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Identificação de genes diferencialmente expressos no cancro da mama

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Resumo

O cancro da mama abrange tumores bastante heterogéneos que têm não só diferentes características clínicas como também diferentes progressões e respostas a tratamentos específicos. Para além disso, o cancro da mama resulta principalmente de alterações genéticas e epigenéticas de células da glândula mamária. A identificação de novos genes diferencialmente expressos no cancro da mama permite a compreensão da biologia da tumorigénese da mama e a identificação de novos biomarcadores ou alvos terapêuticos. O objetivo deste trabalho foi identificar genes diferencialmente expressos no cancro da mama. Neste estudo, foi utilizada a técnica da hibridação subtrativa para gerar dois bancos subtrativos, cada um com 96 clones, os quais foram, posteriormente, digeridos pela enzima Eco*RI*, e foram sequenciados todos os plasmídeos que continham insert. Todos os resultados foram analisados usando o programa Blast e a base de dados GeneBank, identificando-se 37 genes como sendo sobre-expressos e 17 como sub-expressos na linha celular MCF7. Embora alguns destes genes já tenham sido identificados como diferencialmente expressos no cancro da mama, foram encontrados alguns novos genes como diferencialmente expressos nas células MCF7. Estes novos genes podem vir a ser usados como biomarcadores do cancro da mama, ou então, utilizados como possíveis alvos terapêuticos no cancro da mama.

Palavras-chave

Cancro da mama; genes diferencialmente expressos; hibridação subtrativa; sequenciação

Resumo Alargado

O cancro é considerado uma das grandes ameaças à saúde pública em todo o mundo, sendo o cancro da mama a neoplasia maligna mais comum entre as mulheres e a segunda principal causa de morte por cancro entre as mulheres. Em Portugal, o cancro da mama é o tipo de cancro mais comum no sexo feminino, com cerca de 4500 novos casos diagnosticados anualmente. Este tipo de cancro é uma doença heterogénea, que apresenta diferentes características biológicas e clínicas. Os carcinomas da mama resultam principalmente da acumulação de alterações genéticas, como mutações, rearranjos e variações no número de cópias de alguns genes e alterações epigenéticas, como a metilação do promotor e modificação das histonas, em células epiteliais da glândula mamária. Os tumores da mama podem ser classificados em várias categorias, com base nas suas características clínicas, a expressão de marcadores tumorais - recetor de estrogénio (ER), recetor de progesterona (PR) e fator de crescimento humano epidermal-2 (HER-2) - e o seu tipo histológico. O cancro da mama pode então ser classificado como cancro da mama invasivo ou *in situ*. Relativamente à classificação dos tipos moleculares podem ser classificados como luminal A, luminal B, HER-2+, tipo basal e o tipo normal. Existem vários fatores de risco que estão associados a este tipo de cancro, como o estilo de vida e fatores genéticos. Além dos fatores genéticos e reprodutivos, o risco de cancro de mama apresenta uma grande variação étnica e geográfica. Além disso, a dieta alimentar, falta de atividade física, consumo de álcool, a idade da menarca e da menopausa são fatores de risco associados ao desenvolvimento de cancro da mama. A descoberta dos genes BRCA1 e 2, em meados dos anos 90, realçou a importância dos fatores genéticos como causa deste tipo de cancro. Estes fatores podem aumentar substancialmente o risco de contrair cancro de mama e estão associados com o desenvolvimento deste tipo de cancro numa idade jovem. Cerca de 10% dos casos de cancro da mama em países ocidentais é devido a predisposição genética. Outro fator importante é a presença ou não dos recetores de hormonas (ER, PR e HER-2). As mais promissoras descobertas revelam que o ER e o HER-2 podem responder a terapias hormonais, como por exemplo o tamoxifeno e o trastuzumabe, respetivamente. A identificação de genes diferencialmente expressos no cancro da mama tem importantes implicações na compreensão dos processos biológicos deste tipo de cancro, podendo estes serem úteis para o rastreio e diagnóstico do cancro, assim como o desenvolvimento de novas estratégias terapêuticas para o cancro da mama. A identificação de novos genes diferencialmente expressos em tumores da mama irá ajudar a encontrar marcadores específicos para o tratamento e diagnóstico desta doença. Uma das tecnologias usadas para encontrar genes diferencialmente expressos é a hibridação subtrativa (SSH). Este método permite a amplificação seletiva do cADN alvo e, simultaneamente suprime a amplificação de cADN não-alvo. Como resultado, a banco de cADN gerado por SSH contém um elevado número de genes diferencialmente expressos no cancro da

mama. A identificação de alterações nos padrões de expressão genética em células cancerosas é essencial para o diagnóstico precoce e para a identificação de possíveis alvos terapêuticos. Consequentemente, o objetivo deste estudo foi identificar genes diferencialmente expressos nas células MCF7 de cancro da mama. Para tal, foram gerados dois bancos subtrativos, cada um com 96 clones, que foram posteriormente submetidos a uma digestão pela enzima Eco*RI*. De entre todos os clones, foram sequenciados 70 de cada banco, uma vez que eram estes que continham *insert*. Para fazer a identificação dos clones as sequências foram analisadas usando o programa Blast. De seguida, os genes foram agrupados de acordo com as suas funções. Foram identificados 37 genes sobre-expressos e 17 sub-expressos nas células MCF7 de cancro da mama. Alguns destes genes já foram identificados como sendo sobre-expressos (CD24, KRT18, USP32 e DNMT1) e sub-expressos (WISP1, CELSR2, GSN e LALBA) no cancro da mama. Também foram encontrados genes que ainda não foram descritos como sobre-expressos neste tipo de cancro, como por exemplo MTRNR2L 2, MTRNR2L 8, TMPRSS13, RPS25 e LARP1. Para além destes, foram também identificados, pela primeira vez, genes sub-expressos no cancro da mama, tais como CSN1S1, CSN2, CSN3 e OAS1. Este trabalho permitiu a identificação de novos genes diferencialmente expressos no cancro da mama, e que no futuro podem vir a ser usados como possíveis biomarcadores, indicadores de prognóstico, ou utilizados como novos alvos terapêuticos.

Abstract

Breast cancer comprises heterogeneous tumors with different clinical characteristics, disease courses, and responses to specific treatments. Moreover, breast carcinomas result mainly from the accumulation of genetic and epigenetic alterations in epithelial cells of mammary gland. The identification of novel genes differentially expressed in breast cancer has important implications in understanding the molecular mechanisms underlying breast tumorigenesis, and developing new diagnostic and therapeutic targets. Therefore, the aim of this work was to identify genes differentially expressed in the breast cancer MCF7 cells, and to confirm its differential regulation in breast cancer. In this study, we used the suppressive subtractive hybridization technique to generate two subtractive libraries with 96 clones each, which were subsequently digested with Eco*RI* and then, all the plasmids with insert were sequenced. These results were further analyzed by Blast tool and GeneBank. We identify 36 genes as overexpressed and 19 genes as underexpressed in MCF7 cells. Some of these genes were previously identified as over- or underexpressed in breast cancer; however we found several genes that haven't yet been described as up or down-regulated in MCF7 cell line. In the future, these novel genes may be used as possible biomarkers, diagnostic markers or therapeutic targets in breast cancer.

Keywords

Breast cancer; genes differentially expressed; suppressive subtractive hybridization; DNA sequencing

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List of Acronyms

ATM – Ataxia telangiectasia mutated

BRCA1 – Breast cancer gene 1

BRCA2 – Breast cancer gene 2

CHK2 – Checkpoint kinase 2

CIS – Carcinoma in situ

DCIS – Ductal carcinoma in situ

EGFR – Epidermal growth factor receptor

ER – Estrogen receptor

ERα – Estrogen receptor alpha

ERß – Estrogen receptor beta

FSH – Follicle-stimulating hormone

GnRH – Gonadotrophin-releasing hormone

HCC – Hepatocellular carcinoma

HER-2 – Human epidermal growth factor-2

HGF – Hepatocyte growth factor

HN - Humanin

IDC-NOS – Invasive breast carcinoma-not otherwise specified

IDC-NST – invasive breast carcinoma of no special type

KRT – Cytokeratin

LCIS – Lobular carcinoma in situ

LH – Luteinizing hormone

MSPL – Mosaic serine protease large form

MUC-1 – Mucine 1

p53 – Tumor protein 53

Pg – Progesterone

PR – Progesterone receptor

PRA – Progesterone receptor A

PRB – Progesterone receptor B

PRL – Prolactin

PTEN – Phosphate and tensin homolog

RAD51 – DNA repair protein RAD51 homolog 1

SAGE – Serial analysis of gene expression

SSH – Suppressive subtractive hybridization

STAT1 – Signal transducer and activator of transcription 1

TK - Tyrosine kinase

yW - Wybutosine

Chapter I

Introduction

1. Overview

Cancer is a major public health problem in the United States and many other parts of the world (Siegel *et al.,* 2012), being breast cancer the most common malignancy among women (DeSantis *et al.,* 2011). There are several risk factors that are involved in this type of cancer, such as lifestyle and genetic factors (Hadjisavvas *et al.,* 2010).

In the past decade, various methods of gene expression have been described, including serial analysis of gene expression (SAGE) (Nacht *et al.,* 1999; Wu *et al.,* 2010), differential screening (Kuang *et al.,* 1998), suppressive subtractive hybridization (SSH) (Kuang *et al.,* 1998; Yang *et al.,* 1999; Jiang *et al.,* 2002) and microarray techniques (Yang *et al.,* 1999; Jiang *et al.,* 2002; Chen *et al.,* 2008; Selga *et al.,* 2009). These techniques also generated information about the differences and similarities of expression profiles in primary and metastatic breast tumors. For example, combining SAGE and array technology have allowed a rapid identification and validation of the clinical relevance of many genes potentially involved in breast cancer progression (Nacht *et al.,* 1999).

The study of genes involved in the development and progression of breast cancer is of the major importance, mostly because it could be useful to discover new important markers of breast cancer, which could also serve as therapeutic targets for chemotherapy and immunotherapy, and to develop more specific treatments for this cancer. Moreover, identify alterations in gene expression in cancer cells is crucial to the development of more efficient techniques for early diagnosis and for the understanding of the biology of breast tumorigenesis (Jiang *et al.,* 2002).

2. Breast anatomy and physiology

The breasts are the most prominent superficial structures in the anterior thoracic wall (Moore *et al.,* 2006). Each breast extends vertically from the second to the sixth rib, and in the transverse plane, from the sterna edge, medially, almost to the midaxillay line laterally. The superolateral quadrant is prolonged towards the axilla along the inferolateral edge of the pectoralis major, from which it projects a little, and may extend through the deep fascia up to the apex of the axilla (Figure 1) (Standring 2010). The axillary process of the breasts extends upward and laterally toward the axilla, where it comes into close relationship with the axillary vessels. This region of the breast is clinically significant because of the high incidence of breast cancer within the lymphatic drainage of axillary process (Van De Graaff 2002).

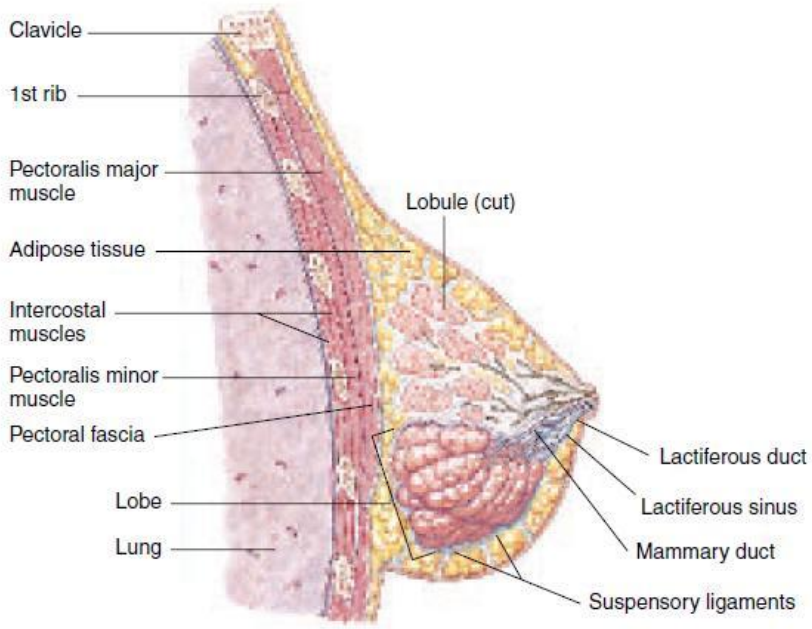

Figure 1 - The structure of the breast and mammary glands - a sagittal section of a mammary gland within the human breast. (adapted from (Van De Graaff 2002).)

Internally, the breast contains glandular tissue, fibrous connective tissue, surrounding the glandular tissue and the interlobar adipose tissue (Standring 2010). Each mammary gland is composed of 15 to 20 lobes, each with its own drainage pathway to the outside. The lobes are separated by varying amounts of adipose tissue. Each lobe is subdivided into lobules that contain the glandular mammary alveoli, which produce milk of a lactating female (Van De Graaff 2002). Breast size is determined by the amount of adipose tissue and has no relationship to the amount of milk the mammary gland can produce (Saladin 2010). Suspensory ligaments between the lobules extend from the skin to the deep fascia overlying

the pectoralis major muscle and support the breasts. The clustered mammary alveoli secret milk into a series of mammary ducts that converge to form lactiferous ducts. The lumen of each lactiferous duct expands near the nipple to form a lactiferous sinus. Milk is stored in the lactiferous sinuses before draining at the tip of the nipple (Figure 2).

The nipple is a cylindrical projection from the breast that contains some erectile tissue (Van De Graaff 2002). Its shape varies from conical to flattened, depending on nervous, hormonal, developmental and others factors. Internally, the nipple is composed mostly of collagenous dense connective tissue with numerous elastic fibers. Smooth muscle cells are also present in and just deep to the nipple, disposed in a predominantly circular direction and radiating out from its base into the surrounding breast (Standring 2010). A circular pigmented areola surrounds the nipple. The surface of the areola may appear bumpy because of the sebaceous areolar glands close to the surface. The secretions of these glands keep the nipple pliable. The color of the areola and nipple varies with the complexion of the woman. During pregnancy, the areolar becomes darker in most women, and enlarges somewhat, presumably to become more conspicuous to a nursing infant (Van De Graaff 2002).

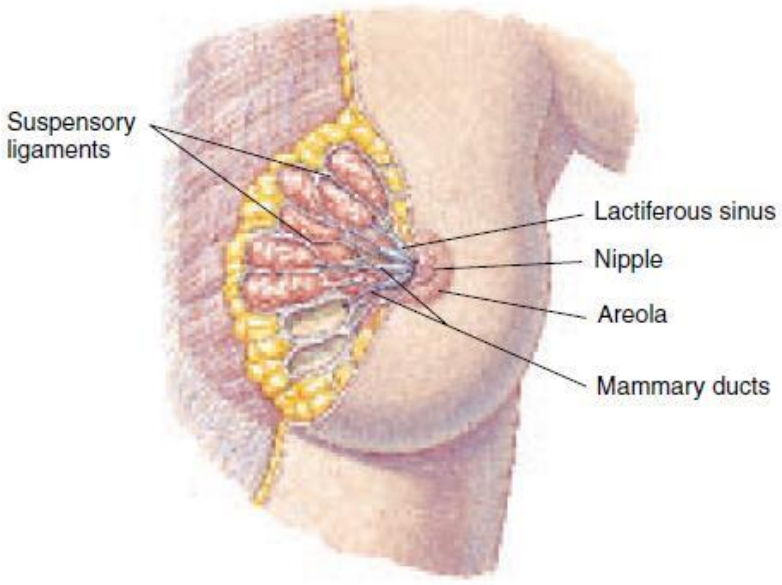

Figure 2 - The structure of the breast and mammary glands - an anterior view partially sectioned (adapted from (Van De Graaff 2002)).

The venous drainage of the breast is mainly to the axillary vein, but there is some drainage to the internal thoracic vein. The lymphatic drainage of the breast is important because of its role in the metastasis of cancer cells. Lymph passes from the nipple, areola and lobules of the gland to the subareolar lymphatic plexus (Moore *et al.*, 2006).

The mammary gland undergoes dramatic tissue remodeling events in response to hormonal stimuli, primarily by estrogens and progesterone (Pg), during puberty and pregnancy (Richert *et al.*, 2000; Lee and Ormandy 2012). Puberty is controlled by the hypothalamus-pituitary-

ovary axis and it begins with the first release of gonadotrophin-releasing hormone (GnRH). GnRH regulates the secretion of two gonadotropin hormones – luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary. Both hormones act on the ovarian follicular cells and induces the production of estrogens and Pg (Van De Graaff 2002; Boron and Boulpaep 2008). Estrogens and growth hormone drive the elongation of the mammary ductal network during puberty (Lee and Ormandy 2012). During pregnancy, gradual increases in levels of prolactin (PRL) as well as very high levels of estrogens and Pg, lead to a full development of the breasts (Boron and Boulpaep 2008) and the co-operation of Pg and PRL during pregnancy stimulate the formation of alveolar structures that produce milk post-partum (Lee and Ormandy 2012). In particular, estrogens stimulates the growth of milk ducts; Pg plays a critical role in inducing ductal side branching of the mammary gland (Atwood *et al.*, 2000), which is essential for lobuloalveolar development during pregnancy (Lee and Ormandy 2012) and, at last, PRL promotes secretion of milk from mammary glands (Boron and Boulpaep 2008).

The breast development (thelarche) can be divided into five separate phases: elevation of the breast bud (phase I); glandular subareolar tissue is present and both nipple and breast project from the chest wall as a single mass (phase II); the areola increases in diameter and becomes pigmented, and there is proliferation of palpable breast tissue (phase III); further pigmentation and enlargement occurs in the areola, so that the nipple and areola form a secondary mass anterior to the main part of the breast (phase IV); a smooth contour to the breast develops (phase V) (Standring 2010).

3. Breast cancer

Breast cancer is a heterogeneous disease, comprising numerous distinct entities that not only have different biological features (Weigelt *et al.,* 2010) but also different clinical characteristics, disease courses, and responses to the specific treatments (Bertos and Park 2011). Moreover, breast carcinomas result mainly from the accumulation of genetic alterations, such as mutations, rearrangements and copy number variations and epigenetic alterations, such as promoter methylation and histone modification, in epithelial cells of mammary gland (Holm *et al.,* 2010; Aboussekhra 2011). Classical pathology has segregated breast tumors into multiple categories (Bertos and Park 2011), based on its clinical features, its expression of tumor markers - estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor-2 (HER-2) receptor status - and its histological type (Li *et al.,* 2005).

3.1. Classification

Breast cancers can be classified into biologically and clinically meaningful subgroups according to histological grade and histological type (Weigelt *et al.,* 2010). The currently used system for histological grade is based on an assessment of three components, including proportion of tubule formation, nuclear pleomorphism (degree of differentiation) and mitotic index (proliferative activity) (Reis-Filho and Lakhani 2008). Histological type refers to the growth pattern of the tumors. There have been identified specific morphological and cytological patterns that were consistently associated with distinctive clinical presentations and outcomes. These patterns are called 'histological types' (Weigelt *et al.,* 2010).

3.1.1. Histological types

Breast cancer can be subdivided in invasive and non-invasive cancer. Most tumors are derived from mammary ductal epithelium, mainly the terminal duct-lobular unit, and up to 75% of the diagnosed infiltrating ductal carcinoma (IDC) are defined as invasive ductal carcinoma, not otherwise specified (IDC-NOS) or of no special type (IDC-NST) (Yerushalmi *et al.,* 2009), which is a diagnosis of exclusion and comprises adenocarcinomas that fail to exhibit sufficient characteristics to warrant their classification in one of the special types (Reis-Filho and Lakhani 2008). The second most common epithelial type is invasive lobular carcinoma which comprises of 5%–15% of the group (Yerushalmi *et al.,* 2009). Breast cancer special types

account for up to 25% of all breast cancers and the latest edition of the World Health Organization classification recognizes the existence of at least 17 distinct histological special types (Weigelt *et al.,* 2010), such as tubular carcinoma, invasive cribriform carcinoma, pure mucinous carcinoma, invasive solid papillary carcinoma, solid neuroendrocrine carcinoma – good prognosis typically ER-positive tumors; medullary carcinoma, secretory carcinoma, adenoid cystic carcinoma, acinic cell carcinoma – good prognosis typically ER-negative; small cell carcinoma, invasive miccropapillary carcinoma – poor prognosis typically ER-positive tumors; and finally metaplastic carcinoma and lipid rich carcinoma – poor prognosis typically ER-negative tumors (Yerushalmi *et al.,* 2009). There has been shown a relationship between histological grade and type, so they provide complementary information (Rakha *et al.,* 2008).

