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Chemoselective Coupling Preserves the Substrate Integrity of Surface-Immobilized Oligonucleotides for Emulsion PCR-Based Gene Library Construction

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Abstract

Combinatorial bead libraries figure prominently in next-generation sequencing and are also important tools for in vitro evolution. The most common methodology for generating such bead libraries, emulsion PCR (emPCR), enzymatically extends bead-immobilized oligonucleotide PCR primers in emulsion droplets containing a single progenitor library member. Primers are almost always immobilized on beads via noncovalent biotin–streptavidin binding. Here we describe covalent bead functionalization with primers ($\sim 10^6$ primers/2.8- μm -dia. bead) via either azide–alkyne click chemistry or Michael addition. The primers are viable polymerase substrates (4–7% bead-immobilized enzymatic extension product yield from one thermal cycle). Carbodiimide-activated carboxylic acid beads only react with oligonucleotides under conditions that promote non-specific interactions (low salt, low pH, no detergent), comparably immobilizing primers on beads, but yielding no detectable enzymatic extension product. Click-functionalized beads perform satisfactorily in emPCR of a site-saturation mutagenesis library, generating monoclonal templated beads (10^4 – 10^5 copies/bead, 1.4-kb amplicons). This simpler, chemical approach to primer immobilization may spur more economical library preparation for high-throughput sequencing and enable more complex surface elaboration for in vitro evolution.

Graphical Abstract

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The authors declare no competing financial interests.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Detailed experimental procedure, compound characterization data, qPCR data, single-bead sequencing data

Emulsion-based in vitro compartmentalization (IVC)¹ has revolutionized contemporary process biochemistry. IVC is an efficient and ultra-miniaturized alternative to microtiter plates in which combinatorial library members (e.g., beads, viruses, single molecules) undergo reaction in separate aqueous compartments of a water-in-oil emulsion. The average compartment volume can be as low as several picoliters, thus modest ~100-μL reaction volumes can be parsed into an enormous ($> 10^8$) number of “virtual” wells. The original IVC disclosure described a new approach to directed evolution in which individual mutant gene library members experience a selection challenge for new or enhanced activity in separate droplets, and which has since driven the discovery of new polymerases, hydrolases, myriad other enzymes, and even whole genetic circuits.^{2–9} IVC also catalyzed the invention of emulsion PCR (emPCR),^{2,10} which is now the bedrock library generation strategy for high-throughput pyrosequencing.^{11–13} With the rise of integrated microfluidic circuitry for droplet-scale combinatorial library screening,^{14,15} BEAMing-derived library beads are now potentially very attractive polyvalent gene delivery vehicles analogous to viral particles¹⁶ for these systems as well.^{17–19}

Library generation via emPCR usually entails single-molecule amplification in the presence of beads, transforming a combinatorial library of nucleic acid molecules into a library of beads. The now-standard beads, emulsions and magnetics (BEAM) protocol²⁰ involves emulsification of PCR reagents, oligonucleotide PCR primer-functionalized magnetic beads, and a limiting dilution of DNA library templates. Thermally cycling the emulsion clonally populates each bead’s surface with many copies of a co-encapsulated progenitor gene library member (if one is present). BEAMing strictly requires the use of streptavidin-coated magnetic beads, which selectively bind a doubly 5′-biotinylated oligonucleotide primer.¹⁰ Despite the exquisite potency of the biotin-streptavidin interaction, the sensitivity and high-temperature conditions of PCR-based amplification demand the additional avidity-driven, near-covalent interaction of the doubly-biotinylated bead-immobilized oligonucleotide. For sequencing-based and genotyping applications,²¹ the biotin-streptavidin interaction is a satisfactory, albeit overly complicated, substitute for a covalent bond. However, other applications (e.g., directed evolution, combinatorial ligand library screening) may invoke denaturing conditions in which the proteinaceous streptavidin linkage may not be viable (e.g., extreme temperature, non-aqueous solvents, proteolytic enzymes).

