MICELLULA DNA
Emulsion & Purification Kit

Kit for creation of emulsions and subsequent DNA purification.
For emulsion PCR (ePCR) and for nucleic acid amplifications in emulsions.

Cat. no. 3600

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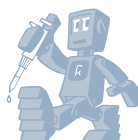

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Kit Contents

- Emulsion component 1,
- Emulsion component 2,
- Emulsion component 3,
- Buffer DX,
- Buffer Orange DX,
- Buffer Wash DX1,
- Buffer Wash DX2,
- Buffer Elution,
- PCR/DNA purification columns.

For maintaining maximum flexibility, any of the following components are *not contained* in this kit and have to be supplied by the user:

- **2-butanol (isobutanol) or butanol** (flammable reagent; 1.0 ml per column required),
- Any kind of beads,
- Acetylated bovine serum albumin (e.g. Cat. No. E4020),
- Thermostable DNA polymerase and 10x buffers
  (See below for a list of tested and supported DNA polymerases),
- Any other DNA-modifying enzyme,
- dNTP ix,
- 2 ml plastic reaction tubes (one tube is required per reaction).

General Notes

Note 1: **Please read through this complete manual at least once before starting**, if not familiar with the underlying concepts of enzymatic reactions in emulsions.

Note 2: Once the kit is unpacked, store components at room temperature. In case of occasional buffer ingredients precipitation, simply warm up in a 37°C water bath, until clarified.

Note 3: All solutions should be kept tightly closed to avoid evaporation and resulting components concentration changes.

Note 4: Do not use surfactant solutions out of shelf-life.

Note 5: The maximum column DNA binding capacity is 20 µg.

Note 6: By standard convention, water-in-oil inclusions are referred to as "reverse micelles", whereas the term "micelle" in a strict sense corresponds solely to oil-in-water emulsions, such as milk. For the sake of readability, the term "micelle" will be used as an abbreviation for "reverse micelles" and thus for water-in-oil inclusions throughout this manual.

Note 7: Emulsion Components 1, 2 and 3 are stable at temperatures up to 100°C.

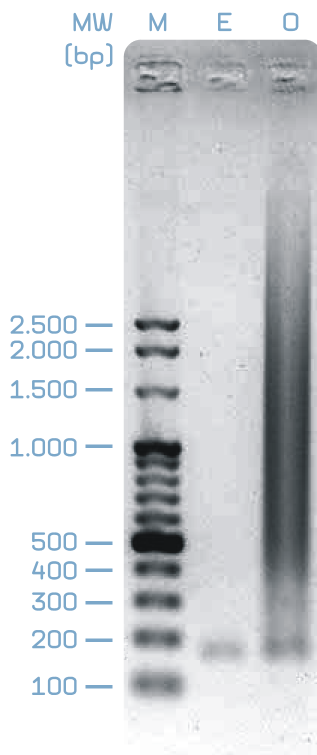

Figure 1: Follow-up amplification of unpurified PCR product from bacterial genomic template DNA. Expected product size: ~200 bp. (M) EURx 100 bp DNA Ladder (Cat. No. E3134), (E) Emulsion PCR, (O) unemulsified, “open” control reaction.

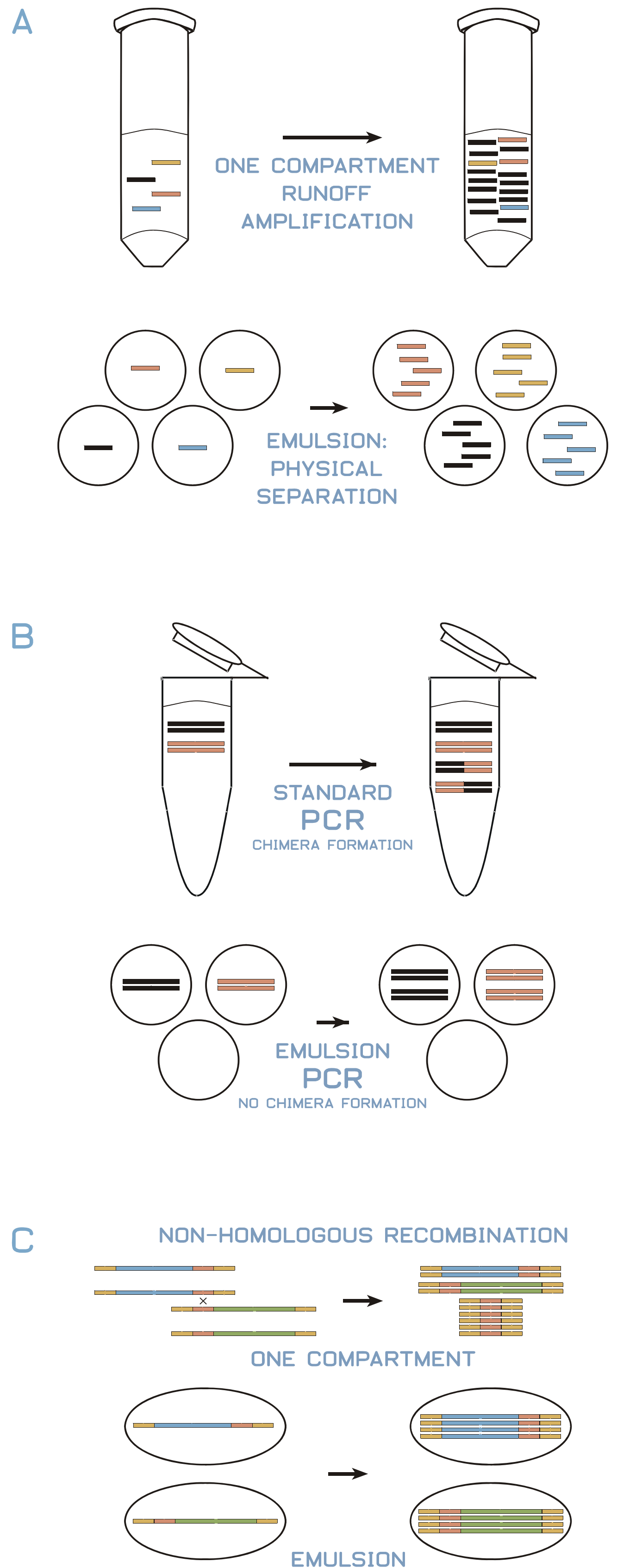

Figure 2: Emulsion PCR (A) reduces the probability of chimera formation by physical separation of template DNA molecules, (B) prevents runoff amplification due to unequal amplification rates and (C) prevents non-homologous recombination. Rare or difficult-to-amplify sample molecules are preserved and high sample diversity is maintained throughout the entire reaction.

