Antibody Design LABORATORIES

fADL-2blue - Trypsin-Sensitive Phage Vector

INSTRUCTION MANUAL

fADL™-2blue Phage Vector for Phage Display

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Description

Introduction

The fADL™-2blue vector is a type 3 phage display vector (Smith 1997) with a cloning site for display on the N-terminal side of the full-length gene III protein. Secretion in the periplasm of the fusion protein is driven by the PelB leader peptide, in place of the wild-type gene III protein leader sequence.

fADL™-2blue derives from the phage vector fd-tet (Zacher 1980) where the tetracycline resistance genes derived from Tn10 have been replaced by the ampicillin resistance beta-lactamase Bla gene. It has a smaller size (7861 bp), making cloning easier. Like fd-tet, the number of double-stranded RF copy number is relatively low, about 60 copies in stationary-phase cells. Therefore fADL-2blue is well tolerated by the host, giving large colonies and small plaques. Yield of physical particles is about $5x10^{11}$ virions/ml, similar to fd-tet; infectivity of the virions is around 5%, depending on the insert sequence. Display is usually multivalent with limited polyphage production, even in the case of large polypeptides such as scFvs. The presence of a trypsin-sensitive site in the linker with protein pIII enables elution of bound phage with trypsin (Smith 2010).

fADL™-2blue differs from fADL™-1e by the presence of a MluI and an SpeI site in the cloning site and a G->A mutation in g8p sequence at position 1403 making the major coat protein VIII sequence identical to M13 protein VIII. This mutation improves cross-reactivity between M13 antibodies and fADL™ virions. fADL™-2blue requires ampicillin for selection. Colonies takes a pale blue color in the presence of IPTG and X-Gal.

Content, Shipping & Storage

Content

VECTOR	COMPOSITION	AMOUNT
fADL™-2BLUE	20 µl at 0.5 µg/µl of DNA vector in DNA Conservation Buffer (Tris-HCL 5 mM, EDTA 0.1 mM, pH 8.5)	10 µg

Shipping & Storage

fADL™-2blue phage vector is shipped on wet ice. Upon receipt, store the vector at -20ºC.

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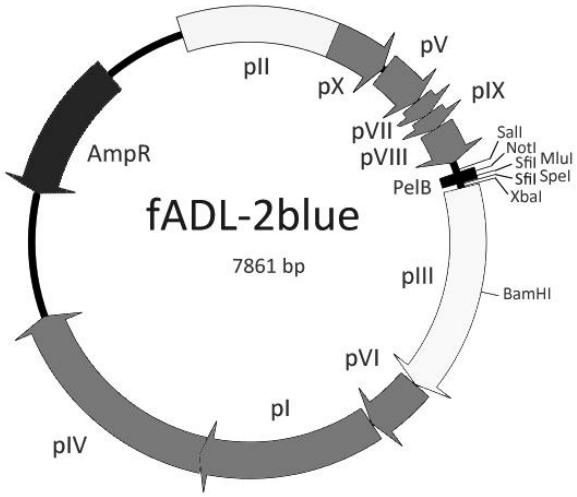
Vector Map

The figure below illustrates the main features of fADL™-2blue vector. The full vector sequence is available online for download in varied formats on the product web page; the total length of the vector is 7993 bp.



Cloning Site

Following is an illustration of fADL-2blue cloning site from the end of the gene VIII sequence to the gene III sequence. The PelB peptide leader sequence (translation *MKYLLPTAAAGLLLLAAQPAMA*) is missing the two last amino acids MA, which will have to be re-introduced during cloning to insure proper reading frame and cleavage (on the C-terminal side of the terminal Alanine).

```
      pVIII terminator                          SalI                          PelB
      ──────────────────────────────────────┐   |                            MetL
1540  AGGCTCCTTT TGGAGCCTTT TTTTTGTCGA CTAACGAGGG CAAATCATGA

                              Not I                                  SfiI/BglI
      leader peptide            |                                      |
      ysTyrLeuLe uProThrAla AlaAlaGlyL euLeuLeuLe uAlaAlaGln
1590  AATACCTATT GCCTACGGCG GCCGCTGGAT TGTTATTACT CGCGGCCCAG

                        MluI      SpeI      Sfi I/BglI
                         |         |          |
      ProAlaMet                             GlyProG lyGlyLeuAr gGlyGlySer
1640  CCGGCCATGT AACGCGTACT AGTGGCCCGG GAGGCCTGCG TGGTGGTTCA

      g3p full length
      ┌──────────···
1690  GCCGAAACTG TTGAAAGTTG TTTAGCAAAA CCTCATACAG AAAATTCATT
```

Feature Table

The main features of fADL™-2blue vector are highlighted in the following table.