There are several alternative chemical strategies to streptavidin–biotin coupling that afford covalent bead surface functionalization. Examples include amide bond formation between an amino-functionalized oligonucleotide and an activated carboxylic acid surface (or activation of a 5′-phosphorylated oligonucleotide and subsequent condensation with an amine surface),²² copper catalyzed azide-alkyne cycloaddition (CuAAC) of an azido oligonucleotide to an alkyne surface,²³ or Michael addition of a mercapto oligonucleotide to a maleimide-modified surface (Figure 1).²⁴ These chemistries utilize coupling partners that are readily prepared or are commercially available as pre-functionalized beads and modified synthetic oligonucleotides, respectively. Additional specific coupling partners include amino-modified oligonucleotide immobilization on a variety of activated glass planar and bead surfaces,^{25,26} radical-catalyzed methacrylate-modified oligonucleotide incorporation in polyacrylamide layers,²⁷ and others.²⁸

Selecting an optimal strategy among these alternatives required confirming that any given reaction efficiently immobilizes sufficient oligonucleotide primers per bead, and that the surface-immobilized oligonucleotide products are still substrates for *Taq* DNA polymerase. Hybridization is the classic strategy for quantitating surface-immobilized oligonucleotide density, which we used to evaluate the above chemistry (Figure 2A). In order to quantitate surface-immobilized oligonucleotide substrate integrity, the beads functionalized with various chemistries were hybridized to an 80-nt ssDNA synthetic template in the presence of *Taq* DNA polymerase and dNTPs to extend the surface-immobilized oligonucleotide. The synthetic template was then denatured from the beads, and the enzymatic extension product probed in a second hybridization with an oligonucleotide complementary to the newly synthesized strand (Figure 2B).

Carbodiimide-mediated -COOH/-NH_2 amide bond formation is the most economical and straightforward of the three approaches. Activation of a -COOH surface using a carbodiimide yields reactive esters that can couple with any number of nucleophiles present in an oligonucleotide. Probe hybridization analysis revealed that $5'$ - NH_2 -modified primer coupling to EDC-activated carboxylic acid beads proceeded reproducibly and covalently, with incorporation of $\sim 26\text{k}$ primers per $1.05\text{-}\mu\text{m}$ -dia. bead and $\sim 180\text{k}$ primers per $2.8\text{-}\mu\text{m}$ -dia. bead, a difference consistent with the surface areas of the two bead sizes (Figure 3A). Side-by-side coupling of both $5'$ - NH_2 -modified oligonucleotide primers and unmodified $5'$ -OH oligonucleotide primers to EDC-activated carboxylic acid beads yielded almost identical numbers of immobilized primers per bead. These results imply that the $5'$ - NH_2 is not strictly necessary for primer immobilization and, while primer immobilization is covalent, it is driven by non-specific interactions on bead surfaces with a high density of reactive sites. Indeed, a broad range of conditions known to abrogate non-specific oligonucleotide binding (e.g., mildly alkaline pH, high $[\text{NaCl}]$, detergent) produced no measurable surface functionalization.

The revelation that non-specific interactions potentially drive the EDC-mediated -COOH/-NH_2 bead-oligonucleotide coupling reaction spurred exploration of alternative covalent and chemoselective primer coupling strategies. Both the CuAAC and Michael addition reactions are chemoselective and suitable for oligonucleotide surface immobilization.^{23,29} Magnetic beads were functionalized to display either a CuAAC alkyne coupling partner or a maleimide Michael acceptor. CuAAC and Michael addition reactions yielded primer immobilization comparable to that of streptavidin/biotin primer immobilization. High salt or detergent did not affect coupling yield for CuAAC or Michael addition reactions, suggesting that non-specific bead interactions do not play a role in these reactions (Figure 3B). Furthermore, assays of primer extension revealed that only specifically immobilized oligonucleotides were viable substrates for *Taq* DNA polymerase. Extension of primers immobilized via either CuAAC or Michael addition was comparable to that of $5'$ -biotinyl primers immobilized on streptavidin-coated beads, with a consistent $\sim 4\text{--}7\%$ extension yield from one thermal cycle and in proportion to the number of sites available. Enzymatic primer extension product is not observed for EDC-mediated -COOH/-NH_2 bead-oligonucleotide functionalization.

Primer-functionalized bead viability in BEAMing-type experiments was evaluated using the various oligonucleotide immobilization strategies and a site saturation mutagenesis library in emPCR. Beads prepared using the $-\text{COOH}/-\text{NH}_2$ bead-oligonucleotide coupling chemistry produced no detectable surface-bound template. Amplification on beads proceeded readily following primer attachment using biotin-streptavidin immobilization, Michael addition, or CuAAC. BEAMing-based generation of monoclonally templated beads from solution-phase gene libraries is dependent on encapsulation of single template molecules with a primer-functionalized bead in an emulsion droplet. Variations in droplet volume preclude any meaningful calculations of bead and template distributions, requiring titration of emPCR input DNA concentration to assess the effects on bead loading. Post emPCR, individual beads were analyzed by qPCR (Figure 4). At 1 bead/well, and assuming every bead is templated, the Poisson distribution predicts 37% of wells will be unoccupied. Although bead clonality is dictated by template titration and emPCR encapsulation, the presence of amplified templates is determined by initial primer loading and enzymatic extension viability. Chemoselectively functionalized beads produced $\sim 10\text{k}$ full-length 1,364-bp trypsinogen templates per bead following 30 cycles of emPCR. The beads were clonally templated at the two lowest emPCR input DNA concentrations and generated clean Sanger sequencing traces, indicative of a successful combinatorial bead library preparation (see Supporting Information).