Introduction

The Micellula Emulsion & DNA Purification System offers you a straightforward, easy and reproducible method for miniaturization of DNA-targeted enzymatic reactions. It allows to scale down high throughput methods and to miniaturize massive parallel reactions to the size of only one single or a few plastic reaction tubes. The kit comes in handy for applications such as emulsion PCR (ePCR) and other nucleic acid amplification methods.

In conventionally conducted molecular biological reactions, most notably in amplification reactions such as PCR, compounds interact within one single body of liquid. As a consequence, either introduced errors (e.g. contaminating DNA) or any errors occurring during early reaction steps (e.g. formation of primer dimers or chimeric molecules) may freely propagate, without any hindrance, through the entire reaction body. This may lead to non-specific or false results, e.g. for PCR: false positives, non-relevant bands in genetic fingerprints, too short amplification products, chimeras, etc. …

Most notably, complex DNA mixtures consist of DNA fragments with partially similar sequence stretches. Typical examples include complex gene libraries with identical linkers or environmental 16S rRNA genes. Starting PCR from a mixture of DNA fragments sharing partially high sequence homology can lead to partial primer extension and incomplete elongation during PCR amplification. In follow-up PCR cycles, eventual homologous recombination of incompletely extended DNA fragments at similar but not identical sequence stretches may occur, resulting in the formation of chimeric molecules.

Else, if assays aim at maintaining an as-high-as-possible diversity, any preferential propagation of one single or of a few quickly propagating molecule species within an homogenous reaction body leads to quick depletion of reaction components. The result is an undesired, effective reduction of diversity at the expense of slower propagating members of the molecule population. Or, in other words, the best amplifying templates may not always be the templates of interest.

The impact of erroneous artifact amplification as well as the bias caused by preferential amplification is minimized by using this Emulsion & DNA Purification System. This kit allows to divide the liquid body into a very large number of minute, physically separate, distinct reaction compartments. Spatial separation of reaction bodies is achieved by creating a water-oil emulsion, containing a very large number (approx. 10^{10} per ml) of very small, physically separated reaction vessels. By choosing an appropriate dilution of sample DNA, almost each single molecule of template DNA is segregated within its own distinct reaction volume, a reverse micelle (throughout this manual briefly referred to as micelle). Each molecule of template DNA is confined with a distinct reaction compartment and is amplified to the extend until all resources contained within this host micelle are depleted – not any further. A large-scale competition for resources within the entire reaction volume is not possible.

Using this kit offers the following advantages over conventional assays:

- Creation of an extremely stable water-in-oil emulsion (from room temperature to 95°C).
- Specifically adjusted, optimized spin column set for extracting DNA from emulsions.
- Ease, speed and reproducibility for creating emulsion reactions.
- Close spatial proximity of reactants and template nucleic acids.
- Protection from common PCR drawbacks (such as false positives, primer dimers, chimeras).
- Run independent reactions in the same reaction vessel (micelles do not merge).
- Greatly increased reliability for genetic fingerprinting techniques.
- Avoids bias due to unequal amplification rates for different DNA templates.
- Enables high numbers of PCR cycles without introducing bias in amplification.
- Possibility for unbiased scale-up of very small template DNA quantities by pre-amplification.
- Facilitates amplification reactions, when starting from small amounts of template DNA.

Implementing and Standardizing Emulsion Reactions

For a successful implementation and standardization of enzymatic emulsion reactions in your lab, conduct the following tasks in this order:

1. Establish working conditions that allow setting up emulsion reactions with maximum reproducibility. We recommend to create emulsion reactions at +4°C. (i.e. to mix oil and enzyme / water phase at this temperature). Thus, the reaction setup facility should be kept in a +4°C environment, either in a cold room or in a fridge. The setup facility should basically contain a vortexer with a holding fixture for plastic reaction tubes
2. Optimize the amount of BSA per reaction by titrating optimum BSA concentration.
3. Estimate the number of micelles per reaction assay (see appendix of this manual)
4. Calculate the number of template copies that will be introduced per reaction.
5. Estimate the average template copy number per micelle.

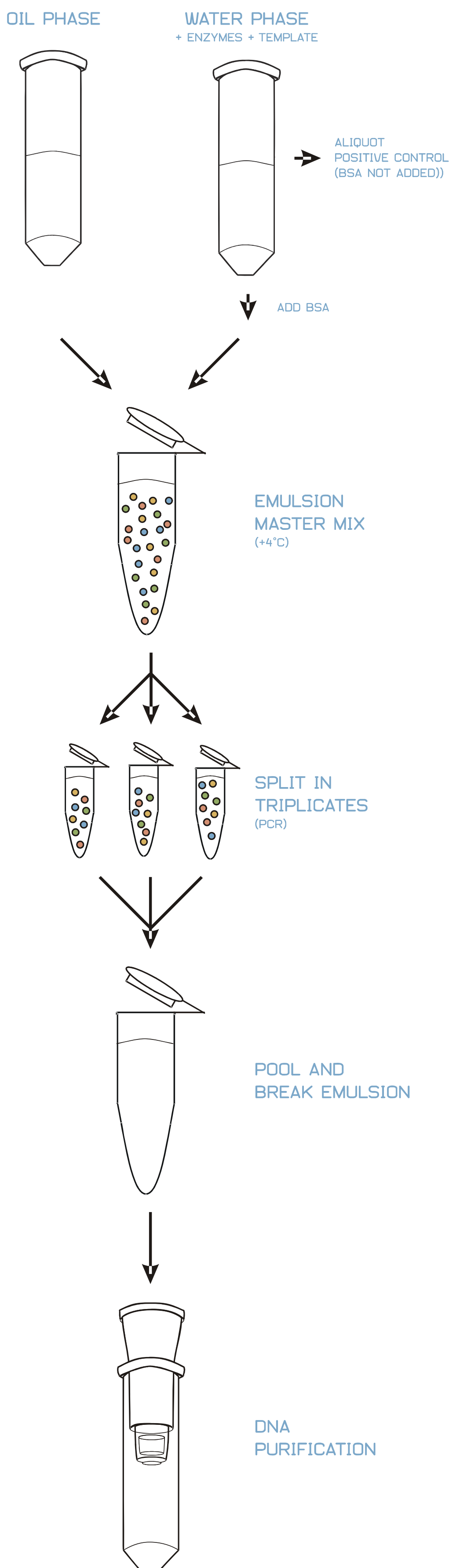

Figure 3: Generic work flow for enzymatic emulsion reactions (e.g. ePCR). Sample volume of the water phase is 50 µl. Total reaction volume increases due to addition of the oil phase. Thus, for thermocontrolled reaction conditions (e.g. ePCR), the total reaction volume is split into triplicates.

