epicPCR (Emulsion, Paired Isolation, and Concatenation PCR)

Manu Tamminen* (mvtammin@mit.edu)
Alm Lab, Massachusetts Institute of Technology
Eric Alm (ejalm@mit.edu)
Alm Lab, Massachusetts Institute of Technology
Sarah Spencer*
Alm Lab, Massachusetts Institute of Technology
Sarah Preheim
Alm Lab, Massachusetts Institute of Technology
Mira Guo
Weitz Lab, Harvard University
Adrian Briggs
AbVitro, Inc.
Ilana Brito
Alm Lab, Massachusetts Institute of Technology
David Weitz
Weitz Lab, Harvard University
Leena Pitkänen
Virta Lab, University of Helsinki
Francois Vigneault
AbVitro, Inc.
Marko Virta
Virta Lab, University of Helsinki

Method Article

Keywords: single cell, parallel sequencing, emulsion, fusion PCR, cell isolation

Posted Date: October 12th, 2015

DOI: https://doi.org/10.1038/protex.2015.094

Abstract

Many microbial communities are characterized by high genetic diversity. 16S ribosomal RNA sequencing can determine community members, and metagenomics can determine the functional diversity, but resolving the functional role of individual cells in high-throughput remains an unsolved challenge. Here, we describe epicPCR \(Emulsion, Paired Isolation, and Concatenation PCR), a new technique that links functional genes and phylogenetic markers in uncultured single cells, providing a throughput of hundreds of thousands of cells with costs comparable to one genomic library preparation. We demonstrate the utility of our technique in a natural environment by profiling a sulfate-reducing community in a freshwater lake, revealing both known sulfate reducers and discovering new putative sulfate reducers. Our method is adaptable to any conserved genetic trait and translates genetic associations from diverse microbial samples into a sequencing library that answers targeted ecological questions. Potential applications include identifying functional community members, tracing horizontal gene transfer networks, and mapping ecological interactions between microbial cells.

Introduction

 epicPCR accepts microbial cells directly from an environmental source, then generates single-cell gene fusions in high-throughput \(Fig. 1). The gene fusions are generated in an emulsion, then amplified in bulk via a nested reaction. Thus each targeted gene should have three conserved sites for primer design \(two for the initial fusion and one more for the nested reaction). The more rare a targeted gene, the more challenging library amplification will become - we recommend controlled titrations to determine a lower limit for your gene of interest. Completing more identical emulsions with the same primers and sample source, then combining the fusion amplicons can also improve performance for rare genes. We are excited about extensions and modifications to this core methodology. Hydrogel encapsulation in particular enables a range of new techniques, including fluorescent sorting of hydrogel beads, covalently attached nucleotides, etc. Please refer to the manuscript for novel designs of synthetic control beads and discussion of future applications. **Primer design** The following schematic \(Fig. 2) describes the gene-specific primers that must be designed for each new functional gene assay. The schematic abbreviations are used in the Protocol section to designate necessary concentrations.  _Fusion primer design_ F1: forward primer targeting a conserved site, distance to R1 should be less than 1,000 bp. R2: reverse primer targeting the 16S rRNA gene; we recommend the universal 1492R primer. R1-F2': bridge primer to connect the genes. R1 is a reverse primer on the target functional gene, and F2' is the reverse complement of a 16S rRNA gene forward primer. For F2' we recommend the universal primer 519R. _Nested primer design_ F3: A primer that nests within the functional gene, ideally < 300 bp from R1. Add an Illumina overhang \(5'-ACACGACGCTCTTCCGATCTYRYR-3') to the 5' end of the primer. R3: A primer that nests within the 16S rRNA gene; we recommend the universal primer 786R. Add an Illumina overhang \(5'-CGGCATTCCTGCTGAACCGCTCTTCCGATCT-3') to the 5' end of the primer. blockF: Add 10 'T' bases to the 5' end of F2 and a 3-carbon spacer to the 3' end. blockR: Add 10 'T' bases to the 5' end of F2' and a 3-carbon spacer to the 3' end. _Illumina adapter primer design_ PE-PCR-III-F: A forward flow cell adapter \(5'-

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT-3') PE-PCR-IV-XXX: A reverse flow cell adapter with a variable 9 bp barcode \(5'-CAAGCAGAAGACGGCATACGAGATNNNNNNNNNCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT-3'); optional barcodes listed in the Supplementary Information of the associated publication, Spencer and Tamminen _et al._, _ISME J_ \(2015).