The observations of this letter lead us to propose a mechanism that explains the rationale for the widely-used reaction conditions of EDC-mediated oligonucleotide immobilization on $-\text{COOH}$ surfaces and the nature of the oligonucleotide modification. Oligonucleotides are only immobilized under conditions that promote non-specific oligonucleotide binding to $-\text{COOH}$ beads. Thus, oligonucleotides likely first adsorb, resulting in greatly increased effective concentration of oligonucleotide and surface-bound active esters, and concomitant rapid, covalent coupling. We observe such covalent coupling for oligonucleotides lacking a 5' amino modification and even for 5'-OH oligo dT, which lacks the exocyclic amines of the other three nucleobases. Taken together with the observations that EDC-mediated coupling also prevents enzymatic extension of the surface-bound products while apparently not disrupting their ability to hybridize, coupling must be occurring through the only remaining nucleophiles, which are the 5' and 3'-OH groups, the latter being the nucleophile that polymerases require for template-directed dNTP polymerization. Ordinarily one would not anticipate cross-reactivity with alcohols (particularly the 3' secondary alcohol) as carbodiimide chemistry finds wide use in solution-phase coupling of myriad functionality with 5' amino oligonucleotide substrates. However, the effective concentration argument above would explain how this transesterification is possible under otherwise mild reaction conditions.

Several previous investigations used carbodiimide chemistry to immobilize oligonucleotides on a variety of surfaces with varying degrees of success. The most prominent example is 454 sequencing emPCR bead preparation, which entails immobilization of amino-modified oligonucleotides to very large $\sim 30\text{-}\mu\text{m}$ -dia. sepharose NHS-ester beads.¹² The reaction occurs at pH 8, disfavoring adsorption, and occurs in large excess of 5' amino-modified oligonucleotide. The large capacity of these beads may also mitigate the otherwise low efficiency of emPCR. Other work describes the mildly acidic carbodiimide chemistry more

or less as above,^{18,30} but with low or unreported efficiency of PCR. If this chemistry is a product of non-specific adsorption as the present data suggest, non-specifically *and* noncovalently bound oligonucleotide primers may be present in emPCR droplets to drive product formation. However, such products confound hybridization and bulk qPCR analysis of bead templating, and ultimately complicate downstream applications that require high-fidelity association of genotype and solid support.

In conclusion, chemoselectively functionalized beads via CuAAC or Michael addition preserve the substrate integrity of the surface-immobilized oligonucleotide whereas EDC-mediated coupling to –COOH beads does not. The widely cited EDC/NHS protocol (which almost ubiquitously invokes activation and coupling in mildly acidic conditions) deserves careful scrutiny as hybridization analysis of the immobilized primer alone was an insufficient measure of effective surface functionalization for emPCR library generation. The CuAAC beads and oligonucleotides are easily prepared using inexpensive reagents and yield highly stable, covalent triazole linkages, providing an attractive alternative to streptavidin-based oligonucleotide immobilization. This is particularly useful for directed evolution experiments involving BEAMing and bead display,^{17,19} where implementation of CuAAC (or other suitably chemoselective coupling) frees the experimenter to use biotin as a selection marker. If our mechanism is correct, chemoselective oligonucleotide immobilization is not merely a matter of surface chemistry control, but a previously unappreciated, strict requirement for applications involving downstream enzymatic transformations of an immobilized oligonucleotide substrate.

