User Manual

LD-Insert Screening Amplimer Sets User Manual

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See List of Components for storage conditions

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I. Introduction

Clontech's LD-Insert Screening Amplimer Sets are convenient tools for characterizing cDNA and genomic inserts using long-distance PCR (LD PCR; Barnes, 1994; Cheng *et al.*, 1994). The LD-Insert Screening Amplimers are longer than conventional insert screening amplimers and have been optimized for use with Clontech's Advantage® 2 PCR Kit and Advantage Genomic PCR Kit. The Advantage Polymerase Mixes are designed for LD PCR—i.e., they contain both primary and proofreading polymerases to permit amplification of virtually any insert, regardless of size. The LD-Insert Screening Amplimers can also be used in conventional PCR reactions, of inserts up to 3 kb, using Clontech's TITANIUM™ Taq Polymerase.

Screening by PCR is a fast and convenient method for carrying out the initial characterization of inserts in positive clones identified by screening cDNA and genomic libraries (Saiki, 1985; Hannon *et al.*, 1994). Within hours, you can determine the size of the inserts, generate simple restriction maps, and eliminate duplicate or partial clones from further consideration. In the past, insert screening by PCR has been limited to relatively small inserts—typically 3 kb or less. This limitation meant the technique was of little use in the characterization of genomic and cosmid inserts. With Clontech's LD-Insert Screening Amplimer Sets, you can amplify genomic inserts of up to 35 kb and virtually any cDNA insert using LD PCR and the appropriate LD Amplimers.

LD-Insert Screening Amplimers can also be used to obtain cDNA or genomic DNA inserts that are not readily recoverable by restriction enzyme digestion. This is particularly useful for large inserts, which often have internal sites for restriction enzymes that might otherwise be used to excise the insert. Now you can simply amplify even very large inserts and clone them directly into a TA-type cloning vector. The higher fidelity of LD PCR (Barnes, 1994) reduces the number of errors introduced when using LD PCR to subclone inserts.

II. List of Components

Store components at –20°C.

Each LD-Insert Screening Amplimer Set contains the following reagents, sufficient for 100 PCR amplifications (based on a 50-µl reaction volume):

- 100 µl 5' primer (20 µM)
- 100 µl 3' primer (20 µM)

III. Additional Materials Required for LD PCR

The following reagents are required but not supplied.

- **DNA template** (see Section IV)
- **50X polymerase mix**

  The LD-Insert Screening Amplimers and this protocol were developed and optimized using Clontech's Advantage® PCR Kits and Polymerase Mixes, but they may also be used with other polymerase mixes suitable for LD PCR (Barnes, 1994; Cheng *et al.*, 1994). Advantage Polymerase Mixes are supplied at 50X concentration and are available separately or as a component in the Advantage 2 and Genomic PCR Kits.

  Note: LD-Insert Screening Amplimers can be used in conventional PCR reactions with TITANIUM Taq Polymerase if the expected size of inserts is less than 3 kb. Conventional PCR with a single polymerase will not amplify inserts over 3 kb.

- **10X PCR reaction buffer**

  Supplied with Advantage PCR Kits and Polymerase Mixes. Otherwise, use the 10X reaction buffer supplied with your primary polymerase.

- **50X dNTP mix** (10 mM each dNTP; for PCR; provided in Advantage PCR Kits). Mix contains:

_____ 10 mM dATP
_____ 10 mM dCTP
_____ 10 mM dGTP
_____ 10 mM dTTP

- **0.5-ml PCR reaction tubes** (e.g., Applied Biosystems GeneAmp® 0.5-ml reaction tubes [Cat. No.N801-0737 or N801-0180])
- **PCR-grade ddH_2O** (sterile, non-autoclaved) We use Millipore-filtered H_2O for most PCR applications. We recommend that you **do not** autoclave H_2O for PCR, as the recycled steam in some autoclaves can introduce salts and other contaminants that may interfere with PCR.

