Chapter 25

Production of Fosmid Genomic Libraries Optimized for Liquid Culture Recombineering and Cross-Species Transgenesis

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Abstract

Genomic DNA libraries are a valuable source of large constructs that can contain all the regulatory elements necessary for recapitulating wild-type gene expression when introduced into animal genomes as a transgene. Such clones can be directly used in complementation studies. In combination with recombineering manipulation, the tagged genomic clones can serve as faithful *in vivo* gene activity reporters that enable studies of tissue specificity of gene expression, subcellular protein localization, and affinity purification of complexes. We present a detailed protocol for generating an unbiased genomic library in a custom pFlyFos vector that is optimized for liquid culture recombineering manipulation and site-specific transgenesis of fosmid-size *loci* across different *Drosophila* species. The cross-species properties of the library can be used, for example, to establish the specificity of RNAi phenotypes or to selectively introgress specific genomic *loci* among different *Drosophila* species making it an ideal tool for experimental evolutionary studies. The FlyFos system can be easily adapted to other organisms.

Key words: Fosmid, Genomic library, High molecular weight genomic DNA, FlyFos, Transgenesis

1. Introduction

Genomic DNA libraries have been widely used for gene cloning and whole genome sequencing (1). A whole new range of application for genomic libraries emerged recently in the fields of cell, developmental, and evolutionary biology. In these research areas, it is often desirable to monitor the behavior of modified transgenes re-introduced into the genome to assay tissue-specific gene expression, subcellular

Virginie Orgogozo and Matthew V. Rockman (eds.), *Molecular Methods for Evolutionary Genetics*,
Methods in Molecular Biology, vol. 772, DOI 10.1007/978-1-61779-228-1_25,

protein localization, or affinity purification of protein–protein or protein–DNA complexes. Traditional methods use tagged cDNA clones under the control of various tissue-specific or inducible promoters (2). However, these reporters typically do not recapitulate the wild-type gene expression specificity of the gene under study nor its expression levels. Unlike in cDNA constructs, large genomic clones can be selected in such a way that they likely include all the regulatory elements required to recapitulate the native gene expression, both qualitatively and quantitatively (3–5).

In tissue culture cells and in many model organisms, transformation techniques exists that allow the integration of large genomic constructs (like fosmids and BACs) into host genomes with ease (6). Moreover, new recombination systems enable precise and high-throughput modifications of BAC or fosmid-sized DNA fragments, and thus make them useful for creating reporter constructs (3–5, 7). The genomic clones can be in principle isolated from any species and re-introduced into any other species where large-clone transformation system is available, opening up new possibilities for functional evo-devo studies of partial hybrids. For instance, it has been shown that genomic clones from *Drosophila pseudoobscura*, a species moderately related to *Drosophila melanogaster*, can rescue RNAi phenotypes when introduced into *D. melanogaster* strain carrying RNAi hairpin constructs (8, 9). The divergence (estimated at 17% (10)) in the primary sequence of the orthologous *locus* in *D. pseudoobscura* is sufficient to render the heterospecific transgene immune to RNAi while complementing the endogenous gene function and providing a convenient proof or RNAi phenotype specificity. Tagged heterospecific transgenes can also be monitored in host species to assess the relative contribution of *cis*-regulatory sequences and *trans*-acting factors to the divergence of gene expression pattern among species, which is an important outstanding question in evolutionary developmental biology. All in all, genomic DNA libraries are a valuable source of rescue constructs and gene activity reporters that can be applied both within and across species.

Here, we present a method for creating a DNA library in a customized fosmid backbone (pFlyFos) designed to enable cross-species transgenesis within the genus *Drosophila*. From the library production stand-point, the fosmid packaging system ensures, in contrast to comparable BAC systems, strict size selection of the clones (on average 36 kb) and relatively low number of chimeric clones, which reduces the costs associated with mapping of the library by end-sequencing (3). The pFlyFos vector provides three features key for streamlining subsequent *in vivo* applications. First, it contains an inducible oriV origin of replication that allows maintenance of the construct in single copy state during recombineering manipulation and induction to high copy necessary for DNA isolation prior to transformation into flies. Second, pFlyFos contains attB sequences recognized by phiC31-integrase that can

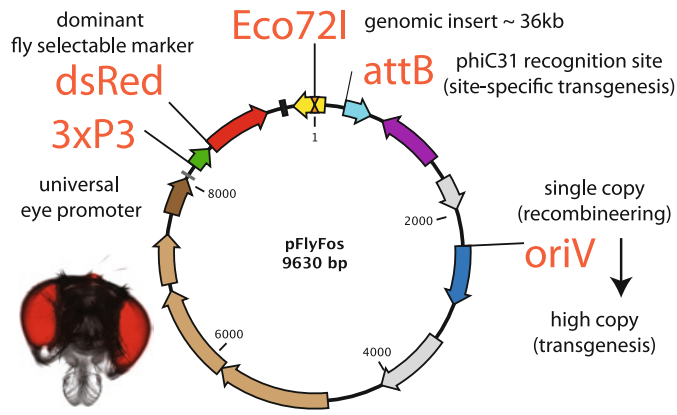

Fig. 1. Overview of the FlyFos vector. Genomic inserts of about 36 kb are cloned into the *Eco*72I site. An inducible origin of replication (oriV) ensures that the fosmid is maintained as single copy during recombineering, but as high copy number for isolation of the fosmid DNA for transgenesis. The attB sequence recognized by phiC31 integrase is used for site-specific integration into the *Drosophila* genome. The dsRed driven by an eye promoter functions as a dominant selectable marker for isolation of transformants.

catalyze site-specific integration into host genomes carrying attP sequence-containing landing sites (11). Third, for selection of transformants pFlyFos carries a dominant 3xP3-dsRed marker cassette that drives the expression of red fluorescent protein into eyes of many species (12, 13) (Fig. 1). The vector can be easily adapted to other model systems and thus the protocol presented here represents a universal approach to generate *in vivo* rescue construct libraries in species where such tools have not yet been introduced.