Experimental Procedures

Buffers and Oils

Bead buffer (BB, 10 mM Tris pH 8.3, 100 mM NaCl, 0.5 mM EDTA, 0.05% KF-6012), breaking buffer (BrB, 10 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 % w/v SDS, 1 % v/v Triton), bind and wash buffer (BW, 5 mM Tris pH 7.5, 1 M NaCl, 0.5 mM EDTA), bind and wash buffer with Tween-20 (2X, BWBT, 10 mM Tris pH 7.5, 2 M NaCl, 1 mM EDTA, 0.1 % Tween-20), saline sodium citrate buffer (2X, SSC, 30 mM sodium citrate pH 7.0, 0.5% w/v SDS, 0.3 M NaCl), and PCR buffer (10X, 100 mM Tris pH 8.3, 500 mM KCl, 15 mM MgCl₂, New England Biolabs) were prepared in DI H₂O and used directly except where noted otherwise. Oil for emulsion PCR (4/20/76, w/w/w, KF-6038/mineral oil/DMF-A-6cs) was prepared gravimetrically and mixed with gentle rotation (2 h, 8 rpm) prior to use.

Oligonucleotides

Oligonucleotides (Integrated DNA Technologies, Inc. Coralville, IA) were purchased as desalted lyophilate and used without further purification unless otherwise noted. Oligonucleotides, indicated by “≈” followed by a bolded numeric identifier in the text, included the following: reverse PCR primer ≈**01**, 5′–AGACCGAGATAGGGTTGAGTGTG–3′; reverse PCR primer hybridization probe (5′-fluorescein labeled) ≈**02**, 5′–CACTCAACCCTATCTC–3′; synthetic template ≈**03**, 5′–CCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAACTGGAA CAACACTCAACCCTATCTCGGTCT–3′; reverse PCR primer extension hybridization

probe (5'-fluorescein labeled) ≈ 04 , 5'-TGGAGTCCACGTTCTTTAAT-3'; T7 expression cassette forward PCR primer ≈ 05 , 5'-TGATGCCGGCCACGATG-3'; mutagenesis overlap reverse primer ≈ 06 , 5'-CAGGAAGCCCACACAGATCATGTT-3'; site saturation mutagenesis forward primer ≈ 07 , 5'-AACATGATCTGTGTGGGCTTCCTGNNSNNSNNSNNTCTTCTTGCCAGGGTGATAGTGGC-3' ("S" degeneracy indicates "G" or "C"); 5' exonuclease assay probe ≈ 08 , 5'-ATGAATACC*TTTGTCTGCTGGCACTGCTG-3' (5'-fluorescein labeled, 3'-Iowa Black quencher labeled, *internal ZEN quencher, HPLC purified at manufacturer).

Probe hybridization of primer coupling

Primer-coupled magnetic resin aliquots (2×10^6 2.8- μ m-dia. beads; 1×10^7 1- μ m-dia. beads) were transferred to separate 1.5-mL tubes and supernatant was removed. Resin aliquots were combined with fluorescein-labeled ≈ 02 (20 pmol in 2X SSC, 20 μ L) vortexed, and incubated (5 min, RT). The probe-hybridized resin was magnetically separated, supernatant was removed, and the resin was washed (2X SSC, 3 x 20 μ L). Probe was eluted (90% formamide in H₂O, 20 μ L, 1 min, 50 °C). Fluorescence of elution fractions and standards (400 nM, 200 nM, 100 nM, 50 nM, 40 nM, 20 nM, and 10 nM in 2X SSC, 20 μ L) was measured (channel 1, CFX96 Real-Time System, Bio-Rad, Hercules, CA). The number of primers per bead was determined by dividing the number of primers measured by probe hybridization by the number of beads in the hybridization reaction.

Enzymatic oligonucleotide extension

Oligonucleotide PCR primer ≈ 03 -functionalized magnetic resin aliquots (1×10^7 2.8- μ m-dia. beads; 4×10^7 1- μ m-dia. beads) were transferred to separate 1.5-mL tubes and supernatant was removed. The resin aliquots were each combined with extension reaction mixture (0.2 μ M synthetic template ≈ 03 , 1X PCR buffer, 0.2 mM each dNTP, 0.05 U/ μ L *Taq*, DI H₂O, 100 μ L total), vortexed, and incubated with shaking (1 h, 65 °C, 900 rpm). Supernatant was removed and all resin types were washed with denaturant to strip ≈ 03 . Streptavidin-functionalized resin was washed with aqueous alkaline solution (0.1 N NaOH, 100 μ L, 1 min, 60 °C); all other resin types were washed with formamide denaturant (90% formamide in H₂O, 100 μ L, 1 min, 60 °C). Denaturing supernatant was removed and resin was immediately subjected to probe hybridization described below.