Designing Your Experiment

The total count of micelles, i.e. the total number of separate reaction volumes, is estimated to a total count of approx. 10^9 to 10^{10} (see appendix on how to estimate total micelle count per reaction). To prevent the inclusion of much more than one template molecule per micelle on average, it is recommended to keep the maximum number of template molecules below 10^8 to 10^9, i.e. to less than 10 % of the total micelle count. If maximum yield is desired, the best opportunity is to increase the total number of PCR cycles, because emulsion PCR effectively prevents the introduction of PCR bias due to unequal amplification rates. It is also possible to increase the total number of template DNA molecules, at the cost of slightly reduced reaction specificity.

In emulsion reactions, **DNA yield is negatively correlated to reaction specificity** (prevention of artifact formation). One cannot gain both goals but has to balance between reaction yield and specificity according to actual experimental requirements. Anyway, as compared to conventional, one-volume-reactions, specificity of emulsion PCR is much higher, even when working with relatively high numbers of template DNA copies.

For optimal results, it is recommended to adjust the number of gene copies carefully to the estimated number of micelles per reaction. See below for further information on how to calculate the gene copy numbers present in 1 µg template DNA and for estimating the number of micelles, i.e. separate reaction compartments per reaction volume (Appendix).

Calculating the Number of Copies per µg DNA

Example: How many copies of a gene or genome are contained in a 1 µg DNA sample?

The number of copies per µg DNA is simply calculated by dividing the product of DNA amount (mass) and Avogadro's number (6.022 x 10^{23} molecules / mole) by the product of gene or genome size, a factor for converting mass units from µg to g (10^6 µg = 1 g; use 10^9 for conversion of ng to g), and the average molecular weight of a base pair.

$$Copy\ Number\ [molecules] = \frac{DNA\ amount\ [\mu g] \cdot 6.022 \cdot 10^{23}\ [molecules\ mol^{-1}]}{gene\ or\ genome\ length\ [bp] \cdot 10^{6}\ [\mu g\ g^{-1}] \cdot 616\ [g\ mol^{-1}\ bp^{-1}]}$$

$$= \frac{DNA\ amount\ [\mu g] \cdot 0.978 \cdot 10^{15}\ [molecules\ bp\ \mu g^{-1}]}{gene\ or\ genome\ length\ [bp]}$$

Since Avogadro's number, mass conversion factor and MW per bp are constants, they can be combined to a single conversion factor for convenient calculation (see examples 1 and 2 below). Simplified conversion factors are 0.978 x 10^{15} [molecules bp µg^{-1}] for calculating number of copies from µg scale DNA values and 0.978 x 10^{12} [molecules bp ng^{-1}] for calculations starting from ng values.

Examples:

(1) Human haploid genome (genome size 3.08 x 10^9 bp). 1 µg DNA contains
:

1 µg DNA x (0.978 x 10^{15} [molec. bp µg^{-1}]) / 3.08 x 10^9 [bp] = ~ 3,2 x 10^5 [molecule copies]

(2) *E. coli* genomic DNA (genome size 4.6 x 10^6 bp). 1000 ng (= 1 µg) DNA contain

1000 ng DNA x (0.978 x 10^{12} [molec. bp ng^{-1}]) / 4.6 x 10^6 [bp] = ~ 2.1 x 10^8 [molec. copies]

(3) 1 µg sample of a 978 bp DNA fragment contains 1 x 10^{12} copies of template DNA

Note 1: When referring to genomic DNA as an entity, the number of copies has to be calculated from the MW of total genomic DNA rather than from the MW of the target DNA fragment size.

Note 2: Pay attention, whether genome size refers to the size of a haploid, a diploid or a polyploid genome. Per convention, genome size is published as haploid genome size.

Note 3: This calculation assumes an average relative molecular weight for a pair of nucleotides of ~ 615.9 and a GC-content of 50 % (Doležel et al. Cytometry, 2003, Vol. 51A, 2, 127-8). Since the molecular weights for an AT-base pair (615.4) and a GC-base pair (616.4) are very similar, any error for extremely AT- or GC- rich sequence stretches will never exceed 1 %.

10^8 - 10^{10} template DNA copies are recommended per reaction. If total template copy number is much smaller than 10^8, consequently, at an estimated total micelle count of 10^{10}, only <1 % of micelles would hold a copy of template DNA. Total reaction yield would be poor. One strategy to increase reaction yield, when starting from low template copy numbers (<10^8), is to scale up the amount of template DNA by pre-amplification. Because ePCR effectively inhibits the introduction of PCR errors, it is possible to conduct a two-step approach by setting up an emulsion PCR on top of a pre-amplified emulsion PCR. See part II of the protocol section for further information.

Estimating the Average Template DNA Copy Number Per Cell

Assuming roughly equal size of individual micelles, copies of template DNA are not distributed equally to micelles, in a one-template-DNA per one-micelle manner. Instead, the allocation pattern roughly follows Poisson distribution (but see Swami et al., Langmuir. 2008 Oct 21; 24(20):11828-33 for a differentiated discussion). This has an interesting consequence: 10^{10} template DNA copies will not be equally distributed to 10^{10} micelles. Rather there will be a fraction of micelles hosting one, two or much more copies of template DNA, whereas another fraction of micelles will remain completely empty. At any given ratio of total template DNA copy count (say, 10^{9}) to total micelle count (say, 10^{10}), the probable percentage of micelles hosting exactly 0, 1, 2, … template copies can be estimated using the Poisson formula

$$f(k;\lambda)=\frac{(\lambda^{k} e^{-\lambda})}{k!}$$

where k is the estimated number of template DNA copies per micelle, e is the base of the natural logarithm (e =~ 2.718…) and λ is calculated by dividing the total number of template DNA copies by the total number of micelles. For an estimated 10^{9} copies of template DNA and 10^{10} micelles per reaction (i.e. on average 10 % occupied micelles), λ equals 0.1. For 20 % average micelle occupation, λ equals 0.2 etc.