We describe all the steps required for generating and characterizing genomic fosmid library using the *D. melanogaster* source material as an example (see Note 1). We first describe a modified protocol for isolation of high molecular weight genomic DNA suitable for library production. This part of the procedure may have to be optimized for other model systems. We next proceed to DNA fragmentation by mechanical shearing, resulting in truly random DNA fragments. For efficient library production these fragments may have to be size selected to narrow down the range of fragment sizes and we provide detailed description on how to achieve that despite the fact that this step is not necessary for *Drosophila* genomic DNA. The actual library production by packaging into phages follows to the large extent the protocol developed by EPICENTRE (14) with minor modifications. Finally, we introduce a highly efficient, 96-well manual and 384-well robotic, protocol for miniprep scale fosmid DNA isolation suitable for end-sequencing and mapping of the clones. This protocol enables DNA isolation from tens of thousands of clones in a matter of days and has proved to be highly effective for relatively affordable mapping of fosmid libraries by end sequencing.

2. Materials

2.1. High Molecular Weight Genomic DNA Isolation

1. 1× PBS.
2. 1× PBT: 0.1% Tween 20 in 1× PBS.
3. 50% Household bleach (2.5% sodium hypochloride final concentration).
4. 100% *n*-Heptane.
5. 100% Methanol.
6. Lysis buffer: 50 mM Tris–HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 0.5% SDS, 50 µg/ml proteinase K, 100 µg/ml RNAse A.
7. Phenol:chloroform:isoamyl alcohol (25:24:1) pH 7.5.
8. Chloroform:isoamyl alcohol (24:1).
9. 3 M Potassium acetate (KAc), pH 5.2.
10. 100% Isopropanol.
11. 70% Ethanol.
12. 1× TE buffer, pH 8.0 (see Note 2).
13. Rotating wheel or nutator.

2.2. Shearing the Genomic DNA

1. Pulse Field Agarose (Bio-Rad).
2. 0.5× TBE.
3. MidRange II PFG Marker (NEB).
4. 10 mg/ml Ethidium bromide.
5. CopyControl™ HTP Fosmid Library Production Kit (EPICENTRE).
6. Hydroshear® DNA Shearing Device (DigiLab) with 4–40 kb shearing assembly.
7. A pulse-field gel electrophoresis (PFGE) system, e.g., CHEF Mapper XA (Bio-Rad).

2.3. Size-Selection of the Genomic DNA

1. Pulse Field Agarose (Bio-Rad).
2. 0.5× TBE.
3. MidRange II PFG Marker (NEB).
4. 10 mg/ml Ethidium bromide.
5. SeaPlaque LMP Agarose (LONZA).
6. CopyControl™ HTP Fosmid Library Production Kit (EPICENTRE).
7. A pulse-field gel electrophoresis (PFGE) system, e.g., CHEF Mapper XA (Bio-Rad).

2.4. Final Purification of the Genomic DNA

1. 3 M Potassium acetate (KAc), pH 7.0.
2. 100% Ethanol.
3. 70% Ethanol.
4. Nuclease-free water.
5. Millipore 0.025 μm VSWP membrane (Merck).

2.5. Preparation of the Fosmid Vector

1. LB + chloramphenicol (25 μg/ml).
2. LB + chloramphenicol (25 μg/ml) + arabinose (0.1%).
3. Plasmid Maxi Kit (QIAGEN).
4. Whatmann filter.
5. 50 ml Syringe.
6. 100% Isopropanol.
7. 70% Ethanol.
8. 1× TE buffer, pH 8.0.
9. *Pml*I (*Eco*72I) restriction enzyme (NEB).
10. NEBuffer 1 (NEB).
11. 10 mg/ml Bovine serum albumin (BSA) (NEB).
12. 0.8% Agarose gel.
13. Crystal violet (Sigma): use 1 μg per 1 ml of gel (optional).
14. QIAquick Gel Extraction Kit (QIAGEN).
15. Antarctic phosphatase (NEB).
16. Antarctic phosphatase buffer (NEB).
17. 5 M Lithium chloride (LiCl) solution.
18. Nuclease-free water.

2.6. Ligation, Phage Packaging, and Infection

1. LB.
2. T4 DNA Ligase (NEB).
3. 10× Ligase buffer (NEB).
4. LB + $MgSO_4$ (10 mM) + maltose (0.2%).
5. CopyControl™ HTP Fosmid Library Production Kit (EPICENTRE).
6. Phage dilution buffer (PDB): 10 mM Tris–HCl pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$.
7. Chloroform.
8. Agar plates with LB + chloramphenicol (15 μM).

2.7. DNA Isolation for Clone Mapping

1. LB + chloramphenicol (25 μg/ml).
2. 50% Glycerol (sterilize the solution before use by passing it through 0.22 μm filter).

3. LB + chloramphenicol (25 μg/ml) + arabinose (0.1%).
4. Plasmid Maxi Kit (QIAGEN).
5. 100% Isopropanol.
6. 70% Ethanol.
7. Nuclease-free water.
8. 96-Well deep square well plates (2 ml) or 384-well deep square well plates (0.2 ml) (Genetix).
9. Air-permeable seal (Corning).
10. Shaker for deep well plates.
11. Centrifuge for deep well plates.
12. A liquid handling robot. The protocol described here uses Biomek FX (Beckman Coulter).
13. Sequencing primers: pCC2FOSfwd (GTA CAA CGA CAC CTA GAC) and pCC2FOSrev (CAG GAA ACA GCC TAG GAA).

3. Methods

3.1. High Molecular Weight Genomic DNA Isolation

Fosmid preparation requires exceptionally high quality, high molecular weight DNA. Our phenol–chloroform protocol for flies is applicable to other organisms, though modifications may be necessary.

1. Set up cages containing a total of about 1,000 adult flies.
2. Collect about 1 ml of embryos that are between 0 and 24 h old. Wash embryos thoroughly with tap water (see Note 3).
3. Dechorionate embryos for 2 min in bleach.
4. Wash embryos with 1× PBS.
5. Wash embryos with 1× PBT.
6. Transfer embryos into a bottle containing 1 volume of PBS and 1 volume of *n*-heptane. Use 20 ml of PBS per 1 ml of embryos. Mix by briefly shaking the bottle.
7. Remove PBS (lower phase). Leave the interphase intact.
8. Add 1 volume of methanol and shake vigorously by hand for 1 min. Embryos should sink to the bottom of the bottle.
9. Remove *n*-heptane and interphase.
10. Transfer embryos into a Falcon tube and wash twice with 1 volume of methanol.
11. Remove methanol completely.
12. Add 1 volume of lysis buffer. Lyse for 2–3 h at 55°C. Gently mix by inverting the tube every 15 min.

