Development and Validation of a Six-Plex Quantitative PCR Potency Assay for an Anti-Breast Cancer DNA Plasmid Vaccine

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Background

Breast cancer represents a major health burden, with over 40,000 deaths annually in the United States alone. This disease encompasses several subtypes, each with distinct genetic profiles and treatment needs. Prevention strategies for breast cancer remain limited, with most interventions either by surgery (e.g., prophylactic mastectomy for BRCA mutation carriers) or targeting only specific subtypes (e.g., tamoxifen for hormone receptor positive breast cancer prevention). Options such as chemotherapy and radiation have serious risk of adverse side effects and are not suitable in the prevention setting. Vaccination offers a promising alternative, leveraging the immune system's ability to mount immune responses specifically to overexpressed tumor-associated antigens (TAAs) without inducing harmful immune responses against normal tissues. Recent studies have demonstrated the potential for vaccines targeting overexpressed self-antigens to safely elicit an immune response, particularly in the context of cancer prevention. The Artemis Project, a collaboration among leaders in breast cancer and immunology, aims to develop a multi-antigen prophylactic vaccine targeting six TAAs: MUC1, HER2, hTERT, Survivin, MAGEA3, and Mammaglobin A, which are overexpressed across all major subtypes of breast cancer, including triple-negative, estrogen/progesterone receptor-positive, and HER2-positive cancers.

Two DNA plasmids were designed to co-express six human antigens in separate constructs (2 and 4 antigens in each construct) using 2A peptide technology (**Figure 1**). Multiantigen gene cassettes were inserted into pUMVC4a vaccine plasmid to create, pBC.1 (pUMVC4a-Her2ECD_Muc1_Her2ICD) and pBC.2 (pUMVC4a-Mamma_Survivin_MAGEA3_hTERT). Expression was validated by transient transfection in HEK293T cells. To support the analysis of these complex plasmids, we have developed a robust pipeline of assays to evaluate potency of the DNA plasmid vaccine during ongoing formulation and stability studies.

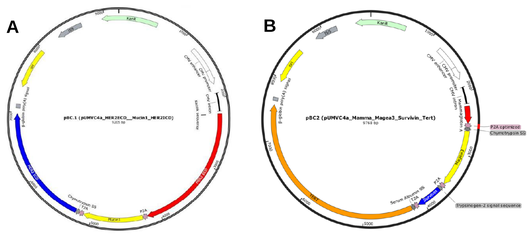

Figure 1. A. pBC.1 Vaccine Plasmid pUMVC4a-HER2ECD_Mucin1_HER2ICD. **B.** Vaccine Plasmid pUMVC4a-Mammaglobin_Magea3_Survivin_Tert

Methods

Cell Culture and Transfection: HEK293T cells were cultured in DMEM (Gibco) with 10% FBS and 1% penicillin-streptomycin at 37°C, 5% CO_2. Cells were seeded in 6-well plates (7.5×10^5 cells/well) ~24 h before transfection to reach 70–90% confluency. Transfections used Lipofectamine™ 3000 per manufacturer's protocol with 2.5 µg plasmid DNA per well. Cells and supernatants were collected 48 h post-transfection.

Primer/Probe Design and gBlock Synthesis: Primers and probes were designed using the IDT PrimerQuest Tool, optimized for product size, Tm, fluorophore compatibility, specificity, and structure. Unique fluorophores were assigned for compatibility with QuantStudio 5 Dx channels. Custom gBlocks (IDT) were designed to match plasmid sequences.

mRNA Isolation and cDNA Synthesis: mRNA was isolated from lysates using Dynabeads™ mRNA Purification Kit (CAT# 61006), quantified via Qubit RNA HS Kit, and either normalized or used as-is depending on yield (≥2.5 µg). cDNA synthesis was performed using SuperScript™ VILO™ Kit (CAT# 11754050), quantified with Qubit 1X dsDNA HS Kit, and normalized to 2 ng/µL for qPCR.