FEATURE	LOCATION	DESCRIPTION
Protein VIII terminator	1538-1564	Transcriptional terminator.
PelB leader sequence	1586-1648	PelB leader sequence for export in the periplasm of the host bacteria. The missing terminal methionine and alanine will have to be added during the cloning to obtain a complete leader peptide (MKYLLPTAAAGLLLLAAQPAMA) necessary for proper removal of the leader during the export process.
g3p fusion coding sequence	1690-2910	Full-length gene III fusion protein coding sequence; the fd g3p protein is fused on its N-terminal side to the linker GPGGLSLE; the exact final sequence of the fusion depends on the cloning strategy (see cloning site).
ß-Galactosidase α fragment	5886-6056	Alpha complementation for making blue colonies in the presence of IPTG and X-Gal.
Amp^R	7007-6147	Ampicillin resistance gene (β lactamase)

Restriction Site Summary

Enzyme	Site	Nb	Position	Strand	Isoschizomers
AlfI	(10/12)GCANNNNNNTGC(12/10)	1	4901		
AloI	(7/12)GAACNNNNNNTCC(12/7)	1	7219	-	
ApaLI	G^TGCAC	1	6887		Alw44I VneI
BalI	TGG^CCA	1	5138		MlsI MluNI Mox20I MscI Msp20I
BamHI	G^GATCC	1	2277		
BcgI	(10/12)CGANNNNNNTGC(12/10)	1	6736		
BciVI	GTATCC(6/5)	1	7063	-	BfuI BsuI
BseRI	GAGGAG(10/8)	1	2065		
Bsp1407I	T^GTACA	1	1021		BsrGI BstAUI
BssSI	CACGAG(-5/-1)	1	6884	-	BauI Bst2BI
CspCI	(11/13)CAANNNNNGTGG(12/10)	1	3067		
DraIII	CACNNN^GTG	1	7170		AdeI
DrdI	GACNNNN^NNGTC	1	7213		AasI DseDI
EagI	C^GGCCG	1	1608		BseX3I BstZI EclXI Eco52I
Eam1105I	GACNNN^NNGTC	1	6215		AhdI BmeRI DriI
Eco57I	CTGAAG(16/14)	1	6902	-	AcuI
EcoNI	CCTNN^NNNAGG	1	2996		BstENI XagI
FalI	(8/13)AAGNNNNNCTT(13/8)	1	2543		
KroI	G^CCGGC	1	1639		
MluI	A^CGCGT	1	1651		
NaeI	GCC^GGC	1	1639		PdiI NgoMIV MroNI
NmeAIII	GCCGAG(21/19)	1	6343		
NotI	GC^GGCCGC	1	1607		CciNI
PacI	TTAAT^TAA	1	4190		
PshAI	GACNN^NNGTC	1	990		BoxI BstPAI
PsiI	TTA^TAA	1	7298		AanI
SalI	G^TCGAC	1	1565		
ScaI	AGT^ACT	1	6698		BmcAI ZrmI
SmaI	CCC^GGG	1	1665		Cfr9I TspMI XmaI

SnaBI	TAC^GTA	1	1268		BstSNI Eco105I
SpeI	A^CTAGT	1	1657		AhlI BcuI
StuI	AGG^CCT	1	1671		Eco147I PceI SseBI
SwaI	ATTT^AAAT	1	7394		SmiI
AlwNI	CAGNNN^CTG	2	2244		CaiI PstNI
		2	4441		
ArsI	(8/13)GACNNNNNNTTYG(11/6)	2	430		
		2	5077		
BaeI	(10/15)ACNNNNGTAYC(12/7)	2	1852	-	
		2	2194		
BbvCI	CCTCAGC(-5/-2)	2	1370	-	
		2	1416		
BglI	GCCNNNN^NGGC	2	1634		
		2	1664		
BmrI	ACTGGG(5/4)	2	5932		BmuI
		2	6265		
BsaBI	GATNN^NNATC	2	1149		Bse8I BseJI
		2	4032		
BsmI	GAATGC(1/-1)	2	1803		Mva1269I PctI
		2	3518		
BspMI	ACCTGC(4/8)	2	1113	-	Acc36I BfuAI BveI
		2	2315		
ClaI	AT^CGAT	2	2584		BsuTUI BspDI BseCI Bsa29I BshVI Bsu15I
		2	7493		
EciI	GGCGGA(11/9)	2	2019	-	
		2	6373		
EcoP15I	CAGCAG(25/27)	2	1368	-	
		2	4930		
FspI	TGC^GCA	2	6040		Acc16I NsbI
		2	6440		
GsuI	CTGGAG(16/14)	2	6305	-	BpmI
		2	7510		
MfeI	C^AATTG	2	715		MunI
		2	5209		
PciI	A^CATGT	2	195		PscI
		2	3775		
PvuI	CGAT^CG	2	6020		Ple19I
		2	6587		
PvuII	CAG^CTG	2	5722		
		2	5990		
SfiI	GGCCNNNN^NGGCC	2	1633		
		2	1663		

