or T7Select10-3 Cloning Kit

OrientExpress Oligo(dT) and OrientExpress Random Primer cDNA Synthesis Kits

- 10 μ g Oligo(dT) or 5 μ g *Hind III* Random Primers
- 4,000 U MMLV Reverse Transcriptase
- 50 μ l 5X First Strand Buffer
- 250 U DNA Polymerase I
- 8 U RNase H
- 250 μ l 5X Second Strand Buffer
- 50 μ l 10X Methylation dNTP Mix
- 100 μ l 100 mM DTT
- 1.5 ml Nuclease-free Water
- 250 μ l 10 mg/ml Glycogen
- 1.8 ml 4 M Ammonium Acetate
- 5 μ g OrientExpress Positive Control RNA

EcoR I/Hind III End Modification Kit

- 25 U T4 DNA Polymerase
- 50 μ l 10X Flush Buffer
- 40 μ l *EcoR I/Hind III* Directional Linkers
- 100 μ l 10X *Hind III* Buffer
- 500 U *Hind III*
- 50 μ l 10 mM dNTP Mix
- 100 μ l 10X *EcoR I* Adjustment Buffer
- 500 U *EcoR I*
- 100 μ l 100 mM DTT
- 50 U T4 Polynucleotide Kinase
- 1.8 ml 4 M Ammonium Acetate



System Components, cont'd

DNA Ligation Kit

- 100 U T4 DNA Ligase
- 500 µl Ligase Dilution Buffer
- 500 µl 10X Ligation Buffer
- 250 µl 100 mM DTT
- 250 µl 10 mM ATP
- 1.5 ml Nuclease-free Water

Mini Column Fractionation Kit

- 5 ml Gel Filtration Resin
- 5 Mini Columns
- 5 ml 10X Column Buffer
- 250 µl 10 mg/ml Glycogen
- 1 ml TE Buffer

λSCREEN-1 *EcoR I/Hind III* Arms plus PhageMaker Kit

- 10 µg *EcoR I/Hind III* digested, dephosphorylated λSCREEN-1 Arms
- 11 PhageMaker Extracts
- 2 µg *EcoR I/Hind III* Control Insert
- 0.2 ml ER1647, BM25.8, and BL21 (DE3)pLysE glycerol stocks
- 2 µg PhageMaker Control DNA

T7Select Cloning Kit

- 5 µg T7Select10-3 or T7Select1-1 *EcoR I/Hind 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



Related products/Available separately	Size	Cat. No.
AP Detection Reagent Kit (NBT/BCIP, 20X AP Buffer)	1X	69264-3
	5X	69264-4
Carbenicillin	5 g	69101-3
Cre Recombinase	250 U	69247-3
DNA Ligation Kit		69838-3
DNase Shotgun® Cleavage Kit		69281-3
Directional <i>EcoR</i> I/ <i>Hind</i> III Linkers	2000 pmol	69366-3
<i>Hind</i> III Random Primers	10 µg	69926-3
λSCREEN-1 <i>EcoR</i> I/ <i>Hind</i> III Arms Kit		69984-3
λSCREEN-1 <i>EcoR</i> I/ <i>Hind</i> III Arms plus PhageMaker Kit		69985-3
Methylation dNTP Mix	50 µl	69346-3
Mini Column Fractionation Kit		69995-3
Pellet Paint™ Co-Precipitant	250 µl	69049-3
Perfect RNA™ Markers, 0.1–1kb	25 lanes	69924-3
Perfect RNA Markers, 0.2–10kb	25 lanes	69946-3
PhageFinder® Immunoscreening Kit, mouse Abs		69260-3
		rabbit Abs 69261-3
PhageMaker <i>in vitro</i> Packaging System	6 extracts	69307-3
	11 extracts	69307-4
Oligo(dT) Primer	20 µg	69896-3
Single Tube Protein System 3, SP6	50 rxn	70207-3
Single Tube Protein System 3, T7	50 rxn	70192-3
SP6 Promoter primer	500 pmol	69349-3
S•Tag primer	500 pmol	69945-3
STP3™-Biotin Kit, SP6	50 rxn	70577-3
STP3-Biotin Kit, T7	50 rxn	70551-3
Straight A's mRNA Isolation System plus Separation Stand		69963-3 69962-3
T4 DNA Ligase	100 U	69839-3
	500 U	69839-
T4 Polynucleotide Kinase	250 U	69248-3
T7 gene 10 primer	500 pmol	69341-3
T7 Promoter primer	500 pmol	69348-3
T7 Terminator primer	500 pmol	69337-3
T7Select Biopanning Kit		70018-3
T7Select1-1 Cloning Kit		70010-3
T7Select10-3 Cloning Kit		70550-3
T7Select Packaging Kit	6 extracts	70014-3
T7SelectDOWN Primer	500 pmol	70006-3
T7SelectUP Primer	500 pmol	70005-3
T7•Tag® Antibody AP Conjugate	50 µl	69999-3
T7•Tag Monoclonal Antibody	50 µg	69522-3
	250 µg	69522-4



