

Linker Amplified Shotgun Library Protocol

Purpose

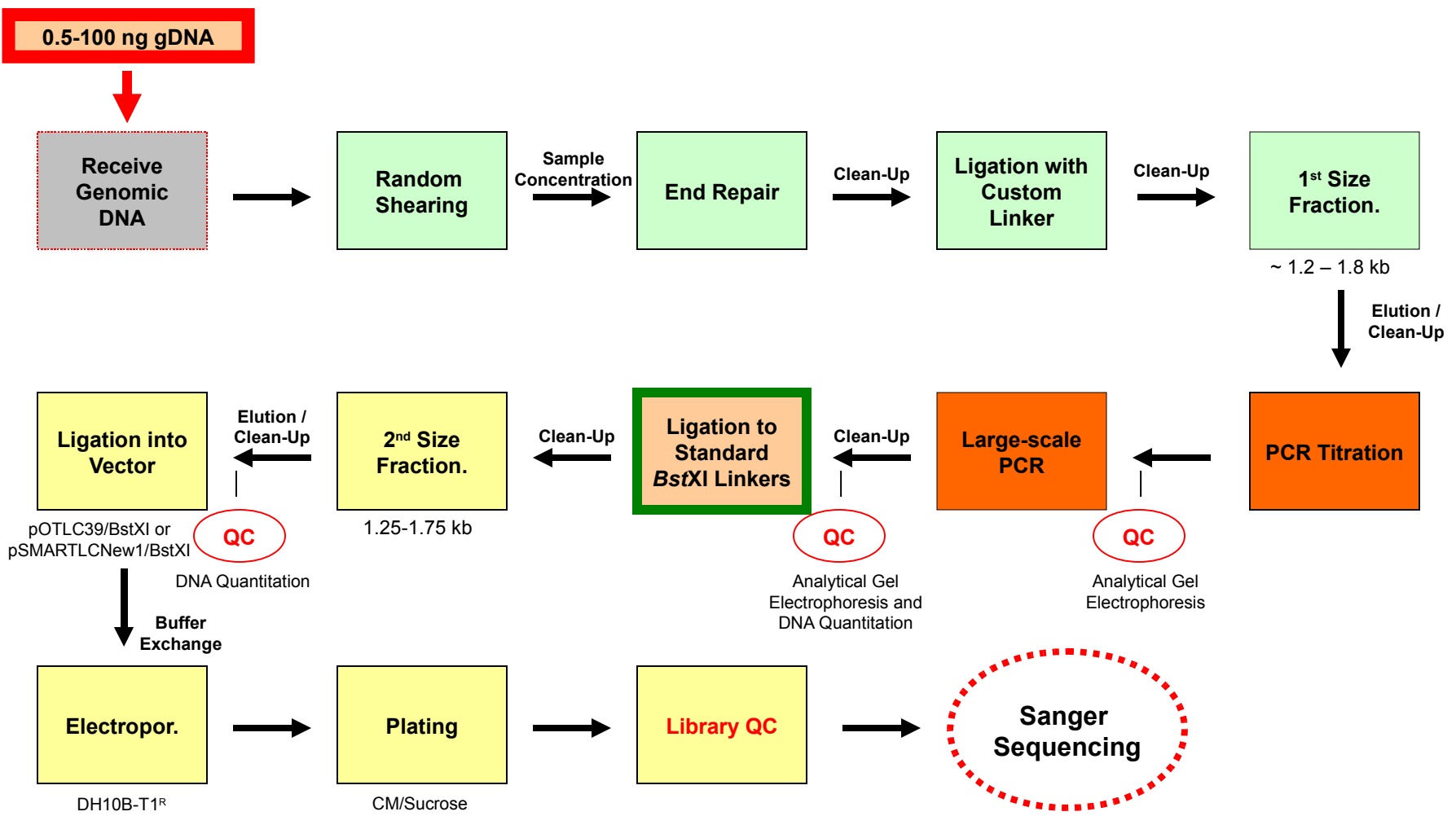
This procedure is intended for the amplification of small quantities of genomic DNA using a linker amplified shotgun library approach in preparation for downstream cloning and sequencing applications.

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Citing Protocol: please contact M. Henn for information regarding citing this protocol ([mhenn\[at\]broadinstitute.org](mailto:mhenn@broadinstitute.org))