It becomes obvious, that there will always remain a percentage of micelles hosting not just one single template DNA molecule, but either zero or more than one template DNA copies. Consequently, due to the high number of empty micelles, at low template DNA concentrations, reaction specificity is high but PCR yield is low, resulting in unused reaction resources. With increasing template DNA concentration, the probability of any micelle remaining completely empty, i.e. not containing any DNA template will decrease. The higher the number of micelles hosting at least one template DNA copy, the more of the supplied resources are used and, in turn, the total product yield of the whole reaction increases. A higher number of template DNA results in a higher yield of reaction products. As DNA concentration increases within a certain range, the fraction of micelles hosting exactly one template molecule increases too, but will never exceed 40 % of total micelle count (see Figure 1).

But one has to be careful: While increasing template DNA concentrations, the number of micelles hosting more than one single DNA molecule increases too. With increasing template DNA concentration, PCR specificity decreases slightly but steadily. Thus it is mandatory to balance reaction yield and reaction specificity exactly according to requirements during experimental design. When speaking of decreasing specificity: Keep in mind, that even when starting from excess DNA concentrations there will be only a few template copies per micelle present (typically below 10). Compared to conventional one-volume reactions with 10^{10} – 10^{12} possibly interacting template DNA copies present in one single reaction compartment, the complexity in emulsion reactions is dramatically reduced. The following table summarizes some values relevant for planning your experiment:

Average no. of template copies per micelle [n] ↓ ↓	[%] micelles statistically not hosting any template copies	[%] micelles statistically hosting one single template copy	[%] micelles statistically hosting more than one template copy
10 %, λ = 0.1	90.5	9.0	0.5
20 %, λ = 0.2	81.9	16.4	1.7
30 %, λ = 0.3	74.1	22.2	3.7
50 %, λ = 0.5	60.7	30.3	9.0
100 %, λ = 1	36.8	36.8	26.4
200 %, λ = 1	13.5	27.1	59.4
500 %, λ = 5	0.7	3.4	95.9

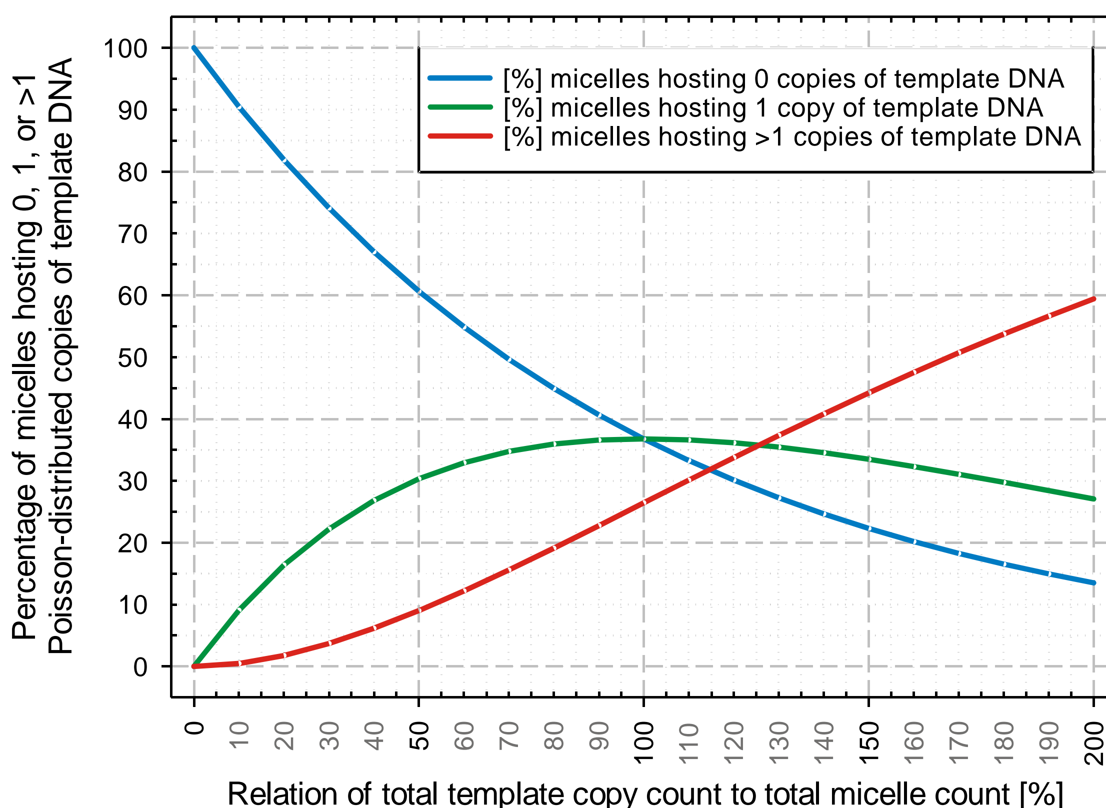

Figure 4: Effect of total template DNA count on the random distribution of template copies per micelle, assuming Poisson-distribution. When template DNA count equals the number of micelles, roughly 37 % of micelles host no template DNA molecule at all, whereas approx. 26 % of micelles host two or more template copies. Aiming at maximum reaction specificity (at the price of low PCR yields) no more than one template molecule should be present per micelle. In this case total template DNA copy count should be kept below 20 %, ideally to 10 % of total micelle count for avoiding any detectable chimera formation. If instead high PCR yield at quite high, but not maximum reaction specificity is desired either the number of PCR cycles (recommended) or the total number of template DNA copies can be increased. The experimenter may balance reaction yield and reaction specificity according to his requirements.

Protocol

Part I: Setting up the Emulsion

1. Create *Oil Surfactant Mixture* (300 µl per reaction):

~73 %	Emulsion Component 1	220 µl
~7 %	Emulsion Component 2	20 µl
20 %	Emulsion Component 3	60 µl
	Total Volume	*300 µl*

- Mix thoroughly by vortexing.
- Keep on crushed ice until further usage.

Note 1: Emulsion Component 2 is very viscous. Mixing of all components is greatly facilitated, when adding Emulsion Component 2 not as first component to the empty reaction tube.