III. Additional Materials Required continued

- **Mineral oil** (Sigma No.M-3516)
- **Thermal cycler**
- **Dedicated pipettors**
- **PCR pipette tips** for the above pipettors and equipped with hydrophobic filters. Do not autoclave pipette tips.
- **DNA size markers** (See Section V.B.9 for specific recommendations)
- **10X Stop/loading buffer** (See Sambrook *et al.* [1989] for recipes)
- **95% Ethanol**

IV. Template Preparation Protocols

A. Preparing Plasmid Templates from *E. coli*

The rapid, boiling method of plasmid isolation from *E. coli* is suitable when screening inserts in MATCHMAKER® vectors grown in *E. coli*.

For most applications, plasmid DNA can be prepared by placing a single colony in 25 µl of deionized H_2O, and then heating the tube at 95°C for 5 min. Use 5 µl of the resulting solution as a template for PCR screening.

B. Preparing Plasmid DNA Templates from Yeast Cells

The MATCHMAKER AD LD-Insert Screening Amplimer Set (Cat No. 630433) is used to amplify inserts in the activation domain (AD) hybrid cloning vectors provided in:

- the MATCHMAKER Two-Hybrid System (Cat No. K1605-1)
- the MATCHMAKER Two-Hybrid System 2 (Cat No. K1604-1)
- MATCHMAKER Libraries (many)
- the MATCHMAKER Random Peptide Library (Cat No. NL4000AA).

Such vectors include pGAD10, pGAD424, pGAD GL, pGAD GH, λACT, pACT, λACT2, and pACT2.

The MATCHMAKER LD Amplimer Set is most often used with plasmid DNA templates prepared from yeast cells. For reliable recovery of plasmids from yeast, we recommend the YEASTMAKER™ Yeast Plasmid Isolation Kit (Cat No. 630441) or one of the protocols in the Clontech Yeast Protocols Handbook (PT3024-1).

There are several yeast plasmid isolation procedures currently in use; for examples, see Ausubel *et al.* (1994; units 13.11 & 20.1.15); Hoffman & Winston (1987); and Kaiser & Auer (1993). These various protocols differ primarily in the method used to break the cell walls. Some procedures utilize lyticase to weaken the cell walls, while others use physical disruption by vortexing with glass beads; some methods use a combination of enzymatic and physical disruption. The various methods also differ in the final steps for cleaning up the plasmid DNA: some use phenol:chloroform extraction, while others recommend CHROMA SPIN™ Columns. Both purification methods work with either cell disruption procedure. However, all yeast plasmid preps contain a significant amount of yeast genomic DNA.

Plasmid DNA isolated from yeast is generally of sufficient yield and purity for use as a PCR template or for transforming *E. coli*. (Typically, 1–5 µl of yeast plasmid prep is used as a template for PCR screening.) However, these plasmid preps are not suitable for sequencing or restriction enzyme digestion. If the DNA is to be used for these applications, it should be isolated from *E. coli* using a standard mini-prep procedure (Sambrook *et al.*, 1989).

IV. Template Preparation Protocols continued

When inoculating overnight yeast cultures for preparation of plasmids, use a minimal synthetic defined (SD) medium that will maintain selection on the desired plasmid, and (if appropriate) on the two-hybrid interaction. For positive clones identified in a GAL4-based MATCHMAKER Two-Hybrid System, use SD/–Leu/–Trp/–His.

Growth in SD/–Leu alone maintains selection for any AD/library plasmid. However, some colonies may contain two or more different AD/library plasmids, in which case the positive interacting plasmid may be lost when the cells are grown without active selection for the positive interaction [i.e., growth on medium lacking His or Leu]. This is particularly likely if the true positive AD/library plasmid expresses a fusion protein that is mildly toxic to the host cell.)