13. Centrifuge at 4,000× *g* for 30 min. Transfer supernatant to a new Falcon tube.
14. Add 1 volume of phenol:chloroform:isoamyl alcohol. Incubate on a rotating wheel or a nutator for 1 h at 4 °C.
15. Centrifuge at 4,000× *g* for 10 min. Transfer aqueous (upper) phase to a new Falcon tube.
16. Repeat steps 13–14.
17. Add 1 volume of chloroform:isoamyl alcohol. Incubate on a rotating wheel or a nutator for 1 h at 4 °C (see Note 4).
18. Centrifuge at 4,000× *g* for 10 min. Transfer aqueous (upper) phase to a new Falcon tube.
19. Add 0.05 volume of 3 M KAc. Mix by gently inverting the tube.
20. Add 0.7 volume of isopropanol. Incubate on a rotating wheel or a nutator for 30 min at 4 °C.
21. Centrifuge at 6,000× *g* for 15 min. Remove supernatant.
22. Wash the pellet twice with 1 volume of 70% ethanol.
23. Air-dry the pellet for 10 min at room temperature.
24. Dissolve the pellet in 1× TE prewarmed to 55 °C. Store DNA at 4 °C.

3.2. Shearing the Genomic DNA

The isolated genomic DNA has to be sheared to fragments of approximately 40 kb in order to be cloned in a fosmid vector. Fragments that are larger than 60 kb or smaller than 20 kb prevent phage assembly. Moreover, DNA fragments smaller than 20 kb that are included in the library production can lead to unwanted chimeric clones.

1. Dilute the genomic DNA to final concentration of 250 ng/μl with water (see Note 2).
2. Shear the DNA using the HydroShear device (DigiLab). Use 4–40 kb (large) shearing assembly. Since every shearing assembly has slightly different shearing properties, test different speedcodes by shearing about 5 μg of the DNA (minimal shearing volume is 50 μl – dilute the DNA accordingly). We have obtained the best results with the following parameters: speedcode 17, retraction speed 40, 25 shearing cycles, 200 μl sample volume.
3. Verify the shearing results by running a pulse-field gel electrophoresis (PFGE) with 1 μg of the sheared DNA. Include Fosmid Control DNA (100 ng) and MidRange II PFG Marker (500 ng) on the gel for reference. The following parameters are suggested for the Bio-Rad CHEF Mapper XA system. Use 0.8% Bio-Rad Pulse-Field Agarose in 0.5× TBE. Setup a two-state

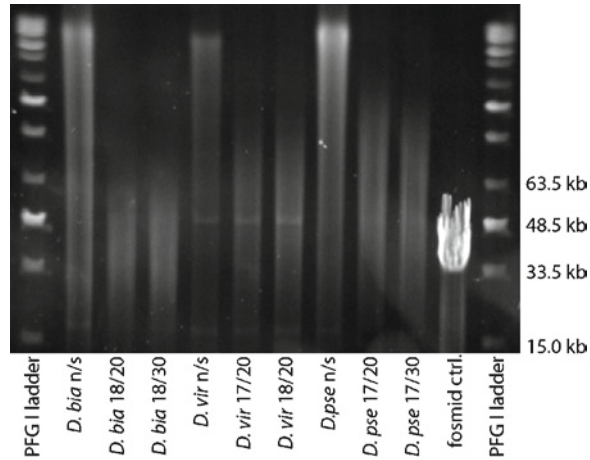

Fig. 2. Sample shearing results for *Drosophila biarmipes*, *Drosophila virilis*, and *D. pseudoobscura DNA*. Electrophoresis was run as described in the text. (n/s) – not sheared gDNA; first number represents speedcode, second shearing cycles, i.e., (18/20) means speedcode 18, 20 shearing cycles. The clear bands visible in the lower part of genomic DNA lanes are the fly mitochondrial DNA. The approximately 50 kb bands visible in *D. virilis* lanes are most likely the yeast mitochondrial DNA contamination (see Note 3).

program at 6.0 V/cm, initial switch at 1.5 s, final switch at 7.0 s, 120° angle, and linear ramping factor. Run the gel at 14 °C for about 20 h. Figure 2 shows sample shearing results.

4. Stain the gel for 30 min with 0.5 μg/ml ethidium bromide in 0.5 ×TBE.
5. Destain the gel for 1 h in 0.5× TBE.
6. Visualize the sheared DNA in UV and determine the best shearing conditions. Choose the speedcode that produces maximal amount of DNA in the range of 30–60 kb and nearly no DNA below 20 kb. Including fragments smaller than 20 kb in the library production process may result in large number of chimeric clones. If shearing under a range of conditions fails to yield DNA that is directly suitable for library production, size-select the DNA as described in Subheading 3.3.
7. Shear 100 μg of the genomic DNA (2 ×200 μl) using the determined conditions. Use the newly sheared DNA for further processing.
8. Setup an end-repair reaction. If PFGE size-selection of the DNA in necessary, use 80 μg of sheared DNA in a 240-μl reaction. Otherwise set up an 80 μl reaction using 20 μg sheared DNA, 8 μl 10 ×End-Repair Buffer, 8 μl 2.5 mM dNTP Mix, 8 μl 10 mM ATP, 4 μl End-Repair Enzyme Mix, and water up to 80 μl.
9. Incubate the reaction at room temperature for 45 min.
10. Heat-inactivate the End-Repair Enzyme Mix at 55 °C for 10 min. If PFGE size-selection is not needed, proceed directly to Subheading 3.4.

3.3. Size-Selection of the Genomic DNA (Optional) (See Note 5)

If shearing under a range of conditions fails to yield DNA that is directly suitable for library production, size-selection of the DNA by preparative pulse-field gel electrophoresis is required.

1. Load the end-repair reaction onto the 0.8% PFGE gel. Run the gel as in Subheading 3.2, step 3. Run both markers (Fosmid Control DNA and MidRange II PFG Marker) on both sides of the gel. In addition, include aliquots (1 μg) of the end-repaired DNA on both sides of the sample for reference (see Fig. 3a and Note 6).
2. Cut off the marker lanes from the gel, and stain them as described in Subheading 3.2. Mark the position between 24 and 73 kb bands of the MidRange II PFG Marker with a razor blade.
3. Reassemble the gel and excise a gel slice containing the sheared DNA between the marked positions. Excise the reference bands containing the sheared DNA as well. Do not expose sample DNA to UV light.
4. Embed the sample DNA gel slice flanked by reference gel slices in 1% SeaPlaque LMP Agarose in 0.5× TBE buffer. See Fig. 3b for reference.