Probe hybridization of primer extension reactions

Primer-extended magnetic resin aliquots (1×10^7 2.8- μ m-dia. beads; 4×10^7 1- μ m-dia. beads) were transferred to separate 1.5-mL tubes and supernatant was removed. Resin aliquots were combined with fluorescein-labeled ≈ 04 (20 pmol in 2X SSC, 20 μ L), vortexed, and incubated (30 min, RT). The probe-hybridized resin was magnetically separated, supernatant was removed, and the resin was washed (2X SSC, 3 x 20 μ L). Probe was eluted (90% formamide in H₂O, 2 x 20 μ L, 1 min each, 50 °C). Fluorescence of elution fractions and standards (400 nM, 200 nM, 100 nM, 50 nM, 40 nM, 20 nM, and 10 nM in 2X SSC, 20 μ L) was measured (channel 1, CFX96 Real-Time System, Bio-Rad). The number of primers per bead was determined by dividing the number of primers measured by probe hybridization by the number of beads in the hybridization reaction.

Site-saturation mutagenesis library construction

Amplification reactions (50 μ L) were prepared in DI H₂O containing trypsinogen expression cassette template (1 ng), PCR buffer (1X), dNTPs (200 μ M) and either oligonucleotide primers $\approx$ 05 and $\approx$ 06 (0.2 μ M each) or $\approx$ 07 and $\approx$ 01 (0.2 μ M each) and *Taq* (0.05 U/ μ L). The reactions were thermally cycled ([95 °C, 15 s; 62 °C, 20 s; 68 °C, 60 s] x 20 cycles, C1000, Bio-Rad) and the products confirmed on agarose gel. Products were purified (MinElute, Qiagen, Valencia, CA) and quantitated by A260. Purified products (5 ng each) were added to an assembly reaction (50 μ L) containing PCR buffer (1X), dNTPs (200 μ M each), *Taq* (0.05 U/ μ L), and oligonucleotide primers $\approx$ 01 and $\approx$ 05 (0.2 μ M each). The assembly reaction was thermally cycled ([95 °C, 15 s; 55 °C, 15 s; 68 °C, 90 s] x 20 cycles, C1000, Bio-Rad). Products were resolved on low melting temperature agarose gel (SeaPlaque, 1.2%, Lonza, Basel, Switzerland), excised, purified (QIAEX II, Qiagen), and quantitated by A260.

Amplification of template on primer-coated beads

PCR amplification mixture (150 μ L) was prepared in DI H₂O containing PCR buffer (1X), dNTPs (0.2 mM each), $\approx$ 05 (8 μ M), butanediol (5%), PEG-8000 (4.8 mM), KF-6012 (0.02%), *Taq* (0.05 U/ μ L), beads functionalized via CuAAC using 5'-N₃-modified $\approx$ 01 (2.5×10^6 beads, 2.8 μ m, Figure S1), and trypsinogen site saturation mutagenesis template (1.1 pg/ μ L, 5.3 pg/ μ L, or 13.3 pg/ μ L). The reaction was transferred to a SafeLock tube (2 mL, Eppendorf, Hauppauge, NY) containing a stainless steel ball (6 mm dia., Thomas Scientific, Miami, FL). The sample was combined with emulsification oil (600 μ L), loaded into a homogenizer (TissueLyser, Qiagen) and emulsified (10 s, 15 Hz; 10 s, 17 Hz). The emulsion was placed on ice immediately and aliquots (50 μ L) were transferred to PCR tubes for thermal cycling ([95 °C, 20 s; 68 °C, 90 s] x 30 cycles; 68 °C, 600 s; C1000, Bio-Rad). Emulsion samples were pooled, combined with BrB (750 μ L), and mixed. The beads were collected by centrifugation (5 min, 3,000 rcf) and magnetically separated. Supernatant was removed and the beads were washed (BrB, 2 x 1 mL), resuspended (BrB, 100 μ L), transferred to a new tube. Washing and resuspension was repeated twice more. Bead particle density was quantitated using a hemocytometer and adjusted (84 beads/ μ L final). PCR amplification reaction mixture (2 mL) was prepared in DI H₂O containing PCR buffer (1X), dNTPs (200 μ M each), *Taq* (0.05 U/ μ L), oligonucleotide primers $\approx$ 01 and $\approx$ 05 (0.5 μ M each), and $\approx$ 08 (250 nM). An amplification reaction mixture aliquot (320 μ L) was reserved for template standards (20 μ L each, prepared in log-scale dilutions from 1×10^5 – 1×10^{-1} fg/ μ L). To the remaining mixture was added emPCR bead product (84 beads), the sample was vortexed, and aliquots (20 μ L) were distributed to 80 wells of a 96-well plate. The plate was thermally cycled ([95 °C, 15 s; 68 °C, 80 s] x 40 cycles; C1000, Bio-Rad) with fluorescence monitoring (channel 1, CFX96, Bio-Rad) and quantitated (CFX Manager, version 3.1, Bio-Rad, C_q method), assigning C_q to each well. Wells with C_q within the standard curve were used to calculate the number of templates per bead.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NHS	N-hydroxysuccinimide
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
SDS	sodium dodecyl sulfate
CuAAC	copper-catalyzed azide-alkyne cycloaddition