Reagents

Acrylamide \(for molecular biology, ≥99.5%, Sigma, St. Louis, MO, USA) BAC \(N,N'-Bis\(acryloyl)cystamine, suitable for electrophoresis, Sigma)* Ammonium persulfate \(for molecular biology, ≥98.0%, Sigma) TEMED \(N,N,N',N'-Tetramethylethylenediamine, ≥99.5%, Sigma) Span 80 \(Sigma) Tween 80 \(Sigma) Triton X-100 \(molecular biology grade, EMD Millipore, Billerica, MA, USA) Mineral oil \(light, suitable for cell culture, Sigma) Diethyl ether \(water-saturated, ≥99.5%, Sigma) UltraPure DNase/RNase-Free Distilled Water \(Life Technologies, Grand Island, NY, USA) Tris-HCl \(pH 7.5, Teknova, Hollister, CA, USA) KCl \(≥99.0%, VWR, Radnor, PA, USA) ABIL EM 90, a surfactant \(Evonik, Mobile, AL, USA) BSA \(molecular biology grade, NEB, Ipswich, MA, USA) Tween 20 \(for molecular biology, Sigma) Deoxynucleotide \(dNTP) Solution Mix \(10 mM each, NEB) Phusion Hot Start Flex DNA Polymerase \(NEB) Ethylenediaminetetraacetic acid \(EDTA, suitable for cell culture, Sigma) Ethyl acetate \(water-saturated, ACS grade, ≥99.5%, BDH, Poole Dorset, UK) Agencourt AMPure XP - PCR Purification \(Beckman Coulter, Danvers, MA, USA) Ethanol \(200 proof, VWR) E-Gel 1 Kb Plus DNA Ladder \(Invitrogen) _Optional_ Ready-Lyse Lysozyme Solution \(Epicentre, Madison, WI, USA) Proteinase K from Tritirachium album \(for molecular biology, Sigma) SYBR Green I Nucleic Acid Gel Stain \(10,000X, Invitrogen, Waltham, MA, USA) *Can be substituted for bisacrylamide \(N,N'-Methylenebis\(acrylamide), 99%, Sigma)

Equipment

1.5 ml Safe-Lock Microcentrifuge Tubes, Polypropylene \(Eppendorf, Hamburg, DE) 2 ml Safe-Lock Microcentrifuge Tubes, Polypropylene \(round-bottom, Eppendorf) PCR 8-Well Tube Strips with Individually Attached Caps \(VWR) Microcentrifuge \(Microcentrifuge 5415D, Eppendorf) Thermal-cycler \(C1000 Touch Thermal Cycler, Bio-Rad, Hercules, CA, USA) BD Falcon 35µm Cell Strainer in 12x75 mm Polystyrene Tube \(Corning, Tewksbury, MA, USA) 2 mm glass beads \(Andwin Scientific, Schaumburg, IL, USA) DynaMag-2 Magnet \(Life Technologies) E-Gel iBase and E-Gel Safe Imager \(Invitrogen) E-Gel EX Agarose Gels, 1% \(Invitrogen) _Optional_ Real-Time PCR System \(CFX96 Real-Time PCR Detection System, Bio-Rad)

Procedure

REAGENT SETUP _Acrylamide solution \(store at 4°C)_ 12% Acrylamide 0.32% BAC \(N,N'-Bis\(acryloyl)cystamine) _1X TK buffer \(recommended filter through 0.2 µm, store at RT)_ 20 mM Tris-HCl \(pH 7.5)* 60 mM KCl* *autoclave the two liquid stocks before combining _STT emulsion oil \(store at RT,