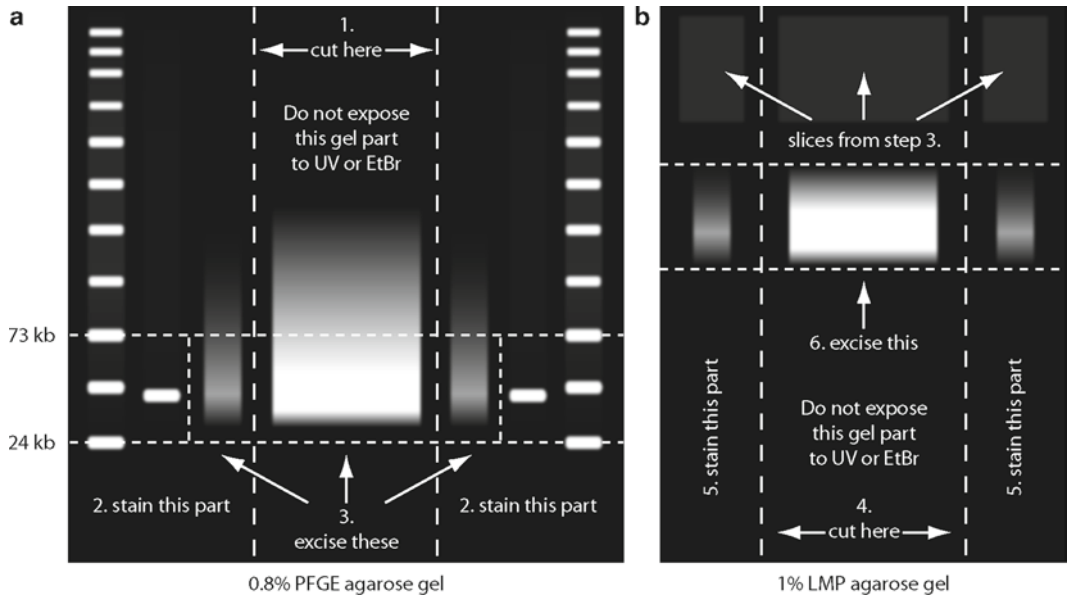

Fig. 3. Running and cutting the PFGE gel and LMP gel. The sheared DNA is run on a PFGE gel (**a**) together with markers (see text for details). After electrophoresis, the marker lanes are cut (*1*) and stained with ethidium bromide (*2*). The identified range is excised from not stained part of the gel containing sample DNA, together with reference lanes (*3*) and run on a LMP gel (**b**). Again, after electrophoresis, the marker lanes are cut (*4*), stained and visualized (*5*). The gel slice containing size-selected DNA in LMP agarose is finally excised (*6*).

5. Run the gel at 5 V/cm at 4 °C for 1.5–2 h to transfer DNA into the LMP agarose.
6. Cut off the reference bands and stain them as described previously. Mark the position of the DNA smear with a razor blade.
7. Reassemble the gel and excise a gel slice containing the sheared DNA between the marked positions. Do not expose sample DNA to UV light.
8. Weight the sample DNA slice in a tared tube.
9. Warm the GELase 50× Buffer to 45 °C. Melt the LMP agarose by incubating the tube at 70 °C for 10–15 min. Quickly transfer the tube to 45 °C.
10. Add the appropriate volume of warmed GELase 50× Buffer to 1× final concentration. Carefully add 2 U (2 µl) of GELase Enzyme Preparation to the tube for each 100 µl of melted agarose. Keep the melted agarose solution at 45 °C and gently mix the solution. Incubate the solution at 45 °C overnight.
11. Transfer the reaction to 70 °C for 10 min to inactivate the GELase enzyme.
12. Remove 500 µl aliquots of the solution into sterile 1.5 ml microfuge tube(s).
13. Chill the tubes on ice for 5 min. Centrifuge the tubes in a microcentrifuge at ≥20,000× *g* for 20 min to pellet any insoluble oligosaccharides. Carefully remove the upper 90–95% of the supernatant, which contains the DNA, to a sterile 1.5-ml tube. Be careful to avoid the gelatinous pellet.

3.4. Final Purification of the Genomic DNA

In the following steps, the sheared (and optionally size-selected) genomic DNA is reprecipitated and dialyzed to remove residual oligosaccharides, protein, salt and to concentrate the DNA solution.

1. Add 0.1 volume of 3 M KAc (pH 7.0) to the end-repaired DNA (Subheading 3.2) or the DNA that has been purified from the LMP agarose gel (Subheading 3.4). Mix gently by inverting the tube.
2. Add 2.5 volumes of ethanol. Mix gently by inverting the tube.
3. Incubate sample at room temperature for 10 min and centrifuge at ≥20,000× *g* for 15 min. Remove the supernatant.
4. Wash DNA pellet with 1 ml room-temperature 70% ethanol, and centrifuge at ≥20,000× *g* for 5 min. Carefully decant the supernatant without disturbing the pellet.
5. Wash DNA pellet again with 1 ml room-temperature 70% ethanol, and centrifuge at ≥20,000× *g* for 5 min. Carefully decant the supernatant without disturbing the pellet. Use a pipet to completely remove the remaining ethanol.

6. Air-dry the pellet for 5–10 min, and redissolve the DNA in 10 µl of warm (55 °C) nuclease-free water.
7. Dialyze the DNA solution against water on the Millipore 0.025 µm VSWP membrane for 1 h. Pour about 20 ml of nuclease-free water into a 10-cm Petri dish. Float the membrane on the water surface (shiny side up) and let it hydrate for 5 min. Apply the DNA solution (10 µl) onto the middle of the membrane. Incubate for 1 h at room temperature. After incubation, recover the DNA into sterile 1.5 ml microfuge tube (see Note 7).
8. Use 1 µl of the solution to determine the DNA concentration by running it on a gel and using 100 ng of the Fosmid Control DNA as a reference. Store the prepared DNA at – 20 °C or use it directly for ligation (recommended).

3.5. Preparation of the Fosmid Vector

The pFlyFos fosmid vector has to be amplified and isolated from bacteria. Isolated fosmid vector is then cut and dephosphorylated. Dephosphorylation of the vector prevents it from self-ligating and greatly increases the cloning efficiency.