Relatively to the non-invasive type, called breast carcinoma *in situ* (CIS), includes ductal carcinoma *in situ* (DCIS), lobular carcinoma *in situ* (LCIS), and other relatively rare forms of breast CIS, with DCIS being the predominant subtype. The term '*in situ*' indicates that neoplastic cells are present but have not spread past the boundaries of ducts or lobules where the tumor initially developed. Unlike DCIS, LCIS indicates neoplastic changes in the breast lobules, a precursor lesion that carries an elevated risk of invasive lobular carcinoma. In contrast, DCIS and other forms of breast CIS are clinically considered pre-invasive lesions that can be associated with the development of invasive breast cancer at the same location in the breast where the CIS is located (Ma *et al.,* 2010).

3.1.2. Molecular types

In the past decade, high-throughput microarray-based gene expression profiling has been extensively applied to the study of breast cancer to unravel the molecular underpinning of biological features such as metastatic propensity or histological grade, and to identify signatures associated with prognosis and response to therapy (Weigelt *et al.,* 2010). Based on several studies on breast cancer cases, distinct molecular subtypes of breast carcinoma were identified with different clinical outcomes. Based on recent gene expression studies, Carey *et al.* characterized five immunohistochemical subtypes as luminal A (ER+ and/or PR+, HER-2-), luminal B (ER+ and/or PR+, HER-2+), HER-2+/ER- (ER-, PR-, HER-2+), basal-like (ER-, PR-, HER-2-, KRT5/6+) and normal breast-cancer (Carey *et al.,* 2006). These molecular signatures have been shown to correlate with clinical features, such as survival, prognosis and treatment sensitivity.

Normal breast-like cancers have been shown to consistently cluster together with fibroadenoma and normal breast samples (Peppercorn *et al.,* 2008). Tumors of this subtype are still poorly characterized and their clinical significance remains to be determined (Correa Geyer and Reis-Filho 2009).

Luminal tumors, both A and B, express hormone receptors, but these two luminal subtypes present distinguishing characteristics. Luminal A cancers have a high expression of ER and PR, HER-2-negative; including the highest proportion of stage I–II (75%) and well/moderately differentiated lesions (97%) (Zaha *et al.,* 2010). Usually, this subtype shows a low histological grade (Weigelt *et al.,* 2010) and presents the most favorable clinical features among the five subtypes. Luminal B tumors express HER-1 and HER-2 in addition to ER/PR and show less favorable clinical outcomes compared with luminal A tumors (Yang *et al.,* 2007). Luminal B cases have shown poorly differentiated cancers, and they are more associated with higher histological grade (Weigelt *et al.,* 2010).

The basal-like and HER-2 molecular subtypes are associated with an aggressive clinical behavior (Yang *et al.,* 2007; Weigelt *et al.,* 2010). Basal-like tumors are characterized by the expression of cytokeratin (KRT) 5/6 and KRT17 and are prevalent in patients with breast cancer gene 1 (BRCA1) mutations (Sorlie *et al.,* 2003). Basal-like carcinomas are usually of high histological grade, have high mitotic indices, pushing borders, conspicuous lymphocytic infiltrate, typical/atypical medullary features and metaplastic areas (Fulford *et al.,* 2006; Livasy *et al.,* 2006; Reis-Filho *et al.,* 2006). This subtype has been reported to be more prevalent in young women of African and Hispanic descent, to be associated with distinct risk factors and to show a high response rate to traditional chemotherapeutic treatments (Carey *et al.,* 2006; Rakha *et al.,* 2008).

HER-2 cancers have high levels of HER-2 expression, with minimal expression of ER and PR (Zaha *et al.,* 2010), and overexpressed genes associated with HER-2 pathway (Weigelt *et al.,* 2010). The HER-2 array subtype is more likely to be high-graded and poorly differentiated, and more likely to involve axillary lymph nodes. Age of patients ranged from 37 to 68 years with a mean age of 52 years, and 80% were premenopausal (Zaha *et al.,* 2010).

Unclassified cancers refer to negative triple tumors where the negative reaction for KRT5 is added; the prognostic of these tumors is slightly better than the basal subtypes (Zaha *et al.,* 2010).

In the past years, at least 3 additional ER-negative molecular subtypes have been described: the 'molecular apocrine' group of tumors, which has been claimed by some to be similar to the HER-2 subtype and appears to have activation of the androgen receptor signalling (Farmer *et al.,* 2005; Doane *et al.,* 2006); the 'interferon' subtype, which is characterized by high expression of interferon regulated-genes, including STAT1 (signal transducer and activator of transcription 1) (Hu *et al.,* 2006); and the 'claudin-low' subgroup, which comprises tumors that have transcriptomic features suggestive of a 'cancer stem cell-like' phenotype (Herschkowitz *et al.,* 2007; Hennessy *et al.,* 2009).

Special types of breast cancer account for up to one quarter of all invasive breast malignancies and their importance should not be disregarded. Understanding the biological

drivers of these entities may lead to a better understanding of the biology of breast cancer cells (Weigelt *et al.,* 2010).

3.2. Epidemiology and Risks factors

Breast cancer is the most common malignancy among women, accounting for nearly 1 in 3 cancers diagnosed among women in the United States, and it is the second leading cause of cancer death among women (DeSantis *et al.,* 2011). In Portugal, breast cancer is the most common type of cancer among women, with nearly 4500 cases per year (Pinheiro *et al.,* 2003).

Numerous epidemiological studies over the last three decades have revealed a number of risk factors associated with breast cancer (Kelsey and Horn-Ross 1993; Lipworth 1995). In addition to genetic and reproductive factors, breast cancer risk displays wide ethnic and geographic variation (DeSantis *et al.,* 2011). Within Europe the incidence varies by almost two-fold. It is highest in Northern European countries, with an estimated 84.6 cases per 100,000 population with intermediate rates recorded in Southern Europe and lowest rates occurring in Eastern Europe with 42.6 cases per 100,000 population (Parkin *et al.,* 2005). Besides to the geographic variation, influences on incidence rates have also been attributed to differences in the use of mammography, diet, physical activity, body size, alcohol consumption and socioeconomic and reproductive factors (Key *et al.,* 2001). The discovery of highly penetrant breast cancer susceptibility genes such as BRCA–1 and 2 - in the mid 1990's (Miki *et al.,* 1994; Wooster *et al.,* 1995) emphasized the importance of genetic factors, but it is currently believed that environmental factors are of greater significance (Hadjisavvas *et al.,* 2010).

3.2.1. Lifestyle/Behavior and diet

There are several factors that influence the risk of breast cancer development. Some of them are created by individual lifestyle and choices like diet, overweight, alcohol intake, age at first pregnancy and physical exercise (McPherson *et al.,* 2000). There are others factors such as age at menarche and age at menopause, that influence the risk of breast cancer (Key *et al.,* 2001).

Migrational data have pointed to nutrition as one of the more relevant environmental factors involved: Japanese women in their home country have a comparatively small lifetime risk for breast cancer. However, after migration to the US, breast cancer incidence assimilates to the risk of Caucasian Americans within one or two generations, a finding which rules out genetic resistance to breast cancer development (Hanf and Gonder 2005). More recent studies have looked at other possible dietary determinants of risk, such as consumption of meat, fiber,

fruit and vegetables, and phytoestrogens. There may be a moderate protective effect for a high consumption of vegetables, but results for meat, fiber, and fruit have been inconsistent, and breast-cancer risk has not been shown to be lower in vegetarians than in non-vegetarians in more developed countries (Key *et al.,* 2001). Although there is a close correlation between the incidence of breast cancer and dietary fat intake in populations, the true relation between fat intake and breast cancer does not appear to be particularly strong or consistent (McPherson *et al.,* 2000). However, there has been much interest in the possibility that phytoestrogens, found at high concentrations in soya and some other foods, may block the effects of the vastly more potent endogenous estrogens and thereby reduce breast cancer risk. However, the results of studies are inconclusive (Key *et al.,* 2001). Regardless of all this, obesity is associated with a twofold increase in the risk of breast cancer in postmenopausal women whereas among premenopausal women it is associated with a reduced incidence (McPherson *et al.,* 2000).

Nulliparity and late age at first birth both increase the lifetime incidence of breast cancer. The risk of breast cancer in women who have their first child after the age of 30 is about twice that of women who have their first child before the age of 20. The highest risk group is those who have a first child after the age of 35; these women appear to be at even higher risk than nulliparous women. An early age at birth of a second child further reduces the risk of breast cancer (McPherson *et al.,* 2000; Parsa and Parsa 2009).

Women who start menstruating early in life or who have a late menopause have an increased risk of developing breast cancer (McPherson *et al.,* 2000). For each 1-year delay in menarche, the risk decreases by around 5%. There is also evidence that, although age at menarche is related to breast cancer risk at all ages, the effect may be stronger in younger (premenopausal) women. Women who experience menopause at a late age are at a higher risk of breast cancer than those who cease menstruating earlier, with risk increasing by about 3% for each year older at menopause (Key *et al.,* 2001; Parsa and Parsa 2009). So, women who have a natural menopause after the age of 55 are twice as likely to develop breast cancer as women who experience the menopause before the age of 45. At one extreme, women who undergo bilateral oophorectomy - surgical removal of the ovaries - before the age of 35 have only 40% of the risk of breast cancer of women who have a natural menopause (McPherson *et al.,* 2000).

The classic risk factors for breast cancer, such as age at menarche, age at menopause, and parity, are not amenable to change for the purpose of reducing risk. However, other behavioral risk factors (obesity, alcohol intake, physical exercise) could be modified and these changes could reduce the risk of breast cancer and would have other health benefits also (Key *et al.,* 2001).

3.2.2. Family history and genetic causes

Genetic and familial factors can substantially increase the lifetime risk of developing breast cancer and are associated with the development of cancer at a young age (Amir *et al.,* 2010). Up to 10% of breast cancer in Western countries is due to genetic predisposition. Breast cancer susceptibility is generally inherited as an autosomal dominant with limited penetrance. This means that it can be transmitted through either sex and that some family members may transmit the abnormal gene without developing cancer themselves (McPherson *et al.,* 2000), in others words most women with the disease do not have a family history of it, and most women with affected relatives never develop breast cancer (Key *et al.,* 2001).

The evidence for genetic predisposition to breast cancer derives originally from observations of cancer clustering in families and cancer risk increasing in individuals with some genetically determined syndromes. Most studies on familial risk of breast cancer have found about two-fold relative risks for first-degree relatives (mothers, sisters, daughters) of affected patients (Pharoah *et al.,* 1997). With affected second-degree relatives (grandmothers, aunts, grand-daughters), there is a lesser increase in risk (Key *et al.*, 2001). Although 10–15% of breast cancer cases have some family history of the disease, only 5% can be explained by rare, highly penetrant mutations in genes such as BRCA1 and BRCA2 (Dunning *et al.*, 1999). So far, at least five germline mutations that predispose to breast cancer have been identified or localized. These include mutations in the genes BRCA1, BRCA2, tumor protein 53 (P53), phosphatase and tensin homolog (PTEN), and ataxia telangiectasia mutated (ATM) (Clague *et al.*, 2011). Two breast cancer genes, BRCA1 and BRCA2, which are located on the long arms of chromosomes 17 and 13 respectively, have been identified and account for a substantial proportion of very high risk families. Both genes are very large and mutations can occur at almost any position, so molecular screening to detect mutation for the first time in an affected individual or family is technically demanding (McPherson *et al.,* 2000). Germline mutations in P53 predispose to the Li-Fraumeni cancer syndrome (including childhood sarcomas and brain tumors, as well as early-onset breast cancer) and those in PTEN are responsible for Cowden disease (of which breast cancer is a major feature) (Key *et al.,* 2001).

3.2.2.1. BRCA1

BRCA1 was cloned in 1994 (Miki *et al.,* 1994) and it is localized at chromosome 17q21 (Hall *et al.,* 1990). It is a large gene, with approximately 100kb that consists of 5592 base pairs (Hofmann and Schlag 2000) in 22 exons, encoding a 220-kilodalton nuclear protein (Nathanson *et al.,* 2001). Studies suggest that BRCA1 accounts for the majority of families containing multiple cases of breast and ovarian cancer, for less than half the families containing breast cancer only, and for few families that include male breast cancer cases (Ford *et al.,* 1998). BRCA1 binds to BRCA2, P53, DNA repair protein RAD51 homolog 1 (RAD51) and many other

proteins involved in cell cycle and DNA-damage response (Scully and Livingston 2000) (Figure 3).

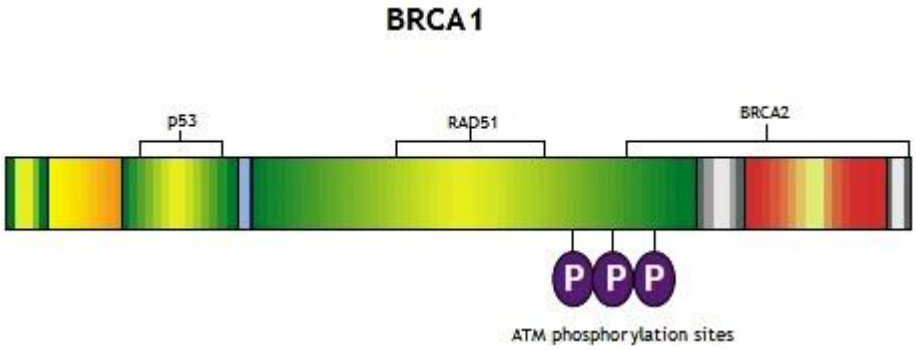

Figure 3 - BRCA1 protein. p53 and RAD51 binding site; P, serines phosphorylated by ATM in response to DNA damage (adapted from (Nathanson *et al.*, 2001))

The involvement of BRCA1 in response to DNA damage is supported by extensive data, including evidence that BRCA1 is phosphorylated by the ATM and checkpoint kinase 2 (CHK2) proteins in response to DNA damage. Cells without functional BRCA1 do not arrest in G2 after DNA damage and are deficient in transcription-coupled repair. In addition, BRCA1 is part of the RAD50–MRE11–p95 complex, an essential component of recombination-mediated repair of DNA double-stranded breaks (Nathanson *et al.*, 2001).

3.2.2.2. BRCA2

BRCA2 was cloned in 1995 (Wooster *et al.*, 1995) and it is localized at chromosome 13q12.3 in 1994 (Wooster *et al.*, 1994) and this gene is even larger than BRCA1, with a 10.3-kilobase including 27 exons encoding a 384-kilodalton nuclear protein (Hofmann and Schlag 2000) (Figure 4).

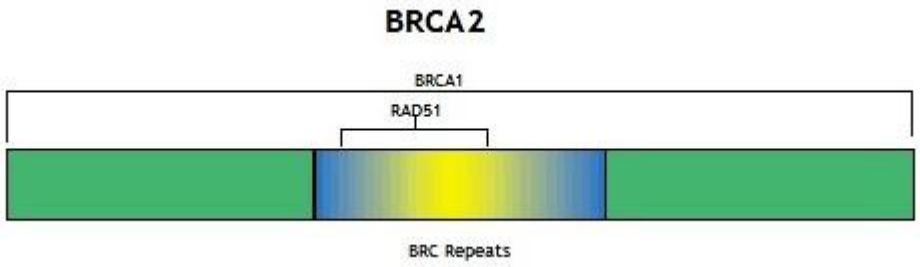

Figure 4 - BRCA2 protein. RAD51 interaction site (adapted from (Nathanson *et al.*, 2001)).

The human tumor suppressor protein BRCA2 plays a key role in DNA repair (Barnes and Antoniou 2012). BRCA2 recruits RAD51 to sites of DNA damage through interaction with eight conserved motifs of approximately 35 amino acids, the BRC repeats (Figure 4). These motifs are highly conserved between mammalian species, and they confer upon BRCA2 the ability to bind RAD51 (Carreira and Kowalczykowski 2011). BRCA2 is also involved in maintaining genomic stability through its interaction with RAD51. Homologous recombination serves to maintain genomic integrity in somatic cells by promoting the repair of breaks in DNA strands. BRCA2 regulates RAD51 function in DNA repair by recruiting it to the sites DNA breaks (Carreira and Kowalczykowski 2011; Barnes and Antoniou 2012).

3.3. Hormone Receptor Status

3.3.1 Estrogen receptor (ER)

Estrogen is an important regulator of growth and differentiation in the normal mammary gland and is also important in the development and progression of breast carcinoma (Gruvberger *et al.*, 2001). The estrogen signal is mediated by the ER, which is a transcription factor belonging to the steroid hormone receptor superfamily. There are two types of ER: ER-α and ER-ß and they are encoded by two different genes, ESR1 on chromosome 6 and ESR2 on chromosome 14q, respectively (Herynk and Fuqua 2004). Both ER-α and ER-ß proteins are expressed in normal breast luminal epithelial cells as well as in breast tumors (Yu *et al.*, 2011). ERα is expressed in approximately 15–30% of luminal epithelial cells (Anderson 2002).

In general, nuclear receptors have a modular structure with six distinct regions, A-F. Of these, region C (corresponding to the DNA-binding domain) and region E (corresponding to the ligand-binding domain) are evolutionally conserved (Mosselman *et al.*, 1996). Although the two ERs are homologous in their DNA-binding and steroid binding domains, the ERß gene is smaller and it encodes a shorter protein (Anderson 2002).

ERα and ERß have been demonstrated to form heterodimers, as well as homodimers, further complicating their individual and/or combined function within a cell. Although both receptors bind estrogen with similar affinities, ERß appears to have a stronger affinity for phytoestrogens (Herynk and Fuqua 2004).

The most promising findings revels that ERα is a very strong predictive factor for response to hormonal therapies, such as tamoxifen. Tamoxifen, which binds ERα and blocks estrogen-stimulated growth, has been shown to significantly reduce disease recurrence and prolong life in patients with ERα-positive invasive breast cancers (EBCTCG 2005). The clinical response to newer types of hormonal therapies, such as the aromatase inhibitors, which suppress the

production of estrogen, is also dependent on the status of ERα, and only positive tumors benefit (Buzdar *et al.,* 2004; Howell *et al.,* 2005). The primary reason for assessing ERα is its ability to predict response to these hormonal therapies (Allred 2010).

3.3.2 Progesterone receptor (PR)

Progesterone has two receptors, progesterone receptor A (PRA) and PRB. These two receptors are transcribed from the same gene, that is located on chromosome 11q13 (Law *et al.,* 1987). PRB is longer than PRA as it contains an additional 164 amino acids at its N-terminal, but otherwise the two proteins are identical (Clarke and Sutherland 1990). PRA and PRB are also members of the steroid hormone nuclear receptor superfamily, and they function as ligand-dependent nuclear transcription factors. PRA as well as PRB can activate gene transcription.

Once expressed, PR is activated by the hormone progesterone to help regulate several important normal cellular functions, including proliferation which, of course, is detrimental in breast cancers. ERα regulates the expression of PR; hence, the presence of PR usually indicates that the estrogen-ERα pathway is intact and functional (Anderson 2002; Clarke 2003; Jacobsen *et al.,* 2003).

There are few studies in the medical literature for PR (Elledge *et al.,* 2000; Love *et al.,* 2002; Mohsin *et al.,* 2004; Viale *et al.,* 2008). Those available show that PR is expressed in the nuclei of 60–70% of invasive breast cancers, that there is a direct correlation between PR levels and response to hormonal therapies, and that tumors with even very low levels of PR-positive cells (≥1%) have a significant chance of responding (Love *et al.,* 2002; Mohsin *et al.,* 2004; Allred 2010).

3.3.3 Human epidermal growth factor-2 (HER-2)

The human epidermal growth factor (HER) family of transmembrane receptors are potent mediators of normal cell growth and development (Hynes and Lane 2005). This family of receptors consists of four closely related type 1 transmembrane tyrosine kinase receptors: HER1 (EGFR), HER-2, HER3 and HER4. Each receptor comprises an extracellular domain where ligand binding occurs, an *a*-helical transmembrane segment and an intracellular protein tyrosine kinase (TK) domain (Baselga 2010).