About the Protocol

This document contains protocols for the synthesis, end modification and fractionation of cDNA using oligo(dT) or random primers. The cDNA will have an *EcoR* I sticky end at the 5' end of the gene and a *Hind* III sticky end at the 3' end, and will be ready for insertion into λ SCREEN-1, T7Select1-1 or T7Select10-3 *EcoR* I/*Hind* III vector arms.

Protocols for cloning the prepared cDNA into the vectors, plating, amplification and screening of cDNA libraries are described in the T7Select System Manual, TB178, and the λ SCREEN Vector Manual, TB119.

Preliminary Considerations

RNA Isolation

The quality of a cDNA library depends on many factors, beginning with the RNA sample. Quality is judged based on integrity of the RNA and on the relative level of contamination by rRNA species. Some amount of rRNA contamination is common with many isolation procedures, which usually are based on a single round of chromatography on oligo(dT) cellulose, poly(U) agarose, or oligo(dT) magnetic particles. Contamination by non-poly(A) containing RNA usually does not pose a significant problem when using oligo(dT) priming strategies for cDNA synthesis, because the primer itself provides a means of selection for mRNA.

Unlike oligo(dT) primers, random primers initiate cDNA synthesis from all RNA species and resulting libraries will contain non-informative cDNA clones corresponding to the proportion of rRNA in the sample. Therefore, we highly recommend that samples to be random primed contain as little rRNA contamination as possible. The classic approach to obtain purer poly(A)⁺ RNA has been to perform an additional round(s) of chromatography on oligo(dT) cellulose; however, in many cases the recovery of poly(A)⁺ RNA is poor after a second round.

We have found that the Straight A's mRNA Isolation System provides superior yields of intact poly(A)⁺ RNA with minimal rRNA contamination from a variety of tissues or from total RNA (McCormick and Hammer, 1994). The Magnetight™ Oligo(dT) particles also can be used for a second round of isolation with minimal losses of material to produce poly(A)⁺ RNA suitable for random primed cDNA synthesis.

Avoiding Ribonuclease Contamination

RNases are ubiquitous in the laboratory and in cells, and precautions should be taken to eliminate the risk of contamination whenever possible (for a discussion see Blumberg 1987). In general, use sterile plastic disposable tubes and pipets, wear gloves, and treat critical solutions with diethyl pyrocarbonate (DEPC). This is done by adding DEPC to 0.1%, stirring vigorously for 10 min, and then heating at 70°C for 1 h or autoclaving to remove the DEPC. Note that DEPC is an acylating agent that reacts with primary amines and sulfhydryl groups so that reagents such as Tris and DTT cannot be treated directly. In these cases make up the solution with DEPC-treated water and sterile filter or autoclave (do not autoclave DTT). Also note that DEPC should always be used in a fume hood and never added to aqueous solutions containing ammonia, which results in the formation of ethyl carbamate, a potent carcinogen (Ehrenberg et al. 1976).