1. Inoculate 50 ml of LB + Cm with a single colony of pFlyFos-transformed EPI300 bacteria. Culture overnight at 37 °C with vigorous shaking.
2. Use 2× 5 ml to inoculate 2× 500 ml LB + Cm + Ara in 2,500 ml flasks. Culture overnight at 37 °C. Shake cultures vigorously – at 250 rpm in a bare minimum.
3. Harvest the bacterial cells by centrifugation at 6,000× *g* for 15 min at 4 °C.
4. Resuspend the bacterial pellet from both flasks combined in 50 ml of Buffer P1.
5. Add 50 ml of Buffer P2, mix thoroughly by vigorously inverting 4–6 times, and incubate at room temperature for 5 min.
6. Add 50 ml of chilled Buffer P3, mix immediately and thoroughly by vigorously inverting 4–6 times, and incubate on ice for 30 min.
7. Centrifuge at ≥20,000× *g* for 30 min at 4 °C. Remove the supernatant containing fosmid DNA promptly.
8. Place folded Whatmann filter in a 50-ml syringe. Prewet and compress filter by passing water through the syringe. Use such prepared syringe for filtering supernatant.
9. Precipitate the DNA by adding 105 ml (0.7 volumes) of room temperature isopropanol to the lysate. Centrifuge at ≥15,000× *g* for 30 min at 4 °C, and carefully decant the supernatant.
10. Redissolve the DNA pellet in 500 µl warm (60 °C) TE buffer, pH 8.0, and add Buffer QBT to obtain a final volume of 12 ml.
11. Equilibrate a QIAGEN-tip 500 by applying 10 ml Buffer QBT, and allow the column to empty by gravity flow.

12. Apply the DNA solution from step 10 to the QIAGEN-tip and allow it to enter the resin by gravity flow.
13. Wash the QIAGEN-tip twice with 30 ml Buffer QC. Allow the QIAGEN-tip to empty by gravity flow.
14. Elute DNA with 15 ml Buffer QF.
15. Precipitate DNA by adding 10.5 ml (0.7 volumes) of room temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at $\geq 15,000\times g$ for 30 min at 4°C. Carefully decant the supernatant.
16. Wash DNA pellet with 5 ml room-temperature 70% ethanol, and centrifuge at $\geq 15,000\times g$ for 10 min. Carefully decant the supernatant without disturbing the pellet.
17. Wash DNA pellet again with 5 ml room-temperature 70% ethanol, and centrifuge at $\geq 15,000\times g$ for 10 min. Carefully decant the supernatant without disturbing the pellet.
18. Air-dry the pellet for 5–10 min, and redissolve the DNA in a suitable volume (250 µl) of warm (55°C) nulcease-free water. Store purified fosmid vector at 4°C.
19. Set up a 100-µl restriction digest of the pFlyFos DNA. Use 10 µl NEBuffer 1; 1 µl BSA; 30 µg pFlyFos DNA; 5 µl *PmlI*; water to 100 µl. Incubate at 37°C overnight.
20. Run the digested vector on a 0.8% agarose gel. Include undigested vector (500 ng) and an aliquot of digested vector (500 ng) as a reference.
21. Cut out the agarose slice containing digested DNA (the linear vector migrates slower than superhelical reference plasmid). Avoid UV exposure. Use undigested and digested vector reference samples to determine where agarose should be cut. As an alternative, crystal violet can be used for gel staining.
22. Weight the agarose slice and isolate DNA using QIAquick Gel Extraction Kit. Use two columns (each per 50 µg of restriction digest). Elute vector DNA from each column with 50 µl water. Combine the eluates.
23. Add 12 µl of antarctic phosphatase buffer and 5 µl of antarctic phosphatase to the eluate. Adjust the volume to 120 µl with water and incubate at 37°C for 3 h. Heat inactivate enzyme at 65°C for 15 min.
24. Precipitate DNA by adding 6 µl of 5 M LiCl and 90 µl of isopropanol. Mix by vortexing and centrifuge at $\geq 20,000\times g$ for 15 min at 4°C. Remove the supernatant.
25. Wash DNA pellet with 1 ml room-temperature 70% ethanol, and centrifuge at $\geq 20,000\times g$ for 5 min. Carefully decant the supernatant without disturbing the pellet.
26. Wash DNA pellet again with 1 ml room-temperature 70% ethanol, and centrifuge at $\geq 20,000\times g$ for 5 min. Carefully decant the

supernatant without disturbing the pellet. Use a pipet to completely remove the remaining ethanol.

27. Air-dry the pellet for 5–10 min, and redissolve the DNA in 10 µl of warm (55 °C) nuclease-free water.
28. Dialyze the DNA solution against water on the Millipore 0.025 µm VSWP membrane for 1 h (see Note 7).
29. Use 1 µl of the solution to measure the DNA concentration and adjust it to 500 ng/µl with nuclease-free water. Store the prepared vector at −20 °C or use it directly for ligation (recommended).

3.6. Ligation, Phage Packaging, and Infection

In the following steps, the genomic DNA library is constructed. Sheared genomic DNA is ligated with the vector and packaged into phage particles, which are used for infecting the bacteria. Upon infection, the fosmid gets injected into bacterial cells and maintained by them similarly to plasmids.

1. Inoculate 50 ml of LB with a single colony of EPI300-T1^R cells. Culture overnight at 37 °C. Store culture at 4 °C for up to 48 h. This culture will be used in steps 3 and 13.
2. Set up a 10-µl ligation reaction with 500 ng of cut pFlyFos from Subheading 3.5, 250 ng to 5 µg of sheared genomic DNA, 1 µl of 10 ×Ligase Buffer, and 1 µl of T4 DNA Ligase. The optimal amount of genomic DNA can differ depending on DNA quality. For our ligations, it was 2 µg. Incubate ligation reaction overnight at 16 °C (see Notes 8 and 9).
3. Inoculate 50 ml of LB + $MgSO_4$ + Maltose with 0.5 ml of the EPI300-T1^R overnight culture. Culture cells at 37 °C with vigorous shaking until OD(600) reaches 0.8–1.0.
4. Thaw on ice one tube of the MaxPlax Lambda Packaging Extract. When thawed, immediately transfer 25 µl of the packaging extract to a new tube. Keep the tube on ice. Return the remaining 25 µl of the packaging extract to −80 °C. Avoid exposing MaxPlax Lambda Packaging Extracts to any source of CO_2.
5. Add 10 µl of the ligation reaction to 25 µl of the packaging extract. Mix by pipetting, avoid introduction of air bubbles. Incubate at 30 °C for 2 h.
6. Add the remaining 25 µl of the packaging extract to the reaction tube. Incubate at 30 °C for 2 h.
7. Add 950 µl of the PDB to the packaging reaction. Mix gently by inverting the tube.
8. Add 25 µl of chloroform to precipitate unassembled phage proteins. Mix gently by inverting the tube.
9. Prepare 1:10, 1:100, and 1:1,000 serial dilutions of the phage particles in PDB.