HER-2 (also referred to as HER-2/neu and erbB2) is a proto-oncogene located on chromosome 17 (Coussens *et al.,* 1985). It encodes a TK receptor residing on the surface membrane of breast epithelial cells (Schechter *et al.,* 1984). HER-2 regulate many normal cellular

functions, including proliferation, survival, and apoptosis (Harari and Yarden 2000; Menard *et al.,* 2000; Allred 2010).

HER-2 overexpression occurs in 15%–20% of patients with breast cancer and is associated with aggressive disease and decreased survival (Baselga 2010; Chang 2010). There is a weak but significant association between poor outcome and amplified and/or overexpressed HER-2 in patients receiving no additional therapy after initial surgery (Allred and Swanson 2000). However, most patients receive some type of adjuvant therapy, and the association between HER-2 status and outcome seems to depend on the type of therapy.

The most promising and useful findings come from recent studies showing that HER-2-positive tumors respond favorably to new antibody-based therapies, which specifically target the HER-2 protein, such as trastuzumab (Engel and Kaklamani 2007) and the main reason for assessing HER-2 status is to identify candidates for targeted therapy. Although trastuzumab was originally demonstrated as being effective in HER-2-positive metastatic disease, more recent clinical trials have demonstrated significant benefit as adjuvant therapy for women with less advanced HER-2-positive breast cancer (Allred 2010).

4. Genes differentially expressed in breast cancer

Identifying novel and known genes that are differentially expressed in breast cancer has important implications in understanding of the biological processes of breast cancer and in discovering cDNAs that encode proteins that could be useful for cancer screening and diagnosis; with the purpose of develop more specific treatment strategies for breast cancer (Jiang *et al.,* 2002; Zheng and Pepe 2007).

Genetic alterations resulting in altered mRNA and protein levels have been described in breast tumorigenesis, such as the activation or amplification of oncogenes or the loss of tumor suppressor genes. Historically, a number of these genes have been identified such as HER-2, a surface growth factor receptor shown to be overexpressed in 15-20% of breast cancers. The p53 gene that normally functions as a tumor suppressor gene has been found to be overexpressed in 57% of breast tumors as an outcome of gene mutation and changes in protein stabilization. Mucine-1 (MUC-1) is another gene that is up-regulated about 10-fold in 90% of breast tumors. Each of these proteins has become the target for novel immunotherapy approaches in the treatment of breast cancer. Identifying additional genes that may be up- or down-regulated in breast tumors will help to find additional markers for treatment and diagnosis of the disease (Jiang *et al.,* 2002).

One of the technologies used to find these genes is the suppression subtractive hybridization (SSH), also known as PCR-based cDNA subtraction, that was developed by (1996). This method allows selective amplification of target cDNAs, while simultaneously suppressing non-target cDNA amplification. An advantage of the PCR-based cDNA subtraction method is that in addition to the recovery of abundant clones regularly obtained by conventional cDNA subtraction, rare transcripts are also recovered due to the incorporated hybridization and PCR steps that normalize sequence abundance. As a result, the subtracted cDNA library generated by SSH technology contains an increased number of differentially expressed genes (Diatchenko *et al.,* 1996).

There are several studies in the identification of genes differentially expressed in breast cancer, and each study identifies quite a lot of genes that are expressed in this type of cancer. It is in this context that it was made a brief summary of some up- (Table 1) and down-regulated genes (Table 2), which had already been studied, in breast tumors (Kuang *et al.,* 1998; Nacht *et al.,* 1999; Yang *et al.,* 1999; Jiang *et al.,* 2002; Chen *et al.,* 2008; Hicks *et al.,* 2012).

Table 1 – Summary of main up-regulated genes in human breast tumors and its biological function

Gene name	Acronym	Accession number	Biological Function	Reference
Fibroblast growth factor 1	FGF-1	NM_001257211	Angiogenesis [GO:0001525]	(Nacht *et al.*, 1999)
Serine/threonine-protein phosphatase 2A regulatory subunit B beta isoform	PPP2R2B	NM_181678	Apoptosis [GO:0006915]	(Hicks *et al.*, 2012)
WW domain-containing oxidoreductase	WWOX	NM_016373		(Hicks *et al.*, 2012)
CD24 molecule	CD24	NM_013230.2	Cell adhesion [GO:0007155]	(Yang *et al.*, 1999)
Fibronectin	FN	NM_212478		(Jiang *et al.*, 2002)
Ephrin type-A receptor 4	HEK8	NM_004438		(Kuang *et al.*, 1998)
Intercellular adhesion molecule 1	ICAM1	NM_000201		(Hicks *et al.*, 2012)
Mucin-1	MUC-1	NM_001204296		(Nacht *et al.*, 1999)
Reelin	RELN	NM_005045		(Hicks *et al.*, 2012)
DNA-3-methyladenine glycosylase	MPG	NM_002434	Cell cycle [GO: 0007049]	(Nacht *et al.*, 1999)
Keratin, type I cytoskeletal 19	KRT19	NM_002276	Cytoskeleton [GO:0005856]	(Nacht *et al.*, 1999; Yang *et al.*, 1999; Jiang *et al.*, 2002)
Bloom syndrome protein	BLM	NM_000057	DNA replication [GO:0006260]	(Hicks *et al.*, 2012)
Replication protein A 32 kDa subunit	RPA2	NM_002946		(Hicks *et al.*, 2012)
Zinc-alpha-2-glycoprotein	AZGP1	NM_001185	Immune response [GO:0006959]	(Nacht *et al.*, 1999)
Deleted in malignant brain tumors 1 protein	DMBT1	NM_017579		(Hicks *et al.*, 2012)
Matrix metallopeptidase 8	MMP8	NM_002424	Metabolic process [GO:0008152]	(Hicks *et al.*, 2012)
Matrix metallopeptidase 15	MMP15	NM_002428		(Nacht *et al.*, 1999)
Tripartite motif-containing protein 45	TRIM45	NM_025188		(Hicks *et al.*, 2012)
Insulin-like growth factor 1 receptor	IGF1R	NM_000612	Protein binding [GO:0005515]	(Hicks *et al.*, 2012)
Heat shock protein beta-1	Hsp27	NM_001540	Response to stress [GO:0006950]	(Kuang *et al.*, 1998)
Trans-acting T-cell-specific transcription factor GATA-3	GATA-3	NM_001002295	Transcription [GO:0006351]	(Yang *et al.*, 1999)
Receptor tyrosine-protein kinase erbB-2	HER-2	NM_001005862		(Nacht *et al.*, 1999)
High mobility group protein HMG-I/HMG-Y	HMG-I(Y)	NM_145905		(Nacht *et al.*, 1999)

Gene name	Acronym	Accession number	Biological Function	Reference
Eukaryotic translation elongation factor 1 alpha 2	EEF1A2	NM_001958.2|	Translation [GO:0006412]	(Kuang *et al.*, 1998)

Table 2 – Summary of main down-regulated genes in human breast tumors and its biological function

Gene name	Acronym	Accession number	Biological Function	Reference
Caspase-8	CASP8	NM_001228	Apoptosis [GO:0006915]	(Hicks *et al.*, 2012)
Galectin-7	LGALS7	NM_002307		(Nacht *et al.*, 1999)
Dystonin	DST	NM_001723	Cell adhesion [GO:0007155]	(Nacht *et al.*, 1999)
Thrombospondin-1	THBS1	NM_003246		(Nacht *et al.*, 1999)
DNA-(apurinic or apyrimidinic site) lyase	APEX1	NM_080649	Cell cycle [GO: 0007049]	(Hicks *et al.*, 2012)
Histone-lysine N-methyltransferase EHMT2	EHMT2	NM_006709		(Hicks *et al.*, 2012)
DNA repair protein complementing XP-G cells	ERCC5	NM_000123		(Hicks *et al.*, 2012)
Methylated-DNA--protein-cysteine methyltransferase	MGMT	NM_002412		(Hicks *et al.*, 2012)
DNA mismatch repair protein Msh2	MSH2	NM_000251		(Hicks *et al.*, 2012)
DNA mismatch repair protein Msh6	MSH6	NM_000179		(Hicks *et al.*, 2012)
DNA-dependent protein kinase catalytic subunit	PRKDC	NM_006904		(Hicks *et al.*, 2012)
Retinoblastoma-associated protein	RB1	NM_000321		(Hicks *et al.*, 2012)
DNA repair protein complementing XP-A cells	XPA	NM_000380		(Hicks *et al.*, 2012)
X-ray repair cross-complementing protein 5	XRCC5	NM_021141		(Hicks *et al.*, 2012)
Keratin, type I cytoskeletal 15	KRT15	NM_002275	Cytoskeleton [GO:0005856]	(Nacht *et al.*, 1999)
Keratin, type I cytoskeletal 17	KRT17	NM_000422		(Nacht *et al.*, 1999)
Tropomyosin beta chain	TPM2	NM_003289		(Nacht *et al.*, 1999)
Replication protein A 70 kDa DNA-binding subunit	RPA	NM_002945	DNA replication [GO:0006260]	(Hicks *et al.*, 2012)
Src kinase-associated phosphoprotein 2	SKAP2	NM_003930	Immune response [GO:0006959]	(Hicks *et al.*, 2012)
Serpin B5	SERPINB5	NM_002639	Metabolic process [GO:0008152]	(Nacht *et al.*, 1999)
S100 calcium binding protein A2	S100A2	NM_005978.3	Protein binding [GO:0005515]	(Nacht *et al.*, 1999)

5. Aim of the study

The identification of alterations in the patterns of gene expression in cancer cells has been an essential tool for development of better and effective approaches for early diagnosis and treatment of human cancer cases. Thus, it is of the major importance to continue looking for novel genes related to breast cancer initiation and progression. Consequently, the aim of this study is to identify genes differentially expressed between normal breast and breast cancer.

Chapter II

Materials and Methods

1. Cell lines

The MCF7 human breast cancer cell line was derived from a pleural effusion taken from a patient with metastatic breast cancer. It is characterized by the expression of ER (Levenson and Jordan 1997) and PR (Horwitz *et al.,* 1975).

For this study, it was used a MCF7 and normal human breast RNA (ref. 636315 and 636163, respectively), that were purchased from Clontech (Mountain View, CA).

2. Supressive subtractive hybridization (SSH)

SSH was performed with the PCR-Select^{TM} cDNA Subtraction Kit (Clontech, Mountain View, CA) according to the manufacturer's protocol.

First, cDNA of the MCF7 RNA (tester) and normal human breast RNA (driver) was synthesized. The cDNA derived from both were digested by *Rsa I* restriction enzyme, to obtain blunt-ends which are necessary for adaptor ligation. The tester cDNA was divided into two portions and a different adaptor was linked to each. Then, two hybridizations were performed. In the first, an excess of driver was added to each sample of the tester. The samples are then heat denatured and allowed to anneal, generating four types of molecules in each sample. In this step, differentially expressed sequences are equalized and enriched. In the second hybridization, the two primary hybridization samples were mixed together to generate differentially expressed sequences. Then, the entire populations of molecules were subjected to a first PCR to amplify the differentially expressed sequences. Finally, the second PCR was performed to reduce any background PCR products and enrich the differentially expressed sequences.

It was generated the forward library, which corresponds to possible up-regulated genes and the reverse library, which corresponds to possible down-regulated genes.

3. Cloning into pGEM-T easy vector

SSH-derived genes were inserted into pGEM-T easy vector (Promega, Madison, WI) and transformed into competent XL1B cells. These were plated in agar plates containing X-Gal, ampicillin and IPTG, and white and blue colonies were formed. The white ones were picked

and grown in a LB-broth medium with ampicillin overnight. Finally, the plasmids were purified using the Wizard® *Plus* SV Minipreps DNA purification Systems (Promega, Madison, WI).

4. Digestion with the restriction enzyme Eco*RI*

To screen the presence of the inserts on vector, the plasmids were digested with the enzyme Eco*RI* for 1hour at 37°C. Then, the digests were electrophoresed in a 1% agarose gel with ethidium bromide for 30 minutes at 100V and it was visualized in the UV transilluminator.

5. DNA Sequencing

The plasmids that contain inserts were sequenced using the GenomeLeb™ Dye Terminator Cycle Sequencing with Quick Start Kit (Beckman Coulter, Fullerton, CA) according to the manufacturer's protocol.

First, the plasmids were denatured by a pre-heat treatment (96°C for 1 minute). Next it was added the primer T7 5'-TAATACGACTCACTATAGGG-3' and the Master Mix and put in the thermal cycling program (96°C for 20 seconds, 50°C for 20 seconds and 60°C for 4 minutes, during 30 cycles). Then, the DNA ethanol precipitation was performed. Initially, it was prepared a fresh Stop Solution, that contains 2 µL of 3M Sodium Acetate (pH 5.2), 2 µL of 100 mM Na2-EDTA (pH 8.0) and 1 µL of 20 mg/mL of glycogen (per sequencing reaction). To each of the sequencing reactions, it was added 5 µL of the Stop Solution mixture and the final samples were mixed thoroughly. Then, it was added 60 µL cold 95% (v/v) ethanol/dH2O from -20°C freezer and was mixed thoroughly. Immediately it was centrifuged at 14,000 rpm at 4°C for 15 minutes. Carefully it was removed the supernatant with a micropipette. Next, it was rinsed the pellet 2 times with 200 µL 70% (v/v) ethanol/dH2O from -20°C freezer. For each rinse, centrifuge immediately at 14,000 rpm at 4°C for a minimum of 5 minutes. After centrifugation carefully remove all of the supernatant with a micropipette and let the samples dry totally. Finally, the samples were resuspended in 40 µL of the Sample Loading Solution. The resuspended samples were transferred to the appropriated wells of the sample plate and each of the resuspended samples was overlaid with one drop of light mineral oil. At last, the sample plate was loaded into the instrument and it was started the desired method.

6. Sequence analysis

The DNA homology searches were performed using the Blast tool (Altschul *et al.,* 1997) at the National Center for Biotechnology Information (NCBI). Next, the genes were grouped according its primary biological function based on Gene Ontology tool (Ashburner *et al.,* 2000).

Chapter III

Results and Discussion

1. Identification of clones with insert

To identify genes differentially expressed in MCF7 cells, the subtractive cloning strategy of SSH generated two subtractive libraries – the forward (FW) one and the reverse (RV) one – with 96 clones each, numbered from A to H and 1-12.

We have performed an enzymatic digestion with the Eco*RI* in all clones of both libraries, to verify which clones containing insert (Figure 5). Seventy of 96 clones of each library contain cDNA insert (Table 3) with sizes ranging from 100 to ~ 800 bp, suggesting that both libraries were well subtracted and normalized.

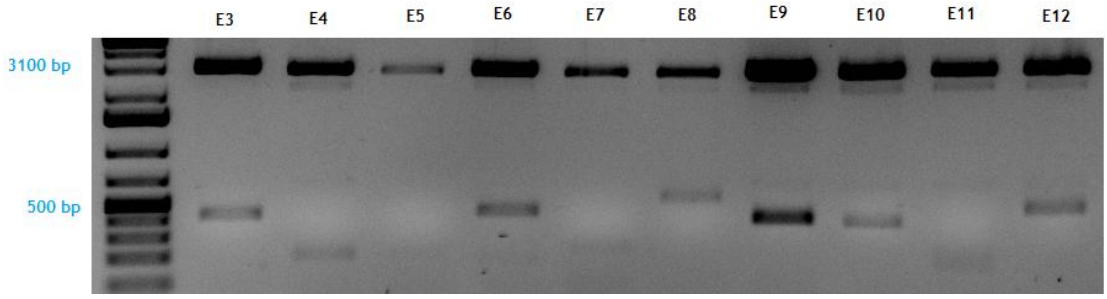

Figure 5 - Enzymatic digestion of the FW clones E3 to E12. The plasmid appears at 3100 bp and several cDNA inserts with different molecular weight can be detected in the clones E3, E4, E6, E7, E8, E9, E10, E11 and E12.

Table 3 – Total number of clones with insert in forward and reverse libraries

	Total	With insert	%
Forward library	96	70	72,92
Reverse library	96	70	72,92

2. DNA sequencing of the inserts and identification of the respective genes

In order to characterize the clone identity, all plasmids with insert were sequenced (Figure 6) and analyzed using the Blast tool (Altschul *et al.*, 1997). In the FW and RV libraries, 49 of 70 and 50 of 70 sequences present high homology with genes deposited at Genebank,

respectively. Although several clones have detected the same gene, it was identified 37 genes as overexpressed and 17 genes as underexpressed in MCF7 cell line.

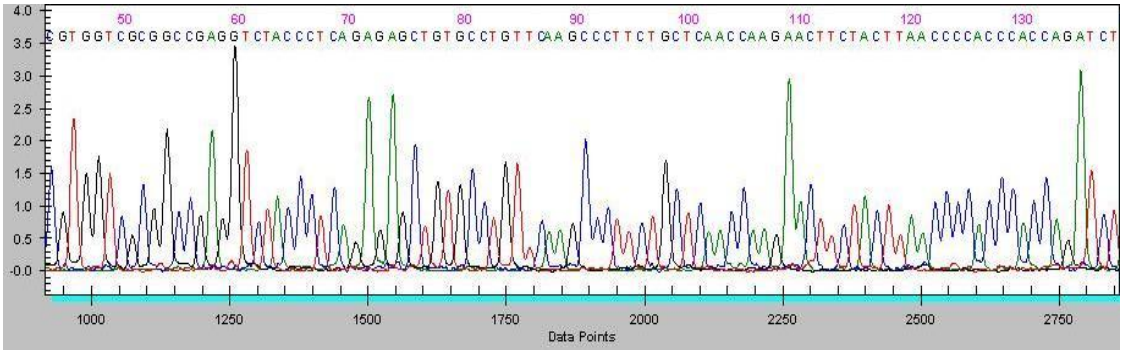

Figure 6 – Representative chromatogram sequencing (clone H10 from RV library).

After all this process, the biological function of each gene was obtained using the Gene Ontology tool (Ashburner *et al.*, 2000), and grouped according its biological function (Table 4 and 5).