In addition, reserve reagents exclusively for RNA work and store them separately. Avoid using any reagents which may have been used for other work. If possible, separate any laboratory procedures such as plasmid preps which require the use of RNase from your RNA work area.

Quick Gel Analysis of RNA

Although the RNA could be checked by electrophoresis under denaturing conditions, such as on formaldehyde or methylmercury hydroxide agarose gels (for RNA > 1 kb), or on formamide or urea polyacrylamide gels, it is usually satisfactory to use the following method, which is faster, safer, and much easier than the others. It is necessary to use an agarose gel apparatus that is RNase-free. Particularly avoid any apparatus that has been used for analysis of plasmid minipreps, since these usually contain copious amounts of RNase. Always run a control lane of a known intact RNA, such as Novagen's Perfect RNA Markers, as size standards and to verify that



the apparatus and gel are RNase-free. RNAs run in this system migrate approximately according to size, but these gels should not be used for accurate size determinations.

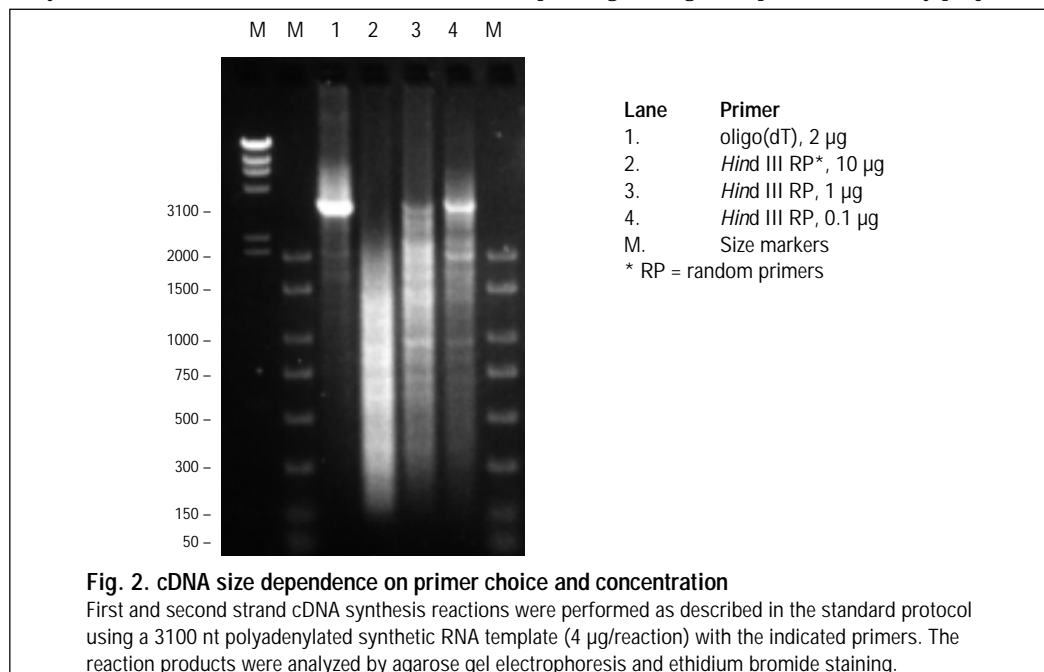
1. Pour a 1% agarose gel in 1X TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA), being careful to use clean glassware and high-quality agarose.
2. Add a sample containing 0.5–2 µg RNA in water or TE to sample buffer (10% sucrose, 90% deionized formamide, 0.05% bromphenol blue, 0.05% xylene cyanole). Then add 1–2 µl of 0.1 mg/ml ethidium bromide to the sample. Up to 50% of the volume can be the RNA sample, although less is better. The final volume should be no more than 20 µl. Prepare the marker sample the same way.
3. Mix the samples, heat at 60–65°C for 3 min, cool to room temperature, and load the gel. Load Perfect RNA Markers in an adjacent well to the mRNA sample.
4. Run the gel at up to 10 V/cm in 1X TBE and photograph under UV illumination as for DNA gels. Two bands, the 28s (4.7–5 kb) and 18s (1.95–2.1 kb) ribosomal RNAs, are often visible to varying degrees of prominence depending on the preparation. mRNA generally appears as a smear between 300–500 nt and 4,000–6,000 nt, with the most intense region from 1,000 to 3,000 nt.

