CLONING AND EXPRESSION PLATFORM FOR PRODUCTION OF RECOMBINANT PROTEINS

BOGUSŁAW LUPA, KRZYSZTOF STAWUJAK, IGOR RÓŻAŃSKI AND JUSTYNA STEĆ-NIEMCZYK
BIOCENTRUM, SELVITA GROUP, BOBRZYŃSKIEGO 14, 30-348 KRAKÓW, POLAND

ABSTRACT

The efficient production of soluble recombinant proteins has become increasingly important due to the increased demand for the target proteins in both basic research as well as in the drug discovery programs. The novel platform of ligation independent cloning for generation of a number of genetic constructs for the production of target proteins based on *E. coli* expression system has been applied. The presented platform relies on the series of linear expression vectors (LEV), which harbor various versions of essential components of expression vectors such as promoters, origins of replications, fusion tags, and regulatory elements. The variations of the vectors allow for relatively rapid selection of the optimal genetic constructs with the highest levels of production of given target protein. Both LEVs and genes encoding the target proteins are generated by the standard PCR method, with specifically designed primers that contain the 15-bp complementary fragments that allow cloning using the specific PCR reaction, termed the circular polymerase extension cloning. The resulting clones are cultivated at the expression inducing conditions and the levels of production of target proteins are assessed in the multi-well plate format. The selected clones, with the highest levels of production of recombinant protein of interest are used for the scaled-up production, which are subsequently purified using the corresponding affinity chromatography based on common fusion tags. Each LEV contains the DNA fragment encoding for the specific protease recognition site, which together with the Clean-Cut, a novel, proprietary, unique and highly specific recombinant serine protease facilitates the efficient removal of fusion tag. The presented approach is cost and time effective and is ideal for the determination of the optimal conditions for the overexpression and production of the range of recombinant, heterologously produced proteins.

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APPLICATION

PRODUCTION OF RECOMBINANT PROTEINS HIGHLY DEPENDS ON:

- TARGET PROTEIN
- PROPERTIES OF GENETIC CONSTRUCT
- CULTIVATION AND EXPRESSION CONDITIONS
- PURIFICATION PROCEDURE

OUR SYSTEM AIMS AT THE DEVELOPMENT OF:

- NOVEL, LIGATION-FREE DNA ASSEMBLY METHODS PROVIDING ROBUST WAY FOR THE CONSTRUCTION OF THE GENETIC CONSTRUCTS (EXPRESSION VECTORS)
- ASSESSMENT OF OPTIMAL EXPRESSION CONDITIONS IN THE MULTI-WELL PLATE FORMAT
- PROTEIN PURIFICATION BASED ON COMMON AFFINITY TAGS REMOVED WITH CLEAN-CUT PROTEASE

ADVANTAGES

- IP FREE SYSTEM COMPARABLE WITH COMMERCIAL EXPRESSION SYSTEMS
- RAPID SCREEN FOR THE OPTIMAL EXPRESSION CONDITIONS (GENETIC BACKGROUND, BACTERIAL STRAIN, CULTIVATION AND INDUCTION CONDITIONS)

PLATFORM DESCRIPTION

CLONING TO EXPRESSION CONSTRUCT:

- SET OF EXPRESSION VECTORS PROVIDE VARIOUS GENETIC BACKGROUND FOR OPTIMAL EXPRESSION

SMALL SCALE EXPRESSION STUDIES:

- TESTING A NUMBER OF EXPRESSION CONDITIONS IN MULTI-WELL PLATE FORMAT
- SELECTION OF OPTIMAL CONDITIONS

PRODUCTION PHASE:

- PILOT CULTURE IN FLASK FORMAT
- SCALABLE UP TO THE REQUIRED VOLUME

PROTEIN PURIFICATION:

- AFFINITY PURIFICATION TAGS: *e.g.* 6x HIS, GST, ETC.
- PURIFICATION BEADS: *e.g.* NINTA, GLUTATHION SEPHAROSE
- FUSION TAG REMOVAL WITH THE PROPRIETARY CLEAN-CUT PROTEASE