Table 4 – Identified up-regulated genes and its primary biological function

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Up-regulated in breast cancer	Up-regulated in others cancers
F9	CD24	Homo sapiens CD24 molecule	6q21	NM_013230.2|	Cell-cell adhesion [GO:0016337]	Yes	Yes
A11 / H2	TUBB2C	Homo sapiens tubulin, beta 4B class IVb	9q34	NM_006088.5	Cell cycle [GO:0007049]	Yes	Yes
B1	NPM1	Homo sapiens nucleophosmin (nucleolar phosphoprotein B23, numatrin)	5q35.1	NM_001037738.2		No	Yes
B8 / D7 / E12 / G10	KRT18	Homo sapiens keratin 18	12q13	NM_000224.2|		Yes	Yes
E1	DNMT1	Homo sapiens DNA (cytosine-5-)-methyltransferase 1	19p13.2	NM_001379.2|		Yes	Yes
F10	MTRNR2L 2	Homo sapiens MT-RNR2-like 2	5q14.1	NM_001190470.1		No	No
F12 / H4	MTRNR2L 8	Homo sapiens MT-RNR2-like 8	11p15.3	NM_001190702.1|		No	No
G3	H3F3B	Homo sapiens H3 histone, family 3B (H3.3B)	17q25.1	NM_005324.3|		No	Yes

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Up-regulated in breast cancer	Up-regulated in others cancers
A2	ACTG1	Homo sapiens actin, gamma 1	17q25.1	NR_037688.1|	Cytoskeleton [GO:0005856]	Yes	Yes
H5	ACTB	Homo sapiens actin, beta	7p22	NM_001101.3		No	No
H12	KRT8	Homo sapiens keratin 8	12q13	NM_002273.3		Yes	Yes
C6	PTGES3	Homo sapiens prostaglandin E synthase 3 (cytosolic)	12q13.3	NM_006601.5|	Immune response [GO:0006959]	Yes	Yes
C12	USP32	Homo sapiens ubiquitin specific peptidase 32	17q23.1	NM_032582.3|	Metabolic Process [GO:0008152]	Yes	No
E10 / F6	GAPDH	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase	12p13	NM_002046.3|		No	Yes
G1	TMPRSS13	Homo sapiens transmembrane protease, serine 13	11q23	NM_001206790.1|		No	No
H3	PGK1	Homo sapiens phosphoglycerate kinase 1	Xq13.3	NM_000291.3		Yes	Yes

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Up-regulated in breast cancer	Up-regulated in others cancers
H11	H2AFZ	Homo sapiens H2A histone family, member Z	4q24	NM_002106.3		Yes	Yes
A1	SNRPD1	Homo sapiens small nuclear ribonucleoprotein D1 polypeptide 16kDa	18q11.2	NM_006938.2|	mRNA processing [GO:0006397]	No	Yes
A9	RPL3	Homo sapiens ribosomal protein L3	22q13	NM_000971.3|		No	Yes
A4 / E8 / H9	eEF1A2	Homo sapiens eukaryotic translation elongation factor 1 alpha 2	20q13.3	NM_001958.2|	Protein biosynthesis or translation [GO:0006412]	Yes	Yes
E2	RPL35	Homo sapiens ribosomal protein L35	9q34.1	NM_007209.3|		No	Yes
E8	eEF1A1	Homo sapiens eukaryotic translation elongation factor 1 alpha 1	6q14.1	NM_001402.5		Yes	Yes
G2	RPL15	Homo sapiens ribosomal protein L15	3p24.2	NM_002948.2|		No	Yes

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Up-regulated in breast cancer	Up-regulated in others cancers
G4 / G12	RPL7	Homo sapiens ribosomal protein L7	8q21.11	NM_000971.3|		No	Yes
G11	RPS25	Homo sapiens ribosomal protein S25	11q23.3	NM_001028.2|		No	No
H6 / H8	EIF2AK1	Homo sapiens eukaryotic translation initiation factor 2-alpha kinase 1	7p22	NM_001134335.1		No	Yes
H7	HSPE1	Homo sapiens heat shock 10kDa protein 1 (chaperonin 10)	2q33.1	NM_002157.2	Protein folding [GO:0051084]	No	Yes
F5	HSP90AA1	Homo sapiens heat shock protein 90kDa alpha (cytosolic), class A member 1	14q32.33	NM_005348.3|	Signal transduction [GO:0007165]	Yes	Yes
G9	LARP1	Homo sapiens La ribonucleoprotein domain family, member 1	5q33.2	NM_015315.3|	RNA binding [GO: 0003723]	No	No
A8	POLR2J	Homo sapiens polymerase (RNA) II (DNA directed) polypeptide J, 13.3kDa	7q22.1	NM_006234.4|	Transcription [GO:0006351]	No	Yes

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Up-regulated in breast cancer	Up-regulated in others cancers
D6 / D10	PSMD7	Homo sapiens proteasome (prosome, macropain) 26S subunit, non-ATPase, 7	16q22.3	NM_002811.4|		Yes	No
E4	PHB2	Homo sapiens prohibitin 2	12p13	NM_007273.3|		No	Yes
A6	RAB10	Homo sapiens RAB10, member RAS oncogene family	2p23.3	NM_016131.4|	Transport [GO:0006810]	No	Yes
E3	KPNA2	Homo sapiens karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	17q24.2	NM_002266.2|		Yes	Yes
F3	LAPTM4B	Homo sapiens lysosomal protein transmembrane 4 beta	8q22.1	NM_018407.4|		Yes	Yes
C11	TYW3	Homo sapiens t-RNA-yW synthesizing protein 3 homolog (S. cerevisiae)	1p31.1	NM_138467.2|	t-RNA processing [GO:0008033]	No	No
E7	RPSAP58	Homo sapiens ribosomal protein SA pseudogene 58 , non-coding RNA	19p12	NR_003662.2|	Unknown process	No	No

Table 5 – Identified down-regulated genes and its primary biological function

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Down-regulated in breast cancer	Down-regulated in others cancers
C2	WISP1	Homo sapiens WNT1-inducible-signaling pathway protein 1	8q24.22	NM_003882	Cell-cell adhesion [GO:0016337]	Yes	No
E9	CELSR2	Homo sapiens cadherin, EGF LAG seven-pass G-type receptor 2	1p21	NM_001408.2|		Yes	No
A3	GSN	Homo sapiens gelsolin	9q33	NM_000177.4	Cytoskeleton [GO:0005856]	Yes	Yes
E7	LYZ	Homo sapiens lysozyme	12q15	NM_000239.2	Defense response to bacterium [GO:0042742]	No	No
B2 / D7 / E10 / F2 / G4 / G8	LTF	Homo sapiens lactotransferrin	3p21.31	NM_001199149.1	Immune response [GO:0006959]	Yes	Yes
B5	OAS1	Homo sapiens 2'-5'-oligoadenylate synthase 1	12q24.2	NM_016816		No	Yes
E3 / H2	PIGR	Homo sapiens polymeric immunoglobulin receptor	1q31-q41	NM_002644.3		No	Yes

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Down-regulated in breast cancer	Down-regulated in others cancers
F8	BTN1A1	Homo sapiens butyrophilin, subfamily 1, member A1	6p22.1	NM_001732.2	Metabolic Process [GO:0008152]	Yes	No
A10	CEL	Homo sapiens carboxyl ester lípase	9q34.3	NM_001807.3		No	Yes
B1 / B10 / F11 / G7 / H5	LALBA	Homo sapiens lactalbumin, alpha-	12q13	NM_002289.2		Yes	No
C4	S100A2	Homo sapiens S100 calcium binding protein A2	1q21	NM_005978.3	Protein binding [GO:0005515]	Yes	Yes
H3	PTPRG	Homo sapiens protein tyrosine phosphatase receptor type G	3q21.14	NM_002841.3		Yes	Yes
A4 / D6 / H1	CSN1S1	Homo sapiens casein alpha s1	4q21.1	NM_001890.1	Transport [GO:0006810]	No	No
A12 / B3 / B8 / B9 / C3 / C5 / C6 / C9 / C10 / D3 / D4 / D8 / D11 / D12 / E1 / E4 / E6 / E11 / E12 / F1 / F5 / F6 / G1 / G9 / G11 / H9 / H10	CSN2	Homo sapiens casein beta	4q21.1	NM_001891.2		No	No

Clone number	Acronym	Gene name	Chromosome localization	Accession number in GeneBank	Biological Function	Down-regulated in breast cancer	Down-regulated in others cancers
A7 / D5	CSN3	Homo sapiens casein kappa	4q21.1	NM_005212.2		No	No
D1	SFRP1	Homo sapiens secreted frizzled-related protein 1	8p11.21	NM_003014.4	Wnt signaling pathway [GO:0016055]	Yes	Yes
A1	IGLL5	Homo sapiens immunoglobulin lambda-like polypeptide 5	22q11.22	NM_001178126.1	Unknown process	No	No

Our study confirmed that breast tumors exhibit a diverse gene expression profile and this can provide a significant increase in our understanding of the main mechanisms that might regulate cancer proliferation.

In this study, we identify some genes as overexpressed in MCF7 cells (Table 4) that were previously described as overexpressed in breast cancers, such as CD24 (Bircan *et al.,* 2006), TUBB2C (Hiser *et al.,* 2006), KRT8 (Kuang *et al.,* 1998), KRT18 (Kuang *et al.,* 1998), ACTG1 (Lacroix *et al.,* 2004), USP32 (Akhavantabasi *et al.,* 2010), PTGES3 (Simpson *et al.,* 2010), PGK1 (Zhang *et al.,* 2005), HSP90AA1 (Cheng *et al.,* 2012), H2AFZ (Svotelis *et al.,* 2010), EEF1A2 (Tomlinson *et al.,* 2005), EEF1A1 (Zhu *et al.,* 2003), PSMD7 (Thompson *et al.,* 2004), DNMT1 (Girault *et al.,* 2003), KPNA2 (Dahl *et al.,* 2006) and LAPTM4B (Fan *et al.,* 2012).

The CD24 is a membrane protein bound that is attached to cell membrane by a phosphatidylinositol anchor and play an important role in the cell-cell adhesion function (Bretz *et al.,* 2012). Bircan *et al.* demonstrated that the expression of CD24 in neoplastic breast tissues (DCIS and IDC) was significantly higher in the neoplastic tissue than in the benign one (Bircan *et al.,* 2006).

The TUBB2C is an important structural protein that participates in the formation of microtubules, which are essential for the separation and segregation of chromosomes during cell division and plays an important role in the cell cycle (Tommasi *et al.,* 2007).

KRT18 is expressed in single-layer epithelial cells of the human body and is localized in the cytoplasm and perinuclear region that is related to cell cycle function (Meng *et al.,* 2009).

DNMT1 is a large enzyme, comprising 1616 amino acids in human, that might regulate cell cycle events (Jurkowska *et al.,* 2011). The main function of DNMT1 is to mediate the establishment and maintenance of DNA methylation (Novakovic *et al.,* 2010), and is consistent with the hypothesis that it plays an important role in cancer (Szyf 2001).

ACTG1 and KRT8 are two proteins necessary to maintain cytoskeletal integrity and its function (Belyantseva *et al.,* 2009; Busch *et al.,* 2012) and they have already been discovered as up-regulated in breast cancer. With this data, we can question the utilization of ACTG1 as a housekeeping gene in some cancer studies.

USP32 is a membrane-bound ubiquitin protease and it is considered that give rise to a proto-oncogene – USP6 - that is highly overexpressed in breast cancers (Akhavantabasi *et al.,* 2010).

PTGES3 is a 23 kDa cytosolic protein that is also known as p23. It is conserved protein characterized as a component of unliganded progesterone receptor complexes. It also controls catalytic activity of certain kinases, regulates protein-DNA dynamics and is up-regulated in several cancers, like breast cancer (Simpson *et al.,* 2010).

Another gene related to metabolic process is PGK1. This is an ATP-generating glycolytic enzyme that forms part of the glycolytic pathway (Wang *et al.,* 2007). Solid tumor cells, like breast cancer cells, employ glycotic enzymes to generate ATP when their supply of oxygen is limited (Zieker *et al.,* 2010). Zhang *et al.* reported that this gene is overexpressed in breast cancer cells (Zhang *et al.,* 2005).

H2AFZ is a histone variant that has been implicated in inducing local changes in chromatin structure and subsequently the regulation of gene expression (Thakar *et al.,* 2009). It was demonstrated by Svotelis *et al.* that this gene is overexpressed in MCF7 cells and its overexpression promotes the cellular proliferation of breast cancer cells (Svotelis *et al.,* 2010).

The primary function of eEF1A2 protein is related with the positioning of the t-RNA on the ribosome, suggesting that may play an important function in protein biosynthesis (Scaggiante *et al.,* 2012). eEF1A1 has a crucial role in protein synthesis, since it is responsible for delivering aminoacylated t-RNAs to the ribosome (Soares *et al.,* 2009). It has been proved that the overexpression of eEF1A1 is correlated with increased metastatic propensity (Zhu *et al.,* 2003).

The protein encoded by the PSMD7 gene, S12, is a regulatory subunit of the proteasome. This protein is homologous to the mouse Mov34 protein. Mutations in Mov34 are lethal in the embryonic stage of development (Thompson *et al.,* 2002). S12 is overexpressed in breast tumor tissue. These observations suggest that S12 may be a key affector of misregulated proteasomal activity in breast cancer and this would lead to a new therapeutic target and diagnostic and prognostic tests (Thompson *et al.,* 2004).

HSP90AA1 is an abundant protein comprising 2% of the total cellular protein content under non stress conditions. It is essential for many signal transduction-regulating cellular proteins. It is also involved in various cellular processes such as cell proliferation, differentiation, and apoptosis (Wang *et al.,* 2009). This gene is overexpressed in breast tumors, and it is required for the stabilization of many proteins that are involved in the main pathways of cancer growth and survival (Cheng *et al.,* 2012).

KPNA2 is a member of the karyopherin family, which is part of the nuclear transport protein complex (Gluz *et al.,* 2008). Its overexpression in breast cancer was identified as a bad prognostic marker, meaning that is an important factor involved in tumorigenesis and progression of breast cancer (Dahl *et al.,* 2006).

LAPTM4B is a member of the mammalian 4-tetratransmembrane spanning protein superfamily, which contains seven exons and six introns, which is strongly expressed in some solid malignancies (Yang *et al.,* 2008). This gene was originally identified as overexpressed in

human hepatocellular carcinoma (HCC) (Shao *et al.,* 2003), but recently it was also demonstrated its up-regulation in breast cancer (Fan *et al.,* 2012).

Regarding to the genes that we identify as down-regulated in MCF7 cells (Table 5), some of them have already been described as underexpressed in breast cancers, such as WISP1 (Davies *et al.,* 2007), CELSR2 (Huang *et al.,* 2005), GSN (Winston *et al.,* 2001), LTF (Campbell *et al.,* 1992), BTN1A1 (La Merrill *et al.,* 2010), LALBA (Klein *et al.,* 2007), S100A2 (Lee *et al.,* 1992), PTPRG (Liu *et al.,* 2002) and SFRP1 (Klopocki *et al.,* 2004).

WISP1 is a member of a family of cysteine-rich proteins called CCN factors (Soon *et al.,* 2003) and it is down-regulated in breast cancer (Davies *et al.,* 2007).

CELSR2 plays a central role in cell-cell adhesion (Takeichi *et al.,* 2000). Huang et al. described that this gene is down-regulated in 7% of breast cancers (Huang *et al.,* 2005).

GSN is an actin regulatory protein which is unique among vertebrates and it is found as both an intrinsic cytoplasmic protein and as a secreted plasma protein (Kwiatkowski *et al.,* 1988).

LTF is an iron-binding glycoprotein that is abundant in exocrine secretions, including milk and the fluids of the digestive tract. Although LTF belongs to a family of transferrins, its biological function is not limited to the regulation of iron metabolism; it also plays multiple roles in host defense, and in immune and inflammatory reactions (Ando *et al.,* 2010). LTF was also been reported as a gene involved in breast cancer (Campbell *et al.,* 1992).

BTN1A1, which is a member of the Ig superfamily, is highly expressed in the lactating mammary gland. It is secreted into milk in association with lipid droplets and is involved in the regulation of milk fat globule secretion (Abeler-Dorner *et al.,* 2012). It is highly expressed in the secretory epithelium of the mammary gland during lactation (Ogg *et al.,* 2004). In La Merrill *et al.* (2010) was reported that BTN1A1 may be one potentially marker of metastasis.

LALBA is a small calcium-binding milk protein and play an important function in mammary secretory cells - it is one of the two components of lactose synthase, which catalyzes the final step in lactose biosynthesis in the lactating mammary gland (Permyakov and Berliner 2000). Moreover, it is involved in the development of breast cancer (Klein *et al.,* 2007).

S100A2 is a calcium-binding protein (Schafer and Heizmann 1996) that is underexpressed in breast cancer (Lee *et al.,* 1992).

PTPRG play a critical role in regulating cellular functions by selectively dephosphorylating their substrates. Dysregulation of PTPRG is associated with a multitude of diseases, and many members of the PTP family have been recognized as potential therapeutic targets (Barr *et al.,* 2009).

SFRP1 was shown to function as receptor for Wnt signaling molecules (Finch *et al.,* 1997) and it was also been shown that this gene has a important function in breast cancer. Klopocki *et al.* showed that SFRP1 is strongly down-regulated in breast tumors (Klopocki *et al.,* 2004).

Our results show that some genes have not yet been described as overexpressed in breast tumors (Table 4). However, they have been described as overexpressed in other types of cancer, suggesting that these genes may also play an important role in breast carcinogenesis. Some of these genes are important in the regulation of cell cycle (NPM1 and H3F3B), in mRNA processing function (SNRPD1 and RPL3), in protein folding (HSPE1), in the translation process (RPL35, RPL15, RPL7 and EIF2AK1), in transcription (POLR2J) and in transport function (RAB10).

The NPM1 is a nucleolar phosphoprotein that is involved in regulation of cell cycle. However, this protein has diverse cellular functions including the processing of ribosomal RNA, centrosome duplication and the control of cellular processes to ensure genomic stability (Lim and Wang 2006). There are studies that reveal that this protein is overexpressed in many types of human solid tumors, such as colon, liver, stomach, ovary and prostate tumors (Lim and Wang 2006). Recent studies point to the overexpression of NPM1 in acute myeloid leukemia (Becker *et al.,* 2010).

H3F3B is a universal histone predominantly incorporated into transcription sites and associated with active and open chromatin (Wong *et al.,* 2010). It is expressed throughout the cell cycle and it is incorporated at all phases of the cell cycle (Drane *et al.,* 2010). It has been reported as overexpressed in gastric (Yasui *et al.,* 2004) and ovarian cancer (Presneau *et al.,* 2005).

The SNRPD1 is a essential component of the nuclear pre-mRNA processing machinery because catalyzes the removal of noncoding sequences from pre-mRNA (Chari *et al.,* 2008). Previously, it was identified as up-regulated in pancreatic tumors (Thakur *et al.,* 2008).

The RPL3 gene encodes a ribosomal protein. It has been demonstrated that RPL3 is associated with alternative pre-mRNA splicing, meaning that has an important role in the mRNA processing (Russo *et al.,* 2011). This gene is also associated with pancreatic cancer (Alldinger *et al.,* 2005).

HSPE1, known also as HSP10, has been proven to be an essential component of the protein folding machinery (Czarnecka *et al.,* 2006). This gene has been associated with several cancers, such as prostate cancer (Cappello *et al.,* 2003).

Other ribosomal proteins, such as RPL7, RPL15 and RPL35, have been also described as up-regulated in several cancers. Several studies point to the main function of these genes in protein biosynthesis (Ko *et al.,* 2006; Chen *et al.,* 2008; Nair and Choi 2011). RPL7 is highly

expressed in colorectal cancer cells (Lai and Xu 2007). RPL15 is overexpressed in gastric cancer tissues, and might play a role in cell proliferation of gastric cancer (Wang *et al.,* 2006). RPL35 may has a role in cervical carcinoma (Huang *et al.,* 2011).

Another gene identified as involved in protein biosynthesis is EIF2AK1(de Haro *et al.,* 1996). Studies demonstrate that this gene is up-regulated in esophageal squamous cell carcinoma (Ling *et al.,* 2010).

POLR2J and PHB2 were identified as up-regulated in some cancers and play an important role in transcription regulation. The first one encodes a subunit of RNA polymerase II, and it is responsible for the synthesis of mRNA (Proshkin *et al.,* 2011). The POLR2J is associated with lung cancer (Campbell *et al.,* 2008). The PHB2 is a highly conserved, and it was identified as a repressor of estrogen activity (Lee *et al.,* 2008). This gene is also up-regulated in some ovarian cancer cell lines (Parker *et al.,* 2009).

At last, the RAB10 belongs to a family of Rab proteins, which are associated to the transport function (Babbey *et al.,* 2006). So, RAB10 is considered as a component of the transport machinery (Schuck *et al.,* 2007) and it is overexpressed in HCC cancer cells (He *et al.,* 2002).

We have identified two other genes, PIGR and CEL, which were previously described as underexpressed in others cancer, but not in breast cancer (Table 5).

PIGR is a transporter of IgA and IgM, which are the first-line antibodies in response to initial infection. It is widely expressed in epithelial cells, and its expression increase in response to pro-inflammatory cytokines triggered by viral or bacterial infections (Ai *et al.,* 2011). The underexpression of this protein has been reported in HCC (Sphyris and Mani 2011) and in lung cancer (Khattar *et al.,* 2005).

CEL is synthesized and secreted by the pancreas. It is also produced by the mammary gland and the liver. The major role of CEL was thought to be the hydrolysis of cholesterol ester, triglycerides and lysophospholipids (Li *et al.,* 2008). It has been found that this gene is down-regulated in intestinal carcinomas (Fijneman *et al.,* 2008).

Our results show for the first time the expression several genes as up-regulated in MCF7 cell line (Table 4), such as MTRNR2L 2, MTRNR2L 8, TMPRSS13, RPS25, LARP1, TYW3 and RPSAP58.