Choice of Priming Method

Previous cDNA synthesis strategies allowed efficient directional cloning only when using oligo(dT)-based primers; therefore, directionality was a factor in primer choice. The OrientExpress System allows efficient cloning using either priming strategy, so that the choice of primer depends more on personal preference or the specific use of the library.

A special feature of random primers is the ability to control the average insert size by adjusting the ratio of primers to sample RNA during cDNA synthesis. Smaller inserts may be desirable when screening for functional domains or other applications. Fig. 2 illustrates this point by comparing the size ranges of cDNA prepared with oligo(dT) and three different random primer:RNA ratios. Note that lower ratios produced longer cDNAs, and that similar cDNA yields were obtained at all three random primer ratios.

As a general rule, both priming strategies can be used to produce quality libraries with large inserts (given appropriate random primer:RNA ratios). While oligo(dT) priming has become the standard for library construction and has allowed isolation of many genes, directional random priming should provide the same benefits and eliminate the 3' bias inherent in oligo(dT)-primed libraries. Since similar reagents and procedures are used for library construction in this system, it may be worthwhile (and economical) to use both priming strategies in parallel for many projects.





cDNA Synthesis

cDNA synthesis is primed with oligo(dT) or *Hind* III Random Primers and 5-me dCTP is incorporated into both strands without extraction or precipitations between first and second strand synthesis. The cDNA is then treated with T4 DNA polymerase to flush the ends and ligated with Directional *EcoR* I/*Hind* III Linkers.

Following linker ligation, the cDNA is digested sequentially with *Hind* III and *EcoR* I. The cDNA is then passed through a small gel filtration column to remove excess linkers and small cDNA products (< 300 bp). It is then ready for insertion into *EcoR* I/*Hind* III digested vector arms.

The following protocol uses 4 µg poly(A)⁺ RNA in a 50 µl first strand reaction and a 250 µl second strand reaction. The reaction can be scaled up or down proportionally as required.

First Strand Synthesis

1. Combine in a sterile, RNase-free 1.5-ml screw-cap microcentrifuge tube:

4 µg	poly(A) ⁺ RNA sample, or 2.5 µg OrientExpress Positive Control RNA
2 µg	Oligo(dT) primer or 1 µg <i>Hind</i> III Random Primers*
x µl	Nuclease-free Water
<hr/>	
20 µl	Total volume

* The amount of *Hind* III Random Primers can be increased to produce cDNA with smaller average size (see Fig. 2, p. 8).

2. Heat to 70°C for 10 min. This step helps to alleviate RNA secondary structure and may allow more efficient priming and cDNA synthesis.
3. Chill quickly on ice.
4. Microcentrifuge briefly to collect contents at bottom of tube.
5. Add the remaining components for first strand cDNA synthesis:

10 µl	5X First Strand Buffer (5X = 250 mM Tris-HCl pH 8.3 at 25°C, 375 mM KCl, 15 mM MgCl ₂)
5 µl	100 mM DTT
2.5 µl	10X Methylation dNTP Mix
x µl	Nuclease-free Water
<hr/>	
50 µl	Total volume, including MMLV reverse transcriptase, added in step 7 below.
6. Mix the components gently and equilibrate for 1 min at 37°C.
7. Add 800 units MMLV RT, mix gently, and continue incubation at 37°C for 60 min. (Optional) To follow first strand synthesis: prior to the 60 min incubation, remove 5 µl of the reaction to a fresh tube containing ~5 µCi (0.5 µl) [α -³²P]dATP (> 400 Ci/mmol). Following the 60 min incubation, process this reaction by standard TCA precipitation and/or alkaline agarose gel techniques to evaluate first strand synthesis.
8. Heat at 70°C for 10 min, chill on ice, and centrifuge briefly to collect the contents at the bottom of the tube.