10. Use 10 µl of each dilution and the undiluted phage individually to infect 100 µl of the EPI300-T1^R cell culture (from step 3). Incubate each tube for 1 h at 37°C. Store the remaining phage dilutions and undiluted phage suspension at 4°C for up to 48 h.
11. Plate cells on plates with LB + Cm[15]. Incubate plates overnight at 37°C. Sometimes longer incubation times (up to 36 h) are necessary to obtain large colonies.
12. Count colonies on the plates and determine the phage titer using the following formula:

$$\frac{(\text{Number of colonies}) \times (\text{dilution factor}) \times 1{,}000}{(\text{Volume of phage extract}[\mu\text{l}])} = x$$

(in colony-forming units per ml).

13. Inoculate 50 ml of LB + $MgSO_4$ + Maltose with 0.5 ml of the EPI300-T1^R overnight culture (from step 3). Culture cells at 37°C with vigorous shaking until OD(600) reaches 0.8–1.0.
14. Dilute phages from step 8 accordingly to obtain 100 colonies from 100 µl of cells infected with 10 µl of phage particles. Infect EPI300-T1^R cells for 1 h at 37°C.
15. Plate the library on plates with LB + Cm. During plating, keep the infected cells on ice to prevent the formation of duplicate clones. Incubate plates overnight at 37°C. Sometimes longer incubation times (up to 36 h) are necessary to obtain large colonies.

3.7. DNA Isolation for Clone Mapping (Manual 96-Well Protocol)

1. Pick fosmid clones into 96-well plates with LB + Cm. Seal plates with air-permeable seal and culture overnight at 37°C with vigorous shaking.
2. Aliquot cultures into backup plates, add 0.2 volume of 50% glycerol, mix, and freeze in −80°C.
3. Use 50 µl of the primary culture to inoculate 1,000 µl of LB + Cm + Ara. Seal plates with air-permeable seal and culture overnight at 37°C with vigorous shaking.
4. Harvest the bacterial cells by centrifugation at 6,000× *g* for 15 min at 4°C. Discard the supernatant by inverting plates over the sink and placing them on a stack of paper towels.
5. Transfer 350 µl of Buffer P1 to each well.
6. Vortex plates vigorously to resuspend bacteria.
7. Transfer 350 µl of Buffer P2. Mix by inverting sealed plate 4–6 times.
8. Incubate plates at room temperature for 5 min.
9. Transfer 350 µl of Buffer P3. Mix by vigorously inverting sealed plate 4–6 times.

10. Centrifuge plates at ≥6,000× *g* for 45 min at 4°C.
11. Transfer 900 µl of supernatant into the new plates. Be careful to avoid touching the precipitate. If transferred supernatant contains precipitate, repeat centrifugation (step 10) and transfer supernatant into the new plates.
12. Precipitate DNA by adding 600 µl (~0.7 volume) of isopropanol into each well.
13. Mix by vortexing and centrifuge plates at ≥6,000× *g* for 45 min at 4°C. Discard the supernatant by inverting plates over the sink and placing them on a stack of paper towels.
14. Wash DNA pellet with 1,000 µl of 70% ethanol, and centrifuge at ≥6,000× *g* for 15 min. Discard the supernatant by inverting plates over the sink and placing them on a stack of paper towels.
15. Wash DNA pellet again with 1,000 µl of 70% ethanol, and centrifuge at ≥6,000× *g* for 15 min. Discard the supernatant by inverting plates over the sink and placing them on a stack of paper towels.
16. Place inverted plates on a stack of paper towels. Allow the remaining ethanol to be completely absorbed through capillary forces. Replace towels when they become wet.
17. Air-dry the plates for 15–30 min.
18. Redissolve the DNA in 200 µl of nuclease-free water.
19. End-sequence clones using pCC2FOSfwd and pCC2FOSrev primers.

3.8. DNA Isolation for Clone Mapping (Automated 384-Well Protocol) (See Note 10)

1. Pick fosmid clones into 96-well plates with LB + Cm. Seal plates with air-permeable seal and culture overnight at 37°C with vigorous shaking.
2. Aliquot cultures into backup plates, add 0.2 volume of 50% glycerol, mix, and freeze in −80°C.
3. Use 5 µl of the primary culture to inoculate 100 µl of LB + Cm + Ara in 384-well plates. Array clones originating from four 96-well plates into each 384-well plate. Seal plates with air-permeable seal and culture overnight at 37°C with vigorous shaking.
4. Harvest the bacterial cells by centrifugation at 6,000× *g* for 15 min at 4°C. Remove the supernatant by aspirating 1 mm from the well bottom at speed of 10 µl/s, move within a well at 50% of speed. Discard the supernatant to the waste container. Wash tips in ethanol and the wash station after pipetting is finished.
5. Transfer 15 µl of Buffer P1 to each well. Wash tips in the wash station after pipetting is finished.