MTRNR2L 2 and MTRNR2L 8 are two recent discovered genes from the family of the humanins (HNs) (Bodzioch *et al.,* 2009). They can act as an anti-apoptotic agent and a neuroprotective factor against Alzheimer's disease. It has been discovered that HNs are an important regulator of male germ cell apoptosis, because of its anti-apoptotic activity (Zapala *et al.,* 2010). Wang *et al.* (2005) reported that the overexpression of HNs decrease the number of cells arrested at the G2/M phase of cell cycle and block them from apoptosis.

TMPRSS13 is a splice variant of mosaic serine protease large form (MSPL), and belongs to the hepsin /TMPRSS subfamily of the type II transmembrane serine protease family. A possible physiological function of TMPRSS13 is as a hepatocyte growth factor (HGF)-converting protease. The activated HGF is involved in proliferation of the epithelial cell. MSPL and TMPRSS13 are expressed in a variety of tissues, and predominantly in lung, placenta, pancreas and prostate. Therefore, MSPL and TMPRSS13 may have physiological functions in these tissues that remain to be explored (Hashimoto *et al.,* 2010).

RPS25 plays a key role in the translation process. This gene encodes a ribosomal protein that is a component of the 40S subunit of the ribosome (Landry *et al.,* 2009; Muhs *et al.,* 2011).

LARP1 is a RNA-binding protein, which the major role is to protect the 3' end of small RNAs from exonuclease digestion (Wolin and Cedervall 2002) and this protein has show to be required for spermatogenesis, embryogenesis and cell cycle progression (Burrows *et al.,* 2010).

TYW3 is a gene that is important to the t-RNA processing. It acts as a component of the wybutosine (yW) biosynthesis pathway. yW is a tricyclic nucleoside with a large side chain found at the 3'-position adjacent to the anticodon of eukaryotic phenylalanine t-RNA. yW supports codon recognition by stabilizing codon–anticodon interactions during decoding on the ribosome (Noma *et al.,* 2006).

Finally, we have identified five novel genes, CSN1S1 (transport), CSN2 (transport), CSN3 (transport), OAS1 (immune response), and IGLL5, as underexpressed in MCF7 cell line (Table 5).

The caseins are the major milk proteins constituting about 40 % of the total protein content in mature human milk (Johnsen *et al.,* 1995). CSN1S1, CSN2 and CSN3 are phosphoproteins complexes that are part of suspended particles or micelles of milk, which bind necessary minerals, such calcium phosphate (Sood *et al.,* 2003), such as CSN1S1. CSN2 is the major protein of the human milk casein fraction (80%) and exists in six calcium-sensitive forms. CSN3 is known for its role in preventing the precipitation of *β*-casein in the presence of Ca^{+2} and covering the micelle surface and thereby stabilizing the micellar structure (Sood *et al.,* 2002).

OAS1 plays an important role in the mechanisms of action of interferon antiviral activity, but is also involved in other cellular processes such as apoptosis and growth control (Rebouillat and Hovanessian 1999; Justesen *et al.,* 2000). Maia *et al.* (2008) had demonstrated that OAS1g, the most abundant gene expressed in rat mammary gland and prostate, is up-regulated by 17β-estradiol in rat mammary gland, but is down-regulated in prostate (Maia *et al.,* 2008).

Chapter IV

Conclusion and Future Perspectives

In this study, several genes were identified for the first time as up-regulated genes in MCF7 cells, such as MTRNR2L 2, MTRNR2L 8, TMPRSS13, RPS25, LARP1, TYW3 and RPSAP58 or down-regulated, such as CSN1S1, CSN2, CSN3, OAS1 and IGLL5. In order to validate these genes as differentially expressed, Real Time PCR will be carried out using the samples used to construct our subtractive libraries. In addition, the study of gene expression should be extended to samples derived from patients with breast cancer.

Studies regarding function and expression of these novel genes identified as differentially expressed in breast cancer cells may reveal new pathways for therapeutic intervention. Moreover, it is possible that some of them may be used as biomarkers, diagnostic markers or prognostic indicators.

Chapter V

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Appendix

Sequences obtained from the clones with insert

Forward:

Clone A1
TCGAGCGGCCGCCCGGGCAGGTACAGCTGGAAACGCTGAGTATTCGAGGAAATAACATTCGGTATTTTATTCTACCAGACAGTTTA
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GAAGAGGAAGAGGACGTGGCCGTGGCAGAGGAAGAGGGGGTCCTAGGCGATAATGTCTCTCAAGATTTCAAAGTCATATGAGATT
TGGGATATTTTTTGTACCTCGGCCGCGACCACGCT

Clone A2
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Clone A4
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CCTGCCCGGGCGG

Clone A6
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Clone A8
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Clone A9
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Clone A11
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AACCWK

Clone A12
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Clone B1
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Clone B4
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CGACCACGCT
Clone B6
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Clone B8
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Clone C5
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Clone C6
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ATCCAAATGATTCCAAGCATAAAAGAACGGACAGATCAATTTTATGTTGTTKACGAAAAGGAGAATCTGGCCAGTCATGGCCAAGGT
TAACAAAAGAAAGGGCAAAGCTTAATTGGCTTAGTGTCGACTTCAATAATTGGAAAGACTGGGAAGATGATTCAGATGAAGACATGT
CTAATTTTAATCGTTTCTCTGAGATGATGAACAACATGGGTGGTGATGAGGATGTAGATTTACCAGAAGTAGATGGAGCAGATGAT
GATTCACAAGACAGTGATGATGAAAAAATGCCAGATCTGGAGTAAGGAATATTGTCATCACCTGGATTTTGAGAAAGAAAAATAACT
TCTCTGCAAGATTTCATAATTGAGAGAATTCCTGAGTTGATAGCTCTAAAGGCAGATATGCTGTATTTACCTACTTTAACCCATTTTT
CAACCTGTTTGTTTTTTAAAAGGCTTCACTAAGGGTTGATATGTACCTG

Clone C7
GCAAACGTCGCATGCACCCGGCCGCCATGGCTGGCCGCGGGAATTCGATKTCGAGCGGCCGCCCGGGCAGGTCGCCAGAACACAG
GTGTCGTGAAAACTACCCCTAAAAGCCAAAATGGGAAAGGAAAAGACTCATATCAACATTGTCGTCATTGGACACGTAGATTCGGGC
AAGTCCACCACTACTGGCCATCTGATCTATAAATGCGGTGGCATCGACAAAAGAACCATTGAAAAATTTGAGAAGGAGGCTGCTGAG
ATGGGAAAGGGCTCCTTCAAGTATGCCTGGGTCTTGGATAAACTGAAAGCTGAGCGTGAACGTGGTATCACCATTGATATCTCCTT
GTGGAAATTTGAGACCAGCAAGTACCTCGGCCGCGTCCACGCTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGG
GAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTC
CTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGA
GCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAAC
GCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCG
AGCGGTATAGCTACTCAAAGGCGGTAATCGGGTATTCACGAATAGGGGATACGCAGGAAGAACTTGTGAGCAAAGGCAGCAAAGGC
AAGAACCGTAAAGGCCGTTGCTGCGTTTTCAATGGCACGCACACGGACGAGCTTCAATAACTACCCTAGCTAAGGGTCAAA

Clone C8
TCGAGCGGCCGCCCGGGCAGGTRCGCRWGRTGRGRCAWCRMMKYWGTYMYSAGTTCAASMAGRRTCSTGSWKRRMSCCYCWYAGT
GCACWSAGAAGGGGTTAGGAGCCTCGCRASWYYCGASSYGCGAWARSGGSMYSMMSAGAASGAAGGRWSACCACGTATTCCMWSAA
SGSGCWSTMWMAWTAATAGAAAGATGTAKGGCACGTACTCCCTCKCGAGTAAGCCATCGACAAGGGGTCACCCCATGCAGTGCCTC
CGGCGGAACMCCCACTGCCTTGATAGTGCTAGCCTTAGGTTCGCAAWTGTGCAGCCAGAGCMSGCCCCGTCGTCCACGCKKCGGA
ACCACTAGTGAATCGAGGGGCAAAKACRCTTTCCGCCCCTAGAACGGGGCTGCTATAGGGGGACTAGCCAAGTTAAAGCCCCTTTC
GGAAGGGCTCAGTCCTGCATCACTTACGGGTCGTTAACATTCCCATCATAAAAGTTTAGGACATTTTTGCGGGCCCGTTGACACTGC
CCACTTGGGGCCTACCGAGTTTACGGCCAACGGCCTATTTTAACTTCGGTGGGGGTAAGGTTAAGGACCATAACTTCCTCGGATTA
TCCCAGTTCCCGCCTTGGTCCGGTGGCAAATTCGGACCAAATTTTAACCGCCCCCATCCTAAGGCCAAAGAATTCAAATTTTTAACC
CTAGAAATAGACCGCCTTGGGGTAACAAACGCCCTATTAAAAAAAATTGATTGGCTTTAATGGGAGGCCCGCCTTAGGAGGTGGCG
GAGTTTGGGCGCCTTTCAAAAATTTATGGGAACAGTTTGGGTTAAAGGTCCCCCTCAATAAGGCCTTTGGCCTTAAGCCAAAAATTT
AATTTAAATAA

Clone C10
TAAGATTCGGCGCCGACGTCGCATGCTACCCGGCCGCCATGGCTGGCCGCGGGAATKCGATKATCACTAGTGAATKCGCGGCCGCC
TGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAA
TCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCC
TGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCT
GCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCT
CGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAA
GAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTG
ACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGC
TCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCCTTCTCAT
AGCTCACGCTGTAGGTATCTCA

Clone C11
TCGAGCGGCCGCCCGGGCAGGTACTCCGGACAGCCAACATAGTTTTTCCTCTCTTTCCCACCGTTATGCCARAGTTCCTGAAACCAG
AATCTATTGCCATGGAATGCAGAATCTGTGCATCCTGCAATTGTCGACACTGCACATGAAGAACAAATGGTTCAAATTTCAAAGTGG
CATCACCATTTGCTTTCTTCAGAGCTACAATCACATMATCTTTTACACAAAGTTTGTGTGTAACCAGTAGCCAGCAACAGTTTTGTTT
CTGAACCTCAAAACCATTTATACCCCGGTCAAGGAGTAGGATGCGGCCAGCGCAGGAGCTGGTGGTGAAAAACTGATCTCGCATGT
TCAGAAACTGCACAAGCTCTACCACATCCTCGTCAACACTGCCCTTCCGGCTGAGGTCCGCTTTGCTCAAACATTGCGCCTTCCATT
TCCTGAACTCCGCGCTGCGATCCATGGGTGACGGACTCAGGGTCTGCGCTCGGACCTCGGCCGCGACCACGCT

Clone C12
AGCGTGGTCGCGGCCGAGGTACTGTGCATACCCGGAAGAAAGACCTATATGATGCGGTTTGGATTCAAGTATCCCGGTTAGCGAGC
CCACTCCCACCTCAGGAAGCTAGTAATCATGCCCAGGATTGTGACGACAGTATGGGCTATCAATATCCATTCACTCTACGAGTTGTG
CAGAAAGATGGGAACTCCTGTGCTTGGTGCCCATGGTATAGATTTTGCAGAGGCTGTAAAATTGATTGTGGGGAAGACAGAGCTTT
CATTGGAAATGCCTATATCGCTGTGGATKGGGATCCCACAGCCCTTCACCTTCGCTATCAAACATCCCAGGAAAGGGTTGTAGATGA
GCATGAGAGTGTGGAGCAGAGTCGGCGAGCGCAAGCCGAGCCCATCAACCTGGACAGCTGTCTCCGTGCTTTCACCAGTGAGGAA
GAGCTAGGGGAAAATGAGATGTACCTGCCCGGGGCGG

Clone D1
AGCGTGGTCGCGGCCGAGGTCAAGAGCGAAACCCCATCTCAAAACCACAACAACAACAACAGGACAACAGAGATGAACGACGGATC
GGGAAAGCCAACCAGACAGCGTGAGGCCAGGACGGAAAGAGGCACAGGGAGCTCTGCTCAGTGTCGCTACAGGGGATCTCTCAGG
CTCACAACGGGCCACTCCTCTAGGGAAGTTCTGGCCTCATCATGATCCTTGTTTGGTCTCACTCCCCATGTCCTTCTCTGTCCCTCC
TCCAACTGCCATTTATTTATTTAACTGAAAAAGTACCTGCCCGGGCGG

Clone D2
CCGGGCCTCGTAAGACTTTCGACCGCACACACGTGCCCATTGCACCCGTGCACGCCATGGTGTYGGCCGCGCSGAATTCGATAWTC
SACGTCAGGTCSRMTCCSGSGCRSGKMCCAGACTAAGCGYACGCACAYATTATGGTGAGAAGCATCTCTCGCRAASGASGYAGGGCA
GTCGACAGKGAGCCTTTGGGTACGATGATTGSGAAYCRAGTGTTGCGYSGGWACGAKAATSCWGGGCCGATGTAGTCMTCGAGAT
GCCATGACGGCTTGGTTTCCCCATCGKGGTCRWWRYTCRTTCMTTYMSSTGCGATCGAACTTTTCTCCACARTCCACACCTATGAAC
ASCACGACASGSTAATTGGYATARACCASTTGKAGACATGCCCAKGCGAGTGTTGCGCTTCAAMTCGAWGTYSAARCCYAARCATACA
TCTAGTGGCAAMWTAKAYGGGYCTTRCCKSGTTGATCCTGTGCGCGCTGTCGATTATGCCCCCAGATTCTGTGTGCATATAACACTT
GGTTACAGGTTTGCCCCTAGAACCCTTTCCACTTTTAAAAACGAAAGTTTTGCGCTCCAATCAGTCCTCCTGTGCGTAGGAGTGCTG
AGTGTGGTGCGCTACTAGGTGGGCAGACTTCCTATCTCTGTCGTTTTCACATCCAGCCCCGGCAACCATAGTATCATGCTGTCGGT
TGTCTGCCATCCAGCGCTCCAGGTTTGCTGGGTCATAGCCGCGACGGAAAGCGGGCTATTGCCATGCCTCGAGCGTTCATAGACGC
GCAGTGTCATATGAGCAAAGAAATTACTCCACGACCTATGCCAAAATACTATGCGAGCGGCAAATACCCACCCGCCCAAGGCCGAAA
GAACAAAGAACAAACAATTTCGGATGGGGGAAGCCCAAGACCAAACCAGGGCTCCCAAAGCCACATAGAAGGAAATTGTTCGCACAT
CGCTTATATCAATAGGTCCTGTTGATTAAAATGGTCCTCTTGTTGCTCCCTAGGGGCGCCACTGCCACCTCGCACTTAGGATCCGAC
GACTTAATGCTAACCACCATACAGGAGTCCCCGGACCCGAAACCCTCCAACCAG

Clone D3
AATGCGCCAGACGTCGCATGCACCCGGCCGCCATGGCGGCCGCGGGAATKCGATKATCACKAGTGAATKCGCGGCCGCCTGCAGGT
CGACCATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGT
CATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTG
CCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAAT
GAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGT
TCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGT
GAGCAAAAGGCCAGCAAAAGGCCAGGAAACCGTAAAAAAGGGCCCGCCGTTTGGGCTGGGGCCGTTTTTTTTTCCCCCATT

Clone D6
AKCGTGGTCGCGGCCGAGGTACATTKCAGKGGAAGAAGTCCATGATGATGGAACTCCAWCCTCGTAMAWCATTTGAACACGTGAC
CAGTGAAATTGGAKCASAGGAAGCTGAGGAAGTYGGAGTTGAACACTTGTTWCGAGATATCAWAGACACGACGGTGGGCACTCTG
TCCCAGCGGATCACAWACCAKGTCCATGGTKTGAWGGGACTGAACTCCAMGCTTCTGGATATCWGGAGCTACCYGGAAAAAGTCG
CCACAGGCAAGCTGCCCATCAACCACCAGATCATCTACCAGCTGCRGGACGTCTCCRRSMTGCKGCCAGATGTCAKCCTGCASGAGT
YCGTCAAGGCCTTYTACCWGAAGACCAATGACCAGATGGTGGTAGTGTACCTGCCCGTGGCC

Clone D7
AGCGTGGTCGCGGCCGAGGTACAGTCCAGTCCTTGGAGATCGACCTGGACTCCATGAGAAATCTGAAGGCCAGCTTGGAGAACAG
CCTGAGGGAGGTGGAGGCCCGCTACGCCCTACAGATGGAGCAGCTCAACGGGATCCTGCTGCACCTTGAGTCAGAGCTGGCACAG
ACCCGGGCAGAGGGACAGCGCCAGGCCCAGGAGTATGAGGCCCTGCTGAACATCAAGGTCAAGCTGGAGGCTGAGATCGCCACCT
ACCGCCGCCTGCTGGAAGATGGCGAGGACTTTAATCTTGGTGATGCCTTGGACAGCAGCAACTCCATGCAAACCATCCAAAAGACC
ACCACCCGCCGGATAGTGGATGGCAAAGTGGTGTCTGAGACCAATGACACCAAAGTTCTGAGGCATTAAGCCAGCAGAAGCAGGGT
ACCTGCCCGGGGG

Clone D8
GGCTCGCCGCGCCGCTCGCTCGGTGGCGCAAGCAAACCAACAAAGACCTGMCTATGGCGGMCGCGGGTTTAMGTATAAGCGTGGT
CGCGGCMGGGAGGTCTTCWGCCGCTCTCTKAGTWGTCMTGGGGTGMCGCAACAGGCACAAAGCTCTCCTCCRGTCCTGGTWTTC
TTTATTTCTTCCCTTCCTTCTCCTTGGTGTATTTGTCCTGTGAGTGTCTGACTCTATCACTTTCAAAGCTGTGCTGTGATTTGGGTC
TTTAGATGAGGCTTCATGCCCTGGCWTAAGCAAAGAGCCTGATACAGAGTGGCCTGCAGACCTGCCCGGGCGG

Clone D9
CGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGA
GAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCT
GTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAG
CTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACG
CGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGA
GCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCA
GCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCC

Clone D10
AGCGTGGTCGCGGCCGAGGTACATTTCAGTGGAAGAAGTCCATGATGATGGAACTCCAACCTCGAAAACATTTGAACACGTGACCA
GTGAAATTGGAGCAGAGGAAGCTGAGGAAGTTGGAGTTGAACACTTGTTACGAGATATCAAAGACACGACGGTGGGCACTCTGTCC
CAGCGGATCACAAACCAGGTCCATGGTTTGAAGGGACTGAACTCCAAGCTTCTGGATATCAGGAGCTACCTGGAAAAAGTCGCCAC
AGGCAAGCTGCCCATCAACCACCAGATCATCTACCAGCTGCAGGACGTCTTCAACCTGCTGCCAGATGTCAGCCTGCAGGAGTTCGT
CAAGGCCTTTTACCTGAAGACCAATGACCAGATGGTGGTAGTGTACCTGCCCGGGCGG

Clone E1
YGATTAGCGTGGTCGCGGCCGAGGTACATGGTGTTTGTGGCCTTGGCTGACATGAAGCTGTTGTGTGAGGTTCGCTTATCAACTAA
TGATTTAGTGATCAAATTGTGCAGTACCTGCCCGGACCTGCCCGGGCG

Clone E2
TCGAGCGGCCGCCCGGGCAGGTGGCGGCTTGTGCAGCAATGGCCAAGATCAAGGCTCGAGATCTTCGCGGGAAGAAGAAGGAGGA
GCTGCTGAAACAGCTGGACGACCTGAAGGTGGAGCTGTCCCAGCTGCGCGTCGCCAAAGTGACAGGCGGTGCGGCCTCCAAGCTC
TCTAAGATCCGAGTCGTCCGGAAATCCATTGCCCGTGTTCTCACAGTTATTAACCAGACTCAGAAAGAAAACCTCAGGAAATTCTAC
AAGGGCAAGAAGTACCTCGGCCGCGACCACGCTAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATAT

Clone E3
AGCGTGGTCGCGGCCGAGGTACCAGTGACAATATTCCCTATGGCTCTKAGGGCAGGAGTCACAATTGGCAATTCAGAAGCTCCTAG
AAGCTTCACAAGTWGGGGCACAACTCCTGTTTTCACCACCATGCCAATTCGTTCATTTGGACCATCAGTAAGGTAGGAAATAGCCCA
GCAKGTATCTGCTAACACTTCTGGATCATCATGATGCAKGAGCCGAACTAAGGTAGGAAGAATCTGCTCAACAGCATCKATCGGGG
GTGCAGGATTCTTGTTGCGGCAAAKATTAGAAAGTGTCCAGGTAAGATTACGTAAGTAGCCACATGCTAAAGATGACATATCAGGAA
CTGCAAGGAGAGCCAACAGTGGGTCAACTGCACCGTACCTGCCCGGGCG