Note: mRNA that contains a high degree of secondary structure can be treated with methylmercury hydroxide to destroy base pairing, although overall cDNA yields will be reduced (Krug and Berger, 1987). Perform all manipulations with methylmercury hydroxide in a fume hood, wearing gloves, and dispose of the waste appropriately. Prior to addition of the primer, mix the RNA (at a maximum concentration of 1 µg/µl) in a total volume of 10 µl with 1 µl of 0.1 M methylmercury hydroxide (Alfa) and incubate at room temperature for 10 min (or boil for 1–3 min and then put on ice, if desired). Inactivate the methylmercury by adding 1 µl of 0.35 M 2-mercaptoethanol and proceed with primer addition.



Second Strand Synthesis

1. Add the following components to the first strand reaction (45 or 50 μ l) on ice:

50 μ l	5X Second Strand Buffer (5X = 200 mM Tris-HCl, pH 7.5 at 25°C, 22 mM MgCl ₂ , 425 mM KCl)
6 μ l	100 mM DTT
2 μ l	10X Methylation dNTP Mix
0.5 μ l	(Optional) ~5 μ Ci [α - ³² P]dATP (> 400 Ci/mmol) to measure second strand conversion
x μ l	Nuclease-free Water
y μ l	(50 units) DNA polymerase I
z μ l	(1.6 units) RNase H
<hr/>	
250 μ l	Total volume
2. Mix gently and incubate at 15°C for 90 min.
3. Add 250 μ l TE-buffered phenol:chloroform:isoamyl alcohol (25:24:1), vortex for 30 sec and centrifuge at 12,000 \times g for 1 min.
4. Remove the aqueous phase to a fresh tube and add 1 μ l Glycogen, 250 μ l of 4 M Ammonium Acetate and 300 μ l of isopropanol. Invert several times to mix.
5. Incubate 5 min at room temperature. Microcentrifuge at 12,000 \times g for 8 min.
6. Carefully remove the supernatant and rinse the pellet with 0.5 ml 70% ethanol and then 0.5 ml 100% ethanol. Centrifuge 3 min for each rinse. Allow the final pellet to dry thoroughly and resuspend in 20 μ l TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Continue with end modification or store at -20°C.

End Modification

Flushing the cDNA Ends with T4 DNA Polymerase

1. Assemble the following reaction:

20 μ l	double stranded cDNA in TE from step 6 above
3 μ l	10X Flush Buffer
1.5 μ l	100 mM DTT
3 μ l	1 mM dNTPs (a fresh 1:10 dilution of 10 mM dNTP Mix prepared in Nuclease-free Water)
1.5 U	T4 DNA Polymerase
x μ l	Nuclease-free Water
<hr/>	
30 μ l	Total volume
2. Mix gently and incubate for 20 min at 11°C.
3. Add 20 μ l TE and then add 50 μ l TE-buffered phenol:chloroform:isoamyl alcohol. Vortex for 30 sec and centrifuge at 12,000 \times g for 1 min. Transfer the top aqueous phase to a fresh tube. Add 50 μ l chloroform:isoamyl alcohol (24:1), vortex and centrifuge as above. Transfer the final aqueous phase to a fresh tube.
4. Add 1 μ l Glycogen, 50 μ l 4 M Ammonium Acetate and 250 μ l ethanol. Mix well.
5. Leave at -20°C for at least 1 h.
6. Centrifuge at 12,000 \times g for 10 min, remove the supernatant and rinse the pellet successively with 0.5 ml 70% ethanol and 0.5 ml 100% ethanol. Allow the pellet to dry and resuspend in 10 μ l TE. Proceed with linker ligation or store at -20°C.