6. Vortex plates vigorously to resuspend bacteria.
7. Transfer 15 µl of Buffer P2. Wash tips in the wash station after pipetting is finished.
8. Incubate plates at room temperature for 5 min.
9. Transfer 15 µl of Buffer P3. Wash tips in the wash station after pipetting is finished.
10. Centrifuge plates at ≥6,000× *g* for 45 min at 4 °C.
11. Transfer 40 µl of supernatant into the new plates. Wash tips in the wash station between each pipetting step. Aspirate 2 mm from the well bottom at speed of 10 µl/s, move within a well at 50% of speed.
12. Precipitate DNA by adding 25 µl (~0.7 volume) of isopropanol into each well.
13. Mix by vortexing and centrifuge plates at ≥6,000× *g* for 45 min at 4 °C. Remove the supernatant by aspirating 2 mm from the well bottom at speed of 10 µl/s, move within a well at 50% of speed. Discard the supernatant to the waste container. Wash tips in the wash station after pipetting is finished.
14. Wash DNA pellet with 75 µl of 70% ethanol, and centrifuge at ≥6,000× *g* for 15 min. Remove the supernatant by aspirating 2 mm from the well bottom at speed of 10 µl/s, move within a well at 50% of speed. Discard the supernatant to the waste container. Wash tips in the wash station after pipetting is finished.
15. Wash DNA pellet again with 75 µl of 70% ethanol, and centrifuge at ≥6,000× *g* for 15 min. Remove the supernatant by aspirating 2 mm from the well bottom at speed of 10 µl/s, move within a well at 50% of speed. Discard the supernatant to the waste container. Wash tips in the wash station after pipetting is finished.
16. Place inverted plates on a stack of paper towels. Allow the remaining ethanol to be completely absorbed through capillary forces. Replace towels when they become wet.
17. Air-dry the plates for 15–30 min.
18. Redissolve the DNA in 20 µl of nuclease-free water.
19. End-sequence clones using pCC2FOSfwd and pCC2FOSrev primers (see Note 11).

4. Notes

1. In able hands, the production of the library takes about 2 weeks. The suggested timing schema is presented in Table 1. Protocols described here are partially based on EPICENTRE CopyControl™ HTP Fosmid Library Production Kit protocol (14), QIAGEN

Table 1
Suggested timeline of the library production

	Genomic DNA preparation	Vector preparation
Tuesday	Embryo collection	Vector preculture (step 1 in Subheading 3.5)
Wednesday	Genomic DNA isolation(Subheading 3.1)	Vector induction (step 2 in Subheading 3.5)
Thursday	Testing DNA shearing conditions (steps 1–2 in Subheading 3.2)	Vector preparation (steps 3–18 in Subheading 3.5)
	Check PFGE (steps 3–6 in Subheading 3.2)	
Monday	Final DNA shearing (step 7 in Subheading 3.2)	
	Genomic DNA end-repair (steps 8–10 in Subheading 3.2)	
	Preparative PFGE (step 1 in Subheading 3.3)	
Tuesday	Isolation of genomic DNA from the gel (steps 2–13 in Subheading 3.3)	Vector digest (step 19 in Subheading 3.5)
Wednesday	Final purification of genomic DNA (Subheading 3.4)	Vector purification (steps 20–29 in Subheading 3.5)
	Preparation of EPI300 cells (steps 1 in Subheading 3.6)	
	Ligation (steps 2 in Subheading 3.6)	
Thursday	Phage packaging (steps 4–8 in Subheading 3.6)	
	Phage titering (steps 3 and 9–11 in Subheading 3.6)	
Friday	Final infection and plating (steps 12–15 in Subheading 3.6)	
Monday	Clone mapping (Subheading 3.7)	

Plasmid Maxi Kit protocol (15), and GeneMachines HydroShear® DNA Shearing Device User Manual (16).

2. Use nuclease-free water to prepare DNA handling buffers and all enzymatic reactions.
3. Since embryos are collected on agar plates covered with yeast, the yeast DNA is a very common contaminant. To avoid yeast DNA contamination, wash embryos well with water or PBS. Make sure that yeast were removed completely. Yeast contamination can be fairly easily detected when genomic DNA is run on the pulse-field agarose gel. The ~85-kb superhelical yeast mitochondrial DNA migrates at the speed of ~50 kb linear DNA and can be seen as a distinct band on the gel (Fig. 2).

4. Any mechanical stress should be avoided during preparation of the high molecular weight genomic DNA. Mix DNA solutions only by slowly inverting the tube. When incubating DNA on a rotating wheel of a nutator use very low rpm settings.
5. We observed that the pulse-field agarose gel electrophoresis ensures good resolution of the genomic DNA fragments. However, the efficiency of the DNA isolation from such gel is extremely low. Therefore, the sheared genomic DNA has to be transferred from pulse-field agarose into a low melting point (LMP) agarose that allows for efficient DNA isolation, but gives poor resolution in pulse-field electrophoresis.
6. Exposure of the sheared genomic DNA to UV light leads to the formation of thymine dimers and as a result DNA lesions. Although moderate exposure to UV is usually acceptable for short DNA molecules, high molecular weight DNA is damaged even upon very short UV exposure. The UV damage occurs with a certain frequency per nucleotide. For short DNA molecules, the chance that majority of molecules will be damaged is low, but this is not the case for long DNA molecules. Therefore, it is crucial to avoid exposing genomic DNA to the UV light. All variations of the protocol presented here where the DNA was exposed to UV failed to produce any colonies after packaging and infection.
7. When placing the membrane on the water additional care should be taken to avoid any water droplets on the membrane's top (shiny) surface. Avoid pushing against the membrane with a pipette tip when applying and removing the DNA solution. Dialyze samples in a safe location, so that the dish does not get disturbed.
8. Always use fresh digested vector and fresh sheared genomic DNA for the ligation reaction. The ligation reaction works best when DNA added to the reaction is dissolved in water. Such DNA is not protected from damage by nucleases (storing DNA at −20 °C decreases but does not completely eliminate the risk). Therefore, it is best to use fresh vector and insert for the ligation reactions.
9. We have found that ligation with NEB T4 Ligase at 16 °C overnight is more efficient than ligation with EPICENTRE Fast-Link Ligase for 2 h at room temperature, as suggested in the EPICENTRE protocol. Therefore, we suggest using the NEB T4 Ligase and an overnight incubation time instead of the kit-supplied enzyme.
10. We have evaluated several different variations of the robotic 384-well miniprep protocol by measuring the absorbance

spectra of the isolated fosmid DNA. In the simplest version of the procedure, we simply dump the supernatant after isopropanol precipitation and during ethanol washes by inverting the plate and pushing the liquid out (Fig. 4c). This version of the protocol results in relatively low yield of DNA template, but it was nevertheless successfully used to map about 15,000 clones of the *D. melanogaster* library. Subsequently, we optimized the robotic miniprep protocol to increase the yield and purity of the fosmid DNA. Removing the solvents robotically during DNA precipitation by transferring 25 µl of liquid from each well in each plate to a waste container improved the DNA yield significantly (Fig. 4b). We remove the solvents by placing the robotic tips 2 mm from the bottom of the plate and aspirating at speed of 10 µl/s while moving within a well at 50% of speed. Additionally, a second 70% ethanol wash dramatically reduced the salt contamination of the isolated DNA and produces very high quality template for sequencing (Fig. 4a).