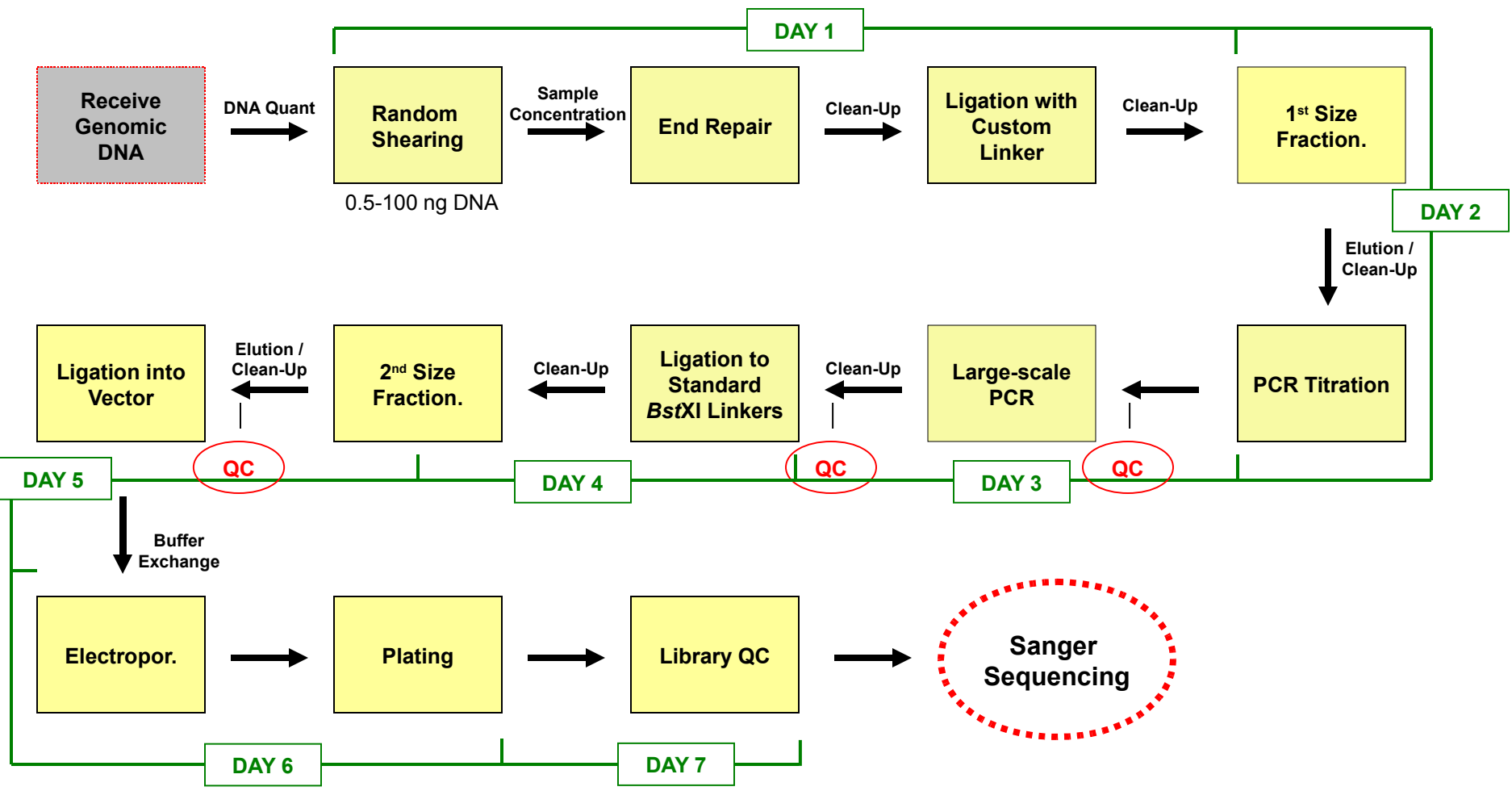
Construction of LASLs

- Process Overview -



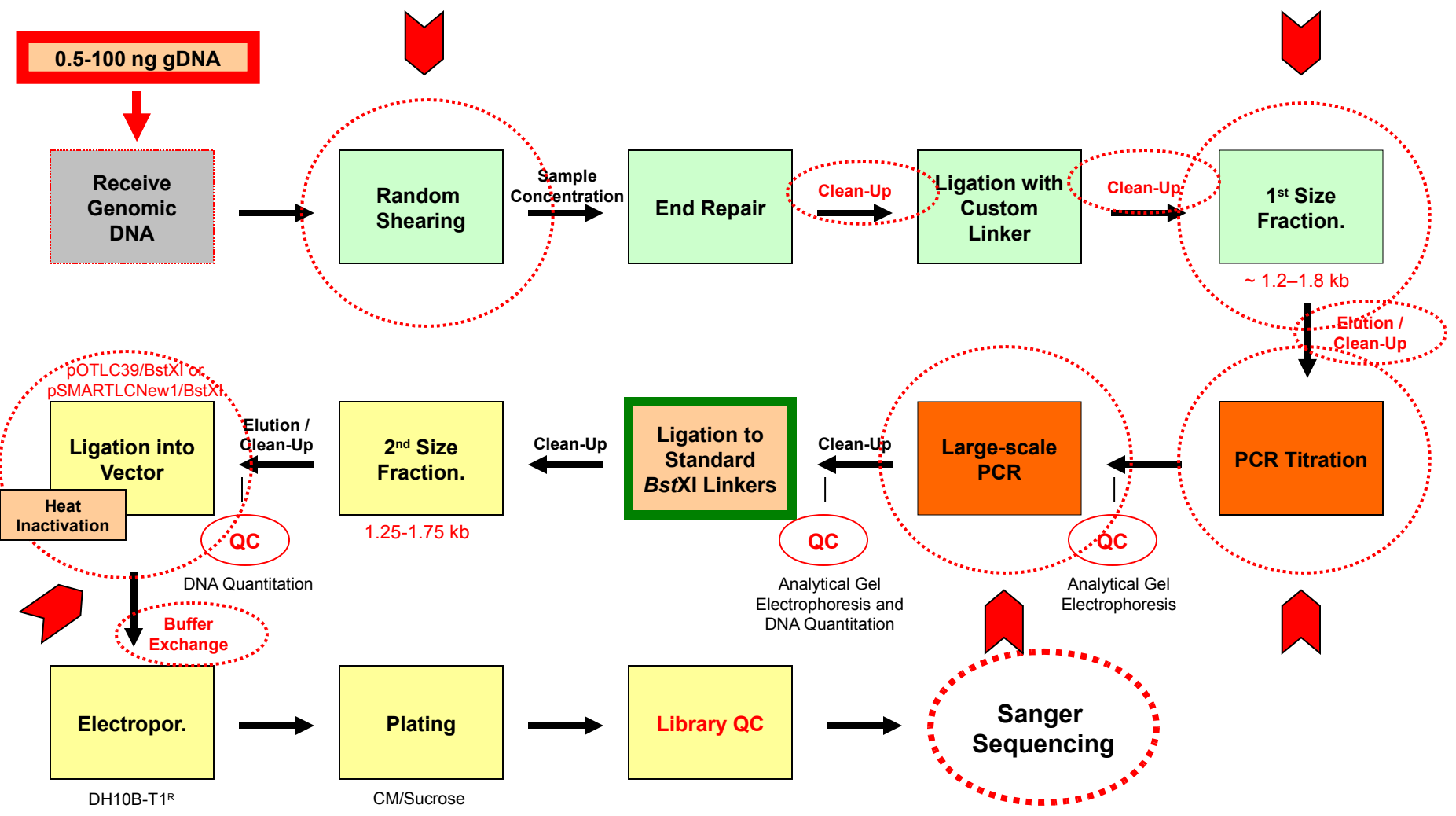
Construction of LASLs

- Current Process (Estimated Work Schedule) -



Construction of LASLs

- Protocol Development & Optimization -



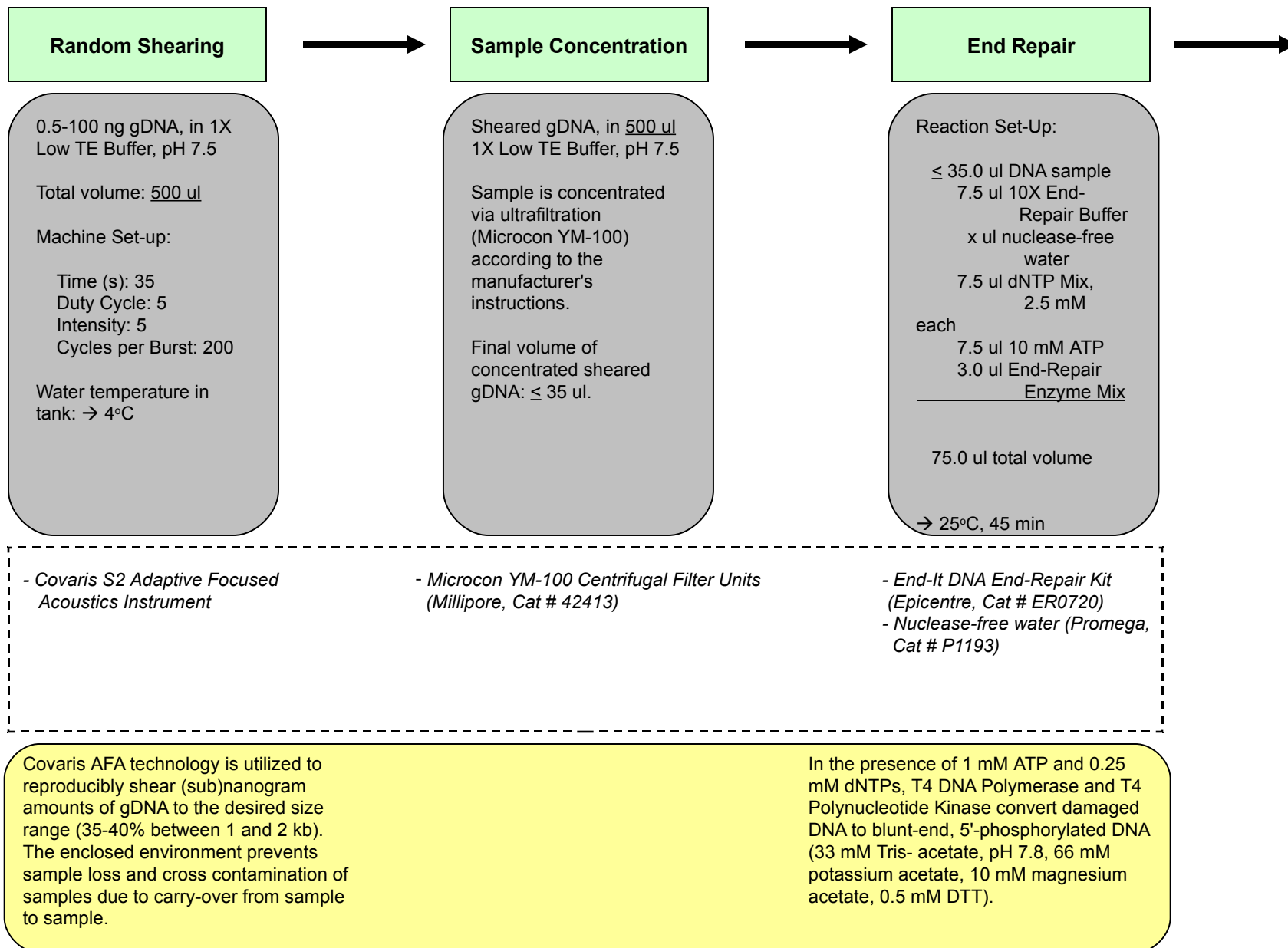
Construction of LASLs

- Protocol Development & Optimization -

- We have implemented the High Frequency Adaptive Focused Acoustics (AFA) technology from Covaris Inc. (Woburn, MA.) to randomly shear the gDNA to the desired size range. The Covaris AFA process works by sending acoustic energy wave packets from a dish-shaped transducer that converges and focuses to a small-localized area. The sample can be introduced into the focal point of the instrument and treated with a non-contact controlled isothermal mechanical energy. Acoustic shearing of nanogram quantities of DNA is highly reproducible. The enclosed environment prevents sample loss and cross contamination of samples due to carry-over from sample to sample.