should be prepared fresh every two weeks)_ 4.5% Span 80 0.4% Tween 80 0.05% Triton X-100 v/v in Mineral oil _ABIL emulsion oil \(store at RT)_ 4% ABIL EM 90 0.05% Triton X-100 v/v in Mineral oil **PROCEDURE** **Stage 1: Polyacrylamide bead formation** TIMING: 4 h 1 Start with 10-20 million cells in 30 µl water. No initial filtering is required for environmental samples. If cells need to be spun down and resuspended, spin at 12,000 _g_ for 1 min in a microcentrifuge or 4,000 rpm \(max speed) for 5 min at 4°C in a benchtop centrifuge. Draw off supernatant and resuspend in 30 µl nuclease-free water. CRITICAL STEP: Be mindful of what liquid the cells are resuspended in. This protocol has been successful with cells resuspended in water and liquid LB, but different salt or chemical concentrations may affect emulsion stability and should be tested. Also consider to what extent cells exist in aggregates vs. in a single-cell state. Vortexing \(Steps 3 and 4) should disperse most cell aggregates, but upstream sonication or filtering may be necessary to ensure predominantly single cells. 2 Combine the following in a 2 ml round-bottom microcentrifuge tube. Vortex gently to mix. • 30 µl suspended cells • 200 µl 12% acrylamide/BAC • 25 µl 10% APS 3 Add 600 µl STT emulsion oil \(shake & mix thoroughly before use). Vortex the combined aqueous and oil phases at max speed \(3,000 rpm) for 30 sec. 4 Add 25 µl TEMED to the emulsion. Vortex at max speed \(3,000 rpm) for 30 sec. 5 Let tube sit on the benchtop at room temperature and polymerize for 90 min. 6 Add 800 µl diethyl ether to the microcentrifuge tube, then immediately close, invert, and flick the microcentrifuge tube to mix the emulsion with the ether. A visible precipitate ought to form. Draw off the ether/oil mixture around the precipitate and discard in a dedicated chemical waste container. CRITICAL STEP: First shake the bottle of diethyl ether in a chemical hood, opening occasionally to prevent pressure from building up as the ether dissolves in water. Draw from the top phase after it settles. Invert and mix the microcentrifuge tube as soon as possible after ether addition. 7 Add 1 ml nuclease-free water to the top of the microcentrifuge tube and mix by flicking and inverting. Transfer suspended mixture to a 1.5 ml eppendorf tube. Centrifuge for 30 seconds at 12,000 _g_. Three layers should form: a bottom layer of beads, a middle cloudy layer of oil/water, and a top milky layer of oil. 8 Using a P1000 pipette, draw off the top oil layer as much as possible and discard it. Add more water \(or the remaining bead suspension from the 2 ml microcentrifuge tube) and repeat this wash \(Steps 7 and 8) five or more times until there is no remaining oil at the top of the tube. 9 Once the oil is removed, aspirate all remaining water without disturbing the bottom bead layer. Resuspend the beads in 1 mL 1X TK buffer. Run this bead suspension through a 35 µm cell strainer; pipette forcefully in small portions to move the liquid through the filter. Transfer the flow-through to a fresh 1.5 ml microcentrifuge tube and store at 4°C. PAUSE POINT: Polyacrylamide beads containing single cells can be stored at 4°C for 1-2 months, although we recommend lysis and fusion PCR as soon as possible \(within a few days) after bead polymerization. **Optional: Cell lysis** TIMING: 1 d 1 Mix polyacrylamide bead tube from Stage 1 by flicking and inverting. Aliquot total desired amount for lysis into 50 µl aliquots in 200 µl PCR tubes. 2 Add 0.4 µl Ready-Lyse Lysozyme \(35,000 U/µl, Epicentre, Madison, WI, USA) to each polyacrylamide bead aliquot. 3 Incubate at 37 °C overnight. 4 Centrifuge each aliquot at 12,000 _g_ for 30 s, discard liquid phase above the beads, and resuspend in 1X TK buffer to 40 µl. 5 Add 10 µl proteinase K \(1 mg/ml, Sigma, St. Louis, MO, USA) and 0.4 µl Triton X-100. 6 Incubate samples at the following temperatures: 37 °C for 30 min, 95 °C for 10 min, 4 °C hold. 7 Pool polyacrylamide bead aliquots in a 1.5 ml microcentrifuge tube. Centrifuge at 12,000 _g_ for 30 s and resuspend in 1X TK buffer three times to

