# Real Vegan Cheese

## Casein production in *Saccharomyces cerevisiae*

### SF BAY AREA DIY BIO

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## INTRO

At the Real Vegan Cheese Project, we are engineering the synthesis of bovine and human casein milk proteins by baker's yeast, *Saccharomyces cerevisiae*, enabling cow-less cheese production. Animal agriculture contributes to animal suffering and a variety of environmental problems, including global warming, water pollution, and deforestation. A vegan cheese that more closely mimics cow cheeses could help many more people switch to a plant-based diet.

Our project fosters public engagement with synthetic biology, and our entire process will be open source and open patent.

We hope to produce cheeses synthetically that are more ethical, sustainable, and, by excluding lactose or allergenic bovine caseins, potentially healthier than traditional dairy cheeses.

## BACKGROUND

The four main proteins in cow's milk cheese are alpha-s1, alpha-s2, beta, and kappa casein (humans do not have alpha-s2). The hydrophobic caseins aggregate into casein micelles, with the hydrophilic tail of κ-casein pointing outwards. During cheese making, this hydrophilic κ-casein tail is cleaved off by chymosin (rennet), making the micelles coagulate into cheese curds. We will also study the Fam20C kinase that phosphorylates caseins in milk [Tagliabracci et al., 2012], thought to be important for formation of casein micelles.

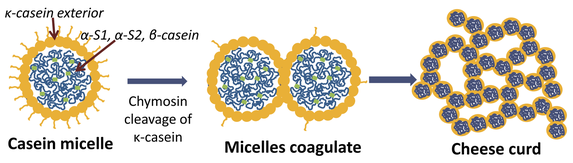



**Fig 1. Model of cow's milk micelles and cheese coagulation.** Adapted from [Horn, 1998].

## METHODS

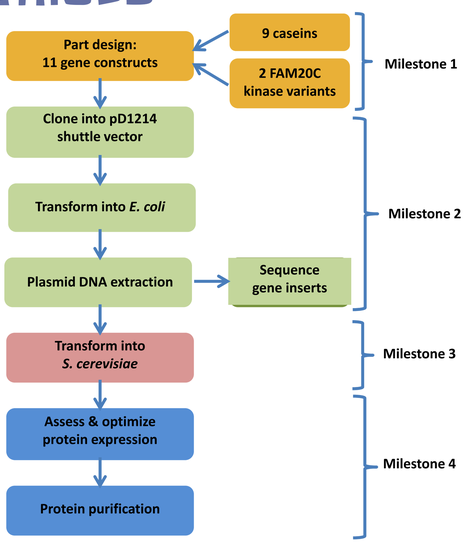



**Fig. 2 Summary of molecular methods.**

**Milestone 1: Part design.**

We started with published sequences for cow and human proteins, as bovine caseins are common human allergens. For the protein kinase, we used sequence from human Fam20C. No animals needed!

First, we codon-optimized gene sequences for expression in yeast using IDT's codon optimization tool. We then added a native yeast secretion signal (alpha-factor) preceding each. The alpha-factor peptide is cleaved off the protein during secretion, by the Kex2 transmembrane protease. Since Kex2 protease recognition sites (KR and RR) also occur in the native sequence of three of our study proteins, we also created "Kex-" constructs in which extraneous Kex2 sites were changed to biochemically similar amino acids (KR -> KK).

Finally, we added flanking regions that include recognition sequences for our cloning method of choice: DNA 2.0's "IP Free" Electra system [1], a simple cloning based on the type IIS 7-cutter, SapI. For Registry submission, the SapI recognition sites are then further flanked by the standard BioBrick pre/suffix for cloning into the pSB1C3. Internal recognition sites for the SapI and BioBrick restriction enzymes were removed during codon optimization.

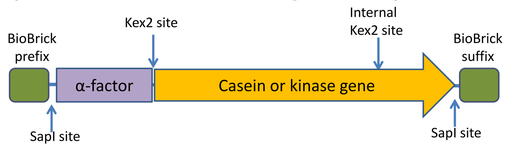



**Fig 3. Representative part insert.**

We created 11 constructs with these core genes:
· Bovine alpha-S1 casein
· Human alpha-S1 casein
· Bovine alpha-S2 casein(Kex + & Kex -)
· Human beta casein
· Bovine beta casein
· Human kappa casein (Kex + & Kex -)
· Bovine kappa casein
· Human Fam20C kinase (Kex + & Kex -)

**Milestone 2: Clone, transform plasmids to *Escherichia coli*, & sequence confirm.**

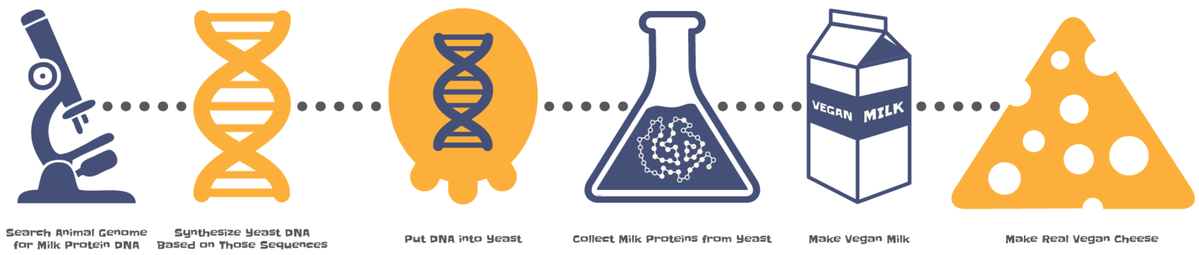
We cloned composite DNA constructs into the DNA 2.0 Electra pD1214 plasmid backbone using a kit that simultaneously digests at the external SapI sites and ligates. This plasmid can be selected in *E. coli* using its Ampicillin-resistance gene. It also contains a strong constitutive TEF promoter for gene expression in yeast and a URA3 marker for selection in Ura deficient yeast strains. After transformation into E. coli, we extracted plasmid DNA and submitted for sequence confirmation.