- Standard protocol steps requiring elevated temperatures are either omitted or performed at high salt concentration (0.3 M NaCl) to increase process yields by reducing potential DNA loss through denaturation. These adjustments generally increase the stability of AT-rich DNA fragments.
- Each enzymatic reaction clean-up prior to the PCR amplification step is performed using QIAGEN's MinElute spin columns with binding and elution properties suitable for nanogram amounts of DNA and small volumes.
- To avoid preferential amplification of very small DNA fragments and to maximize the yield of amplified DNA in the desired size range (~1.2-1.8 kb), an additional size fractionation step (prior to the amplification step) has been introduced.
- A PCR titration is performed prior to the actual large-scale linker-mediated amplification to determine the lowest number of amplification rounds (≤ 28) required to yield sufficient material for the subsequent process steps.
- Performance tests of several high-fidelity DNA polymerases focusing on process robustness and ease-of-use have resulted in selecting Stratagene's *PfuTurbo* Hotstart PCR Master Mix.
- PCR is performed with a phosphorylated primer that anneals to one strand of the custom-made adaptor attached to both fragment ends. The purified PCR fragments can then directly (without the need for a Polynucleotide T4 Kinase step) enter our standard library construction process (at the *Bst*XI linker ligation step).
- After addition of the standard *Bst*XI/*Eco*RI adaptor (Invitrogen) a second size fractionation step is performed to tighten the insert size range and to remove excess linker.
- To increase stability of AT-rich sequences, the DNA fragments are ligated into a low-copy-number version of our existing cloning/sequencing vector pOT.
- To prevent potential contamination with (and subsequent amplification of) non-specific DNA, strict clean lab procedures have been implemented for the sample handling and experimental process steps prior to the linker-mediated whole genome amplification.