dilute the Triton X-100. CRITICAL STEP: Different cell types may require different lysis procedures, or none at all. Any enzymatic and chemical reagents typical for a 16S rRNA gene library preparation can also be applied to polyacrylamide beads, and optimal lysis treatment may vary depending on sample type. In addition to enzymatic and chemical lysis, freeze/thaw cycles are possible if the polyacrylamide beads are separated into 25-50 µl aliquots \(to control the size of ice crystal formation). PAUSE POINT: Polyacrylamide beads containing exposed genomes can be stored at 4 °C for 1-2 months, although we recommend fusion PCR as soon as possible \(within a few days) after lysis treatment. **Stage 2: Fusion PCR** TIMING: 4 h 10 Label a round-bottom 2 ml tube for each sample. Add four 2 mm glass beads and 900 µl ABIL emulsion oil to each tube. 11 Prepare the following mix, multiplied by the number of samples × 1.1: • Nuclease-free water 1 µl • 5X Phusion HF buffer 20 µl • 50 mM $MgCl_2$ 2 µl • 10 mM dNTPs 2.5 µl • F1 \(10 uM) 10 µl • R2 \(10 uM) 10 µl • R1-F2' \(1 uM) 1 µl • BSA 0.5 µl • Tween-20 0.2 µl • Phusion Hot Start Flex 8 µl 12 Aliquot 55.2 µl mix into PCR tubes for each sample. 13 Add 45 µl of polyacrylamide beads \(from Stage 1 with optional lysis treatment) to each sample. Mix by hand, then add to the prepared round-bottom tubes. 14 Emulsify by vortexing at 3,000 rpm for 1 min. 15 Aliquot each sample into 16 PCR tubes, 60 µl each. Run the following cycling program: 94°C 30 s, \[94°C 5 s, 52°C 30 s, 72°C 30 s] for 33 cycles total, 72°C 5 min, 4°C hold. 16 Immediately after the fusion reaction, pool the emulsion aliquots for each sample into a 1.5 ml microcentrifuge tube. Add a total of 1 mM EDTA \(2 µl of 50 mM EDTA) to each pooled sample. PAUSE POINT: Once sample aliquots are pooed and EDTA is added, emulsions can be stored at 4 °C overnight. **Stage 3: Break ABIL emulsions** TIMING: 5 h 17 Centrifuge the pooled samples from Stage 2 at 13,000 _g_ for 5 min at 25 °C. Dispose of the upper \(oil) phase. 18 Shake water-saturated diethyl ether \(1:1 mixture of ether:water) for 30 s and open cap occasionally prevent pressure buildup. Allow the phases to settle before use and draw from the top \(ether) phase. 19 Add 1 ml of diethyl ether to each sample, then vortex gently to mix. Centrifuge samples for 1 min at 13,000 _g_ to separate the phases. Dispose of the upper \(solvent) phase. Repeat. 20 Perform an extraction \(Step 19) with water-saturated ethyl acetate. Ethyl acetate is also less dense than water and will comprise the upper phase. 21 Perform two more extractions with diethyl ether. Dispose of the upper \(solvent) phase. 22 Leave the samples open in a laminar flood hood or covered area for 10 min so the remaining diethyl ether can evaporate. Collect 100-150 µl from the bottom phase. PAUSE POINT: Extracted reactions can be stored at 4 °C for a few hours or at -20 °C overnight. 23 Use AMPure XP beads to purify the fusion PCR products for the nested reaction \(see 'AMPure XP PCR cleanup'). PAUSE POINT: Purified reactions can be stored at 4 °C for a few hours or at -20 °C overnight. **Repeated: AMPure XP PCR cleanup** TIMING: 2 h 1 Shake or vortex the bottle of AMPure XP beads to suspend beads. For each 100 µl sample, aliquot 85.5 µl beads into a 1.5 ml microcentrifuge tube. If samples are >100 µl, scale the volume of beads to add. Let the aliquot sit on the bench during setup and make sure it is room temperature by the end of setup. 30 min max for equilibration to room temperature. 2 Add unpurified DNA samples to the bead aliquots. Mix by vortexing gently until homogenous. Incubate 13 min at room temperature to bind DNA. 3 Separate beads on magnet for 2 min. Then while on magnet, remove and discard the supernatant. 4 Wash beads twice with 70% EtOH, using 500 µl each wash. Keep tubes on the magnet during the entire wash. 5 Open the tubes and air dry the beads for 15-20 min on magnet, in a laminar flow hood or covered area. Check that beads appear dry and there are no droplets on the sides of