Note 2: Use precut pipette tips for a proper transfer of the highly viscous Emulsion Component 2. Use a clean and sterile scissor for cutting off the ends of pipette tips, resulting in a larger pipette tip diameter and an easier transfer of Emulsion Component 2.

Note 3: Do not use any precipitate that may occasionally form in Emulsion Component 1. Do not use surfactant solutions out of shelf life.

Note 4: Do not store assembled Oil Surfactant Mix for more than two days.

Note 5: Due to the high viscosity of components, we recommend to assemble the Oil Surfactant Mixture at room temperature, followed by precooling of the readily assembled mixture to +4°C.

Note 6: Use 300 µl *Oil Surfactant Mixture* per reaction, in case volume of enzymatic water phase equals 50 µl. In case water phase volume does not equal 50 µl, adjust *Oil Surfactant Mixture* volume proportionally.

Note 7: To a certain extent, the proportions of the three components can be varied for obtaining emulsions with different properties. Large deviations from the above given compound composition may result in unstable emulsions.

Note 8: PCR buffers from third parties may contain non-ionic surfactants such as Triton X-100, which are known to affect composition and stability of the emulsion even at low concentrations.

As an example, protocols for emulsion PCR (ePCR) and scale-up emulsion PCR (when starting from low template copy numbers) are given. These protocols serve as a starting point for working with emulsion reactions by illustrating basic concepts and caveats.

Emulsion PCR (ePCR)

2. Create *PCR Water Phase*:
   Mix PCR sample on ice. A single typical emulsion PCR reaction contains a water phase of 50 µl. Per 50 µl water phase, mix*:

10 x PCR Buffer (with or without $MgCl_2$)	1 x	5 µl
$MgCl_2$ [25 mM], if added separately	1.5 mM (or 1 – 5 mM)	3 µl (or 2 – 10 µl)
BSA, acetylated [10 mg/ml] (see Note 1)	0 - 1 mg/ml	0 – 5 µl
Forward Primer [100 µM]	2 µM (or 0.2 – 2 µM)	1 µl
Reverse Primer [100 µM]	2 µM (or 0.2 – 2 µM)	1 µl
dNTP mix [5 mM]	200 µM	2 µl
Thermostable DNA Polymerase [5U/µl]**	2.5 U (or 1.25 – 2.5 U)	0.25 - 0.5 µl
Template DNA	up to 10^9 copies	
Sterile, DNA-free H_2O		@50 µl

Note 1: In most reactions, 0.01 mg/ml EURx BSA (Cat. No. E4020) is the optimum amount for DNA polymerases manufactured by EURx. We recommend EURx Taq (Cat. No. E2500) or OptiTaq (E2600, with “proofreading” activity) for obtaining maximum performance. Acetylated BSA is required for a proper coating of the hydrophilic / hydrophobic interface at the micelle border. This prevents the enzyme (DNA polymerase) from denaturation at the interface border due to orientation of hydrophilic / hydrophobic residues within both liquid phases. In case further BSA addition is necessary, the amount of BSA required for the reaction is dependent on the extent of the micelle borders surface area and thus on the composition of the emulsion, i.e. micelle count and size. Generally, the smaller the micelle size, the higher the total micelle number, the higher the area of the hydrophilic / hydrophobic interface and thus the higher the required amount of BSA. Caution: Excess BSA is an effective inhibitor of PCR reactions. As a consequence, neither too much nor too less BSA should be added to the reaction. The emulsion composition is dependent on variations in the mixture, on vortexing speed and duration. Thus, micelle droplet size and the comprehensiveness of the water / oil phase interface area may vary between labs. Consequently, the amount of BSA required for coating the water / oil phase interface may vary. To prevent ePCR inhibition due to excess BSA, during first establishment of the emulsion technique in your lab, it is mandatory that you once determine empirically the amount of BSA required by performing an appropriate dilution series. Example: During implementation and standardization of the procedure, the following BSA concentrations could be tested: 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, and 1 mg/ml, respectively.

Note 2: When running an “open” (non-emulsified) control reaction, we recommend to proceed as follows:
(1) Prepare a PCR master mix without BSA.
(2) Retain an aliquot for setting up the non-emulsified reaction control.
(3) Add an appropriate amount of BSA.
(4) Proceed setting up the emulsion reaction as described below.

3. Create *Emulsion Reactions*.
- Mix 300 µl precooled (wet ice) Oil Surfactant Mixture
- Add 50 µl precooled (wet ice) PCR Water Phase containing 5 µl BSA stock solution
- Mix PCR assay in a cold room using vortexer for 5 min at max speed or, alternatively, use a bead beater (Caution: Just mix the liquid sample thoroughly, do not use glass beads or similar).
- Dispense each aliquot in equal amounts to three empty, thin-walled PCR tubes ("triplicates").
- Perform regular PCR program in a standard PCR cycler

* Also consult the manual that shipped along with your chosen thermostable DNA polymerase.
** This protocol has been tested and is known to work with any of the following thermostable DNA polymerases:

Standard Taq DNA Polymerases	EURx Taq DNA Polymerase	Cat. No. E2500
	EURx Native Taq DNA Polymerase	Cat. No. E2504
Optimized Enzyme Blends:	EURx OptiTaq DNA Polymerase	Cat. No. E2600
	EURx Amplus DNA Polymerase	Cat. No. E2900
HotStart:	EURx Perpetual Taq DNA Polymerase	Cat. No. E2700
	EURx Perpetual OptiTaq DNA Polymerase	Cat. No. E2720
Proofreading:	EURx Pfu DNA Polymerase	Cat. No. E1114
	EURx PfuPlus DNA Polymerase	Cat. No. E1118
	EURx Hybrid DNA Polymerase	Cat. No. E2950
One-Step RT-PCR:	dART 1-Step RT-PCR Kit	Cat. No. E0803

Note 1: Do not exceed denaturing temperatures of 95°C, since some few buffers tend to destabilize the emulsion, resulting in instability of the emulsion and in possible phase separation.

Note 2: Emulsions may respond extremely sensitive to addition of even minute detergent amounts. Addition of any detergents such as Triton X-100 may result in emulsion instability. Certain manufacturers add detergents to their PCR buffers on a routine basis. Therefore we recommend strongly to rely solely on high quality DNA polymerases from EURx, which ship in detergent-free, plain mineral salt buffers.