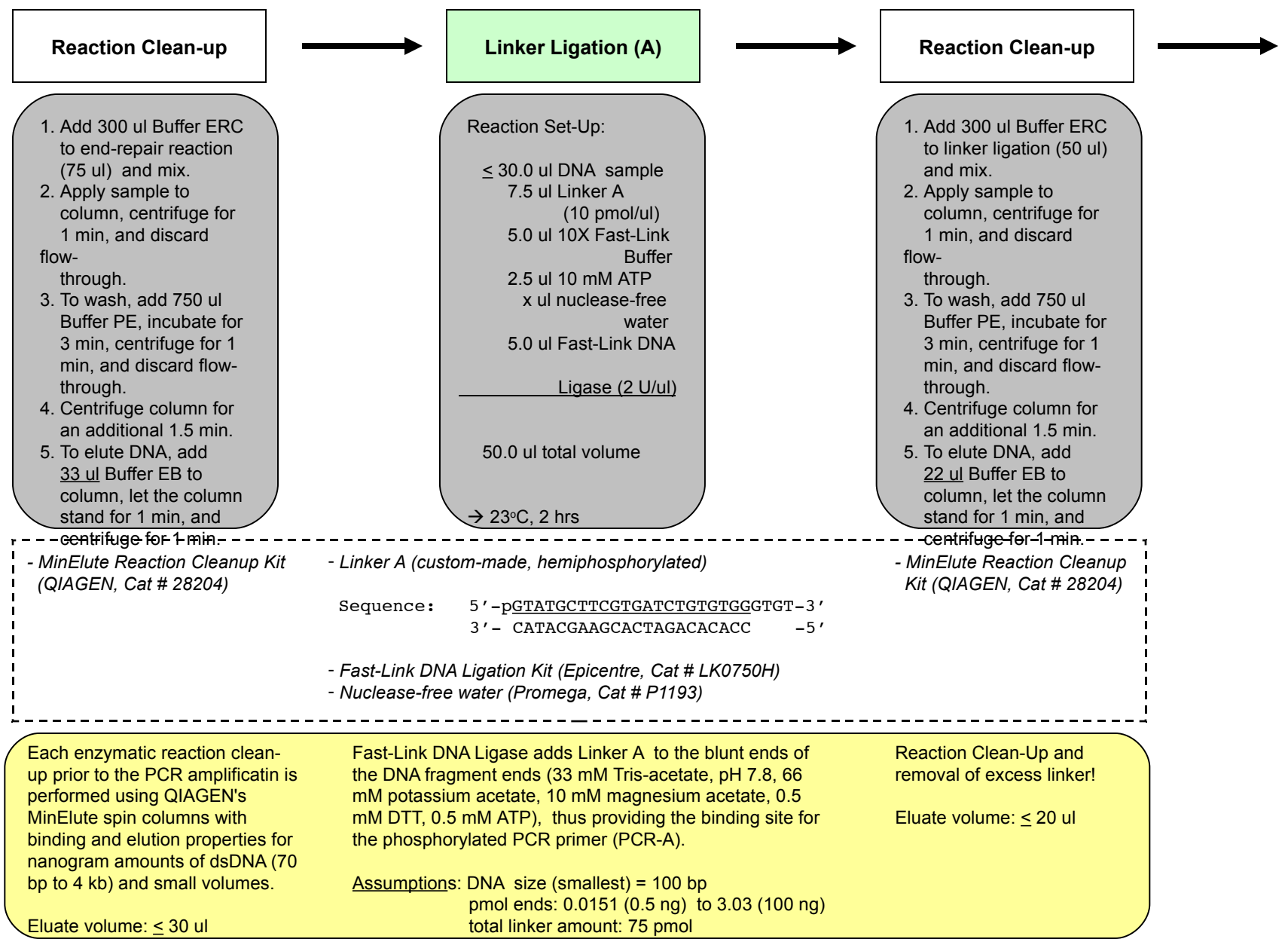
CONSTRUCTION OF LASLs

- Process Map (Page 1) -



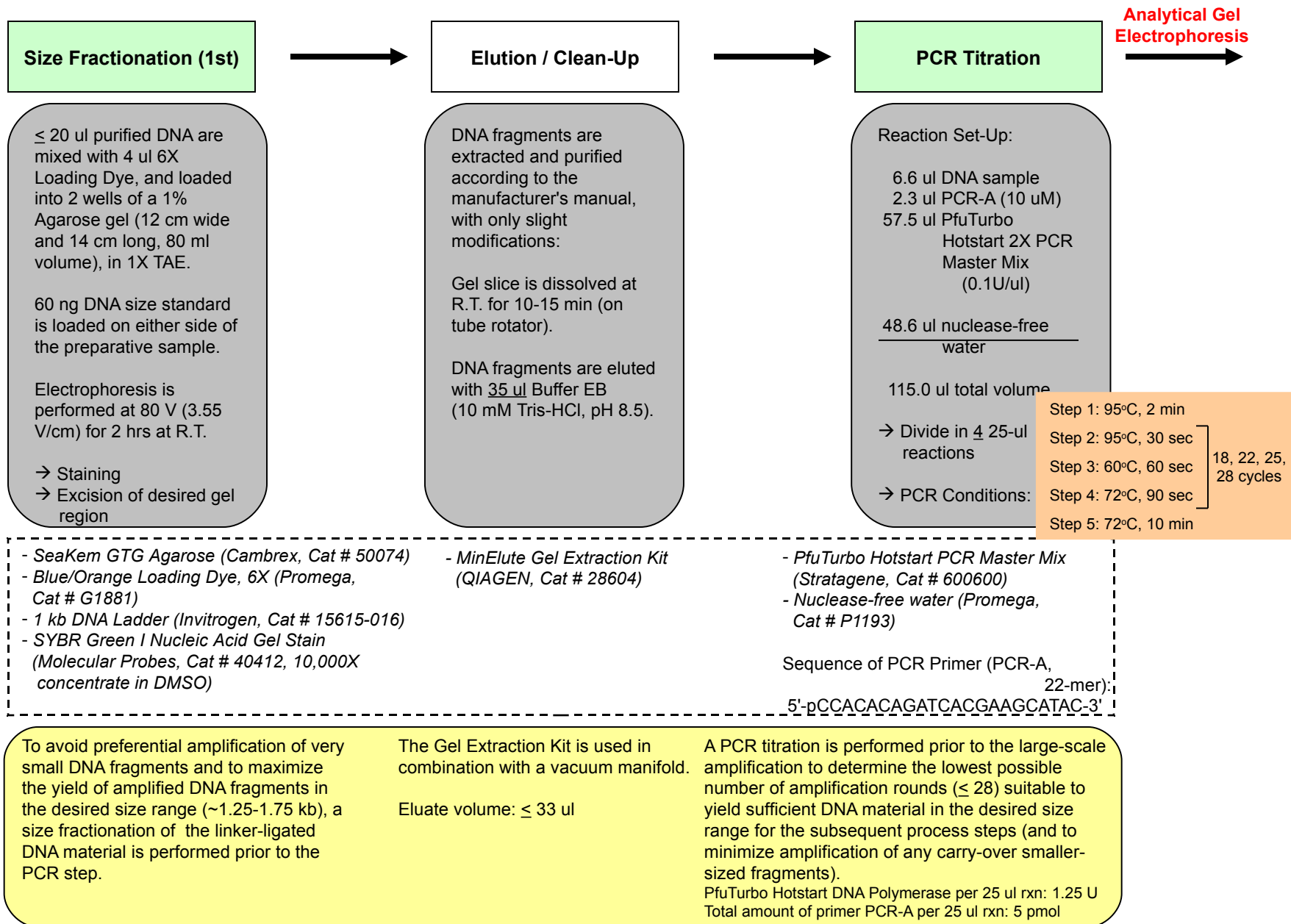
CONSTRUCTION OF LASLs

- Process Map (Page 2) -



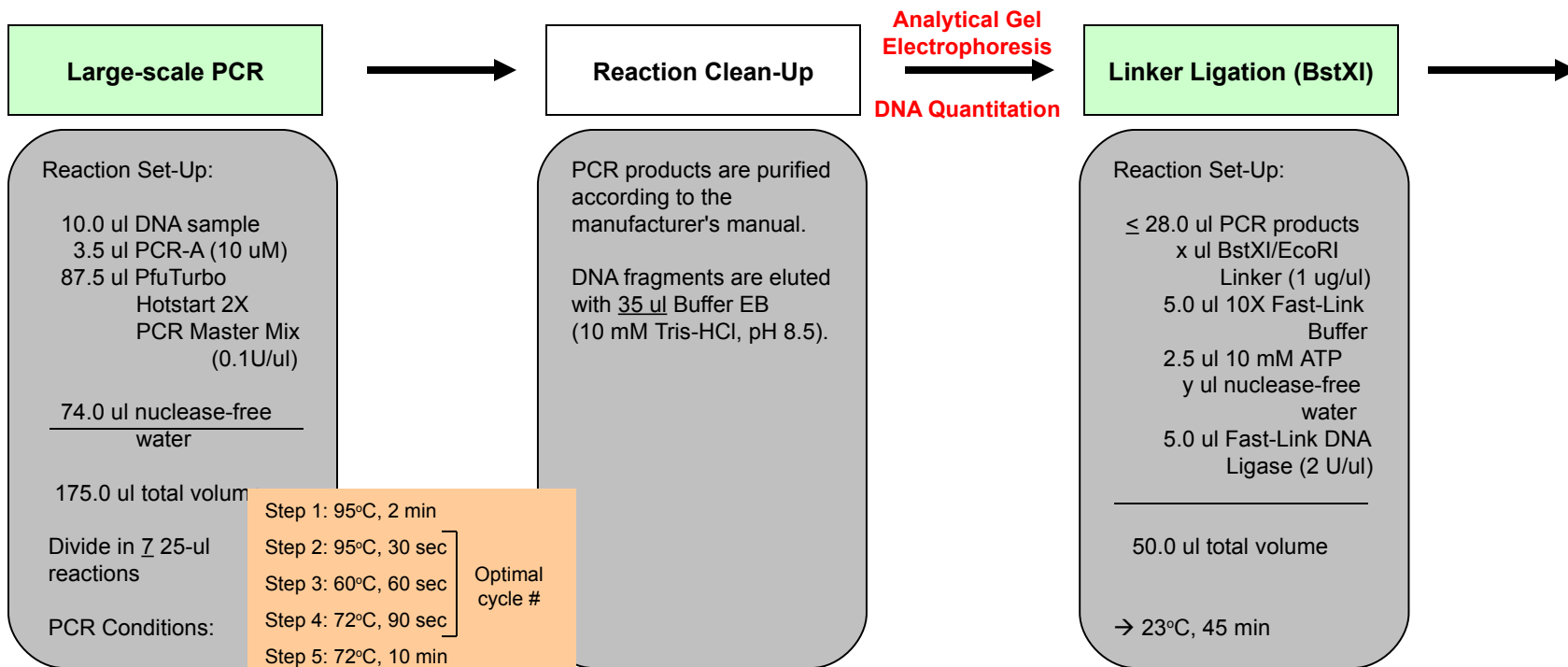
CONSTRUCTION OF LASLs

- Process Map (Page 3) -



CONSTRUCTION OF LASLs

- Process Map (Page 4) -



- PfuTurbo Hotstart PCR Master Mix (Stratagene, Cat # 600600)
 - Nuclease-free water (Promega, Cat # P1193)
 - QIAquick PCR Purification Kit (QIAGEN, Cat # 28104)
 - BstXI/EcoRI Adaptor (Invitrogen, Cat # N418-18)
 - Fast-Link DNA Ligation Kit (Epicentre, Cat # LK0750H)
 - Nuclease-free water (Promega, Cat # P1193)
- Sequence of PCR Primer (PCR-A, 22-mer):
- 5'-pCCACACAGATCAGCAAGCATAC-3'
- Sequence: 5' -pGAATTCCACCACA-3'
3' - CTTAAGGTGp -3'