the tube. Dry up to 30 min or even an hour if necessary. 6 Remove samples from magnet, elute in 40 µl Buffer EB \(or purified water), vortex gently to resuspend. 7 Incubate 7 min at room temperature to elute the DNA. 8 Separate beads on magnet for 2 min. Collect 35-40 µl of the supernatant and store in a fresh 1.5 ml microcentrifuge tube. **Optional: Nested qPCR** TIMING: 4 h 1 Prepare a qPCR mix to determine the optimal nested PCR cycle numbers for different samples. Each sample should have duplicate qPCR reactions and there should also be a no-template negative control \(also in duplicate). Prepare the following mix, multiplied by the number of qPCR samples × 1.1: • Nuclease-free water 7.125 µl • 5X HF 5 µl • 10 mM dNTP 0.5 µl • F3 \(3 µM) 2.5 µl • R3 \(3 µM) 2.5 µl • blockF \(32 µM) 2.5 µl • blockR \(32 µM) 2.5 µl • 2 U/µl Phusion Hot Start Flex 0.25 µl • 100X SYBR Green I 0.125 µl 3 Aliquot 23 µl mix for each reaction into white PCR tubes \(Masterclear Cap and Tube Strips, VWR, Radnor, PA, USA). Add 2 µl purified template from Stage 3 or water \(for negative control). 2 Mix the qPCR reactions by flicking and then spin down. Attach clear cap strips. Run the following cycling program: 98°C 30 s, \[98°C 5 s, 52°C 30 s, 72°C 30 s] for 40 cycles total, melt curve 65°C to 95°C with 0.5°C increment for 5 s, 4°C hold. 3 Use qPCR Ct values to estimate minimum cycle numbers for different samples. If possible, dilute the more concentrated samples so all can be run with the same number of nested PCR cycles. \[Calculate an average Ct from sample duplicates. Calculate delta Ct for each sample using the lowest concentration sample Ct value. Assume 1.75x amplification each cycle.] PAUSE POINT: qPCR reactions can be discarded and templates from Stage 3 can remain stored at 4 °C for a few hours or at -20 °C overnight. **Stage 4: Nested PCR** TIMING: 4 h 24 Prepare 37 µl sample dilutions in water. If the optional qPCR step was not performed, combine 20 µl of Stage 3 product with 17 µl nuclease-free water. If the optional step was performed, dilute according to qPCR results. 25 Prepare the following mix, multiplied by the number of samples × 4 × 1.1: • 5X HF Buffer 5 µl • 10 mM dNTP 0.5 µl • F3 \(3 µM) 2.5 µl • R3 \(3 µM) 2.5 µl • blockF \(32 µM) 2.5 µl • blockR \(32 µM) 2.5 µl • 2 U/ul Phusion Hot Start Flex 0.25 µl 26 Combine 63 µl mix with 37 µl diluted samples from Step 24, mix by hand, then divide into four 25 µl aliquots. \[The quadruplicate aliquots reduce PCR bias.] 27 Run the following cycling program: 98°C 30 s, \[98°C 5 s, 52°C 30 s, 72°C 30 s] for 40 cycles total or variable based on qPCR Ct values, 72°C 5 min, 4°C hold. PAUSE POINT: Amplified reactions can be stored at 4 °C for a few hours or at -20 °C overnight. 28 Pool replicate reactions and use AMPure XP beads to purify the nested PCR products for the final Illumina amplification \(see ‘AMPure XP PCR cleanup’). PAUSE POINT: Purified reactions can be stored at 4 °C for a few hours or at -20 °C overnight. **Stage 5: Illumina PCR** TIMING: 5 h 29 Perform a final PCR reaction to add flow-cell compatible Illumina adapters. Every sample should be prepared in four replicate reactions to reduce PCR bias. Prepare the following mix multiplied by the number of template samples × 4 × 1.1: • Nuclease-free water 8.65 µl • 5X HF Buffer 5 µl • dNTPs 0.5 µl • PE-PCR-III-F \(3µM) 3.3 µl • Phusion Hot Start Flex 0.25 µl • PE-PCR-IV-XXX \(3µM) 3.3 µl 30 Combine 21 µl mix with 4 µl template \(< 250 ng), with four 25 µl replicate reactions for each sample. Mix by hand. 31 Run the following cycling program: 98°C 30 s, \[98°C 30 s, 83°C 30 s, 72°C 30 s] for 7 cycles total, 4°C hold. PAUSE POINT: Amplified reactions can be stored at 4 °C for a few hours or at -20 °C overnight. 32 Pool replicate reactions for each sample and use AMPure XP beads to purify the final Illumina library for sequencing \(see ‘AMPure XP PCR cleanup’). PAUSE POINT: Purified reactions can be stored at 4 °C for a few hours or at -20 °C overnight. 33 Run purified samples on a gel to confirm the band size of the final library. Load a 1% E-gel EX with ladder \(10