ELISA: At 48 h post-transfection, clarified supernatants were analyzed for TAA expression using commercial ELISA kits: Mammaglobin A (AFG Bioscience, EK711654), HER2 (abcam, ab283881), MUC1 (Antibodies Online, ABIN6730894), Survivin (abcam, ab183361), MAGEA3 (CloudClone, SEQ282Hu), and hTERT (abcam, ab285288), following manufacturers' protocols.

Co-transfection of pBC.1 and pBC.2: For simultaneous expression of all six TAAs, 1.25 µg of each plasmid (2.5 µg total) was co-transfected as above. mRNA and protein expression were assessed as described.

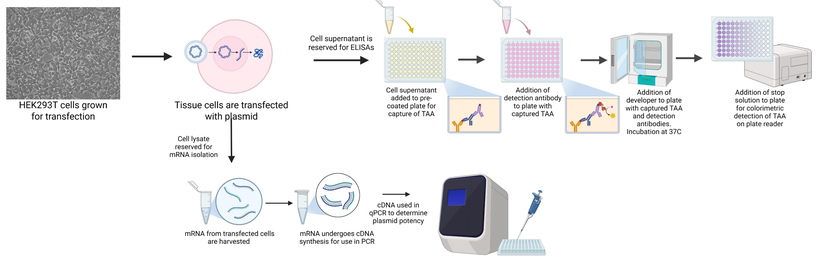

Figure 2. Entire workflow demonstrating cell transfection, to harvest, to cDNA synthesis and qPCR, and ELISAs to determine vaccine potency.

Development of a Multi-Plex qPCR Potency Assay

Development and Optimization of SinglePlex qPCR Assays for the Detection of Each Target

Six custom primer-probe sets were designed using IDT's PrimerQuest Tool, each labeled with a unique fluorophore assigned to a distinct QuantStudio5Dx channel (**Table 1**). Initial singleplex assays used 300 nM primers and 200 nM probes, with standard curves from 5×10^5 to 5×10^1 copies/mL of each gBlock. Baseline results required minimal optimization, achieving sufficient fluorescence and Ct values >30 at the lowest dilution (**Figure 3**). Optimization focused on titrating primer/probe concentrations; final values are listed in **Table 1**.

Assay Name	Fluor	Starting Primer Conc. (nM)	Starting Probe Conc. (nM)	Final Primer Conc. (nM)	Final Probe Conc. (nM)
Survivin	FAM	300	200	300	200
hTERT	JUN	300	200	300	200
Mammaglobin A	Cy5	300	200	400	300
MAGEA3	TAMRA	300	200	300	200
MUC1	VIC	300	200	400	300
HER2	Cy5.5	300	200	400	300

Table 1. Primer and Probe Concentration Optimization.

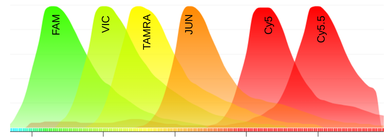

Figure 3. Emission Spectra for Each Fluorophore. Numbers denote wavelength

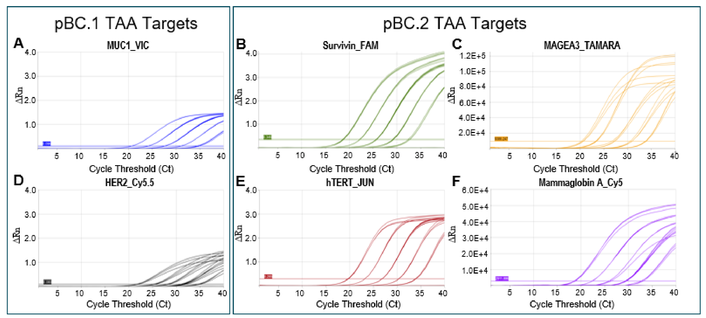

Figure 2 (A-F). Singleplex Assay Baseline Amplification Plots. A. MUC1, **B.** Survivin, **C.** MAGEA3, **D.** HER2, **E.** hTERT, **F.** Mammaglobin A.