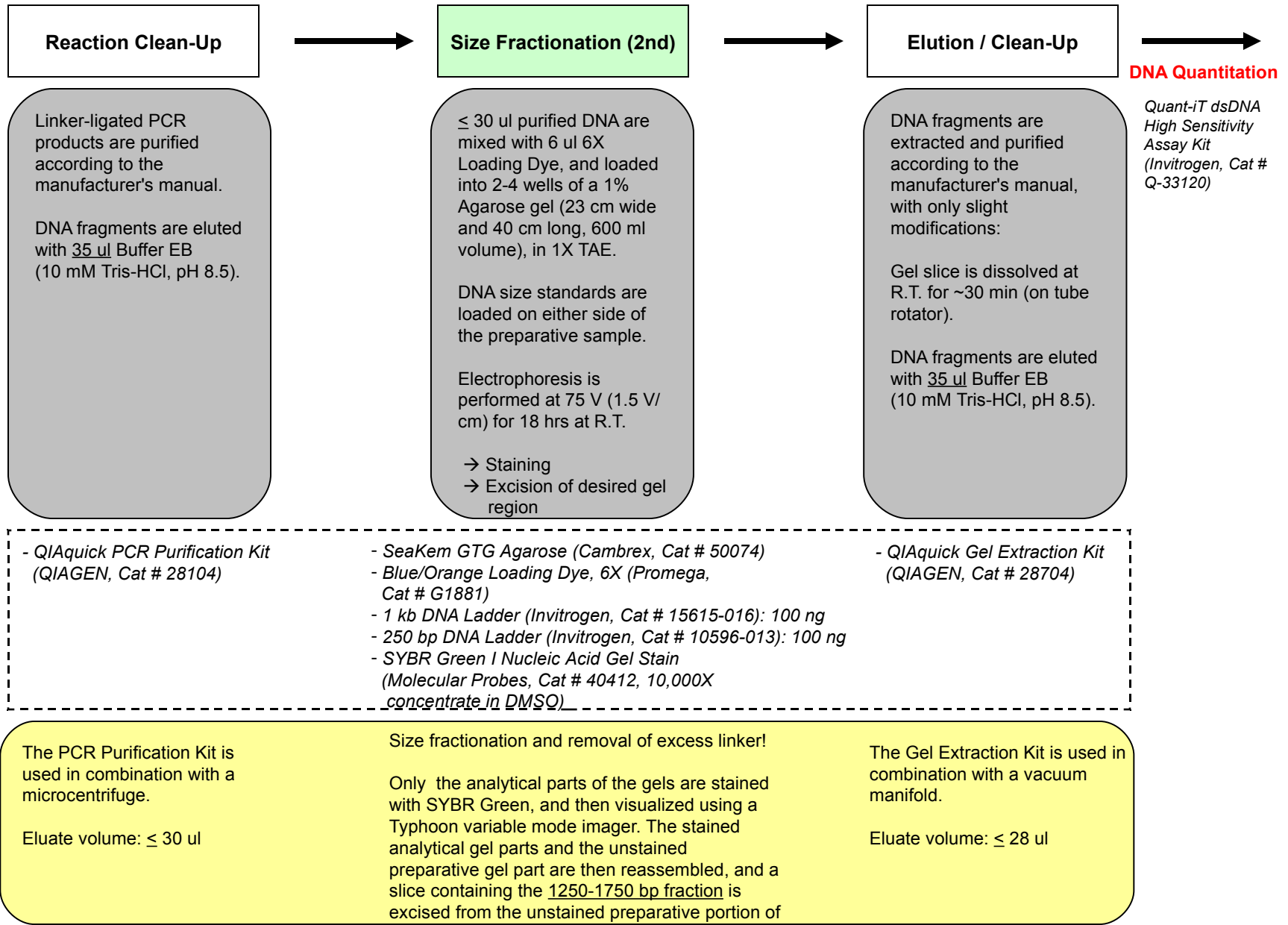
PCR is performed with a 5'-phosphorylated primer that anneals to one strand of the custom-made adaptor (Linker A) attached to both ends of the fragments. The purified phosphorylated PCR fragments can then directly (after a clean-up step) enter our standard WGS LC process at the BstXI linker ligation step.

The PCR Purification Kit is used in combination with a vacuum manifold.
Eluate volume: ≤ 30 ul

Fast-Link DNA Ligase adds BstXI linkers to both ends of the phosphorylated PCR products (33 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 0.5 mM ATP).
Assumptions: DNA size (smallest) = 700 bp
pmol ends: 1.082 (250 ng) to 4.329 (1000 ng)
Linker per fragment end: 150X
Linker concentration: 1 ug/ul, 138 pmol/ul