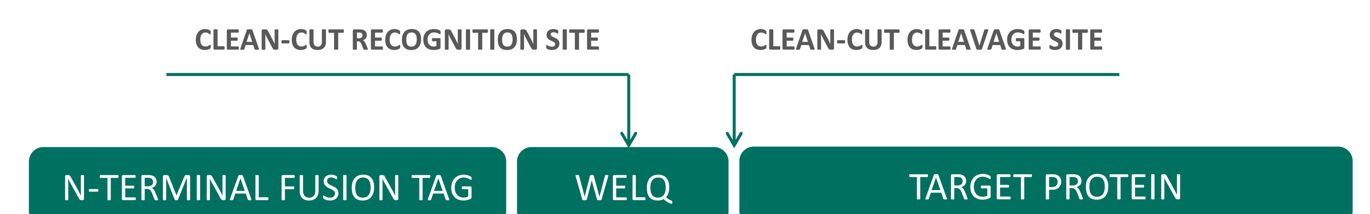
EXTENSIVE SET OF GENERATED PLASMIDS

PROMOTERS	• T5, T7 & TRC promoters • Proprietary, modified promoters: BCMT5, BCMT7 & BCMTRC
ANTIBIOTIC RESISTANCE	• Ampicillin • Kanamycin
ORIGIN OF REPLICATION	• Mutated version of the pBR322 (ORI, high copy, ~ 500 copies per cell) • pBR322 (medium copy, ~ 20 copies per cell) • p15A (low copy ~ 10 copies per cell)
GENE OF INTEREST	• Genes coding for the reporter proteins: GFP and RFP*, LacZ • Genes coding for target proteins or MCS for traditional cloning

*RFP (ROSE FLUORESCENCE PROTEIN) – IS ENCODED BY THE GENE PRESENT IN THE COMMERCIALLY AVAILABLE EXPRESSION VECTOR SantakaRFP PURCHASED FROM THE DNA 2.0 (https://www.dna20.com/)

PROPRIETARY CLEAN-CUT PROTEASE

FOLLOWING THE PURIFICATION OF PROTEINS OF INTEREST USING THE AFFINITY CHROMATOGRAPHIES THE FUSION TAGS CAN BE REMOVED WITH HIGHLY SPECIFIC CLEAN-CUT PROTEASE RECOGNIZING THE AA SEQUENCE **WELQ^** LOCATED DIRECTLY AT N-TERMINUS OF THE TARGET PROTEIN. THE DNA SEQUENCE ENCODING THE WELQ IS PRESENT IN EACH GENETIC CONSTRUCT AND SERVES AS COMPLEMEMTARY DNA FRAGMENT FOR THE JOINING OF MODULES (CLONING).



THE PROPRIETARY CLEAN-CUT PROTEASE:

- RECOMBINANT *S. auerus*–DERIVED ENZYME PRODUCED IN *B. subtilis*
- ESTABLISHED AND OPTIMIZED PURIFICATION PROCEDURE YIELDING THE HIGH PURITY AND HIGH CATALYTIC ACTIVITY
- LOW RISK OF CONTAMINATION WITH OTHER PROTEASES
- HIGH SPECIFICITY
- ABILITY TO FREELY SHAPE N-TERMINUS OF THE PROTEIN OF INTEREST

PRELIMINARY EXPRESSION ANALYSIS

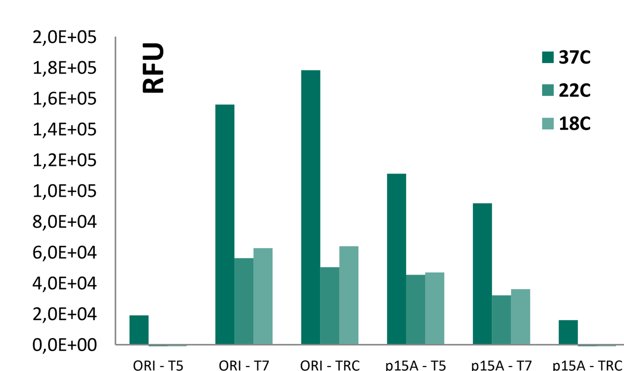


FIG. 1. Comparison of the expression levels of the reporter genes for the select genetic constructs at various cultivation temperatures in BL21 *E. coli* strain.

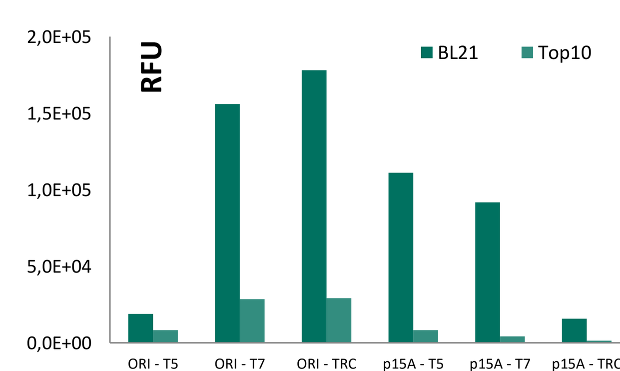


FIG. 2. Comparison of the expression levels of the reporter genes for the select genetic constructs in various strains of *E. coli*.