Recipes for YPD, SD medium, and dropout (DO) supplements are provided in the Clontech Yeast Protocols Handbook (PT3024-1). Yeast growth media are also available from Clontech. Note that yeast cells grow more slowly in triple DO medium than in YPD or single DO medium; they also grow more slowly in SD/Gal/Raf medium than in SD medium with glucose as the carbon source.

V. LD-Insert Screening Protocol

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

1. Thermal cycler

   The guidelines for cycling parameters in this protocol have been developed using a Applied Biosystems DNA Thermal Cycler 480 and Applied Biosystems GeneAmp 0.5-ml PCR reaction tubes. The optimal cycling parameters may vary with different templates, primers, experimental protocols, tubes, and thermal cyclers. Refer to the Troubleshooting Guide (Section VI) for suggestions on optimizing PCR conditions.

2. Template quality

   Because of the exponential nature of PCR amplification, many conventional PCR applications work well with templates of average or even low quality. In many applications such as screening cDNA inserts with Clontech's LD-Insert Screening Amplimers, LD PCR with Advantage® 2 Polymerase Mix will also tolerate a wide range of template quality.

   However, the longer the target, the more important the quality of the template becomes. This is because the number of unnicked, full-length targets decreases as the target length increases, so poor quality DNA will have very few large unnicked targets. Furthermore, some depurination occurs when DNA is denatured during thermal cycling, and this can eventually lead to strand cleavage. Therefore, it is particularly important to prepare high-quality, high molecular weight DNA when amplifying large targets, especially cosmid or genomic DNA inserts.

3. Good PCR practices

   a. Prepare reactions with dedicated pipettors in a dedicated work space

      Due to the tremendous amplification power of PCR, minute amounts of contaminating DNA can produce nonspecific amplification; in some instances, contaminants can cause DNA bands even in the absence of added template DNA. We recommend that you set up your PCR reactions in a dedicated lab area or noncirculating containment hood and use dedicated pipettors, PCR pipette tips with hydrophobic filters, and dedicated solutions. Perform post-PCR analysis in a separate area with a separate set of pipettors.

   b. Pipetting

      Because of the small volumes used in PCR experiments and the potential for tube-to-tube variation, careful pipetting technique is extremely important. Always be sure that no extra solution is on the outside of the pipette tip before transfer. When adding solution to a tube, immerse the tip into the reaction mixture, deliver the solution, and pipet up and down several times.

V. LD-Insert Screening Protocol continued

c. Use a Master Mix

Using a Master Mix greatly reduces tube-to-tube variation whenever multiple PCR reactions are performed. If multiple templates are being tested with the same primers, include the primers in the Master Mix. If one template is being tested with multiple primer sets, include the template in the Master Mix. If running several sets of parallel samples, assemble multiple master mixes (e.g., each with a different set of primers.) The Master Mix should be thoroughly mixed before use (i.e., vortexed without bubbling).

d. Always perform a positive control (provided) and a negative control using PCR-grade H_2O as a template.

4. Use antibody-mediated hot start with TaqStart or TthStart Antibody

Do not use a manual hot start or wax-bead-based hot start when using Advantage 2 or Advantage Genomic Polymerase Mix. Hot start is automatic when using Advantage Polymerase Mixes because the enzyme mix already contains TaqStart or TthStart Antibody. Antibody-mediated hot start with TaqStart or TthStart has been proven to be at least as effective as manual hot start (D'Aquila *et al.*, 1991) or wax-bead-mediated hot start (Chou *et al.*, 1991).

5. Use of additives

TaqStart® and TthStart Antibodies bind their respective DNA polymerases with high affinity under the conditions described in this protocol. The addition of 2–5% DMSO does not interfere with TaqStart or TthStart function and may improve results in some instances. The addition of formamide or other cosolvents may interfere with TaqStart or TthStart function. Excessive glycerol, solutes (e.g., salts), pH extremes, or other deviations from the recommended reaction condition may reduce the effectiveness of the antibody and/or DNA polymerases.