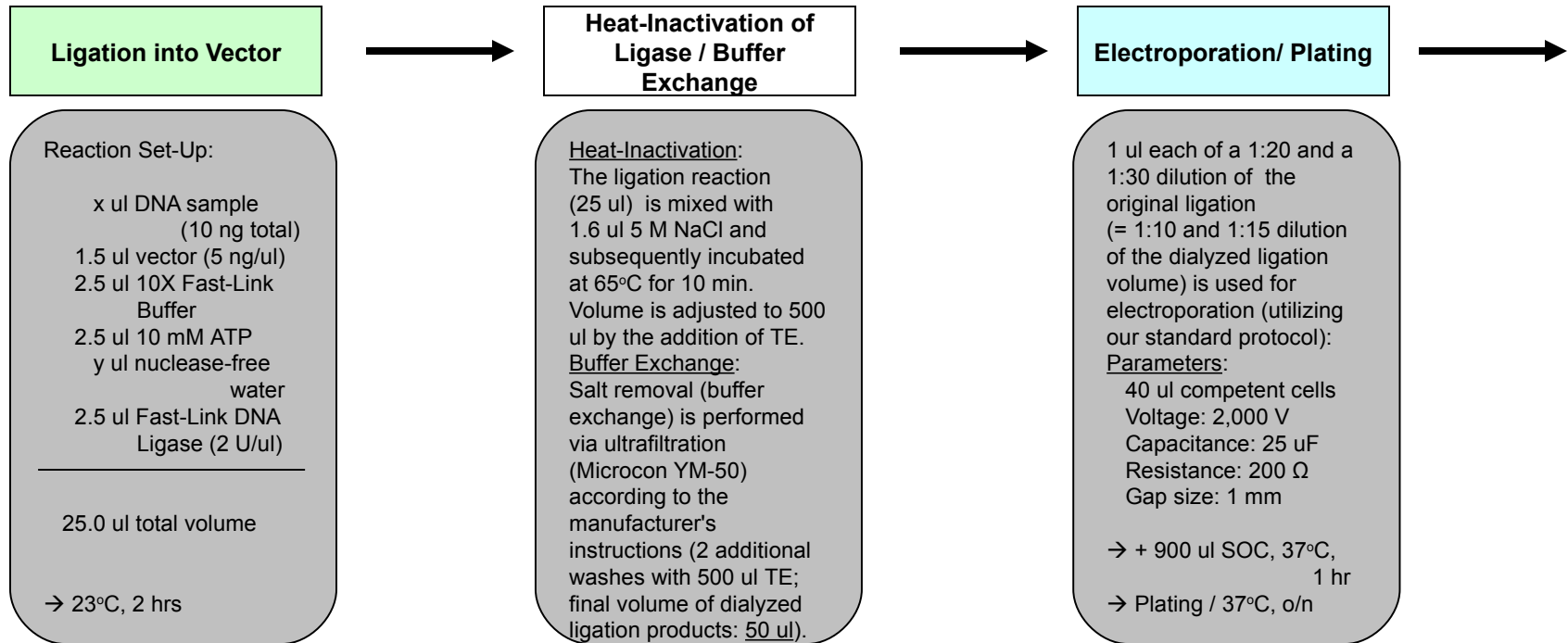
CONSTRUCTION OF LASLs

- Process Map (Page 5) -



CONSTRUCTION OF LASLs

- Process Map (Page 6) -



- *Fast-Link DNA Ligation Kit (Epicentre, Cat # LK0750H)*
 - *Nuclease-free water (Promega, Cat # P1193)*

- *Microcon YM-100 Centrifugal Filter Units (Millipore, Cat # 42413)*
 - *1X Low TE Buffer, pH 7.5*

- *ElectroMAX DH10B-T1^R Electrocompetent Cells (Invitrogen, Cat # 12033-101)*
 - *SOC Medium (Invitrogen, Cat # 15544-034)*
 - *LB/Chloramphenicol/Sucrose Agar Plates (25 mg/ml Chloramphenicol, 5% Sucrose)*
 - *Gene Pulser Cuvettes, 0.1 cm gap (Bio-Rad, Cat # 165-2089)*
 - *GenePulser Xcell System (Bio-Rad, Cat # 165-2660)*

Fast-Link DNA Ligase covalently links DNA fragments with linearized vector molecules (33 mM Tris-acetate, pH 7.8, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 1 mM ATP).
Fragment used: 10 ng total (~1.2 kb = 0.013 pmol)

Vectors (low-copy-number) used:
 pOTLC39/BstXI (1715 bp, 7.5 ng = 0.007 pmol)
 pSMARTLCNew/BstXI (1860 bp, 7.5 ng = 0.006 pmol)

Heat-inactivation of Ligase is performed at high salt concentration (0.3 M NaCl) to prevent DNA degradation and to stabilize DNA fragments with high AT content. The final volume of the dialyzed ligation is 50 ul, representing a 1:2 fold dilution of the original ligation reaction.

3 different ligation dilutions are electroporated to determine the optimal plating conditions (colony density critical for picking efficiency).

CONSTRUCTION OF LASLs

- Process Map (Page 7) -

Library QC

Colony Screen:

To determine the library quality (insert size range, clones without insert), 24-48 clones are analyzed using the Colony Fast-Screen Kit (Procedure B).

- *Colony Fast-Screen (Size Screen) Kit (Epicentre, Cat # FS08250)*
- *Supercoiled DNA Ladder (Invitrogen, Cat # 15622-012)*

For each library that has passed the lab QC, 384-768 clones (1-2 384-well plates) are sequenced with Forward and Reverse Sequencing Primer each (= test sequencing).

CONSTRUCTION OF LASLs

- Supplementary Information -

- Whole Genome Amplification via Linker-mediated PCR:

Adaptor A is ligated to both ends of the end-repaired DNA fragments, thus providing the binding site for the primer PCR-A (5'-pCCACACAGATCACGAAGCATAC-3'). After the size fractionation step, the linker-ligated DNA fragments are amplified via PCR.

- Preparation of Linker A:

Linker A, pre-annealed from oligodeoxynucleotides

(Forward) 5' -pGTATGCTTCGTGATCTGTGTGGGTGT-3'

(Reverse) 3' - CATACGAAGCACTAGACACACC -5'

Oligodeoxynucleotides were pre-annealed in 1X Low TE, pH 7.5, 50 mM NaCl. Annealing was started at 93°C (for 2 min), followed by slow cool down to 4°C at a ramp of 0.1°C per sec.

- Choice of vector system (*Bst*XI):
 - To prevent formation of chimeric clones in the final ligation step
 - For easy integration into standard production sequencing pipeline
 - To eliminate need for special vector preparation and QC