**Milestone 3: Transform plasmids to yeast.**

Our yeast strain is Saccharomyces cerevisiae, UV-Sensitive Strain, G948-1C/U, alpha, rad1 rad18 phr1 ura3 mutant in excision repair. It is Ura deficient, allowing for selection of our desired plasmid transformants on URA negative media. Other yeasts such as Pichia may eventually be used for large-scale industrial production.

**Milestone 4: Verify protein expression and secretion in yeast and purify proteins from yeast growth medium**

We will verify protein expression in yeast culture supernatants via SDS PAGE gel electrophoresis, then explore protein purification methods. We should be able to use the caseins' propensity to become insoluble at specific pH and calcium concentrations to purify the proteins from the supernatant.




## RESULTS

**Milestone 1): Part design.** Done!

**Milestone 2): Clone, transform plasmids to E. coli, & sequence confirm.** 10 done; 100% sequence alignment. No successful transformation of human kappa casein (Kex-) into *E. coli* yet.

**Milestone 3): Transform plasmids to yeast.** Currently optimizing our yeast expression system.

**Milestone 4): Verify protein expression and secretion in yeast and purify proteins from yeast growth medium.** Not yet attempted.

## Ethical, Legal, Societal, and Environmental Issues

Factory farming is environmentally destructive and it entails treating animals less well than most of us would like. We believe our project has the potential to be a more ethical and sustainable way of producing dairy products. Preliminary yield calculations indicate that the production of casein in yeast could reduce greenhouse gas emissions by 40-90% per gram [2-4]; in addition, it is more feasible to capture gases emitted from a bioreactor than from grazing cattle.

· Our practices and proposed methodology takes issues "beyond the bench" into account in several ways:
· choice of a host organism that is already used in food production
· use of a technique that is already widely used in cheese production (>80% of cheese worldwide is made with chymosin produced by GM microorganisms [5])
· purification of the final product so it will not contain any GMOs
· containment of GM yeast by use of an auxotrophic strain · proactive consultation with the FDA and public interest groups
· articulation of a "statement of ethics" that expresses our values and how this project embodies them
· IP strategy of "patent and abandon" to ensure our work remains in the public domain.

## Do-It-Together Biology

Research suggests that public ambivalence surrounding biotechnologies such as GMOs is substantially related to concerns about the institutional context in which they are developed, including concerns about whether institutional actors and processes are truly serving the interests of the public [Marris, 2001].

Our organization responds to these concerns by practicing synthetic biology in a radically open and transparent manner.

We continually welcome new members and interested onlookers to our meetings, which are open to the public.
We also solicit and respond to feedback on social media (including the "Tell Us Why We're Wrong" feature on our Indiegogo campaign).

In our work, we are not only "hacking" biology; we are also tinkering with the kinds of social forms that support scientific innovation. Our team operates with different constraints and motivations than teams from the academic or corporate world. One significant difference is that we are committed to keeping all our work in the public domain so anyone can benefit from and improve upon it.

## NOT JUST A SUMMER PROJECT!

This project goes well beyond iGEM. As a group of mostly non-students, this is not just a fun summer project to put on a resume - we're into this for the long haul! We have raised $37,369 in donations on Indiegogo to make Real Vegan Cheese a reality (including $10,000 for a 50L bioreactor for scaleup) - only a fraction of which has been used on our iGEM deliverables so far. The team is dedicated to carrying this project forwards, and we have set up a non-profit corporation to steward this idea and the funds we've raised for it so far.

## Future Directions

· Continue assessing and minimizing potential environmental & social impacts of our process.
· Continue consulting with regulatory bodies (e.g. FDA) regarding product development and safety.
· Successfully clone human κ-casein (Kex-) into pD1214 plasmid, transform to E. coli, sequence-confirm.
· Optimize our yeast URA3- selection system.
· Transform all constructs into S. cerevisiae.
· Purify and characterize expressed casein proteins from yeast growth media.
· Develop vegan "milk" (including fats and sugar) as base for cheese-making.
· Make Real Vegan Cheese according to traditional culinary methods.
· **Obtain narwhal casein gene sequences; make narwhal cheese.**
· Keep all stages of process open source and open patent.

**Citations**
[1] http://www.dna20.com/products/expression-vectors/electra-system
[2] http://www.progressivedairy.com/index.php?view=article&id=6491
[3] http://www.dairyco.org.uk/market-information/farming-data/cow-numbers/world-cow-numbers
[4] http://www.fao.org/news/story/en/item/41348/icode/
[5] http://www.gmo-compass.org/eng/database/enzymes/83.chymosin.html

## Acknowledgements

**CORPORATE SUPPORTERS**
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**COMMUNITY SUPPORT - THANK YOU!**
Indiegogo Campaign. The campaign had 696 funders raise $37,369. in support

**DIYBIO COMMUNITY**
BioCurious, Counter Culture Labs