Development and Optimization of Plasmid-Specific Multiplex qPCR Assays for the Detection of Plasmid-Specific Targets

Two custom plasmid-specific multiplex reactions were designed to quantify the expression of each antigen in a single reaction. Initial reactions were designed to evaluate specificity for the target of interest when all primer/probe sets were co-mixed. No specificity issues or cross-talk among the fluorophores was observed for any target in the pBC.1 two-plex reactions or pBC.2 four-plex reactions. Minimal optimizations of primer/probe concentrations were required (**Table 1** shows final primer/probe concentrations used in both the single and multi-plex reactions.).

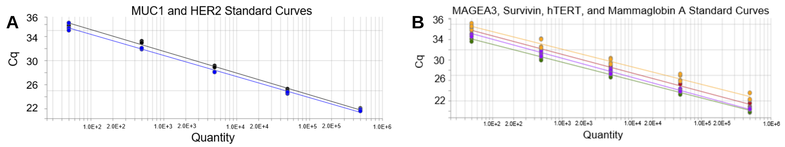

Figure 3. Plasmid Specific Multiplex qPCR Reaction Efficiencies. A. pBC.1 two-plex for HER2 and MUC1. **B.** pBC.2 four-plex for Survivin, MAGEA3, hTERT, and Mammaglobin A.

mRNA Expression in Plasmid Transfected HEK293T Cells using Plasmid-Specific Multiplex Assays

mRNA Expression was assessed in samples transfected with pBC.1, pBC.2, pBC Vector, or mock transfected. HEK293T cells have a low-level basal expression of each TAA of interest, therefore fold over expression was calculated for each target (**Figure 4A-B**). Co-transfection of both plasmids was subsequently performed, and fold mRNA overexpression was determined (**Figure 4C**).

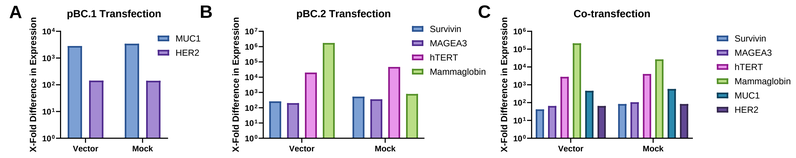

Figure 4. mRNA Expression in HEK293T cells. A. pBC.1 Transfected Samples. **B.** pBC.2 Transfected Samples. **C.** Co-transfected samples. mRNA expression is expressed as fold expression relative to mock or pBC vector transfected negative controls.

Development of a Multi-Plex qPCR Potency Assay Cont.

Initial Development and Trouble Shooting the Six-Plex qPCR Assays for the Detection all TAAs

Initial Six-Plex specificity assays revealed cross-talk between the channel 2 (MUC1) and channel 3 (MAGEA3) probes in channel 2 (**Figure 4**). Cross-talk was not observed in any other channel. Troubleshooting assays (both single and multiplex) were performed to determine if the observed signal was a result of inappropriate signal detection in the wrong channel or non-specific amplification of the wrong target. Amplified material was resolved on a 2% agarose gel (**Figure 5**). No cross-amplification of either MAGEA3 or MUC1 was observed (**Figure 5**). Careful examination of the emission spectra (**Figure 3**) found that VIC and TAMRA probes may be too similar when the emission efficiencies are at 80%. Probe sequence and fluorophore redesign are underway to reduce unwanted cross-talk between the channels. **Figure 6** shows the new overlap between redesigned probe emission spectra, and **Figure 7** shows baseline amplification using the redesigned probes.

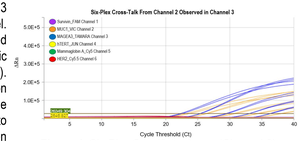

Figure 4. Six-Plex Specificity Assay (Channel 3).