Clone E4
AGCGTGGTCGCGGCCGAGGTACCCCATTATCTATGACATTCGGGCCAGACCTCGAAAAATCTCCTCCCCTACAGGCTCCAAAGACCT
ACAGATGGTGAATATCTCCCTGCGAGTGTTGTCTCGACCCAATGCTCAGGAGCTTCCTAGCATGTACCTGCCCGGGCG

Clone E6
TCGAGCGGCCGCCCGGGCAGGTACCASAGCTKRAWGAAYAKGATTSCACCYAGGCAMYCACATCAACAASYCCATGACTGGGCGGT
CAKSRKSTGAMATCRATGAAGAWCCWSTCWRYAAMKCMAWACGAGGARCTCTGCGGAGGTSGAAAAASMASGGCWCCGGKGAAAS
GGGCCTWTTGATTYCCAARACSTGSGCGGTTCTKCGAGGGATGSGTAYACATGAGTAAGTGTAACATACGTAGCTCCACCTACTCTC
GGGGAATATCATAAAGCAATAATSYWYTCTTAGTACATSWCAMMACCAKATSTCGTRCRWRAKCCYKGYTWYAGATRYTTACATWGT
KWWWGGGGAAKCCRMKATCRAWGATWYATCCCAKCMARCMCCAAGCATASCWRCWGYTKKAGAAAMTTTSAAGAGGTGTCCAWA
GGGTGAAATGCATGCTTCTCCAAAAACATTTTCAATGAACAACACACCAAGCACCTCCAACTGTACCTCGGCCGGCAACCACGCTAA
TCACTAGTGATATACTGCA

Clone E7
TCGAGCGGCCGCCCGGGCAGGTGTCCATACGGCGTTGTTCTGGATTCCCGTCGTAACTTAAAGGGAAACTTTCACAATGTCCGGAG
CCCTTGATGTCCTGCAAATGAAGGAGGAGGATGTCCTTAAGTTCCTTGCAGCAGGAACCCACTTAGGTGGCACCAATCTTGACTTCC
AGATGGAACAGCACCTCGGCCGCGACCACGCT

Clone E8
GCGATAAGCGTTGGTCGCGGCCGAGGTACTGGGAGAAGCTCTCCACACACATGGGCTTTCCCGGCACCATCTCCACGAATGGCCGC
GTCTCCAGACTTCAGGGACTTGGGGTTGTCCTCCAGCTTCTTGCCAGAGCGCCGGTCAATCTTCTCCTTCAGCTCCGCAAACTTGCA
GGCGATGTGGGCTGTGTGGCAGTCGATGACCGGGGAGTAGCCGGCGCTAATCTGCCCCGGGTGGTTCAGGATGATGACCTGGGA
GGTGAACTGAGCAGCCTCCTGCGGCGGGTCAGACTTGCTGTCCCCACACACGTG

Clone E9
TCGAGCGGCCGCCCGGGCAGGTCCCAGGCCATCATCTCCCCACCTCTCCTCCCCTCTCCTCAGTTTTGCCGACTGCTTTTCATCTGA
GTCACCATTTACTCCAAGCATGTATTCCAGACTTGTCACTGACTTTCCTTCTGGAGCAGGTGGCTAGAAAAAGAGGCTGTGGGCAG
GAAAGAAAGGCTCCTGTTTCTCATTTGTGAGGCCAGCCTCTGGCTTTTCTGCCGTGGATTCTCCCCCTGTCTTCTCCCCTCAGCAAT
TCCTGCAAAGGGTTAAAAATTTAACTGGTTTTTACTACTGATGACTTGATTTAAAAAAAATACAAAGATGCTGGATGCTAACTTGATA
CTAACCATCAGATTGTACCTCGGCCGCGACCACGCT

Clone E10
TCGAGCGGCCGCCCGGGCAGGTGCATCTTCTTTTGCGTCGCCAGCCGAGCCACATCGCTGAGACACCATGGGGAAGGTGAAGGTC
GGAGTCAACGGATTTGGTCGTATTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCTGGTAAAGTGGATATTGTTGCCATCAATGA
CCCCTTCATTGACCTCAACTACATGGTTTACATGTTCCAATATGATTCCACCCACGGCAAATTCCATGGCACCGTCAAGGCTGAGAA
CGGGAAGCTTGTCATCAATGGAAATCCCATCACCATCTTCCAGGAGCGAGATCCCTCCAAAATCAAGTGGGGCGATGCTGGCGCTG
AGTACCTCGGCCGCGACCACGCT

Clone E11
AGCAGTGGKCGCGGCCGASGTRYMYARCAAAYCCWCAGGTYCTAAAGCGTWSMWMACCTGCATTAAATAMTTYMRCGTTGGTGGC
GYACCATCKAGAGCMSAACCCMACACTCCGGAGCAKTAYCTGACGCAYGKGCGSWCSMKCTCWGCWMASYWTYSATGTAACGCKTY
GGTYSCAKRYTGGAGCGATAGTAMCTACGCGGCCGCGAAYCAYKCCKAAWTCATYTTAASTTGGRATTTMTKCGGGKCBGTCCGTKG
CCAGGGGTCCGAACCCATTATTGGGGGAAKAAGCCTCCCCCRAACGGYGTTCTGGGAATGCATTAAKCTTTKAAGWTATWYKATAS
GTGTCRCCTRRHT

Clone E12
TCGAGCGGCCGYCCGGGCAGGTACCCTGCTTCTGCTGGCTTAATGCCTCAGAACTTTGGTGTCATTGGTCTCAGACACCWYTTTGC
CATCCACTATCYGGCKGGTGGTGGTCTTTTGGATGGTTTGCATGGAGTTGCTGCTGTCCAAGGCATCACCAAGATTARAGTCCTCG
CCATCTTCCAGCAGGCGGCGGTAGGTGGCGATCTCAKCCTCCAGCTTGACCTTGATGTKCAGCAGGGCCTCATACTCCTGGGCCTG
GCGCTGTCCCTCTGCCCGGGTCTGTGCCAGCTCTGACTCAAGGTGCAGCAKGATCCCGTTGAGCTGCTCCATCTGTAGGGCGTAGC
GGGCCTCCACCTCCCTCAGGCTGTTCTCCAAGCTGGCCTTCWGATTTCTCATGGAGTCCAGGTCGATYTCCAAGGACTGGACTGTA
CCTCGGCCGYGACCACGCT

Clone F3
AGCGTGGTCGCGGCCGAGGTACAAGCAACGCGCAGCCTGGATCATCCCACTCTTCTGTTACCAGATCTTTGACTTTGCCCTGAACA
TGTTGGTTGCAATCACTGTGCTTATTTATCCAAACTCCATTCAGGAATACATACGGCAACTGCCTCCTAATTTTCCCTACAGAGATGA
TGTCATGTCAGTGAATCCTACCTGTTTGGTCCTTATTATTCTTCTGTTTATTAGCATTATCTTGACTTTTAAGGGTTACTTGATTAGC
TGTGTTTGGAACTGCTACCGATACATCAATGGTAGGAACTCCTCTGATGTCCTGGTTTATGTTACCAGCAATGACACTACGGTGCTG

CTACCCCCGTATGATGATGCCACTGTGAATGGTGCTGCCAAGGAGCCACCGCCACCTTACGTGTCTGCCTAAGCCTTCAAGTGGGC
GGAGCTGAGGGCAGCAGCTTGACTTTGCAGACATCTGAGCAATAGTTCTGTTATT

Clone F4
CGGCCACTCATCACTACTCCCACTATCAACTCCGCAATTTTTCGCGCAGACGCCGCRCCACCACCTAGCCATTCCGSCTTTACGCGGA
CCCATTTCCGAAACAATAACATCACCCGTCCAGGAACTCTAYCAGKGACTATCYAATTTCCGCTCGCTAYCGCAGSGACCAACCCGCA
ACTCGACACCGCTCCGCTCAAACAT

Clone F5
TCGAGCGGCCGCCCGGCCAGGTACATGCAGAATTGTCAACTACAGGGAATGAAAAGTTCAAAAAGTAGATCCTACAAGATGTAACG
AATACTTTTCTAAACATCAAGATACAGCTCAGAACACTTCAATAACAAGATTTGGTCTACTTAGGCATCCGGCTTGACAGCTAAACAC
TTTAGACCACAAAGTTAACATCATGTTACATACGTCTTACAGTGCACGTTACCCCAATCTGTGAAAATAAACCAACATGAAACTCAAA
AAGCATTACTAGCTCTGCTTTAGTGCCTAAGGTATCACAGCATCACTTAGTAGACAGAAATCTTATCTTCCCCTTAAAGTAGTTGTCA
TGCCATACAGACTTTTTAATATTAACAAAAATAAAGAAAAACATCCTTGAAAATATATTATCAGAGGAATTGTAGAGTACCTCGGCCG
CGACCACGCT

Clone F6
TCGAGCGGCCGCCCGGGCAGGTACATGACAAGGTGCGGCTCCCTAGGCCCCTCCCCTCTTCAAGGGGTCTACATGGCAACTGTGA
GGAGGGGAGATTCAGTGTGGTGGGGGACTGAGTGTGGCAGGGACTCCCCAGCAGTGAGGGTCTCTCTCTTCCTCTTGTGCTCTTG
CTGGGGCTGGTGGTCCAGGGGTCTTACTCCTTGGAGGCCATGTGGGCCATGAGGTCCACCACCCTGTTGCTGTAGCCAAATTCGTT
GTCATACCAGGAAATGAGCTTGACAAAGTGGTCGTTGAGGGCAATGCCAGCCCCAGCGTCAAAGGTGGAGGAGTGGGTGTCGCTG
TTGAAGTCAGAGGAGACCACCTGGTGCTCAGTGTAGCCCAGGATGCCCTTGAGGGGGCCCTCCGACGCCTGCTTCACCACCTTCTT
GATGTCATCATATTTGGCAGGTTTTTCTAGACGGCAGGTCAGGTCCACCACTGACACGTTGGCAGTGGGGACACGGAAGGCCATGC
CAGTGAGCTTCCCGTTCAGCTCAGGGATGACCTTGCCCACAGCCTTGGCAGCGCCAGTAGAGGCAGGGATGATGTTCTGGAGAGC
CCGCGGCCATCACGCCACAGTTTCCGGAGGGGCCATCCACAGTCTTCTGGGGTGGCAGTGATGGCATTGGACTGTGGTCATGAGT
TCCTCCACGATACCAAAAGTTGTCATTGGATGAACCTGGGCCAGGGGGTGCTAAGCAGTTGGGTGGTGCAGGAGGCATTGCTGAT
GATCTTGAGGCTGTTGTCAACTTCTATTGTCCAAACCATGACGACTTGGGGCATAGCAAGGGGCAGAATATACCCTTTGCTCCCTCA
AATAGCCACCCCTCCAGGCGTCTGC

Clone F9
AGCGTGGTCGCGGCCGAGGTACTTCCAACTCTGGGTTGGCCCCAAATCCAACTAATGCCACCACCAAGGTGGCTGGTGGTGCCCTG
CAGTCAACAGCCAGTCTCTTCGTGGTCTCACTCTCTCTTCTGCATCTCTACTCTTAAGAGACTCAGGCCAAGAAACGTCTTCTAAATT
TCCCCATCTTCTAAACCCAATCCAAATGGCGTCTGGAAGTCCAATGTGGCAAGGAAAAACAGGTCTTCATCGAATCTACTAATTCCAC
ACCTTTTATTGACACAGAAAATGTTGAGAATCCCAAATTTGATTGATTTGAAGAACATGTGAGAGGTTTGACTAGATGATGGATGCC
AATATTAAATCTGCTGGAGTTTCAGGTACCTGCCCGGGCC

Clone F10
TCGAGCGGCCGCCCGGGCAGGTACCGCGGCCGTTAAACATGTGTCACTGGGCAGGCGGTGCCTCTAATACTGGTGATGCTAGAGG
TGATGTTTTTGGTAAACAGGCGGGGTAAGATTTGCCGAGTTCCTTTTACTTTTTTTAACCTTTCCTTATGAGCATGCCTGTGCTGGG
TTGACAGTGAGGGTAATAATGACTTGTTGGTTGATTGTAGATATTGGGCTGTTAATTGTCAGTTCAGTGTTTTAATCTGACGCAGG
CTTATGCGGAGGAGAATGTTTTCATGTTACTTATACTAACATTAGTTCTTCTATAGGGTGATAGATTGGTCCAATTGGGTGTGAGGA
GTTCAGTTATATGTTTGGGATTTTTTAGGTAGTGGGTGTTGAGCTTGAACGCTTTCTTAATTGGTGGCTGCTTTTAGGCCTACTAT
GGGTGTTAAATTTTTTACTCTCTCTACAAGGTTTTTTCCTAGTGTCCAAAGAGCTGTTCCTCTTTGGACTAACAGTTAAATTTACAAG
GGGATTTAGGGGGTTCTGTGGGCAAATTTAAAGTTGAACTAAGATTCTATCTTGGACAACCAGCTATCACCAGGCTCGGTAGGTTT
GTCGCCTCTACCTATAAGTCTTCCCACTATTTTGCTACATAGACGGGTGTGCTCTTTTAGCTGTTCTTAGGTAGCTCGTCTGGTTTC
GGGGGTCTAAGCCTTGGCCCTCCTGCAAAGTTATTTTCTAGTTTAATCATTATGCAGAAGGTATAGGGGGTAGTTCCTGCTATTTAT
GCCTGGGTATAATTTTCATCTTCCCCTGCGGGACCTCGCCGCAAACAGCCTATTCAAGTTAATTC

Clone F12
AGCGTGGTCGCGGCCGAGGTACCTAACAAACCCACAGGTCCTAAACTACCAAACCTGCATTAAAAATTTCGGTTGGGGCGACCTCG
GAGCAGAACCCAACCTCCGAGCAGTACCTGCCCGGGCGG

Clone G1
AGCGTGGTCGCGGCCGAGGTACACCTKCCAGCCCTCCAGGACCTTCTCCCGGGTCACGAAGAAGCAGTGGGCGGYAGTGAGCACC
CACTGGGCGTCAATGAGCGTGCCTCCACAGATGTGGGTGGTGCCGAAGTGCAGACTCACTTGCCAAGGCCACTTGCTATCCGAGGC
CAGCGCCCCTCCCACGATCCGCCCGGTCATGGCCCTCAGTCCGCAGTGGGAACACTGGAGGGAGATATACCGCTGGGAAGGGCAT
TCAGACCTGTGGAGGCTTTCCTGGATGGTGGAKTTGTATCTCAAGATTGAGAAGCTGTTGGCAAAATCCCTGTGGGCAACCTCGGT
TGTCCGGTGAGCACTCTCGAAACCCAGCTGCTGGCAGGTCTTCTCTGAGTAGGAGTCATTCCAGTTGCTGCTACAGATGGGAAGCC
ACTGATGGGAGGACCCAGAGTAGATTTTAAGCAGAGACTTGTCCCAGTCAAACCTCACGCAGCCCAGCTCGTCACTCTTCAGCTTGC
AGTCCACCACCCCGTCACAGCGAACAGCGTGCTTGGGACAGCTCTCCCTCTGCTCCTTGTACCTCCCGGGGG

Clone G2
AGCGTGGTCGCGGCCGAGGTACGTCATTCTTAGTCCAGTCAGTCTTAAAAACATCTTGGGTTACCCACTCTGTCCACTCCCATAGGC
TACAGAAAAAGTCACAAGCGCATGGTTTCCAACCATATGTGTTTTCTGCAGTTATTTCTCTTGTTCTGGCCAAACAACCCTAAAAATC
CTTACCATTCCACAAAGTTGGACCATCACTTGTGCACCCACTTTGACTATGAGTATACCACCACATTGCATTTCTGTTTGCACCATGT
CTTCCAGGAGACTAGACTACTGTTGTCCAGGGTCAACTTGAGTGTAAAGAAAATGTAGACAAGGAATTGCCCAATTTTAAATTCTGA
CTTTGCTGACTTAATTTAAATGCTCGTTCTGAACCAATTTTCTCCTATCTTCTCTAGGGGTTTCAAAAGACTCAGTTAATTGATTTCC
AGGAAGTACCTGCCCGGGGCC

Clone G3
AGCGTGGTCGCGGCCGAGGTACTGTTTCCTCAGCAGAGGAGAAAAACTCAACCTAGTTATGAGACCAACCACACAACACAATGAAAA
GCTGCACTAACTAGTTCAGAATGTTAGTTAAGATGATGCTGGTGTGAATAACTCGTTTTTTCTAGAGCCCTTGTAAATAAAATCCCC
CAGTTAGTGTTTGCATTATCAGCTAGAGGGTTAACATGTGGTAGAATGAGGACTTATGCAAGGTATAAATGCGCATAGCATTTTACT
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Clone G4
TCGAGCGGCCGCCCGGGCAGGTACCCCAATCTGAAGTCAGTAAATGAACTAATCTACAAGCGTGGTTATGGCAAAATCAATAAGAA
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CTATACTGTTGGAAAACGCTTCAAAGAGGCAAATAACTTCCTGTGGCCCTTCAAATTGTCTTCTCCACGAGGTGGAATGAAGAAAAA
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ACCATGATTATTTTTCTAAGCTGGTTGGTTAATAAACAGTACCTCGGCCGCGACCACGCT

Clone G5
TCGAGCGGCCGCCCGGGCAGGTACGTGGCCATCCAGGCCGTGCTGTCCCTCTACGCCTCTGGGCGCACCACTGGCATCGCGATGG
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CGGGACCTGACTGACTACCTCATGAAGATCCTCACCGAGCGCGGCTACAGCTTCACCACCACGGCCGAGCGGGAAATCGTGCGTGA
CATTAAGGAGAAGCTGTGCTACGTCGCCCTGGACTTCGAGCAAGAGATGGCCACGGCTGCTTCCAGCTCCTCCCTGGAGAAGAGCT
ACGAGCTGCCTGACGGCCAGGTCATCACCATTGGCAATGAGCGGTTCCGCTGCCCTGAGGCACTCTTCCAGCCTTCCTTCCTGGGC
ATGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTCCATCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTACCTCGGCCGC
GACCACGCT

Clone G7
AGCGTGGTCGCGGCCGAGGTACTATGTGACTATCATTGATGCCCCAGGACACAGAGACTTTATCAAAAACATGATTACAGGGACAT
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TGCCAGTGGAACCACGCTGCTTGAGGCTCTGGACTGCATCCTACCACCAACTCGTCCAACTGACAAGCCCTTGCGCCTGCCTCTCCA
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Clone G8
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ACCCGGGCAGAGGGACAGCGCCAGGCCCAGGAGTATGAGGCCCTGCTGAACATCAAGGTCAAGCTGGAGGCTGAGATCGCCACCT
ACCGCCGCCTGCTGGAAGATGGCGAGGACTTTAATCTTGGTGATGCCTTGGACAGCAGCAACTCCATGCAAACCATCCAAAAGACC
ACCACCCGCCGGATAGTGGATGGCAAAGTGGTGTCTGAGACCAATGACACCAAAGTTCTGAGGCATTAAGCCAGCAGAAGCAGGGT
ACCTGCCCGGGC

Clone G9
CGAGCGGCCGCCCGGGCAGGTACTACKAGCGTAAGGTGGAACCTTCTCCGCAAAGCCGGATGACAAGTTTTCCTCTCCAGTGAGAA
ATGGGCTCCAAGTTCTTATCTTCTCTGCCATCAGCATGGCTGAAGGGGGAGAGGGTGGGTGTTAGGGAAAATCCAGGGACCCTTCC
ACACAGGAGGTACCTCKGCCGCGACCACGCT