Note 3: Within a certain range it is possible to vary the relative amount of Oil Surfactant Mixture to PCR Water Phase. This can affect micelle size and, as a consequence, have an impact on both DNA yield (positively correlated to micelle size) and reaction specificity (negatively correlated to micelle size) When using less than 250 µl Oil Surfactant Mixture per 50 µl PCR Water Phase, emulsions show a tendency to become unstable and are prone to spontaneous phase separation.

Note 4: Each of the triplicate PCR tubes that were derived from one PCR assay should now contain approx. 113 µl of PCR emulsion reaction, corresponding to approx. 17 µl of PCR Water Phase.

Note 5: In stable emulsions, a merge of individual micelles is rarely observed, if at all. Thus it is possible to merge material from two emulsion reactions containing different water phases (e.g. ePCR reactions targeted at different amplicons) and run those two different reactions in the same reaction vessel. Possible applications are multiplex PCRs or running control reaction and test reaction together in the same reaction tube.

Note 6: The resources of micelles are limited. For example, typical ePCR reactions deplete available resources per micelle within 15, max. 25 cycles, dependent on composition of the emulsion, i.e. on micelle size. For a further increase of ePCR yield (e.g. when starting from small template DNA amounts), conduct a scale-up ePCR as described below.

Note 7: When using genomic DNA as template DNA. Keep in mind that genomic DNA introduces large amounts of DNA to ePCR reactions, but only few copies of the target template, resulting in only a small micelle fraction contributing to amplification and to ePCR yield. A scale-up PCR (page 14) is often necessary.

Excursus: Scale-Up Emulsion PCR, Starting from Small Template DNA Amounts

When starting ePCR from a very small number of template DNA molecules, PCR yield is expected to be low due to the small numbers of micelles occupied with a copy of template DNA. For increasing reaction yield, conduct a two-step approach by setting up an emulsion PCR on top of an emulsion PCR. Two approaches are outlined briefly:

Thorough method:

- In a first step, an emulsion Pre-PCR is conducted to amplify the template copies to a total of max. 10^9 copies followed by breaking the first emulsion.

Note 1: Since resources of individual micelles are limited, it is often sufficient to conduct a total of 10-15 Pre-PCR cycles.

Note 2: It is recommended to prevent the creation of too high DNA amounts in this step, when working with reactions sensitive to homologous recombination. This prevents more than one copy of template DNA being present in a significant fraction of micelles during follow-up PCR (see below).

Note 3: For economic researchers only: Although not recommended, spin columns used for Pre-PCR purification may be re-used for purification of the corresponding follow-up PCR product, when amplifying with identical primers. If necessary, columns can be cleaned with 500 µl sterile DNA-free water, followed by centrifugation (1 min, 12 000 x g, RT).

- Following the first Pre-PCR, the emulsion is broken by addition of 1.0 ml 2-butanol (or butanol) and DNA is purified with spin columns as described in part III and IV of the protocol. The pre-amplified template DNA, ideally close to 10^9 copies per reaction or 10-50 % of total micelle count, is then used for a second scale-up PCR. This follow-up PCR is conducted with identical primers and with the desired number of amplification cycles, typically 15 – 20 cycles. As opposed to conventional PCR, the creation of PCR artifacts during scale-up PCR is effectively inhibited by the compartmentalization of individual template DNA molecules to separate reaction compartments.

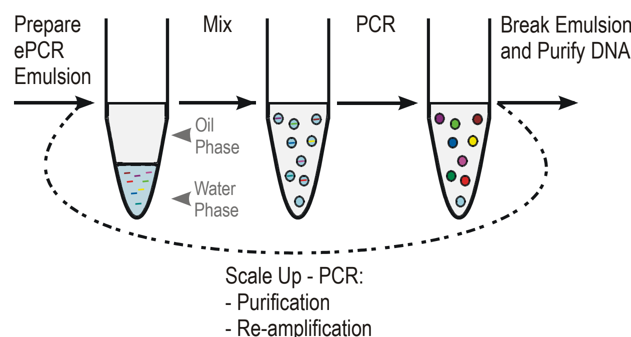

Figure 4: Schematic overview: Performing regular and scale-up emulsion PCR reactions.

Alternate quick method (known to work only with some few buffer systems, please check):

- Prepare 50 µl PCR Water Phase (in a 1.5 ml plastic reaction tube) and 300 µl Oil Surfactant Mixture as outlined above. Keep both mixtures on ice.
- Create Emulsion Pre-PCR by mixing 5 µl PCR Water Phase and 29 µl Oil Surfactant Phase.
- Conduct 15-20 cycles of Emulsion Pre-PCR with 10 min final extension at 72°C, followed by 9 min at 97-99°C.
- Briefly cool Emulsion Pre-PCR on ice, then transfer the whole reaction to the remaining 45 ml PCR Water Phase. Vortex, then spin for 2 min at 14.000 rpm and 4°C. Ideally, this should cause the water-in-oil-emulsion to partly change into an oil-in-water emulsion and allow some pre-amplified DNA to mix with fresh PCR mix. Note that a substantial fraction of (mostly small, non-DNA containing) micelles will not open and remain as a foamy interface.
- Add remaining Oil Surfactant Mixture and assemble Emulsion PCR as outlined above.

Note 1: The quick method may save material (columns) and time. Unfortunately, in most buffer systems this method fails to work due to formation of water-in-oil-in-water droplets, resulting in a lack of water phase mixing.

Part III: Breaking the Emulsion

4. Pool the corresponding triplicates of each ePCR assay into a single 2 ml reaction tube.
   Break emulsion by adding 1.0 ml 2-butanol (or butanol).
   Mix by vortexing.

Note 1: Addition of 2-butanol (or butanol) results in a merger of the water and the oil phase to one single phase. During this step the visual appearance of the emulsion changes from milky, white to clear, transparent.

Note 2: Vortex at high speed. When using a manual vortexer, always use the highest possible speed.

Note 3: At this point, each 2 ml plastic tube should contain 50 µl PCR Water Phase, 300 µl Oil Surfactant Mixture and 1000 µl 2-butanol (or butanol).

5. Add 400 µl of orange-colored **Orange-DX** buffer to the opened emulsion solution.
   Mix opened emulsion solution by gentle agitation (e.g. on a rotator for 2 min).
   Centrifuge for 2 min at maximum speed (e.g. 16 000 x g / approx. 14 000 rpm) for phase separation.

6. Remove most of the the yellow colored organic phase. Since both, water phase and interphase will be used for spin column purification, it is recommended to leave a small rest volume of the organic phase on top of the interphase. All organic phase remains will be removed during the follow-up spin column purification.