B. PCR Protocol for LD-Insert Screening

1. Place all components on ice and allow to thaw completely. Mix each component thoroughly before use.
2. Prepare a Master Mix by combining the specified components in a suitable tube. For screening cDNA inserts with Advantage 2 DNA Polymerase, use Table I. For screening genomic inserts with Advantage Genomic Polymerase Mix, use Table II. See the Advantage protocol for final concentrations of enzyme and buffer.

Note: The LD-Insert Screening Amplimers can be used in conventional PCR reactions with TITANIUM Taq Polymerase if the expected size of inserts is less than 3 kb.

V. LD-Insert Screening Protocol continued

TABLE I: ASSEMBLING MASTER MIXES WITH ADVANTAGE 2 POLYMERASE MIX (Mg^{2+} included in the buffer)

Reagent	1 rxn	10 rxns (+ 1 extra)	25 rxns (+ 1 extra)
PCR-grade deionized H_2O	36 µl	396 µl	936 µl
* 10X Advantage 2 PCR Buffer	5 µl	55 µl	130 µl
5' LD Amplimer (20 µM)	1 µl	11 µl	26 µl
3' LD Amplimer (20 µM)	1 µl	11 µl	26 µl
50X dNTP Mix (10 mM ea.)	1 µl	11 µl	26 µl
* Advantage 2 Polym. Mix (50X)	1 µl	11 µl	26 µl
Total	45 µl	495 µl	1170 µl

TABLE II: ASSEMBLING MASTER MIXES WITH ADVANTAGE GENOMIC POLYMERASE MIX (Mg^{2+} not included in the buffer)

Reagent	1 rxn	10 rxns (+ 1 extra)	25 rxns (+ 1 extra)
PCR-grade deionized H_2O	33.8 µl	371.8 µl	878.8 µl
* 10X Tth PCR Reaction Buffer	5 µl	55 µl	130 µl
* 25 mM $Mg(OAc)_2$	2.2 µl	24.2 µl	57.2 µl
5' LD Amplimer (20 µM)	1 µl	11 µl	26 µl
3' LD Amplimer (20 µM)	1 µl	11 µl	26 µl
50X dNTP Mix (10 mM ea.)	1 µl	11 µl	26 µl
* Advantage Genomic Polymerase Mix (50X)	1 µl	11 µl	26 µl
Total	45 µl	495 µl	1170 µl

* If you are not using an Advantage Polymerase Mix: Use Table I if Mg^{2+} is included in the 10X reaction buffer and Table II if Mg^{2+} is supplied separately (or refer to the manufacturer's recommendations). Use 1 µl of your 50X enzyme mix and 1 µl of the corresponding 10X buffer.

3. Mix **thoroughly** by vortexing and spin the tube briefly to collect all the liquid in the bottom of the tube. Vortex the tube in an upright position to prevent bubbles.

V. LD-Insert Screening Protocol continued

4. For each PCR reaction (including controls), combine the following in a 0.5-ml PCR tube. Use 5 µl of PCR-grade ddH_2O as a template for the negative control.

45 µl	Master Mix
5 µl	DNA template
50 µl	**Total**

5. Spin the tubes briefly to collect all the liquid in the bottom of the tube.
6. Add 1–2 drops of mineral oil to each tube to prevent evaporation during cycling and cap firmly. A good "capping" of mineral oil should have a well-defined meniscus between the two phases.
7. Commence thermal cycling in a Applied Biosystems DNA Thermal Cycler Model 480 (or equivalent model)[a]. Use the following guidelines when setting up your initial experiments with the Advantage Polymerase Mixes. These are general guidelines—the optimal parameters may vary with different thermal cyclers, templates, and other experimental variables.