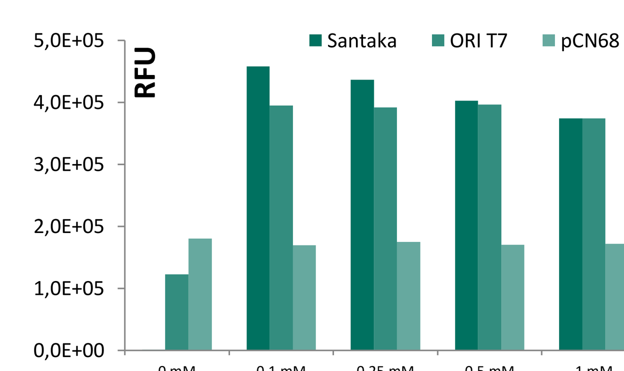


FIG. 3. Induction of the expression of the reporter genes for the select genetic construct with the inducible promoter as compared to the control vector (Santaka) and pCN68 with the constitutive expression of the reporter gene.

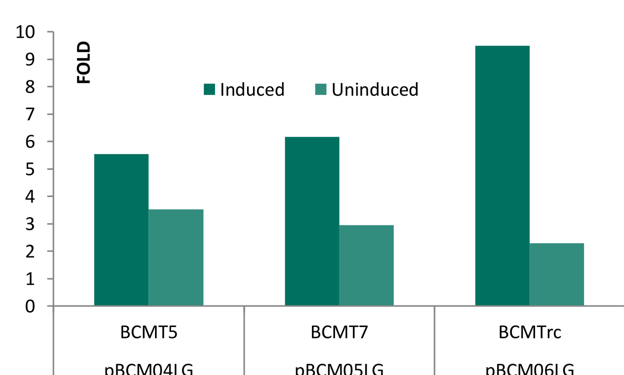


FIG. 4. Relative GST production for three various BCM promoters as compared to the uninduced GST levels from pGEX.

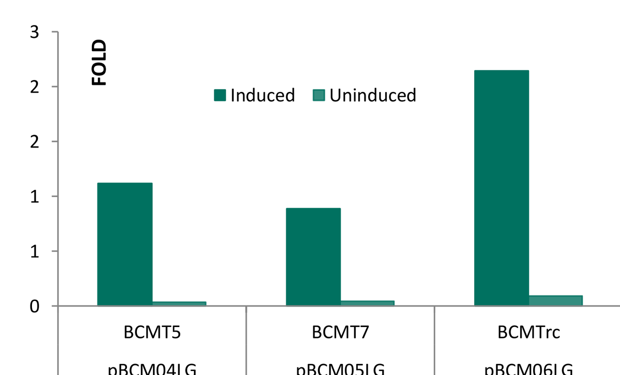


FIG. 5. Relative production of GST fused with human target X for three various BCM promoters as compared to uninduced protein from pGEX.

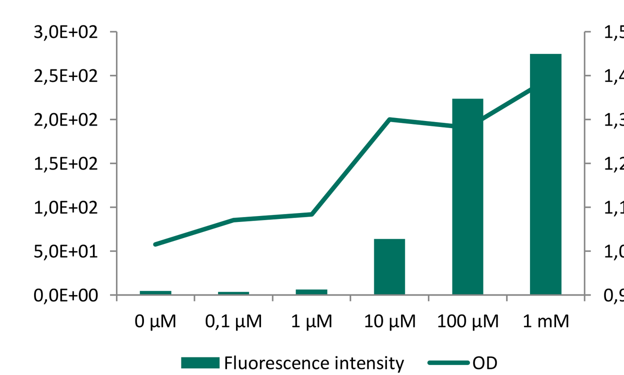


FIG. 6. Induction of the expression of reporter gene as assessed with fluorescence intensity measurements for the control vector Santaka at different IPTG concentrations in BL21 *E. coli* strain.