Absent Sites:
AarI, AatII, AbsI, Acc65I, AccB7I, AccIII, AcvI, AfeI, AflII, AgeI, AjiI, AjuI, AleI, Aor13HI, Aor51HI, ApaI, AscI, AsiGI, AsiSI, Asp718I, AspA2I, AsuII, AsuNHI, AvrII, AxyI, BarI, BbrPI, BbsI, BclI, BfrI, BglII, BlnI, BlpI, BmgBI, BmtI, BpiI, BplI, Bpu1102I, Bpu14I, BsaI, Bse21I, BseAI, BsePI, BsgI, BshTI, BsiWI, BsmBI, Bso31I, Bsp119I, Bsp120I, Bsp13I, Bsp1720I, Bsp19I, Bsp68I, BspEI, BspMAI, BspOI, BspQI, BspT104I, BspTI, BspTNI, BssHII, BssNAI, Bst1107I, BstAFI, BstAPI, BstBI, BstEII, BstPI, BstV2I, BstXI, BstZ17I, Bsu36I, BtrI, BtuMI, Cfr42I, CpoI, CsiI, CspAI, CspI, DinI, Ecl136II, Eco31I, Eco32I, Eco47III, Eco53kI, Eco72I, Eco81I, Eco91I, EcoICRI, EcoO65I, EcoRI, EcoRV, EcoT22I, EgeI, EheI, Esp3I, FbaI, FseI, FspAI, HindIII, HpaI, I-CeuI, I-PpoI, I-SceI, KasI, KflI, Kpn2I, KpnI, Ksp22I, KspAI, KspI, LguI, MabI, MauBI, Mly113I, Mph1103I, MreI, MroI, MspCI, MssI, NarI, NcoI, NheI, NruI, NsiI, NspV, OliI, PI-PspI, PI-SceI, PaeI, PaeR7I, PalAI, PasI, PauI, PciSI, Pfl23II, PflFI, PflMI, PfoI, PinAI, PluTI, PmaCI, PmeI, PmlI, Psp124BI, PspCI, PspEI, PspLI, PspOMI, PspXI, PsrI, PstI, PsyI, PteI, RgaI, RigI, RruI, Rsr2I, RsrII, SacI, SacII, SapI, SbfI, SdaI, SexAI, SfaAI, SfoI, Sfr274I, Sfr303I, SfuI, SgfI, SgrAI, SgrBI, SgrDI, SgsI, SlaI, SphI, SrfI, Sse8387I, SspDI, SstI, Tth111I, Van91I, Vha464I, XbaI, XcmI, XhoI, XmaJI, ZraI, Zsp2I.

Experimental Procedures

Working with fADL-2blue

General Molecular Biology Techniques

Molecular cloning and phage display should be conducted under the supervision of a qualified instructor trained to standard safety practice in a molecular biology laboratory environment. Standard molecular biology procedures can be found in a general molecular biology handbook such as Sambrook (1989).

Working with Filamentous Phage

Keep the bench clean and regularly wiped with 2% bleach to limit phage cross-contamination and only use filtered tips to prevent aerosol contaminations. Phages are known to survive standard autoclaving conditions and are not removed by 0.22 µm filtration. Phages are either killed by heat-treating dry, autoclaved materials in an oven for 4 hours at 105ºC (Phage Display 2001) or by incubation in 2% bleach for at least 1 hour. We recommend to extensively wash with hot water all glass and plastic-ware, then submerge (tubes) or incubate (flasks) with a 2% solution of bleach for at least one hour. Heat-resistant glassware can then be autoclaved in an autoclave that is never used for biological waste while sensitive plastic-ware can be used directly or at best heat-treated as described above.