Note 1: Phases are separated into a water phase containing DNA and a organic phase. The yellow color supplied with buffer Orange-DX moves to the organic phase. Thus, for DNA purification, the colorless water phase will be used, whereas most of the yellow colored organic phase is discarded.

Note 2: In some cases no phase separation is obtained. If only one single, but clear and transparent phase is obtained, continue with purification as described below. If the solution remains milky, the emulsion has not opened completely. In this case DNA cannot bind properly to the column matrix and all attempts to purify DNA will lead to poor results. Try to improve breaking the emulsion by vortexing thoroughly and / or by stepwise addition of 2-butanol.

Note 3: An aliquot of the water phase can be used for a quick reaction control on an agarose gel. Please keep in mind, that for now migration inconsistencies such as gel retardation may occur, because DNA-binding proteins are not yet removed.

7. Apply 40 µl of activation **Buffer DX** onto the spin-column (do not spin) and keep it at room temperature until transferring mixture to the spin-column.

Note 1: Addition of Buffer DX onto the center of the resin enables complete wetting of membranes and maximal binding of DNA.

Note 2: The membrane activation should be done before starting isolation procedure.

8. Pour mixture (aqueous phase + inter phase; max. 600 µl) into a spin-column/receiver tube assembly.
9. Spin down in a micro-centrifuge at 12,000 rpm for 1 minute.
10. Remove spin column, discard flow-through, put the spin-column back on top of the tube.
11. In case total volume of aqueous phase + interphase exceeds 600 µl: Repeat steps 8 – 10 using the same spin column.
12. Add 500 µl of **Wash-DX1** buffer and spin down at 12,000 rpm (~11.000 x g) for 1 minute.
13. Remove spin column, discard flow-through, put the spin-column back on top of the tube.
14. Add 650 µl of **Wash-DX2** buffer and spin down at 12,000 rpm (~11.000 x g) for 1 minute.
15. Remove spin column, discard flow-through, put the spin-column back on top of the tube.
16. Spin down at 12,000 rpm (~11.000 x g) for 2 minutes to remove traces of **Wash-DX** buffer.
17. Place spin-column into new receiver tube (1.5-2 ml). Add 50-150 µl of **Elution-DX** buffer to elute bound DNA.

Note 1: Addition of elution buffer directly onto the center of the membrane improves DNA yield.

Note 2: To improve recovery of larger DNA fragments (above 5 kb) it is recommended to elute with buffer heated to 80°C.

Note 3: For elution of DNA the Elution-DX buffer is highly recommended. The buffer is prepared using ultra-pure water with trace addition of buffering compounds. The Elution-DX buffer will not interfere with subsequent DNA manipulations, such as DNA sequencing, ligation or restriction digestion, among others.

Note 4: It is possible to reduce the volume of eluting buffer below 50 µl (no less than 20 µl). However, recovery of DNA will gradually decrease.

18. Incubate spin-column/receiver tube assembly for 2 minutes at room temperature.
19. Spin down at 12,000 rpm(~11.000 x g) for 1 minute.
20. Remove spin column, cap the receiver tube. Isolated DNA is ready for analysis/manipulations. It can be stored at 2-8°C or (preferred) at -20°C.

Troubleshooting

Problem	Solution
A coarse or unstable emulsion is formed	• Ensure thorough and proper mixing of reaction components at 4-7°C. High quality emulsions have a creamy, white appearance. Low-quality emulsions have a pale, opaque appearance and tend to phase separation. • Check, whether enzyme buffers from any third party might contain compounds affecting the composition and stability of emulsions, even in low concentrations (e.g. non-ionic surfactants such as Triton X-100). • Vortex at high speed. When using a manual vortexer, choose the highest possible speed. • Surfactant stocks are too old. Do not use out-of-shelf-life surfactants.
Little or no product is formed	• The number of template DNA copies may be too low. Check for correct amount of template copies. Keep the number of template copies as close as possible to 10 % of the total micelle number, lower values result in poor yield. In case template copy number exceeds 30 % of total micelle number, yield will increase, but the share of micelles hosting more than one single template DNA molecule will increase as well. To empirically determine the number of micelles, use the calibration procedure outlined above. Note: Even high amounts of genomic DNA introduce only few template copies to ePCR. Conduct a scale up PCR (page 14). • Check, whether the correct amount of BSA was added to the emulsion solution. For DNA polymerases supplied by EURx, 0.01 mg/ml is the optimum final concentration. For DNA polymerases supplied by third parties, own optimizations might be required. BSA is required for a proper coating of the hydrophilic-hydrophobic micelle interface. to prevent any hydrophobic interactions of the enzyme with the micelle surface, which can lead to enzyme impairment or inhibition. On the other hand, excess BSA inhibits PCR reactions effectively. The exact amount of BSA to be added depends on the consistence of the emulsion reaction, which is subject to vary between different labs. Thus the precise amount of BSA to be added per reaction has to be titrated at least once, during establishment of the technique in your laboratory.
PCR artifacts are generated	• Too many copies of template DNA are introduced to the reaction. As a consequence, copies of template DNA are not equally distributed in micelles, but rather follow a Poisson-- distribution. In case of too many copies of template DNA molecules (> 10^9 copies), individual micelles may host more than one copy of template DNA, leading to PCR artifacts. Keep the number of template copies as close as possible to 10 % of the total micelle number. To empirically determine the number of micelles, use the calibration procedure outlined above.
Control, whether micelles are tight or leaky and prone to merge	• Chemical method (Huebner A. et al. (2011) Anal. Chem., 83, 1462–1468): Mix two emulsions, each containing a water phase with one of two colorless salts, KSCN and $Fe(NO3)_3$. Salts react under formation of a dark red complex, indicating leakiness of micelles: $Fe^{3+} + xSCN^{-} \rightarrow Fe(SCN^{-})_x^{(3-x)+}$ • PCR assay: Mix two emulsions. Emulsion 1: PCR water phase with template but without any primers. Emulsion 2: PCR water phase with primer pair but without template DNA. Perform PCR. PCR product can only form upon merger of micelles, indicating leakiness and instability of emulsion.

Appendix

Estimating the Number of Individual Reaction Compartments (micelles)

The following protocol is most useful during first establishment of this technique in your lab. Once you are familiar with the emulsion technique and satisfied with results and reproducibility, it is not necessary to run this protocol on a daily basis.