Clone G10
TCGAGCGGCCGCCCGGGCAGGTACAGTCCAGTCCTTGGAGATCGACCTGGACTCCATGAGAAATCTGAAGACCAGCTTGGAGAACA
GCCTGAGGGAGGTGGAGGCCCGCTACGCCCTACAGATGGAGCAGCTCAACGGGATCCTGCTGCACCTTGAGTCAGAGCTGGCACA
GACCCGGGCAGAGGGACAGCGCCAGGCCCAGGAGTATGAGGCCCTGCTGAACATCAAGGTCAAGCTGGAGGCTGGGATCGCCACC
TACCGCCGCCTGCTGGAAGATGGCGAGGACTTTAATCTTGGTGATGCCTTGGACAGCAGCAACTCCATGCAAACCATCCAAAAGAC
CACCACCCGCCGGATAGTGGGTGGCAAAGTGGTGTCTGAGACCAATGACACCAAAGTTCTGAGGCATTAAGCCAGCAGAAGCAGAG
TACCTCGGCCGCGACCACGCT

Clone G11
AGCGTGGTCGCGGCCGAGGTACCTTGACGAGGCTGCGGTGTCTGCTGCTATTCTCCGAGCKYCGCAATGCCGMCTARGGACGACA
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Clone G12
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TCTATGAAAAAGCAAAGCACTATCAC

Clone H2
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CCTGCGACCCCCGCCATGGCCGCTACCTGACGGTTGCCGCCGTGTTCAGGGGCCGCATGTCCATGAAGGAGGTGGATGAGCAAAT
GCTTAATGTCCAAAACAAAAACAGCAGCWATTTTGTTGAGTGGATCCCCAACAATGTGAAAACGGCTGTCTGTGACATCCCACCTCG
GGGGCTAAAAATGTCCGCCACCTTCATTGGCAACAGCACGGCCATCCAGGAGCTGTTCAAGCGCATCTCCGAGCAGTTCACGGCCA
TGTTCCGGCGC

Clone H3
AGCGTGGTCGCGGCCGAGGTCGTCGGCAGTCGGCTCCCTCGTTGACCGAATCACCGACCTCTCTCCCCAGCTGTATTTCCAAAATG
TCGCTTTCTAACAAGCTGACGCTGGACAAGCTGGACGTTAAAGGGAAGCGGGTCGTTATGAGAGTCGACTTCAATGTTCCTATGAA
GAACAACCAGATAACAAACAACCAGAGGATTAAGGCTGCTGTCCCAAGCATCAAATTCTGCTTGGACAATGGAGCCAAGTCGGTAGT
CCTTATGAGCCACCTAGGCCGGCCTGATGGTGTGCCCATGCCTGACAAGTACCTGCCCGGGCG

Clone H4
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TGCCCGGGGCC

Clone H5
TTGCCGCGGGMATTCGATTTCGAGCGGCCGCCCGGGCAGGTACGTGGCCATCCAGGCCGTGCTGTCCCTCTACGCCTCTGGGCGC
ACCACTGGCATCGCGATGGACTCCGGTGACGGGGTCACCCACACTGTGCCCATCTACGAGGGGTATGCCCTCCCCCATGCCATCCT
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TCCAGCCTTCCTTCCTGGGCATGGAGTCCTGTGGCATCCACGAAACTACCTTCAACTCCATCATGAAGTGTGACGTGGACATCCGCA
AAGACCTGTACCTCGGCCGCGACCACGCTA

Clone H6
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TGTCAAAACTGGAACAGTCCAGTGAGTATGTGCAGGCCCGGGCTTGGGCAGTGACGAGGGCAGGGAGCACACATCAATTTCTGCG
GTACCTCGGCCGCGACCACGCT

Clone H7
CGAGCGGCCGCCCGGGCTGGTACGTAGACTGAAATAAGTCACTATTGAAATGGCATCAACATGATGCTGCCCATTCCACTGAAGTT
CTGAAATCTTTCGTCATGTAAATAATTTCCATATTTCTCTKTTATAATAAACTAATGATAACTAATGACATCCAGTGTCTCCAAAATTG
TTTCCTTGTACCTCGGCCGCGACCACGCTA

Clone H8
TTGCCGCGGGCAATTCGATTTCGAGCGGCCGCCCGGGCAGGTACACTGTTAAGAAGTTGAGCTTTTATCTTAGAGGCAGCAGAAGG
TTTGGAGCCAAGGAATGAAATGATGAGGCGTCCTTCAGGTAATGAACTTCAGCTGCAGTGTGAAAGGGGCAGGAAGACTGGCAGC
TGTCAAAACTGGAACAGTCCAGTGAGTATGTGCAGGCCCGGGCTTGGGCAGTGACGAGGGCAGGGAGCACACATCAATTTCTGCG
GTACCTCGGCCGCGACCACGCTA

Clone H9
TGCCGCGGGCMATTCGATTTCGAGCGGCCGCCCGGGCAGGTACTGGGAGAAGCTCTCCACACACATGGGCTTTCCCGGCACCATC
TCCACGATGGCCGCGTCTCCAGACTTCAGGGACTTGGGGTTGTCCTCCAGCTTCTTGCCAGAGCGCCGGTCAATCTTCTCCTTCAG
CTCCGCAAACTTGCAGGCGATGTGGGCTGTGTGGCAGTCGATGACCGGGGAGTAGCCGGCGCTAATCTGCCCCGGGTGGTTCAGG
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CACCGACACGTTCTTCACATTGAAGCCGACGTTGTCGCCGGGCAGAGCTTCGCTCAGAGCCTCGTGGTGCATCTCCACTGACTTCA
CCTCAGTGGTGATGTTCACTGGCGCAAAGGTCACCACCATGCCCGGCCGCAGGATGCCGGTCTCCACCCGGCCCACGGGCACCGTG
CCAATGCCGCCAATCTTGTACCTCGGCCGCGACCACGCT

Clone H11
AGCGTGGTCGCGGCCGAGGTACAAAACCAAATGTTTGTTACTATAACTTCTGCATCACAATTAAAATCCAAACAGTTTTTTAAAAACA
GTCAACTCAATCAAAACCCACTACTTCAGAATCAATAGCTTCTTTGAAGCCACAGTAACACTTAAATATGGTTAAGACTCGAATGCAG
AAATTTGGTTGGTTGGAAAGCTAATTAAACTTCCAACTTGCTCAAATAGAATTACAAAAAGGCAAAATTGTGACCTGCCCGGGCG

Clone H12
TCGAGCGGCCGCCCGGGCAGGTACAGAGATGGAGAACGAATTTGTCCTCATCAAGAAGGATGTGGATGAAGCTTACATGAACAAG
GTAGAGCTGGAGTCTCGCCTGGAAGGGCTGACCGACGAGATCAACTTCCTCAGGCAGCTATATGAAGAGGAGATCCGGGAGCTGC
AGTCCCAGATCTCGGACACATCTGTGGTGCCGTCCATGGACAACAGCCGCTCCCTGGACATGGACAGCATCATTGCTGAGGTCAAG
GCACAGTACCTCGGCCGCGCCACGCTA

Reverse

Clone A1
AGCGTGGTCGCGGCCGAGGTGCTTTYTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCTRATTGACAWTRARAWTWTTTWTTGAGGGT
TTWTTRAKTGCAGGGARAAGGGCTTRATBCCTTGGGGTGGGARGARARACCCCYCCTCTGGRATCCTSCASCTCTAKTCTCCCKTGG
TGGGGGGTRAGGGTTRARAACCTATGAMCWTTCTGTAGGGGCCACTGTCTTCTCCACGGTGCTCCCTTCWTSCKTRMCCTGSCASC
YKTASCTYCTKTGRACTYCCAYTGCYCAGGCKTYRGGCTCARATASCKGCKGGCCGCKTACCTGCCCGGGCGC

Clone A3
AGCGACGAAGCTCCATGCACCGGCCGCCATGGCGTTGTCCGCGGGAATCGATTYGAGCGGCCGCCCGGGCAGGTACTAGTTGAAT
TAAGATTTGGAAAATGTAAATAGTTCATGAGTCAAATTTCAAATTAAATGAATGACATAATATATAAAATAAATAAAGGGACAAAGTT
CATTTTTTCCATATAAACTCATTCAAACATACTTAATTTGGGGTAACGTGATACAAAGACGGAAAAGGCATCATATTTCCAGTCTCAG
TCAATTCAAAAATAAGGAGGGAAAATTAACTTTGAAATCTTCTTAGACACTAATGGGGTTATGAACTGGGGCAAGTGGCTGAGTCAC
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Clone A4
TCGAGCGGCCGCCCGGGCAGGTCTCTGATAACCATGAGGCTTCTCATTCTCACCTGTCTTGTGGCTGTTGCTCTTGCCAGGCCTAA
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Clone A7
ATTAGCGTGGTCGCGGCCGAGGTACCAACGTAGMCCAGCTATAGCAATTAATAATCCATATGTGCCTCGCACATATTATGCAAACCC
AGCTGTAGTTAGGCCACATGCCCAAATTCCTCAGCGGCAATACCTGCCAAATAGCCACCCACCCACTGTGGTACCTGCCCGGGCGG

Clone A9
TTGCCGCGCGGCAATCCCGCATCCYCSASCSSCCGCCCSGGCASGTACYASYYGAMYCYAACCAYCYYGCSAAMCAYCGTMMMTASTC
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AAMCATACYYAATYBGGGGTAACGTSATSCAMMSACGGAAMASGCATCATATYYCCASTCYCASTCAATYCAAAAATAMSGASGGAAAA
TYAACTTTSAAATCYYCYYASACACYAMTGGGGTTATGAACTACCTCSGCCGCGACCACGCYAHTCACTASTGAATYCS

Clone A10
TCGCATGCACCCGGCCGCCATGGCTTGCCGCGGGAATGCGATTAGCGTGGTCGCGGCCGAGGTACGCCAACGCCGCCGACATCSA
CTATATAGCAGGCACCAACAACATGGACGGTCACATCTTCGCCAGCATCGACATGCCTGCCATCAACAAGGGCAACARGARASTCAC
GGAGGAGGACTTMTACAASCTGGTCAGTGAGTTCACAATCACCAAGGGGCTCAGAGGCGCCAAGACSACCTYYGATGTCTACACCG
AGTCCYGGGCCCAGGACCCWTCCCAGGAGAATAMSAABAASACTGTGGTGGACTTYGAGACCGATGTCCTCTTCCTAKTGCCCACC
GAGATKGCCCTAGCCCAGCACMKAGCCAATGCCAAGAGTGCCWMSACCTACSCCTACCTGTKTYCCCATCCCYCYCGGATGCCCGTC
TACCCCCAAATGGGTGGGGGCCGACCATSCMGATSACATTCAKTACCTGCCCCGG

Clone A12
TCCGCATGCTACCCGCGCCGCCATGGCTTGCCGCGCGGAATCCCGCATTACGCGTGGTCGCGGCCGAGYGTRGTCCCTACASCCYA
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MTTATCGTATAACAYCYCCGCTACCCCTACCCAAKAATAMTTACCCCMTGGTYRTACATYRKCCMWCYWKCCMCWATBMATHMCSM
CATWWGWGTYTWAKAMSATYYCACAAGTTMATATTYCCCTCCWTTATCTYTBCGAMTGSGMCCTGHAGMACCTSGGHAAAWTTAT
TGAATGGCCCCTYTYTTCCCCGTCCCTTTTGGTTATTCACCKTTTYACCCCCCACAWATTCAMGTTATGTTTGGAATGAGTTCTATA
TGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACTATTTACATTTT
CCAAATCTTAATTCAACTAGTACCTGCCCGGGG

Clone B1
CGATTAGCGTGGTCGCGGCCGAGGTACAAGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTAAAGTGGGAMCTTTWTTCAA
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TGRAAAATWGTCTTCAARAATTCGGKRATKTYACTACMGGGCCCAAGGSTCARARACMRATWASCYTKGGGGGAACMRAAARAAACA
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AAASCCACTGTTCCAGCTTCTCAKTGCARARGGCTTTATGGGCCAMCCMGTAKTCAATTCCTTTAATATYCAGRAWCTTCTTGGCAC
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Clone B2
GGCCCCATGTCACTCGGCCGCCACGGTCGGCTCGCGGGCACACTCTTACTTCTSAGWCGGCCGSCGGGCASKWCAGCCCASAATCT
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KGTTCGGTGACTTGTCCTTTCCAAACTYTYCCTGTGCCTGKCGGAKAAGATTCCAGATGGCWTCCTCCTTGTCCATTCACWCTTCST
GCCRCAHCKGCATGASAAGGGACCCGGGCCAGATGGCASTCTTTGAACTYGTCCACTGGCTTCCKASTGTBGTCTGGGCASAGTAAC
TCHTACTCGTSCCTKKCWGCCTCGTCTGACAGGTCCTCAAACACTGTGCTCTCTCTGATAAAAGCCACGTCTCCAGCCCCGTCTCTC
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Clone B3
TATGGCTTGCCGCGTGGAATTCGATTAGCGTGGTCGCGGCYGAGGTGCAGCAGTGTCCCTCAGCCTATTCCTCAGACTCTTGCACT
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CTGTTCAAGCCCTKCTKCTCAACCAAGAACTTCTACTTAACCCCACCCWCCAGATCTACCCTGTGACKCWGCCACTTGCCCCAGTTC
ATARCCCCATTAGTGTCTAAGAAGATKTCAAAKTTAATTTTCCCTCCTTATTTYTGAATKGACTGASACKGGAAATATGATGCCTTTTC
CGTCTTTGTATCACGTYACCCCAAATTAAKTATGTTTGAATGAGTBKATATGGAAAMAATGAACTTYGTCCCTTTATTTATTTYATAT
ATTATGTCATTCATYTAATTTGAAATTTGACTCATGAWCTATYTACMTTTTCCMAATCTTAHTTCAACTASTACCTGCCCGGGC

Clone B4
ACATGTSCASAMATGGGATSGAAMATCAACCACCATAAMSASAMMACGASASGATSATAMACTGGTGGTGSAATGCGTCATGAMASGC
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GATATATYGTTCAMAMASATATBGTTGTYTYCCATGATYTASCAAGCAACTAATYYTCTCCCAASCTGATTTTATTCAATATGGTYACG
TTGGTTAAATAAACTTTTTYYAGATCTAAAAAAAAAAAAAAAAAAAAMMCCTCGGCCGCRACCACSCWAWTCMCTAKTRAWTYCGCGG
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Clone B5
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TCGTTCSGCCACACMACAACGCMACMASAAACGWCGCACCAMACATTATCTAACACACSACACTWAGTCTACCMCTAGMACWCGCA
TCGCGGCTCGGAYCGTCACGCAACACCGC

Clone B7
ACAATCCACCTTTGGAACCGCCAAGGAACGGGTGCCACACCTTGAAACAAAMCCCGHGMCCCVCCACAMCWVGCSACYTTGACMCC
GMCRCSCASCMMCACTYDACMGSACKHSSCSMGGCGACSSCCGARACMCVTCWRCCCVMGHKSVMCMCBCGHMTAMCCMMCVHHTM
CMCAMCMACCMCTMCRCYSCSCMCTCMHTYSAMCYDYVCADMSMVBMCGCSYMCCCCMCMVSNCCGGSGYTSCTMNMMCAYCCDCW
VCSCBANHNMTCWNTCRNVCCCNHSCMSSSCHHCCYHBGKMSSVGGGGGGGVKCMHHMSGCMMHCRABRMSMMMSGHYSMMWCHB
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CTCGGCGTCATAATGGTCATAGTCTGTATAATGTGTGAAAATCGGTCTATGCCGCCTCCCGCATTTACACCCCAAAACCTGGGGAAC
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Clone B8
AGCGTGGTCGCGGCCGAGGTWCTYGCTCTKGCWMKGGAKACCMTWGAWRKCCTTGTCWRSCAGTGAKGAATCTATTACWGAATR
CAAKCACGTAAAGTCTGAGAAAGCGTTAGAWCWTSACGGACCRGCAGMRWGGAGCASGATGAACACCWGGAYAAMGAWYTACCCC
CCCTCCTTTKMCCACGCYCCACAAGTCCCCTCCCTGGTAATTCCYAATTTCCSAATTTTCCTGGTTWGGAAAAACCCCTTTATTTCCY
CCCCTTTATTTGGGGGTTKTYTTCTTTTCCCCAYCACAAARACAYTTSGAGCSCTCTWGCTCACGCCTGCYGTKGTGCYGCMYGTYC
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CAGGTCCCTCAGCCTATGCCTCAGACTCTAGCACTTCCCCCTCAGCCCCTGTGGTCTGTTCCTCAGCCCGAGGTCCTGCCTATCACC
CCAGCAAGTGGTGCCCTATCCTCAGAGAGCTGTGCCTGTATAAGCGCTTCTACTCATCCAAGAACTACTACTTAAGCCCACCACCAG
ATCTATCTAGTGTGTCAGTCCACTTGACTCCAGATACATAGACCTCCATGAAAGCTGTTCTTAACGATAGAAATTCTCAAATAGGTTT
AAAATTTCTTACC

Clone B9
AGCGTGGTCGCGGCCGAGGTGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACT
CATGAACTATTTACATTTTCCAAATCTTAATTCAACTAGTACCTGCCCGGGCG

Clone B10
AGCGTGGTCGCGGCCGAGGTGAGAAGTTGTGAGTGTCTGCTGTCCTTGGCACCCCTGCCCACTCCACACTCCTGGAATACCTCTTC
CCTAATGCCACCTCAGTTTGTTTCTTTCTGTTCCCCCAAAGCTTATCTGTCTCTGAGCCTTGGGCCCTGTAGTGACATCACCGAATT
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GCRACCACSCTAWTCACWAKTRA

Clone B11
TCGAGCGGTCCGCCCGGGCAGGTGGCYAACTCAAYCTACTGCCAWYYWWGACCWGACTGGCAMGAGGAAAGGTGYAATARTSAA
GAGTKTYYTTSTAKTWGTYAATSCCCTGKCATKWAYCYTGCMWTKWKTGGCTGTGGAGGTWCWRAACCAGRWACMACCAKCATKC
CATKAKAWTRATKAAWGACYATWCTATCAGAAAACRRCTCCWTATSTMTCCAATGTATTATGTGCCAAATWGCTATCCTTATYATGG
AACCAATYWGTMCCTCGGCCRCGACCACGCTART

Clone C3
GTTTGAATGAGTTTATATGGAAAAAATGAACTTTGTCCCCTTTATTTATTTTTATATATTAATGTCATTCATTTAATTTGAAATTTTGA
CTCATGAACTATTTACATTTTCCAAATCTTAATTCAACTAGTACCTGCCCGGC

Clone C4
GAAAGGGAGACGAATAAGCGGGCTCAGTCAACCCGGCCGCCATGGCTTGCCGCGGGAATCGATTAGCGTGGTCGCGGCCGAGGTG
TTTGAATGAGTGTATATGGAAAAAATGAACTTTGTCCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCA
TGAACCTATTTACATGTTCCCAAATCTTAAATTTCAACTAAGTTACCTGGCCCCCGGGC

Clone C5
TTATCGCATCGACATGCGCTAGCTCACGGCCGCCATGGCTTGCCGCGGGCAATTCGATTAGCGTGGTCGCGGCCGAGGTGTTTGA
ATGAGTTTATATGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACT
ATTTACATTTTCCAAATCTTAATTCAACTAGTACCTGCCCGGGCGG

Clone C6
TCGAGCGGCCGCCCGGGCAGGTGGAAATATGATGCCTTTTCCGTCTTTGTATCACGTTACCCCAAATTAASTATGTTTGAATGAGTT
TATATGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACTATTTACA
TTTTCCAAATCTTAATTCAACTAGTACCTCGGCCGCGACCACGCTA

Clone C8
TCGAGCGGCCGCCCGGGCAGGTRTSAKWTGTCKKCTSTGCMTTCGCGCGABSCCTCGSYHACTGCACAYACTCCCTAGAKAMTMSCA
TGCTACYMYTWATCSCTCAGTCKTTTTCGACGCTTGTAGTTGTGCTGTCACCTGGTATKTCCCCTCAGAAYGCCTTTATCCTCGTGC

TGCTCTATGCTCATTKGCRCCYCTGCWMKWSWYACWCACGMKWATWCCYTGCATRGTAAYTRTWWTTACMCCAGGGKGCMTYAG
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Clone C9
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CCTTTATTTATTTTATATATTAATGTCATTCATTTAATTTGAAATTTGACTCATGAACTATTTACATTTTCMAATCTTAATTCCAACTA
GTACCCTCGGGCCGCGAACCACGCTA