µl E-Gel 1 Kb Plus DNA Ladder + 10 µl water) and samples \(2 µl sample + 18 µl water). Run for 10 min under the 'E-gel EX 1-2% 10 minute' program. 34 Submit libraries for Illumina 250-250 paired end sequencing, with an 8 bp barcode on the reverse read. Load 20% phiX or other high diversity DNA to offset the low diversity of amplicon samples.

Timing

The times below are estimated for handling 4-8 samples at once. Times would decrease by approximately half if only handling 1-2 samples. **Stage 1: Polyacrylamide bead formation** TIMING: 4 h **Optional: Cell lysis** TIMING: 1 d **Stage 2: Fusion PCR** TIMING: 4 h **Stage 3: Break ABIL emulsions** TIMING: 5 h **Optional: Nested qPCR** TIMING: 4 h **Stage 4: Nested PCR** TIMING: 4 h **Stage 5: Illumina PCR** TIMING: 5 h

Figures

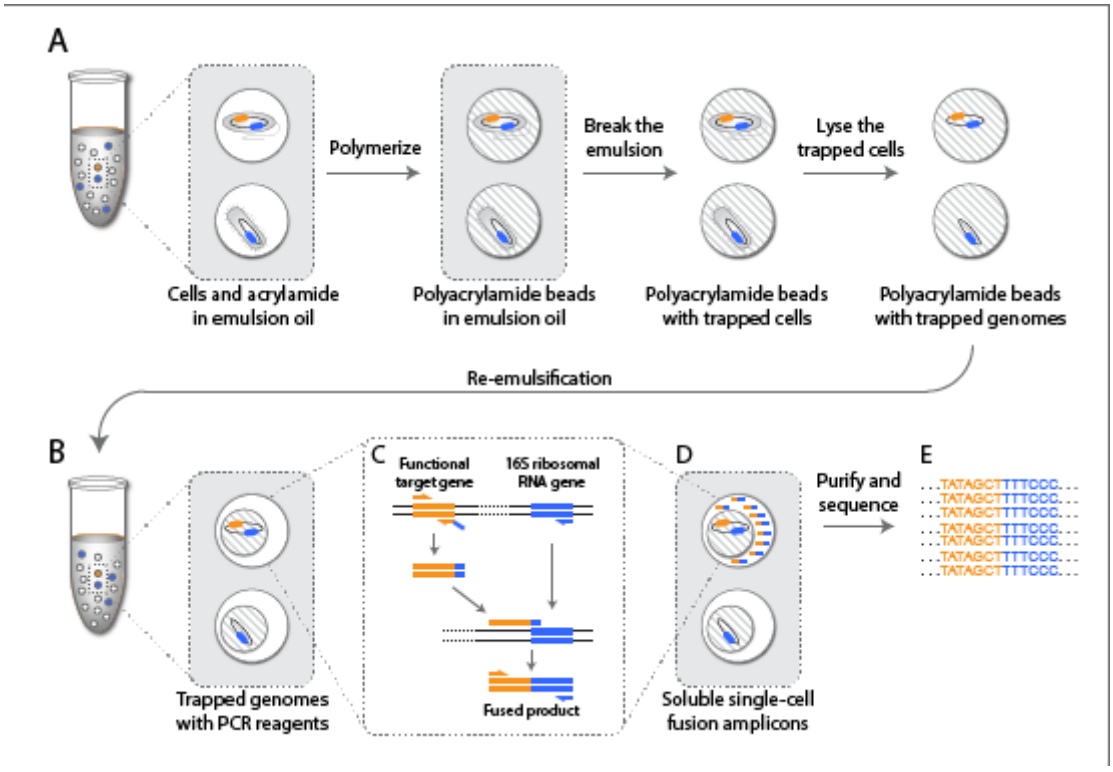

Figure 1

Workflow of epicPCR A) Microbial cells in acrylamide suspension are mixed into emulsion oil. The emulsion droplets are polymerized into polyacrylamide beads containing single cells. The emulsion is broken and the cells in the polyacrylamide beads are treated enzymatically to destroy cell walls, membranes and protein components, and expose genomic DNA. B) Polyacrylamide-trapped, permeabilized microbial cells are encapsulated into an emulsion with fusion PCR reagents. C) Fusion PCR first amplifies a target gene with an overhang of 16S rRNA gene homology. With a limiting