For experimental design, it is important to guess the total micelle count per reaction. The number of individual reaction compartments (micelles) per reaction can be roughly estimated by performing a dilution series with a known number of template DNA copies.

1 µg of a 978 bp DNA fragment contains 1 x 10^{12} template DNA copies. Dilute the template DNA as follows:

(1) 1 µg (1 x 10^{12} copies) per total reaction
(2) 100 ng (1 x 10^{11} copies) per total reaction
(3) 10 ng (1 x 10^{10} copies) per total reaction
(4) 1 ng (1 x 10^{9} copies) per total reaction
(5) 100 pg (1 x 10^{8} copies) per total reaction
(6) 10 pg (1 x 10^{7} copies) per total reaction

Perform emulsion PCR as outlined above. After purification, apply equal volumes of PCR product to an agarose gel.

- Dilution steps (1) – (2) should contain very similar amounts of amplified DNA. Each micelle hosts at least one template DNA molecules and thus the maximum amplification yield is observed.
- In dilution steps (3) – (6) a significant decrease in PCR yield should be observed. Only a diminishing fraction of micelles host at least one template DNA molecule. Thus, maximum DNA amplification yield is not gained. The number of micelles roughly equals the number of template DNA copies at the point, where emulsion PCR yield shows an approx. yield reduction of ~37 %, (≈ 1/e , Poisson-distribution, see Figure 1).
- Depending on the requirements of the application, the optimal DNA concentration is either (1) the highest concentrated dilution step in which the maximum yield is not reached anymore (high yield, good specificity), or (2) the dilution step with only a 10-20 % fraction of micelles hosting a template DNA molecule (determined by experimental design or by choosing dilution steps with 15-30 % DNA yield; moderate to low yield, maximum specificity). In (2), nearly each micelle hosts 0 or 1 copies of template DNA, mostly preventing the formation of any detectable amounts of chimeric amplification products.
- DNA concentrations with even lower yield are too dilute. Either template DNA concentration must be increased or a Pre-PCR amplification step has to be conducted to boost the number of template DNA copies to 10^{8} – 10^{9} copies / reaction.

Note 1: Swami et al. (Langmuir. 2008 Oct 21;24(20):11828-33) showed that embedded DNA influences the size of micelles, inducing the formation of large droplets. According to their finding, coexisting, very small droplets do not contain DNA. Thus, the number of micelles available for DNA amplification has to be determined empirically as outlined above.

Legal Disclaimer:

The Micellula DNA Emulsion & Purification Kit is a dedicated kit for easy and reproducible assembly of particle-/ bead-free emulsion PCR (ePCR) assays and subsequent DNA purification thereof. In addition to particle-/ bead-free emulsion PCR, the flexible design of the kit allows performing a broad variety of DNA-targeted and other enzymatically catalyzed biochemical reactions within water-in-oil emulsions.

Emulsion-based enzymatic reactions are a broad field of ongoing research, development and innovation. New technologies are developed constantly and some of these are covered by patents. Consequently, some emulsion-based applications, such as bead-based ePCR methods or certain in vitro expression technologies, represent intellectual property (IP) owned by third parties. Application of such methodologies may require purchase of a separate license from the holder of rights.

Due to the broad field of additional, potential applications, that are not part of this products' primary focus of application, the Micellula DNA Emulsion & Purification Kit does not include any licenses for technologies covered by third parties IP.

If the intended use of the kit aims at reproducing any methodology covered by third parties' IP rights, it falls within the sole responsibility of the customer to obtain a corresponding license. EURx hereby excludes any direct or indirect liability relating to customer’s use of any technology covered by third parties' IP rights.

The Micellula **Emulsion & Purification Kit Series** allows the reproducible and safe accomplishment of nucleic-acid targeted enzymatic reactions in emulsions. Emulsion reagents and purification columns are carefully adjusted to guarantee implementation and realization of emulsion reactions with high reliability and best results. Nucleic Acids purification is based upon EURx unique, well-proven GeneMATRIX technology.

GeneMATRIX is a synthetic, new generation DNA- and RNA-binding membrane, selectively binding nucleic acids to composite silica structures. Novel binding and washing buffers are developed to take full advantage of GeneMATRIX capacity, yielding biologically active, high-quality nucleic acids. Matrix is conveniently pre-packed in ready-to-use spin-format. Unique chemical composition of the matrices along with optimized construction of spin-columns improve the quality of final DNA or RNA preparation. To speed up and simplify isolation procedure, the key buffers are color coded, which allows monitoring of complete solution mixing and makes purification procedure more reproducible.

As a result, we offer kits, containing matrices and buffers that guarantee rapid, convenient, safe and efficient isolation of ultra pure nucleic acids. Such DNA or RNA can be directly used in subsequent molecular biology applications, such as: restriction digestion, dephosphorylation, kinasing, ligation, protein-DNA interaction studies, sequencing, blotting, *in vitro* translation, cDNA synthesis, hybridization among others. Additional advantage is reproducibility of matrix performance, as component preparation is carried at Eurx Ltd.

The Micellula **DNA Emulsion & Purification Kit** is designed for setting up DNA-targeted enzymatic reactions (such as PCR reactions), followed by subsequent DNA purification. The kit separates individual template DNA molecules in distinct reaction compartments. This facilitates reactions starting from complex DNA fragment mixtures with partially similar sequence stretches such as complex gene libraries with identical linkers or environmental DNA. Common pitfalls such as formation of chimeric molecules due to non-homologous recombination or runoff-amplification of one single molecule species at the expense of other template molecules are avoided and high sample diversity is maintained throughout the reaction. Following the enzymatic reaction, DNA fragments of sizes from approximately 100 bp to over 15 kb can be obtained in ultra pure form. DNA purification is specifically optimized towards emulsion reactions. Furthermore, effectively removed are contaminants such as: ethidum bromide, primers (below 40 nt), short double-stranded DNA (below 20 bp), RNA, Taq DNA Polymerase, Pfu DNA Polymerase, endo- and exonucleases, DNA-binding and modifying proteins, BSA and other enzymes/proteins, lipids, endotoxins, dyes, detergents, nucleotides, radio- and chemical labels, EDTA, problematic restriction and ligation inhibitors, buffers and salts. The kit contains all reagents required for creating emulsions and for subsequent DNA purification. For the operators maximum flexibility, enzymes are not provided with this kit.

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