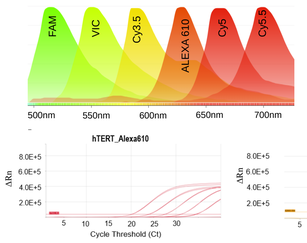

Figure 6. Emission Spectra for Fluorophore in Redesigned Probes. Numbers denote wavelength.

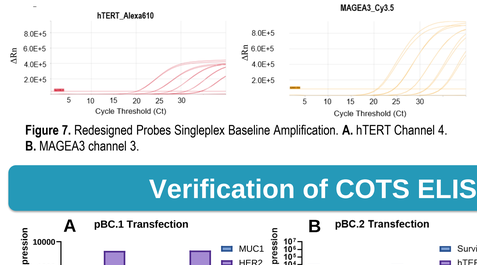

Figure 7. Redesigned Probes Singleplex Baseline Amplification. **A.** hTERT Channel 4. **B.** MAGEA3 channel 3.

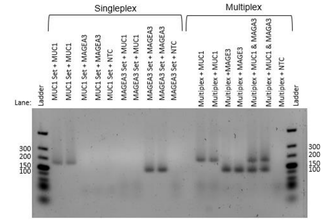

Figure 5. 2% Agarose Gel with Post Amplification Products from MAGEA3 and MUC1 Troubleshooting Assays. MUC1 expected size 180 b.p.; MAGEA3 expected size 150 b.p.

Verification of COTS ELISAs for Potency

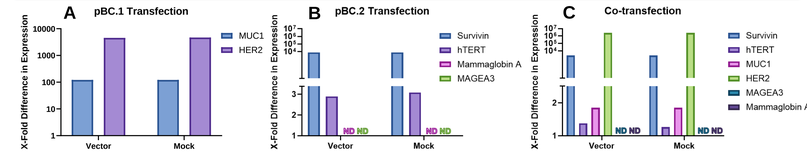

Figure 8. TAA expression in HEK293T cells. TAA expression is expressed as a fold expression relative to mock or pBC vector transfected negative controls.

Six commercial off the shelf (COTS) ELISA kits were purchased per the Methods section. Protein expression was evaluated for each TAA in pBC.1 and pBC.2 transfected samples (**Figure 8A-B**) and co-transfected samples (**Figure 8C**). Protein expression was observed for all pBC.1 targets and Survivin and hTERT on pBC.2 but MAGEA3 and Mammaglobin A. We suspect matrix interference is the cause of the lack of expression for MAGEA3 and Mammaglobin A and Western Blot assays are underway to further evaluate the expression of these two targets.

Conclusion

The mRNA expression of each TAA was quantified using two custom developed multi-plex qPCR assays with a standard curve generated from gBlocks of each antigen. Each multi-plex assay was designed to detect the specific targets expressed on each plasmid. Total protein expression was quantified using a commercially available ELISA kit for each antigen. During development and optimization of the multi-plex qPCR assay we consistently observed <10% CV for each standard curve and contrived samples. Contrived samples were consistently found to have <20% relative error during development. Although a full multi-plex was not created to include all six assays, two plasmid-specific multi-plex assays were created that are fit-for-purpose for the ongoing potency and stability studies. Efforts to create a fully functional and optimized six-plex are ongoing. We observed an 84 to 26,000-fold overexpression of mRNA transcripts for each antigen relative to the vector control and mock transfection controls, indicating suitable transfection efficiencies for both plasmids. Similarly, we observed up to 7,700-fold overexpression of each TAA protein via ELISA. Efforts to quantify MAGEA3 and Mammaglobin A via Western Blot are ongoing. These data indicate that both DNA plasmids are transfected with high efficiencies and all six antigens are overexpressed relative to the mock and vector controls. In conclusion, we developed a novel potency assay workflow to evaluate potency of two DNA plasmid vaccine candidates. These potency assays are integral to evaluating the expression of multi-antigen vaccines for breast cancer prevention and contribute to the ongoing development of safe and effective prophylactic strategies. Our assays provide robust measurements of TAA expression and serve as a key tool for vaccine development.

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