Target Size	Cycle Parameters	Target Size	Cycle Parameters
< 5 kb:	• 94°C for 1 min • 25–35 cycles 94°C 30 sec[a] 68°C 3 min • 68°C for 3 min[b] • Soak at 15°C	10–20 kb:	• 94°C for 1 min • 25–35 cycles 94°C 30 sec[a] 68°C 12 min • 68°C for 12 min[b] • Soak at 15°C
5–9 kb:	• 94°C for 1 min • 25–35 cycles 94°C 30 sec[a] 68°C 6 min • 68°C for 6 min[b] • Soak at 15°C	20–40 kb:	• 95°C for 1 min • 25–35 cycles 95°C 15 sec[a] 68°C 22 min • 68°C for 22 min[b] • Soak at 15°C

[a] Use the shortest possible denaturation time. Exposure of DNA to high temperatures causes some nicking of single-stranded DNA during denaturation and leads to gradual loss of enzyme activity. Minimizing denaturation time is particularly important in experiments with very large templates where total cycling time can exceed 12 hr. For very rapid cyclers (e.g., "air cyclers" or the GeneAmp PCR System 2400 or 9600), reduce denaturation time to 5 sec.

[b] Optional: This final extension may reduce background in some cases.

V. LD-Insert Screening Protocol continued

8. Transfer a 5-µl sample of your PCR reaction to a fresh tube and add 1 µl of stop/loading buffer. (The remaining 45 µl of the reaction mixture can be subjected to further cycling if you do not see a product.) Analyze your sample(s), along with suitable DNA size markers, by electrophoresis on a suitable agarose/EtBr gel. The percentage agarose and the DNA size markers you choose will depend on the expected range of insert sizes.

   Recommendations for agarose gels:

Expected insert size range	Recommended % agarose	Recommended DNA size markers
0.3 – 1.5 kb	1.5	ϕX173/Hae III
0.5 – 10 kb	1.2	1-kb DNA ladder
> 5 kb	0.8	λ/Hind III

VI. Troubleshooting Guide

The following **general** guidelines apply to most PCR reactions.

A. No product observed

PCR component missing or degraded.	Use a checklist when assembling reactions. Always perform a positive control to ensure that each component is functional. If the positive control does not work, repeat the positive control only. If the positive control still does not work, repeat again replacing individual components to identify the faulty reagent.
Too few cycles	Increase the number of cycles (3–5 additional cycles at a time). If you did not add stop/loading buffer to your entire sample, you can simply perform additional cycles with the remaining 45 µl.
Annealing temp. too high	Decrease the annealing temperature in increments of 2–4°C.
Not enough template	Repeat PCR using a higher concentration of DNA.
Poor template quality	Check template integrity by electrophoresis on a standard TBE-agarose gel. If necessary, repurify your template using methods that minimize shearing and nicking.
Denaturation temp. too high or low	Optimize denaturation temperature by decreasing or increasing by 1°C increments. (A denaturation temperature that is too high can lead to degradation of the template, especially for long target sequences.)
Denaturation time too long or too short	Optimize denaturation time by decreasing or increasing by 10-sec increments. (A denaturation time that is too long can lead to degradation of the template, especially for long target sequences.)
Extension time too short	(Especially with longer templates) Increase the extension time in increments of 1 min.
Too little enzyme	Advantage Polymerase Mixes are 50X for most applications. In rare cases, however, 1X Advantage Polymerase Mix may be too low. Always try optimizing the cycle parameters as described above before increasing the enzyme concentration. However, in most cases, increasing the amount of enzyme will lead to high levels of background.

VI. Troubleshooting Guide continued

$[Mg^{2+}]$ is too low	Try adjusting the $[Mg^{2+}]$ in increments of 0.25 mM. (This generally does not apply to the Advantage 2 Polymerase Mix, since the N-terminal deletion mutant Taq DNA polymerase in the mix has a broad optimal range of Mg^{2+} concentrations.)
[dNTPs] is too low	When used as recommended, the 50X dNTP mix provided with the kit gives a final concentration of 0.2 mM of each dNTP. In our experience, this concentration of dNTPs is suitable for a wide range of applications. If you are preparing your own dNTPs, be sure that your final concentration of each dNTP in the reaction is 0.2 mM. Some manufacturers recommend using concentrations higher than 0.2 mM of each dNTP when amplifying large templates. However, we have had no troube amplifying large templates using 0.2 mM for each dNTP. We have successfully amplified up to 35 kb with the Advantage Genomic PCR Kit, so it is unlikely that [dNTP] is limiting. Note that if you do increase the concentration of dNTPs, you will also need to increase the $[Mg^{2+}]$ proportionately.
Difficult target	Some targets are inherently difficult to amplify. In most cases, this is due to unusually high GC-content and/or secondary structure. In some cases, the addition of DMSO to 2–5% may help.