11. The aim of the fosmid libraries described in this protocol is to achieve maximal coverage of the target genome so that most of the genes that can be covered by a fosmid are represented by at least one clone. In order to estimate the number of clones that have to be sequenced in order to achieve such complete coverage, we developed a computer simulation predicting the number of genes cloned in a library of a certain size. The program first randomly shears the DNA into fragments that match the distribution of sizes expected from the packaging step. Subsequently, it uses a random number generator to pick clones, maps them back to the target genome and evaluates the number of genes that are included in each simulated clone. The criteria for inclusion of a gene model into the clone are provided as parameters to the program. We usually require at least 10 kb of DNA upstream and 5 kb downstream of the gene model. By plotting the cumulative number of covered genes against the number of randomly selected clones one obtains an intuitive measure of how many clones need to be sequenced in order to achieve certain coverage of the genome. In our experiments, the actual sequencing of real fosmid clones very closely matched the results of the simulation, suggesting that the method can be used to optimize the library coverage (Fig. 5). The source code for the simulation program is available for download at http://transgeneome.mpi-cbg.de/downloads/shear.c. GNU C compiler (GCC) and GNU Scientific Library (GSL) are required to compile this program.

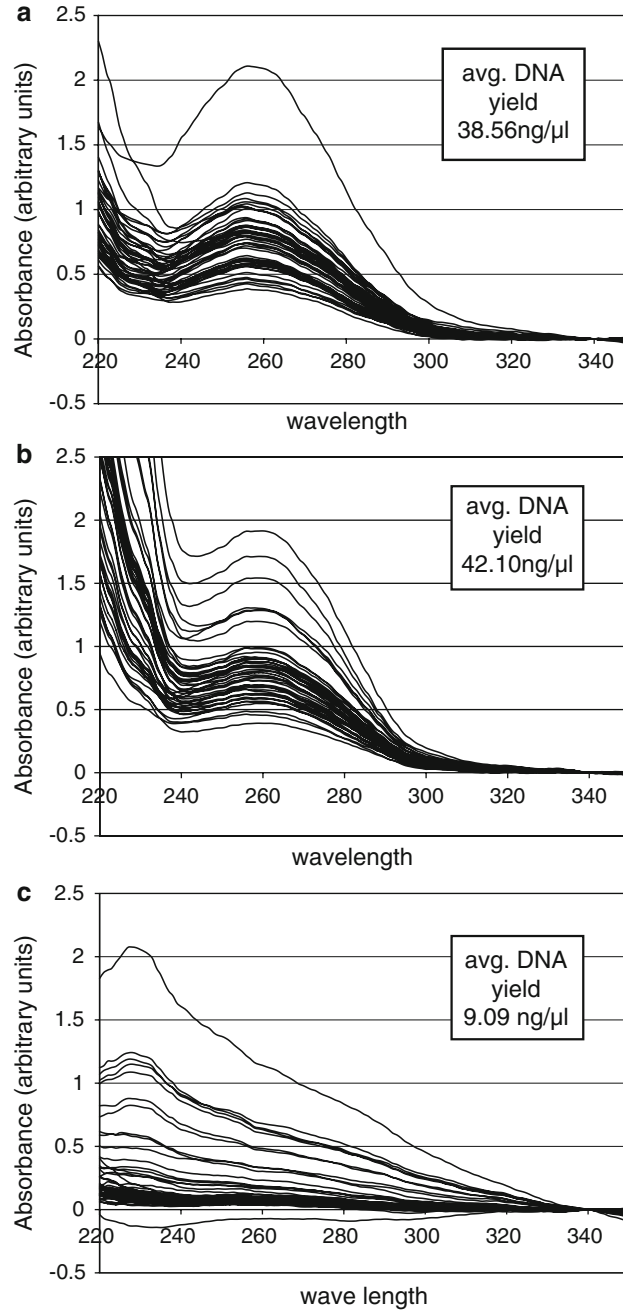

Fig. 4. DNA quality and quantity after high-throughput minipreps. Forty-eight absorbance profiles measured by Nanodrop sampled from a 384-well plate with fosmid DNA isolated using three different versions of the robotic miniprep protocol: robotic removal of the supernatant after protein precipitation step combined with two 70% ethanol washes (**a**), robotic removal of the supernatant combined with single 70% ethanol wash (**b**), and manual removal of the supernatant during a single ethanol wash (by simply tossing the liquid out of the plate) (**c**).

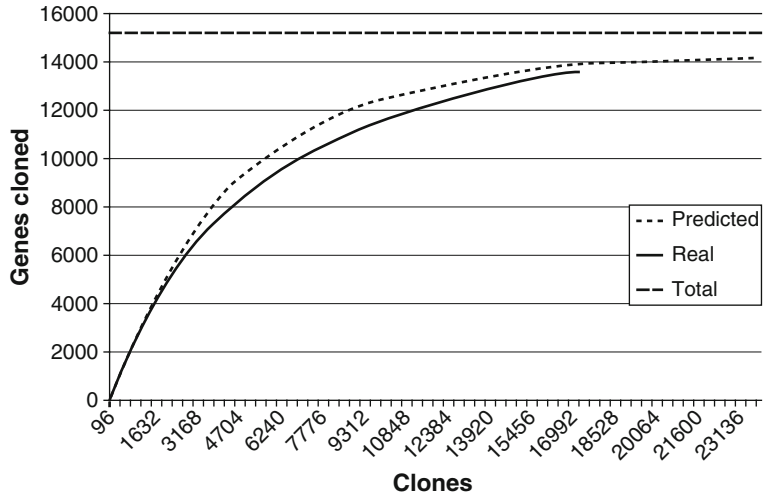

Fig. 5. Comparison of simulated and actual data on library mapping. *Dotted* (in printed version) line shows the cumulative number of genes included in simulated clones as a function of clones picked randomly from the pool of fragments. *Solid* (in printed version) line shows the number of genes included in actual clones as a function of number of clones that were end-sequenced. The *Dashed* (in printed version) line shows the total number of genes in the *Drosophila* genome. Note that about 7% of the genes are larger than a typical fosmid clone and thus cannot be cloned. Data shown for *D. melanogaster* FlyFos library, after Ejsmont et al. (3).

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