Bacterial Strains

In theory, any K12 F^+ *E. coli* strain is suitable for phage display using fADL-2blue, but experience has shown that colony size and virion production of fd-tet derivatives vary between strains. We recommend the strains SS320 and TG1, with which fADL-2blue gives large colonies. SS320 has been widely used for phage display. SS320 derives from MC1061 by introduction of a tet^R F' episome (Sidhu 2000). Like most derivatives of MC1061, SS320 can be made highly competent for transformation by electroporation. We also recommend using the parent strain MC1061 for building libraries to limit contaminations by exogenous filamentous bacteriophage in original library stocks. TG1 is a widely used strain for phage display and is well documented in the literature. TG1 suppresses amber codons and can also be made highly competent for transformation by electroporation.

TG1, MC1061 and SS320 phenotypes are highlighted below:

TG1 *supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5, ($r_K^-m_K^-$)*
F' [traD36 $proAB^+$ $lacI^q$ lacZΔM15]

MC1061 *F^- hsdR2 mcrA0 araD139 Δ(araA-leu)*7697 Δ*lac*X74 *galK16 galE15(GalS) λe14$^-$ rpsL150(Str^R) spoT1 thi*

SS320 *hsdR2 mcrA0 araD139 Δ(araA-leu)*7697 Δ*lac*X74 *galK16 galE15(GalS) λe14$^-$ rpsL150(Str^R) spoT1 thi*
F'[proAB+lacIqlacZΔM15 Tn10 (tet^r)]

Plasmid Maintenance

Propagation and maintenance of fADL-2blue is obtained on any F^-, *recA1, endA1 E. coli* strain using ampicilin 100 µg/ml as a selection marker. We recommend testing DNA production before scaling up the culture volume. The use of an F^+ male strain may select for in-frame mutants that can take over the bacterial culture. We routinely isolate fADL-2blue RF dsDNA using DH10B™ from Life Technologies with yields up to 2 mg/l.

Cloning into fADL-2blue

Primer Design and PelB Leader Sequence

A complete PelB leader sequence *MKYLLPTAAAGLLLLAAQPAMA* is necessary for export in the periplasm and proper removal of the leader peptide by host proteases. In the following schema, where [NNN] represents the insert sequence and [Xxx] the translated amino acid sequence, the short hexanucleotide ATGGCN must be appended immediately to the first *SfiI* site to obtain a complete PelB leader encoding sequence; cleavage will occur on the C-terminal side of the terminal alanine (codon GCN):

```
                        Sfi I                                   Sfi I
                          |                                       |
     euLeuLeuLe uAlaAlaGln ProAlaMetA la [Xxx] GlyProGlyG lyGl
1620 TGTTATTACT CGCGGCCCAG CCGGCCATGG CN [NNN] GGCCCGGGA GGCC
```

Cloning in fADL-2blue Using SfiI/BglI Sites

Large libraries in the 1 x 10^9 range and above can easily be constructed using the double BglI/SfiI cloning site.

The *SfiI* restriction enzyme recognizes rare 8-base-long interrupted palindromes GGCCNNNN/NGGCC and leaves 3-nucleotide-long overhangs after digestion. The fADL-2blue cloning site contains one *SfiI* site close to the end of the PelB leader sequence and a second *SfiI* site 8 nucleotides apart from the first site. The PelB sequence of the empty vector has an early termination by an ochre stop codon and no gene III protein is produced by the vector alone.

The *SfiI* restriction enzyme requires two copies of its recognition sequence for cleavage to occur; cleavage of the two sites happens simultaneously through interaction of two *SfiI* tetramers (Wertzell 1995). Vectors bearing two sites very close to each other are cut in trans and digestion might not complete. Therefore we strongly recommend opening fADL-2blue with the alternative *BglI* restriction enzyme, which cuts the shorter 6-base-long interrupted palindromes GCCNNNN/NGGC and generates identical overhangs.

Sites open with BglI will re-ligate with sites open with SfiI as long as overhangs are complementary. Practically, the pentanucleotide NNNNN must be identical to the original vector sequence to handle both ligation of the complementary overhangs and conservation of the amino acid sequence (PelB sequence and linker to protein III).