Clone C10
ATTGAACCGGGCCAATAGGGCTCAGCACCCGGCCGCCTGGTCTGGCCGCGGSAATTCGATTTCGAGCGGCCGCCCGGGCAGGTGA
GACTGGWTAATATGATGCCTTTTCCGTCTTTGTATCACGTTACCCCAAATTAAGTATGTTTGAATGAGTTTATATGGAAAAAATGAA
CTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACTATTTACATTTTCCAAATCTTAATT
CAACTAGTACCTCGGCCGC

Clone D1
CCAAGACCTTGGGCGCACAGACTGTGGCGCATAGCTACCCGGCCGCCATTGGCTGCCGCGGGAATKCGATYAGCGTGGTCGCGGC
CGAGGTKGGAAATATGATGCCTYYTCCGTCTTTCGYATCACGCYCCACCCCRAATTAAGTATGTTTGATTTSMGTTTATATGGAAAAA
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TCTTAATTCAACTAGTACCTGCCCGGGCGG

Clone D3
AGCGTGGTCGCGGCCGAGGTTGCACTTCCCCCTCAGCCCCTGTGGTCTGYTCCTCAGCCCAAAGTCCTGYCTATCCTCCAGCAAGT
GGTGCCCTACCCTCAGAGAGCTGTGCCTGTTCAAGCCCTTCTGCTCAACCAAGAACTTCTACTTAACCCCACCCACCAGATCTACCCT
GTGACTCAGCCACTTGCCCCAGTTCATAACCCCATTAGTGTCTAAGAAGATTTCAAAGTTAATTTTCCCTCCTTATTTTTGAATTGAC
TGAGACTGGAAATGTGATGCCTTTTCCGTCTTTGTATCACGTTACCCCAAATTAAGTATGTTTGAATGAGTTTATATGGAAAAAATG
AACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACTATTTACATTTTCCAAATCTTAA
TTCAACTAGTACCTGCCCGGGCCC

Clone D4
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ATGAGTTTATATGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACT
ATTTACATTTTCCAAATCTTAATTCAACTAGTACCTCGGCCGCGACCACGCTA

Clone D5
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GAKAATGATGAAAGACCATTCTATCAGAAAACAGCTCCATATGTCCCAATGTATTATGTGCCAAATAGCTATCCTTATTATGGAACCA
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Clone D6
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AACCAACTTCAGCTGCAAGCTGTCCATGCCCAGGAGCAAATTCKCAGAATGAATGAAAACAGCYATGTYCAAGTGCCTTTCCAGCAG
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Clone D7
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Clone D8
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TGCAGATTTTCAAGATCAGTGAGTTTTGGGATTTGAGGGTCAAAAAAGGGTATCGTTGGAGATTTAAGGACAGGCATCACTCTGCC
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Clone D11
AGCGTGGTCGCGGCCGAGGTCCCAGCAAGTGGTGCCCTACCCTCAGAGAGCTGTGCCTGTTCAAGCCCTTCTGCTCAACCAAGAAC
TTCTWCTTAACCCCACCCACCAGATCTACCCTGTGACTCAGCCACTTGCCCCAGTTCATAACCCCATTAGTGTCTAAGAAGATTTCAA
AGTTAATTTTCCCTCCTTATTTTTGAATTGACTGAGACTGGAAATATGATGCCTTTTCCGTCTTTGTATCACGTTACCCCAAATTAAG
TATGTTTGAATGAGTTTATATGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGAC
TCACTGAACTATTTACATTTTCCAAATCTTAATTCAACTAGTACCTGCCCGGGGCG

Clone D12

GCGATTAGCGTGGTCGCGGCCGAGGTATATGATGCCTTTTCCGTCTTTGTATCACGTTACCCCAAATTAAGTATGTTTGAATGAGTT
TATATGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACTATTTACA
TTTTCCCAAATCTTAATTCAACTAGTACCTGCCGGGCG

Clone E1
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TCTTGAAAATCTGCATCTTCCTCTGCCTCTGCTCCAGCCCTTGATGCAGCAGGTSCCTCAGCCTATTCCTCAGACTCTTGCACTTCCC
CCTCAGCCCCTGTGGTCTGTTCCTCAGCCCAAAGTCCTGCCTATCCCCCAGCAAGTGGTGCCCTACCCTCAGAGAGCTGTGCCTGTT
CAAGCCCTTCTGCTCAACCAAGAACTTCTACTTAACCCCACCCACCAGATCTACCCTGTGACTCAGCCACTTGCCCCAGTTCATAACC
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TTGTATCACGTTACCCCAAATTAAGTATGTTTGAATGAGTTTATATGGAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATG
TCATTCATTTAATTTGAAATTTGACTCATGAACTATTTACATTTTCCAAATCTTAATTCAACTAGTACCTGCCGGGGGCTGGCTT

Clone E3
GAGCGGCCGCCCGGGCAGGTGCTGGGAATTATATAGGCATGAGCCACTGAGCCTGGCCCAGAAGCGTTTTTCTCAAAGGCCCTCA
GTGAGATAAATTAGATTTGGCATCTCCTGTCCTGGGCCAGGGATCTCTCTACAAGAGCCCCTGCCCCTCTGTTGGAGGCACAGTTT
TAGAATAAGGAGGAGGAGGGAGAAGAGAAAATGTAAAGGAGGGAGATCTTTCCCAGGCCGCACCATTTCTGTCACTCACATGGACC
CAAGATAAAAGAATGGCCAAACCCTCACAACCCCTGATGTTTGAAGAGTTCCAAGTTGAAGGGAAACAAAGAAGTGTTTGATGGTGC
CAGAGAGGGGCTGCTCTCCAGAAAGCTAAAATTTAATTTCTTTTTTCCTCTGAGTTCTGTACCTCGGCCGCGACCACGCTA

Clone E4
ATGCGATTAGCGTGGTCGCGGCCGAGGTCTGGAAATATGATGCCTTTTCCGTCTTTGTATCACGTTACCCCAAATTAAGTATGTTTG
AATGAGTTTATATGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAAC
TATTTACATTTTCCAAATCTTAATTCAACTAGTACCTGCCCGGGCG

Clone E5
GCGCGCTGGCGCGCGCACCGAGTTTTCCGCCACGTTAAACTCTTCGCGCGCGTGKGGCMCCACCGGAACGCASGATCCCGGGCGS
GTACYTTGCACTCGCSCACGGCCGCCACGCGGCGCGSCTCSTCYSAAACACTCSTGCCGCGCCTTAACTCCTCTCAAACCGGCAASGT
CTTCTCGCCAGCCTCCCGCCGTT

Clone E6
GCGATTTCGAGCGGCCGCCCGGGCAGGTACTAGTTGAATTAAGATTTGGAAAATGTAAATAGTTCATGAGTCAAATTTCAAATTAAA
TGAATGACATAATATATAAAATAAATAAAGGGACAAAGTTCATTTTTTCCATATAAACTCATTCAAACATACTTAATTTGGGGTAACG
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Clone E7
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CCTGCTTCTGTAAAAATATTATTTGTTTGAAGGGGAAATAATGGTAGTGTGACCTTTCCACTTAATTCCTACTCCCCTTAATGTGAGA
GAGACAAAATGAGCTGAAGAAGGAAAATTCTGGAGTTACACTCCACAACCTTGAACATACTGACGGACATCTCTGTT

Clone E8
GCGATTAGCGTGGTCGCGGCCGAGGTCAAGAAGGCTGCTGGAGCTGGCAAGGTCACCAAGTCTGCCCAGAAAGCTCAGAAGGCTA
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AAGTTTAGTAGTAAAAGACTGGTTAATGATAACAATGCATCGTAAAACCTTCAGAAGGAAAGGAGAATGTTTTGTGGACCACTTTGG
TTTTCTTTTTTGCGTGTGGCAGTTTTAAGTTATTAGTTTTTAAAATCAGTACCTGCCCGGGCG

Clone E10
TGAGSGTGGGGTCGCGGGCCCGAAGTACTGTMGCCTACTCTGGTGCCTTCAAGTGTCTGAGAGACGGGGCTGGAGACGTGGCTTT
TATMGAGAGAGCCACAGTGTTTGAGGACCTGTCAGACGAGGCTGAAAGGGACGAGTATGAGTTACTCTGCCCAGACAACACTCGGA
AGCCCAAGTTGGGAACAAGGTTTCAAAAGAACTGGGCCCATTCCTTGGGCCCCGGGGTTCCCTTCCTCATGGCCGTTGTGGGACGA

Clone E11
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Clone E12
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GTTTATATTGGAAAAAATGAACTTTGTCCCTTTATTTATTTTATATATTATGTCATTCATTTAATTTGAAATTTGACTCATGAACTATT
TACATTTTCCAAATCTTAATTCAACTAGTACCTGCCCGGGCGG

Clone F1
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ACGTGATACAAAGACGGAAAAGGCATCATATTTCCAGTCTCAGTCAATTCAAAAATAAGGAGGGAAAATTAACTTTGAAATCTTCTTA
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Clone F2
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CTCGGAAGCCAGTGGACAAGTTCAATAGACTGCCATCTGGCCCGGGTCCCTTCTCATGCCGTTGTGGCACGAAGTGTGAATGGCAA
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CCTAGTGGGCAGAAWGATCTGCTGTTCAAGGACTCTGCCATTGGGTTTTCGAGGGTGCCCCCGAGGATAGATTCTGGGCTGTACC
TCGTGCCGCGACCTACGCTA

Clone F4
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Clone F5
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Clone F6
GCGATTTCGAGCGGCCGCCCGGGCAGGTACTAGTTGAATTAAGATTTGGAAAATGTAAATAGTTCATGAGTCAAATTTCAAATTAAA
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AGAAGGGCTTACCTCGGCCGCGACCACGCTA

Clone F8
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GGTCTGAGCTGTGGAAAAGGGAGAGCAGAGAGGAACTGAGATGAGCAGGGATGAAGGGTCAGGCAGAAAGCGTGATAGAGGAGA
GAATTTTTGACAAAACTCAAAAGTTGTTTGCACAGCTGTTCTTTGTACCTCGGCCGCGACCACGCTA

Clone F9
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GAGGGAAACCCGACAAGAACCTGCGATCGGGCCCTTAGACTACCCACCATTTACGTTGGTTACATTTTCCCATGTTCCCGTGGTGCC
CCCCGCCCGGCCCCT

Clone F11
GCGATKTCGAGCGGCCGCCCGGGCAGGTACAGACCAATTCAGGCAAAGCGATGCCTCCATAACCATCTATGTCTTTCAGCAGCTGG
GACAGCTCACATTTTGTGAATTGCTTGGCCAGGATGGCAGGGAACAGGATGCCCACCAGGAACAGAGGGACAAAGAACCTCATTTT
GGCACCTCGGCCGCGACCACGCTA

Clone G1
GCGATKAGCGTGGTCGCGGCCGAGGTGATGCCTGTCCTTAAATCTCCAACGATACCCTTTTTTGACCCTCAAATCCCAAAACTCACT
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TA

Clone G4

AGCGTGGTCGCGGCCGAGGTACTTCAGCTACTCTGGTGCCTTCAAGTGTCTGAGAGACGGGGCTGGAGACGTGGCTTTTATCWGA
GAGAGCACAGTGTTTGAGGACCTGTCAGACKAGGCTGAAAGGGACGAGTATGAGTTACTCTGCCCAKACAACACTCGGAAGCCAGT
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Clone G5
KAGCGTGGTCGCGGCCGAGGTGCGACRGATGCGAGACAGTCKSATACMAKCTSTGCCRTGMTGRRSYGTGCMGCTKRTGCKRARGR
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CGCGGCTCCCGCYKAASCCYAGGGGTAGGTAGCGAWCGTGAGTAGTTGATGACCGTGCCCTCTGCGGGCTGRCTTTCCCTCCTTTG
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Clone G6
GCGATTTCGAGCGGCCGCCCGGGCAGGTMTKACWGKCWSKAGGAAWGRTKCAATAWTGAAGAGTTGWMYTCTRKTCSTCAATGC
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CCCGG

Clone G7
TCGAGCGGCCGCCCGGGCAGGTACAGACCAATTCAGGCAAAGCGATGCCTCCATAACCATCTATGTCTTTCAGCAGCTGGGACAGC
TCACATTTTGTGAATTGCTTGGCCAGGATGGCAGGGAACAGGACCTCGGCCGCGACCACGCTA

Clone G8
AGCGTGGTCGCGGCCGAGGTACTTCAGCTACTCTGGTGCCTTCAAGTGTCTGAGAGACGGGGCTGGAGACGTGGCTTTTATCAGA
GAGAGCACAGTGTTTGAGGACCTGTCAGACGAGGCTGAAAGGGACGAGTATGAGTTACTCTGCCCAGACAACACTCGGAAGCCAGT
GGACAAGTTCAAAGACTGCCATCTGGCCCGGGTCCCTTCTCATGCCGTTGTGGCACGAAGTGTGAATGGCAAGGAGGATGCCATCT
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Clone G9
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CCCTGTGGTCTGTTCCTCAGCCCAAAGTCCTGCCTATCCCCCAGCAAGTGGTGCCCTACCCTCAGAGAGCTGTGCCTGTTCAAGCCC
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ATTTAATTTGAAATTTGACTCATGAACTATTTACATTTTCCAAATCTTAATTCAACTAGTACCTCCGGGGGGGGG

Clone G11
GCGATTTCGAGCGGCCGCCCGGGCAGGTACTAGTTGAATTAAGATTTGGAAAATGTAAATAGTTCATGAGTCAAATTTCAAATTAAA
TGAATGGCATAATATATAAAATAAATAAAGGGACAAAGTTCATTTTTTCCGTATAAACTCATTCAAACATACTTAATTTGGGGTAACG
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AGAAGGGCTTGAACAGGCACAGCTCTCTGAGGGTAGGGCACCACTTGCTGGGGGATAGGCAGGACTTTGGGCTGAGGAACAGACC
ACAGGGGCTGAGGKACCTCGGCCGCGACCACGCTA

Clone H1
GCGATGAGCGTGGTCGCGGCCGAGGTAAGGCKCTGATAACCATGAGGCTTCTCATTCTCACCTGTCTTGTGGCTGTTGCTCTTGCC
AGGCCTAAACTTCCTCTTAGATACCCAGAACGCCTTCAGAATCCATCAGAGAGCAGTGAGCCTATACCATTAGAATCAAGAGAGGAA
TACATGAATGGTATGAACAGGCAGAGAAACATTCTGAGAGAAAAACAGACTGATGAAATCAAGGATACTAGGAATGAGTCTACTCAG
AACTGTGTTGTGGCAGAGCCTGAGAAGATGGAATCCAGCATCAGTTCATCGAGTGAGGAAATGTCTCTCAGTAAGTGTGCGGAACA
GTTTTGTAGACTGAACGAATACAACCAACTTCAGCTGCAAGCTGCCCATGCCCAGGAGCAAATTCGCAGAATGAATGAAAACAGCCA
TGTCCAAGTGCCTTTCCAGCAGCTCAACCAACTTGCTGCCTACCCCTATGCTGTTTGGTACCTCGGCCGCGACCACGCTA

Clone H2
GCGATTAGCGTGGTCGCGGCCGAGGTACTGGGATTATATAGGCATGAGCCACTGAGCCTGGCCCAGAAGCGTTTTTCTCAAAGGC
CCTCAGTGAGATAAATTAGATTTGGCATCTCCTGTCCTGGGCCAGGGATCTCTCTACAAGAGCCCCTGCCCCTCTGTTGGAGGCACA
GTTTTAGAATAAGGAGGAGGAGGGAGAAGAGAAAATGTAAAGGAGGGAGATCTTTCCCAGGCCGCACCATTTCTGTCACTCACATG
GACCCAAGATAAAAGAATGGCCAAACCCTCACAACCCCTGATGTTTGAAGAGTTCCAAGTTGAAGGGAAACAAAGAAGTGTTTGATG
GTGCCAGAGAGGGGCTGCTCTCCAGAAAGCTAAAATTTAATTTCTTTTTTCCTCTGAGTTCTGTCCTGCCCGGGGCCC

Clone H4
CGAACGTCGGCATGGCTCCCCGGGGCCGCCATGGGCGGGCCGCGGGGAAATGGCGACTGTTTCGAAAGACGGGGCCCGCCCCCG
GGGGACATGGGGTGGGKAAAAWWTTATTTGAGWRACYCCTKGGAAYCTGGCMGGWACCCTGGGTGRRTTATTACATCCRGTTGA
CCCCCCCCCCATAACACTKGTAATGTGTAGTGATTTHGGAGATTGAACGCTCCTAATATTTGGGAGATAATAGATTTGTAGAAAMTT
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AKTTTWGGTAAATAAGTTGWAKAGGYTTACATGTTGCAAAWCCTAAGWTTTATGSAATGAGGTGTCSAAGMGTCACTTTGAAAATC
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AGTAGTTGAGAGTAAGAAGTCTGAACGCTAATCGACGATTTAGGGTAGTGCCAATCACTGACTATTGAAAGACTATTGGGCACGAT

TTAAAGTTTCATTTCAGGCTCCCCAATAAAAGTAACTGGGCTTTTTGGGCACCTGGTTAGGATTTGGCAAATAAGTGGTGGGTATTA
AATGTACCTGGGTCTTATAACCATAGTTTTGGTCAACAAACATAAGCATTTATCCGCCAGGACCCGAGCAAAGTACATTTAAAAAAG
CAGATCAAAT

Clone H5
TCGAGCGGCCGCCCGGGCAGGTGAAGACTATTTTCCAGGGATGCCTGAGTGGTGCACTGAGCTCTAGACCCTTACTCAGTGCCTTC
KATGGCACTTTCACTACWGCACAGATTTCACCTCTGTCTTGAATAAAGGTCCCACTTTGAAKTAWAAAAAAAAAAAAAAAAAAAAAA
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TGGATSCWTAGCTKRAKTATTCTATAGTGTCACCTAAATASCTTGGCKTAATCWTGGTCWTASCTGTTTCCTGTGTGAAATTGTTAT
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GTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCAC
TCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAA
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CCGACCGACTATAAGATCCAGGGTTTCCCCTGAAGCTCCTGTGCGCTTCCGTTCGACCTGCGCTTACGGATCTGTCCCCTTCTCCCT
CGGGAGCGTGGGCCTCTCT

Clone H9
TCGAGCGGCCGCCCGGGCAGGTACTAGTTGAATTAAGATTTGGAAAATGTAAATAGTTCATGAGTCAAATTTCAAATTAAATGAATG
GCATAATATATAAAATAAATAAAGGGACAAAGTTCATTTYTTCCGTATAAACTCATTCAAACATACTTAATTTGGGGTAACGTGATAC
WAAGACGGAAAAGGCATCRTATTTCCAKTCTCAGTCAATYCAAAAATAAGGAGGGAAAATYAMCTTTGAAATCTTCTTAGACACTAA
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GGGCTYKAHCAGGCACAKCTCTCTGAGGGTASGGCACCMCTTGCTGGGGGATABGCAKGACTYTGGGCTGASKAACMGTACCWCA
GTGGGCYGAGGBACCTCGGCYGCGACYMBTCTAATCMCTAKTGAATYYGCGGCYGYCYGCAGGTCGASGATATGGGAAAGCTTTCA
ACGCGTTGGATGCATAGCTGTAGTATTTTATAGTGTACTAGATAGCTGGGTAATTTGGGTGATTACTGTTGTGTAAATGTATTGTTT
CAATTAATGACCGGGAGCATCAGGTAGACGGG

Clone H10
AGCGTGGTCGCGGCCGAGGTCTACCCTCAGAGAGCTGTGCCTGTTCAAGCCCTTCTGCTCAACCAAGAACTTCTACTTAACCCCACC
CACCAGATCTACCCTGTGACTCAGCCACTTGCCCCAGTTCATAACCCCATTAGTGTCTAAGAAGATTTCAAAGTTAATTTTCCCTCCT
TATTTTTGAATTGGCTGAGACTGGAAATATGATGCCTTTTCCGTCTTTGTATCACGTTACCCCAAATTAAGTATGTTTGAATGAKTTT
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TTTCCAAATCTTAATTCAACTAGTACCTGCCCGGGCGG