concentration of overhang primer, the target gene amplicon will anneal and extend into the 16S rRNA gene, forming a fusion product that continues to amplify from a reverse 16S rRNA gene primer. D) The fused amplicons only form in the emulsion compartments where a given microbial cell has the target functional gene. E) After breaking the emulsion the fused amplicons are prepared for next-gen sequencing. The resulting DNA sequences are concatemers of the target functional gene and the 16S rRNA gene of the same cell.

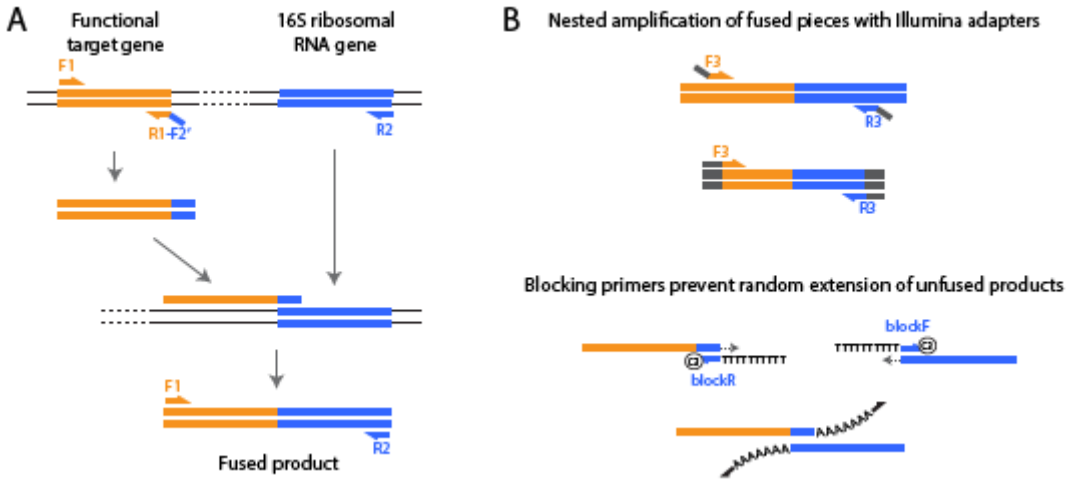

Figure 2

Primer design for epicPCR A) Fusion PCR joins together two amplicons in a single reaction. The amplification first proceeds exponentially for the functional target gene from primers F1 and R1-F2' and linearly for the 16S ribosomal RNA gene from primer R2. Primer R1-F2' adds an overhang to the target gene amplicon that is specific to the start of 16S ribosomal RNA gene. Primers F1 and R2 are in excess over R1-F2', causing its depletion during the early cycles of PCR. After depletion of R1-F2', the 16S ribosomal RNA-specific overhang of the target gene amplicon primes the 16S ribosomal RNA gene creating a fused product. This fused product is subsequently exponentially amplified by F1 and R2. B) In the nested reaction, successful fusion products are amplified with Illumina adapters while partial fusion products are dampened by blocking primers. The blocking primers, added in excess, anneal to the universal 519R sequence but do not extend from the primer end due to a 3' 3-carbon-spacer. Instead, extension occurs from the 3' end of partial fusion products into the overhang region of the blocking primer, adding a string of A bases to the partially fused pieces. This A tail prevents partially fused pieces from annealing, extending, and generating spurious fusion products.