PREPARATION OF VECTOR DNA FOR CLONING

1. On ice add successively water, *BglI* buffer (1x final), fADL-2blue vector and *BglI* enzyme 5 units/µg DNA; make sure the enzyme volume does not to exceed 1/10 of the total reaction volume.
2. Incubate 4 h to overnight at 37ºC.

3. Inactivate for 20 min at 70ºC.
4. Confirm the digestion by DNA analysis on a minigel.
5. Purify the cut vector.

For routine cloning, a standard DNA purification kit can be used directly after the digestion to remove the excess of buffer, the small DNA stuffer and leftover restriction enzyme. For library construction, phenol/chloroform extraction and/or gel purification may be required.

PREPARATION OF INSERTS

SfiI digestion should be rapid and complete in 4 hours especially for fragments longer or equal to 200 bp where sites are cut in *cis*. *BglI* may be used when the insert sequence is known to be free of *BglI* site and therefore is not recommended for building antibody libraries.

Sequencing of Inserts

The following primers give both strong PCR amplification and sequencing traces.

Forward or Sense Primers

phi8S3 5’- CAAGCTGTTTAAGAAATTCACCTCG

phiS2 5’-ATGAAATACCTATTGCCTACGG

Backward, Antisense or Reverse Primers

psiR2 5’-CGTTAGTAAATGAATTTTCTGTATGAGG

psiR3 5’-GCGTAACGATCTAAAGTTTTGTCG

Nested Sequencing

Often it is easier to sequence an insert by PCR on the bacterial culture supernatant or directly from a colony rather than on tediously isolated plasmids. Use the outward primers phi8S3 and psiR3 together with a DNA polymerase not inhibited by bacterial cultures such as TAQ polymerase for the PCR and sequence the insert with the nested reverse primer psiR2. Use less than 1 µl of bacterial culture supernatant per 50 µl-PCR reaction or the touch of a toothpick on a colony as DNA template.

Counting & Amplifying fADL-2blue Virions

Preparation of bacterial cells

1. Peak a single colony from a SS320 agar plate supplemented with tetracycline 20 µg/ml and inoculate a 3-ml culture of 2xYT medium.
2. Incubate overnight at 37ºC with agitation at 250 rpm.
3. Dilute an aliquot of the culture 1:10 v/v with fresh 2xYT medium in a new culture tube and incubate for one hour at 37ºC with agitation at 250 rpm.

4. Take a small aliquot in sterile conditions and measure the absorbance at 600 nm of a 1:10 dilution in 2xYT medium (A_{600}). Absorbance corrected for dilution should be between 0.5 OD and 1.0 OD and cells are ready to be transduced. If necessary adjust the length of incubation to your conditions.
5. Keep the cells at room temperature on the bench and proceed to the transduction within the hour.

Transduction step

1. Dilute the phage with TBS is necessary.
2. Mix gently an aliquot of the phage dilution with an equal volume or more of bacterial culture.
3. Incubate at 37ºC with agitation at 250 rpm for 30 min.
4. Dilute the culture if necessary with fresh 2xYT medium.
5. Plate immediately on 2xYT/agar plates supplemented with ampicillin 100 µg/ml and incubate o/n at 37ºC.
6. In parallel add ampicillin 100 µg/ml final to the culture and pursue the incubation o/n at 37ºC to amplify the phage if necessary.

Counting transducing units (TU)

The day after count the number of colonies, adjust for dilution and express the results indistinctively as either cfu/ml (colony forming unit) or TU/ml (transducing unit). If you know the infectivity of your virions (normally around 5% for small peptide inserts but sometimes much lower for larger inserts), you can deduce the number of virion particles per ml (v/ml) and verify retrospectively than your multiplicity of infection (MOI) at the time of transduction was less than 0.1 (less than one virion particle per 10 bacteria) counting 1.4E9 bacteria/ml at A_{600} of 1.0 OD.

Systematically add controls to your experiments. Plating the bacterial culture on a plate supplemented with ampicillin 100 µg/ml will insure the absence of contamination by one of your phage prior to transduction; plating dilutions of the bacterial culture will count bacteria and help measure the MOI at the time of transduction. Finally always plate in duplicate and repeat the experiment on a different day with a new batch of bacteria and new phage dilutions for accuracy.

Working with fADL-2blue Virions

Phage Production

The phage vector fADL-2blue does not require a helper phage to produce virions. Cultures can be seeded directly from colonies obtained on ampicillin plates. For optimal conditions of growth and virion production, we recommend a rich medium such as 2xYT medium supplemented with ampicillin 100 µg/ml; incubate overnight at 37ºC and 250 rpm.