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Figure 1. Surface functionalization chemistry

Various bead surface functionalization (R₁) and oligonucleotide 5'-terminus modifications (R₂) mediate coupling. Streptavidin-coated beads noncovalently bind biotinylated oligonucleotides (**1**). Carboxylic acid-functionalized beads, once suitably activated (e.g., carbodiimide), covalently couple to amino oligonucleotides via amide bonds (**2**). Carboxylic acid-functionalized beads are similarly activated and functionalized to yield an alkyne surface, which covalently couples to azido oligonucleotides via triazole linkage product of CuAAC (**3**). Amine-functionalized beads are further elaborated to yield a maleimide surface, which covalently couples to mercapto-modified oligonucleotides via thioether linkage product of Michael addition (**4**).

Figure 2. Functionalization density and enzymatic substrate integrity quantitation of surface-immobilized oligonucleotides

(A) Bead functionalization density with oligonucleotide ≈01 coupled via one of several linkages (X) is quantitated by hybridizing fluorescently-labeled (F) complementary probe oligonucleotide ≈02, washing away unbound ≈02, eluting bound ≈02, quantitating fluorescence, and normalizing to the number of beads quantitated. (B) Enzymatic substrate integrity is quantitated by hybridizing an 80-nt synthetic template oligonucleotide ≈03 to beads displaying surface-immobilized ≈01 and enzymatically extending ≈01, eluting synthetic template ≈03, hybridizing fluorescently-labeled complementary probe oligonucleotide ≈04, and quantitating as above. 1) [≈01] = 1 μM in 2X SSC, 5 min, RT; 2) [Taq] = 0.05 U/μL, [dNTPs] = 0.2 mM each, 1X PCR buffer, 1 h, 65 °C; 3) 90% formamide in H₂O, 1 min, 60 °C (streptavidin-coated beads treated with 0.1 N NaOH instead of formamide); 4) [≈04] = 1 μM in 2X SSC, 30 min, RT

Figure 3. Oligonucleotide primer immobilization and enzymatic extension on variously functionalized bead surfaces

(A) EDC-activated carboxylic acid bead surface functionalization with oligonucleotide primer **1** (either 5'-NH₂-modified or native 5'-OH) as quantitated via probe hybridization indicates primer loading, but only under conditions lacking detergent (SDS). The presence of SDS in the functionalization reaction completely abrogates primer coupling to the bead surface, independent of bead diameter (either 1.05- μm dia. or 2.8- μm dia.). (B) Specific addition of 5'-biotinyl (-bio) primers to streptavidin-functionalized resin, 5'-azido (-N₃) primers to alkyne-functionalized resin, or 5'-mercapto (-SH) primers to maleimide-functionalized resin preserves the surface-immobilized oligonucleotides' integrity as a DNA polymerase substrate as measured by probe hybridization of enzymatic extension product. *Taq* DNA polymerase can extend the surface-immobilized primers (4–7%), independent of bead diameter or specific functionalization strategy. Primers (5'-NH₂-modified) covalently immobilized on beads via non-specific reaction with an EDC-activated carboxylic acid bead surface are not viable substrates for *Taq* DNA polymerase.

Figure 4. Azido oligonucleotide primer-functionalized beads amplify in emPCR to yield high-quality library beads

Individual 2.8- μm -dia. beads are analyzed by qPCR at an average of 1 bead/well. The Poisson distribution predicts 63% of the wells will be occupied by at least one bead (the remaining will be empty). As the [input DNA] of emPCR is reduced, fewer beads are coencapsulated with a library gene, and therefore templated. Here, 24/80 wells amplify emPCR [input DNA] = 1.1 pg/ μL , 47/80 wells amplify at 5.3 pg/ μL , and 48/77 wells amplify at 13.3 pg/ μL .