B. Multiple products

Multiple colonies	You may have picked overlapping colonies. Repeat your DNA isolation, being sure to pick well-isolated colonies. If necessary replate or restreak to obtain well-separated colonies.
Too many cycles	Reducing the cycle number may eliminate nonspecific bands.
Annealing temp. too low	Increase the annealing/extension temperature in increments of 2–3°C.
Contamination	See Section D below.

C. Products are smeared

Too many cycles	Reducing the cycle number by 3–5 may eliminate nonspecific bands.

VI. Troubleshooting Guide continued

Denaturation temp. too long	Try increasing the denaturation temperature in increments of 1°C.
Extension time too long	Decrease the extension time in 1–2-min increments.
Poor template quality	Check template integrity by electrophoresis on a denaturing agarose gel. Repurify your template if necessary.
Too much enzyme	Advantage Polymerase Mixes are 50X for most applications; however, 1X Advantage Polymerase Mix may be too high for some applications. If smearing is observed, first try optimizing the cycle parameters as described above, then try reducing the enzyme concentration to 0.5–0.2 X.
$[Mg^{2+}]$ is too high	Try adjusting the $[Mg^{2+}]$ in increments of 0.25 mM. (This generally does not apply to the Advantage 2 Polymerase Mix, since the N-terminal deletion mutant Taq DNA polymerase in the mix has a broad optimal range of Mg^{2+} concentrations.)
Contamination	See Section D below.

D. Dealing with contamination

Contamination most often results in extra bands or smearing. It is important to include an H_2O control (i.e., a control using ddH_2O as the "template") in every PCR experiment to determine if the PCR reagents, pipettors, or PCR reaction tubes are contaminated with previously amplified targets.

If possible, set up PCR reactions and perform post-PCR analysis in separate laboratory areas with separate sets of pipettors.

Laboratory benches and pipettor shafts can be decontaminated by depurination. Wipe surfaces with 1N HCl followed by 1N NaOH. Then neutralize with a neutral buffer (e.g., Tris or PBS) and rinse with ddH_2O.

It is advisable to use one of the commercially available aerosol-free pipette tips.

There is an enzymatic method for destroying PCR product carryover (Longo *et al.*, 1990). It involves incorporation of dUTP into the PCR products and subsequent hydrolysis with uracil-N-glycosylase (UNG).

As noted in Section B above, when performing PCR directly on bacterial colonies, failure to isolate single colonies will also produce multiple bands.

VII. References

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VIII. Related Products

For the latest and most complete listing of all Clontech products, please visit **www.clontech.com**

• Advantage® 2 PCR Kit	639206 639207
• Advantage Genomic PCR Kit	639103 639104
• TaqStart® Antibody	639250 639251
• TITANIUM™ Taq PCR Kit	639210 639211
• cDNA and Genomic Libraries	many
• MATCHMAKER® cDNA and Genomic Libraries	many
• Pretransformed MATCHMAKER® cDNA Libraries	many
• Kits for generating and/or screening two-hybrid libraries	
– MATCHMAKER® Two-Hybrid System 3	630303
– MATCHMAKER® One-Hybrid System	630302
– MATCHMAKER® Library Construction & Screening Kit	630445
• λTriplEx cDNA Libraries	many
• YEASTMAKER™ Yeast Plasmid Isolation Kit	630441

Notes

Notes