Phage Purification

For most applications, including screening of phage libraries, a single PEG precipitation is sufficient as a purification step. Please, consult **Antibody Design Labs** online technical resources at http://www.abdesignlabs.com/technical-resources/ for a purification protocol using PEG precipitation. Sometime, especially when working with large libraries, a second PEG precipitation is indicated to achieve concentrated phage preparations. Note that multiple steps of PEG precipitation will be useful for concentration purposes but will unlikely improve purity; other purification methods beyond the scope of this manual such as purification over a cesium chloride gradient may be necessary. Phages are usually re-suspended in TBS or in PBS; conservatives are not necessary.

Phage Quantification

The two main methods to quantify phages are transduction and spectrophotometry.

Transduction is a functional assay that measures a number of transducing units and is the recommended method to follow a biopanning experiment (the number of eluted TU normally increased rapidly when selection occurs). A detailed protocol is given in the above paragraph Counting & Amplifying fADL-2blue Virions.

Since virions are exclusively made of protein and DNA in a precise ratio, a formula has been devised to convert U.V spectrophometry absorption readings into virion concentrations. Please, consult **Antibody Design Labs** online technical resources at http://www.abdesignlabs.com/technical-resources/ for a protocol for phage concentration determination using spectrophotometry. Using this method, phage concentrations can be expressed in OD using $A_{269-320}$ data. Spectrophotometry is the recommend method to quantify purified phages. For most phages a 10x concentration factor is sufficient during purification; some clones that do not produce a lot of virions may require a 20x or more concentration factor to yield phage concentrated enough for assay purpose. Note that below 0.1 OD (as equivalent phage OD taken in the original bacterial culture), results are usually biased and the concentration of phages is most likely overestimated.

Storage

Phage are extremely resistant particles that will survive almost indefinitely once frozen. On the other hand, peptides and large inserts are more fragile and should not be stored more than a few days in the original culture supernatant at 4ºC, or immediately frozen after purification and stored up to a few weeks at -20ºC or at -80ºC for longer term storage.

Phage preparations can be frozen directly without conservative although some people recommend adding 10% or 15% v/v glycerol. In our hands, in absence of glycerol, TU determination can tolerate up to 2 freeze-thaw cycles and binding assays such as ELISA and Western blots up to 4 freeze-thaw cycles without apparent changes. We normally aliquot libraries and store them at -80ºC for an undetermined period of time and thaw them only once. Beyond those limits, we recommend adding glycerol 10% to the phage preparations.

Contaminations

The possibility of contaminations by other phages, wild-type filamentous phages, helper phages or even fADL-2blue phages from another library should always be in mind. Two reasons favor contaminations; first fd-tet-derived vectors are relatively constrained and make stable superinfections with other phages, in particular ubiquitous wild-type filamentous phage; second contaminations are easily transmitted along successive rounds of screen.

The best treatment of contamination is prevention. Follow precisely the guidelines for working with phages to keep contaminations away; separate vessels and plasticware using other phage systems from the one using fADL-2blue vector; always use an F^- strain such as MC1061 to build the primary libraries.

Sometimes deeper analyses are necessary to diagnostic a contamination. You can try to make plaques with your phage preparation (fADL-2blue makes tiny plaques) or you can prepare RF DNA on an F' recA1 bacterial strain and analyze dsDNA. We find simpler to analyze circular ssDNA from purified phage preparation to verify for the absence of contamination. Please, consult **Antibody Design Labs** online technical resources at http://www.abdesignlabs.com/technical-resources/ for a protocol for analyzing filamentous phage ssDNA by electrophoresis.

Biopanning

Examples of screening workflows and biopanning strategies are illustrated in the MIM™ Phage Display Libraries instruction manual, see product AL101 and AL103.

Appendix

MSDS Information

MSDSs (Material Safety Data Sheets) are available on the **Antibody Design Labs** website at the corresponding product page.

Quality Control

Specifications and quality control are detailed on the online product page. **Antibody Design Labs** certifies that the product will perform according to these specifications.

Technical Support

Visit **Antibody Design Labs'** website at **www.abdesignlabs.com** for technical resources, including manuals, vector maps and sequences, application notes, FAQs, etc.

For more information or technical assistance, call, write, fax, or email us at:

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Fax: 1-858-272-6007 (24 hour)
(Monday – Friday 9:00 AM – 5:00 PM PST)
Email: support@abdesignlabs.com

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