Ligation to Directional *EcoR I/Hind III* Linkers

Directional *EcoR I/Hind III* Linkers are phosphorylated immediately before use by a brief incubation with T4 polynucleotide kinase.

1. Assemble the following reaction:

10 µl	blunt-ended cDNA in TE
2 µl	10X Ligation Buffer (10X = 200 mM Tris-HCl pH 7.6, 50 mM MgCl ₂)
2 µl	1 mM ATP (final conc. is 0.1 mM, which is recommended for blunt end ligation; make a fresh 1:10 dilution of 10 mM ATP in Nuclease-free Water provided with the DNA Ligation Kit)
2 µl	100 mM DTT
2 µl	(100 pmol) Directional <i>EcoR I/Hind III</i> Linkers
x µl	(5 U) T4 Polynucleotide Kinase
y µl	Nuclease-free Water (if needed)
20 µl	Total volume (including ligase added in step 3 below)

2. Incubate for 5 min at 37°C.
3. Place tube on ice and add 6-8 Weiss units of T4 DNA Ligase. Incubate 6-20 h at 16°C.

EcoR I and *Hind III* Digestion

1. Inactivate the ligase by heating the tube at 70°C for 10 min. Allow to cool slowly to room temperature.

2. Add the following:

10 µl	10X <i>Hind III</i> Buffer
x µl	Nuclease-free Water
y µl	(100U) <i>Hind III</i>
100 µl	Total volume

3. Incubate 2 h at 37°C.

4. Add the following:

10 µl	10X <i>EcoR I</i> Adjustment Buffer
100 U	<i>EcoR I</i>

5. Incubate 4 h at 37°C.

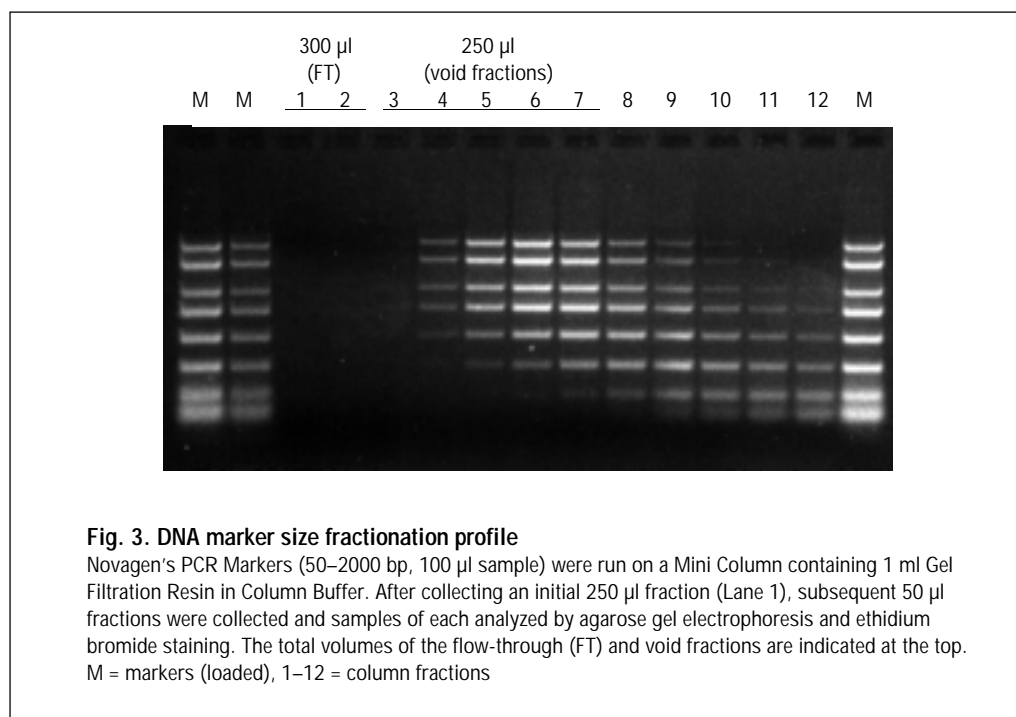
6. Extract with an equal volume of TE-buffered phenol:chloroform:isoamyl alcohol. Transfer the aqueous phase to a fresh tube and use immediately for size fractionation; or extract with one volume of chloroform:isoamyl alcohol (CIAA), remove and discard the bottom organic phase and then add 1 µl Glycogen, 120 µl 4 M Ammonium Acetate and 0.5 ml ethanol. Store at -20°C.