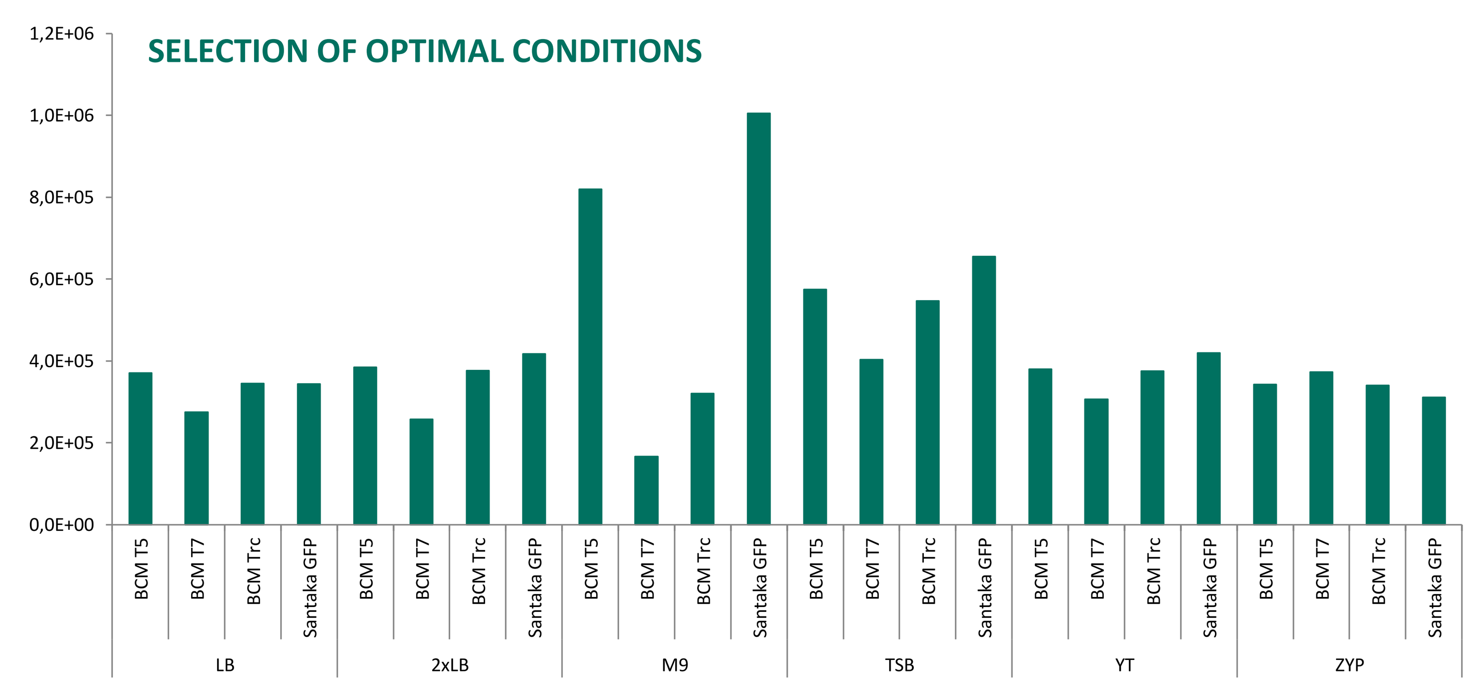


FIG. 7. Selection of optimal culture conditions for GFP expression based on fluorescence intensities for six types of media (including auto-inducing - ZYP and minimal medium – M9) and four selected genetic constructs.

CONCLUSIONS

- ANALYSIS OF THE REPORTER PROTEINS IN MULTI-WELL SYSTEM INDICATED THE EXPRESSION LEVELS OF THE REPORTER GENES HIGHLY DEPENDED ON:
  - THE CULTIVATION CONDITIONS (BACTERIAL STRAIN, MEDIUM TYPE, CULTURE VESSEL, TEMPERATURE, CONCENTRATION OF THE INDUCER);
  - THE GENETIC CONSTRUCT (PROMOTER, GENE OF INTEREST, PRESENCE OF THE REGULATORY ELEMENTS e.g. LacI, COPY NUMBER, ETC.)
- RESULTS OBTAINED IN MULTI-WELL SYSTEM ARE COMPARABLE WITH THE OBTAINED IN STANDARD EXPRESSION TESTS;
- EXPRESSION LEVELS OF GENERATED PLASMIDS ARE COMPARABLE WITH COMMERCIALLY AVAILABLE EXPRESSION SYSTEMS;
  - FURTHER OPTIMIZATION OF THE REGULATION OF EXPRESSION IS REQUIRED;
- MULTI-WELL EXPRESSION TESTS ARE PREREQUISITE FOR SCALE-UP PRODUCTION OF NUMBER OF RECOMBINANT PROTEINS;

GOAL: FULLY FUNCTIONAL PROPRIETARY PLATFORM FOR THE RAPID CLONING AND DETERMINATION OF THE OPTIMAL CONDITIONS FOR PRODUCTION AND PURIFICATION OF THE RECOMBINANT PROTEINS

BioCentrum SELVITA GROUP
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For additional information please feel free to contact us:
- boguslaw.lupa@selvita.com
- www.biocentrum.com.pl