Size Fractionation

A fractionation step is required to remove excess linkers and small cDNA synthesis products. Gel filtration provides a rapid, efficient method that produces cDNA ready for ligation with vector arms.

1. Gently mix the bottle of Gel Filtration Resin by inversion until completely suspended. Transfer slurry (1 ml settled bed volume, approximately 2.0 ml of the slurry) to a Mini Column. You may need to push gently on the top of the column (using a gloved finger or Parafilm®) to start the column flowing. Allow the resin to pack under gravity flow.
2. When the storage buffer drops to the top of the column bed, equilibrate the column with 5×1 ml of 1X Column Buffer.
3. Allow the Column Buffer to drain to the top of the column bed. Carefully pipet the cDNA onto the column. If the cDNA was precipitated prior to this step, centrifuge for 10 min at room temperature, carefully remove the supernatant, rinse the pellet sequentially with 70% and 100% ethanol, allow to dry, and resuspend in 100 μ l TE. Load the entire sample on the column.
4. After the DNA has settled into the resin, gently add 200 μ l 1X Column Buffer without disturbing the gel bed surface. Allow the buffer to flow through.
5. Add 250 μ l 1X Column Buffer and collect the eluate. This is the void fraction of a 1 ml column and represents the largest cDNA molecules (see Fig. 3 below).
6. Add 1 μ l 10 mg/ml Glycogen and 150 μ l isopropanol to the eluate (there is no need to add salt, which is already present in the Column Buffer). Vortex to mix and incubate at room temperature for 5 min.
7. Centrifuge at $12,000 \times g$ for 10 min, remove the supernatant and rinse the pellet successively with 0.5 ml 70% ethanol and 0.5 ml 100% ethanol. Allow the pellet to dry and resuspend in 20 μ l TE. Store at -20°C . The prepared cDNA is now ready for ligation into vector arms.





References

Cited Literature

- Blumberg, D.D. (1987) *Meth. Enzymol.* **152**, 20–24
- Ehrenberg, L., Fedorcsak, I. and Solymosy, F. (1976) *Prog. Nucl. Acid Res. and Mol. Biol.* **16**, 189.
- Krug, M.S. and Berger, S.L. (1987) *Meth. Enzymol.* **152**, 316–325.
- McCormick, M. and Hammer, B. (1994) *inNovations* **2**, 8–10.

General Techniques

- Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1987) "Current Protocols in Molecular Biology" John Wiley & Sons, New York.
- Berger, S.L. and Kimmel, A.R. (eds.) (1987) "Guide to Molecular Cloning Techniques", *Meth. Enzymol.* **152**, Academic Press, Orlando.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) "Molecular Cloning, A Laboratory Manual", Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

cDNA Synthesis and Directional Cloning

- Gubler, U. and Hoffman, B. (1983) *Gene* **25**, 263–269.
- Meissner, P.S., Sisk, W.P. and Berman, M.L. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 4171–4175.
- Okayama, H. and Berg, P. (1982) *Mol. Cell. Biol.* **2**, 161.
- Palazzolo, M.J. and Meyerowitz, E. M. (1987) *Gene* **52**, 197–206.